Yasunari Ogihara · Shigeo Takumi
Hirokazu Handa *Editors*

Advances in Wheat Genetics: From Genome to Field

Proceedings of the 12th International Wheat Genetics Symposium

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Preface

This volume of proceedings is a collection of papers that were presented at the 12th International Wheat Genetics Symposium (IWGS) held in Yokohama, Japan, September 8–14, 2013, containing a selection of invited lectures and representative presentations. All papers were peer-reviewed by the Local Organizing Committee.

On August 29, 1953, 26 wheat geneticists from 19 countries who got together at the 9th International Congress of Genetics held at Bellagio, Italy, initiated the International Organizing Committee of the 1st IWGS. In 1958, the 1st symposium, which Hitoshi Kihara chaired, was held in Winnipeg, Canada. Since then, the IWGS has been held every 5 years at different locations around the world. In 2013, the IWGS turned 60 years old, an age of great traditional importance in East Asia. This volume commemorates an occasion of special celebration.

This proceedings presents the current status of our understanding of wheat genetics and its application to cultivated wheat and related plants. Importantly, it shows that breeding wheat based on genetics has come of age. Despite the large genome size and complexity, molecular breeding has become much more realistic than ever in wheat mainly through development in genomics research. In addition, the global food crisis and the impact of climate change on the food chain have become pressing problems. Consequently, improved production of cereals including wheat is urgently required in order to increase yields for human consumption. The production of wheat is particularly important in this regard as it is the most widely grown crop. We hope that this volume will provide a useful research reference in the field of wheat genetics.

The editors wish to thank Dr. Shuhei Nasuda, who first suggested the idea of preparing this book and collaborated in its initial planning. The editors are also very grateful to Dr. Yoshihiro Matsuoka for his valuable help in the editing work.

Yokohama, Japan — Yasunari Ogihara
Kobe, Japan — Shigeo Takumi
Tsukuba, Japan — Hirokazu Handa

List of the Meetings of International Wheat Genetics Symposium Held to Date

I. Winnipeg, Canada, 1958
II. Lund, Sweden, 1963
III. Canberra, Australia, 1968
IV. Columbia, Missouri, USA, 1973
V. New Delhi, India, 1978
VI. Kyoto, Japan, 1983
VII. Cambridge, UK, 1988
VIII. Beijing, China, 1993
IX. Saskatoon, Canada, 1998
X. Paestum, Italy, 2003
XI. Brisbane, Australia, 2008
XII. Yokohama, Japan, 2013

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Part I
Wheat Genetics: Past, Present, and Future

Chapter 1
Prof. H. Kihara's Genome Concept and Advancements in Wheat Cytogenetics in His School

Koichiro Tsunewaki

Abstract This article introduces Kihara's main achievements in wheat cytogenetics and the succeeding developments in a few fields of wheat cytogenetics, which were founded by Kihara. Following the discovery of polyploidy in wheat by Sakamura (Bot Mag (Tokyo) 32:150–153, 1918), Kihara established the cytogenetics of interploid hybrids, clarifying the meiotic chromosome behavior as well as the chromosome number and genome constitution of their progeny, based on which Kihara formulated the concept of genome. Here, evidence supporting his recognition of the genome as a functional unit is presented. Kihara proposed the methodology for genome analysis and determined the genome constitution of all *Triticum* and *Aegilops* species. Ohta re-evaluated the genome relationships among the diploid species, using the B-chromosomes of *Ae. mutica*. After completing the genome analyses, Kihara's interest was shifted to the genome-plasmon interaction that led to the discovery of cytoplasmic male sterility in wheat. Using the nucleus substitution method elaborated by Kihara, we carried out plasmon analysis of *Triticum* and *Aegilops* species. We classified their plasmons into 17 major types and 5 subtypes and determined the maternal and paternal lineages of all polyploid species. An alloplasmic line, (*caudata*)-Tve, retained male sterility induction and germless grain production for 60 generations of backcrosses with wheat pollen. We are trying reconstruction of the *Ae. caudata* plant from the genome of its native strain and the *caudata* plasmon in the alloplasmic wheat. Two groups of Kihara's school reported paternal transmission of the mtDNA sequences in alloplasmic wheats. Their findings are incompatible with the genetic autonomy of the plasmon, casting a new challenge to the genome-plasmon interaction.

Keywords *Aegilops* • B-chromosomes • Genome analysis • Genome concept • Maternal lineage • Plasmon analysis • Plasmon autonomy • Polyploids • Wheat

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Discovery of Polyploidy and Cytogenetics of Interploid Hybrids in Wheat

Sakamura (1918) who was a graduate student at the Faculty of Agriculture, Hokkaido University, studied both root-tip mitosis and meiosis in PMC's of the following eight *Triticum* species; *T. aestivum, T. compactum, T. spelta, T. turgidum, T. durum, T. polonicum, T. dicoccum*, and *T. monococcum*, finding 2n = 14 for *T. monococcum*, 2n = 28 for *T. turgidum, T. durum, T. polonicum* and *T. dicoccum*, and 2n = 42 for *T. aestivum, T. compactum* and *T. spelta*. This result led him to discover a polyploid series of the diploid, tetraploid, and hexaploid in wheat, with the basic chromosome number of $x = 7$. Sakamura planned a further study on chromosome numbers of the offspring of the hybrids between different ploidies. In 1917 he made crosses between 4*x* and 6*x* wheat in three combinations. At this point, Sakamura was informed from the Ministry of Education, Japan, to go abroad for advanced study. He asked Kihara who had just enrolled in the graduate school to succeed his wheat research and handed the 5*x* hybrids to Kihara (1951). Because of some delay in departure, Sakamura was able to see Kihara's first slide of PMC's of the 5*x* hybrid, and gave a few minutes advice that determined Kihara's later career as the wheat researcher.

Kihara (1924) analyzed the meiotic chromosome behavior of the three 5*x* hybrids of Sakamura and two 3*x* hybrids that he produced. The modal meiotic chromosome configurations of the 3*x* and 5*x* hybrids were 7"+7' and 14"+7', respectively (Kihara 1924, 1930), based on which he assigned genome formulae AA to the diploid, AABB to the tetraploid, and AABBDD to the hexaploid wheat. Later Kihara obtained a new tetraploid wheat, *T. timopheevi*, and analyzed the meiotic chromosome behaviors of its hybrids with one diploid and two tetraploid *Triticum* species. From the results, he designated genome formula AAGG to this wheat (Lilienfeld and Kihara 1934).

Kihara's Genome Concept and Supporting Evidence

Winkler (1920) proposed the term 'genome' for the haploid set of chromosomes. Due to the discovery of polyploidy, this definition required modification, because their gametes contain two or more chromosome sets. Kihara and Lilienfeld (1932) and Kihara (1982) defined the genome concept as follows: (1) Homologous chromosomes have homologous loci identical in sequence as well as in distance. Therefore, when two genomes are homologous, an exchange of homologous partners causes no physiological damage to either the gametes or the zygotes. Non-homologous chromosomes have different loci or the same loci different in sequence or in distance. As a consequence, the homologous chromosomes can synapse in the meiotic prophase, forming bivalents in the metaphase I (MI) by exchanging their homologous parts. On the contrary, non-homologous chromosomes fail to pair,

becoming univalents in MI. (2) Genome has no homologous chromosomes within it. Consequently, a zygote having two homologous genomes forms *x* pairs of bivalents with no univalents, whereas that with two non-homologous genomes forms 2*x* univalents with no bivalents in meiosis. The homologous vs. non-homologous relationship between two genomes can be determined by the number of bivalents formed in meiosis. (3) Genome is a functional unit of life. The deletion of a chromosome or a part of it from a genome causes the loss of life or, at least, a significant loss of functions of the gamete and zygote. In essence, Kihara was first to define the homology of chromosomes by their meiotic behavior, based on which he defined genome homology, and proposed its functional role in life.

Supportive evidence of the functional role of genome in gametic and zygotic development was obtained from the fertility of gametes and the viability of progenies of the 5*x* hybrids, respectively. Fertility of the female and male gametes of the 5*x* hybrids was studied by Kihara and Wakakuwa (1935) and Matsumura (1936, 1940). The transmission rate of a D-genome chromosome was 0.440 when the 5*x* hybrid was backcrossed as female to the 4*x* parent, and 0.673 when the 5*x* hybrid was backcrossed as pollen parent to the 4*x* wheat. Based on these univalent transmission rates, the fertility rates of female and male gametes having zero to seven D-genome chromosomes of the 5*x* hybrid when backcrossed as female or male parent to the 4*x* wheat were estimated, and compared to the observed frequencies. The female gametes of the 5*x* hybrid having no D-genome chromosomes or the complete set of D genome chromosomes took part in fertilization in 3.8 or 5.6 times higher frequencies than the expected ones, whereas those with one to five D genome chromosomes showed fertility rates nearly equal to or lower than the expected frequencies (Fig. 1.1a). Similarly, the male gametes of the 5*x* hybrid having no D genome chromosomes or the complete set of D genome chromosomes took part in fertilization in 391 or 5.7 times higher frequencies, respectively, than expected, whereas those with two to six D genome chromosomes showed fertility rates nearly equal to or lower than the expected frequencies (Fig. 1.1b). These results showed that complete missing or presence of the complete D genome guaranteed both sexes of the gametes for high ability of fertilization. The effect of the genome completeness on the viability of sporophytes was traced for four generations of self-pollination of the 5*x* hybrid (Kihara 1924). The pedigree was converged at two extremes, 2n = 28 with 14" and 2n = 42 with 21", one exception being the stable 2n = 40 (20") progenies called D-dwarfs. This convergence of the pedigrees to 2n = 28 or 2n = 42 plants demonstrated importance of the genome completeness for the continuation of life even in the hexaploid wheat.

Genome Analysis and a Re-evaluation on Genome Homology

Determination of the genome homology is based on the meiotic chromosome pairing between two genomes in comparison. Kihara (1930) illustrated the schemes for genome analysis of an auto- and allotetraploid. Before their application, we need to

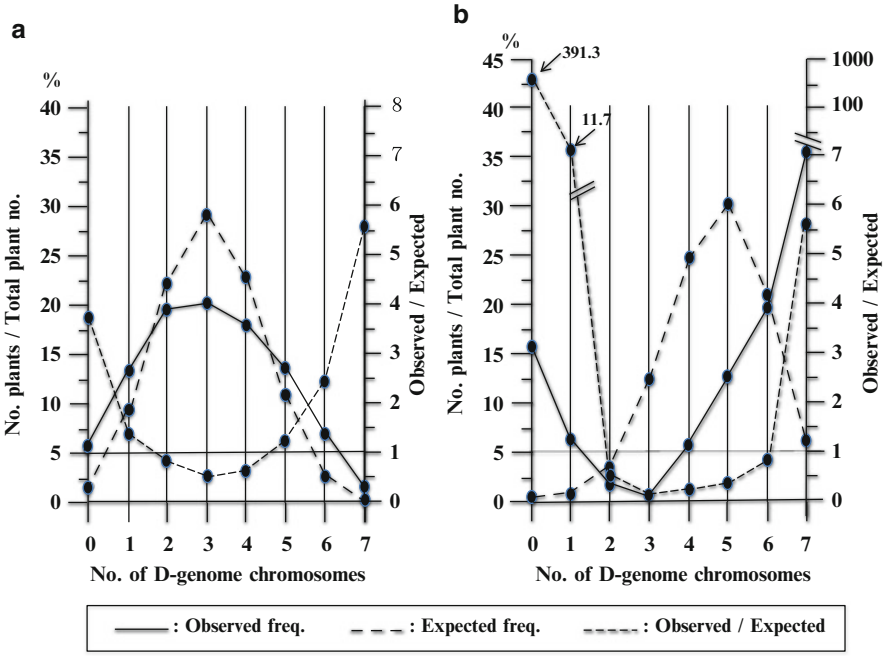

Fig. 1.1 Male and female transmission rates of the D-genome univalents in the crosses between (*T. polonicum* x *T. spelta*) F_1 and *T. polonicum*. (**a**) 5*x* F_1 hybrid x *T. polonicum* (Kihara and Wakakuwa 1935; Matsumura 1940), (**b**) *T. polonicum* x 5*x* F_1 hybrid (Matsumura 1936)

have a set of diploids, each having a genome different from the others, which he called genome analyzers. When the tetraploid is an autotetraploid with A genome, its hybrid with the A genome analyzer forms *x*"', whereas the F_1's with other analyzers form *x*"+*x*', from which results genome constitution of the tetraploid is determined as AAAA. If the tetraploid is an allotetraploid with AABB genomes, its F_1 hybirds with the A and B genome analyzers form *x*"+*x*', whereas the F_1's with other analyzers form 3*x*', thus genome constitution of the tetraploid is confirmed to be AABB. Kihara and his collaborators determined the genome constitutions of all *Triticum* and *Aegilops* species (Kihara 1924, 1945; Kihara and Tanaka 1970; Lilienfeld 1951).

After Kihara's genome analytical works, several genetic factors became known to influence the meiotic chromosome pairing: They are a suppressor, *Ph1*, of the homoeologous chromosome pairing, an enhancer of the homoeologous pairing in *Ae. speltoides* and a suppressor of the homoeologous pairing in B-chromosomes. To distinguish between the homologous and homoeologous pairing, using B-chromosomes of *Ae. mutica,* Ohta (1995) produced hybrids between eight diploid species and an *Ae. mutica* strain having the B-chromosomes, selecting hybrids with zero, one or two B-chromosomes. The presence of two B-chromosomes did not affect the number of bivalents in *Ae. mutica* itself. The T genome of *Ae. mutica* was

highly homologous to S genome of *Ae. speltoides* and D genome of *Ae. squarrosa*, forming five bivalents in the presence of two B's, whereas it was non-homologous to A, C, M, S^b and S^l genomes of the respective species, forming no or one bivalent with two B's. This type of research should be extended to polyploid species for re-evaluation of their genome relationships to the diploid species.

Plasmon Analysis as the Counter Part of Genome Analysis

Later, Kihara's interest shifted to the genome-plasmon interaction. He produced an alloplasmic line of a common wheat, *T. aestivum* var. *erythrospermum* (abbrev. 'Tve') by repeated backcrosses of the F_1 hybrid, *Ae. caudata* var. *polyathera* x Tve, with Tve as the recurrent pollen parent. This alloplasmic line, designated by (*caudata*)-Tve, expressed male sterility in its SB_3 and later backcross generations, leading Kihara to discover the cytoplasmic male sterility in wheat (Kihara 1959).

The pioneering works of Kihara and others suggested the presence of plasmon diversity in the *Triticum-Aegilops* complex. There were three research groups actively working on the plasmon diversity in wheat and its related genera: Maan and Lucken in the North Dakota State Univ., USA, Panayotov and Gotsov in the Wheat and Sunflower Institute, Bulgaria, and Suemoto and Tsunewaki in Kyoto University, Japan. In an international cooperative work, we compared plasmons that were independently introduced by these groups into their own wheat stocks (Mukai et al. 1978). This work showed the wide scope of plasmon diversity in this complex. I obtained 7 plasmons from Maan, 8 plasmons from Panayotov and 15 plasmons from other researchers to enrich our plasmon collection, totaling 46 plasmons, including 16 of our own.

In 1963, I initiated a program to produce alloplasmic lines using a set of 12 common wheat genotypes as the alloplasmon recipients, whose list and reasons of selection are given elsewhere (Tsunewaki et al. 1996). The aim was 10 backcross generations of each alloplasmic line to recover the tester's genotype in 99.9 % purity. The total number of the alloplasmic lines in all combinations between the 12 wheat genotypes and 46 plasmons amounts to 552 NC hybrids, all of which reached SB_{10} or later backcross generation by 1997. A field test of all alloplasmic and 12 euplasmic lines was carried out in the crop season of 1992–1993. By that time, 87 % of the alloplasmics reached at the SB_{10} or later backcross generation. With all lines, 14 vegetative and 8 reproductive characters were observed. The genetic relationships between the 47 plasmons were analyzed (Tsunewaki et al. 2002). With the same plasmons, RFLP analyses of ct and mtDNAs were carried with 13 and 3 restriction enzymes, respectively (Ogihara and Tsunewaki 1988; Wang et al. 2000). Phylogenetic trees depicted from the data on the organellar DNA polymorphisms revealed molecular differentiation between the plasmons in *Triticum-Aegilops* complex. Combining the phenotypic effects on wheat characters and organellar DNA differences revealed by the RFLP analyses, 47 plasmons of this complex were classified into 18 major types and five subtypes (Tsunewaki et al. 2002). The genome

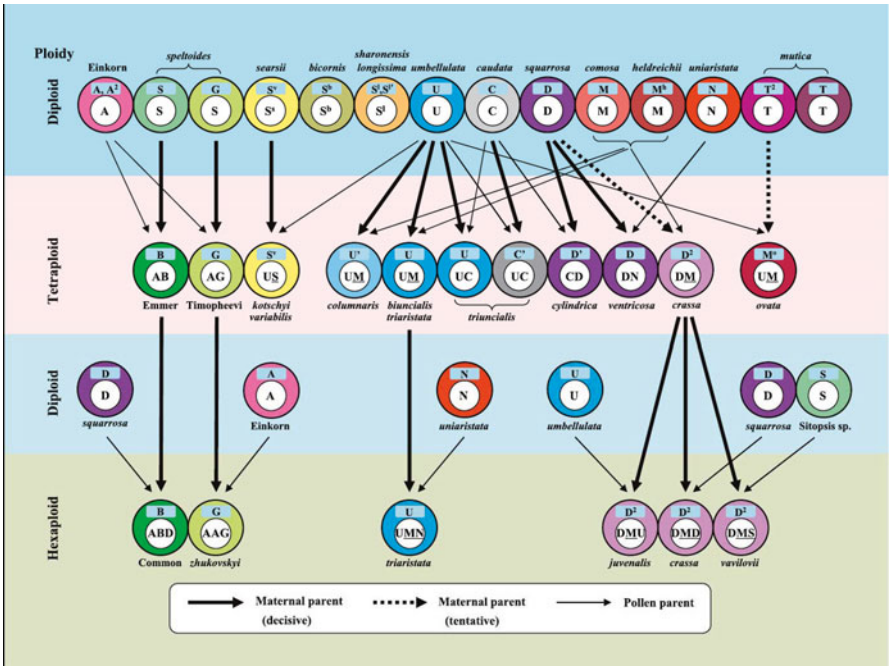

Fig. 1.2 Phylogenetic relationships between the diploid, tetraploid and hexaploid species based on their genome-plasmon constitutions (After Tsunewaki 2009). *Inner* and *outer circle*: genome and plasmon symbol, respectively. Modified genome: *underlined*

and plasmon analyses together clarified the maternal and paternal lineages of all *Triticum* and *Aegilops* species (Fig. 1.2; Tsunewaki 2009).

Persistence of Genetic Effects of *Ae. caudata* Plasmon on Wheat Phenotypes

Kihara crossed in 1949 *Ae. caudata* as female to a common wheat, Tve, and the F_1 hybrid and its progenies were successively backcrossed with the pollen of Tve until SB_{16} (Kihara 1959; unpubl.). I continued the backcrosses with the same pollen parent, up to the SB_{60} generation in 2013. Meiotic pairing was normal, forming 21 bivalents, in an SB_{56} plant.

Selfed and backcrossed seed fertilities of the (*caudata*)-Tve were observed in the entire backcross program. The backcrossed seed fertility (%) was highly variable, with the mean and S. E. of 68.10 ± 16.43. The linear regression to the backcrossed generation calculated using the records of the SB_3 to SB_{60} generations to grasp the tendency of improvement or depression of the backcrossed seed fertility, Y, with progression of the backcross generations, X, turned out to be $Y = 0.181X + 62.3$ (%).

The regression coefficient of 0.181 was non-significant at the 5 % level of probability, meaning the female fertility of (*caudata*)-Tve did not change consistently during 60 years of backcrossing. On the contrary, the selfed seed fertility was completely zero in most backcrossed generations. These results indicated that the *caudata* plasmon induced complete male sterility in Tve wheat, which did not show any sign of recovery by repeated backcrossing with the wheat pollen for 60 generations, proving persistence of the plasmon effect on male sterility induction.

Another prominent effect of the *caudata* plasmon on the wheat phenotype was production of germless grains, which was first noticed in the SB_{15} generation of (*caudata*)-Tve. Since then, occurrence of germless grains in (*caudata*)-Tve was examined in each backcross generation. Its frequency in every ten SB generations was 15.3 % (SB_{15}-SB_{20}), 14.7 % (-SB_{30}), 28.5 % (-SB_{40}), 14.2 % (-SB_{50}) and 5.2 % (-SB_{60}). Overall frequency of the germless grains was 11.8 % in (*caudata*)-Tve, as compared to 0.022 % in normal Tve, indicating more than 500 times increase in its frequency by the *caudata* plasmon. Genetic effect of the *caudata* plasmon to produce germless grains in Tve continued, at least, for 46 generations, from SB_{15} to SB_{60}. These results demonstrated that the genetic effects of the *caudata* plasmon on male sterility induction and germless grain production to 'Tve' persistently expressed during 60 generations of backcrossing with the Tve pollen.

Reconstitution of *Ae. caudata* from Its Genome and Plasmon Separated for Half a Century and Paternal mtDNA Transmission in Wheat

Ae. caudata (genome, CC) was reconstructed from the genome of its native strain and the plasmon originated from it and coexisted with the genomes of Tve wheat (AABBDD) for 50 generations of repeated backcrosses (details of the procedure and the results will be published elsewhere). The reconstructed *caudata* plants resembled those of the *caudata* accession that provided the plasmon to (*caudata*)-Tve SB_{50} in general morphology and showed normal vigor and fertility. We analyzed 8 chloroplast and 5 mitochondrial simple sequence repeat (abbrev. SSR) loci out of 45 loci reported in wheat (Ishii et al. 2001, 2006) (Yotsumoto et al. unpubl.). The tentative results indicated that the reconstructed *caudata* had the same band patterns in most SSR loci, as those of the original *caudata* accession and (*caudata*)-Tve SB_{50}. These results suggested that the *caudata* plasmon remained unchanged during coexistence with the wheat genomes for 50 generations.

Following the report of Laser et al. (1997) with triticale, Tsukamoto et al. (2000), Hattori et al. (2002), Kitagawa et al. (2002) and Kawaura et al. (2011) obtained evidence on the paternal transmission of mtDNA in alloplasmic wheats, which included coding regions of, at least, ten mitochondrial genes. Laser et al. (1997) and Kawaura et al. (2011) showed that the paternally transmitted mtDNAs were rarely transcribed in the heteroplasmic plants. This is compatible with the persistence of phenotypic effects of the *caudata* plasmon during the repeated backcrosses with

wheat pollen. The constancy of mitochondrial SSR loci in the alloplasmon during the repeated backcrosses proved here, however, is hardly compatible with the paternal transmission of mtDNAs, because the majority of mtDNA molecules of an alloplasmic line at an advanced backcross generation is assumed to be the paternal (wheat) molecules, but this is not the case. Further studies are needed to clarify the cause of this discrepancy.

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Chapter 2
How a Gene from Japan Revolutionized the World of Wheat: CIMMYT's Quest for Combining Genes to Mitigate Threats to Global Food Security

Thomas A. Lumpkin

In 1935, the work of Japanese scientist Gonjiro Inazuka to cross a semi-dwarf Japanese wheat landrace with two American varieties resulted in an improved semi-dwarf variety, known as Norin 10. Unlike other varieties, which stood taller than 150 cm, the *Rht1* and *Rht2* genes present in Norin 10 reduced its height to 60–110 cm. In the late 1940s Orville Vogel at Washington State University used Norin 10 to help produce high-yielding, semi-dwarf winter wheat varieties. Eventually, Vogel's varieties ended up in the hands of Norman Borlaug, who was working to develop rust-resistant wheat in Mexico.

In 1953, Borlaug began crossing Vogel's semi-dwarf varieties with Mexican varieties. The result was a new type of spring wheat: short and stiff-strawed varieties that tillered profusely, produced more grain per head, and were less likely to lodge. After a series of crosses and re-crosses, the semi-dwarf Mexican wheat progeny began to be distributed nationally, and within 7 years, average wheat yields in Mexico had doubled. Borlaug named two of the most successful varieties Sonora 64 and Lerma Rojo 64, and it was these two varieties that led to the Green Revolution in India, Pakistan and other countries. This international exchange of ideas and germplasm – starting with genetic resources from Japan – ultimately saved hundreds of millions of people from starvation.

Fifty years on, farmers and societies face new challenges to feed rising populations, and wheat, along with its production and trade, epitomize the difficulties. The world's climate is changing; temperatures are rising in major wheat-growing areas and extreme weather events are becoming more common; natural resources are being depleted; new diseases are emerging; and yields are stagnating. Coupled with these difficulties, ever-increasing demand for wheat from a growing worldwide population

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and changing diets put pressure on grain markets, pushing up prices, disrupting free trade when drought or floods threaten crops in wheat-producing nations.

With offices and research stations in 14 countries including its headquarters in Mexico, the International Maize and Wheat Improvement Center (CIMMYT) is working to address these challenges through scientific research. CIMMYT's research is focused on sustainably increasing wheat and maize productivity, which includes actively contributing to a better understanding of the genetic basis of yield, as well as drought, heat and disease resistance. Through the Seeds of Discovery (SeeD) initiative, and other collaborations, CIMMYT is also broadening genetic variability and discovering new genes that will help increase yields and prevent crop losses to climate change or disease.

The Origins of Norin 10

Upon hearing of Orville Vogel's successes in incorporating the semi-dwarf genes from the Japanese Norin 10 variety into winter wheat, Norman Borlaug wrote to him in 1952 requesting that genetic materials containing the Norin 10 genes be used as parental lines in the Mexican wheat breeding program. A few months later a small number of seeds arrived in Mexico from three different F_2 plants originating from the cross Norin 10 × Baart and a small number of seeds from each of the five F_2 plants from the cross Norin 10 × Brevor (Reynolds and Borlaug 2006). With this simple exchange of communication and germplasm, so began one of the most extraordinary agricultural revolutions in history.

The journey of semi-dwarf wheat from Japan to Mexico may have begun in Korea in the third or fourth century, where short wheat varieties may have originated (Cho et al. 1993). From East Asia, wheat breeders in Italy, the USA and elsewhere began to seek and utilize dwarfing genes to breed for high yield, resistance to lodging and ability to produce more tillers than traditional varieties.

The lineage of the Norin 10 can be traced back to Daruma, a native Japanese short-straw variety (see Fig. 2.1) crossed with one soft "Fultz" and one hard wheat "Turkey Red" variety from the United States. 'Fultz' was first introduced to Japan

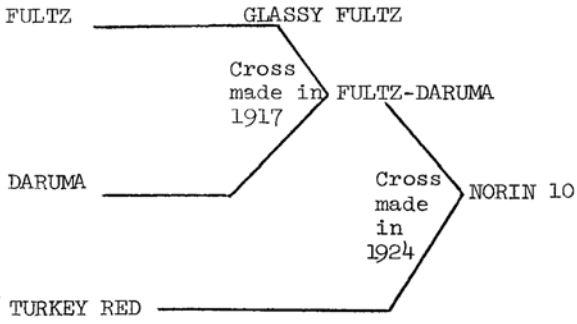

Fig. 2.1 Pedigree of Norin 10 (Reitz and Salmon 1968)

from the USA around 1892. The exact year that 'Turkey Red' arrived in Japan is unknown but it is thought to have been around the same time (Reitz and Salmon 1968). Fultz and Turkey Red were used in two significant crosses at the Central Agricultural Experiment Station in Nishigahara, Tokyo during the early twentieth century leading to the creation of Norin 10. First, in 1917 a variety called 'Glassy Fultz' was isolated from the 'Fultz' variety, and then crossed with Daruma to create 'Fultz-Daruma.' Then, in 1924 Fultz Daruma was crossed with Turkey Red (Inazuku1971). Pedigree lines were then transferred to the Konosu Branch Station, Saitama Prefecture where an F_2 plant was selected in 1926 and then sent to the Iwate-Ken Prefectural Station, in northeast Japan where the F_3 was grown and final selections of this lineage were made. The final selection was released in 1935 and named Norin 10.

The term Norin is an acronym of the first letter of each word of the Japanese Agricultural Experiment Station as spelled out using Latin letters. The successes of Norin 10 are attributed to Mr. Gonjiro Inazuka, a Japanese wheat breeder who was chief of the Wheat Breeding Program at Iwate from 1930–1935 (Iwanaga 2009).

According to Reitz and Salmon (1968) records from the Iwate-Ken Prefectural Station show that Norin 10 was 55 cm tall – 13 cm shorter than the control variety Norin 1. Despite being seeded in rows 50 cm apart and on land that was heavily fertilized and irrigated, the plants only grew about 60 cm high and did not lodge (Reitz and Salmon 1968).

The semi-dwarf stature of the Norin 10 cultivar is controlled by the *Rht1* and *Rht2* genes (Pinthus and Levy 1983). *Rht1* (*Rht-B1b*) and *Rht2* (*Rht-D1b*) have been used extensively over the last 60 years to develop high-yielding varieties that reduce plant height and resist lodging. The *Rht* genes produce shorter cultivars by 'decreasing the sensitivity of reproductive and somatic tissues to endogenous gibberellin' (Sial et al. 2002) which results in decreased internode length and reduced plant height.

From Norin 10 to the Green Revolution

Norin 10 began to attract international attention after a visit by Salmon SD, a renowned wheat breeder in the U.S. Department of Agriculture (USDA), to Morioka Agriculture Research Station in Honshu (Borojevic and Borojevic 2005). Salmon took some samples of the Norin 10 variety back to the United States, which were sent on to a joint Washington State University (WSU) /USDA project at Pullman, Washington (Reitz and Salmon 1968). At WSU in 1949, Dr. Vogel began to use these semi-dwarf varieties extensively and a series of crosses were made, including Norin 10 × Baart and Norin 10 × Brevor.

As previously mentioned, Vogel supplied the Norin 10 × Baart and Norin 10 × Brevor crosses to Dr. Borlaug and the Mexico wheat breeding program. In 1952 and 1953, the Mexican wheat program focused its efforts on tackling the problem of lodging (Borlaug 1981). With varieties such as Chapingo 52, Lerma Rojo 53 and Yaqui 50, the heavy application of nitrogen fertilizer led to severe lodging and yield loss.

After unsuccessfully screening the entire USDA World Wheat Germplasm collection to find shorter and strong varieties, Borlaug wrote to Vogel and requested seed containing the Norin 10 dwarfing genes.

Borlaug detailed in 1981 that the first attempt at incorporating the Vogel genes into the Mexican varieties failed. He attributes this lack of success to the fact that the F_3 plants produced from Dr. Vogel's seed were used as female parents and as a result were highly susceptible to rust.

The second attempt was successful and a new type of wheat with higher yield was evident in the F_1 and F_2 progeny from those crosses. So much so, that from 1957 nearly all of the Mexican wheat breeding efforts focused on the Norin 10 × Brevor derivatives.

> Not only was dwarfness of stature introduced into the crosses from the Norin 10 derivities, but also a number of other genes had been introduced, which increased the number of fertile florets per spikelet, the number of spikelets per head and the number of tillers per plant. (Norman Borlaug 1981)

At first, Borlaug encountered a number of problems with progeny from the cross Norin 10 × Brevor × Mexican varieties:

1. High degree of male sterility leading to promiscuous outcrossing
2. Poor grain that was shriveled, soft and with low gluten content
3. Susceptibility to stem and leaf rust.

The new and innovative shuttle breeding program, introduced by Borlaug, between El Batán, located at an altitude of 2,249 m in the Central Mexican highlands, and Ciudad Obregón located in the irrigated desert in the Northern Sonora Valley, meant that two generations per year could be grown to speed up selection against the above mentioned problems. The exposure of the breeding materials to contrasting locations and diverse environmental constraints also allowed Borlaug and his team to select for a broader range of disease resistances (Ortiz-Garcia et al. 2006).

By 1962, 10 years after Vogel first supplied seed of the Norin 10 Japanese semi-dwarf progeny to Borlaug, two high-yielding semi-dwarf Norin 10 derivatives, Pitic 62 and Penjamo 62, were released for commercial production (Reynolds and Borlaug 2006). As Fig. 2.2 indicates, these wheat varieties then led to a flow of other high-yielding wheat varieties, including Siete Cerros 66, which at its peak was grown on over 7 million hectares in the developing world (Reynolds and Borlaug 2006).

The Impact of the Green Revolution

Although the Green Revolution's roots were in Mexico, South Asia's abundant wheat harvests were its hallmarks and key milestones in global agricultural development.

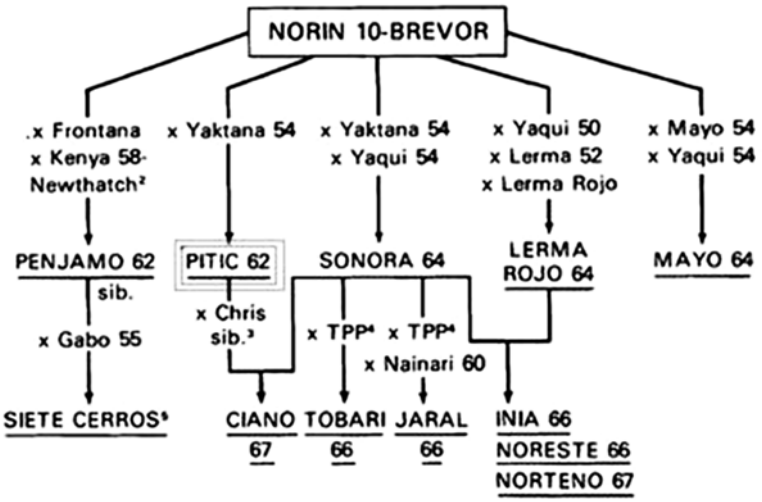

Fig. 2.2 Genealogy of early semi-dwarf CIMMYT wheat varieties (University of Minnesota 2012)

In the early 1960s South Asia was facing mass starvation and extreme food insecurity. To combat this challenge, scientists and governments in the region began assessing the value of the Mexican semi-dwarf wheat varieties for their countries. Trials in India and Pakistan were convincing and demonstrated high yields that offered the potential for a dramatic breakthrough in wheat production (CIMMYT 1989).

Borlaug's first visited the region in 1960, when he toured India and Pakistan as part of a team of FAO-Rockefeller Foundation scientists (CIMMYT 1989). Following the visit, Borlaug agreed to provide training for regional wheat scientists and in response Manzoor A. Bajwa became the first Pakistani researcher to arrive in Mexico in 1961. While working alongside Dr. Borlaug and his team in Obregon, M.A. Bajwa identified a medium-to-hard white grain line with a high gluten content that was ideal for making *chapatis* (flatbread). The new variety also showed promising resistance to rust and powdery mildew. To mark this momentous collaboration, the line was named MexiPak – to indicate that the line selection took place in Mexico by a Pakistani Researcher. In Pakistan, the name MexiPak is synonymous with the successes of the Green Revolution.

Borlaug returned to the region in 1963, at the invitation of a 38-year old Indian wheat cytogeneticist, MS Swaminathan (CIMMYT 1989). Borlaug had sent a few dozen seeds of his high-yielding, disease-resistant semi-dwarf wheat varieties to India to test their resistance to local rust strains. Swaminathan, who like many wheat breeders of the time was interested in testing the semi-dwarf wheat varieties, immediately grasped their potential for Indian agriculture, and wrote Borlaug, inviting him to India. When the unexpected invitation reached him, Borlaug soon boarded a Pan Am Boeing 707 to India.

To accelerate the potential of Borlaug's wheat, in 1967 Pakistan imported about 42,000 t of semi-dwarf varieties from Mexico – including 40,000 t of Indus-66, 1,500 t of MexiPak-65, 200 t of Sonora-64 and 20 t of Inia-66. At the time this was the largest seed purchase in the history of agriculture (CIMMYT 1989).

Wheat yield improvement in both India and Pakistan was unlike anything seen before. In just 4 years between 1963 and 1967, India's wheat harvest doubled to 20 million tons and the nation went from dependence on wheat imports to self-sufficiency. The trend has continued to recent times. In 2012, Indian farmers harvested about 95 million tons of wheat (FAO 2013). A similar effect was experienced in Pakistan. Between 1965 and 1970, wheat production in Pakistan increased from 4.6 million tons to 6.7 million tons (CIMMYT 1989). In 2012, Pakistani farmers harvested over 25 million tons of wheat (FAO 2013).

CIMMYT Today

In spite of these dramatic impacts the world is still facing enormous challenges in wheat production and we must devise new ways to feed a growing population, with less land, less water and under more difficult circumstances than ever. Production must keep up with increasing demands from population growth and changing diets. Extreme weather events, land scarcity, decreasing groundwater supplies, soil degradation, the impact of diseases, pests and weeds, and a decline in yield improvement are challenges facing global wheat food security.

CIMMYT's Global Wheat Program and the CGIAR Research Program on WHEAT are working to boost farm-level wheat productivity while fortifying wheat's resistance to important diseases and pests and to enhance its adaption to climate change. To meet these challenges CIMMYT is working with national agricultural institutions and the private sector to help develop and apply all appropriate technologies, including molecular breeding, biotechnology, precision agronomy, conservation agriculture and cellphone-based decision support tools.

CIMMYT is leading a global affiliation of top-flight scientists that aims to increase wheat's genetic yield potential by 50 %. Approaches being pursued include:

1. Improving photosynthesis to increase total biomass.
2. Improving the partitioning of photosynthates and resistance to lodging, while ensuring that extra biomass is translated to greater grain yield.
3. Stabilizing the physiological and molecular improvements which allow for the expression of agronomic traits responsible for raising wheat yield potential.

CIMMYT's Germplasm Bank constitutes an invaluable resource for wheat improvement. In the Bank, CIMMYT holds in trust one of the largest collections of wheat with 137,692 accessions (CIMMYT 2013). It is a Noah's Ark of genetic

resources for two of the planet's most important crops (maize and wheat) and CIMMYT seed is made freely available to researchers, private companies and public sector agricultural institutions around the world. The CIMMYT Germplasm Bank is one of only three gene banks globally to achieve ISO certification (and the first outside of Europe) and the only one that has global breeding programs directly attached.

The SeeD project led by CIMMYT is discovering new genes that will help increase yield and prevent crop losses from climate change or disease. Over one-third of the Bank's maize and wheat accessions (60,000) have been tested for heat and drought tolerance and the genome of more than 40,000 have been characterized. In close collaboration with Diversity Arrays Technology (DArT) and Cornell University, the SeeD project has developed a genotyping-by-sequencing (GBS) method for the molecular characterization of wheat varieties. Seed of genotyped plants is then collected and multiplied for subsequent study and use.

Conclusion

In 1935, when Gonjiro Inazuka selected the semi-dwarf that became Norin 10, little did he imagine that semi-dwarf genes would not only revolutionize the world of wheat but that they would help save more than one billion lives from hunger and starvation. His work has also improved food security for millions of resource-poor farmers in developing countries.

Accepting the Nobel Peace Prize in 1970, Dr. Norman Borlaug said that "man can and must prevent the tragedy of famine in the future instead of merely trying with pious regret to salvage the human wreckage of the famine, as he has so often done in the past."

Today, CIMMYT is building on Dr. Borlaug's vision and carrying forth groundbreaking wheat research. Through state of the art technologies and sound agronomic practices, wheat production can match demand and contribute significantly to global food security and poverty reduction.

For 50 years CIMMYT has been working to eliminate hunger and poverty. Humanity has the creative potential to eliminate hunger and malnutrition in spite of climate change and rising demand, but we need research investment to empower agricultural systems and farmers. The goal is not simply to avoid another food crisis. The goal is to grow enough food sustainably and efficiently to feed the planet.

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Part II
Germplasm and Genetic Diversity

Chapter 3
Genetic Resources of *Triticum*

Karl Hammer and Helmut Knüpffer

Abstract The political framework and the development of molecular biology and electronic data management caused a general paradigm shift in plant genetic resources (PGR), exemplified here for wheat. (1) *In situ* versus *ex situ* maintenance of PGR. *Ex situ* maintenance lost predominance. Wild wheats are effectively maintained in the wild; landraces do well on farm. New methods did not lead to the expected progress. (2) Inclusion of neglected and underutilized crop species. Some species are probably extinct in traditional cultivation areas, whereas landraces were recently found for others. Wild relatives have gained importance in wheat breeding: besides wild *Triticum* species, also *Aegilops*, *Secale*, *Hordeum* and other genera are used. ×*Triticosecale* reached world importance; ×*Tritordeum* will follow soon. (3) Methods of analysing diversity within and between taxa. New technology yields new insights in the structure and evolution of populations. (4) Genetic erosion is a problem, also inside genebanks. (5) Landraces show complex morphological diversity. Infraspecific classification systems are useful for their characterization and handling, but less recognised by breeders. (6) Methods of evaluation. Molecular markers identify genetic differences on a fairly simple level without reference to ecological adaptation. (7) Genebanks should expand classical evaluation programmes. Pre-breeding will gain importance. (8) Storage and reproduction in genebanks is done effectively and cost-efficiently under long-term conditions, but strategic concepts for reproduction are needed. Traditional methods are often neglected, and modern possibilities over-emphasized. Maintenance of landraces in genebanks and on farm poses challenges. PGR work is conservative. Landraces can be studied by traditional methods; molecular methods can resolve specific questions.

Keywords Crop wild relatives • Diversity • *Ex situ* • Evaluation • *In situ* • Landraces • Neglected crops • Paradigm shift • Wheat

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Introduction

The importance of wheat as a world crop is reflected by the large amount of wheat accessions in the world's genebanks. The FAO (2010) estimated that wheat has the largest number of accessions (856,000), followed by rice (774,000) and barley (467,000). An earlier count (Knüpffer 2009) yielded 732,000 wheat accessions. Large collections have been brought together, especially during the period of the Plant Genetic Resources Movement, described by Pistorius (1997) for ca. 1960–1990. A prominent figure in this Movement has been Erna Bennett (Hanelt et al. 2012) who organised the First Technical Conference on Plant Genetic Resources (Bennett 1967), along with Sir Otto Frankel, Jack Harlan, and Jack Hawkes.

In the beginning of the 1990s, a general paradigm shift (Hammer 2003) was observed in the discipline of plant genetic resources (PGR). The "Convention on Biological Diversity" (CBD 1992) substituted and partly replaced an earlier (1983) instrument, the "International Undertaking on Plant Genetic Resources for Food and Agriculture" of the FAO. A harmonization process between both agreements resulted in the "International Treaty on Plant Genetic Resources" (FAO 2001), still in a state of needing improvements (Moore and Tymowski 2005). Different constituents of plant biodiversity were named differently and, accordingly, treated differently.

Apart from this political framework, a second challenge for PGR arose from the rapid development of molecular biology and of electronic data documentation, management and exchange.

The political and scientific processes together led to a general paradigm shift in PGR, which is here exemplified for wheat.

Paradigm Shifts

In Situ *as Opposed to* Ex Situ *Maintenance of PGR*

The *ex situ* maintenance in genebanks lost its predominance (Brush 2000). For wild wheats, the *in situ* approach has definite advantages. But also for landraces, on-farm maintenance is increasingly being proposed, particularly in their areas of high diversity (Vavilovian gene centres). Since such areas sometimes are suffering from political instability, a loss of genetic resources of wheat is possible. A complementary consideration of the different levels of diversity (infraspecific, species, and ecosystem diversity) is necessary. Wild wheats can be most effectively maintained and protected in the wild, whereas landraces do well on farm, but only if farmers are interested and have the possibilities to take care of them. Here, the methods are still developing but did not lead to the expected progress.

Another shift took place from emphasis on collecting and rescuing landraces and crop wild relatives (CWR), to emphasis on their preservation, evaluation and utilization. Some genebanks still continue collecting, because of the threats of genetic erosion and the expected loss of valuable material for future breeding and utilization. Genetic erosion was an important argument for the Plant Genetic Resources Movement.

Inclusion of Neglected and Underutilized Cultivated Plants

Their importance has been highlighted by Padulosi et al. (2012). Apart from *Triticum aestivum* L., *T. compactum* Host, *T. durum* Desf., and *T. turgidum* L., all other domesticated wheat species can be considered rare, perhaps with the exception of *T. turanicum* Jakubz. ("Kamut") and *T. polonicum* L. with a slightly increasing area of cultivation because of their larger grains for improved and new bakery products. Some wheat species are probably extinct in their traditional cultivation areas, such as *T. ispahanicum* Heslot first described by Heslot (1958), also reported by Kihara's expedition (Kihara et al. 1965) and Kuckuck's FAO missions in 1952–54 (Kuckuck and Schiemann 1957), but later not found again in Iran despite intensive searches (Damania et al. 1993; Khoshbakht and Hammer 2010); *T. jakubzineri* (Udachin et Shakhm.) Udachin et Shakhm., *T. karamyschevii* Nevski, *T. macha* Dekapr. et Menabde, *T. parvicoccum* Kislev, *T. timopheevii* (Zhuk.) Zhuk., and *T. zhukovskyi* Menabde et Ericzjan. Most of them are, however, maintained in genebanks (Table 3.1). Landraces were recently found (re-discovered) for *T. sphaerococcum* Percival in India (Mori et al. 2013), *T. aethiopicum* Jakubz. in Yemen, Oman, and Egypt, *T. dicoccon* Schrank, and *T. monococcum* L.

We want to provide two examples. In Italy from 1980 on, PGR have been collected every year in a collaborative programme between the genebanks of Gatersleben and Bari. In Basilicata province, P. Perrino and K. Hammer found relics

Table 3.1 Total number of accessions of some rare cultivated *Triticum* species in genebanks, and the number of genebanks preserving them

Taxon	Accessions	Genebanks
Triticum aethiopicum	909	17
T. dicoccon	4,775	52
T. ispahanicum	53	16
T. jakubzineri	5	5
T. karamyschevii	71	25
T. macha	232	28
T. monococcum	5,367	54
T. timopheevii	590	37
T. zhukovskyi	64	22

After Knüpffer 2009; Table 5

of emmer and einkorn cultivation (Perrino et al. 1981), which had been considered extinct in Italy. This encouraged other Italian researchers to successfully look for these species, as well as *T. spelta* L., in other parts of Italy (e.g. Laghetti et al. 2009). Discoveries of relic cultivation of hulled wheat species in other European countries and beyond led to a workshop on "Hulled Wheats" in Italy (Padulosi et al. 1996). Since that time, the scientific interest in traditional hulled wheats is unbroken. Their cultivation is gradually increasing.

In the 1930s, *T. aethiopicum* was described as a new species from Ethiopia and Yemen by Vavilov and co-workers. This wheat is recognized as a good species in Flora Ethiopica (Phillips 1995), contrary to other treatments proposing infraspecific recognition at the best. *Triticum aethiopicum* is not yet fully understood, being related with *T. durum* and *T. turgidum*. A large proportion of the wheats grown in Ethiopia still belong to this species (Teklu and Hammer 2006). It was also found in cultivation in Oman (Hammer et al. 2009) and in Egypt, concluding from herbarium sheets in the Vavilov Institute (St. Petersburg) that were re-classified as *T. aethiopicum* (Gowayed 2009). The variable landraces still present in Oman (often mixtures of *T. aethiopicum*, *T. compactum*, *T. aestivum, T. durum*, and *T. turgidum*) and in Ethiopia deserve our special consideration.

Crop wild relatives, i.e. *T. urartu* Thumanjan ex Gandilyan, *T. boeoticum* Boiss., *T. dicoccoides* (Körn. ex Asch. et Graebn.) Körn. ex Schweinf., and *T. araraticum* Jakubz., have gained importance. This is in agreement with increasing priority attributed to CWR (cf. Maxted et al. 2008). – *Aegilops* (Kilian et al. 2011), *Secale*, *Hordeum* and many other genera of the Hordeeae (formerly Triticeae) are increasingly being used for improving yield, adaptation and quality characters in wheat. The diagram (Fig. 3.1) by Bothmer et al. (1992) today needs revision and amplification. ×*Triticosecale* Wittm. (triticale) has already reached world importance (Hammer et al. 2011). ×*Tritordeum* Asch. et Graebn. (Martín et al. 1999) will follow soon. Other grass genera have been included in crossing experiments.

Methods of Analysing Diversity Within and Between Taxa

New technologies are rapidly developing and increasingly provide results towards the status and evolution of populations. Heterogeneity and heterozygosity have characteristic functions inside the genetic structure of populations. Genetic erosion is a specific problem, also inside genebanks: collected samples may be lost during maintenance in genebanks, and the allelic composition of populations may change. In the last 20 years, landraces have gained new interest as sources for extended variation (Zeven 1998). They are usually characterized by complex morphological diversity. For such variation, diagnostic infraspecific classifications have been used (e.g. Percival 1921; Mansfeld 1951; Dorofeev et al. 1979), and they proved useful for characterizing and handling landraces. For example, Dorofeev et al. (1979) (Table 3.2) recognises 27 species with 17 subspecies, 32 convarieties and 1,055 botanical varieties (Knüpffer et al. 2013). If infraspecific forms are not named and

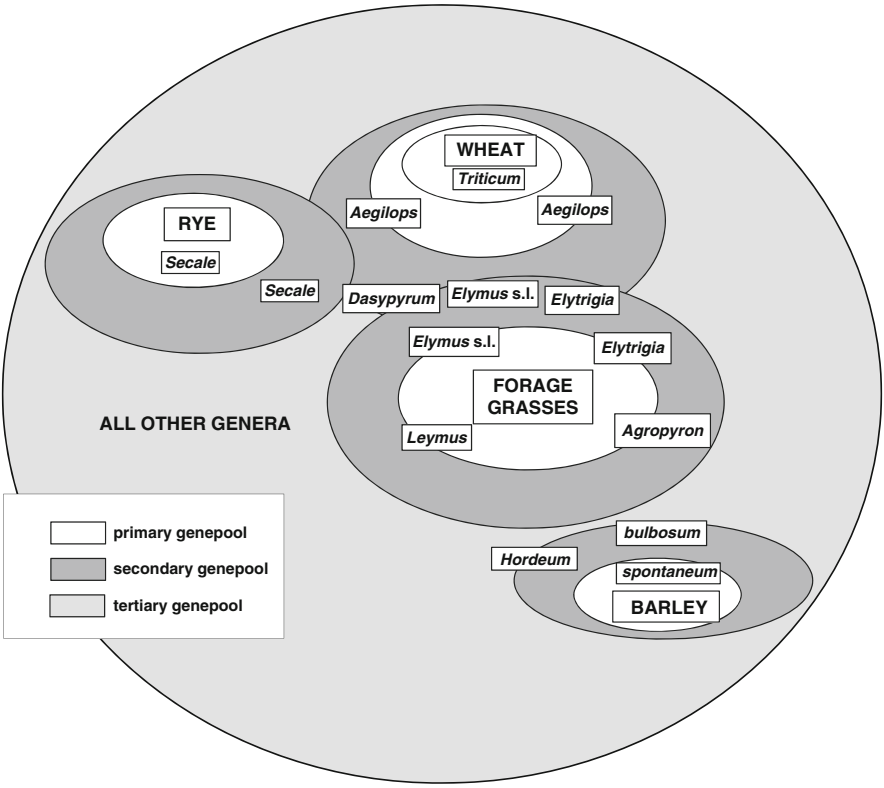

Fig. 3.1 Genepools in Hordeeae (formerly Triticeae) (After Bothmer et al. 1992)

described systematically, their diversity is at risk of being lost. Modern cultivars usually show only few morphologically discernible variants, since breeders selected only a fragment from the previously existing diversity, and, therefore, they do not see the need for traditional classification systems using botanical varieties. Scholz (2008), for example, observed that in *T. aestivum* only a single botanical variety, var. *lutescens* (Alef.) Mansf., is still present in modern cultivars, with very few exceptions.

Methods of Evaluation

Molecular markers in the form of DNA segments, even if they do not always represent functional genes, are used to identify genetic differences on a fairly simple level without reference to ecological adaptation. Traditionally many other evaluations are carried out in the breeding process. Genebanks should increase or newly

Table 3.2 Classification of *Triticum* according to Dorofeev et al. (1979), with minor changes. Authors of scientific names omitted

Subgenus	Section	Species group	Species	2n	Genome	Different genomes
Triticum	*Urartu*	Small spelts	*T. urartu*	14	A^u	1
	Dicoccoidea	Emmer wheats	*T. dicoccoides*	28	A^uB	2
			T. dicoccon	28		2
			T. karamyschevii	28		2
			T. ispahanicum	28		2
		Naked tetraploids	*T. turgidum*	28	A^uB	2
			T. jakubzineri	28		2
			T. durum	28		2
			T. turanicum	28		2
			T. polonicum	28		2
			T. aethiopicum	28		2
			T. carthlicum	28		2
	Triticum	Spelt wheats	*T. macha*	42	A^uBD	3
			T. spelta	42		3
			T. vavilovii	42		3
		Naked hexaploids	*T. compactum*	42	A^uBD	3
			T. aestivum	42		3
			T. sphaerococcum	42		3
			T. petropavlovskyi	42		3
Boeoticum	*Monococcon*	Small spelts	*T. boeoticum*	14	A^b	1
			T. monococcum	14		1
		Naked diploid	*T. sinskajae*	14	A^b	1
	Timopheevii	Emmer wheats	*T. araraticum*	28	A^bG	2
			T. timopheevii	28		2
			T. zhukovskyi	42	A^bA^bG	2
		Naked tetraploid	*T. militinae*	28	A^bG	2
	Kiharae	Spelt wheat	*T. kiharae*	42	A^bGD	3

establish the classical evaluation programmes. Screenings for disease resistance or reaction to abiotic stresses have been carried out in Gatersleben for long time (e.g. Nover 1962 and other publications listed by Hammer et al. 1994; Börner et al. 2006). Pre-breeding (also called germplasm enhancement) will gain importance. It is necessary to bridge the gap between geneticists (aiming at excellent research and high-ranking publications), breeders (aiming at developing new cultivars), and genebanks (aiming at conserving the existing diversity). None of them has the capacity to do pre-breeding alone. Only a combination of efforts developed by all three players can help overcoming this situation.

Storage and Reproduction in Genebanks

Plant genetic resources are usually preserved in genebanks effectively and cost-efficiently under long-term conditions, although the mutation rate may increase during storage, leading to genetic changes (Stubbe 1937). However, strategic concepts are needed for reproduction. This seemingly simple procedure is full of problems and needs higher scientific and technical inputs. For example, genebanks as a rule cannot provide sufficient seed for immediate use of accessions in experiments on larger plots. Perhaps this problem is closely related to pre-breeding.

Outlook

As is the case with all major methodological and technological changes, it is dangerous to neglect the repertoire of methods formerly used and to over-emphasize modern technologies. Landraces of crops are a challenge for maintaining in genebanks and on farm (Maxted et al. 2008). In genebanks, initially diverse landraces may lose rare alleles, due to reproduction and storage conditions, but hundreds and thousands of landraces cannot be efficiently maintained alone on-farm in their regions of origin; costs and logistics requirements are prohibitively high. The historical background and evolutionary history of landraces can be investigated in a first step by using traditional methods. Landraces show the structures for which the traditional methods have been developed. The work with PGR is conservative because we have the task to conserve them. The subsequent examination with the help of molecular methods can resolve specific questions in a satisfactory and meaningful fashion.

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Chapter 4
Development of Core Set of Wheat (*Triticum spp.*) Germplasm Conserved in the National Genebank in India

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Abstract Plant genetic resources, the source of genetic diversity provides a broad genetic foundation for plant breeding and genetic research, however, large germplasm resources are difficult to preserve, evaluate and use. Construction of core and mini core collections is an efficient method for managing genetic resources and undertaking intensive surveys of natural variation, including the phenotyping of complex traits and genotyping of DNA polymorphisms allowing more efficient utilization of genetic resources. A mega characterization and evaluation programme of the entire cultivated gene pool of wheat conserved in the National Genebank, India was undertaken. Wheat accessions with limited seed quantity, were multiplied in the off-season nursery at IARI Regional Station, Wellington during rainy season 2011 and the entire set of 22,469 wheat accessions were characterized and evaluated at CCS HAU, Hisar, Haryana during winter season 2011–12 for 34 characters including 22 highly heritable qualitative, and 12 quantitative parameters. The core sets were developed using PowerCore Software with stepwise approach and grouping method and validated using Shannon-Diversity Index and summary statistics. Based on Shannon-Diversity index, PowerCore with stepwise approach was found better than PowerCore with grouping. The core set included 2,208 accessions comprising 1,770 *T. aestivum*, 386 *T. durum*, and 52 *T. dicoccum* accessions as a representative of the total diversity recorded in the wheat germplasm. The core set developed will

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be further validated at different agro-climatic conditions and will be utilized for development of mini core set to enhance the utilization by wheat researchers and development of climate resilient improved varieties.

Keywords Characterization • Core collection • Genebank • Germplasm • PowerCore • *Triticum* • Wheat

Introduction

Wheat (*Triticum aestivum* L.) is the most widely cultivated food crop worldwide with an area of 220.39 million hectares and production of 704.08 million tonnes reported during 2011–12 (FAOSTAT 2012). In India, it is the second most important staple food crop after rice, grown in an area of 29.90 million hectares with a total production of 94.88 million tonnes and productivity of 3,140 kg/ha in 2011–12 (DWR Annual Report 2013). The Indo-Gangetic plains comprising the states of Punjab, Haryana, Uttar Pradesh and Rajasthan together account for nearly 85 % of total wheat production in the country. India is probably one of the few countries in the world where three wheat types namely *T. aestivum*, *T. durum* Desf., and *T. dicoccum* Schuebl. are grown although the major area (90 %) is under bread wheat (*T. aestivum*). Bread wheat is grown in all the wheat growing areas while durum wheat is largely grown in Central and Peninsular India mostly under rainfed conditions. In recent years, semidwarf durum wheat varieties have also become popular in Northern India, particularly in Punjab and Haryana. The dicoccum wheat is grown in Maharashtra and Karnataka on an area of about 0.5 million hectares.

Wheat originated in the Fertile Crescent area of south-western Asia among the first domesticated food crops around 8,000 years ago. The north-western end of Indian subcontinent, the fold between Hindukush and Himalaya is regarded as the secondary centre of origin of hexaploid wheat (Vavilov 1926). Archaeological records from many parts of India also revealed cultivation of wheat since the Harappan period (2300–1750 B.C.).

Abundant plant germplasm resources, a rich source of genetic diversity provides a broad genetic foundation for plant breeding and genetic research. However, large germplasm resources are also difficult to preserve, evaluate and use (Holden 1984). Therefore, establishing a core collection (CC) is a favored approach for the efficient exploration and utilization of novel variation in genetic resources (Hodgkin et al. 1995; Zhang et al. 2011). The concept of a CC was first proposed by Frankel (1984) and later developed by Brown (1989a, b). Frankel (1984) defined a core collection as a limited set of accessions representing, maximum diversity with minimum

repetitiveness, the genetic diversity of a crop species and its wild relatives. The core collection could serve as a working collection which could be extensively evaluated. It involves the selection of a subset from the whole germplasm by certain methods in order to capture the maximum genetic diversity of the whole collection while minimizing accessions and redundancy. Frankel and Brown (1984) and Brown (1989a, b) developed this proposal further and described methods to select a core subset using information on the origin and characteristics of the accessions. In developing the core collection, the first issue was its size, second, the grouping of accessions in the entire collection and third, the number of accessions to be selected from a group and fourth the sampling theory. Brown (1989a) using sampling theory of selectively neutral alleles, argued that the entries in a core subset should be ~10 % of the total collection with a ceiling of 3,000 per species. This level of sampling is effective in retaining 70 % of the alleles of the entire collection. The hierarchy of grouping begins with the classification suggested by taxonomy (species, subspecies, and races) followed by assigning accessions to major geographic groups (country, state), climate, or agro-ecological regions. The clustering within the broad geographic group could be done to sort accessions into clusters. A germplasm collection with abundant discriminating data would require multivariate clustering to form groups of similar accessions (Zeuli and Qualset 1993). The number of accessions selected from each class will depend on the sampling strategy used. A good core set should capture maximum genetic diversity with a minimal number of genotypically redundant entries and should be small. Brown (1989a) proposed three procedures based on groups sizes, constant (C), proportional (P) and logarithmic strategies (L). Subsequently, Franco et al. (2005, 2006) proposed that efficiency of sampling for allocation of accessions to different groups could be improved by using diversity- dependent (G) strategy. Of the four strategies, strategy G was reported superior to P strategy (Hodgkin et al. 1999; Yonezawa et al. 1995). Since the original concept of Frankel (1984), core collections have been established in many crop species.

The National Genebank of India currently conserves 31,007 accessions of wheat germplasm comprising 19,116 indigenous and 11,891 accessions of exotic origin. However, the available diversity has not been adequately evaluated and extensively used in wheat improvement due to the large size of germplasm collection. Proper evaluation is feasible only for the traits which can be scored easily and do not show genotype by environment (G x E) interactions. Recognizing this, the present study was aimed to develop the core collection of cultivated wheat germplasm conserved in the National Genebank (NGB) based on characterization and preliminary evaluation data at one representative site with a view to reduce the genebank collection to a manageable level for facilitating utilization of germplasm in applied research.

Material and Methods

Experimental Site and Material

The experiment was conducted during winter season 2011–12 at CCS HAU, Hisar, located at 29°–10' N latitude, 75°–46' E longitude and an elevation of 215.2 m asl. The soils were sandy loam having pH range of 7.5–8.0. The study material included entire gene bank holding of cultivated wheat accessions. For the purpose of core set development, bread wheat (*T. aestivum*), durum wheat (*T. durum*), and emmer wheat (*T. dicoccum*) were grown for agronomic characterization. A set of 22, 663 accession of wheat were grown in Augmented Block Design (Federer 1956) with 8 checks representing different species viz, C 306, PBW 343, DBW 17, RAJ 3765, DWR 1006, UAS 415, DDK 1025, and DDK 1029. The checks were replicated in each of the 114 blocks of 200 accessions each. Each accession was grown in three rows of 2 m length and plant to plant spacing of 25 cm. Standard agronomic practices were followed to raise a healthy crop.

Traits Studied

All the accessions were characterized for 34 important traits, 22 qualitative and 12 quantitative, as outlined by NBPGR minimal descriptors and complete set of observation were recorded for 22,469 accessions. The qualitative characters included early growth vigour (EGV), growth habit (GH), flag leaf angle (FLA), foliage colour (FC), waxiness on leaf blade (WLB), waxiness on leaf sheath (WLS), waxiness on peduncle (WP), waxiness on spike (WS), glume pubescence (GP), auricle colour (AC), auricle pubescence (AP), awnedness (WA), awn length (AL), awn colour (AC), glume colour (GC), spike shape (SS), spike colour (SC), spike density (SD), grain colour (GC), grain shape (GS), grain texture (GT) and grain width (GW). The quantitative traits included, days to 75 % spike emergence (SE), days to 90 % maturity (DM), plant height (PH), effective tillers per plant (EF_T), spike length (SL), number of spikelets per spike (SLS), no. of grains per spike (GRS), grain weight per spike (GRW), 1,000 grain weight (TGRW), dry matter yield per m row length (DMY), grain yield of 1 m row length (GY) and harvest index (HI).

Statistical Analysis

The "PowerCore" (http://genebank.rda.go.kr/powercore/) software developed by the Rural Development Administration (RDA), South Korea, that uses the advanced M (maximum) strategy with a heuristic search for establishing core sets possessing

the power to represent all alleles or classes, was used in the present study. It creates subsets representing all alleles or observation classes, with the least allelic redundancy, and ensures a highly reproducible list of entries. This approach has recently been used in developing core set from large rice and foxtail millet collection (Chung et al. 2009; Gowda et al. 2013). It effectively simplifies the generation process of a core set while significantly cutting down the number of core entries, maintaining 100 % of the diversity as categorical variables. Core collections are considered to represent the genetic diversity of the initial collection if the following two criteria are met: (1) no more than 20 % of the traits have different means (significant at $\alpha = 0.05$) between the core collection and the entire collection and (2) Coincidence Rate (CR) is retained by the core collection in no less than 80 % of the traits (Hu et al. 2000). The design, concept and implementation strategy of "PowerCore" and the validation on the outcome in comparison with other methods have been well described by Kim et al. (2007). PowerCore by default classifies the continuous variables into different categories based on Sturges rule (Sturges 1926), which is described as: $K = 1 + \log 2\, n$, where n = number of observed accessions. However, the software also allows modification of this rule to make desired number of classes for the continuous variables. Once classification of the continuous variables is performed, the software takes into account all classes, without omission of any of its variables. It thus, possesses the capability to cover all the distribution ranges of each class.

Results and Discussion

Genebank Material

Characterization of 22,469 wheat accessions revealed skewed distribution for certain qualitative as well as quantitative characters. Among the qualitative traits the gene bank accessions were skewed for absence of glume pubescence, presence of awns, straw coloured awns, white glume colour and tapering spike shape. Among the quantitative characters, the skewness was observed for traits such as grain length (GL) and grain width (GW) that exhibited highly biased distributions.

Core Set Development

Many approaches for selecting core collections have been proposed and used e.g. M-Strat (Gouesnard et al. 2001), Genetic distance sampling (Jansen and Van Hintum 2007), Power Core (Kim et al. 2007) and Core Hunter (Thachuk et al. 2009). Similarly core has been developed using several kinds of data ranging from

Table 4.1 Species wise description of the core collections developed by different approaches out of entire wheat collection

Species	No. of accessions			
	Entire	Core-P	Core-G	Core-M
T. aestivum	18,101	64	1,215	1,770
T. durum	3,871	53	489	386
T. dicoccum	497	31	209	52
Total	22,469	148	1,914	2,208

Core-P core developed by Powercore default approach, *Core-PG* core developed by Powercore with grouping approach, *Core-PM* core developed by Powercore with modified stepwise approach

genealogical data in the Czech spring wheat (Stehno et al. 2006), agronomic data in groundnut (Upadhyaya 2003; Upadhyaya et al. 2003) and molecular data or integration of data in bread wheat (Balfourier et al. 2007) and in rice (Borba et al. 2009; Yan et al. 2007). PowerCore is a new and a faster approach for developing core collection, which effectively simplifies the generation process of a core set with reduced number of core entries but maintaining high percent of diversity compared to other methods used. In this study, core set was developed with agronomic traits using power core with some modifications. The PowerCore could produce only 64 accessions out of entire wheat accessions (22,469) with default programme without any manual classification and forced selection of entry into the core. Therefore, a modified strategy was followed to make around 8–10 % of entire collection including maximum diversity and minimum redundancy. The method was stepwise random selection using PowerCore with cut-off fixed at around 10 %. With this strategy the core set of 2,208 accessions was developed comprising 1,770 *T. aestivum*, 386 *T. durum*, and 52 *T. dicoccum* accessions (Table 4.1).

Evaluation of Core

Evaluation of core was done by comparing with the other approach, classification and grouping of wheat accessions based on passport data and geographical information (stratified random sampling). The accessions without passport data were classified by hierarchical method of clustering using Euclidean distance and Ward's clustering method. Subsequently all the groups were analysed using PowerCore and then the selected accessions were merged to make the core collection. PowerCore successfully selected 1,914 accessions of the entire wheat germplasm. This consisted 1,215, 489, and 209 accessions of *T. aestivum*, *T. durum*, and *T. dicoccum*, respectively (Table 4.1).

Validation of Core

The core sets developed by three strategies [i.e. species-specific PowerCore (Core P), modified PowerCore (Core PM) and PowerCore involving stratified random sampling based on passport and clustering (Core PG)] were validated by different criteria based on summary of statistics. Means of the entire collection and core subset were compared using Newman-Keul's procedure (Newman 1939; Keuls 1952) for the 12 traits. The homogeneity of variances of the entire collection and core subset was tested with the Levene's test (Levene 1960). It is worth noting that the HCC method gave the same range, minimum and maximum values for the core set generated and the entire collection, indicating its capability to capture almost all of the existing variations. In order to compare the efficiency of "PowerCore" for developing core collection with modified stepwise method and PowerCore with grouping approach method, mean and statistical parameters for entire population, core developed using "PowerCore" and core developed using PowerCore with modified strategy of stepwise method and PowerCore with grouping were compared. The results showed that there was no significant difference ($\alpha = 0.05$) for the means of all traits between core and entire collections. The variances of the entire collection and core subset were homogeneous only for five traits viz. days to maturity, plant height, grains per spike, biomass and harvest index. The reason might be due to the large number of germplasm in the entire collection in comparison to that of the core collection. The range of the characters was the same in the entire collection as well as in the core collection implying that the core captured extreme diversity of the total collection (Table 4.2). Four statistical parameters viz., MD (%), VD (%), CR (%) and VR (%), were analyzed using "PowerCore" to compare the mean and variance ratio between core and entire collections. The percentage of the significant difference between the core sets and the entire collection was calculated for the mean difference percentage (MD%) and the variance difference percentage (VD%) of traits. Coincidence rate (CR%) and variable range (VR%) were estimated to evaluate the properties of the core set against the entire collection (Hu et al. 2000).

Mean Difference Percentage (MD %) – which is estimated as:

$$MD(\%) = \frac{1}{m}\sum_{j=1}^{m}\frac{Me - Mc}{Mc} \times 100$$

Where, Me = Mean of entire collection; Mc = Mean of core collection, and m = number of traits.

Variance Difference (VD %) – estimated as:

$$VD(\%) = \frac{1}{m}\sum_{j=1}^{m}\frac{Ve - Vc}{Vc} \times 100$$

Where, Ve = Variance of entire collection, Vc = Variance of core collection, and m = number of traits.

Table 4.2 Descriptive statistics for quantitative traits and their validation in entire and core collection using PowerCore-M approach

Traits	Mean		Sig*	Variance		F value	Sig**	Std. Dev.		Minimum		Maximum	
	Entire	Core-PM		Entire	Core-PM			Entire	Core-PM	Entire	Core-PM	Entire	Core-PM
SE	109.8	111.3	ns	130.8	267.6	8.77	s	11.44	16.36	54	54	164	164. 00
DM	146.5	147.4	ns	81.66	141.4	5.87	ns	9.04	11.89	120	120	199	199
PH	114.9	115.8	ns	517.7	740.4	0	ns	22.75	27.21	33.5	33.5	197.8	197.8
ET	13.79	14.83	ns	14.42	37.26	70.45	s	3.8	6.1	3	3	48.4	48.4
SL	11.22	11.48	ns	4.21	8.2	11.03	s	2.05	2.86	2.72	2.72	24.6	24.6
SS	19.79	20.25	ns	5.65	14.76	51.82	s	2.38	3.84	5.4	5.4	60.4	60.4
GS	45.56	45.45	ns	131.6	276.7	0.78	ns	11.47	16.63	3.8	3.8	111.8	111.8
GW	1.79	1.88	ns	0.37	0.85	19.93	s	0.61	0.92	0.04	0.04	7.16	7.16
TW	39.81	41.93	ns	151.3	355.3	32.42	s	12.3	18.85	2.72	2.72	204.1	204.1
DM	353.4	347.4	ns	11,120	20,376	6.08	ns	105.5	142.7	20	20	1,042	1,042
SY	91.06	88.71	ns	1,463	2,578	9.8	s	38.26	50.77	1.33	1.33	283.2	283.2
HI	23.92	24.44	ns	93.37	156.8	1.76	ns	9.66	12.52	0.81	1.17	58.34	58.15

Core-PM core developed by Powercore with modified stepwise approach
*Significant at 5 % level, **Significant at 1 % level

Coincidence rate (CR %) – estimated as:

$$CR(\%) = \frac{1}{m}\sum_{j=1}^{m}\frac{Rc}{Re}\times 100$$

Where, Re = Range of entire collection, Rc = Range of core collection, and m = number of traits.

CR% indicates whether the distribution ranges of each variable in the core set are well represented.

Variable rate of CV (VR %) – estimated as:

$$VR(\%) = \frac{1}{m}\sum_{j=1}^{m}\frac{CVc}{CVe}\times 100$$

Where, CV_e = Coefficient of variation of entire collection, CV_c = Coefficient of variation of core collection, and m = number of traits.

VR% allows a comparison between the coefficient of variation values existing in the core collections and the entire collections, and determines how well it is being represented in the core sets.

Hu et al. (2000) reported that an MD% smaller than 20 %, in his case 10.07 %, effectively represented the entire collection. The high value obtained for coincidence rate (CR) percentage (95.57 %) suggests that the core attained using the HCC method could be adopted as a representative of the whole collection. In this case, the estimated value for MD% was −6.25, which indicated that there is no difference in the mean values of entire and core collections. VD% was estimated to be 49.04, indicating that the variance for the entire and the core populations are not the same. The CR% obtained was 96.06 which suggests that the core has captured all accessions from all the classes and, thus, is a representative of the entire collection. High VR% (53.87) indicated that the coefficient of variation in the core set is higher compared to entire collections for all the variables. The coefficient of variance in core developed using PowerCore was highest in the case of PowerCore with grouping followed by PowerCore with modified approach and entire collection for all the descriptors. The histogram comparing CV for the entire and core sets is shown in Fig. 4.1. High value obtained for CR% (96.06) suggests that the core obtained using the heuristic approach method could be adopted as a representative of the whole collection.

Shannon-Weaver Diversity Index

The descriptor and descriptor states are parallel to the locus and alleles, respectively, in morphological evaluation. Allelic evenness and allelic richness are the most commonly used parameters for measuring diversity. The allelic evenness in

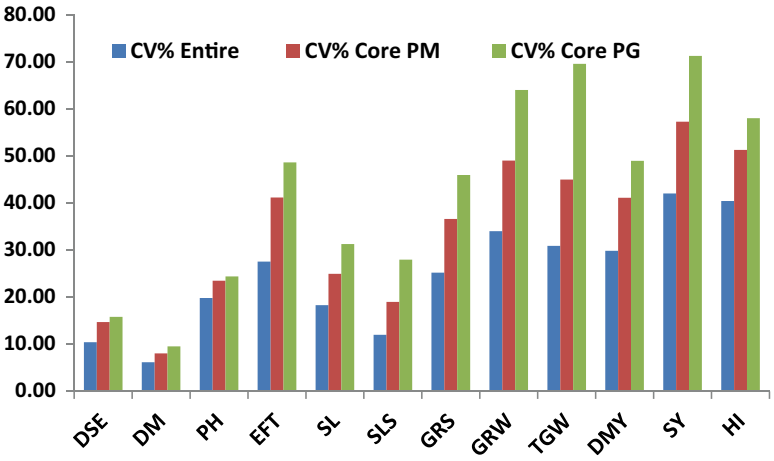

Fig. 4.1 Coefficient of variation (%) in entire, modified core (Core-PM) and group based core collection (Core-PG) for different traits. *DSE* days to 75 % spike emergence, *DM* days to 90 % maturity, *PH* plant height, *EFT* effective tillers per plant, *SL* spike length, *SLS* spikelets per spike, *GRS* grains per spike, *GRW* grain weight per spike, *TGW* 1,000 grain weight, *DMY* dry matter yield per m row length, *SY* seed yield per m row length and *HI* harvest index

this study was measured using the Shannon–Weaver diversity index, whereas the allelic richness was measured by counting the descriptor states for each descriptor without considering their individual frequencies. The Shannon-Weaver diversity index (H') was computed using the phenotypic frequencies to assess the phenotypic diversity for each character.

$$H = -\sum_{i=1}^{n} p_i \cdot \ln p_i$$

where pi is the proportion of accessions in the ith class of an n-class character and n is the number of phenotypic classes for a character. A comparison of Shannon-Weaver (Shannon and Weaver 1949) diversity index for the entire collection, core developed using PowerCore, core developed using modified power core with stepwise approach and PowerCore with clustering method also indicated a high diversity for all the quantitative traits in core developed using PowerCore-M compared to core developed using PowerCore-G approach, except for a few variables, where it was observed at par (Fig. 4.2).

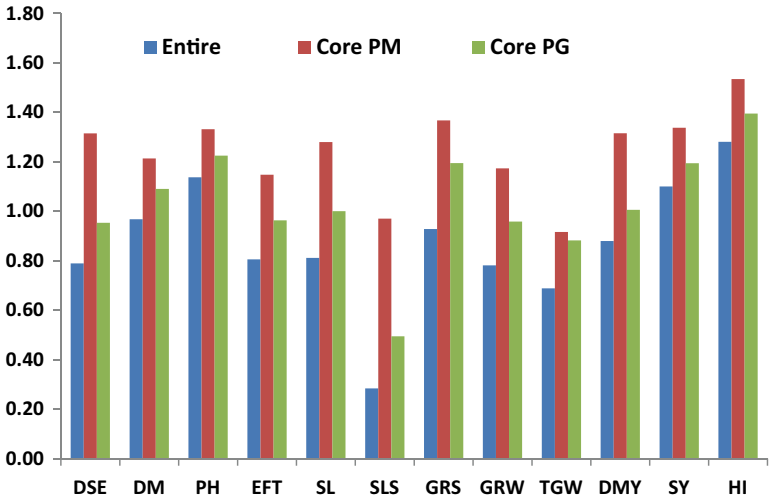

Fig. 4.2 Validation of modified core (Core-PM) and group based core collection (Core-PG) in comparison to entire collection by Shannon diversity index for quantitative traits (traits same as given in Fig. 4.1)

Conclusions

PowerCore is a new and faster approach for developing core collection, which effectively simplifies the generation process of a core set with reduced number of core entries while maintaining high percent of diversity compared to other methods used. Using PowerCore as a tool, three sets of core collections viz. Core P, Core PM and Core PG have been developed. Due to its high Shannon-diversity index, Core PM proved to be the best. These core sets can be further grown with involvement of breeders to select the genotypes with desired background suiting to their requirement. The core sets can be used as a guide for developing trait specific reference/core sets and subsequent allele mining. The best core set could be used as an initial starting material for large-scale genetic base broadening. Thus, it can be concluded that this modified heuristic algorithm can be applied for the selection of genotype data (allelic richness), the reduction of redundancy and the development of approaches for more extensive analysis in the management and utilization of large collection of plant genetic resources.

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Chapter 5
Transfer to Wheat of Potentially New Stem Rust Resistance Genes from *Aegilops speltoides*

Ian Dundas, Dawn Verlin, Peng Zhang, Yue Jin, Jacob Manisterski, and Rafiqul Islam

Abstract Stem rust resistance genes have been found in four different sources of *Aegilops speltoides*. These include diploid accessions AEG357-4 and AEG874-60 and the amphiploids Chinese Spring/*Ae. speltoides* TA8026 and TS01. Stem rust resistance was mapped to the 2S chromosomes derived from each of these lines. The previously reported 2B-2S#3 translocation derived from AEG357-4 was found to carry two stem rust resistance genes, here temporarily named *SrAes2t* and *SrAes3t*. The resistance genes found on the 2S chromosomes each derived from TA8026, TS01 and AEG874-60 are named *SrAes4t*, *SrAes5t* and *SrAes6t*, respectively. Lines carrying genes *SrAes2t* and *SrAes3t* are being distributed to wheat breeding programs around the world.

Keywords *Aegilops speltoides* • Resistance genes • Stem rust

Introduction

The fungal disease stem rust, caused by *Puccinia graminis* f. sp. *tritici*, has long been a scourge of wheat crops since the beginnings of agriculture. Uredia have even been found on ancient wheat spikelets estimated at 3,300 years old (Kislev 1982).

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Recently, the appearance and spread of mutants of the Ug99 stem rust lineage (summarized in Mago et al. 2013) has raised the awareness of the need to search for new resistance genes for use in wheat cultivars. Most of the currently named stem rust genes in wheat originate from related species (e.g., *Secale cereale*, *Triticum monococcum*, *T. turgidum*, *T. timopheevii*, *Thinopyrum ponticum*, *Aegilops speltoides*, *Ae. tauschii* and *Ae. ventricosum*). Many of these genes (e.g., *Sr2*, *Sr22, Sr24*, *Sr26*, *Sr36* and *Sr38*) are widely used in agriculture.

The diploid species *Ae. speltoides* Tausch. (2n = 2x = 14) has provided several resistance genes against stem rust, namely *Sr32*, *Sr39*, *Sr47*, *SrAes1t* and *SrAes7t* (summarized in Mago et al. 2013), and leaf rust (caused by *P. triticina*), namely *Lr28*, *Lr35*, *Lr36*, *Lr47*, *Lr51* and *Lr66* (summarized in McIntosh et al. 2013). To date, none of these genes are deployed in agriculture. This work describes the discovery of potentially new stem rust resistance genes in four different sources of *Ae. speltoides*.

Materials and Methods

Diploid accessions of *Aegilops speltoides* AEG357-4 and AEG874-60 were provided courtesy of the The Harold and Adele Lieberman Germplasm Bank, Tel Aviv University, Israel. Amphidiploids of Chinese Spring/*Ae. speltoides* TS01 and TA8026 were provided courtesy of the Weizmann Institute, Rehovot, Israel and Wheat Genetics Resource Center, Kansas State University, USA, respectively.

Diploid *Ae. speltoides* accessions were crossed with cultivar Angas (from Dr Hugh Wallwork, Urrbrae, Australia) using wheat as the female parent. F_1 seedlings were treated with 0.07 % colchicine (described in Dundas et al. 2008). Hybrids were crossed as females with Angas and later with cv. Westonia up to BC_5. The amphiploid accessions were crossed with Angas then backcrossed with Westonia as the female parent to BC_5.

Identification of backcrossed plants carrying each of the *Ae. speltoides* S-genome chromosomes using RFLP markers has been described in Dundas et al. (2008). PCR markers for 2S chromosomes were obtained from Mago et al. (2009) (Sr39#22r) and Seyfarth et al. (1999) (35R2/ BCD260F1). Backcrossed lines carrying S-genome chromosomes were tested for stem rust resistance at the University of Sydney (*Pgt* 34-1,2,3,4,5,6,7) and at Urrbrae (*Pgt* 343-1,2,3,5,6). Selected lines were screened against Ug99 races TTKSK, TTKST (+*Sr24*) and TTTSK (+*Sr36*) at the USDA Cereal Disease Laboratory, St. Paul, USA. Genomic in situ hybridization (GISH) was conducted on mitotic chromosome spreads using the procedure described in Mago et al. (2013).

Results and Discussion

Ae. speltoides **AEG357-4** Lines carrying the T2BS-2S#3S.2S#3L-2BL recombinant chromosome derived from AEG357-4 (Dundas et al. 2008) showed the loss of distal *Ae. speltoides* 2S#3 chromosome segments and the retention of proximal 2S#3 chromosome segments (Figs. 5.1a and 5.2). This line was found to be resistant to stem rust pathotypes *Pgt* 34-1,2,3,4,5,6,7, *Pgt* 343-1,2,3,5,6 (Dundas et al. 2008) and TTKST (pers. comm., Professor Z. Pretorius, University of Free State, Bloemfontein, South Africa). After crossing this recombinant line with Sears' *ph1b* mutant, we selected five lines derived from a F_3 population of 155 seedlings showing confirmed dissociation of RFLP and PCR markers specific for the 2S#3 chromosome (Fig. 5.2). All lines showed resistance to Australian stem rust pathotypes and Ug99 races TTKSK, TTKST and TTTSK (Table 5.1). The dissociation plants #3 and #20 showed only 2S#3S markers indicating that a stem rust resistance gene locates on the short arm of the 2S#3 chromosome (Fig. 5.2). Dissociation plant #27

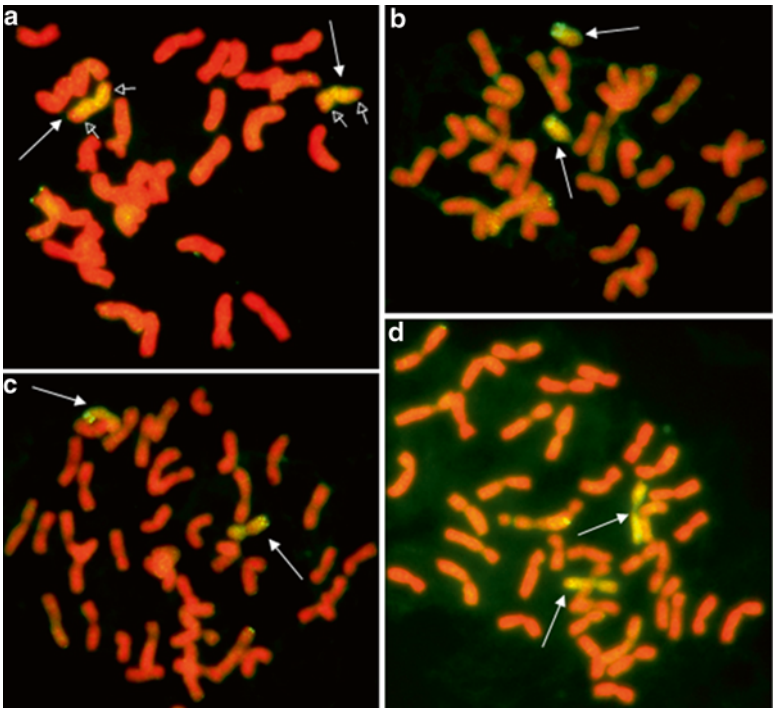

Fig. 5.1 Genomic in situ hybridization using *Aegilops speltoides* genomic DNA as probe on (**a**) T2BS-2S#3S.2S#3L-2BL translocation line, (**b**) 2S#4L ditelocentric addition line, (**c**) 2S#5 disomic addition line and (**d**) 2S#6 disomic addition line. *Solid arrows* show *Ae. speltoides* 2S chromosome segments. *Open arrows* show translocation breakpoints

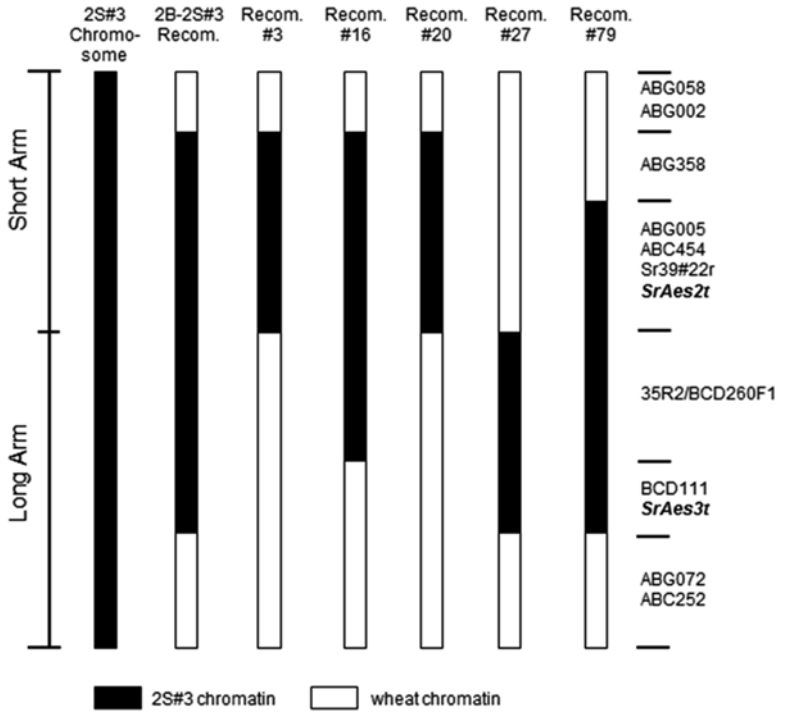

Fig. 5.2 Diagrammatic representation of wheat-chromosome 2S#3 recombinant lines showing the positions of RFLP and PCR markers and stem rust resistance genes. *Aegilops speltoides* and wheat chromatin are represented in *black* and *white*, respectively. The primary recombinant 2B-2S#3 and secondary recombinants #3, #16, #20, #27 and #79 are resistant to stem rust pathotype tested

Table 5.1 Stem rust infection types (ITs) of wheat lines carrying *Aegilops speltoides* 2S chromosomes to Ug99 pathotypes TTKSK, TTKST and TTTSK, and two Australian pathotypes

	Stem rust pathotype				
Line	TTKSK (Ug99)	TTKST (Ug99 + *Sr24*)	TTTSK (Ug99 + *Sr36*)	343-1,2, 3,5,6	34-1,2,3,4, 5,6,7
Westonia + T2B-2S#3				;	2-
Westonia + 2S#3 recomb #3	2+	22+	2	;	
Westonia + 2S#3 recomb #16	;2--	;2-	;2-	;	
Westonia + 2S#3 recomb #20	;2-	-	-	;	
Westonia + 2S#3 recomb #27	2	2-	2-	;	
Westonia + 2S#3 recomb #79	2	;2-	;2-	;	
Westonia + 2S#4 addition	;/;N	;1	;1	;	
Westonia + 2S#5 addition	;1	;/2-	;/;2-	;	
Westonia + 2S#6 addition				;	2-
Westonia	4	4	4	4	
Angas	3	3+	3+		

The 2S#3, 2S#4, 2S#5 and 2S#6 chromosomes were derived from diploid line AEG357-4, CS/*Ae. speltoides* amphiploid TA8026, CS/*Ae. speltoides* amphiploid TS01 and diploid line AEG874-60, respectively. An IT of '3' or '4' is susceptible

showed only 2S#3L markers suggesting that a stem rust resistance gene is located on the long arm of the 2S#3 chromosome (Fig. 5.2).
GISH studies on the five stem rust resistant secondary recombinants failed to reveal 2S#3 chromatin, which suggested that very small segments of 2S#3 chromatin were present on these secondary recombinants. We temporarily name the resistance gene on the short arm of the 2S#3 chromosome as *SrAes2t* and the gene on the long arm as *SrAes3t*.

Chinese Spring/*Ae. speltoides* **Amphiploid TA8026** Stem rust resistant backcrossed lines derived from TA8026 were isolated which carried only the group 2 long arm RFLP markers BCD111, ABG072 and ABC252 specific for the 2S#4 chromosome. Group 2 short arm probes ABG058, BCD221 and ABC454 did not detect the presence of 2S chromatin. GISH analysis of these lines showed the presence of either a wheat-2S#4L translocation or 2S#4L telocentric chromosome (Fig. 5.1b). The line carrying the telocentric 2S#4L chromosome was resistant to Australian and Ug99 stem rust races (Table 5.1). The stem rust resistance gene on the 2S#4L chromosome has been temporarily named *SrAes4t*.

Chinese Spring/*Ae. speltoides* **Amphiploid TS01** Backcrossed lines derived from the amphiploid TS01 were isolated which carried group 2 RFLP markers for the probes ABG002, ABC358 and ABC454, the PCR marker Sr39#22r (short arm), and RFLP markers for probes BCD111, ABG072 and PCR marker 35R2/ BCD260F1 (long arm), specific for the 2S#5 chromosome. Rust tests showed these lines to be resistant to Australian stem rust and Ug99 pathotypes (Table 5.1). GISH analysis of the line showed the presence of an apparently entire *Ae. speltoides* 2S#5 chromosome (Fig. 5.1c). The stem rust resistance gene on that chromosome is here named as *SrAes5t*.

Ae. speltoides **AEG874-60** Dundas et al. (2008) reported that the diploid accession AEG874-60 was resistant to stem rust races *Pgt* 34-2,12,13, *Pgt* 34-1,2,3,4,5,6,7 and *Pgt* 98-1,2,3,5,6. BC_5 plants with cv. Westonia were isolated carrying only the 2S#6 chromosome from AEG874-60 and carried *Ae. speltoides*-specific markers ABG058, BCD221, ABG002, ABG358, ABC454 and Sr39#22r (short arm) and BCD111, ABG072, ABC252 and 35R2/BCD260F1 (long arm). Plants with the 2S#6 chromosome were resistant to stem rust *Pgt* 34-1,2,3,4,5,6,7 and *Pgt* 343-1,2,3,5,6 (Table 5.1). GISH analysis showed an entire 2S#6 chromosome present (Fig. 5.1d). The stem rust resistance gene on that chromosome is here named as *SrAes6t*.

It is unknown if the stem rust resistance genes described herein are different from those previously reported on *Ae. speltoides* 2S chromosomes (namely *Sr32*, *SrAes1t*, *SrAes7t*, *Sr39* and *Sr47*). Lines carrying the T2BS-2S#3S.2S#3L-2BL chromosome with genes *SrAes2t* and *SrAes3t* are being distributed to many wheat breeding programs around the World.

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Chapter 6
Genetic Variation and Its Geographical Distribution in *Aegilops caudata* L.: Morphology, Hybrid Sterility and Gametocidal Genes

Shoji Ohta and Naomi Yasukawa

Abstract *Aegilops caudata* L. is a diploid wild relative of wheat with the main distribution in the northeastern Mediterranean basin from Greece to northern Iraq. Two varieties are taxonomically described in this species based on spike morphology. In the present work, to elucidate the geographical differentiation pattern of the species, the geographical distribution of the two varieties was reviewed, 35 accessions derived from the entire distribution area were crossed with the four Tester lines, two varieties derived from their sympatric stands on the Aegean Islands were crossed with each other, and their F_1, F_2 and/or BC_1F_1 populations were examined. It became clear that the present distribution area of *Ae. caudata* can be divided into the western and eastern regions with the border in the mountains lying between West Anatolia and Central Anatolia: the western and eastern accessions are isolated not only geographically but also reproductively by hybrid sterility caused by gametocidal-like genes, and the morphology of var. *typica* is controlled by two different genotypes in the western and eastern regions. It was suggested that *Ae. caudata* occurred in the two isolated refuges during the maximum glacial period, the Aegean region and the western Levant or some sheltered habitats in the East Taurus/Zagros mountains arc, and that the latter population now occurs in the eastern region while the former now occupies the western region of the distribution.

The genus *Aegilops* L. consists of about 25 species, and the morphological variation and the geographical distribution of the each species and the infraspecific taxa were described in detail in 1920s (Zhukovsky 1928; Eig 1929). And two new taxonomic systems were recently published (Hammer 1980; van Slageren 1994). However, the treatment of infraspecific taxa differed so much from author to author. Genetic reassessment of infraspecific taxa is essential to biosystematic approach to the better

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understanding of the diversity and speciation process of the each species, because some infraspecific taxa are phylogenetically significant but others are not. *Aegilops caudata* L. (syn. *Ae. markgrafii* (Greuter) Hammer) is a diploid wild relative of wheat. Two varieties are taxonomically described in this species based on spike morphology, var. *typica* with a well-developed awn on the empty glumes of only apical spikelets and var. *polyathera* Boiss. with an awn on the empty glumes of both the apical and lateral spikelets.

In the present work, to elucidate the geographical differentiation pattern of *Ae. caudata*, the geographical distribution of the two varieties was reviewed, 35 accessions derived from the entire distribution area were crossed with the four Tester lines, two varieties derived from their sympatric stands on the Aegean Islands were crossed with each other, and their F_1, F_2 and/or BC_1F_1 populations were examined.

Geographical Distribution of the Two Varieties

Ae. caudata distributes from the southern Balkan Peninsula to Afghanistan with its main distribution in the northeastern Mediterranean basin from Greece to northern Iraq (Eig 1929; Bor 1968, 1970; Tutin and Humphries 1980). Based on the geographical distribution of the two varieties described in the literature (de Halácsy 1904; Hayek 1927; Eig 1929; Rechinger 1943; Bor 1968, 1970; Tanaka 1983a) and the collection lists published by Kyoto University (Tanaka 1983b; Sakamoto 1986), the main distribution area of the species can be divided into the three geographical regions according to the geographical division by Davis (1965): (1) The Greek mainland, the Aegean Islands and West Anatolia, where the two varieties occur with the predominant occurrence of var. *typica*, and their sympatric stands are common; (2) South Anatolia, East Anatolia, Iraq and Syria, where the two varieties occur but their sympatric stands are rare; and (3) Central Anatolia, where var. *polyathera* occurs exclusively.

Hybrid Sterility

Cytogenetic Differentiation into Western and Eastern Geographical Groups

Thirty-five accessions derived from the entire distribution area were crossed with the four Tester lines whose F_1 hybrids were highly sterile (Tanaka et al. 1967; Ohta 1992). Pollen fertility in the F_1 hybrids obtained from a total of 134 cross combinations varied from 0 % to 96.3 % (Table 6.1), and it was significantly correlated with their seed fertility by open-pollination ($r = 0.786$). The fertility in the F_1 hybrids was correlated with the geographical origin of the parental accessions. Based on the

Table 6.1 Pollen fertility (%) in the F_1 hybrids between the four Testers and the 35 accessions of *Ae. caudata* from its entire distribution area (Ohta 2000)

Region	Site no.	Accession no. (KU)	Variety[a]	Tester A[b]	Tester B	Tester C	Tester D
Aegean Islands	1	12044B	*P*	0	0	0	25.0
	2	12055B	*P*	0	0	14.5	83.5
	3	12155B	*P*	0	0	0	69.1
	4	12162A	*T*	0	0	0	72.2
	5	12163A	*T*	0.1	0	0.8	96.3
	6	12073A	*T*	0	0.1	0	0
	7	12100B	*P*	0	15.1	42.6	86.3
	8	12177B	*P*	0	0.1	8.9	56.0
	9	12121A	*T*	0	0.3	0.2	0.1
West Anatolia	10	11401	*T*	0	0	13.7	68.8
	11	11403	*T*	0	No obs	22.7	8.9
	12	12169	*T*	0.8	0.2	20.3	36.9
Central Anatolia	13	5853	*P*	0	17.8	0	No obs
	14	5854	*P*	No obs	51.8	0	0
	15	5859	*P*	No obs	65.4	0	0
	16	5860	*P*	13.1	0	0	0
	17	5863	*P*	0.1	31.3	0	0
	18	5888	*P*	0.2	95.5	0	0
	19	5896	*P*	No obs	66.9	0	0
	20	12165	*P*	10.3	1.5	0	0
South Anatolia	21	5867	*P*	14.1	28.9	0	0
	22	5897	*P*	62.5	0	No obs	No obs
	23	5899	*P*	93.3	0.5	0	3.5
East Anatolia	24	5484	*P*	24.2	0	0	0
	25	5485	*T*	0	61.0	0	0
	26	5489	*T*	27.0	19.3	0	0
	27	5490	*P*	69.7	9.4	0	0
	28	5491	*P*	95.3	17.2	0	0
	29	5492	*P*	75.4	19.1	0	0
	30	5885	*T*	65.9	11.9	0	0
	31	5889	*P*	81.3	0.4	0	0
	32	5891	*T*	7.5	54.3	0	0
Northern Iraq	33	5475	*P*	72.8	43.0	0	0
	34	5472	*T*	66.2	6.0	0	0.2
	35	5482	*P*	34.0	4.1	0	0

[a]*P*, var. *polyathera*; *T*, var. *typica*

[b]Tester A, KU6-2, var. *typica* from Syria; Tester B, KU5852, var. *polyathera* from Central Anatolia; Tester C, KU5864, var. *typica* from West Anatolia; Tester D, KU5871, var. *typica* from Greece

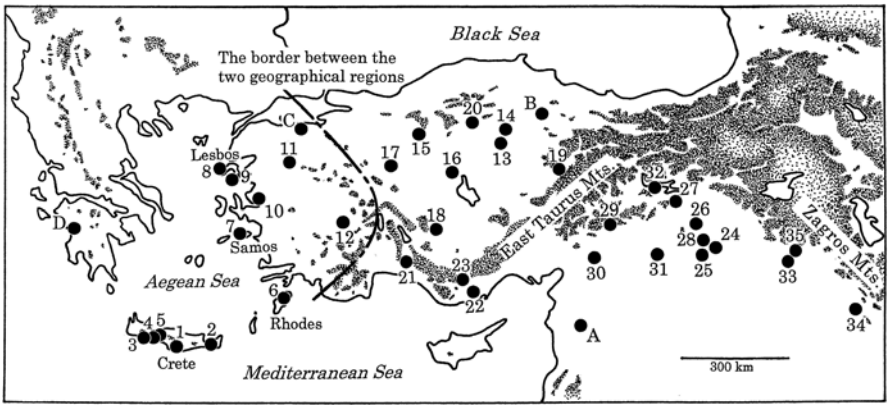

Fig. 6.1 Collection sites of the four Testers (A, B, C and D) and 35 accessions of *Ae. caudata* used in the present study (Ohta 2000). Mountainous area higher than 1,500 m above sea level is *shadowed*. A *broken line* indicates the border between the western and eastern regions defined from hybrid sterility

fertility in the F_1 hybrids, the 39 accessions including the four Testers could be grouped into the western and eastern geographical groups: the former consists of the accessions from the Greek mainland, the Aegean Islands and West Anatolia, while the latter from Central Anatolia, South Anatolia, East Anatolia, northern Syria and Iraq. The two geographical regions are isolated with the mountains lying between West Anatolia and Central Anatolia (Fig. 6.1).

Gametocidal-Like Genes Cause the Sterility in Intraspecific F_1 Hybrids

The highly sterile F_1 hybrids from the two cross combinations between the parental lines derived from the western and eastern regions were backcrossed to their parental lines. The BC_1F_1 plants distinctly restored their fertility when the F_1 hybrids were backcrossed to the western parents, while their fertility was not restored when they were backcrossed to the eastern parents (Table 6.2). A similar sterility has been reported in several *Aegilops* species as the phenomenon that certain *Aegilops* chromosomes, gametocidal chromosomes, were preferentially transmitted in the genetic background of common wheat (Endo and Tsunewaki 1975; Maan 1975; Endo 1990). An *Ae. caudata* chromosome was found to be selectively retained in common wheat (Endo and Katayama 1978). Monosomic addition plants for a gametocidal chromosome to common wheat showed semi-sterility because only gametophytes with a gametocidal chromosome are functional while those without this chromosome are aborted. As a result, gametocidal chromosomes were preferentially transmitted to the next generation. When normal common wheat plants with

Table 6.2 Frequency distribution of the pollen fertility in the BC_1F_1 populations obtained from back-crossing the sterile F_1 hybrids between the western and eastern accessions to their parental lines

Cross group	F_1 hybrid origin	Pollen fertility (%) in F_1	Parental line backcrossed	Total no. of BC_1F_1 plants	Pollen fertility (%) class and no. of BC_1F_1 plants observed									
					<10	<20	<30	<40	<50	<60	<70	<80	<90	<100
Eastern acc.× Western testers	KU5859×C	0	KU5859	2	2									
	KU5859×D	0	KU5859	1	1									
			D	1										1
Western acc.× Eastern testers	KU12169×A	2.4	A	7	3	2			2					
			KU12169	6									5	1
	KU12169×B	7.7	B	1	1									
			KU12169	3										3

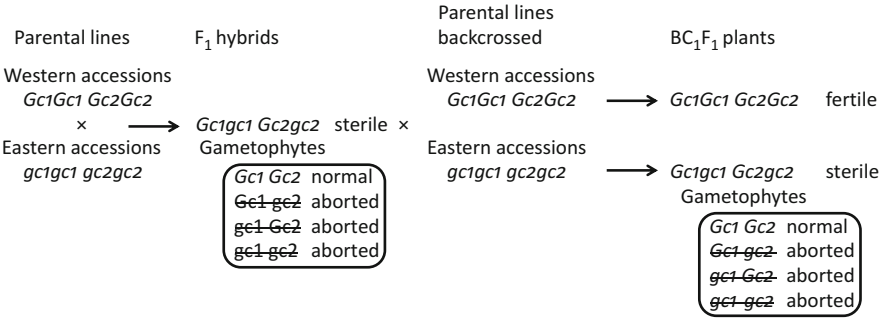

Fig. 6.2 A schema of the present gametocidal gene hypothesis explaining hybrid sterility in the F_1 hybrids between the western and eastern accessions and its restoration in the BC_1F_1 plants obtained from backcrosses to the western parental lines

42 chromosomes were crossed to the monosomic addition plants, the obtained plants had 43 chromosomes (monosomic addition for gametocidal chromosome) and showed semi-sterility again. Disomic addition plants for gametocidal chromosomes showed normal fertility, and such chromosomes were transmitted in stable by self-pollination.

In the present study, a similar phenomenon as that caused by gametocidal chromosomes in wheat genetic background was observed in the F_1 and BC_1F_1 plants of *Ae. caudata* with normal chromosome constitution, $2n = 14$. This result strongly suggests that the sterility in the F_1 hybrids between the western and eastern accessions is caused not by gametocidal *chromosomes* but by certain *gene(s)* on gametocidal chromosomes, and that gametocidal gene(s) cause sterility and their preferential transmission in heterozygotes not only in the genetic background of common wheat but also in the normal genetic background in *Ae. caudata.* A schema of the present gametocidal gene hypothesis explaining hybrid sterility is shown in Fig. 6.2, where we propose that the western and eastern accessions carry dominant and recessive gametocidal alleles, respectively.

Genetic Control of the Diagnostic Morphology of the Two Varieties

Two varieties collected at the nine sympatric stands on the Aegean Islands were crossed reciprocally with each other. All the F_1 hybrids had awnless lateral spikelets and their pollen fertility was normal (Ohta 1992). The segregation of the awn character in the F_2 generation did not significantly deviate from 3 (awnless): 1 (awned) ratio (Table 6.3). This result indicates that a dominant inhibitor controls awnless lateral spikelets characteristic of var. *typica.*

Further, 13 *typica* accessions including Testers A (KU6-2), C (KU5864) and D (KU5871) were crossed with a common *polyathera* Tester B (KU5852) (Table 6.1)

Table 6.3 The segregation of the awn character on lateral spikelets in the F_2 populations derived from fertile reciprocal F_1 hybrids between two varieties collected at their sympatric stands (Ohta 2001)

	No. of F_2 plants observed[a]			
Population no.	Total	Awnless	Awned	$\chi 2$ (3:1)
1982-6-11-1	48	33	15	1.00
1982-6-12-2	86	63	23	0.13
1982-6-12-4	49	35	14	0.82
1982-6-12-7	72	59	13	1.85
1982-6-14-7	89	71	18	2.03
1982-6-14-8	63	49	14	0.26
1982-6-14-9	48	35	13	0.11
1982-6-17-1	69	55	14	0.82
1982-6-23-1	71	53	18	0.04

[a]Data from reciprocal crosses were pooled

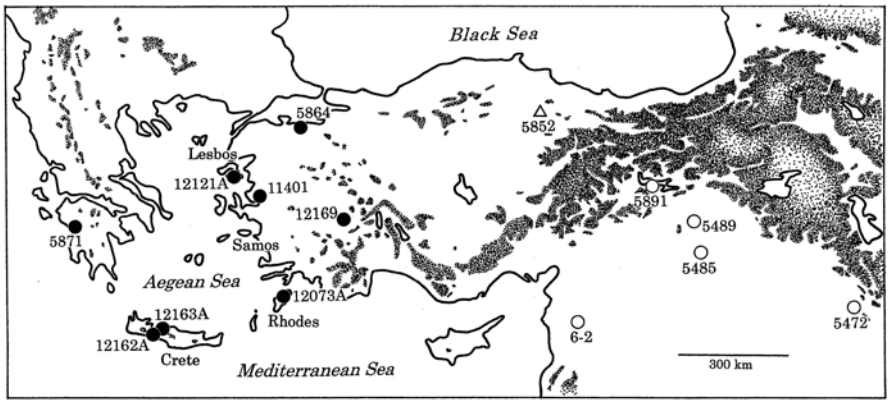

Fig. 6.3 Geographical distribution of the dominant and recessive *typica* accessions (Ohta 2001). *Solid circles*: dominant *typica* accessions, *open circles*: recessive *typica* accessions, and an *open triangle*: a common *polyathera* accession (Tester B: KU5852) used in the crosses. The *figures* indicate the accession numbers of Kyoto University

(Ohta 1992, 2000). These 13 *typica* accessions could be divided into the dominant and recessive *typica* accessions based on the spike morphology of the F_1 hybrids (Fig. 6.3). The F_1 hybrids involving the eight accessions from the western region were var. *typica*, while those involving the five accessions from the eastern region were var. *polyathera*. It is suggested that dominant *typica* accessions in the western region are controlled by a dominant inhibitor of the awns on lateral spikelets, while recessive *typica* accessions in the eastern region are controlled by recessive allele(s) for awn development on lateral spikelets.

Geographical Differentiation and Establishment of the Present Geographical Distribution in *Ae. caudata*

The present distribution area of *Ae. caudata* can be divided into the western and eastern regions with the border in the mountains lying between West Anatolia and Central Anatolia. And the western and eastern accessions are isolated not only geographically by the mountains but also reproductively by hybrid sterility caused by gametocidal-like genes. The morphology of var. *typica* is controlled by two different genotypes in the western and eastern regions, so the division of the two taxonomic varieties based on the awn character on lateral spikelets is not so significant for the phylogeny of the species.

The distinct genetic differentiation between the western and eastern groups strongly suggests that the two geographical groups were isolated for a long term in the past. In the maximum glacial period from 18,000 BP to 16,000 BP, steppe and desert-steppe with *Artemisia* and Chnopodiaceae covered the greater part of the Near East (van Zeist and Bottema 1991), which is now the primary diversity center of the wild *Triticum* and *Aegilops* species. *Ae. caudata* did not occur in Central Anatolia and East Anatolia in that period, and its distribution area might be divided into the two isolated refuges. One was the region surrounding the Aegean Sea where the climate was affected by the sea and was not excessively cold or dry, and the other was the western Levant or some sheltered habitats in the East Taurus/Zagros mountain arc. As the climate became warmer after the last glacial period, *Ae. caudata* penetrated Central Anatolia and East Anatolia with the spread of oak woodland from the latter refuge region, and now occupies the eastern region of the distribution. Variety *polyathera* with many awns on spikes might be advantageous to such a rapid colonization to new open habitats. The western region of the present distribution was established by the populations in the Aegean Sea region during the last glacial period.

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Part III
Cytogenetics and Allopolyploid Evolution

Chapter 7
Wheat Chromosome Analysis

Bikram S. Gill

Abstract The 13th International Wheat Genetics Symposium (IWGS) is being held in the year I begin my phased retirement, marking a career of 40 years in wheat genetics, beginning with a postdoctoral fellowship in 1973 with Ernie Sears and Gordon Kimber at Columbia, Missouri, then the premier center for wheat chromosome research. I was fortunate to have won a DF Jones fellowship for my research proposal, "Exploration and application of the Quinacrine and Giemsa staining technique in the genus *Triticum*" that led to the cytogenetic identification of wheat and rye chromosomes (Gill and Kimber 1974a, b). In 1973, I also attended, for the first time, the meetings of the 4th IWGS in Columbia, Missouri, and was in awe of the research presentations and heated discussions on wheat evolution. In 1979, I established my own research group and laboratory at Kansas State University focusing on wheat chromosome mapping and manipulation for crop improvement under the auspices of Wheat Genetics Resource Center (reviewed in Raupp and Friebe, Plant Breed Rev 37:1–34, 2013). Among the first visitors to my laboratory were Takashi Endo, then at Nara University, Japan, and Chen Peidu, from Nanjing Agricultural University, and presented this research at the 6th IWGS in Kyoto, Japan. Therefore, it is a special feeling to be returning to Japan for a farewell presentation. My intent is to briefly review the history of wheat chromosome research and how our laboratory played a role in advancing wheat chromosome analysis leading to the chromosome survey sequencing paper utilizing telosomic stocks (IWGSC, Science 345:285–287, 2014).

Keywords Aneuploid stocks • C-banding • Evolution • In situ hybridization • Wheat

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Laying the Foundation of Wheat Chromosome Research: Genome Analyzer Method

By 1915, botanists had described three classes of cultivated wheats, the one-seeded monococcum (*Triticum monococcum* L.), the two-seeded emmer (*T. turgidum* L.), and dinkel (*T. aestivum* L.). The one-seeded wild relative of monococcum was reported in Greece and Anatolia between 1834 and 1884. Aaronsohn discovered the two-seeded wild relative of emmer in 1910 in Lebanon, Syria, Jordan, and Israel. Therefore, it was well accepted, as Candolle had suggested in 1886, that since wild wheats grow in the Euphrates basin then wheat cultivation must have originated there. Between 1918 and 1924, Sakamura (1918) and his colleague Hitoshi Kihara (1919), at Hokkaido Imperial University in Japan, and Karl Sax (1922), at Harvard University, reported their classic studies on the genetic architecture of the three groups of wheats (Fig. 7.1). Sax (1922) and Kihara (1924) analyzed meiosis in wheat species and hybrids and were the first to establish the basic chromosome number of seven and document polyploidy in the wheat group. This method of delineating species evolutionary relationships based on chromosome pairing affinities in interspecific hybrids came to be called as the genome analyzer method (Kihara 1954; see also Fig. 7.1 in Gill et al. 2006). These were exciting observations and established polyploidy as a major macrospeciation process and wheat as a great polyploidy genetic model. These interploidy wheat hybrids of course could also be

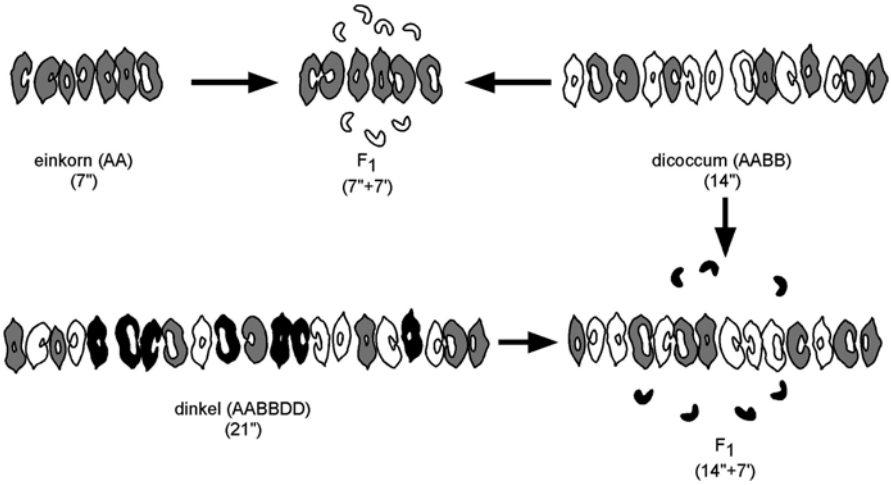

Fig. 7.1 Meiotic metaphase I chiasmate pairing in wheat parents and F_1 hybrids. The F_1 hybrid between einkorn and dicoccum showed 7″ and 7′, indicating that they share one set of chromosomes in common, and it was called a genome. The second genome in dicoccum was called the B genome. The F_1 hybrids between dicoccum and dinkel showed 14″ and 7′ indicating that they share the AB genomes in common, and dinkel wheat had a third genome that was later identified as D genome

exploited in plant breeding for interspecific gene transfers (McFadden 1930; Gill and Raupp 1987).

The crowning achievement of the genome analyzer method was the identification of the D-genome donor of wheat (Kihara 1944; McFadden and Sears 1946) and the production of synthetic wheat (McFadden and Sears 1944). These discoveries are fueling a second green revolution (reviewed in Gill et al. 2006). Kimber practiced the genome analyzer method with passion and developed some quantitative models for measuring genomic affinities. Application of Giemsa staining methods to meiotic preparations allowed the measurement of pairing potential of specific chromosomes, however, perfectly homologous chromosomes may suffer structural aberrations and lose the ability to pair (Gill and Chen 1987; Naranjo et al. 1987). Obviously, the genome analyzer method had reached its limitations, but meiotic pairing analysis remains an important method for monitoring chiasmate pairing and the potential of genetic transfers in interspecific hybrids.

Laying the Foundation for Cytogenetic and Genome Mapping: The Wheat Aneuploid Stocks

Sears (1939, 1954) isolated a range of aneuploid stocks in Chinese Spring wheat that were widely used by the wheat genetics community to dissect and map the polyploid genome of wheat (Fig. 7.2). The monosomic and telosomics were useful for chromosome and arm mapping of genes, and for producing intervarietal substitution lines for the mapping of quantitative traits (Law 1966). Wheat

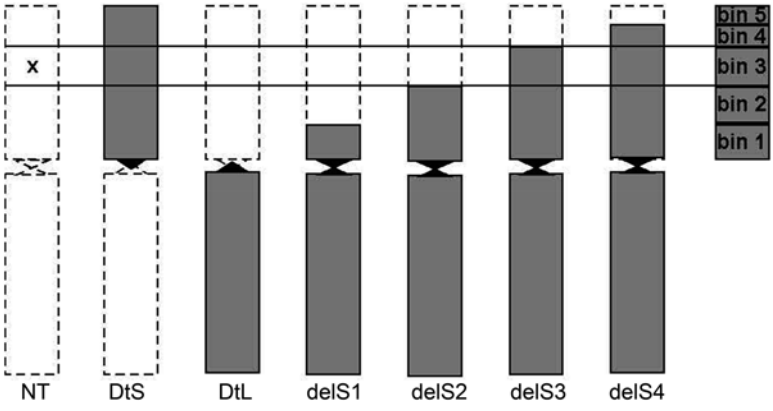

Fig. 7.2 Molecular cytogenetic mapping of wheat phenotypes and the genome. Steps in targeted physical mapping of genes in wheat. Absence of the marker in the critical nullisomic-tetrasomic, long arm ditelosomic deletion stocks, delS1 and delS2, and presence of the marker in the short arm ditelosomic, deletion stocks delS3 and delS4 maps the gene to the chromosome bin 3, which is defined by the deletion interval spanned by delS3 and delS4 (chromosome segments missing in the aneuploid stocks are shown in *hatching*) (Originally published in Faris et al. (2002))

nulli-tetrasomics were useful for comparative genetic mapping and determining homoeologous relationships among chromosomes of different genomic origin (Sears 1966). O'Mara (1940) developed an aneuploid approach for mapping alien chromosomes added to wheat.

Gametocidal chromosomes were used to isolate deletion stocks (Endo and Gill 1996) for mapping traits and ESTs (expressed sequence tags) into deletion bins (Qi et al. 2004). This extensive mapping of chromosomes revealed structural and functional differentiation along the centromere and telomere axis and the unequal distribution of genes and recombination towards the distal ends of chromosomes.

The individual telosomic chromosomes, reported by Sears and Sears (1978), have been sequenced (IWGSC 2014) using chromosome genomic methods pioneered by Dolezel and his group (reviewed elsewhere). Nearly 140,000 wheat genes have been identified and mapped to the genome zipper maps of the 21 chromosomes of wheat for genetic mapping and plant breeding applications. Alien chromosomes, present as additions to the wheat genome, also can be isolated and sequenced, providing unlimited markers for alien gene introgression (Tiwari et al. 2014).

Laying the Foundation for Analysis of Chromosome Structure: Chromosome Banding and In Situ Hybridization Methods

By the 1970s, many genes had been mapped on wheat chromosomes and arms in relation to the centromere but little was known about the chromosome structure, especially chromatin differentiation into biological meaningful heterochromatic and euchromatic regions along the chromosome length. In the early 1970s, Gill and Kimber (1974a, b) applied the C-banding technique to wheat and rye revealing remarkable heterochromatic bands that allowed unequivocal identification of individual chromosomes. Combining C-banding with aneuploid stocks, they constructed cytogenetic maps of the chromosome complements of wheat and rye. A nomenclature system was developed to describe the hundreds of chromosome regions into which the wheat genome was partitioned for mapping, and idiograms of the 21 wheat chromosomes were constructed (Gill et al. 1991; Fig. 7.3). The homoeologous chromosomes belonging to A, B, D, and R genomes showed little conservation of chromosome structure, revealing the fast-paced tempo of cereal chromosome evolution in the Triticeae tribe.

Next, newly developed in situ hybridization methods were used to map DNA sequences on chromosomes on a glass slide (Rayburn and Gill 1985). Repetitive DNA mapped to heterochromatic regions was often genome-specific (Rayburn and Gill 1986). Recently, a robust method of mapping single-copy sequences has been applied to wheat (Danilova et al. 2012). A set of 60 full-length cDNA clones for single-copy FISH was developed to rapidly determine the homoeology of any Triticeae chromosome with wheat chromosomes (Danilova et al. 2014; Fig. 7.4).

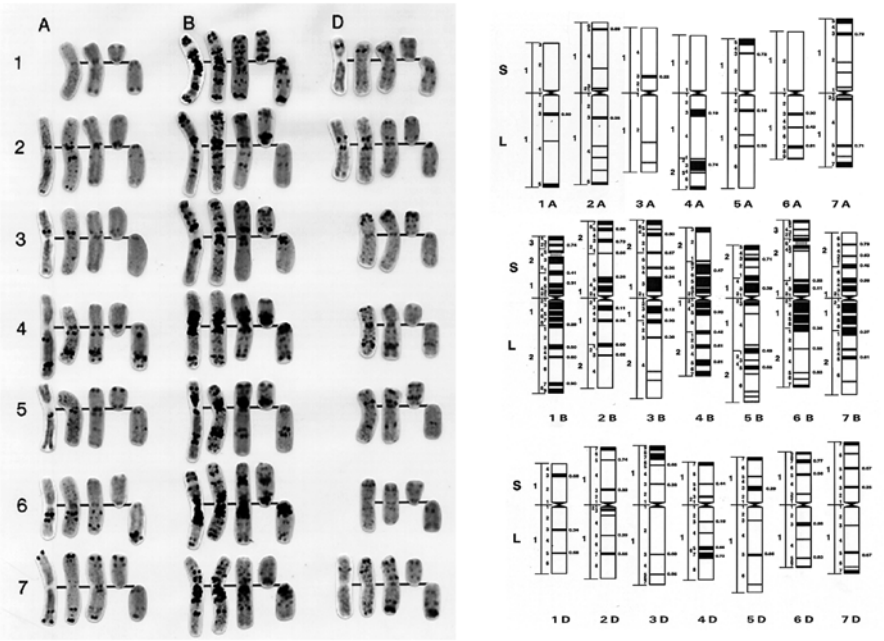

Fig. 7.3 (*Left*) The banded karyotype of Chinese Spring wheat. Each chromosome was stained (from *left* to *right*) by N-banding, modified C-banding, and C-banding (whole and telosomic chromosomes). Because chromosome 1A, and 3D to 6D do not show any N-bands, they were not identified. (*Right*) Idiogram of banded chromosomes of Chinese Spring wheat. Band numbers are indicated on the *left* and FL positions on the *right* of each chromosome. Hatched bands are not numbered as they were not observed consistently (Originally published in Gill et al. (1991). © Canadian Science Publishing or its licensors)

This set also is very useful for detecting chromosomal rearrangements that may have occurred during Triticeae evolution.

Laying the Foundation for Wheat Chromosome Manipulation: An Integrated Approach for Alien Genetic Transfers

Two basic methods of chromosome manipulation for alien gene introgression have been practiced widely in wheat. Sears (1956) used irradiation to transfer a leaf rust resistance gene from *Aegilops umbellulata* Zhuk. into wheat. Riley et al. (1968) interfered with the *Ph1* gene effect (Riley and Chapman 1958) and used induced-homoeologous pairing to transfer yellow rust resistance from *Ae. comosa* Sm. in Sibth. & Sm. into wheat. Later, Sears (1977) produced a mutant at the *Ph1* locus and also used a nullisomic 5B chromosome for induced homoeologous pairing and alien gene transfer (Sears 1973).

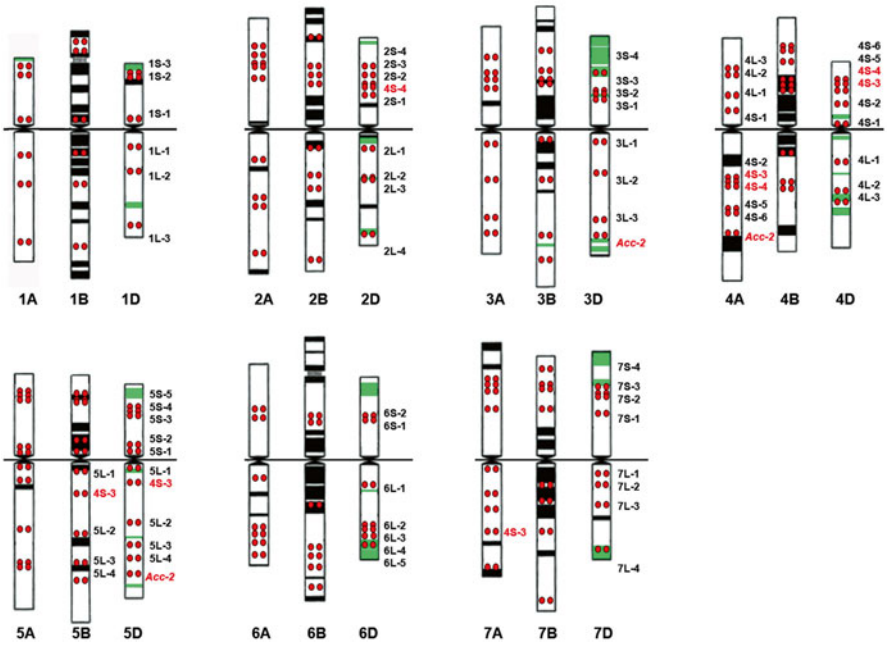

Fig. 7.4 Idiogram of wheat chromosomes where heterochromatic bands that can be visualized with GAA oligonucleotide probes are in *black*, pAs1 bands are in *green*, and cDNA probe positions are shown as *red dots*. Names of probes that hybridize to more than one homoeologous group are highlighted in *red* (Originally published in Danilova et al. (2014))

Chromosome banding and in situ hybridization greatly facilitated the characterization of the alien transfers in terms of the identity of the alien and wheat chromosomes involved in the translocations, the breakpoints of wheat–alien translocations, and the amount of the alien chromatin transferred (Friebe et al. 1991; Mukai et al. 1993; for review see Friebe et al. 1996). Qi et al. (2007) presented an integrated chromosome manipulation protocol utilizing modern cytological and molecular methods that has been widely used in many alien transfer experiments.

Looking to the Future

As the wheat genome is sequenced, it will be important to integrate DNA sequences with chromosome features such as heterochromatin and euchromatin, telomeres, and centromeres to gain insight into chromosome structure, function, and evolution, and, especially, how chromatin structure may control gene expression. The Triticeae tribe contains several 100 species, and only a few have been characterized by modern methods. Ideograms should be developed for all Triticeae chromosomes similar to the wheat chromosome idiogram (Fig. 7.3), including the mapping of single-copy

FISH markers for elucidating syntenic relationships (Fig. 7.4). We also need to produce amphiploids, addition, substitution, and compensating Robertsonian translocations involving complete genomes of Triticeae species added to wheat. For accomplishing such a task, the IWGS provides a great platform for coordination of research and sharing of results.

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Chapter 8
New Aneuploids of Common Wheat

Takashi R. Endo

Abstract Chromosomal structural changes can be induced by the addition of specific alien chromosomes called 'gametocidal (Gc) chromosomes'. In the monosomic addition of the Gc chromosome to common wheat, chromosomal breaks occur in gametes receiving no Gc chromosome, and the broken ends heal and stabilize in the subsequent generations. Thus, by the so-called Gc system, deficiencies and translocations can be induced in common wheat and also in alien chromosome addition and substitution lines of common wheat. Deficiencies of wheat and alien chromosomes were cytologically identified by the chromosome banding and in situ hybridization techniques. The plants carrying deletions or wheat-alien translocations were established as new aneuploid lines of common wheat with sub-arm aneuploidy. Those for wheat chromosomes are called deletion stocks and those for alien chromosomes are called dissection lines. The new aneuploids have been used for cytological chromosome mapping and have corrected some mistakes in genetic mapping.

Aneuploids of Common Wheat

The hexaploid nature of common wheat (*Triticum aestivum* L., 2n = 6x = 42) enables us to produce aneuploids rather easily. Almost 60 year ago Sears (1954) first reported the systematic production of aneuploid lines in common wheat. These so-called conventional aneuploids comprise different series of aneuploid lines of Chinese Spring wheat, such as monosomics, nullisomic, nullisomic-tetrasomics and ditelosomics (Sears 1954; Sears and Sears 1978), which have been used extensively for the genetic and genomic studies of wheat. He allowed everybody to use those aneuploids freely. I believe that his generosity stimulated the subsequent development of not only genetic and cytogenetic studies but also molecular and genomics studies of wheat. Common wheat can also tolerate the addition of alien

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chromosomes from related wild and cultivated species, such as goatgrass, wheatgrass, rye and barley. In some cases, alien chromosomes substitute for wheat chromosomes very well to be established as common wheat cultivars, e.g., those carrying a 1R(1B) substitution.

The new aneuploids described here are not really new, but I would like to distinguish them from the conventional aneuploid lines that have aneuploidy of whole chromosomes or chromosome arms. I would like to define these new aneuploids as 'aneuploids that have sub-arm aneuploidy', which can be induced by two genetic mechanisms, as well as by artificial mutation. By the suppression of the pairing control (*ph*: *pairing homoeologous*) gene, homoeologous pairing is induced between wheat and alien chromosomes to generate wheat-alien recombinant chromosomes in alien substitution lines of common wheat. Also, unique chromosomes called gametocidal (Gc) chromosomes, which were introduced from specific wild species of the genus *Aegilops*, induce chromosomal breakage in common wheat to generate deficient chromosomes and wheat-alien recombinant chromosomes. I named the common wheat lines carrying deficient wheat chromosomes 'deletion stocks' (Endo and Gill 1996), and I proposed to call generically the wheat lines carrying deletions or translocations of alien chromosomes 'dissection' lines (Endo 2007).

Advanced Techniques to Check Aneuploids

The addition of whole alien chromosomes or chromosome arms is generally detrimental to the performance of common wheat. The new aneuploids with dissected chromosomes at the sub-arm level are more useful not only for breeding purposes but also for genomic analysis. Two cytological techniques 'chromosome banding' and 'fluorescence in situ hybridization' are most useful for the identification of chromosomes and sub-arm aberrations. C-banding allows us to identify all common wheat chromosomes (Gill et al. 1991) and not only to verify the conventional wheat aneuploids in mitotic metaphase cells but also to detect deficient chromosomes. Fluorescence in situ hybridization (FISH) is useful for locating specific DNA sequences on chromosomes. The genomic in situ hybridization (GISH), which uses probes of total genomic DNA from alien species, is useful for the detection of alien chromosomes and chromosomal segments introduced into wheat. The combination of chromosome banding and FISH/GISH is an even more powerful cytological technique for identifying alien chromosomes in wheat because we can identify chromosomes and locate specific DNA sequences in the same chromosome preparation (Fig. 8.1).

Gametocidal Mechanism

Some alien chromosomes called gametocidal (Gc) chromosomes ensure their existence in common wheat in a selfish manner. When the Gc chromosome exists in the monosomic condition, two types of gametophyte are produced, those carrying the

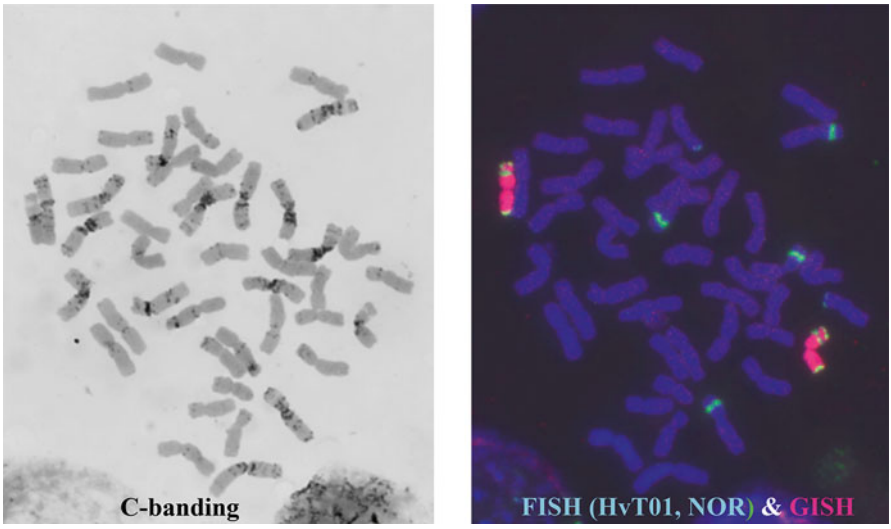

Fig. 8.1 A mitotic metaphase cell of the barley 6H addition line of common wheat depicted by the combination of C-banding and FISH/GISH. The C-banding pattern shows that this line has a chromosomal complement of common wheat, and FISH indicates the presence of rDNA sequences in NORs, and GISH confirmed the disomic addition of barley chromosome 6H

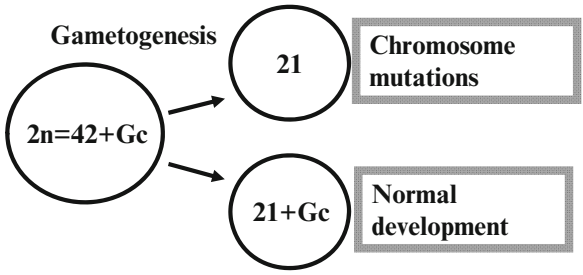

Fig. 8.2 A schematic diagram showing the gametocidal action. Gc stands for 'gametocidal chromosome'

Gc chromosome and those without the Gc chromosome, and chromosome breakage occurs only in the latter gametophytes (Fig. 8.2). Such Gc-induced chromosomal breakage leads to either the sterility of gametes or the production of fertile gametes carrying chromosomal mutations, and the induced chromosomal mutations become stabilized in subsequent generations (Endo 1990).

Deletion Stocks of Common Wheat

Two Gc chromosomes have been used to generate deletion and dissection lines. One is chromosome 2C derived from *Aegilops cylindirica* Host. and the other is chromosome 3C derived from *Aegilops triuncialis* L. (Endo 1988). For the production of

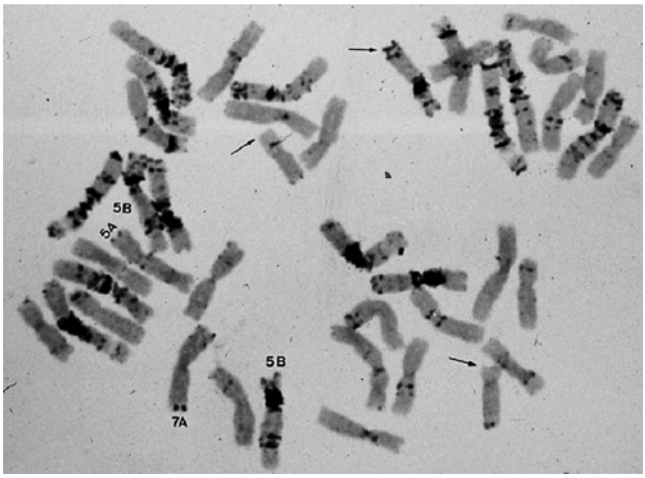

Fig. 8.3 A C-banded mitotic metaphase cell of a progeny carrying three Gc-induced deletions of chromosomes 5A, 5B, and 7A (pointed with *arrows*) (Note that this plant was partial trisomic for 5B)

the deletion stocks, I started cytological selection in the progeny of the Gc (mainly 2C) monosomic addition line crossed with euploid Chinese Spring. Deletions took place in the heterozygous condition and often multiple deletions occurred in single plants as shown in Fig. 8.3. I further selected the selfed progeny of such plants to obtain single-deletion homozygotes. In some cases I ended up getting multiple-deletion homozygous lines and also failed to obtain homozygotes for specific chromosome arms like the short arm of chromosome 4B. Table 8.1 summarizes the distribution of deletion breakpoints among different chromosome arms. I examined about 500 primary progeny, and in the end I identified 436 deletions and established deletion-homozygous lines for about 350 (ca. 80 %) of the deletions. This means that almost one deletion per plant occurred on average. In other words, we can expect to obtain one deletion for a specific chromosome in 4~5 % of plants we examine. The breakpoints of the deletions seem to be randomly distributed, but it is difficult to tell whether or not there are hotspots of Gc-induced chromosomal breaks.

Undoubtedly the deletion stocks are useful for chromosome mapping, especially for deletion mapping of molecular markers. Werner et al. (1992) conducted deletion mapping of RFLP using the deletion stocks for the first time. Since then a series of papers on wheat chromosome deletion or bin mapping of various DNA markers using deletion stocks have been published (e.g. Qi et al. 2004). These deletion-homozygous stocks are distributed from National BioResource Project-Wheat (http://www.shigen.nig.ac.jp/wheat/komugi/).

Table 8.1 Distribution of deletion breakpoints in different genomes, chromosome arms, and homoeologous groups in Chinese Spring wheat

	No. of deletion breakpoints							
	Genomes	A		B		D		
Homoeologous arms	Arms	S	L	S	L	S	L	Total
1		5	6	22	18	5	8	64
2		9	6	13	11	6	12	57
3		4	8	10	12	9	3	46
4		4	13	9	14	5	15	60
5		11	23	9	18	4	12	77
6		5	8	11	15	7	11	57
7		13	25	6	16	6	9	75
Subtotal		51	89	80	104	42	70	
Total		140		184		112		436

Note: Data are taken from Endo and Gill (1996)

Dissection of Alien Chromosomes

The Gc system can also be used to dissect alien (A) chromosomes introduced into common wheat. The cross scheme is simple: First, make a cross between the Gc disomic addition and an alien disomic addition; then, backcross the hybrid to the alien addition to produce plants disomic for the alien chromosome and monosomic for the Gc chromosome (42+A"+Gc') (Endo 2007). In the progeny of this plant, we can find plants carrying structurally changed alien chromosomes, as well as aberrant wheat chromosomes.

By FISH/GISH we can undoubtedly identify deletions and translocations of alien chromosomes and can tell the exact breakpoint of a translocation between alien and wheat chromosomes (Fig. 8.4). The Gc system was successfully applied to produce dissection lines of various alien chromosomes in common wheat, such as barley chromosomes (2H, Joshi et al. 2011; 3H, Sakai et al. 2009; 4H, Sakata et al. 2010; 5H, Ashida et al.2007; 7H, Masoudi-Nejad et al. 2005), rye 1R chromosomes (Tsuchida et al. 2008; Gyawali et al. 2009, 2010) and rye B chromosomes (Endo et al. 2008). All possible kinds of rearrangements of alien chromosomes have been obtained (Fig. 8.5).

PCR-Based Mass Selection of Gc-Induced Deletions for Specific Chromosomes

The chromosomal deficiencies in the deletion stocks of common wheat were cytologically identified and characterized by chromosome banding. Then the breakpoints of the deleted chromosomes were analyzed with DNA markers. Once the

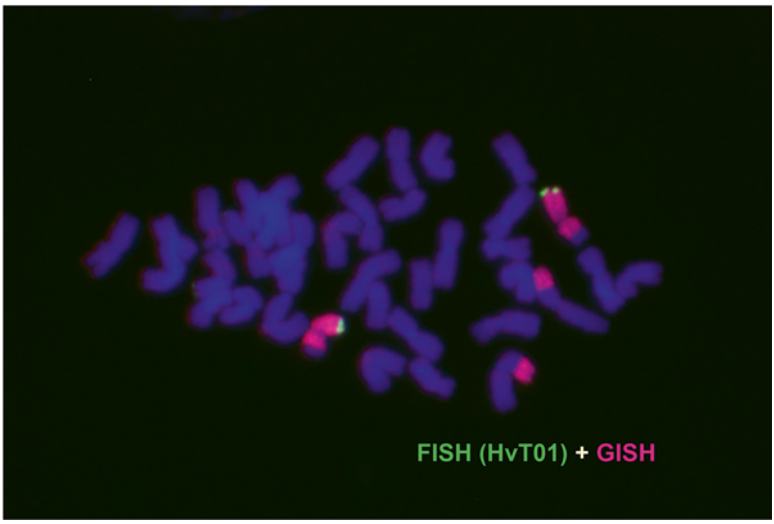

Fig. 8.4 A homozygous reciprocal translocation between barley chromosome 3H and a wheat chromosome

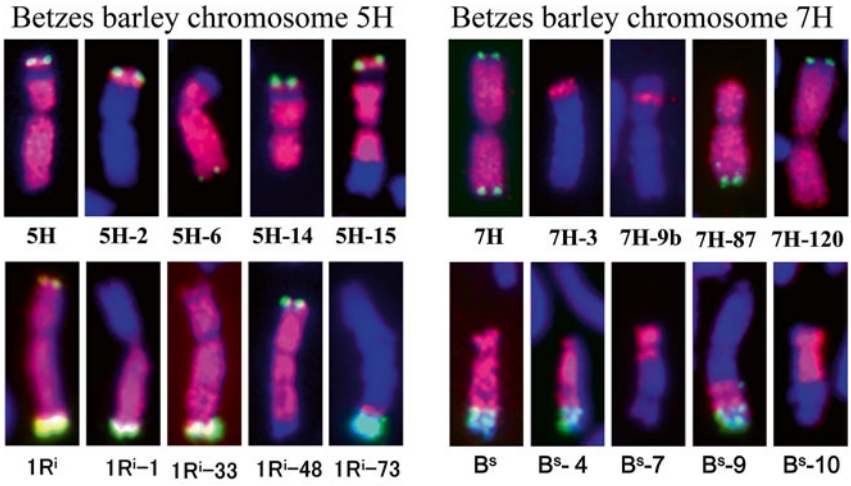

Fig. 8.5 Examples of alien chromosome aberrations induced by the Gc system. The *green* FISH signals indicate the subtelomeric repeats HvT01 for the barley 5H and 7H chromosomes derived from a cultivar Betzes, the pSc200 repeats for the rye 1R chromosome from a cultivar Imperial, and the E1100 repeats for the rye B chromosome from a Siberian cultivar. The GISH signals are shown in *red* for all chromosomes

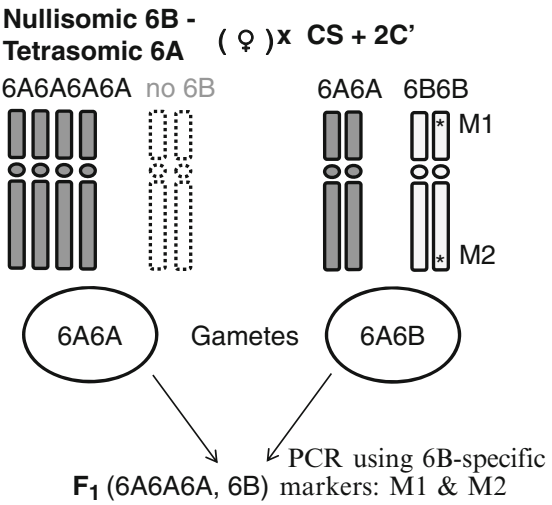

Fig. 8.6 A schematic diagram showing the PCR-based mass selection of Gc-induced deletions for wheat chromosome 6B

positions of the DNA markers are located on a chromosome, more deficiencies of the chromosome can be identified using chromosome-specific DNA markers and appropriate aneuploids. For example, using PCR-based 6B-specific markers, a search was made for deficiencies of chromosome 6B among the progeny from a cross between nullisomic 6B-tetrasomic 6A and a monosomic 2C addition line as the pollen parent (Fig. 8.6). Any deficiencies occurring in the 6B chromosome within the two markers could be detected by PCR because no 6B homologue was transmitted from the female parent. Thus, 102 (5.0 %) of the 2,041 hybrid plants were found to have a deficiency in either or both chromosome arms (unpublished data). In a similar way, Gc-induced deletions of alien chromosomes in common wheat can be identified by PCR. This PCR selection should find deletions overlooked by cytology and moreover probably detect structural changes that do not exist in the root tips but exist in aerial parts of plants. Joshi et al. (2013) conducted PCR analysis in 81 plants carrying a cytologically normal-appearing 2H chromosome in root tips and detected 2H aberration in the leaves of 6 of them. This fact implied the ongoing production of aberrations after fertilization.

Epilogue

Thanks to the next-generation sequencing technologies, sequencing the entire genomes of wheat, barley and rye has become a reality. Several chromosomal landmarks will be needed to assemble contigs into supercontigs or even into chromosomes. The Gc system will help provide a virtually limitless number of such landmarks, namely breakpoints of deleted or translocated chromosomes of the new aneuploids of common wheat.

Lastly, I wish to express my gratitude to Prof. Bikram S. Gill for his encouragement to establish deletion stocks common wheat by using them for his wheat chromosome mapping studies, otherwise I would not have done these works mentioned above.

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Chapter 9
Chromosomal Changes over the Course of Polyploid Wheat Evolution and Domestication

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Abstract Polyploid wheats are represented by two evolutionary lineages – Emmer and Timopheevi. It was reported that the species of these groups differentiated by species-specific translocations; they showed distinct karyotype structures, i.e., the amount and the distribution of heterochromatin. Analysis of more than 1,500 accessions representing 21 wild and cultivated polyploid wheat species using C-banding revealed that intra- and interspecific divergence within these two groups was accompanied by chromosomal rearrangements. Intraspecific diversity was the highest for wild species, followed by landraces and commercial cultivars. Chromosomal rearrangements were more frequent in *T. araraticum* than in *T. dicoccoides* (55.7 % and 35.3 % correspondingly). Altogether, 2 pericentric inversions, 28 single translocations, 13 double translocations, and five multiple translocations were identified in 150 of 270 *T. araraticum* accessions. Sixty types of chromosomal rearrangements (4 inversions, 37 single translocations, 11 double and 6 triple translocations, and two unclassified rearrangements) were found in *T. dicoccoides* (143 of 400 accessions). The range of karyotype diversity decreased in cultivated Emmer: 25 single translocations, 4 pericentric inversions, 6 double and 3 multiple translocations were detected in 119 of 470 accessions (24.5 %). The translocation T5B:7A significantly dominated over other variants; alone or in combination with other translocations, being identified in 51 lines of *T. dicoccum* (Schrank) Schübler, preferentially from Western Europe and Mediterranean countries. Chromosomal rearrangements were also found in common wheat, the translocations T5B:7B and wheat-rye T1RS:1BL being the most frequent (25 and 29 cultivars respectively). In addition to them, 24 variants of chromosomal rearrangements, including inversions, single and multiple translocations and wheat-alien translocations and substitutions were discovered in 112 of 295 cultivars we studied.

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Keywords C-banding • Chromosomes • Domestication • Evolution • Inversions • Polymorphism • Translocations • *Triticum*

Wheat evolution and domestication have been attracting the attention of researchers for over a 100 years, and a broad range of methods have been applied to uncover their genetic mechanisms. These studies have revealed that polyploid wheats are represented by two evolutionarily distinct groups: Emmer and Timopheevi that originated independently from hybridization of two diploid species: *Aegilops speltoides* Tausch. as a female parent and *Triticum urartu* Tum. ex. Gandil. as a male parent (Dvořák 1998). Hexaploid bread wheat arose from hybridization of an unknown tetraploid wheat species with *Aegilops tauschii* Coss. (Kihara 1944).

Examination of meiotic chromosome pairing in F_1 hybrid of *T. turgidum* L. x *T. timopheevii* Zhuk., experiments with Genomic in situ Hybridization (GISH) and the construction of high-density molecular and genetic maps of common wheat and *T. timopheevii* showed that the emergence of polyploid wheats was accompanied by species-specific chromosomal rearrangements: translocation 4A-5A-7B with subsequent pericentric inversion in the chromosome 4A in Emmer group and 1G-4G-6A^t+3A^t-4A^t in Timopheevi group (Jiang and Gill 1994; Liu et al. 1992; Naranjo et al. 1987; Rodríguez et al. 2000; Salina et al. 2006). Further analyses of intraspecific and interspecific diversity in various wheat species revealed differences between species as well as between geographic populations of the same species with respect to nuclear and cytoplasmic genome polymorphisms (Kilian et al. 2007; Mori et al. 2009; Salamini et al. 2002), isozyme and storage protein spectra (Belay 2000; Nishikawa 1984), and C-banding patterns and chromosomal rearrangements (Badaeva et al. 1994; Dedkova et al. 2009; Kawahara 1997; Kawahara and Taketa 2000; Taketa and Kawahara 1996). These studies however included a limited number of species, which does not allow obtaining a general overview of the wheat genome evolution. In the present work we analyzed all *Triticum* species belonging to Emmer and Timopheevi groups using C-banding method to discover karyotype alterations associated with evolution and domestication of polyploid wheat.

Our chromosome analysis demonstrates that intra- and interspecific divergence within Emmer and Timopheevi groups is accompanied by chromosomal rearrangements. Intraspecific diversity is the highest in wild species; and the two evolutionary groups display different mechanisms of karyotype evolution. Chromosomal rearrangements are more frequent in *T. araraticum* Jakubz. than in *T. dicoccoides* (Körn. ex Aschers. & Graebn.) Schweinf. (55.7 % and 35.3 %, lines, respectively). Translocation occur preferentially via the centromeric-breakage-fusion mechanism (63 centromeric vs. 9 interstitial breakage events led to the emergence of totally 28 types of single and 18 complex translocations), whereas the ratio between centromeric/interstitial translocation in wild Emmer is not so extreme (48 centromeric vs. 34 interstitial translocation events). In both lineages translocations among G and B genome chromosomes are the most frequent (66 and 82 variants of rearranged chromosomes are formed by B-B or G-G arm combinations respectively), followed by A^t-G/A-B (44 and 38 variants) or A^t-A^t/A-A – 19 and 26 variants of rearranged chromosomes, respectively).

Altogether, 48 different variants of chromosomal rearrangements, including 2 pericentric inversions, 28 single translocations, 13 double translocations and 5 multiple translocations, are identified in *T. araraticum* (150 of 270 accessions), and among them 13 translocations are novel. Most rearrangements (24 variants) are found in each of the single accessions, whereas 3 types occur more frequently. One of them is pericentric inversion in chromosome $7A^t$ which is present in 15 Iranian and 3 Iraqi (Sulaimaniyah) accessions. The second is a translocation T6G:7G identified in 12 accessions from Salah-ad-Din (Iraq). The third, multiple translocation $T4A^t:7A^t+T2A:4G:7G$ dominates in Nakhichevan, Azerbaijan (13 accessions), however, it has been never observed in other regions of the Caucasus. It might be occurred as a result of three subsequent translocations and, in turn, gave rise to another variant of multiple translocations found in Nakhichevan. Another translocation series was also observed in Armenia (Badaeva et al. 1994).

Triticum dicoccoides also shows high diversity in the C-banding patterns. Two distinct karyotypic groups are able to be distinguished in this species. The first group is typical for the subspecies *judaicum,* which is distributed north and west of the Galilee Sea. These accessions show characteristic C-banding patterns in chromosomes 2A, 4A, 7A, 1B, 3B, 4B, 7B; and, especially, in chromosome 5B (Fig. 9.1). The second group is much larger and includes accessions from all areas of the species distribution. They show an extremely high C-banding polymorphism, however, no distinct karyotypic variant is able to be discriminated among the geographical

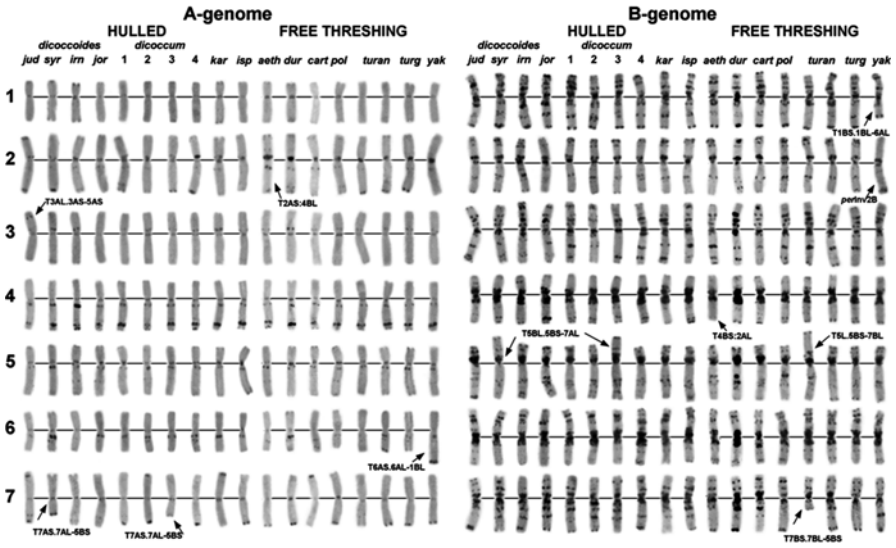

Fig. 9.1 Karyotype diversity in tetraploid wheat species of the Emmer group; *dicoccoides – T. dicoccoides*, *jud* – ssp. *judaicum*, *syr* – Syrian, *irn* – Iranian, *jor* – Jordanian accessions, *dicoccum – T. dicoccum, 1* – Asian type, *2* – Balkan type, *3* – European type, *4* – Ethiopian type, *kar – T. karamyschevii* Nevski, *isp – T. ispahanicum* Heslot, *aeth – T. aethiopicum* Jakubz., *dur – T. durum* Desf., *cart – T. cathlicum* Nevski, *pol – T. polonicum* L., *turan – T. turanicum* Jakubz., *turg – T. turgidum*, *yak – T. yakubzinery* Udacz. et Schachm. *1–7* – homoeologous groups. Chromosomal rearrangements are *arrowed*

populations on the basis of C-banding analysis. The highest karyotype diversity is observed in Jordan Valley, Israel.

Sixty types of rearrangements (4 inversions, 37 single translocations, 11 double and 6 triple translocations, and 2 unclassified rearrangements) are identified in *T. dicoccoides* (143 of 400 accessions). Although most of them are detected in each of the single accessions (40 variants), some are found in two (6 variants), three (6 variants) or more (9 variants) accessions. If the same translocation is identified in several accessions, they are usually assumed to grow in the same geographic region. However, some translocation variants are exceptional. A translocation between 7A and 5B chromosomes is identified in 5 accessions from Iraq, Syria, Lebanon, Israel and Armenia. Another, minor translocation between chromosomes 3A and 5A is found in 34 accessions of *T. dicoccoides* ssp. *judaicum*. In combination with other secondary translocations, three variants of multiple translocations are identified in five other accessions from Israel.

Karyotype diversity decreases in cultivated emmer compared to wild wheat; this is reflected by lower polymorphism in the C-banding patterns, decreased frequencies and narrow spectra of chromosomal rearrangements. Altogether 25 single translocations, 4 pericentric inversions, 6 double and 3 multiple translocations are identified in 119 of 470 accessions we studied. Thirty four translocation types are observed in each of the single accessions, and nine are identified in three and more accessions; among them T6B:7B is found in the accessions from Montenegro, and two related translocations T1B:4A + T4B:6B + T1A:6A and T1B:4A + T4B:6B:T1A:6A – are found in those from Morocco. The most frequent translocation T5B:7A is identified in 45 accessions, and also in combination with secondary translocations, in other six accessions. It is found in 22 countries in Europe, Asia and Northern Africa, but is the most abundant in Western Europe and Mediterranean countries (25 % of the population). Although T5B:7A is detected in two *T. dicoccum* lines from Turkey, it is totally absent in Turkish population of wild emmer (63 accessions). As is mentioned above, a similar translocation has been identified in five Palestine accessions of *T. dicoccoides.* Based on this fact we assume that 5B:7A translocation was either transferred from wild to cultivated emmer owing to intercrossing of these species, or European emmer was independently domesticated from the local Palestine population of *T. dicoccoides*.

Comparison of tetraploid species of the Emmer group does not reveal any significant differences among them in karyotype structures and C-banding patterns (Fig. 9.1). Species with broad distribution are more polymorphic compared to the endemic species (*T. karamyschevii, T. ispahanicum, T. turanicum, T. carthlicum, T. aethiopicum*). The species *T. yakubzinery* is represented by a single accession, which carries a 1B:6A translocation and pericentric inversion in the chromosome 2B (Fig. 9.1). A translocation between chromosomes 2A and 4B is common in *T. aethiopicum* and is observed in 70 % (present study) up to 100 % of the accessions (Kawahara and Taketa 2000). Two of six *T. turanicum* accessions we studied (TRI 4326 and TRI 11533), one of 400 *T. dicoccoides* accessions (IG 117890, Syria), and one of 470 *T. dicoccum* accessions (PI 308879, Spain) possess a 5B:7B translocation, which is very similar to the most widespread translocation of the European bread wheat in the structure of rearranged chromosomes.

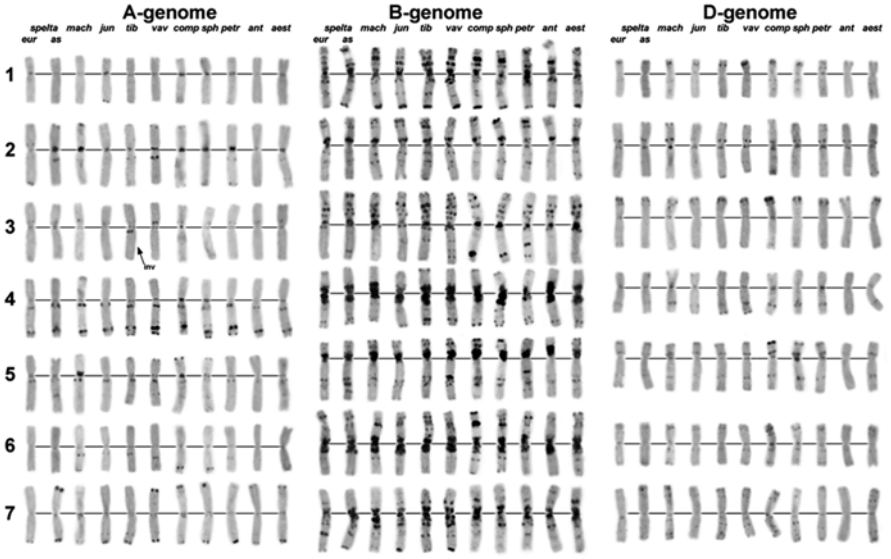

Fig. 9.2 Karyotype diversity in hexaploid wheat species of the Emmer group; splt- *T. spelta* L., eur – European type, as – Asian type, mach – *T. macha* Dekapr. et Menabde, jun – *T. aesticum* ssp. *junnanense*, tib – *T. aestivum ssp. tibetianum* Shao, vav – *T. vavilovii* (Thum.) Jakubz., comp – *T. compactum* Host, sph – *T. sphaerococcum* Perciv., petr – *T. petropavlovskyi* Udacz. et Migush., ant – *T. antiquorum* Heer ex Udacz, aest – *T. aestivum* L. em Thell. *1–7* – homoeologous groups. Chromosomal rearrangements are *arrowed*

Hexaploid wheat species are similar to each other in the C-banding patterns of the A, B and D genome chromosomes (Fig. 9.2). Three species – bread wheat (*T. aestivum*), spelt (*T. spelta*) and club wheat (*T. compactum*) are highly polymorphic, whereas *T. macha, T. vavilovii, T. sphaerococcum, T. petropavlovskyi,* and two subspecies of common wheat *T. aestivum* ssp. *junnanense* and ssp. *tibetianum* show limited polymorphism; all these wheats possess characteristic banding patterns allowing discrimination among them. Two of the two *T. aestivum* ssp. *tibetianum* accessions we studied here carry the same modification of the chromosome 3A (Fig. 9.2). C-banding analysis reveals clear differences between European and Asian groups of spelt wheat; the C-band distribution on the chromosome 2A of European spelt is similar to that of the European Emmer, which further confirms that European spelt was a secondary crop that originated from hybridization of common wheat with *T. dicoccum.* The most frequent translocation in spelt is T4A:1B, which is found in 8 accessions from Europe and Iran.

Polymorphism level decreases in common wheat compared to that in spelt, commercial varieties are less polymorphic than local varieties and landraces. Thirty five variants of chromosomal rearrangements and/or wheat-alien introgression are identified in 112 out of 295 common wheat cultivars. Translocation T5B:7B is the most widespread, being found in 25 cultivars studied and in at least 16 other cultivars described in literature (Friebe and Gill 1994; Schlegel 1996).

Other rearrangements have much lower frequencies. Many cultivars and breeding lines of *T. aestivum* have been reported to carry wheat-alien substitutions and/or translocations (Friebe et al. 1996; Schneider et al. 2008). The wheat-rye translocation T1B:1R is the most frequent one followed by T1A:1R. Wheat-*Agropyron*, wheat-*T. timopheevii,* and wheat-*Aegilops* substitutions and translocations are also identified in several Russian wheat cultivars and breeding lines, most of them being associated with pest resistance.

Our study shows that the evolution and domestication of polyploid wheat is associated with karyotypic changes due to C-banding polymorphisms and chromosomal rearrangements. Variation of Giemsa banding patterns is highest in wild species. Geographic populations within *T. araraticum* and *T. dicoccoides* diverged as a result of emergence of region-specific polymorphic Giemsa bands. Domestication of wheat led to a significant decrease of C-banding polymorphisms. Among cultivated wheats endemic species show the lowest variation of the C-banding patterns which can be due to geographic isolation. Our results revealed an important role of chromosomal rearrangements in interspecific and intraspecific divergence of polyploid wheats. The spectra and frequencies of chromosomal rearrangements are species-specific and geographical region specific and can be used as cytogenetic markers in phylogenetic and evolutionary studies of wheat. On the other hand, broad occurrence of some chromosomal rearrangements can be an indicative of their adaptive significance.

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Part IV
Toward Whole Genome Sequencing

Chapter 10
Comprehensive Functional Analyses of Expressed Sequence Tags in Common Wheat

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Abstract Although shotgun sequences of the genomic DNA of common wheat and its ancestors are available, gene discovery in common wheat is primarily based on proof sequencing of expressed full-length (FL) cDNAs. Use of expressed sequence tag (EST) databases including FLcDNA has been recognized as an important method for gene annotation in common wheat. In the large repetitive genome of common wheat, a transcriptome approach is complementary to whole genome sequencing. We have initiated a wheat EST project in Japan and constructed cDNA libraries from various tissues and strains of wheat, including biotic and abiotic stress treatments. We have also generated a high quality full-length cDNA resource for common wheat, an essential element necessary for the ongoing curation and annotation of the wheat genome. After several rounds of screening of CAP-trapped cDNA libraries, 21,408 FLcDNAs have been fully sequenced. The origins of these FLcDNAs were estimated through examination of the RNAseq data of three ancestral diploids, namely, *Triticum urartu*, *Aegilops speltoides*, and *Aegilops tauschii*. In addition, 51 cDNA libraries were constructed with an accumulation of 0.9 million ESTs. The ESTs, including the FLcDNA data, were assembled into contigs with stringent bioinformatic tool parameters. In total, 41,003 gene clusters were classified, in which 27,943 (68.1 %) had homology with other cereal genes. The digital monitoring system was utilized to identify characteristic gene expression patterns among various tissues and stress treatments in common wheat. These transcriptome data comprise a substantial reference for wheat genome sequencing.

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Keywords Common wheat • Expressed genes • Full-length cDNA • Homoeoalleles • Standardization of transcripts

Large-Scale Collection of Genes Expressed in Common Wheat

Wheat is characteristically polyploidic in nature and harbors large complex genomes. Therefore, accumulation of expressed sequence tags (ESTs) for wheat is particularly important for enabling functional genomics and molecular breeding studies. We obtained large collections of ESTs from various tissues in the wheat life cycle and from tissues subjected to stresses. Since full-length cDNAs are indispensable for certifying the ESTs collected and for annotating genes present in the genome, we performed a systematic survey of and sequencing of full-length cDNA clones. The strategy for the collection of ESTs in common wheat is shown in Fig. 10.1. First, total RNAs were extracted from the ten tissues over the course of the wheat life cycle. Subsequently, RNAs were extracted from biotic and abiotic-stressed tissues. The cDNA libraries were constructed from these RNAs by using standard methods. Colonies were randomly picked and sequenced from both ends. At present, 894,756 unbiased ESTs are available. More than 1.2 million wheat ESTs

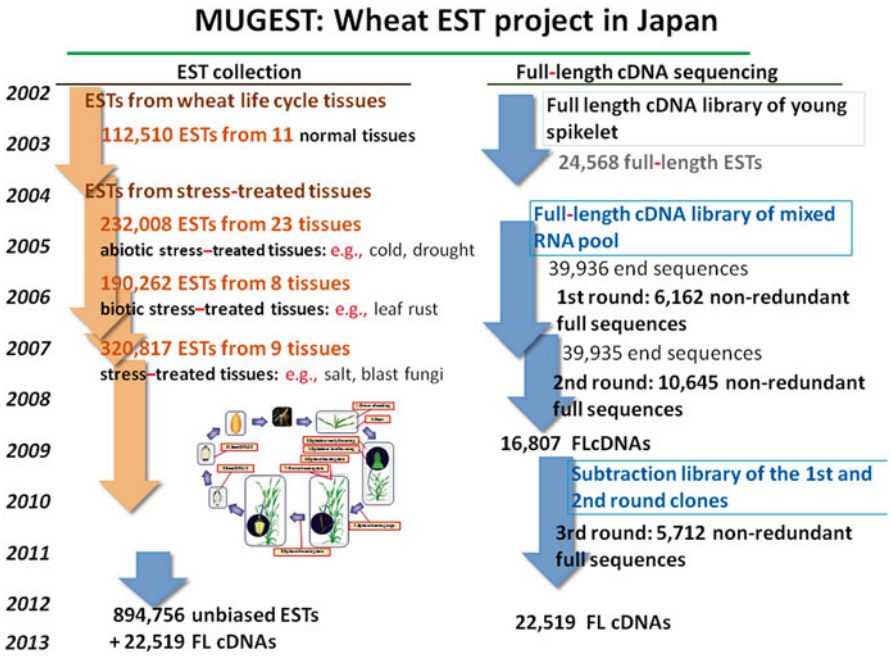

Fig. 10.1 MUGEST: Wheat EST project in Japan

are registered in the NCBI EST database, 70 % of which were contributed from Japan. For full-length cDNAs, the cDNA library was constructed with the CAP-trapper method (Kawaura et al. 2009). After one-path sequencing of cDNA clones, independent clones were selected three times, and the inserts of the cDNA clones were verified. Finally, 22,519 sequence-verified full-length cDNAs were obtained.

Functional Analysis of ESTs in Common Wheat

About one million ESTs comprising 125.3 Mb nucleotides were collected from the 51 cDNA libraries constructed from various tissues (including biotic and abiotic tissues) and organs under a range of conditions (Manickavelu et al. 2012). ESTs were assembled with stringent parameters so as to produce 37,138 contigs and 215,199 singlets, 10.6 % of which had no homology with those in the public databases. Using these EST data, we developed the correspondence analysis (CA) method (Yano et al. 2006), as shown in Fig. 10.2. The CA method enables identification and comparisons of significant gene expression with the specific library. This method was applied for comparison of gene expression analysis of susceptive and resistant near-isogenic lines in common wheat infected by *Puccinia triticina* (Manickavelu et al. 2010). In Fig. 10.2, four libraries developed for powdery mildew and leaf rust (two susceptible and two resistant) have been compared. Using this method, common and specific genes related to treatments or the library were easily selected.

Based on the gene ontology, characteristic proteins were classified according to molecular functions, cellular localization, and biological processes. Furthermore, the unigenes were classified into susceptible and resistant classes based on the EST members assembled from the respective libraries. Several genes showing specific expression in the resistant and susceptible lines could be selected. The molecular pathogenicity of leaf rust after infection in wheat was evaluated, and the EST data were supplied for future studies.

Full-Length cDNA Collection in Common Wheat

For construction of full-length cDNAs, total RNAs were extracted from the 17 tissues studied (Kawaura et al. 2009). Tese RNAs were mixed together to construct a cDNA library with the CAP-trapper method. We used three rounds of cDNA selection. At first, 19,968 clones were supplied for one-path sequencing from both ends. These sequences were clustered and non-redundant groups were selected. In this step, 6,162 sequence-verified full-length cDNAs were obtained after using the primer-walked Sanger sequencing method. Subsequently, data were obtained for an additional 10,645 cDNAs. After the second round, the already sequenced clones were subtracted from the library. An additional 5,712 redundant cDNAs were

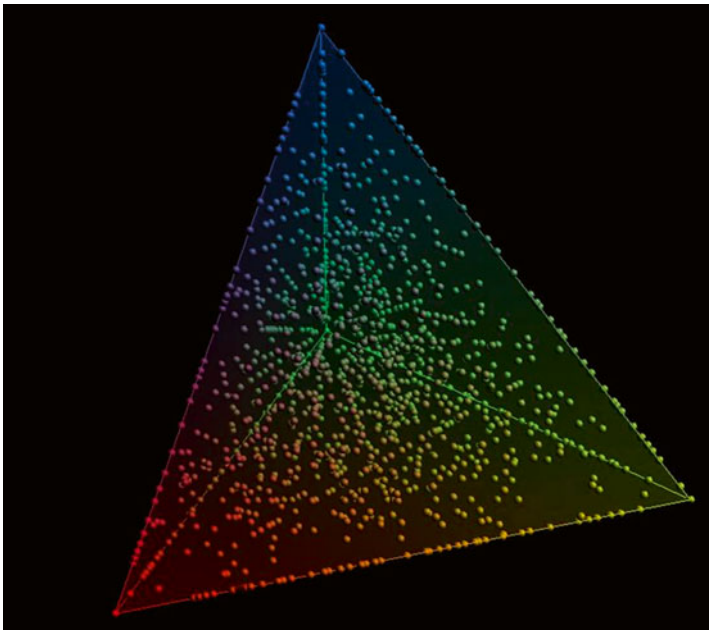

Fig. 10.2 Correspondence analysis for characterization of gene expression profiles in four libraries. Four cDNA libraries were developed for powdery mildew and leaf rust (two susceptible and two resistant). Each *corner* refers to each library; the significant genes related to specific libraries are positioned near, and in the same color as, the corresponding library

sequenced with the 454 FLx + instrument. Finally, 22,519 sequence-verified full-length cDNAs are now available. These FLcDNAs showed a wide size distribution with a range of 64–8,983 bp, and the mean size of these FLcDNAs was 1,848 bp larger than those of barley (1,711 bp; Matsumoto et al. 2011) and rice (1,746 bp; Kikuchi et al. 2003).

We performed RNAseq analysis of diploid ancestors to assign these FLcDNAs to three genomes, namely A, B, and D. Total RNAs were extracted from seedlings, roots and spikes of *Triticum urartu* Tum., *Aegilops speltoides* Tausch, and *Aegilops tauschii* Coss. These RNAs were utilized for RNAseq by using the Illumina Hiseq 2000 system. For each RNA sample, more than 50 Gb were read. These RNA data were grouped to generate contig clusters for each species. In this case, we used data for 16,807 FLcDNAs. These FLcDNA data were compared against contigs from each species. Top hits were selected based on homology and SNP patterns amongst genes from the three genomes. Thus, 4,759 cDNAs were assigned to the A genome, 2,849 to B, and 5,343 to the D genome. In total, 77 % of genes could be assigned. The remaining 23 % had no counterpart in the diploid RNAseq data pool. The B genome had a lesser number of expressed genes than the other genomes. This finding suggests that the lesser sequence homology of genes between the B genome of common wheat and the S genome of *Ae. speltoides* might disturb, to some extent, the ability to assign groups.

Identification of Standard Transcripts in Common Wheat

We attempted to identify standard transcripts in common wheat from the EST data; our data for 805,544 ESTs, including those for FLcDNAs, were assembled in each of the ten strains of common wheat. After assembly with MIRS (http://chevreux.org/projects_mira.html), 191,988 contigs and 326,851 singlets were obtained. The resultant contigs and singlets were clustered by the CD HIT software by setting the criteria at an identity of 99.5 % to obtain homoeologs (6×), and 95 % to obtain homologs (2×). Consequently, 162,874 gene clusters (2×) were grouped. Out of these clusters, 81,694 clusters were matched to expressed genes of barley (Matsumoto et al. 2011), *Brachypodium* (Mochida et al. 2013), and rice (http://ricegaas.dna.affrc.go.jp/rgadb/). By removing redundant results, 27,943 gene clusters that had homologies with Poaceae transcripts could be established at the diploid level; 81,180 clusters had no homologs among cereal genomes. Therefore, we conducted a "blat search" against 5× draft wheat genome sequences (Brenchley et al. 2012); 54,446 clusters were found to have homologies with the wheat genome sequences. By grouping these clusters at 80 % identity, 13,060 wheat-specific genes were obtained. From these analyses, 41,003 gene groups were clustered in wheat. Out of the 41,003 genes, 27,943 wheat genes had common homologies to Poaceae-expressed genes.

Chromosome Assignment of Expressed Genes in Common Wheat

We assigned these expressed homoeologs to each chromosome based on the "Survey sequence" organized by the International Wheat Genome Sequence Consortium (http://www.wheatgenome.org/). From this rough assignment, we can estimate the number of expressed genes from each chromosome and/or chromosome arm (Fig. 10.3). Chromosome 3B and 5BL were highly expressed. Conversely, genes located on 6B, which is the Japanese sequencing target, were less commonly expressed.

Conclusion

Snapshots of gene expression profiles in the wheat life cycle and/or in response to environmental stresses were developed; 22,519 sequence-verified full-length cDNAs were obtained. Of these, 77.4 % were assigned into the A, B, and D genomes with the RNAseq data from their diploid ancestors. We obtained 284,822 homoeo-clusters (6×) from 805,544 ESTs of common wheat. These homoeo-clusters were classified into 27,943 (Poaceae common) and 13,060 (wheat specific) gene groups

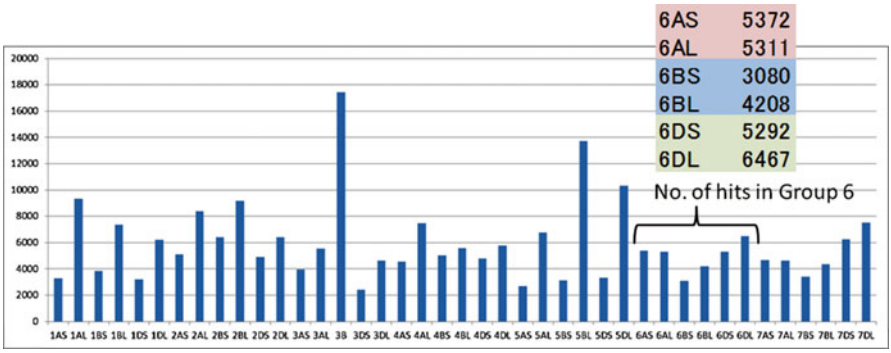

Fig. 10.3 Chromosome assignments of wheat-expressed genes by using the Chinese Wheat "Survey-sequences" of IWGSC

(2×). In total, 41,003 wheat transcripts (2×) were classified; 284,822 homoeoclusters were assigned into 21 chromosomes using "Survey-sequences" from the IWGSC.

Thus, the words of Prof. Kihara (1946), "The history of the earth is recorded in the layers of its crust. The history of all organisms is inscribed in the chromosomes" hold true even now, in the genomics era.

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Chapter 11
Development of the BAC Physical Maps of Wheat Chromosome 6B for Its Genomic Sequencing

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Genome Sequencing Project for Chromosome 6B

The International Wheat Genome Sequencing Consortium (IWGSC) has been promoting the decoding of the wheat genome sequence to enhance our knowledge of the structure and function of the wheat genome. The vision of the consortium is

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to establish a high quality reference sequence with a strategy to sequence its whole genome using BAC (bacterial artificial chromosome) clones aligned on the physical map of each chromosome. Under the framework of IWGSC, we are in charge of the chromosome 6B with the financial support from the Japanese Ministry of Agriculture, Forestry and Fisheries ("Genomics for Agricultural Innovation" project and "Genomics-based Technology for Agricultural Improvement" project) and Nisshin Flour Milling Inc. The "KomugiGSP" (Genome Sequence Program) (http://komugigsp.dna.affrc.go.jp/index.html) currently consists of the following four main research subjects: (1) Construction of 6B-specific BAC library and development of DNA markers, (2) comprehensive analysis of transcripts for gene annotation, (3) construction of the BAC physical map and genome sequencing (BAC-by-BAC and whole chromosome survey sequencing), and (4) development of annotation pipeline. Here, we report our current progresses on the construction of a physical map for chromosome 6B using chromosome arms-specific BAC libraries with DNA markers and BAC-based genomic sequencing.

Chromosome 6B-Specific BAC Libraries

Common wheat (*Triticum aestivum* L.) has a large genome, of approximately 17 Gb, and is allohexaploid with three homoeologous genomes (2n=6x=42, genome formula AABBDD). Chromosome 6B is one of the largest chromosomes among its 21 chromosomes, with an estimated size of 914 Mb, in which the short-arm (6BS) and long-arm (6BL) contain 415 Mb and 498 Mb, respectively (Šafář et al. 2010). Because of the large size, individual chromosomes or chromosome arms can be isolated using the flow cytometry to reduce the complexity of its genome for the construction of physical maps and genomic sequencing as well. The sorting of single chromosomes or chromosome arms from common wheat cultivar 'Chinese Spring' (CS) and its aneuploid lines has enabled the construction of chromosome (arm)-specific BAC libraries (Šafář et al. 2010), that have served as the critical resources for the development of physical maps and map-based genome sequencing by IWGSC. A double ditelosomic 6B line of CS was supplied for the flow sorting of mitotic chromosomes to construct chromosome arm-specific BAC libraries by collecting more than five millions of each chromosome arms. Using the chromosomal DNA extracted from each arm, two BAC libraries with the specificities to 6BS and 6BL, comprising 57,600 and 76,032 BACs with an average insert length of about 130 kb to represent 15.3 and 18 times equivalents of their estimated sizes of each arm, respectively, were successfully constructed.

BAC Contig Construction

For physical mappings of chromosome 3B, chromosome arm 1AL and whole genome of *Aegilops tauschii* Coss., BAC clones were fingerprinted through the method of SNaPshot™ (Paux et al. 2008; Lucas et al. 2013; Luo et al. 2013), which

is an automated fingerprinting technique by sizing restriction fragments of each BAC (Luo et al. 2003). However, restriction fragment sizes shared randomly between two non-overlapping BACs often lead to chimerical contigs or mis-assembled BACs particularly in the large and repetitive genomic regions. Whole Genome Profiling (WGP™) is an alternative method for the establishment of the chromosome physical maps developed based on a next-generation sequencing-based technology (van Oeveren et al. 2011). The efficiency of WGP on physical mappings of wheat chromosomes has been demonstrated by comparison with that of SNaPshot (Philippe et al. 2012).

In our project, chromosome 6B BAC clones were fingerprinted by WGP for a robust physical map. Pooled BAC DNAs were digested with the two restriction enzymes, *Eco*RI and *Mse*I, and the restriction fragments with adaptors were sequenced using the Illumina HiSeq2000 sequencer to yield WGP tags. Deconvolution enables the assignment of the WGP tags to individual BACs. We excluded the low quality BACs during the contig building process in order to construct the physical map with high accuracy. After filtering BACs, assembly of the fingerprints representing nine and ten times equivalent of 6BS and 6BL, respectively, was performed with the FingerPrinted Contigs (FPC) software (Soderlund et al. 1997). A stepwise method was used for the assembly to further improve the quality of BAC contigs, which was defined by Paux et al. (2008) during the construction of chromosome 3B physical map and finally modified by Philippe et al. (2012). The physical maps of chromosome 6B have been successfully established to have an estimated chromosomal coverage of more than 90 %. Overlap analysis between the neighboring clones within BAC contigs enabled us to select minimal tiling path clones along each chromosome arm, which were subjected to the genomic sequencing.

Development of DNA Markers for Anchoring BAC Contigs to the Specific Genomic Regions on Chromosome 6B

DNA markers are essential to anchor BAC contigs onto their specific genomic positions. Information of publically available markers was collected from the databases such as “GrainGenes” and “National BioResource Project-Wheat Japan”. There are only about 200 markers such as SSR and RFLP available currently for chromosome 6B within these databases. Considering the chromosome size and number of BAC contigs, it is obvious that this number of markers is far enough to anchor BAC contigs for a physical map with high chromosome coverage. We thereafter used other public marker resources, like PLUG (PCR-based Landmark Unique Gene) marker, that were EST-PCR markers developed by taking advantage of the syntenic gene conservation between rice and wheat (Ishikawa et al. 2007, 2009). The barley GenomeZipper (chromosome 6H) (Mayer et al. 2011) and syntenic relationship among rice (chromosome 2) and *Brachypodium* (chromosome 3) were also used as a good resource with more than 2,200 markers. Insertion Site-Based Polymorphism (ISBP) marker, which is developed using the junction sequences between

transposable elements and their flanking sequences, has been reported as a powerful tool for wheat studies (Paux et al. 2006). Using our survey sequences (http://wheat-urgi.versailles.inra.fr/Seq-Repository) obtained from 6BS and 6BL, we identified more than 40,000 ISBP marker candidates from both arm survey sequences, and then a higher success rate of markers assigned to chromosome 6B was exhibited than those of other sequence resources (Kaneko et al. in preparation). All markers assigned to chromosome 6B were used for PCR screening of BAC libraries, leading to anchor BAC contigs on the chromosome 6B, which cover about 86 % of the entire chromosome. In general, the ISBP markers were scattered while genic markers tended to be clustered within the gene-baring BAC contigs. These results might reflect the features of wheat genome organization that it is predominantly composed of repetitive elements around small genic region, indicating the efficiency of ISBP markers as anchors because of their randomly distributed pattern on the wheat genome.

The BAC contigs have been assigned to their specific genomic regions using the anchoring markers, which is extremely important because the order of the contigs provides essential information to create a pseudomolecule sequence to reach the final goal of the IWGSC. A genetic map using a recombinant inbred line derived from a cross between CS and winter cultivar 'Mironovskaya 808' (M808) was already developed (Kobayashi et al. 2010), which provided the good frame map with SSRs (Fig. 11.1). We have added the markers mentioned above showing polymorphism between CS and M808 to the 6B genetic map. DNA makers on the genetic map has led to assign BAC contigs covering about 33 % of chromosome 6B in length. Because the pericentromeric region is devoid of recombination event, the marker density of genetic map around the centromere is very low. Aiming to overcome this problem, we employed a method using the chromosome deletion lines caused by the gametocidal (Gc) system or γ-ray irradiation to map more DNA markers on chromosome 6B. The Gc system-induced chromosomal breakage of 6B was derived from a cross between CS with monosomic addition of a chromosome 2C from *Aegilops cylindrica* Host and a nullisomic 6B-tetrasomic 6A (N6BT6A) line of CS. Chromosome mapping using both the new developed deletion lines and previously produced lines by Endo and Gill (1996) led to localize DNA markers more than on 70 loci (Sakaguchi et al. in preparation). Radiation hybrid (RH) mapping is known as a powerful tool for a high-resolution mapping in wheat (Tiwari et al. 2012). The RH panel was produced through a cross between the N6BT6A and CS with the pollen freshly irradiated by γ-ray (Watanabe et al. in preparation). Using the above RH panel, we have successfully assigned the BAC contigs that represented more than 80 % of the entire physical length of chromosome 6B. From the genic marker information, the contig order along the RH map could provide a virtual gene order on the chromosome 6B. The results thus allow us to study the relationship between the position of genic markers present on the chromosome 6B BAC contigs and the rice gene order on chromosome 2, which will reveal the microscale rearrangements with a higher degree of resolution than previously identified by comparative mapping using EST (La Rota and Sorrells 2004).

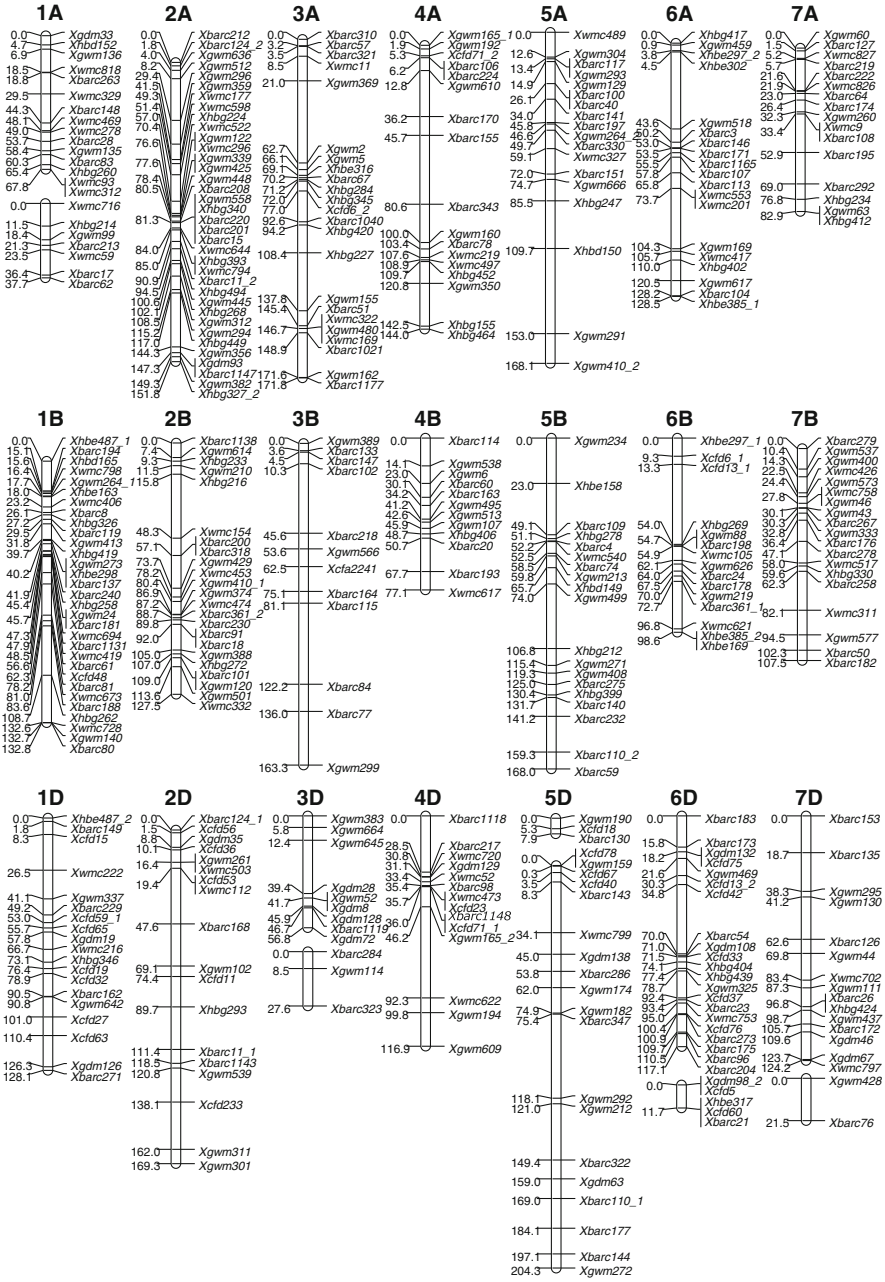

Fig. 11.1 A linkage map of M808 and CS constructed using RILs. Linkage analysis was performed using MAPMAKER/EXP version 3.0b. The threshold for log-likelihood score was set at 3.0, and the genetic distances were calculated with the Kosambi function. The linkage map provides 410 loci of SSR markers, and the total map length is 2814.5 cM with average spacing of 6.9 cM between markers

Concluding Remarks

We have successfully established a physical map of chromosome 6B integrating the BAC contigs and genetic or RH maps using a large number of DNA markers. Our physical map has a high quality and a high resolution, and provides important information on the robust BAC contigs necessary for its genomic sequencing to conduct comparative analysis and prediction of the genomic structure. Currently BAC sequencing is underway using the next-generation sequencer. We will provide a high quality reference sequence to offer an unlimited source on marker development and genome organization of wheat chromosome 6B.

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Part V
Structural and Functional Genomics

Chapter 12
Sequencing of Wheat Chromosome 6B: Toward Functional Genomics

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Abstract International Wheat Genome Sequencing Consortium (IWGSC) decided to adopt the strategy of chromosome sorting and short read assembly to overcome difficulties of wheat genome sequencing derived from the hexaploid status, the large genome size (about 17 Gb) and high repeat contents (more than 80 %). Our Japanese group was responsible for the sequencing of wheat chromosome 6B. Using DNAs from the flow-sorted chromosome arms, we conducted whole-chromosome shotgun sequencing of chromosome 6B. We sequenced more than 12 million reads obtained from the short and long arms by GS-FLX Titanium, and assembled contigs of 235 Mb for 6BS and 273 Mb for 6BL were generated by GS assembler 2.7 (Roche). These assemblies cover 56.6 % and 54.9 % of estimated sizes of 6BS (415 Mb) and 6BL (498 Mb), respectively. We annotated repetitive regions covering more than 80 % of contigs, 4,798 possible expressed loci, and various kinds of RNA genes using our annotation pipeline. We also found the evolutionary conserved regions

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among syntenic chromosomes from four grass genomes. For application of the 6B sequences to wheat genomics, various kinds of markers, such as simple sequence repeat (SSR) and insertion site-based polymorphism (ISBP) markers were constructed. Combination of the marker data with the comparative genome analysis will lay a strong foundation of functional genomics of the group-6 chromosomes in wheat.

Keywords Annotation • Chromosome 6B • Genome sequencing • Marker construction • RNA gene • Synteny

Chromosome by Chromosome Sequencing

Completed rice genome sequence in 2004 by International Rice Genome Sequencing Project was the first cereal genome sequence (IRGSP 2005), and then sorghum and maize genome sequences were followed (Paterson et al. 2009; Schnable et al. 2009). For the rice genome sequencing, because BAC by BAC sequencing method using Sanger sequencing was adopted, the sequencing accuracy was less than one error in 10 kb. This accuracy was validated by genome resequencing by Next Generation Sequence (NGS) data (Kawahara et al. 2013), and it showed that the rice genome sequence is the most accurate one in the cereal genomes sequenced so far. Sorghum genome was determined by whole-genome shotgun sequencing method and maize genome sequence was achieved by the combination of the minimum tiling path (MTP) method and BAC by BAC sequencing. However, their genome sequences were less accurate than rice genome and were still fragmented into many scaffolds.

In *Pooideae*, *Brachypodium distachyon* Bd21 genome was sequenced in 2010 by whole-genome shotgun sequencing method, because of its small genome size (272 Mb) (The International Brachypodium Initiative 2010). However, compared with the *B. distachyon* genome sequencing, the sequencing of other *Pooideae* genomes, such as wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) has fallen behind due to the complexity of their genome structures. First, the wheat and barley genome size was 17 and 5.1 Gb, respectively. They are more than 40-times and 13-times larger than rice genome. Second, repeat regions occupied more than 80 % of the genome hamper their genome assembly. Third, in particular, since wheat is a hexaploidy, it is quite hard to distinguish homoeologous sequences from A, B and D genomes.

To overcome these problems, the various new methods were applied. NGS technology enables us to assemble large sized genome with the low cost. Even if the NGS read length is several hundred bp, millions of NGS reads can be used in one analysis (the total read length is up to several Gb) so that assembly of large genomes can be conducted. For the barley genome sequencing, BAC by BAC sequencing and NGS sequencing methods were combined, and then 1.9 Gbp of the sequences were released in 2012 (The International Barley Genome Sequencing Consortium 2012). In 2013, the genome sequences of *Aegilops tauschii* and *T. urartu* were determined (Jia et al. 2013; Ling et al. 2013; Luo et al. 2013). Wheat genome was also sequenced

by whole-genome shotgun technology with NGS data (Brenchley et al. 2012). However, because of the hexaploidy, whole genome assembly was not achieved as same as other diploid genomes of *Triticeae*.

To solve the genome complexity, chromosome sorting by flow cytometry was developed in cereal genomics (Doležel et al. 2007). This method can reduce sample complexity, such as the hexaploid status of the wheat genome, therefore International Wheat Genome Sequencing Consortium (IWGSC) decided to apply this technologies to their activity. Single chromosomes or chromosome arms were sorted by the flow cytometric analysis and chromosome (arm)-specific BAC libraries were constructed. Progress of physical map construction and genome sequencing of each chromosome and chromosome arms can be seen on the IWGSC website (http://www.wheatgenome.org/) and URGI wheat portal site (http://wheat-urgi.versailles.inra.fr/).

Survey Sequencing and Annotation of Chromosome 6B

Under the framework of IWGSC, sequencing project of chromosome 6B was started in Japan in 2011 and the first survey sequences of 6B was released in 2013 (Tanaka et al. 2014). In this analysis, the DNA libraries of sorted 6B chromosome arms were constructed and sequenced independently using the 454 GS-FLX Titanium (Roche, CT, USA). The sequence reads (454 reads) from each arm were assembled by GS assembler 2.7 (Roche). From more than 12 million reads for each arm, 234 and 273 Mbp were assembled comprising 262,375 and 173,655 contigs for 6BS and 6BL, respectively. They correspond to 56.6 % and 54.9 % of the estimated lengths of both arms (415 Mbp for 6BS and 498 Mbp for 6BL).

As described before, the wheat genome is composed of abundant repetitive elements. Known classes of repeat elements were detected using the repeat libraries, such as TREP and MIPS repeat libraries. In addition, to detect novel repeat elements, we constructed a new repeat library by RepeatModeler (http://www.repeatmasker.org/RepeatModeler.html). Using a repeat masking program, censor (http://www.girinst.org/censor/index.php) with TREP and the new repeat library (Jurka et al. 1996), 76.6 % and 85.5 % of 6BS and 6BL assembly were masked, respectively. Since 63.6 % and 72.2 % of 6BS and 6BL assemblies were masked by TREP library, around 13 % of repetitive regions may be novel repeat elements detected only by the new library.

After repeat detection, we identified transcribed regions by mapping many transcripts in public domains. In addition to mRNA and millions of ESTs in DDBJ/EMBL/GenBank, wheat full-length cDNAs (FLcDNAs) were available from TriFLDB (http://trifldb.psc.riken.jp/index.pl) (Mochida et al. 2009). In combination with transcriptome mapping and an ab initio gene prediction program, 4,798 transcribed regions were determined. We found several genes that were known to locate on chromosome 6B, such as α-gliadin gene, the stripe rust resistance gene *Yr36*, the grain protein content gene *Gpc-B1*, α-amylase gene, the genes for three

low-temperature-responsive dehydrins, *Wcs120*, *Wcs66* and *Wcor410*, the flowering time gene *TaHd1-2* and the gene involved in vernalization *TmVIL2*.

Our assemblies also showed the conservation of syntenic genes between monocots. First, 2,399 of 2,573 high-confidence barley genes on chromosome 6H could be mapped on our assemblies (E value $<10^{-5}$). Second, 3,772 syntenic loci were detected from homology search of syntenic genes from chromosome 2 of *O. sativa*, chromosome 3 of *B. distachyon* and chromosome 4 of *S. bicolor*. Since 57.4 % of the syntenic regions had wheat transcriptome evidence, which was significant higher than that of non-syntenic regions (32.7 %), we concluded that wheat 6B has a conserved synteny with the chromosomes of other grass species.

Our annotation pipeline included detection of RNA genes, rRNAs, tRNAs, and miRNAs. It is known that chromosome 6B has a locus for ribosomal DNA (rDNA) containing approximately 5,500 rRNA genes. Moreover, non-protein coding RNAs, such as microRNAs (miRNAs) are currently recognized as biologically important genetic components. We found that some RNA genes were associated to a particular repetitive element. For example, 83 of 131 $tRNA^{Lys}$ were located in an LTR retrotransposon, Gypsy, and *de novo* repeats. Almost predicted miRNAs were also located in repeat-masking regions, especially DNA transposons, Mariner and CACTA. In case of rRNA genes, the quite small number of contigs with rRNA genes could be explained by high read depth of contigs. Because of the high sequence similarity, rRNA regions were degenerated during the assembly so that a few contigs with high depth reads existed in our data. This result is quite similar to that of repetitive regions. These results suggested that RNA genes were distributed in the wheat genome with the diffusion of transposons and repetitive elements

Application of Chromosome 6B Sequences to Wheat Genomics

Decipher of genome sequences enables us not only to know representative gene set containing many novel genes, but also to prepare resources for genomics and breeding, such as maker information. In case of wheat, chromosome information is quite useful to distinguish homoeologous genes. For example, there are three homoeologs of flowering time genes, *TaHd1-1*, *TaHd1-2* and *TaHd1-3*. Our 6B assembly can distinguish *TaHd1-2* transcribed from 6B and other two homoeologs from 6A and 6D in the sequence similarity level. In addition, since exon-intron structures are determined on wheat genomes, constructions of transcript-based markers, such as PLUG markers, are easier and more accurate than the previous situation using rice genome data.

Insertion site-based polymorphism (ISBP) marker can be constructed using genome sequences (Paux et al. 2010). Genome wide survey of simple sequence repeat (SSR) is applied to construct SSR markers on non-genic regions that have not been focused by the transcript-based marker constructions. As same as the genome

zipper analysis (Mayer et al. 2009, 2011), virtual order of the markers would be speculated by sequence homology of the flanking regions of the markers to closely related species, such as barley and *Brachypodium*. In fact, we found 16,728 SSRs on non-repetitive regions of 6B and at least 1,354 SSRs of them were positioned on barley chromosome 6H. Since more than 80 % of the SSRs were located in intergenic regions of 6H, the new SSR markers can be efficiently used for the gap filling between known markers.

Survey sequences of wheat chromosome 6B provided the various types of novel information, e.g. repeat information, genome annotation including genes and RNA genes, and marker information. However the current genomic sequences of the chromosome 6B are fragmented and not completely covered so that improvement of genome assembling should be needed. Sequencing of chromosome 6B is ongoing with MTP method and BAC by BAC sequencing using Roche 454, and more accurate and physical positioned sequences will be available in near future.

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Chapter 13
Genetic Mechanisms of Vernalization Requirement Duration in Winter Wheat Cultivars

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Abstract Wheat is cultivated across more land area than any other grain crops. Wheat cultivars are classified as two general types: winter wheat with variable low temperature requirement for a proper flowering time (vernalization) and spring wheat without the requirement, based on their qualitative vernalization requirement. Winter wheat cultivars are classified as three types, weak winter, semi-winter and strong winter, according to their quantitative vernalization requirement to reach a vernalization saturation point or achieve the maximum vernalization effect. Three vernalization genes, *VRN1*, *VRN2*, and *VRN3*, were cloned using a positional cloning approach and a one-gene model of qualitative variation in vernalization requirement between spring and winter wheat. A major gene for the vernalization requirement duration in winter wheat was mapped using a population of recombinant inbred lines (RILs) that were generated from two winter wheat cultivars, 'Jagger' and '2174'. Furthermore, the cloning population was developed using a RIL to backcross with 2174, which was segregated in a 3:1 ratio of the early flowered plants and the late flowered plants after the population was vernalized for 3 weeks. The wild type Jagger *vrn-A1a* allele for less vernalization was dominant over the 2174 *vrn-A1b* allele for more vernalization, and the two alleles encoded the vrn-A1 proteins with two point mutations. A third haplotype with one of the point mutations was found in common wheat. Gene markers were developed to direct breeding of semi-winter and strong winter wheat cultivars to adapt to different geographical areas and changing climates.

Keywords Flowering time • Gene cloning • Semi-winter wheat • Vernalization requirement duration • Winter wheat

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Wheat (*Triticum aestivum*, 2n = 6x = 42, AABBDD) is one of the mostly cultivated crops in different geographical areas in the world. Wheat is traditionally divided into two types, winter wheat with vernalization requirement and spring wheat without this requirement. Vernalization is an exposure of the plant to a few weeks of low temperature in order to accelerate its ability to transition from vegetative to reproductive development, providing an adaptive mechanism for winter wheat to synchronize its developmental transition with seasonal changes in temperature (Rawson et al. 1998). Winter wheat is sown in autumn, whereas spring wheat is sown in autumn or spring season. Advances in understanding the genetic basis and molecular mechanisms of winter wheat development are critically important to ensure that winter wheat flowers at a proper time.

Vernalization has significant effects at typically 2–10 °C, with dramatic decline at temperatures above 11 °C and an apparent loss of effects above 18 °C (Brooking 1996). Based on their various vernalization requirement durations with a combination of the amplitude and duration of low temperatures in different geographic areas, winter wheat cultivars are typically categorized into three types: a weak winter type that is stimulated to flower by brief exposure to low temperature, a semi-winter type that requires 2–4 weeks of cold exposure for flowering, and a strong winter type that needs 4–6 weeks of cold exposure (Crofts 1989). The winter wheat cultivar Yeoman reportedly requires up to 12 weeks at low temperature to attain a vernalization saturation point (Berry et al. 1980).

Recent studies suggest that global climate will inevitably produce significant changes in air temperatures (Körner and Basler 2010). Average global surface air temperature rose 0.5 °C in the twentieth century or 4–7 °C in the past million years between the ice ages and the warm interglacial periods (Solomon et al. 2007). The global mean temperature is projected to continue its increase by roughly 3 °C (Kerr 2007) or 5 °C by the end of the twenty-first century (Semenov and Halford 2009). Various crops, requiring specific environmental cues for growth and development, will inevitably respond differently to changing climate (Craufurd and Wheeler 2009; Lanning et al. 2010). As various simulation models have shown, winter wheat is more vulnerable to changing climate due to its higher sensitivity to temperatures for proper flowering time and successful grain reproduction (Morison and Long 1995). Higher temperatures in a winter season will lead to insufficient or failed vernalization hence delayed reproductive development for successful grain production of winter wheat.

Three genes controlling vernalization requirement in wheat were cloned, including *VRN1* (Yan et al. 2003), *VRN2* (Yan et al. 2004), and *VRN3* (Yan et al. 2006). *VRN1* is an orthologue of *AP1* in *Arabidopsis*, and a dominant *Vrn1* allele originated from mutations in the promoter or first intron of a recessive wild type *vrn1* gene. *VRN2* encodes a novel transcription factor containing a Zinc finger and a CCT domain, and a recessive *vrn2* allele had a point mutation in coding region resulting in an alteration of an amino acid at the CCT domain or complete deletion, compared to the dominant allele as a wild type. *VRN3* is an orthologue of *Arabidopsis FT*, and allelic variation at *VRN3* is related with mutations in its promoter or intron one. *VRN1* and *VRN3* were cloned in populations that were segregated as a 3:1 ratio of

early and late flowering plants, indicating that both *VRN1* and *VRN3* are promoters of flowering. *VRN2* was cloned in a population that was segregated as a 1:3 ratio of early and late flowering plants, indicating that this gene is a repressor of flowering. Heading date in each of these populations was controlled by one gene, whereas the other two were fixed at the same allele.

In our previous studies on winter wheat, we found that the *vrn-A1* locus was associated with variation in the stem elongation in the winter wheat Jagger × 2174 RIL population (Chen et al. 2009), and this locus influenced subsequent timing of heading and physiological maturity when characterized in the field for 3 years (Chen et al. 2010). This locus is very sensitive to temperature change and bears close association with variation in development in the winter wheat population that may be caused by vernalization requirement duration across years. The present study aimed to genotype wheat germplasm and to establish genetic models accounting for strong winter, semi-winter, and weak winter wheat cultivars.

Materials and Methods

In our previous study, we found a C/T polymorphism in exon 4 that is responsible for the amino acid change at Leu^{117}/Phe^{117} in *Ta*VRN-A1 between the Jagger allele and the 2174 allele. A PCR marker for this polymorphism showed an association with developmental variation in the Jagger × 2174 RIL population tested in the field (Chen et al. 2009). Primers used for the SNP in exon 4 are vrn-A1F4F 5′-CAACTTGTTTGGGACTAAAGGC-3′ and vrn-A1F4R 5′-CTGCAACTCCTTGAGATTCAAAG-3′. PCR was performed for 40 cycles (90 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s per cycle) followed by a 10-min final extension at 72 °C. PCR products were digested with *Dpn*II and run on a 1 % agarose gel.

In this study, we found that there was another SNP in exon 7, and we developed the PCR marker for the SNP in exon 7 to screen wheat germplasm available in our laboratory. The hexaploid wheat cDNA sequences in GenBank for each of three homoeologous *VRN1* genes were divided into three groups, and genomic DNA sequences for each group were derived from the wheat genome sequence database (http://www.cerealsdblished.uk.net/). Specific primers for each homoeologous *VRN1* were designed. Chinese Spring nullitetrasomic lines, N5AT5D, N5BT5D or N5DT5B missing each of three homoeologous group five chromosomes were used to determine specificity of the primers. Primers vrn-A1F7B (5′-GTGGAGAAGCAGAAGGCGCATG-3′) and vrn-A1R7 (5′-CCGACAGAACTGCATAGAGACC-3′) were designed to detect the SNP encoding A^{180}/V^{180} between the Jagger *vrn-A1a* allele and 2174 *vrn-A1b* allele. The two primers amplified a 221 bp fragment using an annealing temperature of 55 °C and extension time for 1 min. The PCR products digested with restriction enzyme *Sph*I were run on a 1 % agarose gel, showing polymorphic bands between the *vrn-A1a* allele (199 bp) and *vrn-A1b* allele (221 bp).

Results and Discussion

The vrn-A1 *Gene Controlling Vernalization Requirement Duration in Winter Wheat Cultivars*

Jagger required 3 weeks to reach the maximum vernalization effect on flowering, whereas 2174 required 6 weeks to reach the maximum vernalization effect on flowering under the same condition (Li et al. 2013). We generated a BC_1F_2 population using RIL23 to backcross with 2174. When vernalized for 3 weeks, heading date of 90 F_2 plants showed a clear segregation. On average, 24 plants homozygous for the Jagger *vrn-A1a* allele headed at 110 days after planting, 20 plants homozygous for the 2174 *vrn-A1b* allele headed at 138 days, and 46 plants heterozygous at *VRN-A1* headed at 118 days (Fig. 13.1a). The 70 plants either homozygous or heterozygous for the Jagger *vrn-A1a* allele for early heading showed a significant difference from the 20 plants homozygous for the 2174 *vrn-A1b* allele for late heading ($p<0.001$). The observed segregation ratio between the earlier heading and later heading groups was not significantly different from a 3:1 ratio ($X^2=0.37$, df = 1, $p=0.54$) and fit a one-gene model.

A Critical Point Mutation in vrn-A1 at the Protein Level

We used the positional cloning strategy to prove that quantitative vernalization requirement in winter wheat is controlled by vrn-A1 at the protein level (Li et al. 2013). There were 29 SNPs in *vrn-A1* between the Jagger allele (11,922 bp, JQ915055) and the 2174 allele (11,921 bp, JQ915056), including 464 bp from the start codon for translation, the complete gene from the start codon and the stop codon, 41 bp after the stop codon. These SNPs were confirmed by sequencing two

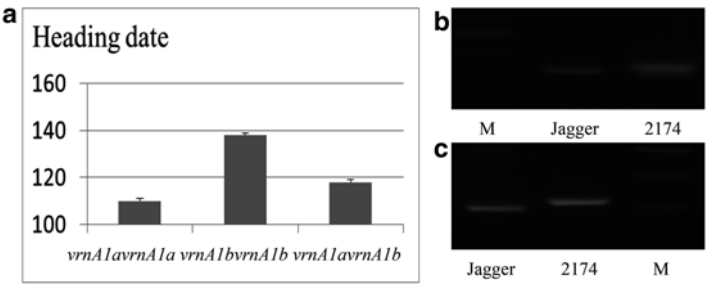

Fig. 13.1 Allelic variation and genetic effects of *vrn-A1*. (**a**) The segregation of heading date in plants carrying different *vrn-A1* alleles in a BC_1F_2 population. *vrn-A1a* for the Jagger allele and *vrn-A1b* for the 2174 allele. (**b**) PCR marker for the SNP in exon 4 of *vrn-A1* using primers vrn-A1F4F and vrn-A1F4R. (**c**) PCR marker for the SNP in exon 7 of *vrn-A1* using primers vrn-A1F7F and vrn-A1F7R

independent PCR products or digesting PCR products using restriction enzymes wherever appropriate. No difference was observed between the Jagger *vrn-A1a* allele and 2174 *vrn-A1b* allele in the previously identified regulatory sites in the promoter or intron one that accounted for allelic variation between the winter *vrn-A1* allele and the spring *Vrn-A1* allele.

There were two SNPs in exon 4 and exon 7, both of which resulted in an altered amino acid in the conserved domain between the Jagger and 2174 alleles. An amino acid Ala^{180} encoded in exon 7 in the Jagger *vrn-A1a* allele controlling less vernalization or early flowering was mutated to Val^{180} in the 2174 *vrn-A1b* allele. The Ala^{180}/Val^{180} substitution accounted for the differential interactions of vrn-A1a and vrn-A1b with *Ta*HOX1 in pull-down assays and protein immune-precipitation analyses (Li et al. 2013).

A Novel Haplotype of vrn-A1 at the Protein Level

The *vrn-A1* sequences were used to search GenBank expressed sequence tags (EST) databases. Diploid wheat *T. urartu* (DQ291016) and tetraploid wheat *T. turgidum* ssp. *durum* (AY747598) have the same sequences at SNPs at both exon 4 and exon 7 as Jagger (Fig. 13.2), suggesting that the *vrn-A1a* allele in Jagger is the wild type. This result was confirmed using a PCR marker for another two accessions of diploid wheat *T. urartu* and 11 accessions of tetraploid wheat *T. turgidum* ssp. *durum*, ssp. *dicocoides*, and ssp. *dicoccum* (Table 13.1).

There are five hexaploid wheat cultivars, which *VRN-A1* sequences are deposited in GenBank (Fig. 13.2). Chinese Spring was found to carry the *vrn-A1a* allele. Two winter wheat cultivars, Norstar and Recital, were found to carry the *vrn-A1b* allele. Norstar carrying the *vrn-A1b* allele was one of the most cold-hardy wheat cultivars. Interestingly, another two cultivars, IL369 and Nongda 3338, were found to have a novel haplotype at the protein level. The two cultivars have the mutation at Ala^{180}/Val^{180} but not at Leu^{117}/Phe^{117}. Therefore, the third haplotype differing from the

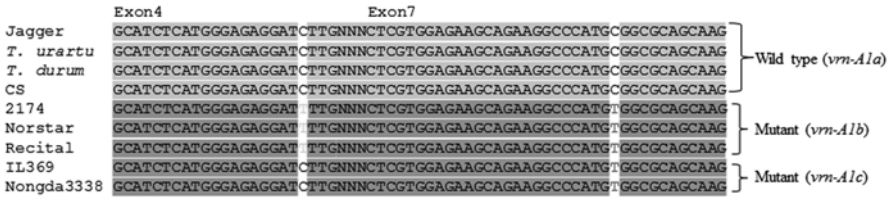

Fig. 13.2 Multiple sequence alignment of the *VRN-A1* genes in different wheat species. The sequences of Jagger and 2174 were obtained in this study, and other sequences were from GenBank. *T. urartu*: DQ291016; *T. durum*: Langdon, AY747598; CS: Chinese Spring, AM502869; Norstar, DY761363; Recital: CD872434, CD936812; IL369: AY747599; Nongda 3338: DQ512342. The two SNPs in exons 4 and 7 resulting in alternation of amino acids are *highlighted*. N indicates sequences between exon 4 and exon 7

Table 13.1 The *vrn-A1* alleles in wheat cultivars

Allele		Species	Cultivar/line	Source
vrn-A1a	L^{117}/A^{180}	2X	DQ291016	GenBank
			PI428180, PI428183, PI428323	NGRP
		4X	AY747598	GenBank
			Rugby and Maier (ND)	CAP
			PI14082, PI113393, PI191654, PI265004, PI286061, PI347135, PI352541, PI366990, PI384392	NGRP
			Chinese Spring (AM502869)	GenBank
			Jagger (OK, NE), UC1110 (CA), ID0556 and Zak (ID), McNeal and Thatcher (MT), Louise and Panawawa (WA), GRN*5/ND614-A (MN), Heyne and KS01HW163-4 (KS), Jupeteco (TX)	CAP
		6X	Jagalene, Overley, Santa Fe, Shocker, Protection	SGT
vrn-A1b	F^{117}/V^{180}	6X	Norstar (DY761363), Recital (CD872434, CD936812)	GenBank
			2174 (OK), CIMMYT-2 (PI610750) (CA), Platte and CO940610 (CO), SS550 and PIONEER 26R46 (GA), Rio Blanco and IDO444 (ID), Harry and Wesley (KS), Stephens and OR9900553 (OR), TAM105 (NE), P91193 and P92201 (IN), NY18/ Clark's Cream 40–1 (MN), Pio 25R26 and Foster (OH), Cayuga and Caledonia (NY), Weebill (TX), USG3209 (VA), Finch and Eltan (WA)	CAP
			Above, Centerfield, Custer, Cutter, Danby, Deliver, Doans, Duster, Endurance, Fannin, Fuller, Guymon, Hatcher, Intrada, JEI 110, Lakin, Neosho, Okfield, Ok102, Ripper, TAM 110, TAM 111, TAM 112, Trego	SGT
vrn-A1c	F^{117}/A^{180}	6X	IL369 (AY747599), Nongda 3338 (DQ512342)	GenBank
			Jaypee (VA), Pio 26R61 (NY)	CAP
			OK Bullet	SGT

CAP coordinated agriculture project, *SGP* southern great plains, *NGRP* national genetic resources program (NGRP), U.S.

Jagger *vrn-A1a* allele and the 2174 *vrn-A1b* allele was referred to the *vrn-A1c* allele. These observations suggested that mutations from the wild type *vrn-A1a* allele to the *vrn-A1b* allele occurred twice, and the *vrn-A1c* allele is an intermediate type between them. The mutation in exon 7 should occur preceded to the mutation in exon 7 of *vrn-A1*.

The disassociation between Leu^{117}/Phe^{117} and Ala^{180}/Val^{180} suggested that the previously reported marker for Leu^{117}/Phe^{117} (Fig. 13.1b) (Chen et al. 2009) cannot be used for the Ala^{180}/Val^{180} mutation found in this study. A new marker has been devel-

oped for Ala^{180}/Val^{180} PCR markers for *vrn-A1* were developed to distinguish the SNP in exon 7 (Fig. 13.1c) between the Jagger and 2174 alleles.

Diverse VRN-A1 Proteins in Winter Wheat and Spring Wheat Cultivars

The two PCR markers, the SNPs in exon 4 and exon 7 between the Jagger and 2174 alleles, were used to determine the genotypes of wheat germplasm (Table 13.1). Among 73 cultivars of hexaploid wheat, 18 cultivars carry the *vrn-A1a* allele, 50 cultivars carry the *vrn-A1b* allele, and 5 cultivars carry the *vrn-A1c* allele. The higher frequency of the *vrn-A1b* allele in contemporary wheat cultivars indicated that breeders in the southern Great Plains have inadvertently selected this allele, contributing to delay stem elongation and extend the vegetative phase of the dual purpose wheat to produce more biomass for cattle.

Five cultivars carrying the *vrn-A1c* allele, Jaypee (VA), Pio 26R61 (NY), OK Bullet, IL369, and Nongda 3338, do not have the same pedigree, indicating that the *vrn-A1c* allele in these cultivars has evolved independently. The possibility that these cultivars have the same tetraploid wheat donor carrying the *vrn-A1c* allele cannot be excluded, though none of the tetraploid wheat accessions tested in this study carried the *vrn-A1c* allele.

When vrn-A1 *acts as a protein form, it should appear as a* VRN-A1-*Ta*HOX1 protein complex because of a direct binding between them. VRN-A1 protein and *Ta*HOX1 protein should also have direct interaction in spring wheat cultivars. It was found that both *vrn-A1a* and *vrn-A1b* existed in spring wheat cultivars. Nine spring wheat cultivars in the CAP group carry the *vrn-A1a* allele, including UC1110 (CA), ID0556 and Zak (ID), McNeal and Thatcher (MT), Louise and Panawawa (WA), GRN*5/ND614-A (MN), Jupeteco (TX); whereas four spring wheat cultivars carry the *vrn-A1b* allele, including CIMMYT-2 (PI610750) (CA), OR9900553 (OR), NY18/ Clark's Cream 40–1 (MN), and Weebill (TX).

Allelic variation in the dominant *Vrn-A1* locus also indicated pleiotropic genetic effects in spring wheat cultivars (Blake et al. 2009; Santra et al. 2009; Zhang et al. 2008), supporting that the *VRN-A1* gene has different mechanisms in controlling wheat development. There were three CAP populations, UC1110 and CIMMYT-2 (PI610750) (CA), GRN*5/ND614-A and NY18/ Clark's Cream 40–1 (MN), and Jupeteco and Weebill (TX) that have different VRN-A1a and VRN-A1b forms. It would be interesting to investigate how allelic variation in VRN-A1 is associated with variation in development in spring wheat populations. Similarly, Jaypee (VA) and Pio 26R61 (NY), which have a VRN-A1c form, can be used to investigate effects of a single point substitution Ala^{180}/Val^{180} on development in wheat.

Application of Multiple Molecular Markers for VRN-A1

Molecular breeding is a new strategy that uses contemporary methods of molecular genetics and genomic sequencing to assist in all steps throughout the procedure of conventional breeding. Most of the molecular markers currently being applied in breeding are SSR (simple sequence repeat) markers due to their relative simplicity for breeding applications. However, the 'repeat' feature of a SSR marker results in multiple and inconsistent locations of the same marker among divergent wheat cultivars. SNP markers would provide more powerful tools to assist selection in breeding (Akhunov et al. 2009). In most cases, however, a SNP marker is derived from the sequence of an EST that is randomly distributed among genomes; thus a SNP marker may not associate with a given trait. A gene marker (perfect marker) that is developed for the specific regulatory site of a functional gene can provide the ultimate resolution to select for a given trait.

Four markers have been developed for allelic variation in *VRN-A1*. It is not yet known if the allelic variation in exon 4 is associated with any trait, though the point mutation occurred in the K-box, a critical domain for protein-protein interaction. Two markers for allelic variation in the promoter (Yan et al. 2004) and intron one (Fu et al. 2005) should be used to distinguish between spring wheat and winter wheat, whereas the marker for the SNP intron 7 should be used to distinguish semi-winter and strong winter wheat cultivars.

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Chapter 14
Building Ultra-Dense Genetic Maps in the Presence of Genotyping Errors and Missing Data

Yefim Ronin, Dina Minkov, David Mester, Eduard Akhunov, and Abraham Korol

Abstract Recent advances of genomic technologies have opened unprecedented possibilities in building high-quality ultra-dense genetic maps. However, with very large numbers of markers available for a mapping population, most of the markers will remain inseparable by recombination. Real situations are also complicated by genotyping errors, which "diversify" a certain part of the markers that would be identical in error-free situations. The higher the error rate the more difficult is the problem of building a reliable map. In our algorithm, we assume that error-free markers can be selected based on the presence of "twins". There is also a probability of an opposite effect, when non-identical markers may become "twins" because of genotyping errors. Thus, a certain threshold is introduced for the selection of markers with a sufficient number of twins. The developed algorithm (implemented in MultiPoint software) enables mapping big sets of markers (~10^5–10^6). Unlike some other algorithms used in building ultra-dense genetic maps, the proposed "twins" approach does not need any prior information (e.g., anchor markers), and hence can be applied to genetically poorly studied organisms.

Introduction

Recent advances of genomic technologies have opened unprecedented possibilities in building high-quality ultra-dense genetic maps. However, with very large numbers of markers available for a mapping population, most of the markers will remain inseparable by recombination and will represent groups of co-segregating, or absolutely linked markers (AL markers). In such cases, only one marker from each

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group could be placed on the map that can be referred to as a framework, skeleton, or bin map; the remaining markers can then be attached to the skeleton map (Mester et al. 2003; Korol et al. 2009; Ronin et al. 2010). The real situation is significantly complicated by genotyping errors, which "diversify" a certain part of markers that would be identical in the ideal situation of no errors. The higher the error rate and the number of markers, the more difficult it is to build a reliable map (Buetow 1991). An additional complication is when a part of data points is missing, which is common in the genotyping-by-sequencing (GBS) approach and cannot always be compensated for by the imputation of missing scores.

Several approaches have been suggested for constructing high-density genetic maps aimed at overcoming the aforementioned difficulties. The dominating strategy includes various ways of building hierarchical framework maps (Isidore et al. 2003), e.g. by combining the irresolvable markers of a linkage group into bins (groups of "bound together markers") in the first phase followed by joint ordering of the representatives of these groups and singleton markers. Our approach to the ordering problem is based on reducing it to the traveler salesperson problem (TSP) and employing Guided Evolutionary Strategy heuristics for building the framework or skeleton map (Mester et al. 2003, 2010; Ronin et al. 2010). An interesting alternative possibility of phasing the mapping analysis is by constructing a minimum spanning tree of a graph followed by improvement of the initial solution based on TSP-inspired heuristics (Wu et al. 2008). For situations of ultra-dense mapping, with thousands and dozens of thousands of markers per chromosome "contaminated" by typing errors, we propose a simple "twins" approach for selecting reliable skeletal markers. Combined with our powerful discrete optimization heuristics, this approach enables the mapping of very big sets of markers (e.g. 10^5), i.e., suitable to wheat genotyping with the 90 K iSelect chip as well as with the GBS approach. The corresponding algorithms implemented in MultiPoint software were intensively tested using simulated data and a set of 420,000 SNP and GBS markers of a wheat DH population.

Geometry of Genotyping Space in the Presence of Marker Typing Errors

The sample size (N) of mapping populations limits the marker density in the map. Thus, for a DH population with $N=200$, the minimal non-zero recombination rate between two adjacent markers cannot be less than 0.5 %. In the absence of errors, all markers should appear in AL groups, with the distance between the groups ≥0.5 cM. Typing errors will lead to the erosion of these groups into "clouds" of falsely different markers. Figure 14.1 illustrates the formation of such a cloud from a set L of 11 AL markers in a multi-dimensional space of markers scored for a sub-sample of 16 individuals from the mapping population. In an ideal error-free situation, all 11 markers would vary identically across the shown 16 genotypes: in the 16-dimensional space these markers are in the same state (**aababbbaaaabbaba**)

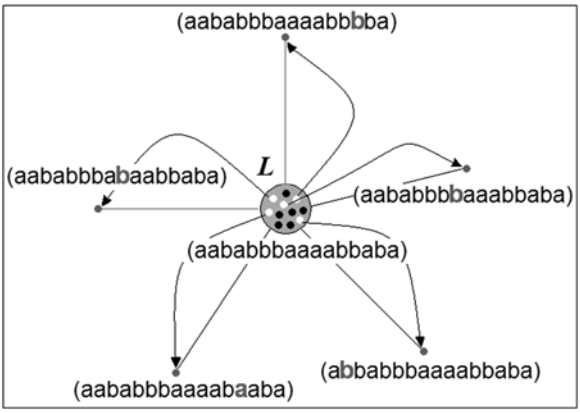

Fig. 14.1 A geometric model of erosion of AL marker groups due to scoring errors (only 11 markers scored for 16 genotypes are shown)

and belong to the set *L* (represented as dots within the grey circle). Due to typing errors, some of the markers change their 16-dimensional states and leave the set *L* (white holes); corresponding genotypes will be erroneously recorded as "recombinants". The problem is how to select markers for building a reliable genetic map in a challenging situation when the data set includes thousands of markers per chromosome while a certain proportion of markers are contaminated by erroneous data points and a part of the data points are missing.

The Proposed Method and Algorithm

We propose a method of addressing these problems based on a simple idea that with very large numbers of scored markers (e.g., thousands or dozens of thousands per chromosome) and small-to-moderate population size, many markers will be irresolvable by recombination and should appear as groups of AL markers. But some of AL markers will appear as "recombinants" if even a small proportion of scores per marker are erroneous. Thus, we can trust more markers from groups of absolutely linked markers compared to singleton markers. For sample size N and a proportion of genotyping errors p per marker, the probability that in all individuals both alleles of a marker will be unmistakably identified can be estimated under the assumption that the typing errors are independent, as $P = (1\text{-}p)^N \approx e^{-Np}$. Assuming 1 % error rate within a group of AL markers, about a third will still remain error-free. In a DH population of $N = 100$ individuals, for a chromosome length of 100 cM the minimum interval length will be 1 cM. Consequently, the density of the map cannot be greater than 101 markers. If we genotyped 10,000 markers of this chromosome, only 100 markers (referred to as skeletal markers) can be ordered, whereas the rest will remain absolutely linked to the skeletal markers. Thus, for building a skeleton map one can select presumably error-free markers based on the presence of "twins" in the sample, although there is also a small probability that non-identical markers may become "twins" because of genotyping errors. Therefore, a certain threshold is

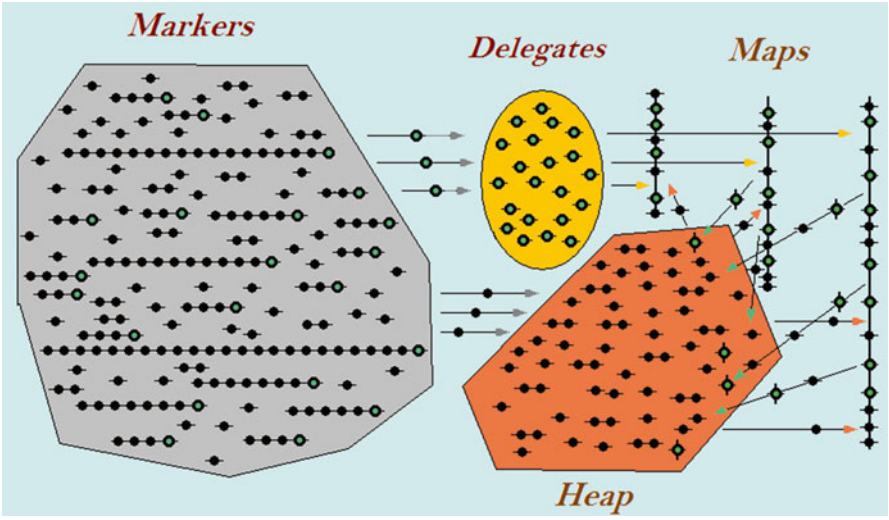

Fig. 14.2 Scheme of the "twin" algorithm. Illustrated is the marker information flow in the process of map construction

introduced in our algorithm for the selection of markers with a sufficient number of twins. In regions with a lower density of recombination events (e.g., affected by the centromeric effect on recombination), the map will be less affected by typing errors.

The major steps in our algorithm for building ultra-dense genetic maps (Fig. 14.2) implemented in MultiPoint software (www.multiqtl.com) include: **(a)** Forming groups of markers with zero distance and selecting a "delegate" from each group containing no fewer twins than the predefined threshold (equal 3 in Fig. 14.2); **(b)** Except for twins of the candidates, all remaining markers are removed to the Heap; **(c)** Clustering the delegate markers and ordering the obtained linkage groups (LG); **(d)** Filling gaps and extending LG ends using markers from Heap; **(e)** Removal of markers violating map stability and monotonic growth of distance from a marker and its subsequent neighbors.

Results and Discussion

Various algorithms have been proposed for building dense genetic maps, including the stepwise increase of the map density (Jansen et al. 2001; Isidore et al. 2003; Mester et al. 2003, 2010; Wu et al. 2008). This problem becomes especially challenging with the current widespread transition from a few hundred to tens or even hundreds of thousands of typed markers per genome. It is well recognized that in such a reality even 1 % of typing errors may lead to a dramatic reduction of map quality, i.e., "more" (markers) may imply "less" (confidence in map quality, at least on a microscale). The problem includes a few aspects: (i) computational

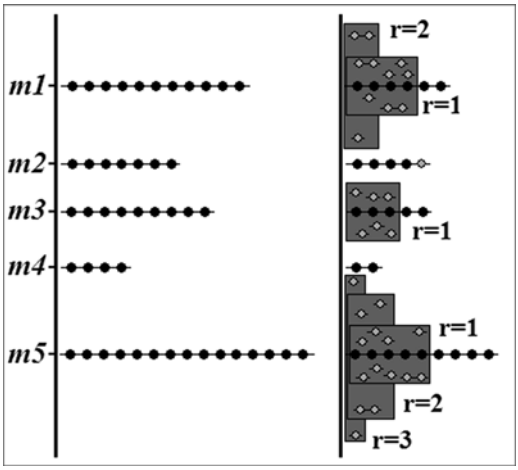

Fig. 14.3 The structure of clouds with markers with scoring errors

complexity, related to the exponential growth of the number of potential marker orders to be tested, (ii) the impossibility to resolve the vast majority of markers by recombination under reasonable population sizes, and (iii) high impact of typing errors on map quality. Our approach is based on the assumption that upon high excess of irresolvable compared to resolvable markers and a low level of typing errors, members of "twin" groups with minimum missing scores can be considered as more credible markers compared to singleton markers.

For an illustration of the efficiency of our "twins" approach, two examples are provided here: simulated data for one chromosome with 10,000 markers for a DH population with N = 200 (two variants of the same marker set were considered, with and without marker typing errors), and real DH data on ~24,000 markers of wheat chromosome 3B (the whole genome set included ~420,000 markers). In the first example, the map length was 212 cM. For error-free data, the skeleton map included 197 markers. For data with 1 % typing errors, about 1/8 of the markers appear as AL groups, while 7/8 of the markers appear as clouds surrounding AL groups, as explained in Fig. 14.1 and illustrated by Fig. 14.3 (grey dots). Figure 14.3 illustrates the distribution of markers with errors relative to the skeleton map (when it is known, as with simulated data).

The analysis of simulated data with 1 % errors (Table 14.1) demonstrates how a meaningful map can be obtained for such data when nothing is known about the order of markers, which is a standard situation with non-model species. Obviously, the result may depend on the threshold size of the AL groups to be represented in the skeleton map. Thus, with threshold = 4, AL groups with two and three markers are excluded from consideration together with singletons (moved to heap) and the first variant of the skeleton map is constructed (stage 1 of the procedure). Stage 2 is cleaning the map. MultiPoint package enables the detection and removal of markers violating the order stability and monotonic growth of distances in the skeleton map (Ronin et al. 2010). After cleaning, markers from the heap can be checked as candidates for filling in the gaps (if gaps are present in the obtained skeleton map). The

Table 14.1 Building dense multilocus maps based on selection of twin markers

Stage		Threshold size of AL groups		
		2	3	4
1	M	318	122	98
	L	384	218	208
2–3	M	158	141	145
	L	218	219	218

M number of markers in the skeleton map, *L* skeleton map length (cM), the skeleton map build using error-free marker data included 197 markers (L = 212 cM)

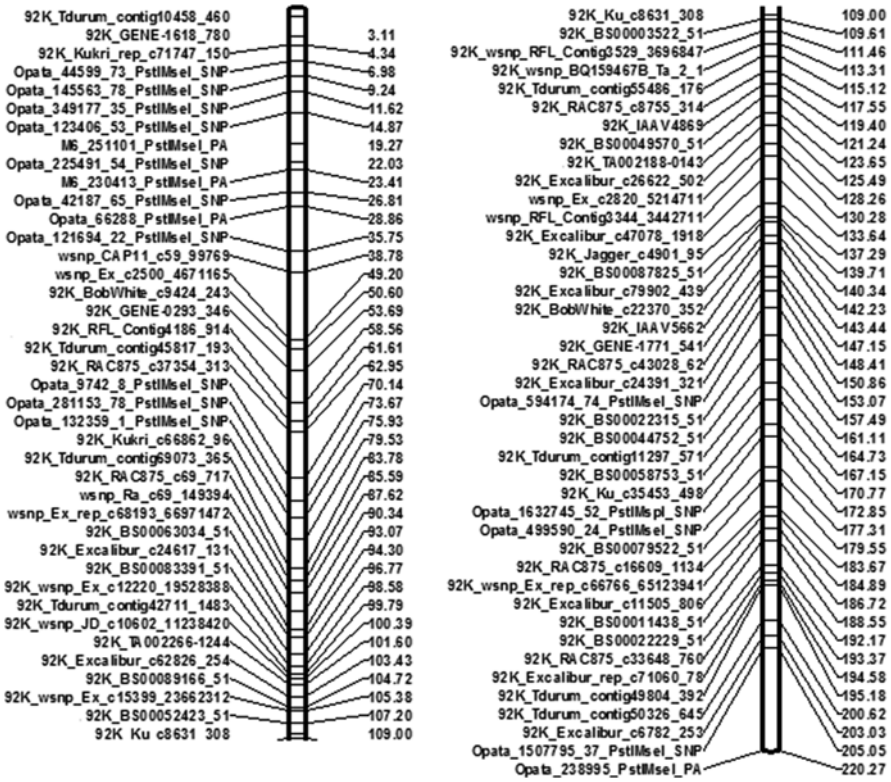

Fig. 14.4 Map of wheat chromosome 3B, the largest in the wheat genome (the figure is split into two parts to fit the page size limits)

results in Table 14.1 show a relatively weak dependence on the arbitrarily selected threshold of the AL group size and very good correspondence between the map characteristics (the number of skeletal markers and length of the map) obtained under zero and 1 % marker typing errors. Clearly, each of the remaining >9,800 markers can be attached to the corresponding interval or marker on the skeleton map. Figure 14.4 shows the skeleton map of the second example, on wheat chromosome 3B (DH population, the total set included ~420,000 markers).

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Part VI
Functional Gene Analysis and Molecular Tools

Chapter 15
Exploiting Comparative Biology and Genomics to Understand a Trait in Wheat, *Ph1*

Graham Moore

Abstract For hexaploid wheat to be highly fertile, only true homologues must pair at meiosis, rather than the highly related chromosomes present. The mechanism, which restricts this pairing, must have arisen rapidly on wheat's polyploidisation, to ensure stability and fertility. From the analysis of *Ph1*, which is the major locus restricts this pairing, tweaking Cdk-type phosphorylation levels is one way to provide such a control.

Introduction

Meiosis is a cell division process that ensures gametes carry the correct number of chromosomes, without a doubling of chromosome number. During meiosis, chromosome numbers are halved, leading to haploid gametes, a process that is crucial for the maintenance of a stable genome through successive generations. The process to achieve an accurate segregation of the homologous chromosomes (homologues) starts in pre-meiosis as each homologue is replicated and the respective products, sister chromatids are held together as via specific cohesion proteins. Then at the start of meiosis, each chromosome must recognize its homologue from amongst all the chromosomes present in the nucleus and associate or pair, and then recombine with that homologue. The homologues are observed as paired at Metaphase I. The homologues are then separated and segregated to two different daughter cells. In the next round of division, the sister chromatids are then separated, moving to one of four haploid cells. The accuracy of recognition and segregation of the homologues has a profound effect on overall fertility. This is more complicated in polyploids because of the greater number of related chromosomes. Polyploid fertility depends on the efficiency by which they behave as diploids during meiosis by restricting pairing to true homologues, despite the presence of related chromosomes (homoeologues). The mechanism, which restricts this pairing, must arise rapidly on polyploidisation

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to ensure fertility of the new polyploid plant. So what controls the difference between diploid and polyploid pairing in a polyploid species such as wheat?

The hexaploid wheat (AABBDD, 2n = 42) carries a diploid set of 42 chromosomes composed of three ancestral genomes, A, B and D. The 42 chromosomes can be divided into seven groups of six chromosomes (two chromosomes from each of the ancestral genomes). In hexaploid wheat, chromosome 1A must pair with 1A at meiosis and not with 1B or 1D. In wheat, a single locus, *Ph1*, (*P*airing *h*omoeologous *1*) on the long arm of chromosome 5B has a major controlling effect (Okamoto 1957; Riley and Chapman 1958; Sears and Okamoto 1958). Both Riley and Sears experimented with both haploids and wheat-wild relative hybrids, which lack homologues and only possess homoeologues. They observed that, when chromosome 5B was deleted in both haploids and wheat-wild relative hybrids, that there was a level of pairing between the related chromosomes at Metaphase I. They recognized that a locus on chromosome 5B was responsible for the major effect on polyploid pairing in wheat. Using deletions, Sears further defined the effect to the long arm of wheat chromosome 5B (Sears 1977). Thus *Ph1* is defined as a deletion phenotype.

The Basic Chromosome Pairing and Recombination Process

In many species, there are chromosome movements at the onset of meiosis, which enable the telomeres of the chromosomes to cluster as a telomere bouquet. Within this cluster of telomeres, the terminal regions of homologous chromosomes find their correct homologous partner. The two homologues then "zip up" from the telomere regions. This process of "zipping up" or synapsis involves the placement of a protein complex, a synaptonemal complex, between the homologues. Essentially this is equivalent to "gluing" the chromosomes together. Within this synaptonemal complex structure, double strand breaks can be repaired. Meiotic recombination occurs through the generation and repair of double strand breaks (DSBs) using the homologues or homoeologues. Essentially the double strand break is formed early during meiosis and is then resectioned to generate a single strand end. The pairing process involves the single strand end finding and invading the homologous region of the corresponding homologue or homoeologue. Successful invasion results in strand displacement in this region, generation of a Double Holiday Junction, a cross-over event (chiasmata) leading to recombination. The single strand invasion occurs during late zygotene, and double holiday formation occurs during pachytene. The synaptonemal complex (SC) starts to be disassembled during pachytene. The formation of chiasmata, physical links which together with sister chromatid cohesion, still hold the homologues together after the disassembly of the SC, so the chromosomes are visualized as paired at metaphase I. If single strand invasion is unsuccessful, the double strand break can be repaired through using its own sister chromatid, leading to the chromosomes being visualized as unpaired at metaphase I.

The Power of a Cell Biological Experiment

After 50 years, the cell biological tools (antibodies to key meiotic proteins) finally became available to answer two fundamental questions about *Ph1*.

Firstly, the question as to whether *Ph1* actually blocks chromosome pairing between related chromosomes (homoeologues). The locus is named homoeologous pairing 1 (*Ph1*) because it is always assumed that it reduces pairing or synapsis between homoeologues, and that this then subsequently affects the levels of recombination between such chromosomes. However, our recent cell biology data reveals that in wheat-rye hybrids, where there are no homologues, only homoeologues, the related chromosomes pair or synapse to a similar level, whether *Ph1* is present or absent (Martin et al. 2014). Therefore *Ph1* doesn't suppress homoeologues pairing in the hybrid. This implies that in wheat itself where there are both homologues and homoeologues, the overall effect of *Ph1* on chromosome pairing as distinct from recombination must be the promotion of homologue pairing rather than specifically suppressing pairing between the related chromosomes.

Secondly at what stage does *Ph1* block recombination between homoeologues, a process that occurs on both the male and female sides from leptotene to diplotene? The major surprise of our recent study is that double strand breaks are formed at similar levels, and are processed with similar kinetics into Double Holliday Junctions between the paired homoeologues whether *Ph1* is present or absent (Martin et al. 2014). This results in a similar number of Double Holliday Junctions at diplotene in the hybrid as revealed by immunolabelling with the MLH1 antibody. In all other species so far studied, MLH1 marks sites on paired chromosomes that will become crossovers. However, these studies have been performed on paired homologues rather than paired homoeologues as in the case of the wheat-rye hybrid. Based on the number of MLH1 sites, 21 crossovers would be expected between the paired homoeologues in the wheat-rye hybrid whether *Ph1* is present or absent, yet only seven crossovers on average occur in the absence of *Ph1,* and one or none in the presence of *Ph1*. Therefore the resolution of Double Holliday Junctions to crossovers between the paired homoeologues fails in both the presence and absence of *Ph1*, but this failure is partially alleviated by deleting *Ph1* or increasing Cdk2-type activity. Thus *Ph1* suppresses recombination between homoeologues by preventing the resolution of Double Holliday Junctions as crossovers (Martin et al. 2014).

The MLH1 protein complex involved in this resolution has been characterized in other species. It contains two mismatch repair proteins, MLH1 and MLH3, EXO1 (a nuclease), CDK2 (which is activated by a meiotic specific cyclin), and finally an E3 Ubiquitin ligase (HEI10), which may be involved in the degradation of other cyclins, enabling the meiotic cyclin to activate CDK2. Various recent reports have provided indirect evidence that CDK2 regulates the activity of the MLH1 complex and crossovers (Martin et al. 2014).

Thus this cell biological study reveals that *Ph1* has two distinct effects on chromosome pairing and recombination. Firstly it promotes homologue pairing rather

than prevent homoeologue pairing, and secondly it prevents recombination between paired homoeologues by stalling Double Holliday Junctions from being resolved as crossovers (Martin et al 2014). Interestingly subsequently a number of Cdk2 studies have reported that it has two distinct effects on chromosome pairing and recombination. It affects chromosome pairing through altering the function of the telomere bouquet, and recombination via crossover resolution (Liu et al. 2014; Viera et al. 2015). So what is *Ph1* locus?

Ph1 Locus at a Molecular Level

Given *Ph1* is a deletion phenotype effect, its mapping required the screening of mutagenised hexaploid and tetraploid wheat populations to identify a set of overlapping deletions covering chromosome 5B. Over a 15-year period ten mutagenised populations were developed and screened in hexaploid wheat (Roberts et al. 1999), and one mutagenised population in tetraploid wheat with the help of Shrahryar Kianian. The deletion breakpoints needed to be located and the gene content of the regions covered by these deletions revealed. The hexaploid wheat genome is 5x larger than the human genome and was unsequenced. To solve this problem, the use of the small rice genome, as a model for the larger wheat genome was postulated, should there be conservation of gene order (Moore et al. 1993). This needed to be confirmed before the *Ph1* cloning strategy could be implemented (and funded). Conservation in gene order, was confirmed first at the genetic and then at the physical level (Moore et al. 1993; Kurata et al. 1994; Foote et al. 1997; Griffiths et al. 2006). *Brachypodium* with its small genome was also added to this concept (Moore et al. 1993; Foote et al. 2004; Griffiths et al. 2006). Taking this approach further, it should be possible to reconstruct the ancestral genome from which the genomes of present day cereals and grasses have evolved (Moore et al. 1993). To this end, mapping data for rice from Japan, for maize and sorghum from North America, for sugarcane from France and finally for rye, wheat and millets from the UK was used. From these datasets, there was indeed a pattern of genomic building blocks or groups of genes within the rice genome, which could be used to describe the structure of all the other cereal chromosomes (Moore et al. 1995). The comparison of the order of blocks within the different cereal chromosomes, revealed that they could all be derived from the cleavage of a single structure, a hypothetical 'ancestral' genome, formed from the blocks, and a diagrammatical framework for comparing the order of all the major cereal genomes unified cereal genetics (Moore et al. 1995). This concept was purposefully termed "Synteny", which in classical genetics had been used in a different context, but which is today the widely used term for the concept, indicating its cereal origin (see Encyclopaedia Britannica). With the development of this concept, funding was made available from BBSRC for the *Ph1* cloning strategy, in particular the development of genomic libraries for wheat and *Brachypodium* in order to generate a physical contig of the *Ph1* locus. A 1.2 million clone BAC library was constructed with INRA (Allouis et al. 2003). The *Ph1* deletion effect

region was delineated by phenotyping the deletion lines and mapping the deletion breakpoints using Synteny. The breakpoints of deletions lacking *Ph1* clustered non-randomly either side of a 2.5 Mb region carrying a large segment of satellite DNA, located within an amplified Cdk locus (Griffiths et al. 2006). There is also an anther specific gene within the delimited *Ph1* region, which is the homologue of RA8 in rice, now named Raftin1 protein. We initially named the annotated wheat gene, as RA8, then Raf1 in subsequent analyses (Griffiths et al. 2006; Al-Kaff et al. 2008). The Raftin genes are anther specific and have been extensively characterized in rice, and now in wheat (Jeon et al. 1999; Wang et al. 2003; Sheng et al. 2011). They are mainly expressed in tapetal cells and are responsible for transporting lipids and cell wall proteins to the developing meiocytes. Mutation or deletion of the genes produces male steriles, as a result of the microspores becoming stressed (dehydrated). Stressed meiocytes exhibit chromosome clumping or clustering at metaphase I. Thus the genes have been patented in rice, maize and wheat for making male steriles in hybrid production. We excluded this gene as being responsible for the *Ph1* effect because: it is only expressed on the male side and not on the female side; it is not expressed during the stages when recombination occurred on the male side; and finally if the 5B copy is functional, its deletion would result in male steriles, which are not observed with deletion of the *Ph1* region. Consistent with this observation, the 5B copy of RA8/Raf1 carries an early stop codon, and the transcripts derived from this copy are antisense and not sense. The transcripts run into the promoter regions and contain exonintron junctions in the incorrect orientation.

Subsequently, we identified two additional deletion mutants which possessed wild type pairing in wheat itself, and therefore both retained the *Ph1* locus (Al-Kaff et al. 2008). One of these deletions encompassed the RA8/Raf1 gene. Thus by a process of exclusion, the analysis delineated the *Ph1* locus to a region where nearly half the genes are a cluster of kinases, including Cdk2-like genes. Expression analysis revealed that many of these Cdk2-like genes are expressed during meiotic prophase I, where the processes of pairing and recombination occur. To take the molecular study further required a working hypothesis for *Ph1's* mode of action. Given nearly half the genes in the delimited region are kinases, our hypothesis is that *Ph1* affected kinase activity and hence overall phosphorylation levels. Amongst these kinases is a cluster of defective kinase genes (Cdk-like), with similarity to Cdk2 (Griffiths et al. 2006; Al-Kaff et al. 2008; Yousafzai et al. 2010). Therefore the deletion of *Ph1* region could result in either an increase or decrease Cdk activity and phosphorylation levels, and that this altered phosphorylation levels could induce pairing between related chromosomes. We were able to test whether increasing Cdk-type activity phenocopies the effect of deleting the *Ph1* locus. Treatment with okadaic acid, a serine-theonine phosphatase inhibitor increases Cdk-type activity. Treatment of detached tillers from *Ph1* wheat-rye hybrids with okadaic acid from the onset of meiosis, does indeed phenocopy the effect of *Ph1* deletion by inducing metaphase I pairing between related chromosomes (Knight et al. 2010). Thus increased phosphorylation levels overcomes the stalling of MLH1 sites on paired homoeologues in the presence of *Ph1*, enabling some of the sites to progress to crossovers which are visualized as pairing between related chromosomes at meta-

phase I. However does deleting the *Ph1* region actually increase phosphorylation levels during meiosis? Our mapping of *Ph1* region reveals the presence of a defective Cdk2-like kinase complex, which therefore could suppress active Cdk2-like genes via a dominant negative effect. Consistent with this proposal, phosphoproteomics revealed that phosphorylation at Cdk2 consensus sites on Histone H1 is increased in the absence of *Ph1* (Greer et al. 2012). As indicated previously, phosphatases directly dephosphorylate proteins including Cdks, and are inhibited by Okadaic acid, which therefore can increase Cdk2 type activity and hence Cdk2-type phosphorylation. Okadaic acid treatment during meiosis mimics the effect of deleting *Ph1* by inducing pairing and recombination between homoeologues even in the presence of *Ph1* (Knight et al. 2010). This treatment also increases phosphorylation of the same Cdk2 consensus sites on Histone H1 as deleting *Ph1* (Greer et al. 2012). Thus the reduced phosphorylation levels at Cdk2 consensus sites (hence Cdk2-type activity) in the presence of *Ph1* and the stalling of MLH1 complex (which in other species has been shown to contain CDK2) on Double Holliday Junctions between paired homoeologues, are all entirely consistent. The *Ph1* data implies that the MLH1 complex needs to be more active to resolve junctions on paired homoeologues than it does for junctions between paired homologues. This is consistent with the observations of Dvorak and colleagues. They found that in the absence of *Ph1*, recombination occurred between a pair of wheat chromosomes composed of combinations of homoeologous and homologous segments, but in the presence of *Ph1*, recombination was restricted to homologous segments (Dubcovsky et al. 1995). Interestingly mutating the *Ph1* Cdk homologue in *Arabidopsis* also affects meiotic chromosome pairing (see Wen 2011 for initial studies on models).

What Pairing in Euploid Wheat Itself Tells Us?

Is the effect on chromosome exchange the whole explanation of *Ph1's* action? As stated previously from analysis of hybrids, *Ph1* can't prevent related chromosomes synapsing (Martin et al. 2014), however *Ph1* does prevent related chromosomes from synapsing in wheat itself. Wheat synapsis studies reveal that the chromosomes are essentially synapsed as bivalents at pachytene when *Ph1* is the present, but synapse as multivalents when *Ph1* is absent (Holm 1986; Holm and Wang 1988). This implies that in wheat itself where there are both homologues and homoeologues, the overall effect of *Ph1* on chromosome pairing as distinct from recombination must be the promotion of homologue pairing rather than specifically suppressing pairing between the related chromosomes. How does this work? Euploid wheat homologues synchronously elongate prior to pairing at the onset of meiosis (Prieto et al. 2004; Colas et al. 2008). The degree of homologue elongation reflects the level of homology between the two parental chromosomes (Colas et al. 2008). This promotes homologue pairing which is disrupted in the absence of *Ph1* leading to incorrect pairing. Thus the level of chromosome homology in the presence of *Ph1* influences conformational changes required for initial pairing, which determines the extent of

chromosome pairing in wheat. Thus if two segments within homologous chromosomes are too distinct with respect to each other, they can't associate or synapse at all and if they are related, they can partially pair or synapse. Recent studies have reported a similar effect in *C. elegans* suggesting that this is a general meiotic phenomenon (Nabeshinia et al. 2011). They are identified some of the proteins involved, whose activity will depend on phosphorylation levels. The data suggests that these proteins bind along the chromosome triggering the conformational changes at a regional level. The increased Cdk2 activity through deleting *Ph1* will also affect the phosphorylation of the protein SUN1 early in meiosis. This may well affect the functioning of the telomere bouquet, and therefore pairing of chromosomes via the telomeres early in meiosis. Reduced shaking of chromosomes by the telomere bouquet will enable homoeologous associations to be maintained, while more rigorous shaking will rip such associations apart leaving just homologous associations between the chromosomes. We are currently studying the effect of *Ph1* on telomere bouquet function, and its subsequent effect on chromosome pairing early in meiosis (Richards et al. 2012).

Independent Centromere Pairing

Finally proper segregation of chromosomes to daughter cells requires that the paired chromosomes correctly orientate themselves so that the spindle fibres attach to the centromeres and pull the chromosomes in opposite directions. To achieve balanced gametes, the homologous centromeres must be correctly paired. We isolated an element Hi-10, which is found at the centromeres of all cereals (Aragon-Alcaide et al. 1996) and exploited it as an in situ marker for studying centromere pairing behaviour during meiosis. We reported that wheat centromeres pair independently from the rest of the chromosome, which associate and synapse from the telomeres during the telomere bouquet stage at the onset of meiosis. Our studies were controversial at the time, however it has been since reported that centromeres pair independently from the rest of the chromosome for meiosis in *Arabidopsis*, rice, *Brachypodium* and maize. It has been elegantly demonstrated in some of these systems that the centromeres synapse homologously, and independently of the rest of the chromosome (Da Ines et al. 2012). Tetraploid and hexaploid wheat possess 28 and 42 chromosomes respectively, or two and three copies of seven sets of chromosomes. During anther development in wheat-wild relative hybrids, tetraploid and hexaploid wheat, the centromeres associate as pairs (Aragon-Alcaide et al. 1997; Martinez-Perez et al. 1999; Martinez-Perez et al. 2003). During premeiotic replication, the pairs engage in a sorting process reducing to seven centromere sites at the onset of meiosis, again as the telomeres cluster to form a telomere bouquet (Martinez-Perez et al. 2003; Greer et al. 2012). *Ph1* increases the stringency of this independent centromere pairing process (Martinez-Perez et al. 2001), and therefore will affect the correct segregation of chromosomes, and the production of balanced gametes.

Summary

We have shown that *Ph1* has two distinct effects on chromosome pairing and recombination. It promotes homologous pairing through: influencing conformational changes required for initial pairing, increasing the stringency of independent centromere pairing, and finally altering telomere bouquet formation (and possibly function, we are currently studying this). *Ph1* also stalls Double Holliday Junctions from resolving as crossovers on paired homoeologues. The *Ph1* locus has been delineated to a region containing a cluster of Cdk2-like genes containing a large segment of heterochromatin. Interestingly recent mouse studies also reveal that Cdk2 has two distinct effects on this system, one on telomere bouquet function, and one at the stage when Double Holliday Junctions are being resolved.

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Chapter 16
The Specific Features of Anthocyanin Biosynthesis Regulation in Wheat

Olesya Y. Shoeva and Elena K. Khlestkina

Abstract Anthocyanins are flavonoid pigments important for plant adaptation under biotic and abiotic stress conditions. In bread wheat (*Triticum aestivum* L.), purple pigmentation caused by anthocyanins can be present on leaves, culm, auricles, glumes, grains, coleoptile, and anthers. Since the first mentions on expression of purple color traits in wheat, the studies into inheritance of these characters have made big steps toward revealing molecular-genetic mechanisms of anthocyanin pigment biosynthesis and its regulation in wheat. Most of the structural genes, encoding enzymes of the biosynthesis, have been cloned and localized in wheat genome. The genetic mapping data suggest that different pigmentation patterns in wheat are determined by genetic loci, distinct from the enzyme encoding loci. The data on functional role of the genes underpinning phenotypic variation together with results of inter-genera comparative mapping suggest these genes to encode transcriptional activators of the anthocyanin biosynthesis structural genes. Here, a brief review is provided of recent findings in the genetic regulation of anthocyanin biosynthesis in wheat.

Keywords Purple pigmentation • Comparative mapping • Regulatory genes • Structural genes • Transcription analysis • *Triticum aestivum* L

Introduction

Anthocyanin pigmentation of different parts of plants is related with their adaptation to environment stress conditions (reviewed by Chalker-Scott 1999; Khlestkina 2013a). In addition, anthocyanins are important for human health maintenance, preventing cardiovascular diseases, carcinogenesis, inflammation and many others human pathological states (Lila 2004). All these findings stimulated intensive investigations of different aspects of anthocyanin biosynthesis in plants, and nowadays,

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it is considered to be one of the best characterized secondary metabolite pathways (Winkel-Shirley 2001). Identification of the anthocyanin biosynthesis regulatory and structural genes in the model plant species (maize, Arabidopsis, snapdragon, and petunia) (Mol et al. 1998) facilitates homology-based cloning of their orthologues in cultivated species with complex genomes, such as bread wheat (*Triticum aestivum* L., $2n=6x=42$).

In wheat, purple colour of coleoptile, culm and anthers is reportedly related with resistance to bunt (Bogdanova et al. 2002). Comparative analysis of wheat near-isogenic lines differing by anthocyanin content in the coleoptile and pericarp showed higher drought tolerance of intensely colored seedlings (Tereshchenko et al. 2012a). The relationship between accumulation of anthocyanins in wheat coleoptiles and cold treatment has been shown (Gordeeva et al. 2013). Furthermore, the purple-grained NILs had better viability after accelerated ageing compared to the recurrent parent lacking anthocyanins (Gordeeva and Khlestkina 2013). The knowledge about specific features of anthocyanin biosynthesis regulation in wheat can be useful for improvement of its adaptation to biotic and abiotic stress conditions.

Structural Genes of Anthocyanin Biosynthesis in Wheat

The anthocyanin biosynthesis pathway (ABP) is one of the branches of the whole flavonoid biosynthesis pathway (Winkel-Shirley 2001). The genes encoding enzymes are referred as structural genes. Most structural genes needed for anthocyanin biosynthesis have been studied in wheat (Table 16.1).

Two copies of phenylalanine ammonia-lyase gene (*Pal*) have been isolated from the same phage clone of the wheat genomic library (Li and Liao 2003). A total of six loci for the *Pal* gene have been mapped to chromosomes of homoeologous group 3 and 6 using Southern blot hybridization method with the nucleotide sequence of the maize *Pal* gene as a probe (Li et al. 1999; Table 16.1). Similarly, six loci for the chalcone synthase gene (*Chs*) have been identified in homoeologous group 1 and 2 chromosomes (Li et al. 1999). Only four full-length nucleotide sequences of this gene have been isolated thus far (Yang et al. 2004).

Three loci for chalcone-flavanone isomerase (*Chi*) have been assigned to homoeologous group 5 chromosomes using Southern blot hybridization method with nucleotide sequence of the maize *Chi* gene as a probe (Li et al. 1999). One partial sequence of the *Chi* gene was reported by Himi et al. (2005). Then, three homoeologous full-length *Chi* copies were isolated and precisely mapped to the long arms of 5 group chromosomes (Shoeva et al. 2014a).

Four copies of the flavanone 3-hydroxylase (*F3h*) gene are present in wheat genome (Khlestkina et al. 2008, 2013; Himi et al. 2011). These copies have been mapped to chromosomes 2AL, 2BL (two copies) and 2DL (Khlestkina et al. 2011).

The genes for flavonoid 3′5′-hydroxylase (*F3′5′h*) and flavonoid 3′-hydroxylase (*F3′h*) belong to the gene family of cytochrome P450 monooxygenases (Tanaka

Table 16.1 Known structural genes encoding enzymes needed for anthocyanin biosynthesis in wheat

Gene	Cloning method	Number of cloning copies	GeneBank accession number, references	Mapping/ chromosome location, references
Pal	Phage library screening	2 full-length, genomic DNA	X99705 (Li and Liao 2003)	3A, 3B, 3D, 6A, 6B, 6D (Li et al. 1999)
Chs	Cloning of the PCR-product + RACE	4 full-length, cDNA	AY286093, AY286095, AY286096, AY286097 (Yang et al. 2004)	1A, 1B, 1D, 2A, 2B, 2D (Li et al. 1999)
Chi	Cloning of the PCR-product	3 full-length, genomic DNA	AB187026 (Himi et al. 2005); JN039037, JN039038, JN039039 (Shoeva et al. 2014a)	5AL, 5BL, 5DL (Li et al. 1999; Shoeva et al. 2014a)
F3h	Cloning of the PCR-product	4 full-length, genomic DNA	EF463100, DQ233636, EU402957, EU402958 (Khlestkina et al. 2008); AB223024, AB223025, AB223026 (Himi et al. 2011); JN384122 (Khlestkina et al. 2013)	2AL, 2BL (2 genes), 2DL (Himi et al. 2011; Khlestkina et al. 2011)
F3'5'h	Cloning of the PCR-product	1 partial, cDNA	AY519468 (Yang et al. 2004)	–
Dfr	Cloning of the PCR-product + RACE	3 full-length, cDNA	AB162138, AB162139, AB162140 (Himi and Noda 2004)	3AL, 3BL, 3DL (Himi and Noda 2004)
Ans	Cloning of the PCR-product + RACE	5 full-length, cDNA	AB247917, AB247918, AB247919, AB247920, AB247921 (Himi et al. 2006)	6AS (2 genes), 6BS (2 genes), 6DS (Himi et al. 2006)
Ufgt	Cloning of the PCR-product	1 partial, genomic DNA	– (Ahmed et al. 2006)	–
3Rt	Cloning of the PCR-product	2 partial, genomic DNA	EU815627 (Khlestkina et al. 2009b)	5BL, 5DL (Khlestkina et al. 2009b and unpublished)

et al. 2009). There are no data on cloning and/or mapping of these genes in wheat with the exception of one partial nucleotide sequence of *F3′5′h* (Yang et al. 2004).

Three copies of the dihydroflavonol-4-reductase gene (*Dfr*) have been isolated from wheat genome and localized in homoeologous group 3 chromosomes (Himi and Noda 2004). Five copies of the anthocyanidin synthase gene (*Ans*) assigned to chromosomes 6A (two copies), 6B (two copies) and 6D (one copy) have been sequenced (Himi et al. 2006).

The genes participating at the latest stages of anthocyanin biosynthesis encode for different transferase enzymes. From these genes, only two have been partially isolated from wheat genome thus far: UDP-glucose: flavonoid 3-*O*-glucosyltransferase (*Ufgt*; Ahmed et al. 2006) and UDP-rhamnose:anthocyanidin-3-glucoside

rhamnosyltransferase gene (*3Rt*; Khlestkina et al. 2009b). Two *3Rt* gene copies have been mapped to chromosomes 5BL and 5DL (Khlestkina et al. 2009b; unpublished results).

The genetic mapping data suggest that the ABP structural genes locations (Table 16.1) are different from that of the genes underpinning phenotypic variation in coloration traits (see below).

Genes Determining Anthocyanin Pigmentation in Different Parts of Wheat Plant

In bread wheat, anthocyanin pigments determine purple (culm, leaf blades, leaf sheaths, glumes, anthers, and grain pericarp), red/purple (coleoptile and auricles) or blue (aleurone layer) coloration (Fig. 16.1). Most genes determining anthocyanin pigmentation of different parts of wheat plant have been already identified and mapped (Khlestkina 2013b; McIntosh et al. 2013).

Mapping of the Genes Determining Anthocyanin Pigmentation Traits

Three genes for red coleoptile (*Rc*) localized on chromosomes 7A (Sears 1954), 7B (Gale and Flavell 1971), and 7D (Jha 1964) have been precisely mapped in homoeologous positions of the chromosome arms 7AS, 7BS, and 7DS and designated *Rc-A1*, *Rc-B1*, and *Rc-D1*, respectively (Khlestkina et al. 2002). Three homoeologous genes for purple culm (*Pc-A1*, *Pc-B1*, *Pc-D1*), three homoeologues for purple leaf sheaths (*Pls-A1*, *Pls-B1*, *Pls-D1*) and three homoeologues for purple leaf blades (*Plb-A1*, *Plb-B1*, *Plb-D1*) have been mapped in close linkage with red coleoptile genes *Rc-A1*, *Rc-B1*, and *Rc-D1* (Khlestkina et al. 2009a, 2010b). Two genes determining purple anther (*Pan-A1* and *Pan-D1*) have been mapped on chromosomes 7A (Blanco et al. 1998) and 7D (Khlestkina et al. 2009a) at a short distance from *Rc-A1* and *Rc-D1*, respectively.

Two complementary genes for purple pericarp, *Pp1* and *Pp3*, have been mapped in chromosomes 7B and 2A of bread (Arbuzova et al. 1998; Dobrovolskaya et al. 2006) and durum (Khlestkina et al. 2010a) wheat. For the durum gene *Pp1* (*Pp-B1*), the homoeologue has been identified on chromosome 7D (*Pp-D1*) of bread wheat (Tereshchenko et al. 2012b). They have been mapped on the short arms of chromosomes 7B and 7D close to the *Rc-B1* and *Rc-D1*, respectively. The *Pp3* gene of durum wheat is closely linked to the gene for purple glume (*Pg*), however, unlike purple pericarp, the purple glume color is a monogenically inherited trait (Khlestkina et al. 2010a).

Chromosome locations and a number of the genes for red auricles (*Ra*) still remain a matter of contention. The genes determining this trait have been assigned

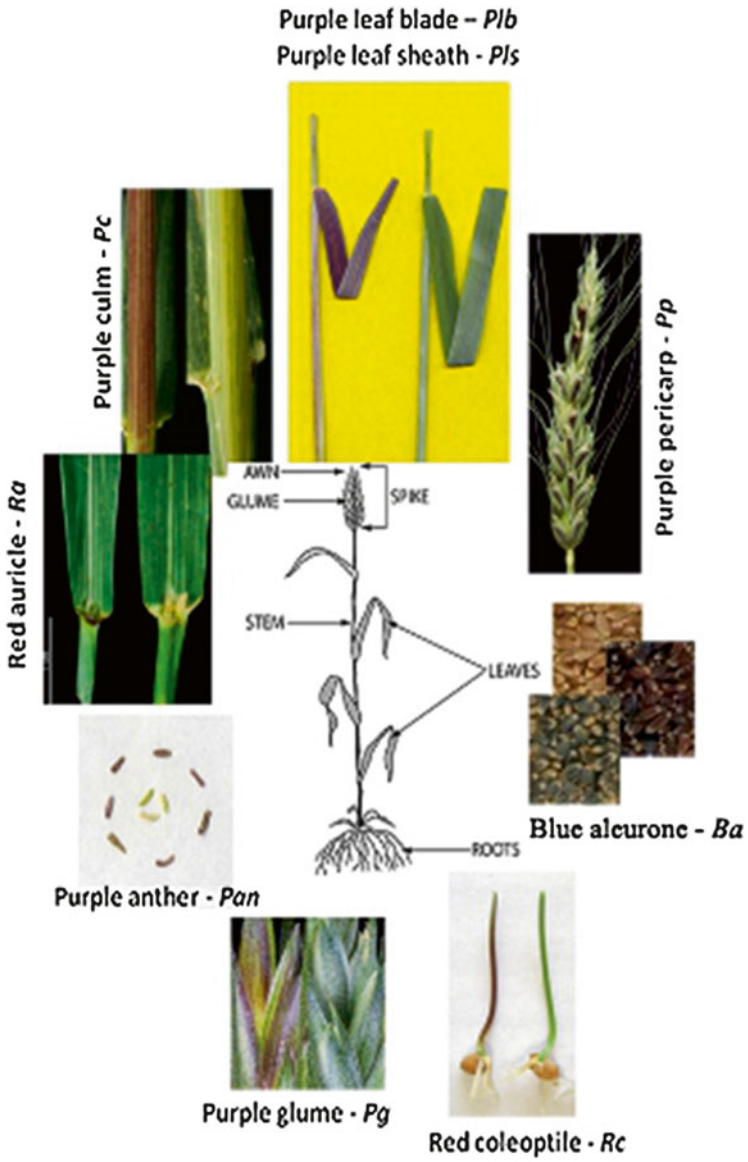

Fig. 16.1 Anthocyanin pigmentation of different parts of wheat plant. The picture was taken from (Khlestkina 2012) with modifications

to chromosomes 7A and 7D (Jha 1971), 1D (Gulyaeva 1984), 4B or 6B (Melz and Thiele 1990). None of these loci has been mapped. The reason for inconsistency in determining chromosome location and inability of mapping of the *Ra* genes is unstable expression of this trait. Near-isogenic lines and modern DNA-based genotyping approaches provide a powerful means of chromosome localization and fine mapping of genes with unstable expression. Using this approach we localized recently the gene *Ra-D1* in a vicinity of *Rc-D1* on chromosome 7DS (Khlestkina et al. 2014). There is a good agreement between our data and that reported by Jha (1971).

The blue aleurone (*Ba*) color had been inherited by wheat from its related species (Zeven 1991). For instance, the *Ba* genes have been identified in *Thynopirum ponticum* (*Ba1*; Keppenne and Baenziger 1990), *Th. bessarabicum* (*BaThb*; Shen et al. 2013), *T. monococcum* (*Ba2*; Dubcovsky et al. 1996), *T. boeoticum* (*Ba2*; Singh et al. 2007). In blue-grained wheat lines, alien substitutions or introgressions into homoeologous group 4 chromosomes are usually observed (Zeven 1991; Arbuzova et al. 2012; Shen et al. 2013).

Comparative mapping data in wheat, rice, and maize indicate that loci for anthocyanin pigmentation, mapped to homoeologous group 7 chromosomes, are orthologous to the maize gene *C1* and rice gene *OsC1*, encoding Myb-like transcription activators of anthocyanin biosynthesis (Saitoh et al. 2004; Khlestkina 2013b). Furthermore, the maize *C1* gene was used as a probe in Southern hybridization-based mapping in wheat, and its homologue has been mapped to chromosome 7D (Li et al. 1999) in position highly comparable with that of the wheat *Rc/Pc/Pls/Plb/Pan/Pp1* genic cluster.

Similarly, comparative mapping data demonstrate that the wheat *Pp3* gene is orthologous to rice *Pb* /*Ra* (Hu et al. 1996; Wang and Shu 2007) and maize *Lc* /*R* (Ludwig et al. 1989), encoding Myc-like protein needed for anthocyanin biosynthesis regulation. Recently nucleotide sequence of the candidate gene for *Pp3* was isolated from wheat genome (Shoeva et al. 2014b).

Thus, the inter-genera comparative mapping suggests the anthocyanin biosynthesis genes on homoeologous group 7 chromosomes to encode Myb-like (C1-like) regulatory factors and that on chromosome 2A to encode Myc-like regulatory factors. Following this suggestion, the effect of different alleles of the *Rc*, *Pc*, *Pls*, *Plb*, and *Pp* genes on transcriptional activity of the ABP structural genes was investigated using wheat precise genetic stocks (see below).

Transcriptional Analysis of Anthocyanin Biosynthesis Structural Genes in Different Wheat Organs

Using comparative transcriptional approach, regulatory role of the genes, determining anthocyanin pigmentation of wheat organs, has been investigated. Ahmed et al. (2006) compared expression of the ABP structural genes in the red and green coleoptiles of the chromosome substitution line ‘Chinese Spring’(‘Hope’ 7A) and cv.

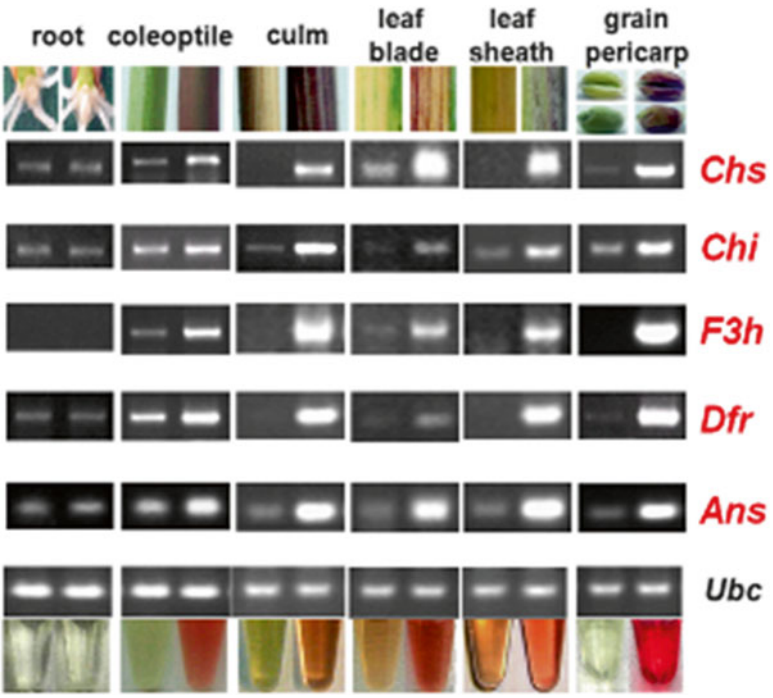

Fig. 16.2 RT-PCR analysis of the anthocyanin biosynthesis structural genes in different organs of wheat 'Saratovskaya 29' (*left* in each pair) and its near-isogenic line 'i:S29Pp1Pp3^{P}' (*right* in each pair). Anthocyanin extracts from the corresponding organs are shown below. *Ubc* (ubiquitin) – endogenous control

'Chinese Spring', respectively, and concluded that the *Rc-A1* gene activates expression of the structural genes *Dfr*, *Ans*, and *Ufgt*. Later the regulatory role of the *Rc-A1*, *Rc-B1*, and *Rc-D1* genes has been demonstrated using a wide range of wheat precise genetic stocks: near-isogenic and introgression lines, chromosome substitution and recombinant lines (Khlestkina et al. 2008, 2010b; Tereshchenko et al. 2013). In addition, it has been found that multiple dominant alleles of the same regulatory gene (*Rc-A1*) have different effects on dynamics and intensity of the structural gene expression (Khlestkina et al. 2010b).

Regulatory role of the *Pc*, *Pls*, *Plb*, and *Pp* genes has been demonstrated using near-isogenic lines (Fig. 16.2; Tereshchenko et al. 2013). It has been noted that the *F3h* gene is expressed only in colored tissues and is not expressed in non-colored ones such as roots of both lines or pericarp of 'Saratovskaya 29' (Fig. 16.2). The other structural genes are still transcribed in the absence of anthocyanin pigments, but at the lower level in comparison with the intensively colored tissues (Fig. 16.2; Tereshchenko et al. 2013). This specific regulation of *F3h* was also observed earlier by Khlestkina et al. (2009b) in coleoptiles of wheat-rye addition lines.

In some plant species, the whole set of anthocyanin biosynthesis genes is regulated as a single unit (Dooner 1983; Meldgaard 1992; Mato et al. 2000; Honda et al. 2002; Mano et al. 2007). In other plant species, anthocyanin biosynthesis can be regulated at different stages of the pathway (Boss et al. 1996; Ramazzotti et al. 2008; Zhao et al. 2012). However, the regulation of the anthocyanin biosynthesis at the stage of the *F3h* gene expression has been observed in wheat only and this may be a species-specific peculiarity of the anthocyanin biosynthesis regulation in *Triticum*. Such peculiarities of flavonoid biosynthesis regulation provide a basis for taxonomic distinguishing among plants (Bell 1980). However, biological meaning of the flavonoid biosynthesis interruption in non-colored tissues of wheat at the stage of F3H enzyme action, when flavanones are converted to dihydroflavonols, is not clear yet.

Conclusion

Anthocyanin pigments are reportedly the universal defense compounds produced in response to a wide range of biotic and abiotic stress factors. Most of the regulatory and structural anthocyanin biosynthesis genes have been mapped in wheat. The majority of the structural ABP genes and one of the two complementary genes determining purple grain trait have been sequenced. The other regulatory genes can be isolated and sequenced in the near future based on the data provided from investigations of their functions and from inter-genera comparative mapping. The knowledge of genetic basis of anthocyanins biosynthesis in wheat and the availability of wheat precise genetic stocks provide a highly appropriate basis for exploring the changes in expression of the ABP genes under stress conditions. These data will be useful in future for improvement wheat adaptation properties.

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Chapter 17
Association of Wheat miRNAs with Hybrid Incompatibility in Interspecific Crosses of *Triticum* and *Aegilops*

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Hybrid Incompatibility in Higher Plants

For successful speciation through allopolyploidization, normal growth and fertility of interspecific hybrids are essential. However, hybrid plants frequently fail to produce a next generation due to lethality and sterility. Such hybrid incompatabilities are considered a postzygotic reproductive barrier, and play important roles in differentiation and establishment of new genealogical lineages in plants. The Dobzhansky-Müller (DM) model simply explains the process for generating genetic incompatibility in hybrids between two diverging lineages (Bomblies and Weigel 2007). This model proposes that reduction of fitness in hybrids generally occurs due to interaction between at least two epistatic loci derived from divergent parents.

The molecular nature of the causal genes for DM-type hybrid incompatibilities, including hybrid sterility and hybrid lethality, was recently elucidated in some plant species (Bomblies and Weigel 2007). A nucleotide binding leucine rich repeat-type disease resistance (*R*) gene is necessary for induction of hybrid necrosis in some intraspecific crosses of *Arabidopsis thaliana* L. (Bomblies et al. 2007; Alcázar et al. 2009). The epistatic interaction of *RPP1*, the NB-LRR-type *R* gene, and *SRF3*, a receptor-like protein kinase gene, corresponds to the DM relationship for induction of hybrid necrosis in *Arabidopsis* (Alcázar et al. 2010). Therefore, it has been postulated that hybrid necrosis is caused by particular alleles of the *R* locus inducing autoimmune-like responses when interacting epistatically with particular alleles of genes elsewhere in the genome (Bomblies and Weigel 2007). On the other hand, gene duplication followed by reciprocal gene loss sometimes results in DM-type hybrid incompatibilities. Loss or silencing of different copies of a duplicated gene in genealogically separated populations contributes to reduced fitness of F_2 progeny

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derived from F_1 hybrids, which functions to accelerate genetic differentiation between the genealogically separated populations (Taylor et al. 2001). In intraspecific hybrids of *A. thaliana*, arrested embryo development and root growth impairment are induced by a lack of both duplicated gene copies (Bikard et al. 2009). Similarly, pollen sterility is induced by independent disruption of each copy of two paralogs in intersubspecific hybrids between *Oryza sativa* L. subspecies *indica* and *japonica* (Mizuta et al. 2010). These epistatic interactions of the paralogous DM genes result in segregation distortion in the progeny.

Some reproductive barriers in interploidy crosses are established in the endosperm, and parent-of-origin specific gene expression in the endosperm is related to reproductive barriers (Schatlowski and Köhler 2012). A maternally expressed WRKY transcription factor controls hybrid lethality during seed development in interploidy crosses between a tetraploid accession of *A. thaliana* and a diploid accession of *Arabidopsis arenosa* L. (Dilkes et al. 2008). Embryo arrest occurs, accompanied by abnormal proliferation of endosperm in interspecific crosses between this tetraploid accession of *A. thaliana* and diploid accession of *A. arenosa*, and increased expression of target genes of an imprinted Polycomb group protein gene is observed during seed development in the incompatible crosses (Walia et al. 2009). Therefore, precise expression of imprinted genes in the endosperm plays an important role in successful development and establishment of hybrid seeds, and seed arrest in incompatible crosses is explained by deregulation of the imprinted gene expression during endosperm development (Schatlowski and Köhler 2012). In addition, recent studies have shown association of small RNAs with failure of seed development in *Arabidopsis* hybrids (Ng et al. 2012). Paternal expression of the *ATHILA* retrotransposon is related to maternally expressed p4-siRNAs in developing endosperm (Mosher et al. 2009), which is essential for normal seed development in interploidy hybrids of *Arabidopsis* (Josefsson et al. 2006). Thus, disruption of parental genome balance in interploidy crosses could result in developmental failure of endosperm through imbalanced regulation of siRNA-mediated transcripts (Ng et al. 2012).

Abnormal Phenotypes in Wheat Hybrids

Wheat type I necrosis, caused by *Ne1-Ne2* complementary genes, is a good example of a DM incompatibility (Tsunewaki 1960). Necrotic cell death induced by the *Ne1-Ne2* epistatic interaction is accompanied with generation of reactive oxygen (Sugie et al. 2007). Because the *Ne2* gene seems to be located in a chromosomal region closely linked to the *R* gene against rust fungus, it has been postulated that type I necrosis is due to autoimmune-like responses triggered by the *Ne1-Ne2* interaction (Bomblies and Weigel 2007). The *Ne1-Ne2* interaction results in segregation distortion of molecular markers around the *Ne1* and *Ne2* chromosomal regions in mapping populations of common wheat even if the necrotic effect is weak (Takumi et al. 2013; Iehisa et al. 2014).

Sometimes ABD triploid hybrids between tetraploid wheat and wild diploid *Aegilops tauschii* Coss. show abnormal growth phenotypes such as germination failure, hybrid necrosis and hybrid sterility (Matsuoka et al. 2007). In particular, the abnormal growth phenotypes in hybrids between the tetraploid wheat cultivar Langdon and *Ae. tauschii* accessions have mainly been divided into the following four types: two types of hybrid necrosis (type II and type III), hybrid chlorosis, and severe growth abortion (Mizuno et al. 2010). In hybrid lines showing type III necrosis, cell death occurs, gradually beginning with older tissues, as observed in type I necrosis. Type III necrosis is presumed to be due to interaction of *Nec1* and *Nec2* complementary genes located on the D and AB genomes, respectively. Plants exhibiting type II necrosis show necrotic symptom and marked growth repression only under low temperature. A previous report assumed that complementary genes located on the AB and D genomes, respectively named *Net1* and *Net2*, trigger type II necrosis (Nishikawa 1962). A hypersensitive response-like reaction might be associated with necrotic cell death in type II and III necrosis (Mizuno et al. 2010, 2011). Therefore, the two types of hybrid necrosis in wheat triploid hybrids at least partly share similar responses.

In addition to necrotic symptom, a significant decrease in cell cycle- and division-related gene expression occurs at the crown tissues including the shoot apical meristem (SAM) of plants displaying type II necrosis (Mizuno et al. 2011). Severe growth abortion, which is hybrid lethality with developmental arrest at the early seedling stage in ABD wheat hybrids, might be caused by abortion of mitotic cell division and meristematic activity at the SAM (Hatano et al. 2012). In severe growth abortion, the related cell death induced by an autoimmune response might be a secondary event; arrest of cell division at SAM seems to occur prior to the autoimmune response (Hatano et al. 2012). Thus, dramatic alteration of gene expression profiles at the SAM induced by the AB and D genome interaction could be significantly associated with the growth abnormalities in triploid wheat hybrids.

Interestingly, tiller number is dramatically increased at normal temperatures in type II necrosis, although plant height is significantly shorter (Mizuno et al. 2011). An extremely bushy dwarf phenotype, called grass clump, can also be induced by epistatic interaction of *Net1* and *Net2*. Therefore, phenotypic effects of the *Net1*-*Net2* interaction at the crown tissues show plasticity strongly dependent on plant growth temperature (Takumi and Mizuno 2011; Fig. 17.1). At the normal growth temperature, transcriptome analysis of the crown tissues of plants with type II necrosis showed downregulation of wheat *APETALA1*-like MADS-box genes, which are considered to act as flowering promoters (Matsuda et al. in preparation). The downregulation of the MADS-box genes corresponds with the delayed flowering phenotype in plants showing type II necrosis. On the other hand, disease resistance-related genes are not upregulated under normal temperature conditions (Matsuda et al. in preparation). Thus, dramatic alteration of gene expression profiles at the SAM induced by DM gene interaction could be significantly associated with the growth abnormalities in triploid wheat hybrids.

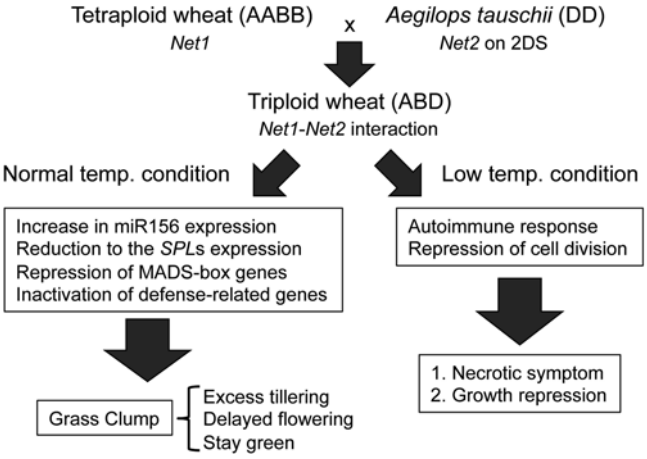

Fig. 17.1 Putative mechanism underlying the temperature-dependent phenotypic plasticity observed in ABD wheat hybrids

Wheat microRNAs and Their Association with Hybrid Incompatibility

Recently, the association of microRNA (miRNA) networks with developmental plasticity in higher plants has been discussed (Rubio-Somoza and Weigel 2011). miRNAs have been defined as a highly conserved class of small non-coding RNA molecules acting in post-transcriptional gene repression (Bartel 2009). The mature miRNA coupled with an RNA-induced silencing complex directs repression of mRNAs containing the complementary sequence, usually by mRNA cleavage in higher plants. Modified expression levels of small RNAs including miRNAs have been reported in the allopolyploidization process of *Arabidopsis suecica* Fries (Ha et al. 2009; Ng et al. 2012). A number of miRNA molecules have been identified in common wheat by next generation sequencing of small RNA molecules (Kanter et al. 2012; Yao and Sun 2012). The percentage of small RNAs corresponding to miRNAs increases with wheat polyploidy level, with the abundance of most miRNA species similar to midparent values in an interspecific wheat hybrid between tetraploid wheat and *Ae. tauschii* (Kenan-Eichler et al. 2011). Levels of accumulation of some miRNAs, such as miR168, miR156 and miR390, respective to the midparent values were distinct in ABD hybrids (Kenan-Eichler et al. 2011), though a direct relationship between the altered miRNA levels and phenotypic changes during allopolyploid evolution of wheat has not been demonstrated.

To clarify temperature-dependent changes in expression profiles of miRNAs in type II necrosis plants, we conducted deep sequencing using small RNAs isolated from crown tissues (Matsuda et al. in preparation). A comparative study of miRNA expression profiles showed that growth temperature dramatically changed the

expression profiles of miRNAs, and that more than 200 (15 %) of the identified 1,600 miRNAs were differentially expressed between the wild type and type II necrosis plants. Among the differentially expressed miRNAs, miR156 was upregulated in the crown tissues of type II necrosis plants under normal temperatures. In maize, the grass clump phenotype of *Corngrass1* mutants is caused by the overexpression of miR156, which induces altered expression of SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) transcription factor genes (Chuck et al. 2007). *SPL* genes, post-transcriptionally regulated by miR156, control tillering in higher plants (Xie et al. 2006; Fu et al. 2012). Overexpression of miR156 was also observed in synthetic hexaploid wheat relative to the miR156 midparental value (Kenan-Eichler et al. 2011). These observations imply significant association of miRNAs with temperature-dependent phenotypic plasticity in the *Net1-Net2* interaction at the crown tissues. In fact, transcript accumulation of some wheat *SPL*s containing the miR156 target site were significantly reduced in the crown tissues of type II necrosis plants only at the normal temperature (Matsuda et al. in preparation). Therefore, we presumed that, at the normal temperature, *Net1-Net2* epistatic interaction increased the miR156 level, and that the enhanced levels of miR156 led to digestion of *SPL* transcripts, resulting in an excessive increase in tiller numbers in type II necrosis (Fig. 17.1). Therefore, gene expression profiles including miRNAs in SAM in response to growth temperature could be dramatically altered in wheat hybrids and allopolyploids, resulting in phenotypic plasticity. Further studies of interspecific hybrids in *Triticum* and *Aegilops* species should offer new knowledge about hybrid incompatibility.

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Chapter 18
High Efficiency Wheat Transformation Mediated by *Agrobacterium tumefaciens*

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Abstract Wheat is one of the most important cereals for humans but quite recalcitrant in transformation. We have thoroughly examined every aspect of the wheat transformation protocols mediated by *Agrobacterium tumefaciens* and we were able to identify and optimize the key factors. Immature embryos isolated from healthy plants grown in a greenhouse were pre-treated with centrifuging and co-cultivated with *A. tumefaciens*. The frequency of transformation (independent transgenics/explant) was between 50 % and 60 % were routinely observed and higher than 90 % were recorded in the best cases. Not surprisingly, the key factors did not differ much from those in other cereal plants such as rice and maize. Both bar and hpt genes were good as selection markers. Fielder, a spring wheat cultivar, constantly showed high efficiency of transformation by our protocol. We have been able to obtain transgenic plants from the embryos harvested from the greenhouses throughout the year. Most of the transformed plants were normal in morphology and fully fertile. More than 40 % of the transformants had a single copy of the transgenes, which were inherited in a Mendelian fashion in most of the lines analyzed. Transgenic wheat has been generated at high frequency by several research groups by our protocol by now. Therefore, wheat has finally joined the list of cereals that can be efficiently transformed.

Keywords *Agrobacterium tumefaciens* • Cereal • Genetically modification • Transformation • *Triticum aestivum* • Wheat

Introduction

Transformation is an essential technology in both applied and basic studies in wheat. The first transgenic wheat was produced by particle bombardment method in the early 1990s (Vasil et al. 1992). Soon after efficient protocols of transformation mediated by *Agrobacterium tumefaciens*, which can generally transfer low copy

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numbers of large DNA segments with defined ends to plant chromosomes with few rearrangements, were developed in rice (Hiei et al. 1994) and maize (Ishida et al. 1996), wheat was also transformed by *A. tumefaciens* (Cheng et al. 1997) and quite a few reports followed. However, the progress thereafter made in wheat was slow (Przetakiewicz et al. 2004), while significant improvement in the efficiency of gene transfer and in the range of transformable genotype was made continuously in other cereals (Hiei et al. 2006; Ishida et al. 2003; Frame et al. 2006; Bartlett et al. 2008). Embryos and cultured cells of several wheat genotypes were tested, and various factors were examined in the early 2000s, however, the frequency of transformation was mostly less than 5 % of the inoculated tissue pieces. Even in the recent reports (He et al. 2010; Bińka et al. 2012), the frequency of transformation in wheat did not change much from those described in the early reports.

We examined any and all factors possibly involved in wheat transformation. Since we have an experience of development of efficient transformation protocol for rice and maize, we tried to include the factors, which were important in transformation of rice and maize and not tested in wheat. We were able to find a good combination of the parameters and to develop a highly efficient protocol for wheat transformation, which is hereby described.

Materials and Methods

Spring wheat cultivars, Bobwhite and Fielder, were cultivated in a greenhouse. Immature seeds were collected from panicles about 2 weeks after anthesis, sterilized with 70 % ethanol and 1 % sodium hypochlorite and then washed three times with sterilized distilled water. Immature embryos were isolated from the seeds under stereoscopic microscope.

Agrobacterium tumefaciens strain EHA101 and EHA105, and the vectors pIG121Hm (Hiei et al. 1994), pLC41bar and pLC41Hm were mainly used in this study. The vectors pLC41bar and pLC41Hm had the T-DNAs, which carried a gene for β-glucuronidase (GUS) that contained an intron in the coding sequence and a phosphinothricin (PPT) resistance gene for pLC41bar and a hygromycin (Hm) resistance gene for pLC41Hm respectively.

Isolated immature embryos were treated with centrifuging at various strength in the liquid medium and then inoculated with *Agrobacterium*. The embryos were placed on co-cultivation medium that contained 5 μM of $AgNO_3$ and/or $CuSO_4$ with the scutellum-side up and incubated at 23 °C in the dark for 2 days. Embryo axis was excised and the embryos were transferred to resting medium and incubated at 25 °C in the dark for 5 days. Some of the embryos after resting culture were examined histochemically for transient expression of GUS gene according to the procedure described by Ishida et al. (2007). The embryos without used for GUS assay were placed on the first selection medium that contained 5 mg/L of PPT or 15 mg/L of Hm and incubated for 2 weeks. Each of embryos was cut into two pieces, which were then transferred to the second selection medium that contained 10 mg/L of

PPT or 30 mg/L of Hm and incubated for 3 weeks. The cell clumps proliferated from the pieces were placed on regeneration medium including 5 mg/L of PPT or 30 mg/L of Hm and incubated at 25 °C under continuous illumination (35 μmol m^{-2} S^{-1}) for 2 weeks. Regenerated shoots were transferred to regeneration medium that contained 5 mg/L of PPT or 15 mg/L of Hm and incubated for 2 weeks. Regenerated plants were transferred to soil in pots and grown in a greenhouse.

Results

Preliminary Study

Agrobacterium strain EHA105 and LBA4404 were compared in the preliminary study. Because the GUS expression in the embryos infected with LBA4404 was generally weaker than that in the embryos infected with EHA101 and EHA105, LBA4404 was not examined further.

The preliminary study also revealed that centrifugation of the immature embryos at 5,000 × g or 20,000 × g for 10 min before the infection and the excision of the embryo axes from the immature embryos 2 days after the infection resulted in higher expression of GUS in the embryos after the co-cultivation. These conditions were taken into the design of the optimization experiments described below.

Immature embryos that were between 2.0 mm and 2.5 mm in length along the axis of Bobwhite and another cultivar, Fielder, were infected with EHA101 (pIG121Hm) in the optimization experiments. Firstly, further addition of salts to the co-cultivation medium was examined. Co-cultivation medium that contained 5 μM each of $AgNO_3$ and $CuSO_4$ was better than that without both or either one of the salts in the level of transient expression of GUS in and of callus formation from the embryos after the resting culture in both genotypes, employed in the rest of the optimization experiments, and taken into the recommended protocol.

Then, the timing of the removal of the embryo axes and the strength of centrifugation before the infection were revisited. The excision of axes 2 days after the infection and centrifugation at 20,000 × g were good in terms of both the transient expression of GUS in and the callus formation from the embryos after the resting culture in both genotypes. Thus, these processes were taken into the final protocol.

Production of Transgenic Wheat

Immature embryos that were between 1.0 mm and 3.0 mm in length along the axis of Fielder were co-cultivated with EHA105 (pLC41bar) or EHA105 (pLC41Hm) according to the protocol determined in the optimization experiments, and transgenic wheat was produced. In these experiments, the immature embryos in different size ranges were compared.

Table 18.1 Typical results of transformation

Vector	Size of immature embryo (mm)	Immature embryos inoculated (A)	Embryos produced resistant plants (B)	Frequency of transformation (B/A, %)
pLC41bar	1.5–2.0	17	8	47.1
	2.0–2.5	46	34	73.9
		90	67	74.4
		40	38	95.0
		17	13	76.5
pLC41Hm	1.0–2.0	14	3	21.4
	2.0–2.5	12	7	58.3
		17	14	82.4
		35	24	68.6
	2.0–3.0	47	28	59.6

Transformation of wheat was conducted efficiently under most of the combinations of the factors listed in Table 18.1, and the highest frequencies of transformation, ranging between 58.3 % and 95.0 %, were observed when the immature embryos of between 2.0 mm and 2.5 mm of Fielder were tested and the selection was made by the bar gene and hpt gene.

Characterization of the Transgenic Wheat

The transformants of wheat thus produced (T_0 generation) were all normal in morphology and fully fertile (Fig. 18.1). The GUS was well expressed in the tissues of leaves, roots, reproductive organs and the seedlings of the next generation of the transformants (Fig. 18.1).

Some of the transformants were analyzed by Southern hybridization. The integration of the T-DNA was clearly demonstrated, and the six transformants out of the 14 shown had a single copy of the transgene. The copy number of the transgene in the other lines was mostly two or three.

The inheritance of the drug resistance was examined in the T_1 generation. The hygromycin resistance segregated in 3:1 ratio in the progeny of all four Bobwhite transformants examined. The phosphinothricin resistance segregated in 3:1 ratio in the progeny of six Fielder transformants, in 15:1 in four lines and in 63:1 in three lines. One line showed none of these patterns, but, because presence of such a pattern at a low frequency among the transformants is quite normal, it was not investigated further to see whether the sensitiveness was linked to the absence of or loss of the expression of the transgene. Overall, it is evident that the expression of the transgenes was inherited to the progeny in Mendelian fashion.

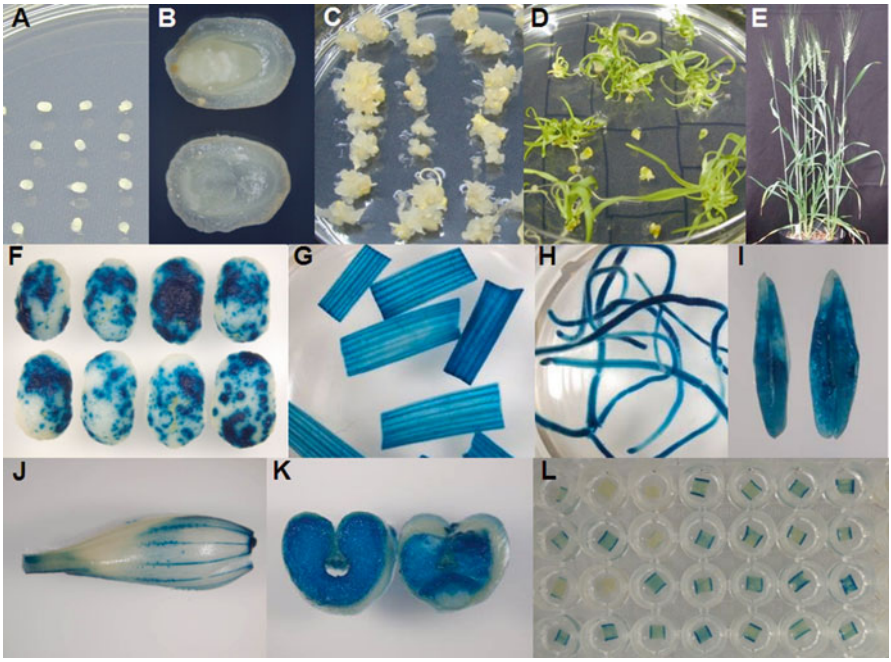

Fig. 18.1 Cells and plants derived from immature embryos of cv Fielder infected with *A. tumefaciens* EHA105 (pLC41bar) and expression of GUS gene in transgenic Fielder. (**a**) Immature embryos inoculated with EHA105 (pLC41bar). (**b**) Immature embryos 2 days after inoculation. Upper, before excision of embryo axis; Lower, after excision of embryo axis. (**c**) Immature embryos at the end of 2nd selection culture. (**d**) Plant regeneration and rooting from phosphinothricin (PPT) resistant explants under light condition**.** (**e**) Transgenic plant at flowering. (**f**) Transient expression in immature embryos after the resting culture. (**g**) Leaves detached from young regenerants. (**h**) Roots detached from young regenerants. (**i**) Spikelet. (**j**) Anthers. (**k**) Endosperm of immature seed. (**l**) Leaves detached from T_1 seedlings

Discussion

The transformation protocol hereby reported is quite efficient. The frequency of transformation of immature embryos, which was constantly over 50 % and as high as 95.0 % in the best case, was higher than the ones previously reported by roughly ten times. Therefore, one of the issues of wheat, which was a low efficiency of transformation methods, appeared to be resolved now.

Many factors needed to be examined and adjusted in order to achieve this much of improvement. The list of key factors per se in wheat transformation, including choice of genotype, quality of immature embryos, media composition, strain of *A. tumefaciens*, pre-treatment of embryos and handling of tissues, was not much different from those studied in rice and maize, but the details and specific parameters for the factors were quite different. For example, both centrifuging and heating as pre-treatments were effective in rice and maize, but only centrifuging was effective

in wheat. With respect to the handling of tissues, the process of the excision of embryo axes two days after the co-cultivation was not included in the protocols for transformation of other cereals.

The composition of the medium for co-cultivation of embryos and *A. tumefaciens* was a factor not well optimized before the present study, and a lot of experiments were needed. A combination good for wheat transformation was found by primarily looking after conditions suitable for stronger transient expression of the GUS gene in and the callus induction from the immature embryos after the co-cultivation and was further optimized.

It should also be noted that the windows of optimal ranges of parameters for the key factors in wheat seemed to be narrower than those in rice and maize. We experienced that, although the frequency of transformation in wheat was higher than that reported for maize when all the factors were optimal, the frequency dropped more drastically than maize when one or more of the factors became suboptimal.

The fact that too many factors with narrow optimal windows were involved in wheat transformation may explain both low frequency of transformation and low reproducibility of a protocol at a different laboratory before the present study. In addition, critical importance of the use of healthy immature embryos harvested at the right stage from wheat plants vigorously growing in a well-conditioned greenhouse, like any other cereal species, may explain them further. A greenhouse could be different in many ways from another no matter how similar they are and no matter how well the conditions are controlled. Therefore, a certain adjustment of the protocol by trials and errors may be inevitable for immature embryos from other greenhouses.

Commonly recognized advantage of *Agrobacterium*-mediated transformation, such as low copies of transgenes in the transformants and the stable Mendelian inheritance of the expression of transgenes, were well demonstrated in wheat. Thus, wheat has finally joined the list of cereals that can be transformed efficiently by *A. tumefaciens*. The next challenge for the present protocol is how reproducible it is at other laboratories. Chances are good because the many factors were well optimized now and the very high frequency of transformation was recorded in the present study. Finding of Fielder as a suitable genotype is another positive factor. In fact, the protocol recommended by the present study has already been tested successfully in more than ten leading laboratories in the world, and in this process, hands-on guidance provided by scientists experienced in wheat tissue culture was very helpful (personal communication). It is also likely that efforts will be made to develop methods for transformation of genotypes other than Bobwhite and Fielder, making use of the current protocol as a starting point. Therefore, the protocol must be very useful in basic and applied study of molecular biology, genomics, biotechnology and breeding in wheat.

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Chapter 19
Extra Early-Flowering (*exe*) Mutants in Einkorn Wheat Generated by Heavy-Ion Beam Irradiation

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Abstract Four extra early-heading mutants, named *extra early-flowering1* (*exe1*), *exe2*, *exe3*, and *exe4*, were identified in diploid einkorn wheat (*Triticum monococcum* L.) following heavy-ion beam mutagenesis. Based on their phenotypes in the field, the four *exe* mutants were classified into two groups: Type I (moderately extra early-heading type; *exe1* and *exe3*) and Type II (extremely extra early-heading type; *exe2* and *exe4*). Analysis of *VERNALIZATION 1* (*VRN1*), a flowering promoter gene, showed that it was more highly expressed at earlier stages of vegetative growth in Type II mutants than in Type I mutants. Our analyses indicate that the difference in earliness between Type I and Type II mutants is associated with differences in the expression level of *VRN1*.

Introduction

Improving our understanding of the molecular mechanisms of flowering, the phase transition from vegetative to reproductive growth associated with heading time, is one of the most important goals for wheat breeding at the present time. In bread wheat (*Triticum aestivum* L.), heading time is genetically determined by three characteristics: vernalization requirement; photoperiod sensitivity; and narrow-sense earliness (earliness *per se*). Three genes have been identified to determine the requirement of vernalization, namely *VERNALIZATION 1* (*VRN1*), *VRN2* and *VRN3*.

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VRN1 encodes an APETALA1/FRUITFULL-like MADS-box transcription factor that is up-regulated by vernalization (Yan et al. 2003; Murai et al. 2003; Trevaskis et al. 2003; Danyluk et al. 2003). Recent studies revealed that expression of *VRN1* is epigenetically suppressed in seedlings at an earlier stage of the vegetative growth phase, and that the repressive histone state is modified by the vernalization signal, leading to the up-regulation of *VRN1* (Oliver et al. 2009; Diallo et al. 2012). The level of *VRN1* transcription gradually increases during the seedling growth stage without the need for further vernalization (Murai et al. 2003; Kitagawa et al. 2012), suggesting that the epigenetic status of *VRN1* is also modified by aging. Furthermore, *VRN1* shows a diurnal expression pattern that is affected by daylight, with a long photoperiod producing up-regulation of its expression level (Shimada et al. 2009). In summary, these observations indicate that the *VRN1* expression is also controlled by autonomous and photoperiodic pathways, as well as the vernalization pathway.

The *VRN2* locus consists of two linked *ZCCT* genes, *ZCCT1* and *ZCCT2*, which encode a protein with a zinc finger motif and a CCT domain (Yan et al. 2004). Natural variations have been identified in the *VRN2* locus. Simultaneous deletion or non-functional mutations of these two *ZCCT* genes result in a plant showing the spring habit (Distelfeld et al. 2009), indicating that *VRN2* is a flowering repressor gene. A high level of *VRN2* expression is observed in seedlings at the 1-leaf stage, while expression is down-regulated by vernalization and aging; by contrast, *VRN1* shows the opposite pattern with low expression in seedlings and up-regulated expression after vernalization (Shimada et al. 2009). It has also been reported that *VRN2* shows a diurnal expression pattern and that a long photoperiod up-regulates its expression level (Dubcovsky et al. 2006; Trevaskis et al. 2006), suggesting that the *VRN2* expression is affected by photoperiod as well as vernalization.

VRN3 encodes a Raf kinase inhibitor-like protein with a high similarity to the Arabidopsis FLOWERING LOCUS T (FT) protein, which is a florigen (Yan et al. 2006). Transgenic wheat plants overexpressing *VRN3* show an extra early-flowering phenotype without the need for vernalization (Yan et al. 2006; Shimada et al. 2009), indicating that *VRN3* is a strong flowering promoter. Under long day conditions, *VRN3* shows a diurnal expression pattern; however, expression is very low under short day conditions (Shimada et al. 2009; Kitagawa et al. 2012).

Based on data from expression, transgenic and mutant analyses, we developed a gene network model for the interaction of *VRN1*, *VRN2* and *VRN3* in leaves (Shimada et al. 2009). In this model, *VRN1* is upstream of *VRN3* and activates *VRN3* expression under long day conditions. Thus, *VRN1* is proposed to play a role as an integrator of the vernalization and photoperiodic signals. Trevaskis (2010) put forward an alternative gene network model for *VRN1*, *VRN2* and *VRN3*; this model was based on the results of investigations using barley. This alternative model postulates that *VRN1* and *VRN3* mutually up-regulate each other: *VRN1* first activates *VRN3* expression, and then *VRN3* further activates *VRN1*. The model was referred to as "the flowering model for temperate cereals" in a review paper on flowering in plants (Andres and Coupland 2012). More recently, a third model was suggested by Chen

and Dubcovsky (2012). This model proposes that *VRN1* is activated by *VRN3* and then suppresses *VRN2* expression. In this model, *VRN1* is not essential for flowering; this conclusion was drawn from an analysis of a *VRN1* mutant line. However, it is not certain that the mutant line is a true *VRN1* knock-out, because its genotypic alteration is a point mutation and *VRN1* mRNA is transcribed.

To obtain more information about the flowering mechanism in wheat, we are developing a large-scale panel of mutants induced by heavy-ion beam mutagenesis; these mutants are being systematically screened for effects on flowering time (Murai et al. 2013). Heavy-ion beam irradiation is effective at producing gene deletion mutants (null mutations) (Kazama et al. 2011, 2013). Here we describe four newly identified extra early-flowering mutant lines in diploid einkorn wheat, which have been named *extra early-flowering 1* (*exe1*), *exe2*, *exe3*, and *exe4*.

Identification of *exe* Mutants

Seeds of the diploid einkorn wheat (*Triticum monococcum* L., 2n = 2x = 14, genome constitution A^mA^m) strain KU104-1 were given 50 Gy of 50 keV μm^{-1} LET (linear energy transfer) carbon ion beams and then sown in the field. The spikes of M_1 plants were bagged and the harvested selfed seeds of each spike were used to produce the M_2 lines. From approximately 1,200 M_2 lines, we identified plants showing an abnormal extra early-heading phenotype; we termed these mutants *extra early-flowering* (*exe*). The original wild type (WT) strain KU104-1 is a spring habit einkorn wheat having a dominant *VRN1* allele and a null *VRN2* allele. Therefore, the *exe* mutants, *exe1*, *exe2*, *exe3*, *exe4*, identified in this study have no active gene at the *VRN2* locus.

Table 19.1 shows the heading time of the WT and *exe* mutants in the field. Based on the heading time, the *exe* mutants were classified into two groups: Type I showed moderately extra early-heading type (*exe1* and *exe3*); and Type II showed extremely extra early-heading type (*exe2* and *exe4*). In the field, Type I and Type II headed about 30 and 45 days earlier than the WT, respectively.

Table 19.1 Heading time of the *exe* mutants and wild type (WT) plants grown in the field

Lines	Heading time	Difference from WT	Type
WT	6 June	–	–
exe1	7 May	−30	Type I
exe2	22 April	−45	Type II
exe3	7 May	−30	Type I
exe4	25 April	−37	Type II

Data from season 2011/2012

Morphological Characteristics of the *exe* Mutants

WT plants and *exe* mutants were grown in the experimental field at Fukui Prefectural University and their morphological phenotypes were characterized during the maturation stage. Three agronomic characters were assessed: internode length, spike length, and spikelet number per spike.

The internode lengths of *exe* mutants were shorter than those of WT plants (Fig. 19.1). In particular, Type II *exe* mutants showed a significantly shorter first internode length than WT plants. Compared to WT plants, *exe* mutant plants produced smaller spikes with fewer spikelets (Fig. 19.2). As a consequence of the smaller numbers of spikelets, spike lengths in *exe* mutants were shorter than in WT plants. Furthermore, the spikes of Type II *exe* mutants were smaller than those of Type I mutants. These observations indicate that shortened culm lengths and fewer spikelets per spike are associated with the extra early-flowering phenotype in the *exe* mutants.

Hypothetical Model for Extra Early-Flowering Phenotype

The expression analysis of *VRN1* indicated that *VRN1* is highly expressed at earlier stages in Type II mutants than in Type I mutants under both short day (SD) and long day (LD) conditions (data not shown). This clearly indicates that the difference in earliness between Type I and Type II mutants is associated with the level of *VRN1* expression. Thus, *VRN1* is essential for flowering in wheat, and the level of expression of *VRN1* determines flowering time (Fig. 19.3).

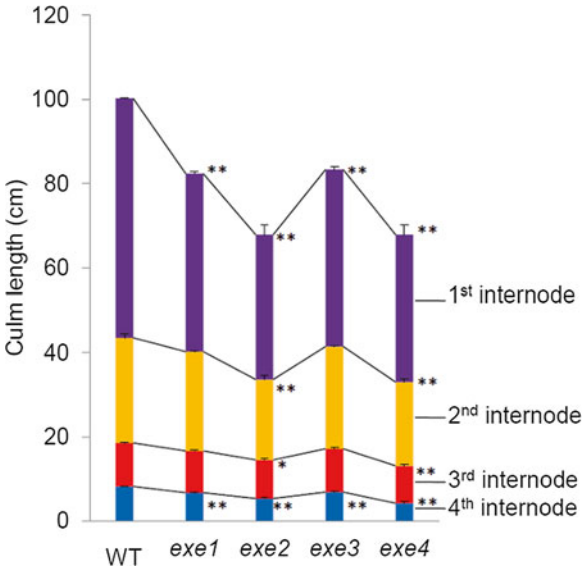

Fig. 19.1 Comparison of internode lengths of *exe* mutants and wild type (WT) plants grown in the field. First to fourth internode lengths are shown. The mean from the main culms of five plants of each line are shown. *Asterisks* indicate significant *P* values (Student's *t*-test): $*p<0.05$ and $**p<0.001$ vs WT

Fig. 19.2 Spikes of *exe* mutants and wild type (WT) plants grown in the field. The *exe* mutants showed a significantly decreased spike length compared to the WT

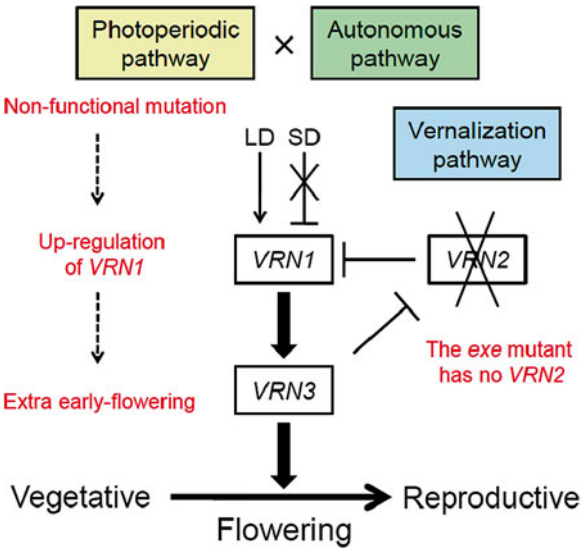

Fig. 19.3 Schematic outline of the proposed mechanism for the extra early-flowering phenotype in *exe* mutants. This is based on the model of Shimada et al. (2009) in which *VRN1* acts as an integrator of the vernalization and photoperiodic pathways that are coordinated with the autonomous pathway. *VRN1* acts by up-regulating the florigen gene *VRN3*. In *exe* mutants, the mechanism for suppressing expression of *VRN1* under SD conditions must be disrupted. Levels of accumulation of *VRN1* transcripts induce *VRN3* expression, resulting in the extra early-flowering phenotype. Note that the *exe* mutant has no *VRN2* gene, because the original strain KU104-1 lacks *VRN2* locus. *Arrows* and *T-bars* indicate promotion and suppression effect, respectively. *Arrows* indicated by *bold lines* show stronger effects

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Part VII
Biotic Stress Response

Chapter 20
Stem Rust Resistance: Two Approaches

Colin Hiebert, Wolfgang Spielmeyer, Curt McCartney, Mulualem Kassa, Tom Fetch, Frank You, Jim Menzies, Gavin Humphreys, and Brent McCallum

Abstract Stem rust, caused by *Puccinia graminis* f.sp. *tritici* (*Pgt*), is a destructive disease of wheat that has historically caused significant yield losses in much of the global wheat production area. Over the past 50 years, stem rust has been effectively controlled by deploying cultivars carrying stem rust resistance (*Sr*) genes. With the emergence of new *Pgt* races, namely Ug99 and its variants, there has been a reinvestment in stem rust research. This includes discovery, characterization, genetic mapping, and cloning of *Sr* genes. Here we investigated two such examples of genetic characterization and mapping of stem rust resistance. In the first example, a region on chromosome 6DS harbouring resistance to Ug99 was examined in several populations and from several sources. In the second example, a less typical genetic model of resistance was studied in which seedling resistance was activated by an independent locus exhibiting an apparent "nonsuppressing" effect. The knowledge gained by these and other lines of research will contribute to the goal of durable resistance to stem rust.

Introduction

Stem rust, caused by *Puccinia graminis* Pers.:Pers. f. sp. *tritici* Eriks. & E. Henn. (*Pgt*), is a disease of wheat that has the potential to cause devastating losses in grain yield. The last epidemic of stem rust in North America occurred from 1953 to 1955 (Peturson 1958). Since then, resistant cultivars have successfully controlled the disease.

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However, the appearance of *Pgt* race TTKSK, also known as Ug99, rendered most of the globally grown wheat cultivars vulnerable to stem rust (Singh et al. 2011; Fetch et al. 2012). The distinguishing characteristic of this race is its virulence to *Sr31*, a broadly deployed stem rust resistance (*Sr*) gene (Pretorius et al. 2000). Furthermore, Ug99 has continued to evolve and accumulate different virulence combinations (Jin et al. 2009; Park et al. 2011). This has led to renewed efforts to find and deploy new *Sr* genes (e.g. Njau et al. 2010). More generally, this threat from *Pgt* has stimulated investment in several aspects of wheat, *Pgt* and their interactions. This has also underscored the need to employ multiple strategies to achieving resistance and utilise diverse resistance. To meet these needs it is critical to understand the resistance present in breeding material and cultivars, understand the relationship between different sources of resistance (e.g. allelism, linkage, breadth of resistance, target in the pathogen etc.), and develop tools to implement responsible breeding strategies.

It is in the context of renewed and diverse investigation into the wheat stem rust host-pathogen system that this paper was presented. Two lines of research were presented: (1) comparative mapping and fine-mapping of a chromosome region conferring qualitative resistance and (2) genetic interactions that enhance stem rust resistance at the seedling stage and in field conditions across multiple environments. Here, the pertinent literature for these studies is reviewed and our preliminary findings are discussed.

Comparative Mapping of Ug99 Resistance on Chromosome 6DS

SrCad

Most Canadian hexaploid cultivars are susceptible to *Pgt* races from the Ug99 race group and all of the cultivars that are grown on significant acreage are susceptible (Fetch et al. 2012). A screen of Canadian cultivars in Njoro, Kenya, where Ug99 races predominated, revealed that two hard red spring wheat cultivars, Peace and AC Cadillac, showed high levels of resistance. Peace and AC Cadillac are closely related cultivars and were believed to carry the same source of resistance to Ug99. Two populations were generated to study the inheritance and determine the chromosome location of the resistance (Hiebert et al. 2011). A doubled haploid (DH) population from the cross RL6071/Peace and an F_2 population from the cross LMPG-6S/AC Cadillac both showed that a single gene was responsible for the Ug99 resistance detected at the seedling stage. Mapping with SSR markers showed that the resistance in both populations mapped distally on chromosome 6DS in the same genetic interval. A gene for common bunt resistance, *Bt10*, has been mapped to a similar chromosome region (Menzies et al. 2006). DNA marker FSD_RSA,

a dominant marker that was developed to select *Bt10* (Laroche et al. 2000), was added to the genetic maps of the Peace and AC Cadillac derived populations (Hiebert et al. 2011). FSD_RSA showed tight linkage to the *Sr* gene, which was given the temporary designation *SrCad*. Both AC Cadillac and Peace carry *Bt10* which was inherited from the breeding line BW553. In the Canadian Prairie Spring (CPS) class of hexaploid wheat, BW553 was a parent to several cultivars and used as a donor of *Bt10*. A survey of CPS cultivars revealed that all of the cultivars that carried the positive allele (the allele found in BW553, Peace and AC Cadillac) were also resistant to Ug99 while all other cultivars were susceptible (Hiebert et al. 2011). In the germplasm screened with FSD_RSA, the positive allele is rare and has been linked to Ug99 resistance in every instance to date (Hiebert et al. 2011; Ghazvini et al. 2012). Thus, it appears that the presence of FSD_RSA is indicative of *SrCad* though it would be too large an assumption to deem the marker diagnostic as recombinants between FSD_RSA and *SrCad* were recovered in mapping populations (Hiebert et al. 2011; Hiebert et al. unpublished data). Interestingly, the CPS cultivars that carry *SrCad* showed a lower level resistance to Ug99 in field nurseries in Njoro, Kenya compared to Peace and AC Cadillac. Both Peace and AC Cadillac carry *Lr34* while none of the CPS cultivars carrying *SrCad* carry *Lr34*. Hiebert et al. (2011) showed the combination of *SrCad* and *Lr34* improved the level of Ug99 resistance in field conditions.

Sr42

Given the map location of *SrCad*, there was interest in determining the relationship between *SrCad* and *Sr42*. *Sr42* was reported to be carried on chromosome 6DS however there were no published genetic maps. *Sr42* was found in Norin 40, a Japanese winter wheat (McIntosh et al. 1995). Using a DH population from the cross LMPG-6S/Norin 40, Ghazvini et al. (2012) mapped *Sr42* using SSR markers and FSD_RSA. *Sr42* mapped to the same genetic interval as *SrCad*. Norin 40, like Peace and AC Cadillac, carries the positive allele of FSD_RSA. Given that this marker allele is rare, it seems likely that Norin 40 and BW533, the donor of *SrCad* to all Canadian carriers, have a parent in common however no such parent could be traced. The sizes of the DH populations used to map *SrCad* (n = 295; Hiebert et al. 2011) and *Sr42* (n = 248; Ghazvini et al. 2012) yielded good map resolution and their respective map positions are not significantly different (Fig. 20.1). Both *SrCad* and *Sr42* conferred resistance to *Pgt* races Ug99 and RTQSC. While very limited, this does show an additional similarity between these resistances. New markers developed in ongoing fine-mapping experiments were unable to show differences in map position between *SrCad* and *Sr42* (unpublished data). It is likely that *SrCad* and *Sr42* are allelic and probably represent the same allele.

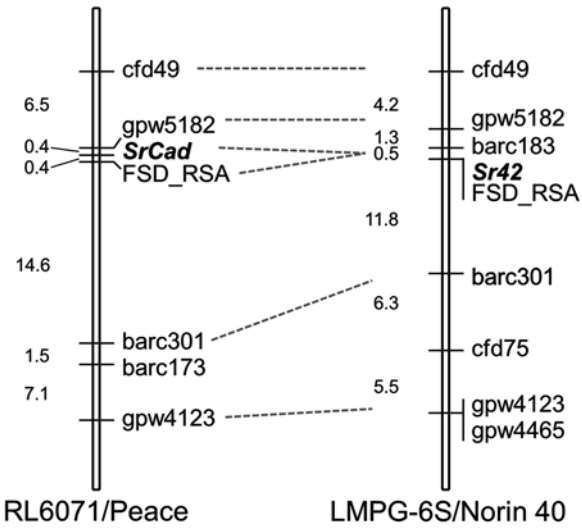

Fig. 20.1 Ug99 resistance on chromosome 6DS mapped in the RL6071/Peace (Hiebert et al. 2011) and LMPG-6S/Norin 40 (Ghazvini et al. 2012) DH populations. Genetic distances are in cM

Other Sr *Genes on Chromosome 6DS that Confer Resistance to Ug99 Stem Rust*

There have other *Sr* genes mapped to 6DS in a similar chromosome region as *SrCad* and *Sr42* that confer resistance to Ug99. One such example was reported by Olsen et al. (2013) where an *Sr* gene, temporarily designated *SrTA10187*, identified in *Aegilops tauschii* was transferred to common wheat and was mapped to chromosome 6DS. In that study, few DNA markers were mapped, however two of the three markers were also used to map *SrCad* (Hiebert et al. 2011). The map positions of *SrCad* and *SrTA10187* cannot be differentiated based on the data published to date. Recently, seedling resistance to Ug99 was mapped in five CIMMYT spring wheat cultivars and one US winter wheat cultivar (Lopez-Vera et al. 2014). In all six populations the resistance mapped to a similar region of chromosome 6DS however the map order varied slightly between the various maps. Whether the map differences are the result of experimental error such as misclassified progeny, or the detection of different genes is unknown presently. The authors postulate that two different genes were mapped, one that is near or allelic to *SrCad*/*Sr42* and one that is distal to these genes but closely linked. It was suggested that the resistance postulated to be allelic to *SrCad*/*Sr42* was *SrTmp* derived from the cultivar Triumph 64 and our preliminary map of *SrTmp* that would agree with this assertion. While Lopez-Vera et al. (2014) are basing their hypothesis on gene postulation experiments, we used a DH population from the cross LMPG-6S/Triumph 64 to generate a population to directly determine the map position of *SrTmp*. Using the LMPG-6S/Norin 40 and

LMPG-6S/Triumph 64 DH populations we were able to show that *Sr42* and *SrTmp* differ in the breadth of resistance they confer. Thus, even if the two are allelic, they represent different allelic forms.

Conclusion

Resistance to Ug99-type stem rust races has been mapped to chromosome 6DS in a number of studies from a number of sources. Understanding the relationships between these genes is important for developing strategies to deploy these genes. If the genes are allelic it is prudent to determine which allele is the most relevant for a given growing region and the local *Pgt* population. If the genes are non-allelic it could be worthwhile to attempt to recover recombinants carrying gene combinations to generate a gene stack carried in a tight linkage group. The DNA markers developed from the fine-mapping of *SrCad* (unpublished) could be useful in delineating the *Sr* genes mapped to this chromosome region. To further characterize these genes phenotypically, near-isogenic lines (NILs) should be produced to allow an accurate comparison of their range of effectiveness against a panel of *Pgt* races.

Chromosome 7D: Carrier of a Suppressor and a Nonsuppressor

A Suppressor of Stem Rust Resistance

Canthatch is an old Canadian hard red spring wheat cultivar that is a derivative of Thatcher (McCallum and DePauw 2008). Canthatch is susceptible to several races of *Pgt* and was used in many genetic studies by Dr. Eric Kerber (retired, AAFC, Winnipeg, Canada). In an early experiment, the A and B genome component of Canthatch was isolated to generate an 'extracted tetraploid' which had the genome constitution of $2n=4x$, AABB (Kerber 1964). The extracted tetraploid, named Tetra Canthatch, lacked vigour, had low fertility, and was morphologically different from Canthatch and common tetraploids. Furthermore, Tetra Canthatch had seedling resistance to some *Pgt* races that were virulent to Canthatch. This implied that the D genome or a component of the D genome was suppressing *Sr* genes present on the A and/or B genomes in Canthatch. Using nullisomic and ditelosomic stocks in a Canthatch background, it was demonstrated that the suppression of *Sr* genes was caused by the long arm of chromosome 7D (Kerber and Green 1980). Induced mutants showed the same phenotype as Tetra Canthatch and Canthatch ditelo 7DS. Furthermore, the suppression was caused by a single gene on chromosome 7DL (Kerber 1991). Telocentric mapping showed that the suppressor locus was independent of the centromere.

Adult-Plant Resistance Genes Can Act as a Nonsuppressor

Lr34 is a well-known adult-plant resistance (APR) gene that confers quantitative resistance to all of three of the rust disease of wheat and is located on the short arm of chromosome 7D (Dyck 1987; Singh et al. 2012). In addition to conferring APR, *Lr34* was associated with nonsuppression of seedling stem rust resistance in a Thatcher background (Dyck 1987; Kerber and Aung 1999). Comparing Canthatch, Thatcher, Tetra Canthatch, Canthatch-nulli 7D and RL6058 (a Thatcher NIL carrying *Lr34*) with four races of *Pgt*, Dyck (1987) showed that (1) Canthatch and Thatcher were susceptible to all four races of *Pgt*, (2) Tetra Canthatch and Canthatch-nulli 7D were resistant to three of these races, (3) RL6058 was resistant to the same three races as Tetra Canthatch and Canthatch-nulli 7D, and (4) one race was virulent on all five genotypes. Thus, it appears that *Lr34* negates the effect of the suppressor locus and that that the stem rust resistance expressed in Thatcher-type wheats by either removing the suppressor or adding *Lr34* is race-specific. Figure 20.2 shows a conceptual drawing of the relationship between the suppressor locus and *Lr34*, although the actual mechanisms are not presently understood.

Another Thatcher NIL, RL6077, showed the same pattern of seedling stem rust resistance as RL6058 (Dyck 1987). RL6077 carries the gene *Lr67* which is similar to *Lr34* in its phenotype and its multiple-disease resistance (Hiebert et al. 2010). A preliminary study showed that *Lr67* was responsible for the expression of seedling stem rust resistance in RL6077 and appeared to act as a nonsuppressor like *Lr34* (Hiebert et al. 2012). Thus, two unique APR genes, which both confer multiple pest resistance, also appear to act as nonsuppressors of seedling stem rust resistance genes found in Thatcher-type wheats.

We presented preliminary data in three areas to (1) assess *Lr34* as a nonsuppressor, (2) preliminary map position of seedling stem rust resistance in Thatcher expressed in the presence of *Lr34*, and (3) preliminary map position of stem rust resistance in the field that is expressed in the presence of *Lr34*. Using mutants of *Lr34* in RL6058 (Spielmeyer et al. 2013), it appears that mutant lines that have lost *Lr34* activity

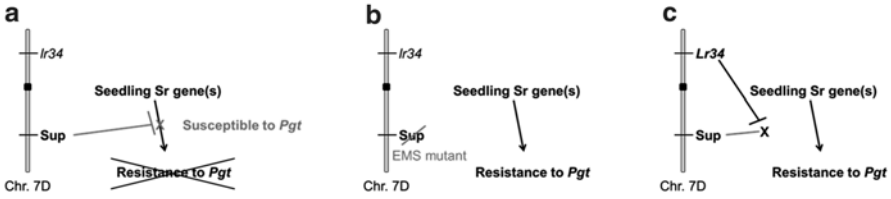

Fig. 20.2 A conceptual summary of the interaction of the suppressor (Sup) and *Lr34* on chromosome 7D. (**a**) The suppressor prevents the expression of seedling *Sr* genes from the A and/or B genomes of Thatcher-type wheats if the susceptible allele of *Lr34* is present. (**b**) Knocking out the suppressor by mutagenesis allows the expression of the otherwise suppressed Sr genes. (**c**) The resistant allele of *Lr34* appears to negate the effect of the suppressor and allows the expression of the *Sr* genes

have also lost the seedling stem rust resistance observed in the presence of a normal *Lr34* allele. Thus, we tentatively claim that it is *Lr34* that is responsible for the nonsupressing character observed in RL6058 and not a gene tightly linked to *Lr34*. We developed two populations that are fixed for *Lr34* but segregate for Thatcher-derived seedling stem rust resistance and resistance in the field. Seedling resistance and field resistance were mapped as QTL in these preliminary analyses and chromosome 3B significantly contributed to both traits. These preliminary findings need to be confirmed and experiments are ongoing to do so.

Conclusions

One aspect of the relationship between the suppressor on 7DL and *Lr34* that is presently unknown is whether removing or inactivating the suppressor leads to the expression of the same *Sr* genes that are expressed in the presence of *Lr34*. Similarly, we do not know if *Lr67* and *Lr34* are interacting with the same genes. These lines of investigation are ongoing and should fill in the gaps in our knowledge of these interactions. From a practical standpoint, understanding these genetic interactions could be important for wheat breeders. It would easier to retain *Sr* genes (i.e. the suppressed genes) in elite breeding material and add, for example, *Lr34* compared to the effort of introducing new or different *Sr* genes to enhance to stem rust resistance in new cultivars. In Canada, Thatcher comprises a large component of coefficient of parentage for most of the widely grown hard red spring wheat cultivars (McCallum and DePauw 2008). Thus, identifying the genes in Thatcher that are expressed in the presence of *Lr34* is directly applicable.

Concluding Remarks

An array of strategies can be employed to achieve resistance to stem rust. There is not a "one size fits all" approach that must be followed. However, genetic resources for resistance to stem rust are valuable and implementing a strategy that prolongs the usefulness of *Sr* genes is the responsible approach. In order to accomplish this we must understand which genes are present in our germplasm, determine the relationships between genes and their breadths of effectiveness, develop tools for molecular breeding approaches, and unravel the genetic interactions that can lead us to more durable resistance. Here we examined two examples of the lines of research that can help geneticists, pathologist and breeders reach the goal of effective and durable resistance. With the recent investment in various aspects of wheat stem rust research more tools and resources are becoming available. It is now our responsibility to translate the advances made in the lab into rust resistant wheat cultivars in the field.

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Chapter 21
Germplasm Enhancement for Resistance to *Pyrenophora tritici-repentis* in Wheat

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Abstract Yellow spot (syn. tan spot), caused by *Pyrenophora tritici-repentis*, is an important foliar disease of wheat in Australia that causes losses exceeding 50 % when conditions are favourable for disease development. Although good progress has been made internationally to understand yellow spot resistance, relatively few resistance genes have been identified and mapped in Australian germplasm and only one (*tsn1* on chromosome 5BL) is in general and known use in Australian breeding programs. Although *tsn1* is an important yellow spot resistance gene, it doesn't explain the full spectrum of resistance and there is a significant opportunity to enhance expression of yellow spot resistance through identification of resistance factors other than *tsn1*. Six doubled haploid (DH) mapping populations (five of which were fixed for *tsn1*) were screened for yellow spot resistance at the seedling/tillering and adult plant stages at the Department of Agriculture and Food, Western Australia (DAFWA) and the Department of Environment and Primary Industries Victoria (DEPIVic) from 2009 to 2012. Four of the above populations were screened at the Department of Agriculture, Fisheries and Forestry Queensland (DAFFQ). Frequency distribution of individuals within each population for various levels of yellow spot resistance was continuous indicating that resistance is conditioned by several genes with partial effects. A few lines within each population consistently showed high levels of resistance probably resulting from a combination of several genes with additive effects. Nine new loci for yellow spot resistance were mapped

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by the Australian Wheat and Barley Molecular Marker Program (AWBMMP) at the University of Adelaide on chromosomes 1AS, 2AS, 5AS, 5AL, 4B, 6BS, 7BL, 2D and 7D in five of the six populations phenotyped so far. High LOD scores of 9–11 have been obtained for some of the QTL with a percentage disease reduction of 24–42 %. Efforts are now focused on identifying additional yellow spot resistance genes using newly developed populations and rapid phenotyping methods and developing a series of fixed lines, each carrying yellow spot resistance genes from various sources, in elite Australian backgrounds. These materials will provide proof-of-concept for achieving better resistance by pyramiding resistance genes, and they should be directly useful as parents for wheat breeding.

Keywords Phenotyping • Quantitative trait loci • Resistance • Tan spot • Yellow leaf spot

Pyrenophora tritici-repentis is a necrotophic fungus that causes yellow spot of wheat. The disease is also known as yellow leaf spot or tan spot. There has been an increased prominence of this disease in recent years due to implementation of new farm practices such as minimum tillage, stubble retention, increased wheat-after-wheat cropping and use of susceptible varieties. In Australia, Shipton (1968), Rees et al. (1982), Rees and Platz (1983), and Bhathal et al. (2003) measured 20–50% losses in grain yield of bread wheat from this disease. In Western Australia this disease often occurs as a complex with *Stagonospora nodorum* blotch.

P. tritici-repentis is known to produce at least three host specific toxins (HSTs) that interact with specific host sensitivity genes to cause necrosis and/or extensive chlorosis (Ali et al. 1999; Lamari et al. 1995, 2005; Strelkov et al. 2002). These are Ptr ToxA, for which sensitivity is conditioned by the gene *Tsn1* on chromosome arm 5BL (Anderson et al. 1999; Faris et al. 1996); Ptr ToxB, for which sensitivity is conditioned by the gene *Tsc2* on chromosome arm 2BS (Friesen and Faris 2004), and Ptr ToxC, for which sensitivity is conditioned by the gene *Tsc1* on chromosome arm 1AS (Effertz et al. 2002). Isolates of the pathogen can be classified into eight theoretical races based on the presence or absence of each of the three toxins. All eight of these combinations have been found among North American isolates. Recently, however, ToxA and ToxB gene-deficient isolates from Arkansas have been found not to fit this race classification system (Ali et al. 2010). These isolates may contain different toxin compounds that produce ToxA-like symptoms on some cultivars of wheat.

Improved resistance to the disease is the most economical and effective method of control and is therefore a key target for Australian wheat breeding programs. Resistance can be both qualitative (Gamba and Lamari 1998; Lamari and Bernier 1989) and quantitative (Elias et al. 1989; Friesen and Faris 2004). Genes such as *tsn1*, *tsn2*, *tsn3*, *tsn4*, *tsn5*, *tsn6*, and *tsn-syn1* and *Tsn-syn2*, are known to affect toxin sensitivity and to confer race-specific resistance to necrosis (Anderson et al. 1999; Singh et al. 2006, 2008; Tadesse et al. 2006a, b, 2010) and the recessive genes *tsc1* and *tsc2* condition resistance to chlorosis (Abeysekara et al. 2010; Effertz et al.

2002; Friesen and Faris 2004). Non-race-specific quantitative trait loci have been mapped on almost all chromosomes of wheat (Chu et al. 2008, 2010; Faris and Friesen 2005; Faris et al. 2012; Li et al. 2011; Singh et al. 2012; Sun et al. 2010; Patel et al. 2013). Incorporating both qualitative and quantitative resistance is essential to develop cultivars with high levels of durable resistance.

Although good progress has been made internationally to understand yellow spot resistance, only one gene (*tsn1* on chromosome 5BL) is in general and known use in Australian breeding programs. While *tsn1*, is an important YS resistance gene, it doesn't explain the full spectrum of resistance in the Australian germplasm. Furthermore, Faris et al. (2012) have shown that the amount of variation explained by *tsn1* can vary considerably (5–30 %) with different isolates and suggest possible variability in ToxA gene regulation amongst isolates.

The Department of Agriculture and Food Western Australia (DAFWA) has been conducting research for the past 3 years on genetic enhancement of resistance to the disease and improved germplasm development under a national project involving several collaborating organisations and co-funded by the Grains Research and Development Corporation. The project aims to provide improved genetic solutions to yellow spot management by: (i) development of improved phenotyping methods, (ii) identification of new resistance genes, (iii) improved germplasm and associated genetic knowledge that enhance resistance expression and diversity and (iv) improved parental stocks which can be readily utilised within breeding programs.

Phenotyping methods have been improved in three areas. Firstly, enhanced spore production techniques have been developed based on a modified method of Evans et al. (1993) which allow the production and storage of large amounts of yellow spot inoculum. This has greatly improved inoculation of a large number of plots both in the field and glasshouse. Secondly improved field phenotyping methods have been developed based on Shankar et al. (2008) which overcome the effect of maturity and height on disease expression allowing the assessment of true adult plant resistance. This basically involves inoculating individual plots at heading and rating at a specific thermal time after inoculation. Thirdly, a rapid assay for evaluating adult plant resistance has been developed under controlled environmental conditions under which the same plant can be assessed at both seedling and adult stages. Under this method plants head within 5–8 weeks under controlled environment as compared to 11–16 weeks in the field. Moderate to good correlations ($r = 0.7–0.8$) are obtained between assessments made under controlled environmental conditions and field conditions.

Twelve doubled haploid mapping populations have been developed or made available to the project. Of these, six populations (five of which were fixed for *tsn1*) were phenotyped for yellow spot resistance from 2009 to 2012 at the seedling/tillering and adult plant stages at DAFWA and the Department of Environment and Primary Industries Victoria (DEPIVic). Four of the above populations were phenotyped at the seedling/tillering and adult plant stages at the Department of Agriculture, Fisheries and Forestry Queensland (DAFFQ). Frequency distribution of individuals within each population for various levels of yellow spot resistance was continuous indicating that resistance is conditioned by several genes with partial effects. A few

lines within each population consistently showed high levels of resistance probably resulting from a combination of several genes with additive effects.

Genetic maps were developed by the Australian Wheat and Barley Molecular Marker Program (AWBMMP) at the University of Adelaide using DArT, SSR and SNP markers. Nine major QTL other than *tsn1* have been mapped on chromosomes 1AS, 2AS, 5AS, 5AL, 4B, 6BS, 7BL, 2D and 7D in five of the six populations phenotyped so far. Of these, QTL on 2A and 6B appear commonly between two populations but it is yet to be determined if they are at the same chromosomal positions. Statistical analyses have been completed on two of the populations and good correlations ($r = 0.6–0.8$) and high heritability estimates ($H^2 = 0.7–0.9$) were obtained for yellow spot severity at various growth stages, environments and sites. High LOD scores of 9–11 have been obtained for the resistance QTL detected in these populations with a percentage disease reduction of 24–42 %.

Germplasm development is being carried out collaboratively by DAFWA and AWBMMP. The strategy involves pyramiding at least three resistance loci from different sources into elite Australian backgrounds. In a first set of crosses QTL on 2A and 5BL detected in one population are being pyramided with QTL on 1A detected in another population. Resistant parents were selected using both phenotypic and genotypic data and a cross was made in the spring of 2011. Although all the 100 F_1s produced turned out to be grass clumps 194 F_2 seeds were produced off these grass clumps by growing them at a constant 26 °C temperature and gibberellic acid treatment. The F_2s were then grown out in single seed trays and leaf material sent to AWBMMP for marker-assisted selection. F_2 and F_3 segregation indicated that the grass clump trait was controlled by two to three complementary genes.

Currently 16 F_4 lines, homozygous at the three resistance loci as well as fixed at the *Vrn-A1* locus have been selected. Fourteen of these are mid to late maturing while two are early maturing. In addition 28 other F_4 lines, also homozygous at the three resistance loci but segregating for either grass clumps or at the *Vrn-A1* locus, have been selected. These are being further selected against the grass clump trait and are being fixed for the *Vrn-A1* locus. All F_4/F_5 selections will be grown out for a bulk F_5/F_6 harvest and phenotyped for proof of concept. These are potential parental stocks enriched for resistance in wheat breeding.

This year crosses are being made to pyramid up to five resistance loci in both short season and long season backgrounds by crossing lines with three stacked genes with parental selections involving two additional genes from other available populations.

A spin off research activity under gene pyramiding for yellow spot resistance is developing sister lines with different resistance QTL combinations. For this, individuals which are heterozygous at the three resistance loci are being selected in each generation while allowing the rest of the genome to become homozygous. The aim is to obtain different QTL combinations within sister lines so that effects of individual loci and loci in various combinations can be compared within a similar background. Currently 25 F_4 lines heterozygous at the three resistance loci have been selected and will be further selected in F_5 for various QTL combinations.

In another activity within the project, genetic variability within the Australian germplasm is being combined by developing a large multiparent advanced generation intercross (MAGIC) population from a complex cross involving different sources of yellow spot resistance. The four parents include GBA Ruby, H45, King Rock and Magenta. The four-way cross was completed last spring and 583 F_1s produced. Two F_2 seeds from each of these F_1s are being progressed through SSD to F_5. Four way crossing is continuing this year with the aim to produce a total of around 2,000 F_5 lines. This population will provide a unique opportunity to maximise genetic recombination, generate potential parental stocks enriched for yellow spot resistance and create opportunities for fine mapping resistance loci.

Another major activity within this project is identification of elite yellow spot resistant lines amongst material acquired from CIMMYT and ICARDA and amongst various mapping populations. Of over 1,000 lines screened so far 42 lines representing targeted yellow spot resistant material were tested under a ring test at various growth stages across Australia and 19 of these have been identified as having good levels of broad spectrum yellow spot resistance.

New resistance genes, germplasm and screening methodologies developed under this project are important resources that can be used by breeders for rapid development of varieties with high levels of resistance at both seedling and adult plant stages that is effective in various environments and across various national sites. Newly developed resistant varieties will greatly reduce the estimated annual losses of $212 M per annum caused by this disease in Australia (Murray and Brennan 2009).

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Chapter 22
Next Generation Sequencing Enabled Genetics in Hexaploid Wheat

Ricardo H. Ramirez-Gonzalez, Vanesa Segovia, Nicholas Bird, Mario Caccamo, and Cristobal Uauy

Abstract Next Generation Sequencing (NGS) is providing new methodologies to improve and complement traditional genetic approaches. These strategies, collectively termed NGS-enabled genetics, consist of identifying variation in bulks of plants that have been assembled based on a specific phenotype of interest. We examined NGS-enabled genetics in hexaploid wheat by using near isogenic lines (NIL) differing across a specific disease resistance locus. RNA-Seq of NILs allowed the identification of SNPs across this locus and helped distinguish allelic SNPs from homoeologous variants. F_2 bulks were assembled based on opposing disease resistance phenotypes and the frequency of the informative allelic SNPs was examined across bulks using RNA-Seq. Variants enriched in the corresponding bulks are expected to be most closely linked to the phenotype of interest and were prioritized for validation. Recent advances in cereal genomics in the form of wheat gene models, sequenced diploid progenitors, and the advances in the Chromosome-based Survey Sequencing Project enabled us to develop a pipeline to automatically design SNP-based markers. These high-throughput assays were used to genotype the original individuals used to assemble the bulks and to generate a genetic map across the target locus. Linked markers are now being incorporated into marker assisted selection programs by breeders.

Keywords Bulk frequency ratio • Bulked segregant analysis • Genotyping • KASP • Marker assisted selection • Near isogenic lines • Next generation sequencing • RNA-Seq

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Introduction

Wheat is among the most important crops in the world providing over 20 % of the world's calorie and protein intake (FAO 2012). Genetic improvement and breeding, however, are hampered by the large proportion of repetitive elements (>80 %, Flavell et al. 1974) across its large genomes and the polyploid nature of hexaploid wheat which often leads to functional redundancy among homoeologous genes (Avni et al. 2014). The advances in next generation sequencing (NGS) technologies are creating new opportunities to exploit the variation in the wheat genome for crop improvement, both in repetitive regions (insertion site-based polymorphism (ISBP) markers, Paux et al. 2010) and in low copy regions (gene based single nucleotide polymorphisms (SNPs), Allen et al. 2013).

The challenges that are thus emerging are how to prioritize the large number of SNPs available both in public databases, for example Cerealsdb.uk.net (Wilkinson et al. 2012), and those obtained with Next Generation Sequencing (NGS), as well as how to convert these into high-throughput (HTP) markers for breeding. One option is to exploit synteny between grass species (Moore et al. 1995), although this approach is limited to gene-based SNPs. The recent release of the Wheat Chromosome-based Survey Sequencing Project coordinated by the International Wheat Genome Sequencing Consortium (IWGSC) allows broad scale localisation to a particular homoeologous chromosome arm, although resolution is limited.

Wheat Genetics

A complementary approach is to exploit genetics to decrease the background complexity of the wheat genome. Near isogenic lines (NILs) that only differ across a specific target region can be a powerful resource for this purpose. A great example is the set of Avocet-S NILs for major disease resistance (*R*) genes against yellow rust (*Yr*) developed by Colin Wellings and colleagues at the University of Sydney. We have used the Avocet S-*Yr15* NILs (Fig. 22.1a) to develop a segregating BC_7F_2 population that was phenotyped through virulence assays to the wheat yellow rust pathogen (*Puccinia striiformis*). Resistant plants, composed of both homozygous and heterozygous individuals across the *Yr15* locus, were pooled into a resistant bulk. In an analogous manner, susceptible plants were grouped into a susceptible bulk (Fig. 22.1b). This strategy, first published as bulked segregant analysis (BSA) in 1991 (Michelmore et al. 1991), can now be further exploited with NGS approaches. An initial complexity reduction is achieved with the Avocet NILs whose main difference is across the *R* gene locus. This list of SNPs can then be further refined by the BSA approach. In this study we highlight how the combination of genetic and genomic resources, along with methods such as BSA, can be used to leverage new biological research and develop HTP markers for breeding.

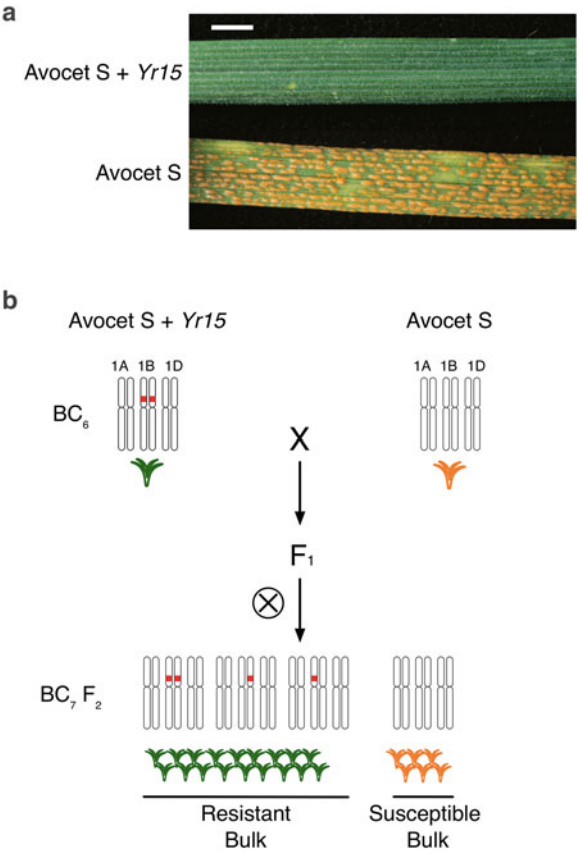

Fig. 22.1 Segregating population for *Yr15*. (**a**) Resistant and susceptible phenotypes of Avocet S and Avocet S + *Yr15* challenged with the yellow rust pathogen (*Puccinia striiformis*), respectively. *Scale bar*; 1 cm. (**b**) Segregating lines were developed by crossing a homozygous resistant NIL, Avocet S + *Yr15*, to Avocet S. Heterozygous plants were then self-pollinated to produce a segregating F_2 population with an expected 3:1 segregation ratio for resistant and susceptible individuals, respectively. The resistant and susceptible plants were grouped by phenotype into the corresponding bulks. The resistant bulk includes both homozygous and heterozygous individuals across the *Yr15* locus as exemplified by the red squares on the wheat chromosomes

Wheat Genomics

A high quality reference genome of bread wheat hasn't yet been completed. In the past few years, however, the wheat community has produced valuable genomic resources that can be used as a proxy reference: a whole genome shotgun (WGS) assembly of Chinese Spring based on 454 technology (Brenchley et al. 2012); the reference genomes for the diploid progenitor species *Triticum uratu* (2n = 14;AA) (Ling et al. 2013) and *Aegilops tauschii* (2n = 14;DD) (Jia et al. 2013); an assembly of the close Triticeae relative *Hordeum vulgare L.* (2n = 14;HH) (International Barley Genome Sequencing et al. 2012); and the genomic contigs from the Chromosome-based Survey Sequencing (CSS; International Wheat Genome Sequencing Consortium 2014). We can use the listed collections as supporting information, bearing in mind that discrepancies are expected for the related organisms and that specific genes being studied may not be present in Chinese Spring. Nevertheless, using the available genomic sequences facilitates NGS analyses that depend on a reliable genome reference.

SNPs that can be used to map genes of interest are becoming increasingly available. The CerealsDB website holds ~100,000 SNPs from British varieties (Allen et al. 2013) obtained by transcriptome and exome sequencing, although only a fraction (<8 %) have been converted into functional HTP assays. Recently a subset of these have been incorporated into the ~82,000 iSelect array coordinated by Eduard Akhunov at Kansas State University (Wang et al. 2014) which follows the 9,000 SNP array (Cavanagh et al. 2013). These SNP arrays are providing an extremely valuable resource for the community in diversity and association studies. Their use however is limited for large mapping populations because of their cost, the difficulty in interpreting data when using diploid or alien introgressions, and the difficulty to reliably call heterozygous individuals. Because of these reasons, alternative approaches are sometimes required to identify polymorphisms across target regions.

NGS is providing different methodologies to identify putative variations. WGS sequencing consists on sequencing random fragments of genomic DNA without any selection except for the fragment size. The reads are then aligned to a reference and SNPs can be called from the alignments. In several model organisms, these re-sequencing NGS approaches have been combined with bulks/pools of phenotypically distinct individuals to identify SNPs that are closely linked with the gene of interest within a single experiment.

These approaches, collectively termed as NGS-enabled genetics (Schneeberger and Weigel 2011), are rapidly evolving and have used different strategies. MutMap produces segregant populations from seeds with induced mutations and then bulks are sequenced using WGS (Abe et al. 2012; Takagi et al. 2013b). QTL-seq takes two plants with opposite phenotypes and the progeny with extreme phenotypes are bulked and their DNA is extracted for sequencing (Takagi et al. 2013a). Renseq, focuses on *R*-genes by designing baits from known resistance genes and performing targeted resequencing (Jupe et al. 2013). The mentioned techniques do not scale well in wheat because they rely on having a reliable reference sequence and relatively small genome size. A full Illumina HiSeq 2500 can produce 600-Gbp in a single run, providing 35-fold coverage of the complete wheat genome. However, this translates into less than two reads for each position per individual for a bulk of 20 plants.

To reduce the complexity of the data, we propose to focus on sequencing the wheat transcriptome using RNA-Seq (Westermann et al. 2012) instead of genomic DNA. By sequencing the transcribed RNA we can use short read aligners and use transcript assemblies as reference (Fig. 22.2a). At first we used the wheat UniGenes set available NCBI, which consist of collapsed homoeologous transcripts from a

Fig. 22.2 (continued) reference sequence, with matches indicated by *dots*, and polymorphisms at positions 181 and 184 indicated by the corresponding nucleotide variants at those positions. The SNP index is calculated as the frequency of the informative allelic SNP in each bulk. The Bulk Frequency Ratio is the quotient of the resistant and susceptible bulk SNP Indexes. (**c**) Primer design: The allelic SNP (G184A) is used at the 3′ end of the differentiating primers. For the common primer, a homoeologous SNP is selected for the 3′ end to make the marker genome-specific. (**d**) The KASP assay output shows the intensities of the HEX and FAM fluorescence of individual plants as a single dot. The clusters near the X and Y-axis are composed of homozygous individuals, while the central cluster contains heterozygous plants

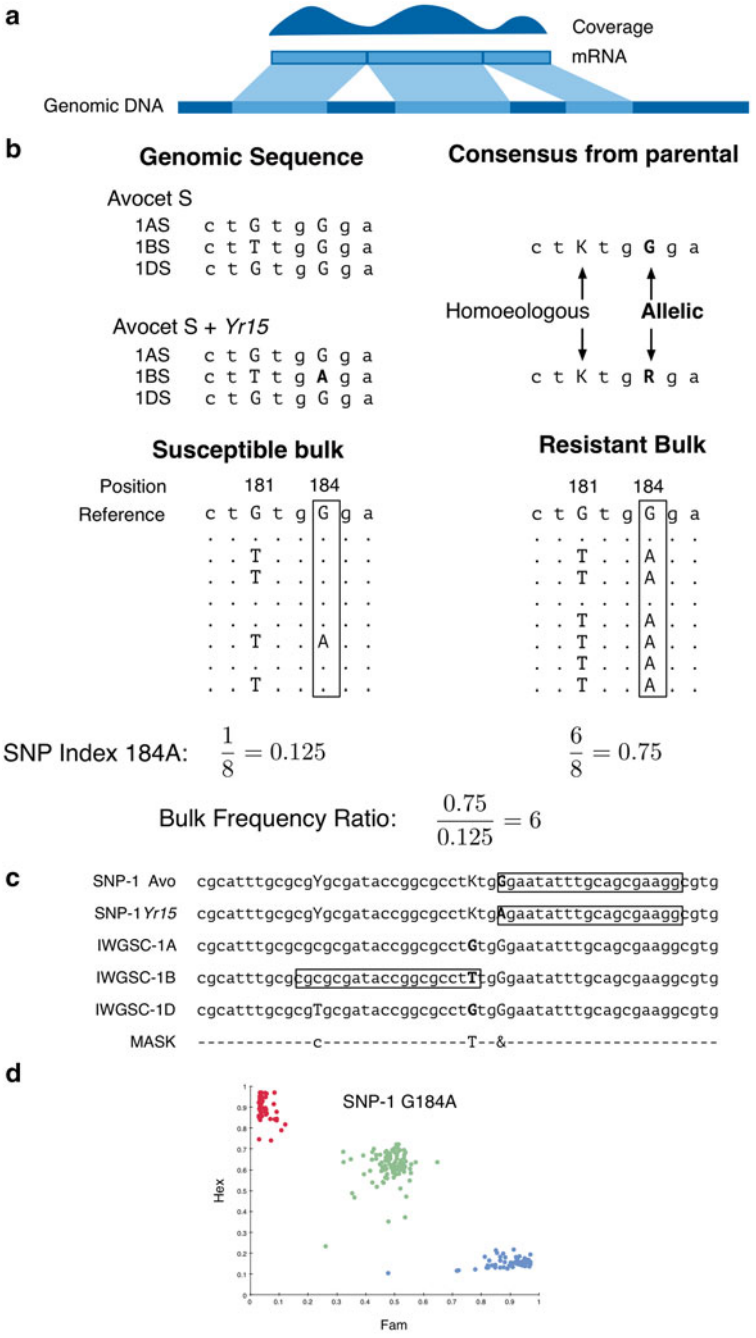

Fig. 22.2 RNA-Seq NGS-enabled genetics. (**a**) Representation of a typical RNA-Seq output; the method excludes non-coding regions from the genome and the coverage is correlated to the expression of the gene. (**b**) Illustration of a non-informative homoeologous SNP (G181T) present in both *parental lines*, and an informative allelic SNP (G184A), only present in the resistant progenitor Avocet S+*Yr15*. The consensus sequences from the parental genotypes include this information in the form of ambiguity codes (K and R, respectively). In the bulks, the individual reads align across the

myriad of sources (Pontius et al. 2002). More recently, we have shifted to a phased transcriptome which has gene models separated by the corresponding genome (Krasileva et al. 2013).

RNA-Seq captures the full range of the dynamic spectrum of the transcriptome, an advantage when compared to array platforms that are restricted to the pre-defined set of variants incorporated into the array design. SNPs can be identified either by aligning to a known transcriptome or by *de novo* assembly over the transcriptome (Grabherr et al. 2011). With the use of F_2 populations it is possible to create a panel of putative SNPs that enables haplotype analysis without a priori knowledge of the positions of the loci (Trick et al. 2012). RNA-Seq allows rapid access to SNPs in wheat and it scales well as the transcriptome is several orders of magnitude smaller than the genome, ~80 Mb compared to 17 Gb, respectively. In principle, it is possible to sequence the transcriptome with an average coverage over 900x (keeping in mind variations of coverage due to expression) in a single HiSeq 2,500 lane, as opposed to just over 1.5x of genomic sequence.

The original purpose of RNA-Seq is to characterize expression levels of genes, which can bias the SNP calling, as the assumption of uniform coverage is not valid. To overcome these biases and analyse the volume of data produced by NGS, bioinformatics pipelines and access to high performance computing are required. Although this is a potential barrier for adoption, new web-based user-friendly graphical interfaces, such as Galaxy (Goecks et al. 2010), are empowering new users to access high-performance computing facilities. For NGS-enabled genetics in wheat, we propose a pipeline that integrates BSA (Michelmore et al. 1991; Trick et al. 2012), syntenic information from related grasses, and the use of the CSS to aid in the design of genome specific primers from putative SNPs.

SNP Selection and Marker Design

To identify loci linked to a trait we use BSA and extend it by using RNA-Seq instead of targeting known markers. We sequence the parental genotypes (Avocet S and Avocet S+*Yr15*, Fig. 22.1a, b) and generate a consensus reference by aligning the reads to the UniGenes and gene models described above (Krasileva et al. 2013). Although genome-specific references are used when possible, there are still cases where multiple homoeologues will align to a common reference (as illustrated in Fig. 22.2b). These homoeologous variants (exemplified by the G>T variant at position 181; K in consensus) will generate ambiguity codes within both parental consensus sequences and can therefore be excluded. Real allelic SNPs between the parental genotypes (exemplified by the G>A variant at position 184; R in consensus) are distinguished by the presence in one, but not the other parental consensus sequence (Fig. 22.2b).

These allelic SNPs are then examined further in the alignments of the RNA-Seq reads from the susceptible and resistant bulks. To identify enriched and depleted SNPs we first calculate the SNP Index, which is the proportion of the non-consensus

base at the positions previously identified (A at position 184 according to the example in Fig. 22.2b; Takagi et al. 2013a). Then, we calculate the bulk frequency ratios (BFR), which is the ratio between the SNP Indexes of the resistant and susceptible bulks (Trick et al. 2012). The BFR helps reduce the noise generated by differential expression of homoeologous genes and accounts for the presence of alternative bases at any given position. A closely linked SNP to the *R*-gene should generate a very high BFR since the resistant bulk will carry exclusively plants with the resistant allele, whereas the susceptible bulk should be devoid of any plants carrying the resistant allele. As one moves further away from the *R*-gene, recombination events occur between the gene and the candidate SNPs decreasing the BFR.

Once a list of SNPs with the corresponding BFRs is obtained, these can be prioritized in several ways before independent validation as genetic markers. First and foremost is the BFR value itself; the higher the BFR the most likely the SNP is genetically linked to the gene. Second, we can align the candidate genes to the genome of syntenic species such as *Hordeum vulgare*, *Brachypodium distachyon* and *Oryza sativa* to identify the orthologous genes and start to identify syntenic regions with high BFRs. More recently, we have started to align the genes to the CSS assemblies to locate the chromosome arms with highest BFR. The putative SNPs with enriched BFR can then be converted into HTP SNP assays (see next section) and genotyped across the individuals that were used to assemble the bulks. This generates a genetic map with markers across the *R*-gene locus. If the interval is syntenic to one of the sequenced grass genomes, an additional round of SNP selection can be performed based on synteny and using slightly more relaxed parameters to establish the BFR cut-off.

High-Throughput Genotyping

A key issue is to move from *in silico* SNPs into a HTP SNP assay that can be amenable to both researchers and wider applications such as marker assisted selection (MAS) in breeding programs. In this regard, it is particularly important to have genome specific SNP assays that allow the screening of germplasm where heterozygotes need to be easily identified. The IWGSC CSS assemblies facilitate this objective by allowing us to generate a multiple alignment of the reference sequence containing the SNP of interest with the assemblies from the three homoeologous genomes (Fig. 22.2c). In this manner, homoeologous SNPs can be readily identified and incorporated into the primer design to assay the SNP only in the genome of interest. This is particularly applicable to end point fluorometric assays, such as KASP (Allen et al. 2013), that identify SNPs using two differentially labeled primers with the SNP at the 3′ end of the primers (boxed sequences with SNP in bold; Fig. 22.2c). A common reverse primer can be designed across a homoeologous SNP to generate the genome specific amplification. This results in a HTP assay that can be readily used to genotype heterozygous individuals (Fig. 22.2d). In the past, the generation of genome-specific assays has been a time consuming task (Chao et al.

2008) but with the use of this pipeline the creation of allele specific SNPs has been simplified. We developed a web-based user-friendly interface (PolyMarker, Ramirez-Gonzalez et al. 2015a) to make this pipeline readily available to the community (http://polymarker.tgac.ac.uk).

Final Remarks

Despite the complexity of the wheat genome and the draft status of the genomic reference, it is possible to advance in the development of NGS-enabled genetic approaches in hexaploid wheat. RNA-Seq of NILs facilitates the identification of SNPs and helps distinguish these as informative allelic SNPs or non-informative homoeologous variants. RNA-Seq of NIL-derived F_2 bulks helps identify those SNPs that are most closely linked to the phenotype of interest. With the aid of the recently released CSS, putative SNPs can be rapidly converted into HTP assays that can be incorporated into MAS improvement programs by breeders (Ramirez-Gonzalez et al. 2015b). These approaches will continue to improve in resolution as physical maps improve in wheat and homoeologous relationships between transcripts are more precisely defined.

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Part VIII
Abiotic Stress Response

Chapter 23
Genomics Approaches to Dissect the Genetic Basis of Drought Resistance in Durum Wheat

Roberto Tuberosa and Marco Maccaferri

Abstract A better knowledge of the genetic basis of the mechanisms underlying the adaptive response to drought will be instrumental to more effectively deploy marker-assisted selection (MAS) to improve yield potential while optimizing water-use efficiency. Genomics approaches allow us to identify and clone the genes and QTLs that underlie the adaptive response of durum wheat to drought. Linkage and association mapping have allowed us to identify QTLs for traits that influence drought resistance and yield in durum and bread wheat. Once major genes and QTLs that affect yield under drought conditions are identified, their cloning provides a more direct path for mining and manipulating beneficial alleles. While QTL analysis and cloning addressing natural variation will increasingly shed light on mechanisms of adaptation to drought and other adverse conditions, more emphasis on approaches relying on resequencing, candidate gene identification, 'omics' platforms and reverse genetics will accelerate the pace of gene/QTL discovery. Genomic selection provides a valuable option to improve wheat performance under drought conditions without prior knowledge of the relevant QTLs. Modeling crop growth and yield based on the effects of major QTLs offers an additional opportunity to leverage genomics information. Although it is expected that genomics-assisted breeding will enhance the pace of durum wheat improvement, major limiting factors are how to (i) phenotype genetic materials in an accurate, relevant and high-throughput fashion and (ii) more effectively translate the deluge of molecular and phenotypic data into improved cultivars. A multidisciplinary effort will be instrumental to meet these challenges.

Keywords Consensus map • Drought • Genomics • Durum wheat • Marker-assisted selection • Modeling • Phenotyping • QTL • Roots • Yield

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Introduction

Among all abiotic stresses most affected by climate change, drought is the major one curtailing global wheat production. Although conventional breeding has steadily increased crop productivity under drought conditions and across a broad range of environmental constraints, the present rate of increase in wheat productivity is insufficient to meet food security globally. Genomics-assisted crop improvement provides novel opportunities to enhance the yearly rate of increase in wheat yield while advancing our understanding of the genetic and functional basis of the adaptive response to water-limited conditions (Habash et al. 2009; Fleury et al. 2010; Able and Atienza 2014; Tuberosa et al. 2014). This chapter illustrates how genomics approaches have been applied to genetically dissect durum wheat performance under water-scarce conditions and, more in general, how this information might help to mitigate the negative effects of drought on wheat productivity. In view of the genetic and functional similarity shared by durum and bread wheat, a number of relevant examples for the latter have also been considered.

Dissecting the Genetic Basis of Drought Resistance in Durum Wheat

The genetic basis of grain yield and the morpho-physiological traits that determine durum wheat performance under drought involves a myriad of quantitative trait loci (QTLs) of widely different effects, mostly too small to be detected experimentally. One of the reasons accounting for the modest impact of genomics-assisted breeding on the release of drought-tolerant cultivars is that screening conditions adopted under controlled conditions (e.g. growth chamber) usually provide a rather poor surrogate of the dynamics of the drought episodes that crops are exposed in the field (Passioura 2007; Tuberosa 2012). Additionally, the high context-dependency of QTL effects according to the genetic background and environment (Maphosa et al. 2014) further limits the effectiveness of marker-assisted selection (MAS) for improving field performance under drought conditions.

Until the introduction of association mapping, QTL identification has been pursued via linkage mapping based on the evaluation of biparental populations of recombinant inbred lines (RILs). Notably, the availability of maps obtained with different crosses and sharing common polymorphisms allows for the construction of a consensus map (Maccaferri et al. 2014) that in turn enables an even more accurate comparative analysis (e.g. meta-analysis) of QTL positions.

Association mapping (AM) based on sets of unrelated accessions provides additional opportunities to identify the loci (genes and/or QTLs) for target traits. In durum wheat, the evaluation of a panel of elite accessions characterized by high LD (>1 cM) has allowed for a genome-wide search using a limited number of markers (Maccaferri et al. 2005, 2011). Although AM has mostly targeted traits with a

genetic basis less complex than drought tolerance (e.g. resistance to biotic stress), some studies have targeted drought-adaptative traits and grain yield in durum wheat grown under varying water regimes (Sanguineti et al. 2007; Maccaferri et al. 2011; Canè et al. 2014; Graziani et al. 2014).

Notwithstanding the clear advantages of AM as compared to biparental linkage mapping, a major limitation of the former is the high rate of false positives (i.e. Type-I error rate) due to the presence of hidden population structure. Additionally, for highly integrative and functionally complex traits such as yield, particularly under drought conditions, the effectiveness of AM is reduced by the fact that different genotypes may show similar phenotypes due to trait compensation (e.g. yield components), which inevitably undermines the identification of significant marker-trait association. Although the number of studies is clearly too limited to draw more certain conclusions on the validity of AM in identifying QTLs for yield, the results reported by Maccaferri et al. (2008, 2011) in durum wheat suggest that the identification of yield QTLs under different water regimes should also be pursued via biparental mapping. This is particularly true whenever the investigated trait (e.g. yield under drought conditions) is strongly influenced by flowering time, in which case the overwhelming effects on yield of this phenological covariate will overshadow the effects due to the action of yield per se QTLs, hence reducing the possibility of identifying them.

QTLs for Drought-Adaptive Traits

On the discovery side, the past two decades have witnessed remarkable progress in several areas as shown also by the manuscripts in this special issue. The pivotal role of phenotyping in drought-related research is now universally recognized and receiving renewed attention (Tuberosa 2012; Araus and Cairns 2014). This revival in phenotyping has been sparked by the recent availability of new phenotyping technologies and highly automated platforms coupled with a better appreciation of the role of phenotyping in accelerating the response to selection for drought resistance, either through conventional or non-conventional approaches.

Dehydration avoidance and dehydration tolerance are the main mechanisms that contribute to maintain yielding ability under water-limited conditions (Blum 1988). Deep rooting and osmotic adjustment – classified under dehydration avoidance – enable the plant to maintain better hydration while other biochemical and physiological features (e.g. accumulation of molecular protectants, remobilization of stem water-soluble carbohydrates, etc.) classified under dehydration tolerance enable the plant to sustain metabolism even under severely dehydrated conditions. Notably, most genes induced under extreme dehydration have been shown to belong to metabolic pathways with doubtful functional significance under the water-limited field conditions encountered by wheat (Passioura 2007). Conversely, exploitation of naturally occurring variation for yield and/or drought-adaptive traits has allowed for

slow albeit unequivocal progress in wheat performance under drought conditions (Reynolds and Tuberosa 2008).

Given the quantitative nature of abiotic stress tolerance, QTLs have been the main target of studies attempting to identify the loci regulating the adaptive response of crops to environmentally constrained conditions. In very few cases, major QTLs affecting yield and other drought-adaptive traits across a broad range of soil moisture conditions have been identified (Quarrie et al. 2005; Maccaferri et al. 2008).

QTLs for Root Architecture and Size Among the traits that affect the water balance of the plant, roots play a key role in conditions of limited soil moisture (Richards 2008). Roots show a high level of morphological and developmental plasticity, a peculiarity that allows plants to adapt to moisture-limited conditions (de Dorlodot et al. 2007; Den Herder et al. 2010). An example is provided by root aerenchyma and root angle, root features that are receiving increasing attention for their effects on the response to drought and other abiotic stresses (Christopher et al. 2013). Other root features remain much more challenging to investigate, particularly under field conditions, such as in the case of root depth, a trait that has repeatedly shown a key role in crop adaptation to drought conditions when residual moisture at maturity is mainly available in deeper soil layers (Blum 2009, 2011; Watt et al. 2013). In bread wheat, soil coring down to 2 m depth revealed a broad range of genetic variation in deep root traits and showed that root features of high-performing genotypes were superior to those of low-performing genotypes or commercial varieties (Wasson et al. 2014). Since direct measuring of root depth remains an unresolved challenge, large-scale phenotyping for this trait can only be addressed through the use of proxies (e.g. canopy temperature depression) that through aerial remote sensing allow for monitoring the water status of a large number of genotypes in the field (Lopes and Reynolds 2010; Lopes et al. 2014).

In wheat, root metaxylem diameter is another feature that has shown an association with yield under drought conditions (Schoppach et al. 2014). Notably, selection for higher water-use efficiency (WUE) has shown merits in Australian environments where the crop prevalently grows on moisture stored in the soil prior to planting. Under these conditions, a wheat plant using water conservatively is able to complete grain filling with greater amount of water available in the soil. The adoption of this conservative strategy led to the release of two cultivars ('Drysdale' and 'Rees'; Condon et al. 2004) characterized by yield increases of up to 23 % when compared with control cultivars. The final effects of root architecture and size on yield will depend on the distribution of soil moisture and the level of competition for water resources within the plant community.

A most challenging aspect is to define the most desirable root ideotype able to optimize yield according to the prevailing dynamics of soil moisture profile but also accounting for the concurrent presence of gradients in the soil profile for other abiotic factors (e.g. salinity, toxic elements, high pH, etc.) that may impair plant growth. Therefore, each root ideotype should be established based upon the prevailing soil features in the target environment, a good understanding of the root architectural features that limit water uptake, and the metabolic cost required to develop

and functionally sustain the root system. Along this line, loci that affect root growth under particular abiotic (e.g. boron toxicity) and biotic (e.g. nematode resistance) constraints are interesting targets for MAS aimed at improving drought resistance through a more vigorous root system of wheat grown in problematic soils.

QTLs for Carbohydrate Accumulation and Relocation In wheat, the accumulation of carbohydrates and their relocation to the ear are key factors for optimizing yield under adverse environmental conditions (Blum 1998; Reynolds et al. 2009). In bread wheat, QTLs for stem reserve, water-soluble carbohydrates (WSC) remobilization and leaf senescence have been reported across well-watered and water-stressed conditions (Snape et al. 2007; Rebetzke et al. 2008; Bennett et al. 2012; Zhang et al. 2015). Although these studies showed an important role for WSC in assuring stable yield and grain size, Rebetzke et al. (2008) concluded that the small effects of many independent WSC QTLs may limit their direct use for MAS. A combined QTL analysis for yield of several wheat populations evaluated across different environments and seasons enabled Snape et al. (2007) to identify QTLs showing stable and differential expression across irrigated and non-irrigated conditions. Variation for stem water-soluble carbohydrate reserves was associated with the chr. 1RS arm of the 1BL/1RS translocated (from rye to wheat) chromosome, and was positively associated with yield under both irrigated and rainfed conditions, thus contributing to general adaptability (Snape et al. 2007). The beneficial role of this translocation on wheat performance under drought-stressed conditions has already been reported (Ehdaie et al. 2003).

QTLs for Other Traits of Interest for the Control of Water Balance Measurement of traits such as stomatal conductance, canopy temperature and leaf rolling provides indications of water extraction patterns and the water status of the plant. Therefore, measuring these traits together with soil moisture may help in selecting deep-rooted germplasm in environments where water is available at depth (Blum 1988; Reynolds et al. 2009). Stomatal conductance integrates important environmental and metabolic cues and allows the plant to modulate and optimize its transpiration and WUE (Brennan et al.2007). A study conducted on a series of successful bread wheat cultivars released from 1962 to 1988 showed a strong and positive correlation between stomatal conductance and grain yield ($r = 0.94$; Fischer et al. 1998), suggesting that the more modern cultivars extract more water from the soil. These results indicate the possibility of raising the yield potential using stomatal conductance as proxie and suggest the value of identifying the relevant QTLs. Canopy temperature is an integrative trait that reports on the water balance at the leaf and whole-plant level, thus providing a proxie of the capacity of the plant to extract soil moisture (Blum 1988, 2009; Reynolds and Tuberosa 2008). Canopy temperature depression (CTD) is mainly useful in hot and dry environments, with measurements preferably made on recently irrigated crops in cloudless and windless days at high vapour pressure deficits (Blum 1988; Reynolds et al. 2009). Under these circumstances, CTD can be a good predictor of grain yield in bread wheat (r varying from 0.6 to 0.8; Reynolds et al. 2009), where yield progress has been associated with cooler

canopies, hence higher transpiration (Fischer et al. 1998). Genetic gains in yield have also been reported in response to direct selection for CTD (Reynolds et al. 2009).

QTLs for Yield Under Different Water Regimes As global climate change intensifies, the identification of loci with consistent per se effects on yield (i.e. not loci for flowering time) across a broad range of soil moisture regimes becomes increasingly important to raise yield potential (Maccaferri et al. 2008; Pinto et al. 2010; Reynolds et al. 2011; Turner et al. 2014). Major QTLs for grain yield and its components across a broad range of soil moisture regimes have all been reported in bread wheat (Quarrie et al. 2005; Kirigwi et al. 2007; Snape et al. 2007) with only one notable exception in durum wheat where Maccaferri et al. (2008) searched for QTLs for grain yield in RILs evaluated in 16 environments with a broad range in grain yield values (from 0.56 to 5.88 t ha^{-1}), mainly consequent to different soil moisture availability. Two major QTLs on chr. 2BL and 3BS (*QYld.idw-2B* and *QYld.idw-3B*, respectively) showed highly significant and consistent effects in eight and seven environments, respectively. In both cases, an extensive overlap was observed between the LOD profiles for grain yield and plant height, but not with those for heading date, thus indicating that the effects of these two QTLs on yield were not due to escape from drought, a well-known factor in determining yield under terminal drought stress conditions that typically characterize Mediterranean environments (Araus et al. 2008). Accordingly, this population was originally chosen because it had shown limited variability in flowering time. For plant height and grain yield, a strong epistasis between *QYld.idw-2B* and *QYld.idw-3B* was detected across several environments, with the parental combinations providing the higher performance. These two QTLs evidenced significant additive and epistatic effects also on ear peduncle length and kernel weight (Graziani et al. 2014). As a prerequisie to positional cloning, progeny derived from the cross of isogenic lines have been evaluated for fine mapping of both QTLs (Maccaferri et al. unpublished).

Improving Drought Resistance via Marker-Assisted Selection

Several factors limit the possibility of obtaining reliable QTL data and, most importantly, their deployment in breeding programs through MAS (Tuberosa et al. 2007). Among such factors, the environment dependence of QTL expression is of utmost importance in order to obtain reproducible data and effectively assess the value of a particular QTL. This aspect is particularly relevant for stress tolerance traits since the effect of the same QTL can markedly differ according to the prevailing environmental conditions (Collins et al. 2008). Although many studies have described QTLs that influence tolerance to drought, MAS has so far contributed marginally to the release of drought-resistant cultivars. Improving crop performance under water-limiting conditions via MAS may also require considering QTLs for tolerance to abiotic (e.g. high boron) and biotic (e.g. nematodes) factors that impair root growth and functions. A common feature of cereal responses to drought near flowering and

during early stages of seed growth is a reduction of reproductive fertility due to partial sterility and/or early abortion. This loss of fertility has been attributed to different factors acting alone and more likely on reproductive fertility. The QTL approach attempts to dissect out the genetic and physiological components affecting source-sink relationships under abiotic stress and to what extent these may influence yield (Miralles and Slafer 2007). Major QTLs for seed weight and grain yield at different moisture conditions have been identified in durum wheat (Maccaferri et al. 2008) and are being introgressed in different genetic backgrounds. In bread wheat, Fleury et al. (2010) have implemented a strategy where a specific environment is targeted and appropriate germplasm adapted to the chosen environment is selected, based on extensive definition of the morpho-physiological and molecular mechanisms of tolerance of the parents. This information was then used to create structured populations and develop models for QTL analysis, MAS and positional cloning.

Future Perspectives

Increasing attention has been devoted to the use of crop modeling for elucidating the genetic basis of genotype × management × environment (G × M × E) interaction at the level of the entire genotype and, more recently, at the level of single loci (Ludwig and Asseng 2010; Richards et al. 2010; Tardieu and Tuberosa 2010; van Eeuwijk et al. 2010; Parent and Tardieu 2014). The objective is to predict, via modeling, yield differences among genotypes grown under different environmental conditions (Cooper et al. 2009; Tardieu and Tuberosa 2010). The benefits accrued by modeling studies are expected to increase as the complexity of the genetic control of traits increases provided it is possible to account for the effects of genetic interactions for predicting trait variation (Cooper et al.2009). Ultimately, modeling aims to predict the best combinations of QTL alleles able to optimize yield. The main underlying assumption of the modelling approach is that yield and other functionally complex traits can be analyzed and improved by dissecting it into simpler processes, and then by re-assembling such processes to reconstruct via modelling higher order of plant functionality and ultimately yield itself. Models have been used to generate an index of the climatic environment (e.g. of drought stress) for breeding program trials. In wheat grown in northern Australia, this has shown that mid-season drought generates large genotype by environment interaction (Chapman 2008).

With only a few exceptions as listed above, the vast majority of loci that affect crop yield per se have a rather small effect, particularly under drought conditions. Therefore combining the favorable alleles by MAS to achieve a significant improvement quickly becomes impractical and would excessively constrain the potential for achieving yield gain due to the action of other loci. In this case, MAS for mapped QTLs (Randhawa et al. 2013) can be replaced by genome-wide selection (Bernardo 2010; Storlie and Charmet 2013). Nowadays, genome selection is facilitated by the

availability of large numbers of markers, particularly Single Nucleotide Polymorphysms (SNPs; Wang et al. 2014) that are amenable to high-throughput profiling at very low cost.

Conclusions

The release of cultivars better adapted to a broader range of environmental conditions will become an increasingly important goal of breeding projects worldwide. Compared to conventional breeding practices, the contribution in this direction of molecular breeding has somehow fallen short of expectations (Blum 2014; Tuberosa et al. 2014). Nonetheless, genomics approaches and sequence-based breeding will expedite the dissection of the genetic basis of abiotic stress tolerance while providing unprecedented opportunities to tap into wild relatives of wheat. To what extent this will actually impact the release of improved cultivars will largely depend on a more complete and comprehensive understanding of the adaptive response of crops to abiotic stress and our capacity to integrate this information into breeding programs via modeling or other approaches such as genomic selection. In view of the complexity of yield, particularly under drought, we foresee that genomic selection will provide the most effective way to raise the yield potential to the levels required to keep up with the fast-increasing demand in food worldwide. However, MAS will remain a valid option for major loci (genes and/or QTLs) as long as their effects will be sufficiently predictable and economically viable (Tuberosa and Pozniak 2014). Additionally, QTL cloning will become a more routine activity thanks to a more widespread utilization of high-throughput, accurate phenotyping (Tuberosa 2012), sequencing and the identification of suitable candidate genes via 'omics' profiling. Ultimately, reducing wheat vulnerability to drought will require a multidisciplinary and integrated approach that will eventually allow breeders to more effectively select drought-resistant cultivars.

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Chapter 24
Hybrid Breeding in Wheat

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Abstract Despite promising superior performance, hybrid wheat currently occupies only a niche sector in commercial wheat production. However, with the recent development of practicable hybrid seed production systems, a switch from line to hybrid breeding in wheat seems realistic. Here, we discuss what consequence this may have for wheat breeding programs and provide suggestions on how quantitative genetic analysis can contribute to design optimal selection strategies.

Status Quo of Wheat Hybrid Breeding

Hybrid cultivars with improve yield and other favorable agronomic traits are widely used in plant production. For wheat, one of the most important staple crops, commercial hybrid breeding and seed production is still restricted to a niche sector in comparison to other cereals like maize or rice (Longin et al. 2012; Whitford et al. 2013). Currently, there is a limited number of wheat hybrid cultivars based on chemical hybridization agents (CHAs, gametocytes) registered for the European market (Hybridwheat 2013). In China and India, hybrid wheat is produced based on cytoplasmic male sterility (CMS) systems or photoperiodic sensitivity sterility systems (Longin et al. 2012). Major practical limitations for a more widespread use of hybrid wheat are seed production capacities and costs, but progress has been made to improve the availability and economic competitiveness of hybrid wheat (reviewed by Kempe and Gils 2011; Whitford et al. 2013). Eventually, economically successful broad implementation of hybrid wheat will need the combination of a practicable

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low cost hybrid seed production system, high performance in traits of interest such as grain yield and yield stability and an efficient breeding scheme for further improvement (Longin et al. 2012).

Hybridization Systems in Wheat

Hybrid seed production requires the enforcement of an efficient cross-pollination between wheat inbred lines that overcomes the naturally autogamous pollination mode of wheat. This is practically achieved by planting male-sterile maternal plants with good pollen recipient properties in close proximity to paternal plants with good pollen shedding properties. Thus, effective male-sterility of the maternal plants is a general requirement that can be based on different mechanisms.

Some major efforts have been made to deploy cytoplasmic male sterility (CMS) for hybrid wheat breeding. While CMS is functional in other important cereals such as rice and rye, it has turned out to be difficult to develop, complex to maintain and marginally reliable for wheat. Although sterility-inducing cytoplasms were identified (e. g. from *Triticum timopheevii*), reliable restorer genes are not yet available for wheat (Angus 2001) and CMS systems are often sensitive to environmental factors, in particular to temperature and photoperiod (Kaul 1988; Murai et al. 2008). Thus, no wheat CMS system with more than regional application is currently available for hybrid seed production.

Rather, contemporary commercial hybrid wheat production is mainly based on the in-field application of CHA preventing the formation of viable pollen on the maternal crossing partner (Cisar and Cooper 2002). On a production site, the intended maternal line is planted in strips alternating with strips of the intended pollen donor lines and maternal plants are sprayed with CHA, while treatment of paternal plants is strictly avoided. Hybrid seeds are then harvested from the pollinated mother plants to give rise to F_1 progeny that then displays the heterosis (hybrid vigor) effect (Kempe and Gils 2011). The plant growth regulator Croisor®100 (Sintofen, former Dupont-Hybrinova, Saaten Union Recherche, France) is currently the only CHA for wheat registered in Europe for commercial production (Hybridwheat 2013). Although modern CHAs are in principle functional for a broad spectrum of genotypes and display relatively low phytotoxicity in wheat, they still have limitations such as compromised seed set on treated plants (Adugna et al. 2004) or variation in field-efficiency depending on the weather conditions at the time of application.

Alternatively, the exploitation of transgene technologies may be promising for the establishment of hybrid wheat production systems (Kempe and Gils 2011; Whitford et al. 2013). As an example, a recessive split-gene transgene system was suggested that utilizes complimentary fragments of barnase to induce male-sterility in maternal plants with simultaneously retaining pollen fertility and thus grain yield in the resulting F_1 hybrids (Gils et al. 2008; Kempe et al. 2009). Functional barnase formation in the tapetum layer of anthers and corresponding male-sterility should

exclusively be effective in heterozygous plants, which can eventually serve as the mother plants in the hybrid cross. With both barnase gene fragments located on allelic chromosomal positions and thus "linked in repulsion", hybrid F_1 plants inherit only one of the barnase fragments and, as a result, remain fully fertile (Kempe and Gils 2011).

Moreover, the modification of the naturally closed inflorescence structure of wheat adapted to self-pollination to a more open structure allowing more efficient pollen reception as well as shedding will be an important breeding objective (Whitford et al. 2013). With these recent developments in hybrid seed production in mind, it seems now timely to devote some thought on how to develop optimal parental combinations for highest yield and quality improvements in wheat hybrid breeding.

Advantages of Hybrids in Comparison to Lines

In several outcrossing crop plant species, hybrid varieties have already largely replaced population varieties (Coors and Pandey 1999). The major benefits of hybrids compared to population varieties are increased yields due to an optimal exploitation of heterosis, higher biotic and abiotic stress resistance, and enhanced yield stability (Hallauer et al. 1988). In wheat as an autogamous species, the amount of midparent heterosis for grain yield is less pronounced, and commercial heterosis, the contrast between hybrids and the best commercially available line variety, is lower in comparison to outcrossing cereal species such as maize (Longin et al. 2012). Concerning yield stability, Léon (1994) concluded that for autogamous crops, findings in hybrids versus lines are contrasting, ranging from higher yield stability of hybrids (Borghi et al. 1988; Oury et al. 2000; Oettler et al. 2005; Gowda et al. 2010) to no differences (Borghi and Perenzin 1990; Peterson et al. 1997; Bruns and Peterson 1998; Koemel et al. 2004).

Recent large scale-phenotyping activities involving extended collections of inbred lines and hybrids, however, revealed a positive commercial heterosis for grain yield in hybrid winter wheat adapted to Central Europe (Gowda et al. 2012; Longin et al. 2013). Thus, a change from line to hybrid varieties has the potential to break the yield barriers in wheat breeding. Using a broad base of data from multi-location field trials, Mühleisen et al. (2013) also re-evaluated grain yield stability in wheat, triticale, and barley hybrids in comparison to lines. The authors observed consistently higher yield stability for hybrids in all three autogamous crops (Fig. 24.1). Moreover, the lack of consistencies of results on yield stability of hybrids versus lines in previous studies is most likely owing to an unbalanced definition of the environmental index caused by exploiting exclusively data from inbred lines but not from hybrids. Thus, in summary, current experimental studies clearly underline the advantages of hybrids in comparison to inbred lines in terms of higher yield and yield stability. Due to the expected climate change with more extreme weather conditions, such improved robustness of performance will become even more important in the future.

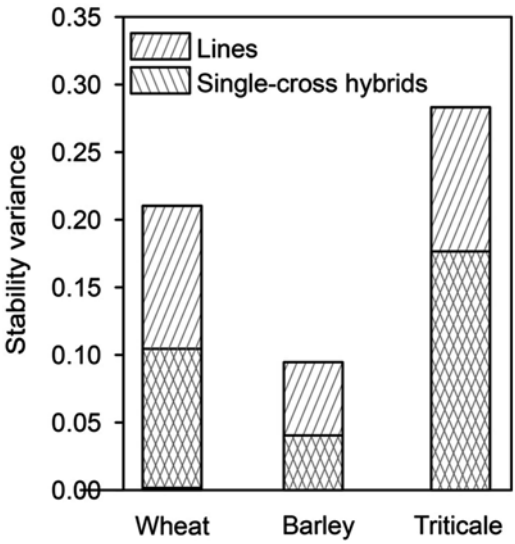

Fig. 24.1 Yield stability measured based on the stability variance of wheat, barley, and triticale single-cross hybrids (descending hatching) versus lines (ascending hatching) based on the data published by Mühleisen et al. (2013). Low values of stability variance correspond to high yield stability

Beyond improved grain yield and yield stability, hybrid wheat also outperforms inbred lines in sturdiness to abiotic and biotic stress. Longin et al. (2013) observed a positive midparent (albeit negative better parent) heterosis for frost tolerance as well as resistance against leaf rust, stripe rust, septoria tritici blotch, and powdery mildew for hybrid winter wheat. In two companion genome-wide mapping studies, Zhao et al. (2013a) and Gowda et al. (2013) investigated the degrees of dominance of putative QTL underlying the above outlined abiotic and biotic stress resistances. For most QTL with large effect on the genotypic variation, the degree of dominance was in the range of partial dominance. Thus, if particular traits are in focus, superior genotypes should carry resistance QTL in the homozygous state, favoring breeding of lines instead of hybrids. However, if pyramiding of multiple relevant QTL is desired, hybrid breeding will offer far more flexibility to combine favorable partially dominant alleles originating from either maternal or paternal inbred lines. This advantage of hybrids is illustrated by calculating a stress susceptibility index combining data on frost tolerance and resistance to leaf rust, stripe rust, septoria tritici blotch, and powdery mildew for an extended set of winter wheat inbred lines and hybrids derived from them (Fig. 24.2). Hybrids show overall lower stress susceptibility than related inbred lines, which might also be contribute the higher yield stability observed for hybrids as mentioned above (Mühleisen et al. 2013).

Beside the described advantages of hybrid versus line varieties in terms of yield, yield stability and biotic as well as abiotic stress resistance, it is pivotal to point out that the recurrent selection gain in hybrid breeding is at least as high in hybrid as compared to line breeding. The expected recurrent selection gain depends on quantitative genetic parameters such as the ratio of additive variance which can be exploited in hybrid versus line breeding. Besides quantitative genetic parameters,

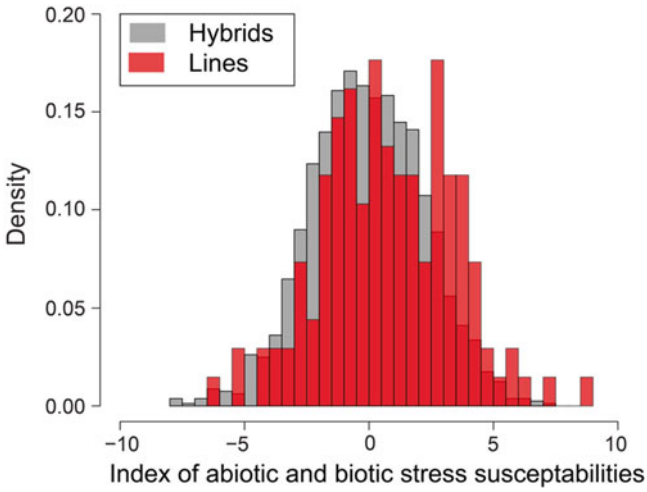

Fig. 24.2 Distribution of 1.604 wheat hybrids (*grey*) and 135 parental lines (*red*) for a stress susceptibility index combining frost tolerance and resistance to leaf rust, stripe rust, septoria tritici blotch, and powdery mildew. Calculation was done assuming equal weight based on data published by Longin et al. (2013)

the economic framework such as the return in investment is pivotal to determine the selection gain of line versus hybrid breeding. Longin et al. (2012) performed a first rough theoretical comparison of the expected selection gain for line versus hybrid breeding under the simplifying assumption of an equal budget. The model calculations indicate that if the dominance effect is substantial, hybrid breeding can result in a competitive selection gain as compared to line breeding (Longin et al. 2012, 2014). These theoretical considerations are corroborated by one study comparing hybrid versus line breeding using experimental data of official variety tests in hard winter wheat across 20 years in the U.S. (Koemel et al. 2004). The authors observed a higher selection gain across time for hybrids compared to line breeding. These findings are very stimulating but further experimental data are needed to substantiate the long-term selection gain for hybrid versus line breeding in autogamous cereals.

Prediction of Hybrid Wheat Performance

In a traditional wheat breeding program, several thousand inbred lines are produced every year. Superior genotypes are efficiently identified by applying multi-stage selection programs. Selection of superior hybrids, however, is afflicted by the vast number of potential single-cross combinations among available elite parental lines (Zhao et al. 2013a). Consequently, field evaluation of all potential hybrid combinations is unfeasible, leading to a strong demand for hybrid prediction approaches.

For complex traits such as grain yield, midparent performance is only moderately associated with hybrid performance (Longin et al. 2012). Predicting hybrid performance based on general combining ability (GCA) effects of their parents is accurate in situations with predominance of variance due to GCA (σ^2_{GCA}) versus variance due to specific combining ability (SCA) effects (σ^2_{SCA}). A recent large scale experimental study in wheat revealed that σ^2_{SCA} is quite substantial for grain yield in wheat (Longin et al. 2013), which suggests that field testing of single-cross combinations is needed at least in the final stages of a selection program. Therefore, robust methods to predict hybrid wheat performance are urgently required.

The potential of genomic selection to predict hybrid wheat performance has been investigated for grain yield with a small factorial mating design comprising 90 hybrids fingerprinted with a 9k SNP array (Zhao et al. 2013b). The results of this cross validation study point towards a high potential of genomic selection to predict hybrid wheat performance. This finding was further substantiated in further studies predicting hybrid wheat performance with genomic selection focusing on several agronomic traits (Miedaner et al. 2013; Zhao et al. 2013a, 2014). Further empirical data analyses are needed, however, to finally judge the prospects of genomic selection models for predicting hybrid wheat performance.

Upcoming Challenges for Wheat Hybrid Breeding

In conclusion, the most urgent challenges for wheat hybrid breeding will be (i) development of a stable hybridization system to reduce the costs of hybrid seed production compared to the use of CHA, (ii) identification of the genetic architecture of pollination capability for knowledge-based improvement of cross-pollination among elite lines, (iii) optimum design of hybrid wheat breeding programs including dimensioning of multi-stage selection programs including genome wide prediction approaches and (iv) development of heterotic pools in wheat.

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Chapter 25
Broadening the Genetic Diversity of Common and Durum Wheat for Abiotic Stress Tolerance Breeding

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Abstract An increase in cereal production is required if we are to support a world population of more than nine billion people expected in 2060. For this purpose, plant breeding will serve as a key technology as it did during the Green Revolution of the 1960s. However, the changing climate and decrease in agricultural resources are new challenges that will require consideration. We developed common and durum wheat populations expressing the intraspecific diversity of wild species. We collectively named these populations 'multiple derivative lines', and specifically 'multiple synthetic derivatives' (MSD) in the case of common wheat. The germplasm in the MSD population shows diverse morphology in regular genetic background enabling accurate selection of desired genotypes/phenotypes associated with abiotic stress tolerance. Accordingly, such plant materials can subsequently be used for various breeding purposes. Abiotic tolerance, especially drought tolerance, is largely determined by the interaction between genotype and environment, making drought-tolerance breeding difficult. Wild species, even the self-pollinating ones, possess high diversity which allows them to adapt to the diverse and fluctuating environments. Accordingly, during wheat breeding for abiotic tolerance, it may be better to consider using diverse populations such as the MSD.

Keywords Abiotic stress • *Aegilops tauschii* • Germplasm enhancement • Synthetic wheat • Wheat

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In the next 50 years, an increasing world population will require cereal production to double under radically changing climate conditions. Plant breeding, which played an essential role in the Green Revolution of the 1960s, will serve as a key technology in addressing this problem. However, wheat breeding is becoming increasingly difficult due to the limited genetic variation in the wheat gene pool. This narrow genetic variation is in part a consequence of a bottleneck effect that occurred through very few interspecific hybridization events during the origin of common wheat (*Triticum aestivum* L., 2n = 6x = 42, AABBDD) 8,000 years ago. Novel alleles can be introduced by crossing with wild relatives; however, the difference in ploidy and morphology between wild and common wheat often hinders proper evaluation of traits, particularly quantitative ones. To date, examples of the successful use of genes or alleles derived from wild species have mostly been limited to qualitative traits such as disease and insect resistance. Here, we aim to produce breeding populations containing the intraspecific variation of the wild species *Aegilops tauschii* Coss. (2n = 2x = 14, DD) and *Triticum dicoccoides* Koern. (2n = 4x = 28, AABB) in the genetic background of common and durum wheat varieties. It is hoped that these populations will be useful initial materials during the selection of quantitatively controlled traits such as abiotic stress tolerance.

To develop a breeding population with the genetic background of common wheat, the genetic diversity of 81 *Ae. tauschii* accessions collected from a range of natural habitats was analyzed using 4,449 polymorphic diversity array technology (DArT) markers (Fig. 25.1; Sohail et al. 2012). The results identified three intraspe-

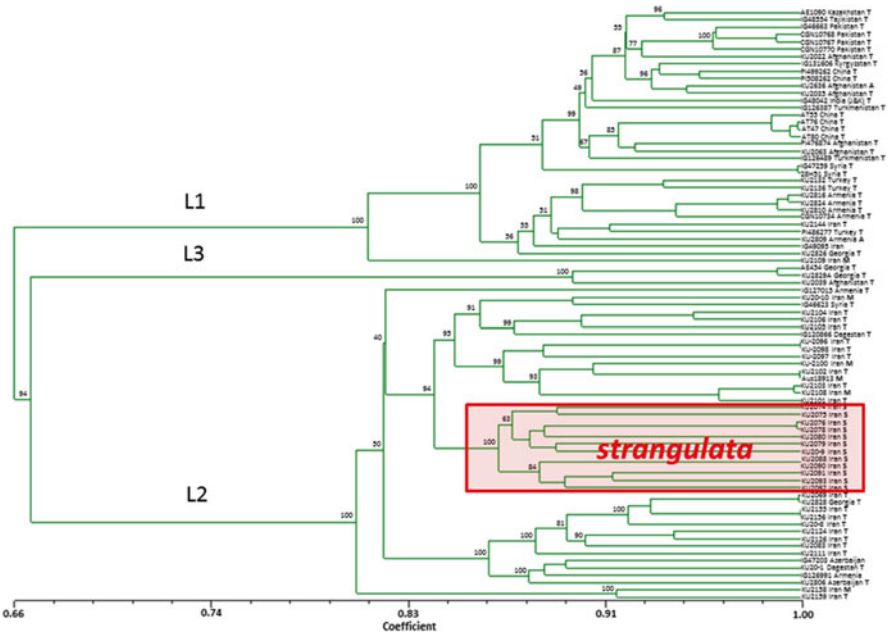

Fig. 25.1 Phylogenetic tree of the 81 accessions of *Ae. tauschii* constructed using 4,449 DArT markers. Lineages 1, 2 and 3 are indicated by L1, L2 and L3, respectively. The clade of *Ae. tauschii* ssp. *strangulata* is boxed

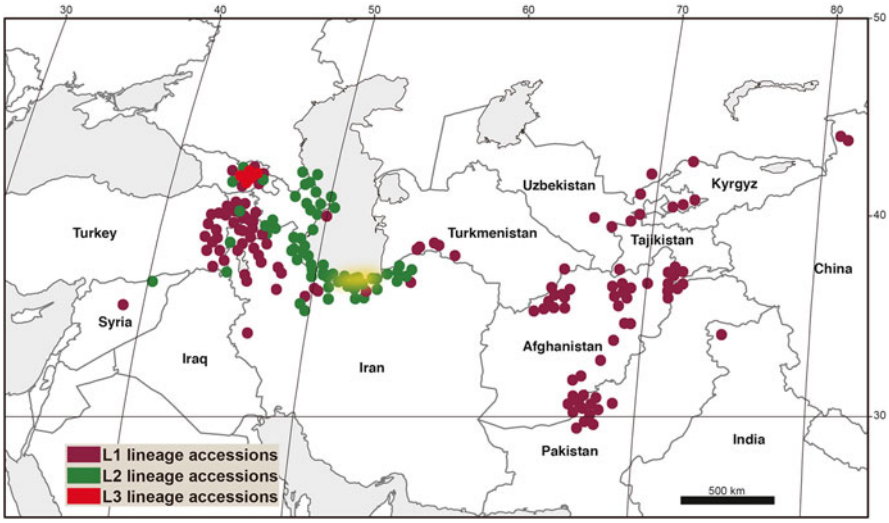

Fig. 25.2 Distribution of *Ae. tauschii* accessions belonging to Lineages 1, 2 and 3. The habitat area of subspecies *strangulata* is shaded in *yellow*

cific lineages: Lineage 1, distributed widely in Central and Western Asia; Lineage 2, distributed in Transcaucasia and northern Iran; and Lineage 3, found in a specific region of Armenia (Fig. 25.2). Because Lineage 2, which includes *Ae. tauschii* ssp. *strangulata*, has a close genetic relationship with the D genome of *T. aestivum* relative to Lineage 1 (Matsuoka et al. 2013), common wheat may show reduced genetic variation compared to the overall interspecific diversity of this species. Fifty-one of the 81 accessions were then randomly selected and crossed with durum wheat (*Triticum durum* Desf., 2n = 4x = 28, AABB), cv. 'Langdon', to produce 51 amphidiploids, designated 'primary synthetics' or 'synthetic hexaploid wheat' (SHW).

The drought tolerance-related traits of these SHWs were then examined together with those of the original *Ae. tauschii* accessions under well-watered and drought conditions. The growth performance of the parental *Ae. tauschii* accessions and their derivative SHWs was then compared (Table 25.1; Sohail et al. 2011). Under well-watered conditions, intercellular CO_2, transpiration rate, root dry weight and water use efficiency were significantly correlated in both diploid and hexaploid plants. Under drought conditions, however, no correlation was observed, indicating that the degree of drought tolerance cannot be predicted by the performance of the wild species. In general, wild species have specific mechanisms that allow them to cope with environmental stresses, but if such mechanisms are not transferable to common wheat, they cannot be utilized in breeding programs. The expression of drought-tolerance traits must therefore be evaluated in hexaploid wheat. In contrast, traits that are controlled by a small number of major genes could be screened first using accessions of the wild species. Furthermore, the SHW lines were often unsuitable for evaluation of drought tolerance, because they usually presented a

Table 25.1 Correlation between the morphological and physiological traits of the *Ae. tauschii* accessions and their derivative SHW lines under well-watered and drought conditions (Sohail et al. 2011, modified)

Trait	Well-watered conditions	Drought conditions
Photosynthesis	0.09	0.02
Stomata conductance	0.15	–0.11
Intercellular CO_2	0.49**	0.32
Transpiration rate	0.37*	0.05
Chlorophyll content	0.07	0.34
Leaf water potential	–0.24	0.20
Root dry weight	0.36*	0.30
Shoot dry weight	0.25	0.15
Water use efficiency	0.36*	0.24

**$P<0.01$; *$P<0.05$

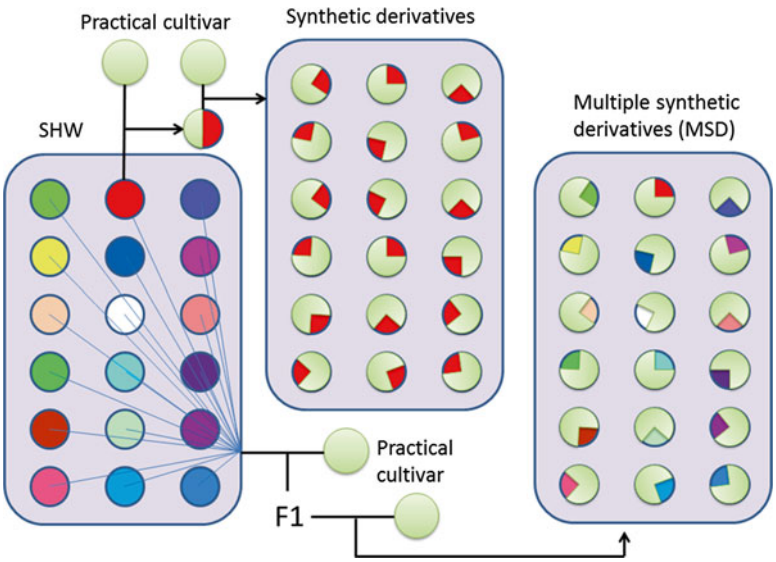

Fig. 25.3 Production of a multiple synthetic derivative population containing the genetic diversity of *Ae. tauschii*. SHW, synthetic hexaploid wheat

robust appearance with long and tough spikes, very different from the morphology of common wheat cultivars.

In general, an SHW line is crossed twice with a common wheat line and the resulting derivative populations used as initial selection materials for breeding (Fig. 25.3). In this protocol, however, only one *Ae. tauschii* accession can provide the genes of the derivative population. Thus, to obtain material populations

containing the wide genetic diversity of *Ae. tauschii*, we crossed each SHW with 'Norin 61' (N61), a Japanese standard cultivar that has wide adaptability, and Sephadak Ishkashim W (SIW), a landrace from the Ishkashim region of Tajikistan and Afghanistan. As a result, we obtained 43 and 40 F_1 hybrids, respectively. The F_1 progenies were then backcrossed, resulting in 1,893 and 2,546 BC_1F_1 plants, respectively. These plants were then self-pollinated to produce BC_1F_2 seeds. Ten seeds from 10 BC_1F_1s from each line were mixed, resulting in seed-stock populations containing the genetic diversity of *Ae. tauschii* in the N61 and SIW backgrounds (Fig. 25.3). Plants from these mixed-seed stock populations showed a diverse morphology, allowing concise evaluation of the traits associated with abiotic stress tolerance. Genomes of more than 40 accessions of *Ae. tauschii* contributed to these populations and as a result, it is believed they contain most of the genetic diversity of *Ae. tauschii* within the common wheat genetic background. Furthermore, by obtaining seeds derived from different crosses mixed in one population, the handling of materials for screening was made easier. In fact, the BC_1F_1 plants showed a different response to drought stress.

In addition to the above, we also crossed *T. durum* cv. 'Miki 3', a leading cultivar from ICARDA provided by Dr. M. Nachit, with nine accessions of wild tetraploid Emmer wheat, *T. dicoccoides*. The F_1 plants were then backcrossed with Miki three, giving a population consisting of 236 BC_1F_1 plants, all showing a diverse morphology (although some with brittle spikes). The BC_1F_1 plants were then selfed and the seeds (BC_1F_2) were mixed to produce a seed stock population.

We collectively named these hexaploid and tetraploid stock populations 'multiple derivative lines' (MDLs), and specifically the hexaploid populations were designated 'multiple synthetic derivatives'. MDL populations can be used for various breeding purposes, because they show potentially valuable traits derived from diverse wild species in the cultivar-derived genetic background. Moreover, the morphological similarity of each plant in the population makes the selection of complex traits feasible.

In general, the phenotype of any individual is influenced by genotype, environmental factors, and the interaction between the two. Because the genotype-environment interaction is thought to play a significant role in the expression of drought tolerance in wheat, finding genotypes well adapted to dryland environments is usually a difficult task, especially under conditions that fluctuate greatly from year to year. As a result, common modern breeding practices; that is, determining the "best" genotype that outperforms in a set of material accessions under given conditions, may not be useful in wheat drought tolerance breeding, since the "best" drought-tolerant genotype may differ depending on the cropping year and location. Traditionally, wheat farmers cope with unpredictable environmental changes by intentionally cultivating various landraces in the mix (Matsuoka et al. 2008). Accordingly, one alternative approach in wheat drought tolerance breeding would be to produce a genetically diverse material population and perform sowing-harvesting cycles at a local site for several years. This way, an optimal genetic structure could be realized in the descendant population through the natural process of local adaptation. We believe the MDLs could provide useful materials for wheat

drought tolerance breeding using such an approach. By monitoring allele frequency transition in a MDL population under cultivation using molecular markers, the genetic mechanisms that underlie the local adaption could subsequently be studied in depth. In principle, the MDL-based approach relies on the natural adaption process that wheat landraces must have undergone. For this reason, the above approach may allow development of a novel field of breeding science we call 'evolutionary breeding'. Furthermore, the MDL-based approach would also provide a great opportunity for participatory breeding of drought-tolerant wheat.

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Chapter 26
Early Maturity in Wheat for Adaptation to High Temperature Stress

Suchismita Mondal, Arun K. Joshi, Julio Huerta-Espino, and Ravi P. Singh

Abstract High temperatures pose a serious threat to productivity maintenance and enhancement in wheat. A strategy that has come forward in the CIMMYT breeding program is the development of high yielding early maturing lines that are adapted to high temperature stress especially for South Asia. The high temperature stress in South Asia is classified into terminal high temperature stress where the high temperatures stress occurs during grain filling stages, and continual high temperature stress, where high temperature persists across the wheat growing season. The new high yielding, early maturing and heat tolerant CIMMYT wheat lines were evaluated for grain yield and adaptation across diverse locations in South Asia and Mexico. Trials were conducted for three consecutive years 2009–2010, 2010–2011, and 2011–2012. The results suggest that CIMMYT lines with high yields and early maturity, selected under normal and late sown condition in Cd. Obregon, Mexico, have the potential to adapt and outperform normal maturing check varieties under terminal and continual high temperature stress in South Asia. Earliness favored the plants to escape terminal high temperature stress and also promoted an efficient utilization of available resources under continual high temperature stress to achieve higher grain yield. The simultaneous enhancement of grain yield potential and heat stress tolerance of early maturing wheat lines is likely to be beneficial in enhancing productivity under high temperature stress across South Asia.

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Keywords Early maturity • Heat stress • Heat tolerance • South Asia • Wheat

Temperature Stress and Wheat Production

Increasing variation in climate across the globe has become a serious concern to crop production. Wheat, a temperate crop, prefers a cooler climate for growth and reproduction. High temperatures during crop growth and grain filling stages are a major concern to its production. South Asia, comprising of India, Nepal, Pakistan and Bangladesh is one of the major wheat producing and consuming area in the world. This region suffers significant losses each year due to high temperature stress (Kumar et al. 2013). A recent study by World Bank predicts that a 2 °C rise in world average temperature may lead to extreme heat conditions in South Asia. With estimated losses of 6–20 % per degree rise in temperatures in South Asia, (Mondal et al. 2013; Lobell et al. 2008) high temperatures are a serious threat to wheat production.

The wheat producing areas in South Asia are grouped into mega environments (ME) based on the classification system developed by CIMMYT (Braun et al. 1992). ME1 is defined as the optimally irrigated highly productive environment where wheat grows in cool temperatures but suffers from terminal high temperature stress, such as North Western Gangetic Plain. ME5 is a rainfed, warm regions, where continuous high temperature stress is a major concern and comprises of eastern Gangetic plain, peninsular India, plains of Nepal and Bangladesh. ME1 has a cooler climate during crop growth which gradually increases during reproductive and grain filling stages in March and April. ME5 has warmer temperatures across the crop season. Similar trends are seen for maximum and minimum temperatures for ME1 and ME5 locations in South Asia (Fig. 26.1). Thus it is imperative to develop wheat varieties that are high yielding as well as tolerant to high temperature stress.

Heat Adaption Strategies

A number of traits have been studied in wheat for adaptation under high temperature stress. One of the strategies being followed in CIMMYT is breeding for early maturing high yielding heat tolerant wheat varieties. Earliness or early maturity is an adaptation strategy where early heading lines complete the initial seed setting and grain filling under favorable temperatures and avoid the late incidence of heat stress. Earliness has been suggested as a good approach for wheat breeding in the eastern Gangetic plains that suffers from high temperature stress during grain filling (Joshi et al. 2007). Tewolde et al. (2006) evaluated a diverse set of US wheat

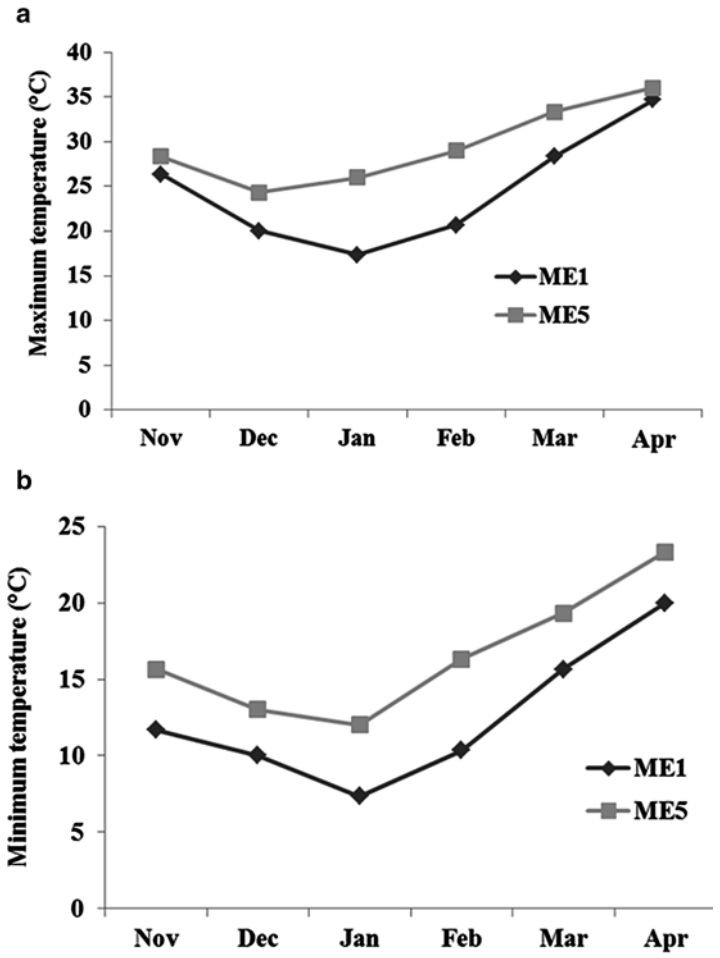

Fig. 26.1 Maximum (**a**) and minimum (**b**) temperatures during the wheat crop growing cycle (November, 2012–April, 2013) across ME1 and ME5 locations in South Asia

cultivars for their adaption to high temperature stress and concluded that early maturity was the single effective trait that defined adaptation under post heading high temperature stress. Al-Karaki (2011) observed that the length of pre heading period in durum wheat affects grain yield under heat stress conditions. They reported that the early maturing high yielding durum lines were suitable for the heat stress affected areas in semi-arid environment. Thus the early maturing high yielding CIMMYT wheat lines developed in Mexico were evaluated for grain yield and adaptation to high temperature stress in South Asia.

Performance of Early Maturing Wheat Lines

High yielding early maturing wheat lines were developed and tested in CIMMYT research station, Cd. Obregon, Sonora, Mexico. The best performing lines were selected for evaluation in South Asia and Mexico. Each trial had 28 entries, with one CIMMYT check 'Baj' and a local check which was the best locally adapted variety at each location. The trials were grown in 9, 13 and 15 locations across South Asia in 2009–2010, 2010–2011 and 2011–2012 respectively. Each year the trials were also planted at Cd. Obregon, Mexico in two environments, normal sowing in November and late sowing in February for heat stress. The locations were grouped in to mega environments, ME1 and ME5.

In 2009–2010, 20 % of the lines had grain yield 1–4 % higher ($p < 0.05$) than the local check across all locations (Fig. 26.2). In the next two cycles (2010–2011 and 2011–2012) nearly 50 % of the lines yielded 1–20 % higher ($p < 0.05$) than the local checks. A similar trend was seen when compared to the CIMMYT early maturing check line 'Baj'. In 2009–2010 a few lines were performing above 'Baj', but a gradual increase was seen in 2010–2011 and 2011–2012. Thus early lines bred in Mexico were able to outperform the locally adapted checks in South Asia. The performance of the lines in the individual MEs was also interesting. The mean grain yield of the trial had significant reduction in the warm ME5 compared to cooler ME1. When the entries that performed above local check in both MEs in each year were grouped together, it was observed that each year these entries had around 5–10 % yield advantage ($p < 0.05$) over the local checks in each ME (Fig. 26.3). Thus the early maturing high yielding lines were able to adapt under both terminal and continual high temperature stresses.

High temperatures affect both grain filling duration and grain filling rate. Studies have reported an increase in grain filling rate and reduction in grain filling duration due to high temperatures (Farooq et al. 2011). But the increase in grain filling rate has been reported to not compensate for the shorter grain filling duration (Wardlaw et al. 1980; Stone et al. 1995). The results from this study show otherwise. The early maturing short duration wheat lines were able to outperform the best locally adapted varieties. A comparison of grain accumulation rate based on grain filling duration of the five top high yielding stable lines in each year with the local checks showed that the early maturing lines had higher grain accumulation rates as well as higher grain yields (Fig. 26.4).

Conclusions

With high temperature becoming a major issue in wheat producing areas, there is an enhanced focus on developing heat tolerant high yielding wheat varieties. The results show that simultaneous enhancement of yield potential and heat tolerance is possible and that the CIMMYT strategy to develop high yielding early maturing wheat lines is promising for South Asia. The early maturing lines were able to

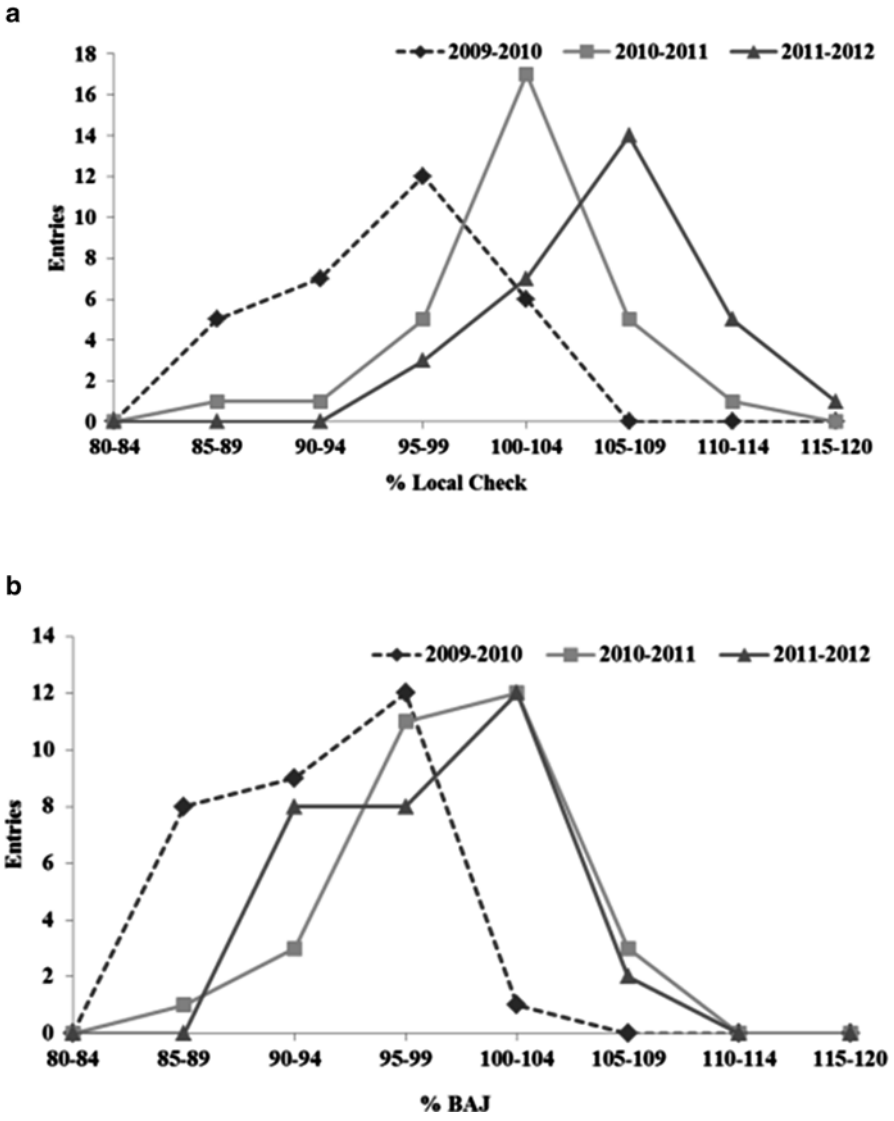

Fig. 26.2 Mean grain yield of the 30 lines across South Asia in 2009–2010, 2010–2011 and 2011–2012. Grain yield is expressed as % above (**a**) local check and (**b**) CIMMYT check line BAJ

outperform the locally adapted high yielding lines under both terminal and continual high temperature stress. Thus early maturity while maintaining yield superiority is beneficial for South Asia in adapting to high temperature stress and enhancing productivity. The identified heat tolerant, high yielding and early maturing Lines are available for the national wheat improvement programs in South Asia for testing and further development of superior heat tolerant wheat varieties.

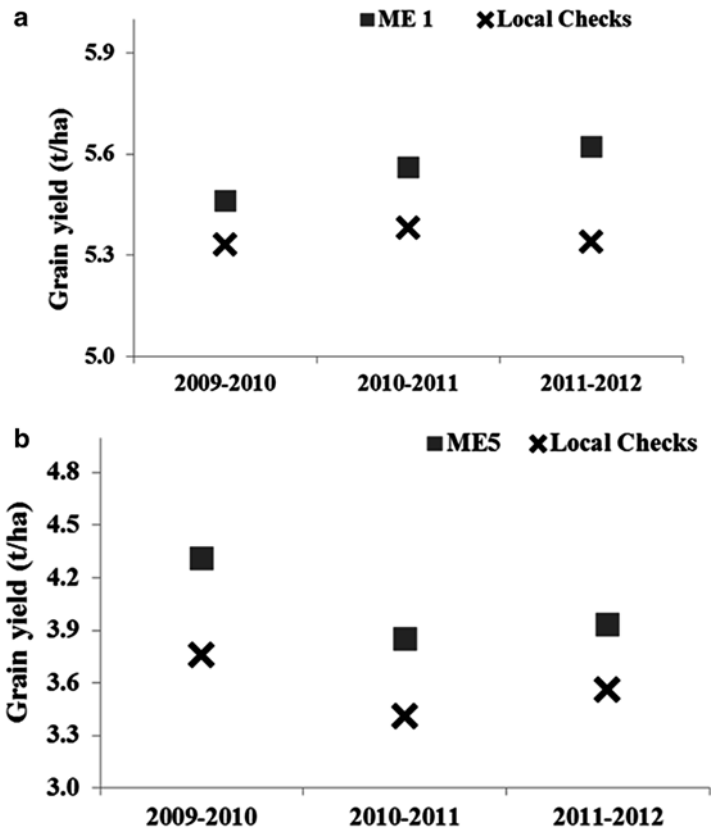

Fig. 26.3 Grain yield of the top five lines and local checks in (**a**) ME1 and (**b**) ME5 of South Asia in 2009–2010, 2010–2011 and 2011–2012

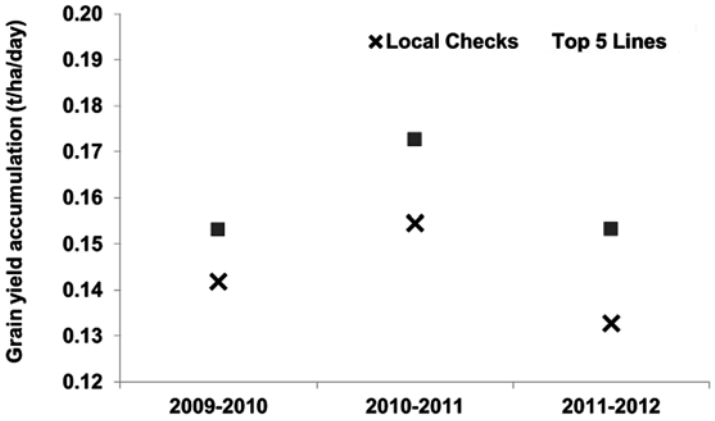

Fig. 26.4 Grain yield accumulation rate (t/ha/day) of the top five lines and local checks in South Asia in years 2009–2010, 2010–2011 and 2011–2012

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Chapter 27
Gene Expression Profiles Involved in Development of Freezing Tolerance in Common Wheat

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Wheat Cold Acclimation and Freezing Tolerance

Exposure of plants to low, nonfreezing temperatures leads to an increase in freezing tolerance, and this adaptive process, called cold acclimation, involves drastic physiological, biochemical and metabolic changes. Most of these alterations are regulated through changes in gene expression. One of the mechanisms behind development of freezing tolerance is induction of the *Cor* (cold-responsive)/*Lea* (late-embryogenesis-abundant) gene family (Thomashow 1999). In common wheat, major loci controlling freezing tolerance (*Fr-1* and *Fr-2*) have been assigned to the long arm of group five chromosomes (Galiba et al. 1995; Snape et al. 1997). *Fr-2* is coincident with a cluster of genes encoding C-repeat binding factors (CBFs) in wheat and barley (Miller et al. 2006; Francia et al. 2007), which directly induce the downstream *Cor*/*Lea* gene expression during cold acclimation (Takumi et al. 2008). In expression quantitative trait locus (eQTL) analysis of *Cor*/*Lea* and *CBF* genes, four eQTLs controlling cold-responsive genes were found, and the major eQTL with the greatest effect was located on the long arm of chromosome 5A (Motomura et al. 2013). The 5AL eQTL region, which plays important roles in development of freezing tolerance in common wheat (Motomura et al. 2013), coincides with a region homoeologous to a frost-tolerance locus (*Fr-A^{m}2*) reported as a *CBF* cluster region in einkorn wheat (Vágújfalvi et al. 2003; Miller et al. 2006). Allelic differences at *Fr-A2* might be a major cause of cultivar differences in extent of freezing tolerance in common wheat (Motomura et al. 2013). It was recently reported that

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large deletions in the *CBF* cluster at *Fr-B2* significantly reduced frost tolerance in tetraploid and hexaploid wheat (Pearce et al. 2013). In barley, two QTLs for low-temperature (LT) tolerance, *Fr-H1* and *Fr-H2*, are found on the long arm of chromosome 5H (Francia et al. 2004), and the *Vrn-H1*/*Fr-H1* genotype affects both the expression of *CBF* genes at *Fr-H2* and LT tolerance (Stockinger et al. 2007; Chen et al. 2009). Thus, the barley *Vrn-H1*/*Fr-H1* and *Fr-H2* regions function to develop freezing tolerance through *Cor*/*Lea* gene expression during cold acclimation. In contrast to barley, the functions of *Vrn-A1*/*Fr-A1* and *Vrn-D1*/*Fr-D1* in regulation of cold-responsive gene expression in common wheat remain unclear.

A lot of other genes, including *Wlip19* and *Wabi5* bZIP transcription factor genes (Kobayashi et al. 2008a, b), contribute to cold acclimation and freezing tolerance in common wheat. These transcription factors, which act in abscisic acid (ABA) signaling, bind to ABA-responsive elements in the promoters of *Cor*/*Lea* genes. Thus, ABA induces expression of a variety of genes that function in the regulation of gene expression, signal transduction and abiotic stress tolerance in common wheat. In fact, ABA sensitivity strongly affects the basal levels of freezing tolerance (Kobayashi et al. 2006, 2008c), and some QTLs on wheat chromosomes controlling ABA sensitivity at the seedling stage are also related to *Cor*/*Lea* gene expression and putatively associated with freezing tolerance (Kobayashi et al. 2010). Recent reports showed that QTLs for ABA sensitivity at the seedling stage could be also associated with dehydration tolerance, seed dormancy and preharvest sprouting tolerance (Iehisa et al. 2014a, b). The QTLs for ABA sensitivity do not correspond to *Fr-1* and *Fr-2*, and the two *Fr* loci act independently of ABA signal transduction pathways (Fig. 27.1).

Transcriptome Analysis During Cold Acclimation

Wheat *CBF* gene expression is temporal and upregulated at least two-fold by LT (Kume et al. 2005). The first upregulation occurs within 1–4 h, which might correspond to the rapid response to LT, while the second upregulation occurs between 2 and 3 weeks after the start of cold acclimation. Maintenance of a high *CBF* transcript level in freezing tolerant cultivars might represent a long-term effect of cold acclimation (Kume et al. 2005). Effects of long-term LT treatment on gene expression profiles could be distinct from rapid changes in response to cold stress. A comprehensive image of transcriptome alteration in cells and tissues of common wheat during cold acclimation and subsequent freezing stress conditions is not yet available. The above-ground tissues of wheat plants become wilted and wither under freezing conditions. However, cold-acclimated seedlings of freezing tolerant wheat cultivars rapidly recover from freezing stress and develop new shoots from surviving meristems of the crown tissues (Ohno et al. 2001). Therefore, biologically important events in the development of freezing tolerance should occur in the crown tissues.

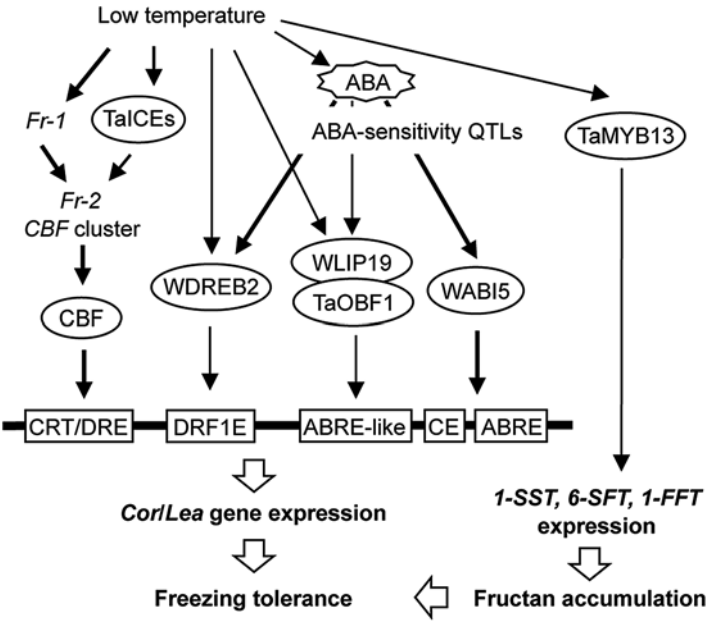

Fig. 27.1 Cold stress signaling pathways in common wheat. Low temperature leads to accumulation of transcription factors (indicated by *ovals*) through ABA-dependent and -independent pathways. Specific binding of each transcription factor to *cis*-acting elements (indicated by *boxes*) activates *Cor*/*Lea* gene expression. *TaMYB13* activates fructan biosynthesis-related genes

Freezing stress treatment significantly alters gene expression profiles of more than 400 genes in the crown tissues of cold-acclimated wheat plants (Skinner 2009). This transcriptome analysis revealed that 68 genes, including CBF, WRKY and zinc finger transcription factor genes, were more than fivefold upregulated by freezing stress. The upregulated genes also encoded kinases, phosphatases, calcium trafficking-related proteins and glycosyltransferases. This observation implied the presence of genetic variation among wheat cultivars in the ability to alleviate the damage to crowns exposed to freezing stress (Skinner 2009). Thus, many genes besides the *CBF* and *Cor*/*Lea* genes presumably participate in each step to develop freezing tolerance in the crown tissues of wheat.

To identify other LT-responsive genes related with cold acclimation in hexaploid wheat, we compared comprehensive gene expression patterns of a synthetic hexaploid line under normal and LT conditions using a wheat 38k DNA microarray (Yokota et al. 2015). For hybridization, total RNA samples were extracted from 3-week-old seedling leaves exposed to LT for 12 weeks, and from crown tissues exposed to LT for 6 weeks. The microarray analyses showed that *TaWRKY45*, *TaWRKY72*, and *TaMYB73* transcription factor genes and two fructan synthesis-related genes, *Ta1FFT* and *Ta6SFT*, were highly upregulated by long-term LT treatment, in addition to a number of *Cor*/*Lea* genes (Yokota et al. 2015). The transcript accumulation levels of these upregulated genes reflected the freezing tolerance

levels of two distinct lines of synthetic hexaploid wheat. Our observations suggest that, in addition to COR/LEA proteins, the WRKY and MYB transcription factors and fructan biosynthesis play important roles in development of freezing tolerance.

Fructan Biosynthesis Pathway and Freezing Tolerance

Severe abiotic stresses induce detrimental changes in cellular compounds, and sugars are regarded as one of the metabolites preventing detrimental changes (Valluru and Van den Ende 2008). In particular, long-term stress conditions lead to higher soluble sugar concentrations and lower amounts of starch (Silva and Arrabaca 2004). Fructans, soluble fructosyl polysaccharides, are storage carbohydrates in a large number of higher plants. Fructans accumulating in perennial grasses can be considered as longer-term reserve carbohydrates to survive the winter period (Yoshida et al. 1998). Transgenic perennial ryegrass plants with an increased amount of fructans showed significantly increased levels of freezing tolerance (Hisano et al. 2004). Genetic transformation of two wheat fructan-synthesizing enzymes conferred fructan accumulation and enhanced chilling tolerance in rice (Kawakami et al. 2008). Therefore, fructans play important roles as anti-stress agents in overwintering plants (Kawakami and Yoshida 2005), and are considered to function in membrane stabilization through formation of a fructan-lipid interaction under water stresses such as cold and drought (Valluru and Van den Ende 2008).

In wheat and barley, three enzyme families, sucrose:sucrose 1-fructosyltransferrase (1-SST), sucrose:fructan 6-fructosyltransferase (6-SFT) and fructan:fructan 1-fructosyltransferase (1-FFT), synthesize graminian-type fructans consisting of β-2,6 linked fructosyl units with β-2,1 branches (Ritsema and Smeekens 2003). The TaMYB13 transcription factor binds to the promoters of wheat *1-SST* and *6-SFT* genes and activates fructosyltransferase gene expression (Xue et al. 2011). Overexpression of *TaMYB13* results in upregulation of *1-SST*, *6-SFT* and *1-FFT* and enhances fructan accumulation and yield-related traits under water-limited conditions in transgenic wheat plants (Kooiker et al. 2013). Snow mold resistant cultivars accumulate and maintain higher fructan levels in the crown tissues from autumn to the end of winter (Yoshida et al. 1998). Yoshida et al. (1998) also reported that fructan may increase freezing tolerance, although its efficiency is lower than mono- and disaccharides in common wheat. Livingston (1996) suggested that fructan is indirectly involved in freezing tolerance of oat and barley. Therefore, fructans surely play important roles in development of water stress tolerance.

As mentioned above, our transcriptome analysis showed that fructan biosynthesis-related genes were significantly upregulated during long-term LT treatment in crown tissues of wheat synthetics (Yokota et al. 2015). In fact, fructan accumulation levels also reflected the distinct freezing tolerance levels of two synthetic wheat lines (Yokota et al. 2015). These observations support a significant association of fructan biosynthesis with development of freezing tolerance in common wheat

(Fig. 27.1). The relationship between carbohydrate accumulation in crown tissues and wheat freezing tolerance and winter hardiness should be elucidated in more detail in future studies.

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Part IX
Improvement of Grain Quality

Chapter 28
Coping with Wheat Quality in a Changing Environment: Proteomics Evidence for Stress Caused by Environmental Changes

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Abstract High temperatures (HTs) during grain filling adversely impact grain yield and its end-use quality for wheat. HTs strongly reduce the expression of major enzymes associated with starch synthesis, whereas enzymes associated with defence against stress and protein folding are dramatically increased. Using proteomics tools, the effect of different temperature regimes on storage protein (SP) accumulation was investigated. HT significantly decreased the quantity per grain of individual gliadin and glutenin spots, but at maturity the ratio of gliadin to glutenin was not modified. HT during grain filling strongly reduced starch accumulation, modified the size distribution of starch granules, and to a much lesser extent, reduced the quantity of total proteins per grain. The aggregation and polymerisation of SP was investigated using asymmetric flow field flow fractionation. Previous analyses of near-isogenic hard/soft lines showed that characteristics of glutenin polymers were significantly influenced by puroindoline alleles (*Pina-D1a* and *-D1b*), and proteomics analysis showed that a typical mechanism of unfolded protein response occurs in ER, resulting from stress during protein accumulation. Effects of alleles encoding puroindolines, HMW-GS and LMW-GS, and temperature during grain development on glutenin polymer characteristics, dough rheological properties, and bread loaf volume were investigated for 40 cultivars grown in six environments in France. A difference of only 2 °C in average daily air temperature between locations during the grain-filling period resulted in increased molecular mass of the glutenin

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polymers and dough tenacity, but decreased dough extensibility and bread loaf volume. To compensate these adverse effects, some solutions are suggested.

Keywords Dough properties • Endosperm • Gliadins • Glutenins • Glutenin polymers • Heat stress • Proteomics • Starch • Temperature

High Temperature Is a Major Factor Affecting Wheat Production

Cereals and particularly winter crops are influenced by ongoing climate changes. Drought may strongly affect plant physiological development resulting in fewer fertile ears per plant and fewer grains per spike. Among abiotic stresses, heat stress, particularly during grain filling, is the main factor responsible for a reduction in grain weight, and hence in yield (Jenner 1994; Ottman et al. 2012). High temperatures when storage compounds are accumulating in the grain are one of the main factors influencing wheat end-use value (Wardlaw and Wrigley 1994). Although a gradual rise in daily maximum temperatures causes less damage to grain compounds than a sudden temperature rise during grain filling, wheat cultivars may also respond differently to high temperatures (Stone and Nicholas 1994; Stone et al. 1997). Whatever the cultivar studied, high temperatures have been shown to shorten the effective grain filling period, and may significantly increase protein concentration because of more dramatic effect on starch than on protein accumulation (Altenbach et al. 2003; Blumenthal et al. 1991a, b, 1995; Corbellini et al. 1998; Triboï et al. 2003). Many studies have addressed the responses of wheat grain to high temperatures during grain filling using either transcriptomics tools (Altenbach and Kothari 2004; Altenbach et al. 2007; Hurkman et al. 2003; Perrotta et al. 1998) or proteomics approaches (Hurkman et al. 2009; Laino et al. 2010; Majoul et al. 2003, 2004; Majoul-Haddad et al. 2013; Skylas et al. 2002; Vensel et al. 2005). In the present study, major events occurring in the wheat endosperm in response to high temperatures during grain filling were investigated using proteomics approaches.

The present report describes major variations in individual storage proteins (SP), metabolic enzymes and proteins involved in carbohydrate metabolism and glutenin polymer formation in response to HT, based on three experiments carried out under controlled conditions and one multi-location field trial in France.

Experiments Used for Heat Stress Studies

Three experiments were carried out at INRA Clermont-Ferrand (45° 46'N, 03° 09'E, 329 m a.s.l.) in the Crop Climate Control and Gas Exchange Measurement (C3-GEM) platform (Triboi et al. 1996) to analyze wheat (*Triticum aestivum* L.) grain responses to heat treatments. Crops were grown outside in 2-m^2 containers

0.5 m deep. Seeds were sown in mid-November at a density of 578 seeds m^{-2}. Crops were watered and fertilized to avoid any growth limitation with an objective of grain protein concentration of 12.5 %. At anthesis the containers were transferred under transparent enclosures under natural light in the C3-GEM platform and exposed to the following conditions:

Experiment 1 Moderate high temperature (MHT) treatment. Cultivars Arche and Tamaro were maintained at 23 °C/11 °C (day/night) for the control and at 28 °C/15 °C for the MHT treatment. Grains were sampled at seven stages from 163 °C days (cumulative degree-days after anthesis) to 781 °C days (physiological maturity) after anthesis.

Experiment 2 Very high temperature (VHT) treatment. Cultivar Thésée was maintained at 18 °C/10 °C for the control treatment and 34 °C/10 °C for the VHT treatment. Results of proteomics analysis of endosperm responses to VHT based on this experiment for total proteins were reported by Majoul et al. (2003) and for albumins-globulins (AG) by Majoul et al. (2004).

Experiment 3 Heat shock (HS) treatment. Cultivar Récital was grown with a day/night temperature of 18 °C/10 °C. One container was subjected to 4-h periods at 38 °C for four consecutive days (HS treatment) between 300 and 400 °C days after anthesis. Total proteins and AG were analysed just before and 1, 8, and 26 (ripeness) days after HS were applied. Proteomics analysis of grain responses to HS based on this experiment was reported by Majoul-Haddad et al. (2013).

The Multi-location Field Trial A total of 68 genetically diverse wheat cultivars provided by INRA and by 11 wheat private breeding companies were grown in three locations in France in 2009 and 2010. The cultivars were grown in conventional conditions with full mineral supply and fungicide protection. The aim of the multi-location trial was to better understand the genetic and environmental factors which influence three parameters: dough tenacity (P) and extensibility (L) and bread loaf volume. The grain composition and quality characteristics of 240 samples (40 cultivars × 6 environments) were analyzed.

Main Proteomics Responses of Developing Wheat Grain to High Temperature

Major Impacts of High Temperature on Energy Metabolism and Starch Synthesis

In all experiments average single grain mass was significantly reduced with the temperature treatments. In experiment 3, the HS treatment (38 °C for 4 h on 4 consecutive days) caused a 25 % reduction in single grain dry mass at maturity (26 days after the HS period; Majoul-Haddad et al. 2013). Although here the amount of starch per grain was not determined, previous studies have shown that reduced grain

masses in response to elevated temperature results from reduced accumulation of starch (e.g. Altenbach et al. 2003; Hurkman et al. 2009). Most proteomics studies have reported a significant decrease in the amount of the small subunits and/or large subunits of the ADP glucose pyrophosphorylase (AGPase, also named glucose 1 phosphate adenyl transferase), the enzyme that catalyses the first committed step in starch biosynthesis pathway, in response to elevated temperature. In experiment 2, at maturity, the AGPase was reduced by 50 % for VHT compared to control. However, in experiment 3, no significant decrease was observed in response to HS. Surprisingly, the abundance of enzymes involved in amylopectin synthesis (starch synthase SSI and SSII; starch branching enzymes SBE I and SBE II; and starch debranching enzyme) was not reduced by elevated temperatures. The abundance of some of these enzymes was slightly, but not consistently, decreased for HS compared to control. This discrepancy may be associated with the specific procedure required for the extraction of these enzymes, a procedure that is rarely performed in proteomic studies (Bancel et al. 2010). The abundance of granule-bound starch synthase (GBSS I) was increased fivefolds for HT compared to control, in good agreement with the higher amylose content previously observed for grain exposed to elevated temperature (Shi et al. 1994).

Transcriptomic and proteomic analyses of grain exposed to HT revealed an increase of transcripts and proteins involved in glycolysis. Here, the amount of phosphohexose isomerase, aldolase, triose phosphate isomerase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, and enolase increased in response to VHT (experiment 2), or were transiently present in response to HS (experiment 3). Grains exposed to HT require more energy (ATP) as evidenced by the higher abundance of the glycolysis enzymes, reducing the amount of glucose available for starch accumulation. The β-amylase present in the late stage of grain filling (Hurkman et al. 2009) was also reported to have increased threefolds at maturity for VHT compared to control in experiment 2. The over expression of β-amylase, usually found in germinating grain, occurs to provide the endosperm with energy. The above findings indicate that starch accumulates less due to higher glycolysis and is also partly hydrolysed by β-amylase, probably to provide the endosperm cells with energy. In HT samples, the consequences were a reduced volume of B and C starch granules and correlatively an increased proportion of A granules (Table 28.1).

Table 28.1 Percent volume of large (A-type, diameter > 15 μm), intermediate (B-type, diameter = 5–15 μm) and small (C-type, diameter <5 μm) starch granules determined using laser granulometry for mature grains of cv. Récital grown under normal temperature regime (control) or exposed to 38 °C for 4 h on for 4 consecutive days (heat shock, experiment 3)

	Percent volume (%)		
Temperature treatment	A	B	C
Control	71.0 ± 0.3	27.8 ± 0.3	1.2 ± 0.01
Heat shock	78.9[a] ± 0.2	20.1[a] ± 0.2	1.0[a] ± 0.01

[a] Significantly different from control at $P < 0.0001$
Data are means ± 1 SD for $n = 3$ independent replicates

Synthesis of Wheat Storage Proteins Is Not Drastically Affected by High Temperature

MHT treatment (experiment 1) caused variation in the amount of only few individual SP of cv. Arche: two ω-gliadins were increased, whereas one γ-gliadin and one LMW-GS were reduced. No HMW-GS was affected by MHT. However, MHT resulted in a significant decrease in the amount of SP expressed in micrograms of protein per grain, as only 65.7 % and 65.5 % of the total amount of gliadin and glutenin, respectively, were accumulated at maturity, compared to control.

VHT treatment increased (2- to 27-folds) the amount of only three α-gliadins in the mature grain of cv. Thésée (Majoul et al. 2003) and HMW-GS and LMW-GS were not modified by the treatment.

HS treatment affected the kinetics of accumulation of 13 SPs. Ten of these proteins still differed significantly at maturity: four α-gliadins, two γ-gliadins, and two LMW-GS were decreased and one β-gliadin and one ω-gliadin were increased compared to control. In an early study, one HMW-GS encoded by *Glu-A1* significantly increased when expressed as a percentage volume (Branlard et al. 2008). Reassessing the analysis of all 2D gel images failed to confirm this higher abundance when expressed in micrograms of protein per grain (Majoul-Haddad et al. 2013), again providing evidence that the accumulation of HMW-GS was not affected by the HS treatments.

In addition to the remarkable stability in abundance exhibited by HMW-GS in response to HT and considering that more than 80 SP spots were detected on 2D gels whatever the cultivar analysed, it should be noted that only few SP were influenced by HT treatments. In all three experiments, the kinetics of individual SP was analysed. During grain filling, the ratio of gliadin to glutenin was different at one (640 °C days after anthesis) out of seven sampling stages for cv. Arche (medium quality wheat) whereas no difference was found for cv. Tamaro (high quality wheat). At maturity, no difference between heat exposed and control grains were detected for the ratio of gliadin to glutenin. Altogether, the amount of individual SP during grain filling demonstrated remarkable stability in response to HT treatments, indicating the existence of regulatory mechanisms, which could differ between cultivars. This underlines the need for a better understanding of the regulatory gene networks involved in SP synthesis when breeding future high quality cultivars.

Glutenin Polymers Are Strongly Impacted by High Temperature

The molecular mass of glutenin polymers was previously shown to be significantly influenced by grain hardness (Lesage et al. 2011). Further investigations (Lesage et al. 2012) of the puroindoline function provided clear evidence that the endoplasmic reticulum (ER) is the site of the unfolded protein response (UPR), a phenomenon first described in animal cells (Schröder and Kaufman 2005). Protein folding is

known to be inhibited by several factors including excess proteins in ER, temperature, magnetic field, and electric field. It was thus necessary to investigate the influence of the characteristics of the polymers for cultivars of different genetic origin and grown in different locations. The polymer characteristics (molecular mass Mw2, polydispersity index Mw2/Mn2 and radius of polymers Rw2) of the 240 samples from the multi-location field trial were evaluated using asymmetric flow field flow fractionation (AFFFF; Lemelin et al. 2005). Glutenin alleles and puroindoline alleles, as well as their interactions were shown to significantly influence Mw2, which, unexpectedly, varied from 5×10^6 to 49×10^6 Da (Lesage et al. 2013). In addition, it was shown that the sum of daily mean air temperatures for June and July (i.e. during the grain filling period; SumT-JuJy), differentially affected Mw2 in the three hardness classes (hard, medium and soft). A difference of 110 °C in SumT-JuJy was found across the six environments, which was correlated with an increase of Mw2 from 10×10^6 to 20×10^6 Da in hard cultivars and from 13×10^6 to 33×10^6 Da in soft cultivars. Partial least square regression used to explain Mw2 with several grain characteristics including glutenin and puroindoline alleles and SumT-JuJy, provided clear evidence for the major influence of SumT-JuJy on phenotypic variations in polymer characteristics (Fig. 28.1a). Why does temperature have such a strong effect on the characteristics of glutenin polymers? All the proteomics studies on grain response to HT reported that two to four of the redox enzymes ascorbate dismutase, ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase were increased by HT. This, together with evidences of UPR, suggests that grains exposed to HT undergo sever oxidative stresses causing higher polymer mass.

Variations in Dough Properties Resulting from High Temperature

The rheological properties of dough and bread loaf volume measured on the 240 samples revealed that SumT-JuJy had a strong impact on these important quality parameters. Indeed, the increase of the molecular mass of glutenin polymers in response to temperature was correlated with an increase in dough tenacity (Fig. 28.1b) and a decrease in both dough extensibility (Fig. 28.1c) and loaf volume (Fig. 28.1d). The standardized PLS coefficients attributed to SumT-JuJy were among the highest in explaining phenotypic variations in dough tenacity, dough swelling, and bread loaf volume. Dough tenacity was, as expected, positively influenced by grain hardness, *Glu-B1* 7-8, *Glu-D1* 5-10 and *Glu-B3*c, and negatively influenced by *Glu-B1* 6-8, *Glu-B1* 6.1-22, *Glu-D1* 2-12, *Glu-B3*b'(bp in Fig. 28.1) but surprisingly, also negatively influenced by *Glu-A1* 2* and *Glu-B1* 17-18. Dough extensibility increased with grain protein content (GPC) as is usually the case, but also increased with some glutenin alleles like *Glu-A1* 2*, *Glu-B1* 17-18. The glutenin subunits *Glu-D1* 5-10 were negatively correlated with dough extensibility and *Glu-D1* 2-12

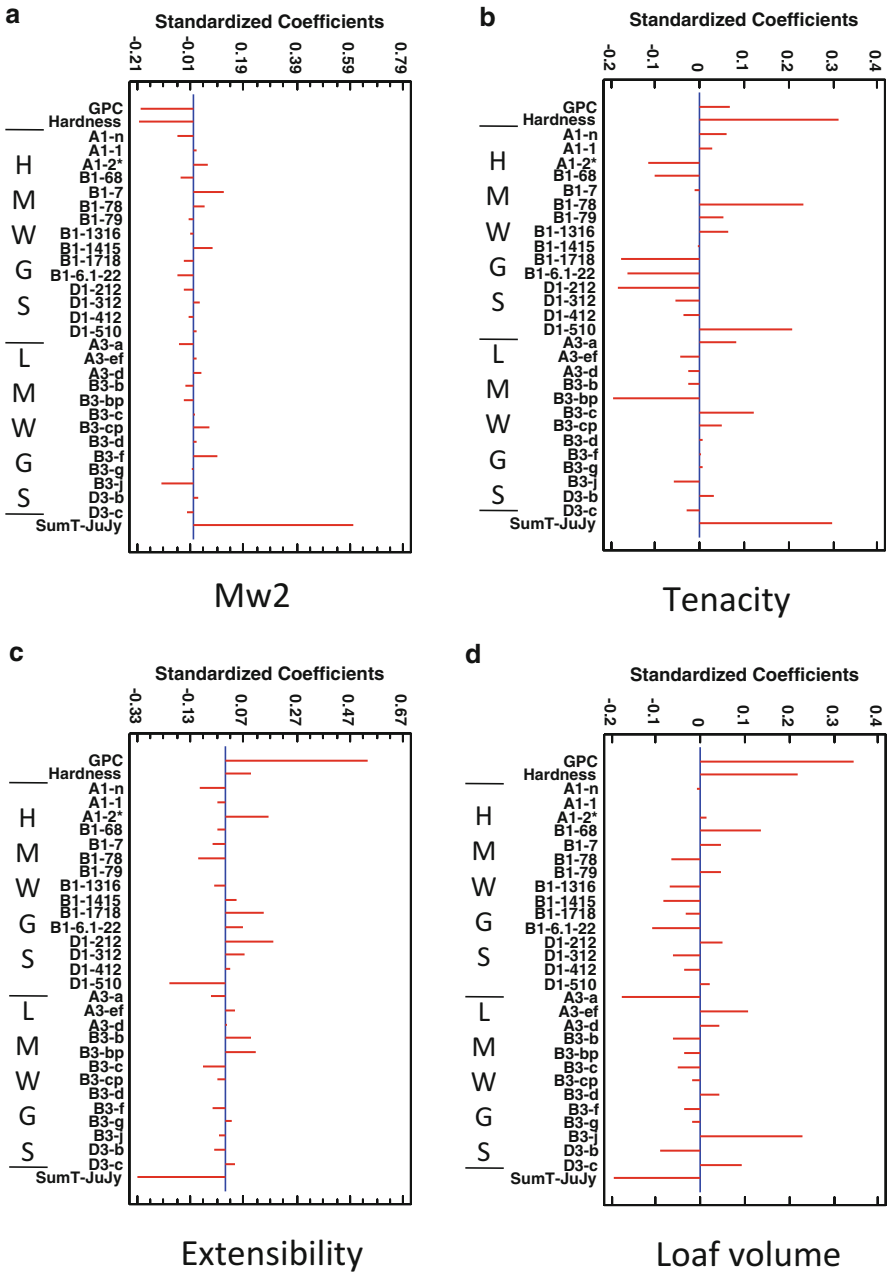

Fig. 28.1 Partial least square regression performed to explain the molecular mass of gluten in polymers, Mw2 (**a**), dough tenacity (**b**) and extensibility (**c**), and bread loaf volume (**d**): the negative (*left horizontal bar*) and positive (*right horizontal bar*) standardized coefficients were computed for grain protein concentration (GPC), grain hardness (Hardness), HMW-GS and LMW-GS alleles, and the sum of mean daily temperature during the grain filling period (SumT- JuJy: June and July)

was positively correlated with dough extensibility. In addition to the well-known positive effect of GPC and grain hardness on bread loaf volume, the standardized coefficients attributed to glutenin alleles clearly evidenced that the molecular mass of glutenin polymers had a negative impact on loaf volume. This result was supported by the positive correlation between loaf volume and glutenin alleles known to have a very negative effect on dough rheological properties (*Glu-B1* 6-8, *Glu-A3ef*, and *Glu-B3*j).

Some Proposals to Cope with Wheat Quality in a Changing Environment

High temperatures during grain filling have a more pronounced effect on the amount of starch than on the amount of protein accumulated per grain. All genetic factors associated with higher starch accumulation including light saturated photosynthesis, awned ears, and prolonged photosynthesis in grain peripheral layers, must be regarded as potentially favorable for maintaining higher amounts of starch per grain during HT events (reviewed by Cossani and Reynolds 2012). The influence of HT on the genetic regulation of major starch enzymes and particularly AGPase and β-amylase should now be investigated in wheat to identify potential markers for breeders. Whatever the potential benefit of using those markers, determination of the test weight, negatively impacted by HT during grain filling, is the easiest way to phenotype wheat progenies capable of completing grain filling even in a HT environment.

Only few individual SPs are influenced by HT or heat shock during the linear phase of grain filling. The ratio of gliadin to glutenin was not significantly affected by the HT treatments used here. However, higher temperatures (daily maximum >35 °C) have been reported to increase the gliadin to glutenin ratio (Blumenthal et al. 1995). Although individual SP may not be affected, we showed that daily maximum temperature lower than 35 °C can strongly increase the molecular mass of the glutenin polymers. The resulting effect is to increase dough tenacity and decrease extensibility. This may explain why, in the last decade, the ratio of tenacity to extensibility has often been reported to have increased in many countries where the alveograph test is used. To tackle such quality consequences, it is advisable not to discard alleles previously associated with dough extensibility like *Glu-A1* 2*, *Glu-B1* 17-18 and *Glu-D1* 2-12. The influence of SP diversity on the molecular mass of glutenin polymers and wheat properties also needs to be reassessed in relation with puroindoline alleles, as judiciously proposed by Ikeda et al. (2013). The molecular regulation of redox enzymes such as dehydroascorbate reductase, glutathione reductase, and glutathione transferase also need to be investigated to improve the stability of the molecular mass of glutenin polymers in HT environments.

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Chapter 29
Starch Modification: A Model for Wheat MAS Breeding

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Abstract In the past two decades, three types of starch mutants, *waxy* (*Wx*), *high amylose* (*HA*) and *sweet type wheat* (*SW*) have been developed in Japan. Each of these lines was obtained by identifying "partial" mutants with mutations in one or two homeoelogous genes derived from the A, B or D-genomes, followed by crossing to produce fully null mutants. The *Wx* line lacks the three granule-bound starch synthase I (GBSSI) proteins responsible for amylose synthesis, and HA lacks the three starch synthase IIa (SSIIa) enzymes that are involved in amylopectin synthesis. SW lacks all active GBSSI and SSIIa enzymes. "Partial" null mutants have also been used to obtain more subtle modifications in starch quality. For example, the *GBSSI-B1* single null lines produce starch which is slightly reduced in amylose. This suggested that the identification of other desirable lines using MAS would allow us to further fine-tune starch characteristics. Sixty-four homozygous lines differing in *GBSSI* and *SSIIa* composition can be selected from progeny of crosses between *HA* and *Wx*. Co-dominant markers for all *GBSSI* and *SSIIa* genes enabled us to select all 64 lines quickly and effectively. The modulation of starch characteristics serves as a model that demonstrates the utility of mutation identification in combination with MAS in hexaploid wheat. The availability of genome sequence information, combined with new methods of mutation detection, reduces the amount of work involved in finding single mutations in wheat, and one can easily see the potential for expanding this methodology to other traits.

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Introduction

Starch is one of major components of cereal seed, and starch composition greatly influences the quality of cereal-based food products. Starch is composed of amylose, a linear chain of glucosyl units joined by α-1,4 linkages, and amylopectin, which is a highly branched molecule with α-1,6 linked side chains. The key enzyme in amylose synthesis is granule bound starch synthase (GBSSI, alternatively called waxy protein), and lines lacking *GBSSI* have amylose-free starch. In contrast, many enzymes are involved in amylopectin synthesis, including several types of starch synthases, as well as branching and debranching enzymes. Starch synthase IIa (SSIIa), which elongates the outside branches of the amylopectin molecules, appears to play a particularly important role in amylopectin synthesis.

Null mutations of the genes encoding the enzymes involved in starch synthesis can result in changes in starch structure and properties, which may affect the processing and cooking properties of starch. In diploid cereals such as maize and rice, many starch mutants have been identified and used extensively in both basic and applied research (Boyer and Hannah 2001; Vandeputte and Delcour 2004). However, although starch mutants of common wheat could potentially provide interesting new materials for the food industry, none of the typical starch mutants identified in maize and rice have been available until recently. In the past two decades, three types of starch mutants were developed in Japan; *waxy* (*Wx*, 1995), *high amylose* (*HA*, 1999) and *sweet type wheat* (*SW*, 2006).

Waxy Wheat (*GBSSI* Mutant)

The initial screening of wheat germplasm to detect genetic variation in the gene encoding GBSSI was performed using protein gel electrophoresis. When starch granule-bound proteins are run on standard SDS-PAGE gels, the waxy or GBSSI protein can be identified as a distinct band. Because wheat is a hexaploid crop, this band includes the proteins derived from the A, B and D genomes. By modifying SDS-PAGE conditions, we were able to separate the single band into three isoforms (Nakamura et al. 1993). We then used nullisomic-tetrasomic analysis to determine what genome each isoform was derived from. Germplasm screening identified several lines that were missing *Wx-A1* and *-B1* proteins, and one line, Kanto107, that lacked both the *Wx-A1* and *-B1* proteins.

These results suggested several methods for obtaining fully waxy lines. To produce tetraploid waxy wheat, Kanto 107 (K107) could be crossed with tetraploid wheat to remove the D genome. Hexaploid waxy lines could be developed by identifying a line that had a null mutation in the D gene, and combining it with K107, or alternatively mutagenic treatment of K107 could be used to inactivate the functional D gene. We were successful in selecting a fully *waxy* tetraploid wheat line from a

cross between K107 and a durum wheat variety (Nakamura et al. 1995). Identification of a line with a naturally occurring mutation in the *Wx-D1* gene proved more difficult, and hundreds of lines were screened before the identification of a line (Bai-Hao) missing the *Wx-D1* protein. A fully *waxy* hexaploid line was then selected from the progeny of a cross between Bai-Hao and K107 (Nakamura et al., 1995). Later, mutagenic treatment of K107 also resulted in the identification of a fully waxy hexaploid line (Yasui et al. 1997).

Registered varieties of waxy wheat have already been developed in several countries and many studies characterizing the chemical and rheological properties of this wheat type have been reported. Waxy wheat showed substantial differences in flour properties compared to control lines, as evidenced by rapid viscosity analyzer (RVA) and differential scanning calorimetry (DSC) analysis (Yoo and Jane 2002). Despite the intensive studies on waxy wheat, few commercial uses for waxy wheat flour have been developed to date.

Conversely, "partial" waxy mutants, lacking one or two GBSSI proteins, appear to be much more practically useful, particularly B-null and AB-null lines (Ishida et al. 2003; Yamamori et al. 1994; Zhao et al. 1998). The amylose contents in these lines are reduced by a small but significant amount, making them particularly suitable for the production of Japanese salted noodles. However, the selection of these partial lines by measuring amylose content requires substantial time and effort by breeders, and we felt that the development of a marker- assisted selection protocol would streamline this process. Our first step towards this goal was the molecular characterization of the mutations in the three waxy genes (Vrinten et al. 1999). This was followed by the development of DNA marker sets that would work well under the same PCR conditions and were co-dominant, making them easy to use in practical breeding programs (Nakamura et al. 2002; Saito et al. 2009). Subsequently, these markers were adapted in Japanese wheat breeding programs, and this essentially marked the beginning of MAS wheat breeding in Japan.

During our work with the waxy mutants, we made several observations which proved useful in our further work on starch modification. First, we noted that early efforts to obtain starch mutants using mutation breeding led to the successful isolation of waxy lines and other starch mutants in diploid plants such maize or rice, but not in wheat. It appeared that wheat had a high resistance to mutagenic treatment, but in fact, mutations likely occurred but were masked by the presence of active homoeologues, and the probability of concurrently mutating all three genes was extremely low. However, combining "partial" null lines represented a simple but effective method of obtaining fully null mutants. Naturally occurring single null mutations can often be identified in material from germplasm databases, and mutagenesis treatments can be used to increase mutation frequency. We also realized that not only fully null mutants but "partial null" lines with mutations in one or two homoeologous genes might prove useful, as was seen for the Waxy genes. The ability to create "partial" mutants can be considered an advantage of working with a hexaploid crop such as wheat.

High Amylose Wheat (*SSIIa* Mutant)

Screening for lines missing the SSIIa proteins was performed by Yamamori et al. (2000) using similar methodology as described above for waxy proteins. After *SSIIa-A1*, *–B1* and *-D1* null lines were identified, these lines were crossed and a fully null *SSIIa* mutant was selected. The amylose content in this line was increased by about one-third as compared to wild-type (Yamamori et al. 2000; Shimbata et al. 2005). Although no commercially available HA variety has been released yet, quality studies have shown dramatic changes in flour properties for this type of wheat (Yamamori et al. 2000; Shimbata et al. 2005). *HA* seed also contains a higher proportion of resistant starch (Yamamori et al. 2006), which is thought to have a beneficial influence on health. Although *HA* flour used alone does not appear to be suitable for baking or noodle products, combinations of *HA* and regular flour are giving promising results in ongoing quality tests.

However, our interest has focused more on the "partial null" *SSIIa* genotypes. We were curious to know if there were significant effects due to the presence of one or two null genes, and if the contributions of the three *SSIIa* genes were different. Again, the use of MAS appeared to be the most efficient way to obtain the desired genotypes. We first characterized the mutations in the three *SSIIa* genes at the DNA sequence level, then used this information to develop PCR-based markers that were co-dominant and could be multiplexed (Shimbata et al. 2005). After reconstituting the *HA* line by MAS, we used MAS to develop near-isogenic lines of the eight possible homozygous genotypes for the *SSIIa* gene, using a leading variety from the southern area of Japan as the recurrent parent.

In less than three years, we obtained BC_5F_2 NIL lines for wild (type 1) and HA (type 8) lines, as well as all "partial" lines (types 2–7). Of the eight lines, only the high amylose line showed significant differences in kernel weight and amylose content as compared to wild-type (Shimbata et al. 2012). However, differences in starch characteristics including chain length distribution, relative viscosity, retrogradation, pasting, and enzymatic hydrolysis properties were observed among partial null lines. Generally, larger effects were observed in lines carrying two null homoeologous genes than in lines carrying a single null gene. Significant differences were also observed among the three lines carrying two null *SSIIa* genes (Shimbata et al. 2012). These results largely concurred with results from similar studies using doubled haploid lines (Konik-Rose et al. 2007). We were also able to determine that the effect of *SSIIa* genes on amylopectin synthesis follows the order *SSIIa-B1* > *SSII-D1* ≥ *SSIIa-A1* (Shimbata et al. 2012), which parallels the differential effects of the *waxy* genes on amylose synthesis (*Wx-B1* > *Wx-D1* ≥ *Wx-A1*).

Sweet Wheat (*GBSSI* and *SSIIa* Mutant)

With two fully null starch mutant lines available, creating a double mutant became possible. Since we had co-dominant markers for wild-type and null alleles of all six genes, selection of the fully null *SSIIa*/*GBSSI* double mutant was fairly straightforward. Immature seed of the double mutant showed a higher sugar content, with significant increases in sucrose, glucose, maltotriose and particularly maltose (Nakamura et al. 2006). The seeds of the double mutant appeared to develop normally until seed desiccation began, when the seeds became shrunken, resulting in a large decrease in seed weight, as is seen in some sweet corns. The wheat *SSIIa*/*GBSSI* double mutant also resembles sweet corn in terms of its increased sugar content, therefore we refer to it as "sweet wheat" (SW). Mature seed of SW also showed a high sugar content, with increases in fructan and total dietary fiber levels (Shimbata et al. 2011).

The endosperm starch granules of SW are very small and appear degraded, even at earlier stages of seed development, when SW seeds still appear plump (Vrinten et al. 2012). Starch granules from the *HA* line also have a somewhat deflated appearance in comparison to the normal pancake-like shape found in wild-type granules, suggesting that the lack of functional SSIIa protein may play a major role in this phenotype.

The structure of starch from SW seeds also showed large changes in comparison to starch from wild-type or parental lines. When pasting properties of normal wheat starch are subjected to relative viscosity analysis, a strong peak indicating increased viscosity due to starch swelling is usually observed. However, in SW flour, this peak was essentially absent. As well, the amylopectin from SW seeds had a reduced molecular weight and an increased proportion of short branch chains (Vrinten et al. 2012).

Product tests conducted with SW have indicated that mixing SW flour with regular flour can impart a slightly sweet taste and a crispy texture to baked goods. However, SW flour does not seem to be suitable for use in pasta or noodles. Product tests are still ongoing, and other uses, such as using fresh immature seed as a topping or vegetable, are being considered.

The dramatic changes in starch and seed phenotypes seen in SW can predictably lead to certain problems in the field. For example, SW seed is very light and is therefore easily lost during harvesting, necessitating modification of harvesting machinery. SW also appears to be very susceptible to pre-harvest sprouting, probably due to the high levels of sugar in seeds.

Expanding Genotype Availability: Combining Mutations

While the fully null mutants *Wx*, *HA* and *SW* have starch characteristics that are of interest to the food and flour milling industries, the dramatic changes resulting from these mutations may lead to some less than desirable characteristics, as outlined above. However, one major advantage in working with wheat is that its hexaploid composition allows us to make more subtle changes in starch characteristics. An example of this is the *Wx-B1* mutation in wheat, which is widely present in lines used in Asian noodles. Although the reduction in amylose content in the *Wx-B1* mutant is small (approximately 27 % of total starch vs 29 % in wild-type; Yamamori and Quynh 2000), it results in a change in viscoelasticity that imparts improvements in noodle quality.

Using the six available mutations covering the *GBSSI* and *SSIIa* genes, 64 homozygous genotypes can be created (Fig. 29.1). By using the co-dominant, "perfect" markers described above, we were able to select all 64 genotypes. We have begun quality testing on these lines, and have identified lines which have significant differences in starch characteristics, yet maintain similar starch levels and seed weights as wild-type lines. Interestingly, we noted that the shrunken phenotype is not limited to the SW genotype (null alleles for all *SSIIa* and *GBSSI* genes); certain lines that are null for all *SSIIa* genes but have a single wild-type *GBSSI* gene also produce shrunken seeds. When starch granules from all 64 lines were observed using

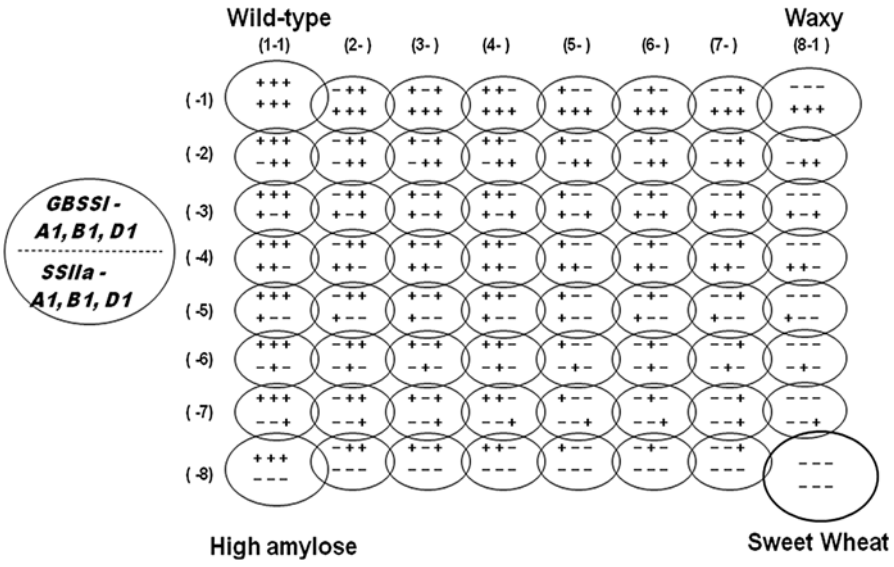

Fig. 29.1 Two- dimensional array of homozygous genotypes developed from *GBSSI* and *SSIIa* wild-type and mutant alleles. For each of the six genes, the homozygous wild type allele is indicated by "+" and the homozygous mutant allele by "−". Homoeologous genes are indicated as A1, B1 or D1

scanning electron microscopy, most lines had normal pancake shaped granules, except for lines missing all SSIIa proteins, which had starch granules with flattened shapes. This indicates the importance of amylopectin on starch granule architecture, whereas amylose does not seem to play such a significant role. However, a decrease in the number of functional waxy genes compounded the effects of the null *SSIIa* genotype.

In contrast to changes in seed shape or starch granule appearance, differences in starch quality characteristics between the lines show a wider range of effects. RVA analysis showed small but significant differences between lines; for example, a line with three null waxy genes and two null *SSIIa* genes showed a decrease in peak viscosity temperature and an increase in peak viscosity as compared to waxy wheat. Although this is essentially a waxy line, fine-tuning of starch characteristics has resulted from the introduction of two null *SSIIa* genes, thereby producing a new type of waxy wheat. Similar subtle but significant differences were also observed in other lines, indicating that we have developed a pool of variation for starch quality characteristics simply by making combinations among two sets of homoeologous genes using marker-assisted selection. The chances of developing this amount of variation for starch characteristics naturally in any breeding program would be very small.

Adding a third set of homoeologous genes and making the array "3-dimensional" would increase the number of genotypes exponentially, resulting in a total of 512 genotypes. Although certainly not all of these genotypes would have useful changes in starch characteristics, we expect that some lines would be commercially useful.

Future Directions

Work with the starch mutations outlined above has demonstrated three main points: (1) Development of fully null mutants of wheat can be achieved by the simple but effective method of identifying individual lines with null mutations in the genes derived from the A, B and D genome, and combining these lines by traditional hybridization. The use of markers greatly facilitates this work. (2) "Partial" mutants, with homozygous null alleles for one or two of a set of three homoeologous genes, can have phenotypes that are significantly different from wild-type. The practical usefulness of these "partial" null lines can exceed that of fully null lines. (3) By creating combinations of homozygous wild-type and null alleles for two or more sets of homoeologous genes, we can create a range of variability for a trait that is unlikely to be present by chance in a breeding program.

The availability of genome sequence information, combined with new methods of mutation detection, significantly reduces the amount of work involved in finding single null mutations in wheat. Although starch synthesis represents a good model for analyzing the effects of null and partial null genotypes in wheat, one can easily see the potential for expanding this methodology to other traits. However, after the detection of single mutants, crossing and analyzing mutant lines remains laborious. Our focus now is on streamlining this process as much as possible.

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Chapter 30
Quality Characteristics of Soft Kernel Durum; A New Cereal Crop

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Abstract Production of crops is in part limited by consumer demand and utilization. In this regard, world production of durum wheat (*Triticum turgidum* subsp. *durum*) is limited by its culinary uses. The leading constraint is its very hard kernels. Puroindolines, which act to soften the endosperm, are completely lacking in durum. Currently, durum grain is milled on highly specialized mills which produce as their primary objective coarse semolina. Morris and co-workers (2011) described the development of soft kernel durum wheat. The soft kernel trait (*Hardness* locus) was introgressed into Langdon via *Ph1b*, and crossed to the Italian durum cv. Svevo (producing 'Soft Svevo'). Soft Svevo behaves much like a soft hexaploid wheat with somewhat lower break flour yield, higher water absorption, and smaller cookie diameter. Pilot scale spaghetti manufacture indicated that hydration levels could be reduced to 26–27 % for soft durum, as opposed to about 32 % for commercial semolina. Cooking trials indicated equal-or-better texture, cooking loss and tolerance. Soft Svevo flour performed well in a range of baked goods. These studies demonstrate the stable transfer of the *Puroindoline* genes from *T. aestivum* to *T. turgidum* subsp. *durum*. As such, the processing and utilization of durum was dramatically altered. The creation of soft durum therefore increases the potential for wheat production under marginal cropping conditions, while establishing a new wheat class with expanded and novel uses.

Keywords Durum wheat • Hardness • Kernel texture • Pasta • Puroindoline

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Production of specific crops is in part limited by consumer demand and utilization. In this regard, world production of durum wheat (*Triticum turgidum* subsp. *durum* (Desf. [Husn.]) is limited by its culinary uses, which are primarily pasta and couscous. Why are the culinary uses of durum wheat more restricted than bread wheat (*T. aestivum* L.)? Two main genetic systems are responsible: very hard kernels and moderate gluten strength/extensibility. These grain quality traits have influenced the utilization of durum wheat since its domestication, and these traits have defined cultural and social aspects of food in different societies.

Kernel hardness (texture) in wheat is primarily conditioned by the *Hardness* locus on 5DS which is comprised of *Puroindoline a* and *Puroindoline b* (Morris 2002; Morris and Bhave 2008). Puroindolines, which act to soften the endosperm, are completely lacking in durum. Soft wheat has a fully "functional" haplotype, *Pina-D1a/Pinb-D1b*. Hard hexaploid wheats result from loss-of-function mutations in either *Pina* or *Pinb*. To assess kernel texture phenotype, one of the most common methods is the Single Kernel Characterization System (SKCS) (Perten Instruments). The SKCS crushes individual kernels and thus provides data on a "population" of kernels. The mean and standard deviation are diagnostic with regard to interpreting the texture and homogeneity of a particular grain lot. Common results from the SKCS would be ~25 for soft wheat, ~65 for hard, and ~85 for durum.

Currently, durum grain is milled on highly specialized "durum only" mills that can accommodate the very hard kernel texture. Further, these mills have as their primary objective the production of coarse semolina with minimal bran contamination. Some 'flour' is produced, but tends to have high levels of starch damage. Attempts to reduce the particle size of semolina raises starch damage to unacceptable levels. Semolina has about 4–5 % starch damage and a particle size 3–4 times larger than flour. These larger particles require longer hydration times, whereas smaller particles with more starch damage have a higher water requirement. Non-uniform particle size distribution results in uneven hydration and pasta defects. In theory, having a durum wheat with soft kernels would reduce its culinary constraints, and thereby expand its use, demand and production. Since durum can better compete with bread wheat on more marginal lands, soft durum could enhance global food security.

Morris et al. (2011) described in detail the development of soft kernel durum wheat. The soft kernel trait (*Hardness* locus) originated from Chinese Spring and was introgressed via *Ph1b* homoeologous recombination. Initially, 14 independent recombination lines were isolated in the durum cv. Langdon and assigned a series of numbers, 674–688. Of these, 10 were crossed to the Italian durum cv. Svevo. Soft kernel progeny were obtained from five. A progeny plant from the 674 cross was selected and used in developing a BC_3-soft kernel derivative of Svevo. The soft derivative is termed 'Soft Svevo', and has been deposited in the American Type Culture Collection as Accession No. PTA-10087.

Soft Svevo was grown in 2010 at Bozeman, MT, USA under irrigation. Soft Svevo behaves much like a soft hexaploid wheat with somewhat lower break flour yield, higher water absorption, and smaller cookie diameter. Grain protein was 14.2 %, with an SKCS hardness of 23.2, compared to typical durum values of >80.

Modified Quadrumat milling produced a break flour yield of 40.5 % and a straight-grade (SG) yield of 68.7 %. SG flour protein was 12.0 % with a 0.52 % ash content. Mixograph water absorption was 59.7 %. Sugar-snap cookie diameter was 8.97 cm, and sponge cake volume was 980 cc. Polyphenol oxidase (PPO) (L-DOPA substrate) was 0.124 AU. Flour SDS sedimentation volume was 11.9 mL/g. Solvent Retention Capacities (SRC) were Carbonate SRC 74.8 %, Lactic Acid SRC 97.8 %, Water SRC 59.1 % and Sucrose SRC 94.1 %. For comparison, similar data for cv. Xerpha soft white winter wheat grown at a number of Washington State locations were, break flour yield averaged 44.1 % and SG flour yield was 67.0 %. SG flour protein was 8.80 % with a 0.37 % ash content. Mixograph water absorption was 57.0 %. Sugar-snap cookie diameter was 9.28 cm. Flour SDS sedimentation volume was 8.5 mL/g. Carbonate SRC was 69.5 %, Lactic Acid SRC 87.3 %, Water SRC 56.0 % and Sucrose SRC 79.8 %. Soft Svevo was grown in 2012 at three different locations and management systems in Washington State. Resulting grain proteins ranged from 12.0 % to 15.0 %, SKCS hardness ranged from 12.4 to 20.9. Kernel weights ranged from 34.5 to 39.3 mg. PPO levels were 0.128–0.140 AU.

The Langdon recombination lines were also crossed to the Italian durum cv. Creso and to the Canadian durum cv. Kyle. In both cases, progeny with soft kernels were obtained, consistent with the presence of the *Hardness* locus and expression of the soft, wild-type *Puroindolines* from Chinese Spring. Soft Svevo was used as a parent to transfer the soft kernel trait to the adapted high-yielding durum cultivars Alzada, Havasu and Strongfield. Initial and backcross progeny segregated in an expected 1:2:1 ratio for soft kernel indicating stable and predictable inheritance. Recently we have obtained F_2 progeny kernels from crosses between Soft Svevo and eleven of the CIMMYT International Durum Yield Nursery (IDYN) entries. All show normal segregation of F_2 kernels in the spike (see Morris and Beecher 2012).

Pilot scale spaghetti manufacture indicated that hydration levels could be reduced to 26–27 % for soft durum, as opposed to about 32 % for commercial semolina –a reduction of some 15 or more per cent. Less water added means less water to remove with expected significant energy savings. Cooking trials indicated equal-or-better texture, and cooking loss and tolerance.

Soft Svevo flour was used in various baked goods: baguette and batard hearth breads, pan bread, muffins, refrigerated doughs, croissants, and pizza crust. Results were generally very promising. All products showed the creamy yellow color of the durum endosperm carotenoids. Informal taste panelists commented on a very appealing flavor, appearance and texture. More detailed, controlled studies are certainly in order.

These studies demonstrate the stable transfer of the *Puroindoline* genes and *Hardness* locus from *T. aestivum* to *T. turgidum* subsp. *durum*. As such, the processing and utilization of durum were dramatically altered. Milling can be performed on "standard" wheat mills. As opposed to coarse semolina, a low starch damage, fine particle size flour can be produced. Flour quality for the first soft durum, 'Soft Svevo' appears to be intermediate between hard and soft hexaploid varieties. Further breeding, selection and testing will be needed to resolve end-use quality in greater detail. However, pilot-scale pasta trials have indicated excellent spaghetti quality of

soft durum. A number of baked goods, traditionally made from hexaploid wheat flour, have been successfully made. Durums can also be freely crossed with wild emmer wheat, providing direct access to an enormous gene pool of disease resistance and stress tolerance traits. The creation of soft durum therefore increases the potential for wheat production under marginal cropping conditions, while establishing a new wheat class with expanded and novel uses.

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Chapter 31
Proposal of International Gluten Research Group

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Abstract In a scenario of climate change and rapidly rising urban populations demanding processed foods, it is necessary to develop new wheat cultivars combining high yield potential, disease resistance, and stability for yield and processing quality, even under heat or drought stress conditions. Allelic variation for gluten proteins (glutenin subunits and gliadins) is a major determinant of differences in dough viscoelastic properties observed between cultivars of both bread wheat and durum wheat. Technical difficulties in allelic identification due to the complexity of the protein profile produced by each cultivar and the use of different nomenclature systems in different laboratories has historically interfered with information exchange between research groups, a situation exacerbated by the vast number of possible profiles found in different cultivars due to the multi-allelic nature of the principal loci encoding gluten proteins (*Glu-1*, *Glu-2, Glu-3*, *Gli-1* and *Gli-2*). For the *Glu-3* alleles, we have collaborated to unify criteria across laboratories and to

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compare four different methods of allelic identification (SDS-PAGE, 2-DE, MALDI-TOF-MS and PCR), and have shown that the four methods can be regarded as complementary techniques for allelic identification. We seek to continue addressing remaining analytical challenges, place the findings in the context of the Catalogue of Gene Symbols for Wheat, and, with unified criteria, initiate work to define better the relationship between specific gluten proteins and processing quality attributes. Therefore, we propose a new system to share materials through public gene banks in collaboration with the Catalogue, and the formation of a wider international group aimed at facilitating the resolution of the remaining problems in the field. We also propose to extend our collaboration by forming a wheat quality expert working group under the Wheat Initiative.

Current Status of *Glu-3* Allele Nomenclature

It has been shown that allelic variation for the high-molecular-weight glutenin subunits (HMW-GSs) and low-molecular-weight glutenin subunits (LMW-GSs) affects the properties of dough made with different wheat cultivars. LMW-GS composition in common wheat is one of the critical determinants of gluten properties (Branlard et al. 2001; Eagles et al. 2006; Gupta et al. 1994; Liu et al. 2005; Maruyama-Funatsuki et al. 2005). Gupta and Shepherd (1990) assigned the individual LMW-GSs to *Glu-A3*, *Glu-B3* and *Glu-D3* loci and selected standard cultivars that covered the allelic variation observed. However, subsequent use of *Glu-3* nomenclature has not been consistent among laboratories, due to the complexity of the LMW-GSs, different separation methods and different standard cultivars used by researchers (Branlard et al. 2003; Ikeda et al. 2006; Singh et al. 1990). It is

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necessary to unify the various *Glu-3* allelic nomenclature systems in use, to allow information to be shared regarding the effects of individual alleles on gluten properties and to be applied in breeding programs aimed at improving gluten properties. In previous studies, four laboratories plus an international institution shared cultivars and compared results. We confirmed that there were inconsistencies to identify *Glu-3* alleles between laboratories due to differences of separation and identification methods (Ikeda et al. 2008). Using 2-DE analysis, we found new *Glu-3* alleles among these materials (Ikeda et al. 2009). Combining SDS-PAGE, 2-DE, MALDI-TOF-MS and PCR analyses, we showed that a combination of methods was required to identify certain alleles, and that these methods would be especially useful when characterizing new alleles. We also recommended 30 cultivars as standards for the determination of LMW-GS alleles (Liu et al. 2010).

Sharing Materials

It is very important to share materials to unify nomenclature among research groups. However, it is not always possible to obtain cultivars representing *Glu-3* alleles listed in Catalogue of Gene Symbols for Wheat (McIntosh et al. 2013). Therefore, we need to establish a system to share materials internationally. We propose to deposit cultivars representing particular alleles in public gene banks (e.g. Germplasm bank in CIMMYT, Genebank in VIR in Russia, NBRP in Japan, and GRIN in the USA). The registered alleles will be available publicly through these gene banks. New alleles can be evaluated by curators of the Catalogue and other researchers for registration in the catalogue. This system also helps to refine the catalogue (Fig. 31.1). At present CIMMYT Genebank performs seed multiplication of a *Glu-3* common wheat master set.

Sharing Methods

It is also important to use common methods to identify *Glu-3* alleles. For SDS-PAGE, Peña proposed the use of separation gels containing Tris buffer of pH 8.5 instead of pH 8.8 for better separation of LMW-GS bands (Ikeda et al. 2008; Peña et al. 2004). Lowering bis-acrylamide concentration and using larger size gels also helps better separation (Branlard et al. 2003). Further evaluation for creating a standard SDS-PAGE method is necessary. For PCR markers, as the number of known alleles increases, we need to reconfirm the usefulness of PCR markers to identify *Glu-3* alleles. For example, a PCR primer set, which was developed to identify the *Glu-B3i* allele (Wang et al. 2009), identified the *Glu-B3ad* allele instead. It is necessary to select a standard PCR primer set to identify *Glu-3* alleles.

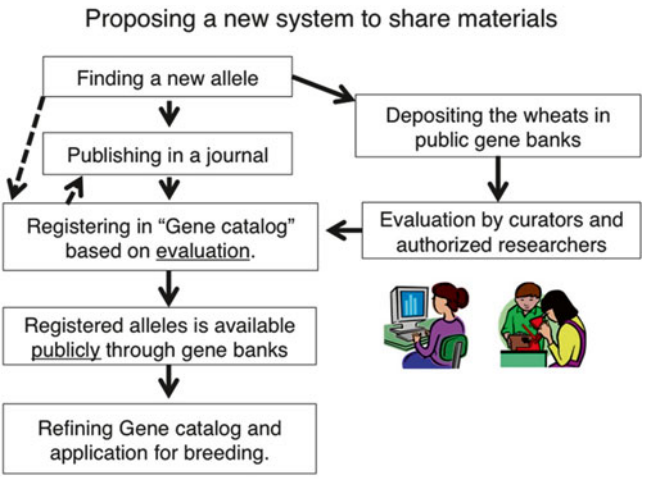

Fig. 31.1 A new system to share materials for gluten analysis

Functional Analysis of Gluten Proteins

By sharing materials and methods among international research groups, it becomes possible to define better the relationship between specific gluten proteins and processing quality stability, even under heat or drought stress wheat growing conditions. We will set an international framework to evaluate allelic effects on quality attributes under various environmental conditions.

Unification with Durum *Glu-3* Alleles

Durum *Glu-3* alleles were classified independently of those of common wheat (Martinez et al. 2004; Nieto-Taladriz et al. 1997). In the Catalogue, the durum *Glu-3* alleles were originally assigned separately and subsequently combined into one provisional list. Since tetraploid durum wheat shares common ancestral species with common wheat, we would expect some alleles to be identical to those of common wheat. We shared standard cultivars and studied *Glu-3* alleles by SDS-PAGE, 2-DE and PCR. Some alleles seemed to share the same alleles with common wheat, but some were unique in durum wheat (data not shown). This means that durum allele might widen the genetic diversity of common wheat alleles, and vice versa. Further analysis is necessary to clarify durum *Glu-3* alleles and produce a definitive list in the Catalogue for use by the wheat community. This is also important for *Glu-1* alleles.

Gliadin Analysis

Gliadin consists of α/β/γ/ω-gliadins, which contain many proteins having a range of molecular weights and pI values. Variation in the gliadins also effects dough properties (Branlard and Dardevet 1994). Gliadins are also known to contain epitopes involved in wheat gluten related disorders (Sapone et al. 2012). Gliadin analysis was mainly carried out using A-PAGE. The analysis of gliadin proteins using SDS-PAGE allows the determination of the banding patterns associated with the close linkage existing between *Gli-1* and *Glu-3*, and, therefore, this approach further contributes to the identification of specific *Glu-3* LMW-GS in both common and durum wheat. With increasing genome sequence data availability, it is important to identify gliadins by proteomic techniques to clarify correspondence between gliadin proteins, the epitopes of allergen and coding genes (Juhasz et al. 2012).

Forming an International Gluten Research Group

To carry out these tasks, we propose to form an international gluten research group. Using the same materials under different environmental conditions makes it possible to evaluate the effects of *Glu-3* alleles on dough properties in such conditions. From this collaboration, we can share advanced knowledge of gluten function for further study, accelerating the development of new cultivars maintaining good quality under climate change and responding to quality demands from industries and consumers (Fig. 31.2). It is also possible to use gluten protein alleles for cultivar identification to protect breeder's rights.

Further Perspective

The field of gluten research overlaps other wheat quality related fields, e.g. allergy, nutrition and carbohydrates. Therefore, it is logical to attempt extending our collaboration to other researchers related to wheat quality. Currently we work to form a wheat quality expert working group under the Wheat Initiative (http://www.wheatinitiative.org/about/expert-working-groups). We would like to invite other colleagues related to wheat quality to join our collaboration.

Fig. 31.2 Objectives and targets for forming an international gluten research group

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Chapter 32
Enlargement of the Genetic Diversity for Grain Quality in Bread Wheat Through Alien Introgression

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Abstract Diverse technological purposes of bread wheat grain requires a broadening the genetic base of selection for quality traits. Introgressions potentially may affect technological properties of grain and flour and add to a genetic variability of the trait. The aim of this work was to investigate the influence of introgressions from exotic tetraploid wheat *Triticum timopheevii* and wild cereal species, *Aegilops speltoides* and *Aegilops markgrafii* into bread wheat on grain quality. The introgression from *T. timopheevii* including the microsatellite marker *Xgwm636* into 2AS chromosome of cv. Saratovskaya 29 resulted in a significant increase of gluten content in grain. The effect was confirmed by re-introducing the recombinant chromosome again into the same genetic background. The analogous effect was observed in the line of winter cultivar Alcedo with the similar alien fragment in 2AS chromosome inherited from *Ae. markgrafii*. Introgression of 5S chromosome of *Aegilops speltoides* carrying the gene *Ha-Sp* for grain softness into hard-grain bread wheat cultivars resulted in obtaining the genotypes with soft grain texture. Combining two dominant genes for grain softness *Ha* and *Ha-Sp* in one genotype allowed us to obtain the plants with grain having the new milling properties – very low vitreousness (about 30 %) and small particle size (about 10 μm).

Keywords *Aegilops markgrafii* • *Aegilops speltoides* • Bread wheat • Gluten content • Introgression • Milling properties

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Diverse technological purposes of bread wheat grain require a broadening the genetic base of selection for quality traits. The wild cereal species are widely used for improvement of bread wheat in respect to resistance to fungus diseases through a wide hybridization. The resulted genotypes often carry the alien genetic material with known chromosomal location and rearrangements indicated with molecular markers. Such introgressions potentially may affect technological properties of grain and flour and add to a genetic variability of the trait. Alien cereal species often have a high protein and gluten in grain but, unfortunately, the quality of their gluten is low. Often it has dark unattractive color and sticky in a consistence. For these negative features introgression lines are not considered as possible sources of genetic variability for quality traits. At the same time, the limited introgressions may positively effect on technological properties of grain and flour. It was demonstrated with discovery and investigation of *Gpc-B1* gene for high protein content in grain introgressed from *Triticum dicoccoides* into durum and bread wheat (Carter et al. 2012). The aim of this work was to investigate the influence of introgressions from exotic tetraploid wheat *Triticum timopheevii* and wild cereal species, *Aegilops speltoides* and *Aegilops markgrafii* into bread wheat on grain quality.

The collection of lines with introgressions from *T. timopheevii* were obtained by crossing and one backcrossing with several spring bread wheat cultivars followed by multiply selection on resistance to fungi diseases (Budashkina 1988). Two lines, 821 and 842, were obtained on the genetic background of cv. Saratovskaya 29 (S29). As was determined using microsatellite markers they carry very similar fragments of introgressions (Leonova et al. 2001). The line 821 carries an entire short arm introgression in 2A chromosome while the line 842 carries a shorter fragment which does not include the distal markers *Xgwm636* and *Xgwm296*. Gluten content in grain was studied in the lines and their parents using a standard method of washing in tap water (Anonymous 1988). Compare of the lines with the parents showed that donor had the highest gluten content, about 57 %, while the recipient cv. S29 had a significantly lower meaning, 34.5 % (Fig. 32.1a). From the two lines, the line 821 had high gluten content (45 %) while the line 842 had the gluten content comparable with the recipient S29. It was supposed that the gene responsible for high gluten content in grain introgressed from *T. timopheevii* is situated in a distal part of 2AS chromosome in the region of microsatellite marker *Xgwm636*.

To verify this, the recombinant 2A chromosome was introduced from the line 821 again in the genetic background of S29. The line was crossed and backcrossed to the monosomic line for 2A chromosome of the recipient and the disomic population was developed. Again gluten content in grain was studied in the new line. It was found (Fig. 32.1b) that the gluten content was significantly higher in the line than in the recipient and was comparable to 821 line.

Introgression with the similar position on 2AS chromosome was detected in the line M483/98 obtained by crossing of winter cultivar Alcedo and *Aegilops markgrafii* (Weidner 2004). The introgression includes the region of *Xgwm636* and limited by *Xgwm296* marker (Iqba et al. 2007). Again the wild cereal had the highest gluten content in grain, more than 40 % (Fig. 32.1c). The mean of the trait in the line

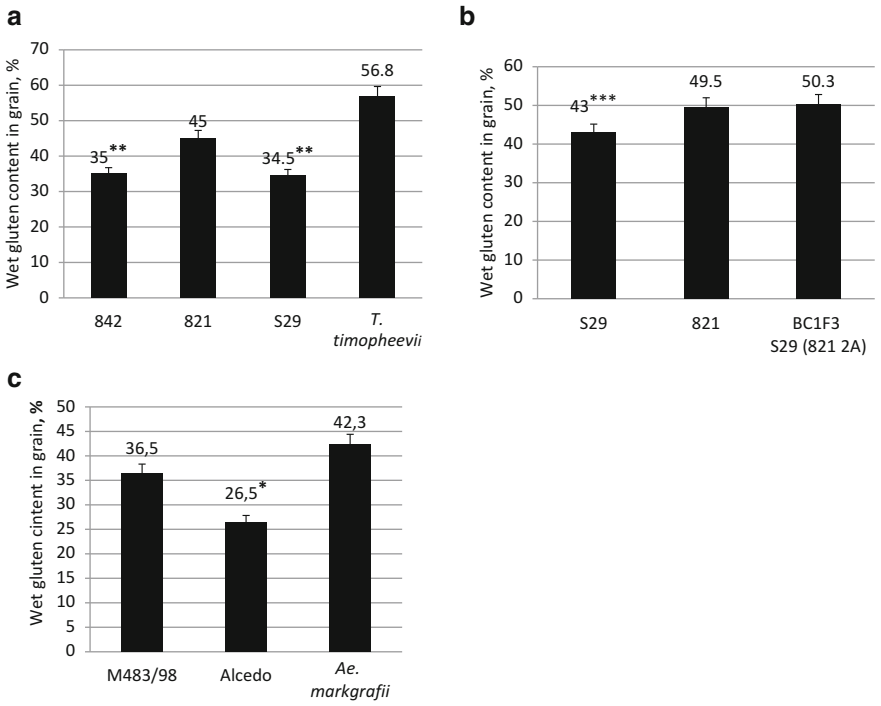

Fig. 32.1 Wet gluten content (GC) in grain of bread wheat lines with introgressions from wild cereals: (**a**) introgression lines 842 (low GC) and 821 (high GC), cv. S29 (recipient), *T. timopheevii* (donor); (**b**) cv. S29 (recipient), introgression line 821 (high GC), substitution line S29 (821 2A); (**c**) introgression line M483/98, cv. Alcedo (recipient), *Ae. markgrafii* (donor)

483/98 did not reach the donor meaning but it was on 10 % higher than in the parental cultivar Alcedo. The difference was statistically significant. Now this chromosome is being introduced from introgression line into S29 background in order to compare its effect with introgression from *T. timopheevii*.

Earlier, the quantitative trait locus *QPro.inra-2A* for a high protein content in grain was positioned in the region of *Xgwm636* using the mapping population of bread wheat Renan × Recital (Groos et al. 2003). The donor of high meaning of the trait was cv. Renan. A close correlation of protein content in grain with gluten content and the similarity of the marker positions associated with manifestation of the traits in different genetic material suggest the presence of the responsible locus on the short arm of 2A chromosome.

Milling parameters are determined by endosperm texture of wheat grain. Vitreous grains give a hard-grain flour with big particles and floury grain gives soft flour with small particles. Different kinds of flour are used for different technological purposes

(Peña 2002). Today all the variability is provided by mutations in one locus *Ha* on 5DS chromosome (Morris 2002). Milling parameters show a quantitative variability but usually cultivars retain the certain characteristics of the trait.

The effect of introgression of 5S chromosome from *Ae. speltoides* on expression of milling parameters was studied in the work. Vitreousness was determined on cut halves of grains visually; flour particle size was determines instrumentally using PSH-4 device (Anonymous 1988). The line 84/98^{w} from 'Arsenal' collection (Lapochkina et al. 2003) obtained from a wide cross of *Ae. speltoides* with spring wheat cultivar 'Rodina' was used. Winter growth habit and presence of awns indicated the introgression into 5A chromosome. Additionally, the line was characterized with a significantly lower vitreousness of endosperm and smaller flour particle size comparing to the initial hard-grained cultivar (Table 32.1).

In order to prove that just this introgression is responsible for changes in milling parameters this chromosome was introduced into two hard-grain cultivars S29 and Diamant 2 using monosomic lines for 5A chromosomes. It was found that this chromosome significantly decreases vitreousness and particle size to the meanings compared with the meaning of the introgression line (Table 32.2). The introgressed gene was designated with a temporarily gene symbol *Ha-Sp* (Pshenichnikova et al. 2010).

Crossing of introgression line with cultivar Chinese Spring – carrier of the dominant allele of *Ha* gene in 5DS chromosome resulted in emergence of wide variability in milling parameters relatively parental forms (Fig. 32.2). Among them the plants were found carrying two dominant genes for grain softness with new milling properties – very low vitreousness (0–30 %) and small particle size (11–12 μm). These plants have become the progenitors of F_6-F_7 lines which retain these properties. Their flour may be used for special technological purposes.

Table 32.1 The effect of introgression from *Ae. Speltoides* on milling parameters of wheat flour

Milling parameters	cv. Rodina	*Ae. speltoides*	Introgression line 84/98^{w}
Vitreousness, %	87**	26	50
Particle size, μm	24**	–	12.1

*$P<0.05$ (vs. introgression line), **$P<0.01$ (vs. introgression line)

Table 32.2 Effect of introducing of 5S chromosome of *Ae. speltoides* into genetic backgrounds of hard-grain cultivars S29 and Diamant 2 on milling parameters of grain

Milling parameters	Genotypes				
	Introgression line 84/98^{w}	S29	F_3 mono S29 (84/98^{w} 5S)	Diamant 2	F_3 mono Diamant 2 (84/98^{w} 5S)
Vitreousness, %	50	99	51**	84	52**
Particle size, μm	12.1	21.5	11.4**	16.5	10.8*

*$P<0.05$ (vs. corresponding recipient), **$P<0.01$ (vs. corresponding recipient)

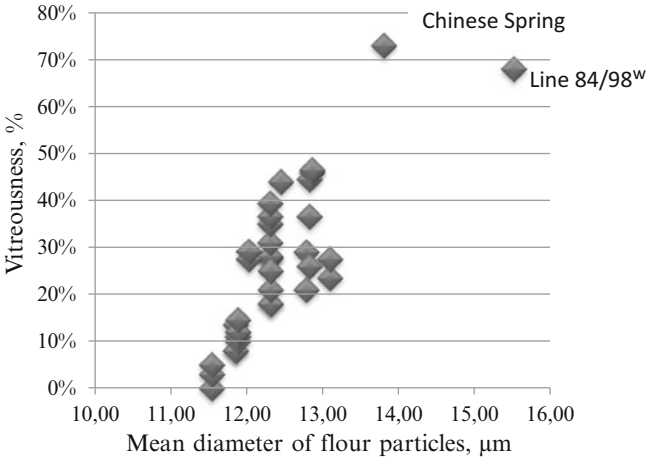

Fig. 32.2 Milling parameters of F_{6-7} lines carrying two dominant genes *Ha* and *Ha-Sp* for soft endosperm texture

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Chapter 33
Complex G × E Interactions and QTL Clusters Govern End-Use Quality Traits in Hexaploid Wheat

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Abstract Evaluation of wheat end-use quality in terms of loaf volume (LV) requires enormous time and labor inputs. Hence, many studies have attempted to use grain, flour and dough properties to predict LV. Many quantitative trait loci (QTL) underlying these traits have also been identified to facilitate breeding. However, correlations between such predictive tests and LV as well as their QTLs could be influenced by the environment. In this chapter, we review recent literature on the correlations and G × E interaction (GEI) of the bread making quality traits grain protein content (GPC), sodium dodecyl sulphate sedimentation volume (SV), dough rheological traits (DRT) and LV. We briefly discuss our results from the evaluation of a hexaploid wheat recombinant inbred line population for GPC, SV, LV and nine DRT by mixograph analysis in six year-location environments in India, which revealed that correlations between DRT and LV were not stable across environments. In addition, GEI measured in terms of principal components using Additive main effects and multiplicative interaction model showed up to 47 % contribution to the total variation of the traits, which was reflected in the location-specificity of QTLs expressed in single as well as multiple environments. Even though 16 QTL clusters for four to seven traits were identified, only one of them involved LV. The strong influence of the environment on complex interrelationships between DRT and the other end-use quality traits suggested that during breeding for wheat end-use quality, marker-based selection of these traits would be more efficient if specific agro-climatic zones are targeted separately.

Keywords AMMI analysis • Bread • Dough rheology • Interaction • Loaf volume • QTL • Quality

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Selection for End-Use Quality Traits in Hexaploid Wheat: The Challenges

Improving wheat grain quality to cater to its diverse end uses is necessary to make its cultivation profitable. The quality of the industrial products made from hexaploid wheat such as leavened loaf bread depends critically on the properties of the grain, flour, dough and its responses during baking. Selection in the early generations for the most perceptible indicator of bread making quality (BMQ) namely, loaf volume (LV) is difficult due to the requirement of substantial quantities of grain and lack of high-throughput in evaluating it. BMQ is determined by many complex traits like grain protein content (GPC), gluten strength, dough viscosity, extensibility and elasticity. Hence, to predict LV, dough rheological traits (DRT); gluten strength often determined by sodium dodecyl sulphate sedimentation volume (SV) and GPC have been evaluated. However, determining simple and reliable predictive tests for LV and identification of consistent quantitative trait loci (QTL) with substantial contribution for such traits to aid marker-assisted selection (MAS) have proved difficult. This is due to the complex inter-relationships among the traits, large number of QTLs governing them, their co-location and possible pleiotropic effects as well as genotype × environment interactions (GEI) (Mann et al. 2009; Li et al. 2013). In this Chapter, we discuss the recent literature on these aspects and present a brief summary of our work on QTL and GEI analyses of the BMQ contributing traits.

Predictive tests for LV need to have high correlations with it that are stable in diverse environmental conditions and are simple, quick, require small sample size and should be able to clearly distinguish between genotypes. The breeding material thus selected can eventually be evaluated for LV in advanced stages. Various instruments such as farinograph, alveograph and mixograph have been employed to assess dough rheological changes during processing for the quality control of the end products. Among these, the computerized mixograph instruments can measure DRT when flour-water blend is subjected to a fixed mixing time. Though mixograph characters like dough development time, peak height and width are often employed to evaluate BMQ, single or few mixograph parameters as predictors of loaf volume have not been conclusively established (Mann et al. 2009; Caffe-Treml et al. 2010). More recently, Li et al. (2013) suggested small-scale tests like swelling index of gluten and lactic acid retention capacity to predict LV; however, further studies on the stability of their correlations with LV are needed.

For both LV and its potentially predictive parameters, many QTLs have been identified; however, their consistent expression across environments is necessary for their effective use (Li et al. 2012; El-Feki et al. 2013 and references therein). We performed detailed GEI, correlation and QTL analyses of end-use quality traits in a recombinant inbred line (RIL) population of 105 individuals derived from a bread wheat cross HI977 × HD2329. HI977 (good BMQ) possesses Glu-A1 (2*), Glu-B1 (17 + 18) and Glu-D1 (5 + 10) and HD2329 has Glu-A1 (2*), Glu-B1 (7 + 9) and Glu-D1 (2 + 12) and is poor in BMQ. Phenotypic evaluations were performed in three agro-climatically diverse locations in India namely, Karnal, Kota and Pune for two consecutive years (2003–04; 2004–05) and 12 BMQ related traits, *viz*. GPC,

Table 33.1 Dough rheological traits measured by mixograph analysis in HI977 × HD2329 RIL population

Trait	Abbreviation
Envelope peak integral	EPI
Midline right integral	MRI
Midline curve tail integral	MTI
Midline right width	MRW
Midline curve tail width	MTW
Midline peak time	MPT
Midline right value	MRV
Midline curve tail value	MTV
Weakening slope	WS

SV, LV and nine mixograph traits that could quantitatively reflect the energy used during the mixing process, changes in dough consistency and elasticity, optimum dough development time and the rate of the gluten network breakdown during over-mixing were recorded (Table 33.1) (Elangovan et al. 2008, 2011; Prashant et al. 2015).

Contributions of G, E and GEI Effects for DRT and BMQ Traits

The contributions of genotype (G) and environment (E) main-effects can be preliminarily judged by ANOVA (Tsilo et al. 2011; Li et al. 2013); however, its third component could include the GEI effects and the error. The Additive main effects and multiplicative interaction (AMMI) analysis (Nachit et al. 1992) combines additive and multivariate approaches and improves the analysis of non-additive GEI effects. In our studies on the HI977 × HD2329 population, LV indicated the highest contribution from G (38 %). However, for all the traits, either E (47–74 %) or GEI (42–57 %) were the main contributors. AMMI analysis showed 6–47 % contribution from GEI to total variation in terms of 1 to 4 principal component axes (Prashant et al. 2015). Similarly, Hristov et al. (2010) showed significant GEI contribution to LV, SV and GPC by AMMI analysis. It is possible that GEI could influence correlations among LV and its predictive tests thereby making it necessary to examine them individually in year-location combinations.

Trait Correlations and the Influence of the Environment

Using the average of the data from different years and locations (environments) to calculate trait correlations masks the effect of GEI and suppresses the patterns of trait inter-relationships across environments (Tsilo et al. 2011; Li et al. 2012). Mann et al. (2009) evaluated genetic correlations among DRT and BMQ traits in three

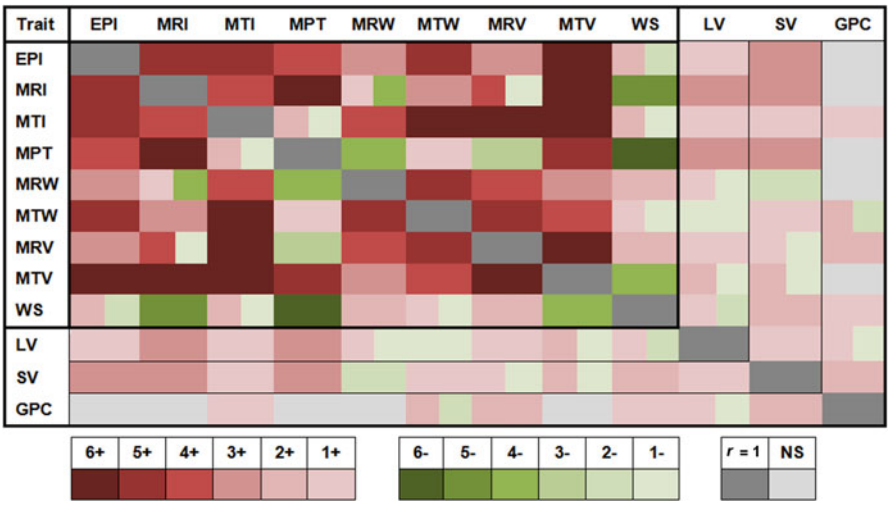

Fig. 33.1 Pattern of correlations among dough rheological traits, loaf volume, SDS-sedimentation volume and grain protein content in HI977 × HD2329 population. *ns*- non-significant

individual environments that indicated consistent negative correlation between Maximum height of the Extensograph curve and LV. However, the stability of the correlations across multiple years in different locations could not be conclusively assessed since data were recorded at two locations, but in single year in one of them. We could efficiently document the repeatability of the correlations among LV, GPC, SV and DRT in HI977 × HD2329 RIL population as we assessed them in six individual year-location environments (Fig. 33.1). We found that correlations between 15 mixograph trait pairs were highly consistent since they were significant in five or in all the six environments. Among the rest, significant correlations were observed in one to four environments. MRI and MPT with LV and EPI, MRI and MPT with SV were significant in three environments and were the only DRT-BMQ trait pairs that showed fairly consistent correlations. However, majority of the correlations that were significant in only one or two environments involved LV, SV or GPC. Eleven trait pairs showed correlations that were inconsistent since they were positive or negative in different year-locations (Prashant et al. 2015). The environmental influence on correlations between predictive tests and LV, as well as significant GEI contributions suggested that even if a QTL with high contribution to predictive tests is detected, its stability across environments is important if it has to be considered for use in MAS for LV.

Clustering and Location-Specificity of BMQ and DRT QTLs

Composite Interval Mapping (CIM) determined QTLs on all the chromosomes of bread wheat for LV, GPC, SV and DRT (Li et al. 2012; El-Feki et al. 2013; references therein). In addition, QTL clusters mainly for GPC, SV and DRT (Li et al.

2012) and for DRT and LV (Mann et al. 2009; Tsilo et al. 2011; Simons et al. 2012) suggested that as the traits are correlated, they could be controlled by common QTLs. But it needs to be noted that the HMW glutenin loci *Glu-B1* or *Glu-D1* often showed overlap with the largest of such clusters for DRT and LV and suggested that extensive studies to identify novel loci on other chromosomes are necessary for further improvement of wheat quality. In our analyses on HI977 ×HD2329 population, 158 QTLs for LV, SV, GPC and DRT were identified with 9–46 % contribution to phenotypic variance. There were 16 QTL clusters on nine chromosomes with co-locating QTLs for four to seven traits. Only a single QTL cluster on chromosome 5B showed co-location for LV and DRT.

In many of the previous studies, large number of DRT and BMQ QTLs were detected in single environments (Mann et al. 2009; Tsilo et al. 2011; Li et al. 2012; Simons et al. 2012) and stable QTLs were very few, which often co-located with HMW glutenin loci (Mann et al. 2009; Tsilo et al. 2011). Examining their specificity to individual locations and pattern of detection across years within a location can help reveal their responses to GEI. In our study, 69 % of the QTLs were identified in single environments and only 15 % were detected in three or more environments. The significant influence of GEI was reflected in the pattern in which QTLs were detected across environments. We identified 109 QTLs for the traits analyzed, which were specifically expressed in single years in Karnal, Kota and Pune (Fig. 33.2).

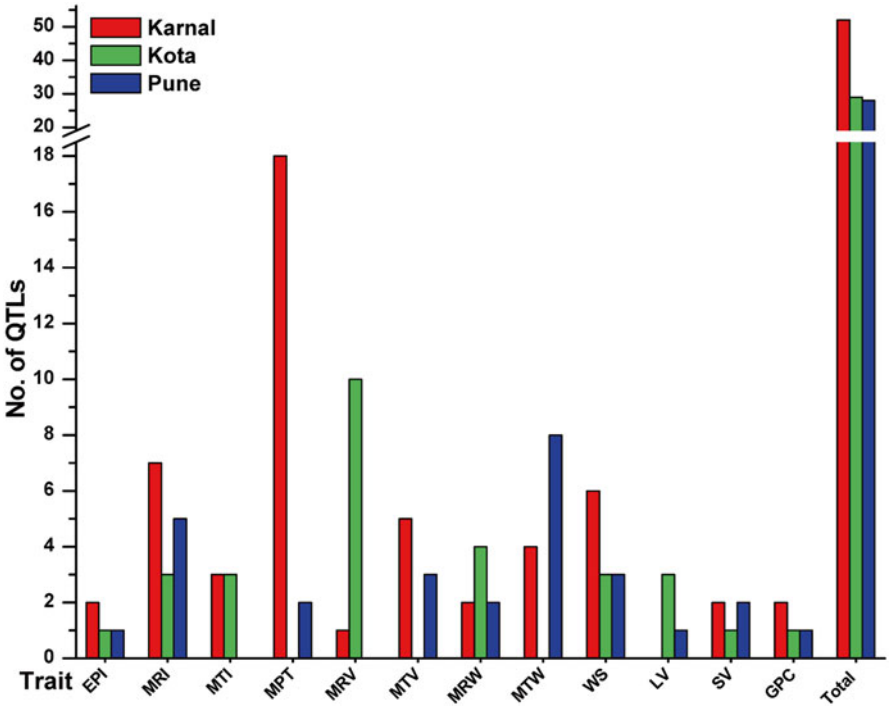

Fig. 33.2 Location-specificity of QTLs for dough rheological traits, loaf volume, SDS-sedimentation volume and grain protein content detected in single years in HI977 × HD2329 population

Interestingly, majority of them (47 %) were specific to Karnal and the rest were equally distributed in other two locations. In addition, the individual traits showed differential location-specificity for such QTLs. Furthermore, when the QTLs expressed in two or more environments were examined, 62 % showed expression in consecutive years in Karnal, followed by 34 % in Kota and only 4 % at Pune (Prashant et al. 2015).

Conclusions

The GEI contributions, trait correlations and QTL clusters reinforce the view that wheat quality is achieved by complex gene repertoire and action underlying the levels and composition of grain constituents. The QTL clusters provide leads for characterization of genomic regions controlling end-use quality traits in wheat. In the coming years, genetic maps with better resolution and the wheat genome sequence would help resolve pleiotropic gene effects and multiple genes with close linkage. The location-specificity of QTLs detected in both single-environment and across years in HI977 × HD2329 population suggested that for traits exhibiting high GEI, it might be beneficial to target specific agro-climatic zones while breeding varieties for quality traits. In addition, QTL validation in those zones in multiple years using many wheat genotypes is necessary for their use in MAS.

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Chapter 34
A Consistent QTL for Flour Yield on Chromosome 3B in the Soft Winter Wheat Variety, Kitahonami

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Abstract The soft winter wheat variety, Kitahonami, shows a superior flour yield in comparison to other Japanese soft varieties. In order to map quantitative trait loci (QTL) associated with the flour-yield trait, association mapping was performed using panel lines in Kitahonami's pedigree, along with leading varieties and advanced breeding lines. Using a mixed linear model corrected for kernel types and familial relatedness, 62 marker-trait associations were identified and classified into 21 QTLs. Five out of eight QTLs tested were validated by linkage analyses using three sets of doubled haploid populations from crosses in which Kitahonami was used as a parent. Among them, QTLs on 3B and 7A chromosome showed highly significant effects and consistency across the three populations. A joint linkage map of 3B showed that the QTL on this chromosome was located at the same interval across the populations. By applying a meta-analysis approach, we have succeeded in identifying QTLs with consistent contributions to high flour yield across various genetic backgrounds.

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Introduction

Flour yield is of great interest to milling companies. Thus, breeding of wheat varieties with higher flour yield, in addition to enhancement of milling techniques, is important to flour milling industries. In 2006, a soft winter wheat variety, Kitahonami, was released in the Hokkaido prefecture of Japan (Yanagisawa et al. 2007). It has a superior flour yield compared to other Japanese soft varieties (Fig. 34.1) and has become a leading variety in the Hokkaido area. Kitahonami is also being used as a source of the high flour-yield trait in all Japanese wheat breeding programs. Mapping of quantitative trait loci (QTL) associated with the flour-yield trait and identification of linked markers would accelerate the development of varieties with high flour yield.

QTL studies using bi-parental populations have been conducted within hard wheat populations or within populations derived from hybridizing hard and soft wheat. These studies have revealed that reliable QTLs for flour yield are located on 16 out of 21 chromosomes: 1B, 1D, 2A, 2B, 3A, 3B, 4A, 4B, 4D, 5A, 5B, 5D, 6B, 6D, 7A and 7D (Parker et al. 1999; Campbell et al. 2001; Smith et al. 2001; Lehmensiek et al. 2006; Fox et al. 2013). Interclass hybridization between soft and hard wheat indicated that the hardness locus *Pinb* on 5D had a strong influence on flour yield (Campbell et al. 2001). Fox et al. (2013) detected a QTL explaining the highest phenotypic variance close to the plant height locus, *Rht-D1* located on 4D. For soft wheat populations, only a few studies have been reported to date. Using an association mapping approach, Breseghello and Sorrells (2006) detected weak QTLs associated with flour yield and break flour yield on 2D and 5B. One bi-parental population derived from two soft wheat cultivars revealed QTLs for flour yield, flour protein, softness equivalent, and solvent retention capacities (Smith et al. 2011), the majority of which were located on 1B and 2B. Carter et al. (2012)

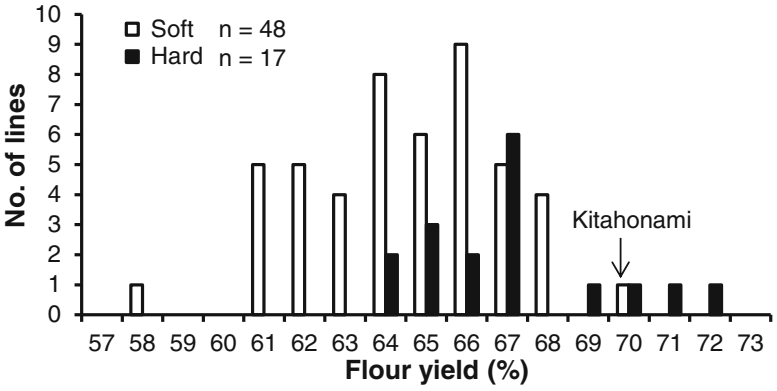


Fig. 34.1 Distribution of flour yield for 65 winter wheat accessions used in this study

found that a large number of QTLs including QTLs for flour yield were located on 3B and 4D and coincided with traits for milling quality and starch functionality. Although these QTLs were detected with high confidence, few were detected in more than one study, indicating that flour yield is a complex trait strongly influenced by genetic background.

The objective of this study was to dissect genetic factors contributing to the high flour-yield trait of Kitahonami and to find effective QTLs and associated markers that can be used for MAS in our breeding programs.

Materials and Methods

Plant Materials Sixty-five winter wheat accessions consisting of Kitahonami and its related lines were used. Lines were field-grown with two replications at three locations, Kitami (Hokkaido island), Tohoku (Northern Honsyu island) and Nagano (Central of Honsyu island) during three successive cropping seasons from 2008/2009 to 2010/2011. Grain samples were harvested from each replicated plot.

Milling Samples were milled on a Quadrumat Junior mill (Brabender Co.). Flour yield was expressed as the percentage of total flour weight to initial sample weight.

Genotyping All accessions were genotyped by SNP (Cavanagh et al. 2013), SSR (GrainGenes 2.0), DArT (Diversity Arrays Technology, Pty Ltd.) and established diagnostic markers for *Pina-D1*, *Pinb-D1 Wx-A1*, *Wx-B1*, *Ppo-A1*, *Ppo-D1*, *Psy-A1* and *Psy-B1* (review in Liu et al. 2012). After removing data with minor allele frequencies of less than 0.1, genotypes from 3,815 markers were used for association analysis.

Association Analysis Association between markers and trait was tested with TASSEL 3.0 (Bradbury et al. 2007) using the mixed linear model. Since a different distribution pattern was observed between soft and hard kernel type (Fig. 34.1), the effect of kernel type was considered in the model. Kinship matrix calculated by TASSEL was used for considering familial relatedness of accessions. To take into account multiple comparisons, significance was tested using a 0.5 false discovery rate implemented in the q value software (Storey and Tibshirani 2003).

QTL Validation QTLs were validated using three doubled haploid (DH) populations from crosses in which Kitahonami was used as a parent. DH populations were field-grown without replication during the 2010/2011 season. Flour yield values were obtained with the same method described above. Differences between allele mean values were tested for each combination of QTL and population. For the 3B chromosome, linkage map construction and QTL analysis were conducted by MapDist 1.74 (Lorieux 2012) and QTL IciMapping 3.25 (Li et al. 2007), respectively.

Results and Discussion

Analysis of variance indicated there was a significant genetic variation in flour yield among accessions compared to residual errors (location, year, interaction) (data not shown). Correlations across nine environments ranged from 0.394 to 0.891 (average 0.682), indicating that relative differences among accessions were consistent over the environments. Thus, accession means of all environments were used for association analysis.

By association analysis using a mixed linear model corrected for kernel type and familial relatedness, 62 marker-trait associations were identified. Based on the locations of the markers, they were classified into 21 QTLs (Table 34.1). Due to the lack of common markers, it was difficult to compare positions of QTLs detected in this study to those of previous reports. However, based on the microsatellite consensus map (Somers et al. 2004), it is possible that the QTLs on 2B.1, 2B.2, 3B.2, 6A.2 and 7A observed here are the same as those reported by

Table 34.1 Flour yield QTLs detected by genome-wide association analysis

QTL	No of markers	Chromosome[a]	Position (cM)[a]	Effect[b]	R^2 (%)	
1B[a]	1	1B	18	−2.6	17.0	
2B.1	2	2B	56	1.8	9.4	[c]
2B.2	5	2B	210–217	1.5–2.5	9.4–20.5	[c]
3B.1[a]	1	3B	53	1.6	9.3	[c]
3B.2	4	3B	62–72	1.6–2.2	9.2–14.2	[c]
3B.3	1	3B	91	2.0	9.3	
3D[a]	1	3D	51	1.7	11.0	
4B[a]	1	4B	20	1.9	10.3	
5A	1	5A	28	2.2	11.3	
5B	1	5B	213	−2.1	9.7	
5D.1	4	5D1cult	43–48	1.7–2.2	9.1–15.4	[c]
5D.2	4	5D3cult	8–11	2.1–2.3	9.2–10	
6A.1	1	6A	76	2.7	14.5	
6A.2	21	6A	115–117	1.6–2.1	8.9–12.6	[c]
6B	1	6B	60	1.8	9.8	
7A	2	7A	59	2.3	9.2–12.0	[c]
7B.1	1	7B	73	1.9	11.0	[c]
7B.2	1	7B	116	1.9	9.6	
7B.3	1	7B	165	−2.1	10.3	
7B.4 (Psy-B1)	1	7B	Unknown	2.4	12.9	
7D[a]	1	7D	2	2.4	11.4	

[a]Genetic positions are based on the consensus map of Cavanagh et al. (2013), except for positions of QTLs on 1B, 3B.1, 3D, 4B, and 7D, which are based the on DArT consensus map of Huang et al. (2012)

[b]Values of effect indicate increasing effect of Kitahonami alleles

[c]QTLs were subjected to validation in segregating populations

Smith et al. (2001), Lehmensiek et al. (2006), Carter et al. (2012), Fox et al. (2013) and Lehmensiek et al. (2006), respectively.

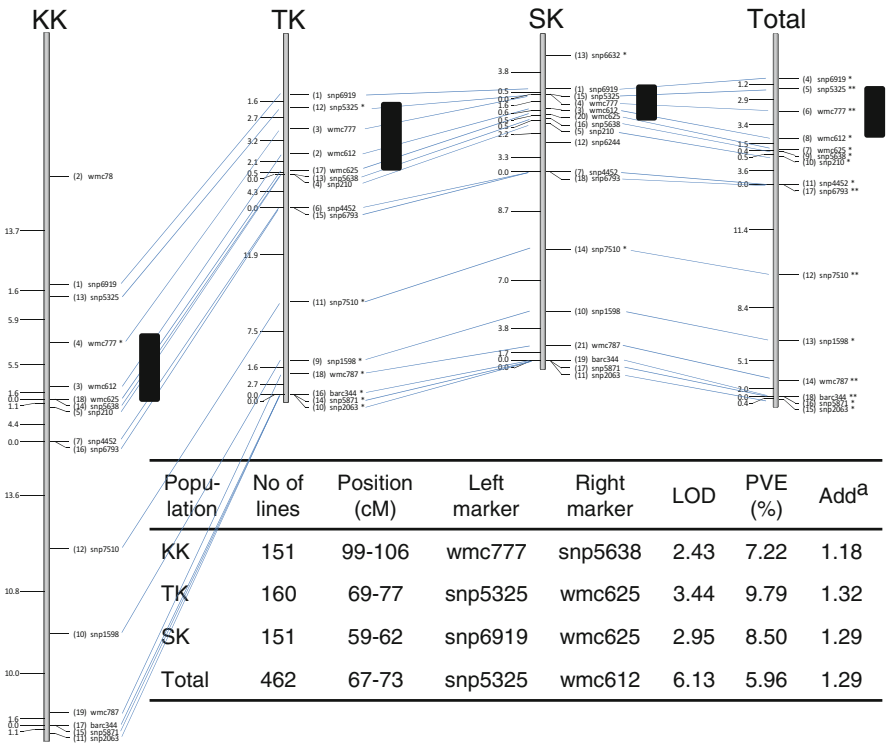
Segregation analysis revealed five out of eight QTLs tested had significant effects on flour yield in at least one of three populations (Table 34.2). QTLs on 3B and 7A showed highly significant effects and consistency across the populations. A joint linkage map from the three populations showed that the 3B QTL interval was around 6 cM, located between the markers snp5325 and wmc612 (Fig. 34.2). This QTL (LOD score 6.1) explained 6.0 % of the total variation. In addition to the DH populations, the 3B QTL was also detected among materials derived from crosses with Kitahonami in three separate breeding programs (data not shown).

Performing milling tests is time-consuming and requires a fairly large amount of grain. Thus, it can be a rate-limiting step in wheat breeding programs. By applying a meta-analysis approach, we have succeeded in identifying a QTL on 3B which was consistently associated with high flour yield across different genetic backgrounds. Introducing this QTL into Japanese soft varieties by marker-assisted selection is a promising method of improving flour yield. The results obtained in this study also provide us with a starting point for the isolation of candidate gene(s), which will lead to a better understanding of the mechanisms governing flour yield.

Table 34.2 QTL effects on flour yield in three doubled haploid populations

		KK			TK			SK		
QTL	Allele	No of lines	Mean (%)		No of lines	Mean (%)		No of lines	Mean (%)	
2B.1	A	79	63.0	ns	NA			73	66.7	ns
2B.1	B	72	63.3					78	66.5	
2B.2	A	86	63.7	**	80	64.6	ns	76	66.8	ns
2B.2	B	65	62.4		80	63.7		75	66.3	
3B.1	A	70	63.8	*	68	65.2	***	69	67.6	***
3B.1	B	81	62.6		92	63.4		82	65.7	
3B.2	A	76	63.6	ns	67	64.8	*	70	67.4	**
3B.2	B	75	62.7		93	63.7		81	65.9	
5D.1	A	81	63.2	ns	87	64.2	ns	NA		
5D.1	B	70	63.1		73	64.1				
6A.2	A	74	63.6	ns	75	64.2	ns	74	66.8	ns
6A.2	B	77	62.7		85	64.1		77	66.4	
7A	A	71	64.1	***	76	64.9	**	81	67.2	**
7A	B	80	62.3		84	63.5		70	65.9	
7B.1	A	82	63.4	ns	75	64.5	ns	81	67.3	***
7B.1	B	69	62.8		85	63.8		70	65.8	

"A" indicates Kitahonami alleles, while "B" is the other parental allele. T-tests between allele mean values were performed. *ns* not significant; *, **, ***: significant at $p = 0.05$, 0.01 and 0.001 level, respectively. *NA* not available, *KK* Kinuhime/Kitahonami, *TK* Tohoku224/Kitahonami, *SK* Shunyou/Kitahonami



Population	No of lines	Position (cM)	Left marker	Right marker	LOD	PVE (%)	Add[a]
KK	151	99-106	wmc777	snp5638	2.43	7.22	1.18
TK	160	69-77	snp5325	wmc625	3.44	9.79	1.32
SK	151	59-62	snp6919	wmc625	2.95	8.50	1.29
Total	462	67-73	snp5325	wmc612	6.13	5.96	1.29

Fig. 34.2 A consistent QTL for flour yield on 3B in three doubled haploid populations. *KK* Kinuhime/Kitahonami, *TK* Tohoku224/Kitahonami, *SK* Shunyou/Kitahonami. [a]Values of additive effect indicate increasing effect of Kitahonami alleles. *LOD* limit of detection, *PVE* phenotypic variation explained by the marker

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Part X
Marker-Assisted Breeding

Chapter 35
Recent Improvements in Japanese Wheat Varieties

Shunsuke Oda

Abstract In Japan, the breeding of new wheat varieties for use in bread, Chinese noodles, as well as other noodles, is an urgently required objective if domestic wheat production and food self-sufficiency ratio have to increase. Many molecular markers are now available; those used in wheat breeding programs in Japan are generally to assess the amylose content, dough strength, grain hardness, wheat yellow mosaic virus, preharvest sprouting, and *Fusarium* head blight. Hard and extra-strong wheat varieties have been released using marker-assisted selection.

Background: Domestic Wheat in Japan

Figure 35.1 shows the trends in wheat production area and yield in Japan after World War II. The disorder in the immediate aftermath of the war resulted in a sharp decrease in the production area, which recovered to a maximum level in 1949. However, from 1949 until about 1975, the production area decreased to less than 100,000 ha, in particular with a sharp decrease from 1963. After 1975, the production area began to gradually increase, peaking by around 1988, before decreasing until 1994. Recently, the production area has stabilized at approximately 200,000 ha. Rice production is the main influence on these trends in wheat production area.

Rice is the staple food in Japan. Therefore, following World War II, the Japanese Government boosted rice production. As a result, the area covered by wheat production decreased. However, in about 1970, farmers produced too much rice, and the Government consequently introduced a rice production adjustment in 1970. This adjustment contained two policies: the first was non-cropping, and the second was to changing some rice production area to cultivation of other crops. The second policy led to many farmers beginning to cultivate wheat instead of rice, which led to increase in wheat production.

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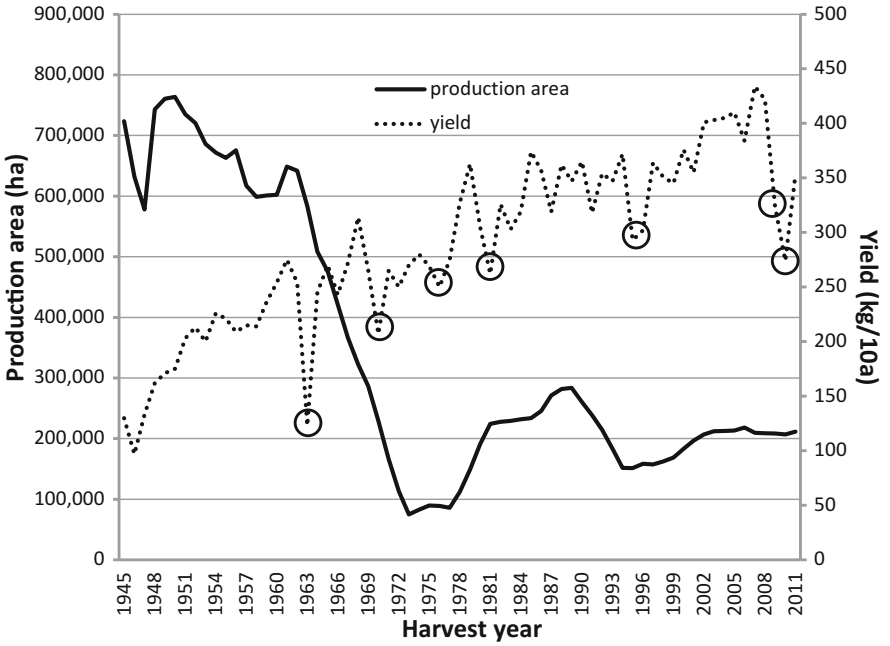

Fig. 35.1 Production area and yield of Japanese wheat

Despite the gradual increase in production after the end of World War II (Fig. 35.1), we can see occasional sharp drops in yield (circles in Fig. 35.1); the drop in 1963 was one of the triggers for a sharp decrease in production area. Yield is unstable, primarily because of degradation in crop quality because of humid and wet weather conditions during the harvest season, specifically causing preharvest sprouting and *Fusarium* head blight damage.

Various products are made from wheat flour in Japan. Chinese noodles are more yellow and elastic than Japanese noodles because the dough is kneaded with kansui, a sodium-carbonate-infused alkaline mineral water, rather than plain water. Instant noodles are also produced. These are dried or precooked and often sold with packets of flavoring, including seasoning oil. Wheat flour is also used in domestic cooking. Each product has a different self-sufficiency ratio (Fig. 35.2). Domestic wheat is mainly used for Japanese noodles, the self-sufficiency ratio of which is already 70.5 %. In contrast, the self-sufficiency ratio of bread, and Chinese and other noodles, is very low.

Wheat Breeding in Japan

Wheat breeding stations are classified into three types in Japan. The first is the National Agriculture and Food Research Organization (NARO) (Fig. 35.3), which manages five wheat breeding stations. NARO was originally a national institute, prior to the privatization of national agricultural institutes in 2001. The second comprises Prefectural Research Institutes, and the third is the Institute of Agricultural

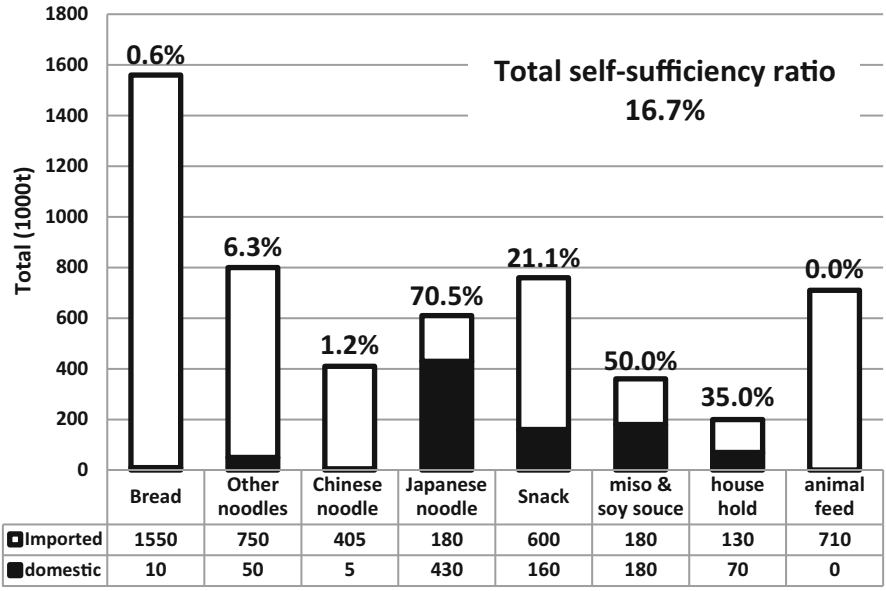

Fig. 35.2 Self-sufficiency ratio for Japanese wheat

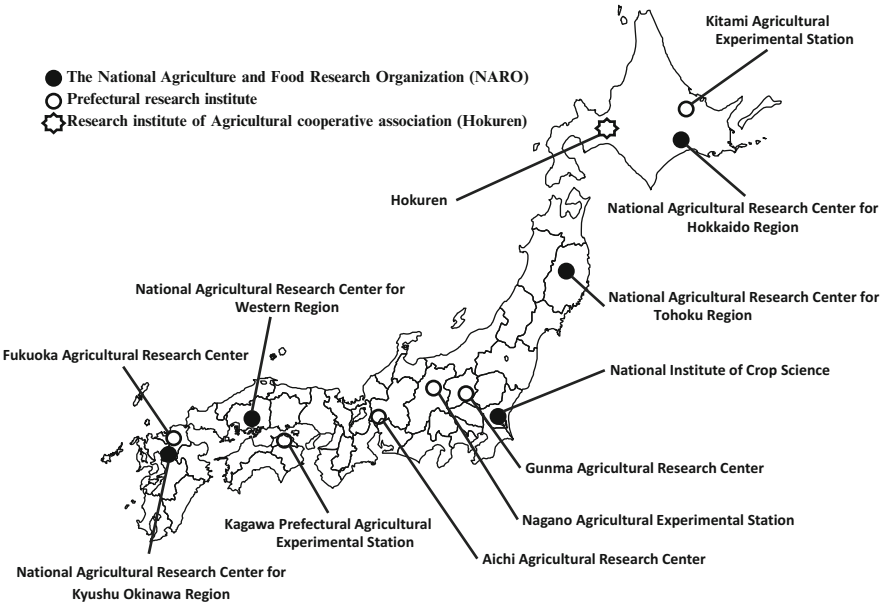

Fig. 35.3 Wheat breeding stations in Japan

Cooperative Associations. No private seed companies in Japan are operating a wheat breeding program; most of the wheat breeding stations are public institutes. Therefore, breeding objectives are always influenced by Government policy.

Every 5 years, the Ministry of Agriculture, Forestry and Fisheries sets the provisions contained in the Basic Law on Food, Agriculture and Rural Areas. These provisions determine the policy basis for food and agriculture. The most recent version, which was approved on May 30, 2010, for the first time set a target for the food self-sufficiency ratio; the target is to achieve a ratio of 50 % in 2020 (40 % in 2008). In order to achieve this, the Government drew up a new policy plan in which domestic wheat production is set to increase from 880,000 t in 2008 to 1,800,000 t in 2020. Current wheat breeding objectives have been established to enable this target food self-sufficiency ratio to be achieved.

To increase domestic wheat production from 880,000 to 1,800,000 t, there needs to be an increased use of domestically grown wheat in wheat products with a currently low self-sufficiency ratio. The target products are bread, Chinese noodles, and other noodles, which are made from hard wheat flour (Fig. 35.2); therefore, breeding of hard wheat is an urgent objective. Hard wheat was not a breeding objective prior to 1999 because domestic wheat was mainly used in the production of Japanese noodles, which are made from soft wheat flour. Hard wheat breeding is however now a higher priority than soft wheat. The required quality standard is equivalent to the Hard Red Winter (HRW) class. However, soft wheat cultivars are still in demand for making Japanese noodles (Udon). The required quality is equivalent to the Australian Standard White (ASW) class; currently, domestic wheat flour yield and color is inferior to ASW.

The wheat harvest coincides with the start of the rainy season, other than in Hokkaido (Fig. 35.4). This means there is always a risk that domestic wheat will be

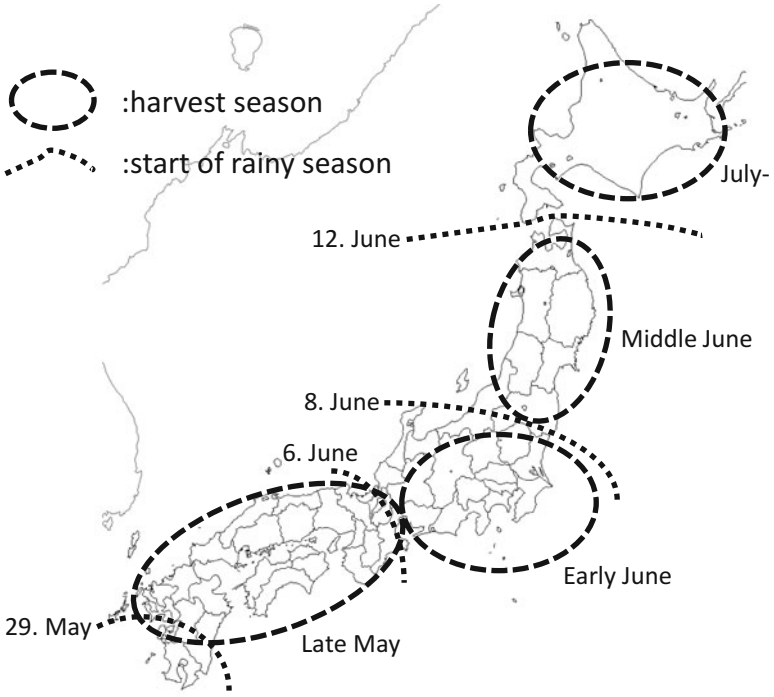

Fig. 35.4 Wheat harvest and rainy seasons in Japan

exposed to conditions of high humidity and wet weather during the harvest season in Japan. Resistance to preharvest sprouting and *Fusarium* head blight resistance are required for both hard and soft wheat.

Marker-Assisted Selection of Wheat Breeding in Japan

Varieties of wheat produced outside of Japan are much better suited to producing good quality bread, whereas domestic wheat is produced to be suitable for making Japanese noodles. In contrast, domestic varieties are superior in terms of agricultural performance (excluding yield, early maturity), *Fusarium* head blight resistance, and preharvest spouting resistance, as they are well adapted to Japanese climate conditions. Overseas varieties provide a useful genetic resource for improving the quality of domestic wheat for bread making. However, it is difficult to breed good quality wheat for bread making that also has high yield and resistance to both *Fusarium* head blight and preharvest sprouting from a single cross. Therefore, the backcross method is considered more reliable in improving domestic wheat quality for bread making.

'Setokirara' was released in 2013, having been bred using the backcross method and marker-assisted selection (Fig. 35.5). 'Fukuhonoka' was chosen as the recurrent parent. 'Fukuhonoka' is a soft wheat cultivar for Japanese noodles, and is well adapted to the temperate climate conditions in Japan. It has a high yield, good preharvest sprouting resistance, and acceptable *Fusarium* head blight resistance. To improve its bread quality, a triple homozygous genotype (*Glu-D1d*, *Glu-B3h*, and *Pinb-D1c*) was selected using a Polymerase Chain Reaction (PCR) marker. 'Setokirara' showed agricultural performance similar to that by 'Fukuhonoka' and quality of the same standard as that by HRW for bread making.

A combination of glutenin subunits *Glu-D1a* and *Glu-B3g* results in extra-strong flour (Maruyama-Funatsuki et al. 2004; Tabiki et al. 2006). 'Yumechikara' is an extra-strong Hard Red Winter wheat cultivar released in 2009 with a marker-assisted selection of these two subunits (Tabiki et al. 2011). Extra-strong flour has a unique bread-making quality. The bread-making quality score for 'Yumechikara' flour was superior to Canadian western No. 1 (1CW) when blended with domestic soft wheat flour (Fig. 35.6).

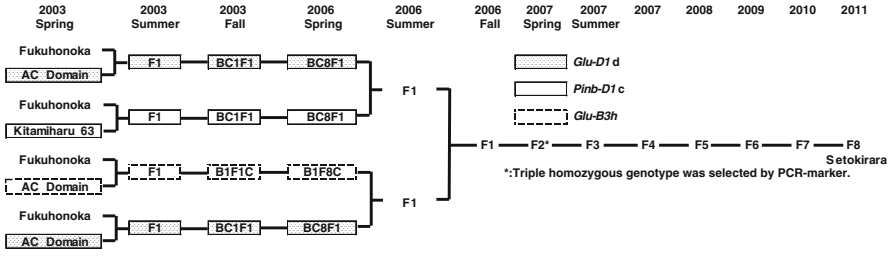

Fig. 35.5 Breeding of 'Setokirara' with good bread-making quality

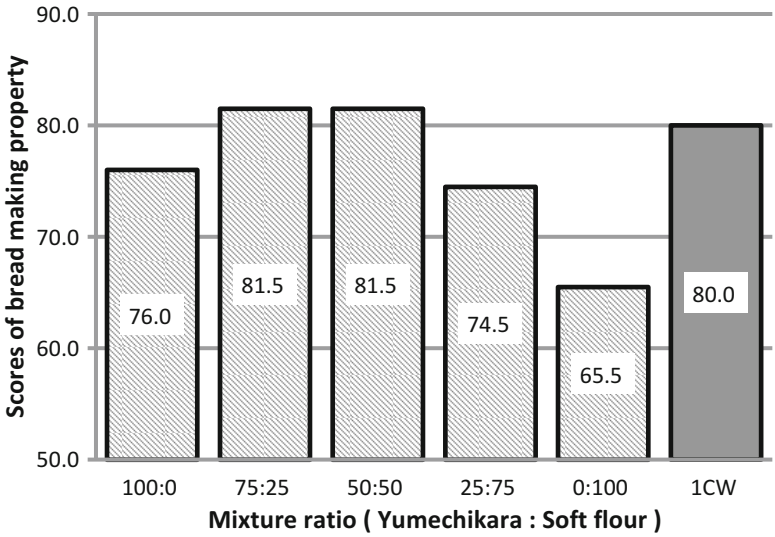

Fig. 35.6 Bread-making quality of 'Yumechikara' blended with soft flour

Wheat yellow mosaic virus is a soil borne disease; outbreaks have been reported on the mainland since 1936, and in 1991, an outbreak was reported in Hokkaido, where half the quantity of domestic wheat is produced. There are many genetic varieties resistant to this disease; for example, 'Yumechikara' is highly resistant. In 2010, a gene, *YmIb*, located on the long arm of the 2D chromosome, was reported as conferring resistance to wheat yellow mosaic virus (Nishio et al. 2010). Markers for this gene were also reported, making it particularly useful, and a backcross program to breed resistant varieties is now in progress.

Preharvest sprouting (PHS) resistance is required for Japanese cultivars. In Japan, breeders use 'Zenkouzikomugi' as a genetic resource for PHS resistance. 'Zenkouzikomugi' has two quantitative trait loci (QTLs) for PHS resistance located on chromosomes 3A and 5A. One of these QTLs was assumed to be the *Mother of FT and TFL1* (*MFT*) (Nakamura et al. 2011). Mapping analysis showed that *MFT* is colocated on chromosome 3A with the PHS-resistant QTL (*Qphs.ocs-3A.1*). Precocious germination of isolated immature embryos was suppressed by transient introduction of *MFT* driven by the maize ubiquitin promoter. This and further evidence showed that *MFT* is a germination repressor and may be the causal gene for *Qphs.ocs-3A.1*. A comparison of the genomic sequences of *MFT-3A* from Chinese spring (which is less PHS-resistant) and 'Zenkouzikomugi' (highly PHS-resistant) revealed single nucleotide polymorphism (SNP). A cleaved amplified polymorphic sequence (CAPS) marker was developed in this SNP.

The Zenkouzikomugi-type allele, which is highly PHS-resistant, is very popular in domestic wheat varieties. In contrast, the Zenkouzikomugi-type allele is very rare in foreign varieties, and the less resistant Chinese spring-type allele is more common. When only domestic varieties were used as cross parents to breed soft wheat for Japanese noodles, this *MFT* CAPS marker was useless because there was no

polymorphism. However, when varieties from overseas were used to improve bread-making quality, the *MFT* CAPS marker revealed its usefulness, and it has now started to be used alongside other markers (e.g., *Glu-1*, *Glu-3*, *Gli-1*, *Pina-1*, and *Pinb-1*). In the near future, a new variety will be released with the *MFT* CAPS marker.

There are three types of *Fusarium* head blight (FHB) resistance in wheat.

Type 1: Resistance to FHB initial infection
Type 2: Resistance to FHB spread within the spike derived from the initial infection
Type 3: Decomposition or lack of accumulation of mycotoxins of FHB

In 2011, 'Wheat Norin PL-9' was bred from a cross between 'U24' (cleistogamous [closed flowering]) and 'Saikai 165' (chasmogamous [open flowering]) (Kubo et al. 2012). 'U24' is a cleistogamous line with type 1 FHB resistance (Kubo et al. 2010, 2013). However, its agricultural performance is poor (late maturity, long culm length). 'Saikai 165' is a derivative of 'Sumai 3', which is globally the most popular FHB-resistant genetic resource. 'Saikai 165' and 'Sumai 3' have a QTL (*Fhb1*) for type 2 FHB-resistance located on the short arms of chromosome 3B. 'Wheat Norin PL-9' has a cleistogamous characteristic that increases its resistance to initial infection by FHB; it also incorporates a Saikai 165 (resistant) genotype in *Fhb1*, which was selected by a PCR-marker. It showed similar levels of resistance to the spread of FHB and mycotoxin accumulation as 'Saikai 165', and a better agricultural performance than 'U24'. However, we still need to see an improvement in agricultural performance before releasing a new variety with both type 1- and type 2-resistance to FHB.

Table 35.1 shows the molecular markers used in wheat breeding in Japan. Molecular markers for flour color and yield will be developed in the next few years. Grain yield is an important character for increasing domestic wheat production. However, much further research is needed in order to elucidate the mechanisms driving high yield under Japanese climate conditions before a molecular marker can be developed.

Table 35.1 Use of molecular markers in wheat breeding in Japan

Trait	Marker	Status of application
Amylose content (stickiness, shelf life)	*Waxy*	In current application
Dough strength	*Glu-1*, *Glu-3*, *Gli-1*	In current application
Grain hardness (damaged starch)	*Pina-1*, *Pinb-1*	In current application
Wheat yellow mosaic virus	*YmIb*	In current application
Preharvest sprouting	*MFT* (*Mother of FT and TFL1*)	In current application
Fusarium head blight	*Fhb1*	In current application
Flowering	*Vrn*, *Ppd*	In validation
Flour color	Unknown	Basic research/development
Flour yield	Unknown	Basic research/development
Grain yield	Unknown	Not yet

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Chapter 36
Determining the Order of Resistance Genes *Qsng-3BS*, *Fhb1* and *Sr2* and Combining Them in Coupling on Wheat Chromosome 3BS

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Abstract A variety of diseases of wheat (*Triticum aestivum* L.) occurs every year in the U.S. leading to significant grain yield losses. *Stagonospora nodorum* blotch (SNB), fusarium head blight (FHB) and stem rust (SR) are caused by the fungi *Stagonospora nodorum, Fusarium graminearum* and *Puccinia graminis*, respectively. These diseases penalize both grain yield and quality. Three resistance factors, *Qsng.sfr-3BS*, *Fhb1* and *Sr2* conferring resistance, respectively, to SNB, FHB and SR, each from a unique donor wheat parent line, have been mapped to chromosome 3BS of wheat and are believed to be closely linked. Based on previously published analyses, *Sr2* is on the distal end, *Fhb1* is on the proximal end and *Qsng.sfr-3BS* is in the middle of *Sr2* and *Fhb1* in the 3BS wheat genome. Thus, the objectives of this project are to determine the gene order of *Qsng.sfr-3BS*, *Fhb1* and *Sr2*, in a linkage block on chromosome 3BS and combining them in coupling. The linkage relationships were determined through analysis of a three-way cross between parental lines Arina, Alsen and Ocoroni86, containing the resistance genes *Qsng.sfr-3BS*, *Fhb1* and *Sr2,* respectively. A total of 1,600 F_2 plants was screened, along with the parental lines, using KASPar genotyping technology via single-nucleotide polymorphism markers to identify the recombinant progeny. Phenotypic screening for SNB was performed on the entire F_2 population. Knowing the positional order of these

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resistance genes will enable the development of a wheat line with three genes in coupling to provide durable and broad-spectrum resistance against three major diseases of wheat.

Introduction

Stagonospora nodorum blotch (SNB), fusarium head blight (FHB) and stem rust (SR) of wheat are caused by the fungi *Stagonospora nodorum*, *Fusarium graminearum* and *Puccinia graminis*, respectively. Each of these diseases can cause yield losses up to 50 % or more during severe epidemics and when environmental conditions are favorable (Chester 1943; Roefls 1978; Stakman and Harrar 1957; Wicki et al. 1999). In addition to yield reduction, FHB reduces quality due to production of a mycotoxin called vomitoxin (deoxynivalenol) produced by *F. graminearum* (Bai et al. 2001; Gilbert and Tekauz 2000), which is harmful to both humans and livestock. FHB also reduces test weight and lowers market grade. Thus, FHB is one of the most feared fungal diseases of wheat because an entire crop can be rejected for human consumption due to mycotoxin contamination. SR has a capacity of destroying millions of hectares of healthy, high-yielding wheat in less than a month by reducing fields to a mass of bare stalks supporting only small, shriveled grains by harvest time (Singh et al. 2008). There have been several epidemics of SR during the past 80 years that have reduced the yield by 50 % in the Great Plains (Chester 1943; Roefls 1978; Stakman and Harrar 1957). SNB is one of the major foliar and glume diseases of wheat and the most yield loss occurs when the flag leaf and the two leaves below the flag leaf become infected by the time the wheat flowers in late May.

The objective of this study is to find the gene order of *Fhb1*, *Sr2* and *Qsng.sfr-3BS* with the long-term goal of combining them in a linkage block on wheat chromosome 3BS. The first objective was achieved by crossing three unique parental lines with resistance genes *Fhb1*, *Sr2* and *Qsng.sfr-3BS* to combine them into one background. An F_2 population segregating for all three genes was genotyped with single-nucleotide polymorphism (SNP) markers to validate the presence of markers linked to the resistance genes and also to determine the gene order. The F_2 population was also phenotyped for level of resistance to SNB.

Materials and Methods

The mapping population consisted of 1,600 F_2 progeny derived from a three-way cross between wheat cultivars Arina, Alsen and Ocoroni86 providing the resistance genes *Qsng.sfr-3BS, Fhb1,* and *Sr2*, respectively. The F_2 population, its parents and cultivar Chinese Spring as negative control, were planted in January, 2013 in a greenhouse at Purdue University in West Lafayette, Indiana, U.S.A.. The primary

spike after spike emergence (Feekes growth stage 10.3) of an adult plant of every F_2 progeny and the parental lines was phenotyped in a greenhouse using an isolate of *S. nodorum* obtained from an infected wheat field in Indiana. The disease severity scores were recorded 21 days after inoculation on a 0–9 scale. The F_2 plants were screened along with the parental lines using KASPar genotyping technology to identify the recombinant plants. Single-nucleotide polymorphism markers were utilized to identify the recombinants, determine the gene order, to make the genetic map and for quantitative trait loci (QTL) analysis. Linkage analysis was performed and the map was made with JoinMap 3.0.

Results and Discussion

Phenotyping of all 1,600 F_2 progeny was performed and the results utilized the full range of the 0–9 disease-rating scale. The frequency distribution of the percent diseased glume tissue of F_2 progeny derived from the three-way cross of Arina (containing the *Qsng.sfr-3BS* SNB resistance QTL), Alsen and Ocoroni86 inoculated with a field isolate of *S. nodorum* in a greenhouse showed a continuous distribution with a skew towards susceptibility, indicating the presence of one or more QTL for resistance (data not shown).

The preliminary genetic linkage map suggests that the stem rust resistance gene (*Sr2)* is the most distal, the fusarium head blight resistance gene (*Fhb1)* is proximal, and with the *S. nodorum* resistance gene (*Qsng.sfr-3BS)* is between *Sr2* and *Fhb1* on chromosome 3BS of wheat (Fig. 36.1). This result supports our hypothesis made

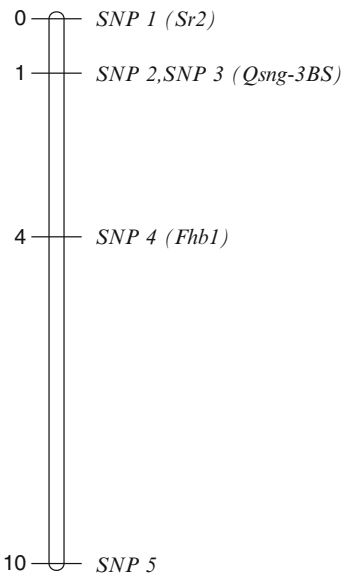

Fig. 36.1 Preliminary linkage map of wheat chromosome 3BS for markers SNP 1, SNP 2, SNP 3, SNP 4, and SNP 5 in the F2 population of 1,600 individuals derived from a three-way cross of wheat cultivars Arina, Alsen and Ocoroni86. The map was generated with JoinMap 3.0 at LOD = 9.0. Numbers to the *left* of the vertical bar indicate the total distance in centimorgans, and positions of mapped markers are indicated on the *right*. The approximate positions of disease resistance genes are indicated by *brackets*

from analyses of previously published marker positions. Knowing the correct order and relative distance between the three resistance genes indicates how a linkage block can be created. Markers for *Sr2* and *Qsng.sfr-3BS* were placed 1 cM apart in our mapping population while the predictive marker for *Fhb1* was located 4 cM proximal from *Sr2*. Thus, recombinant plants having both *Sr2* and *Qsng.sfr-3BS* were recovered at a lower frequency than plants having *Fhb1* and *Qsng.sfr-3BS* in coupling. These plants having two resistance genes in coupling will be used in further crosses to obtain recombinants having all three resistance genes in a linkage block. The tight linkage between *Sr2*, *Qsng.sfr-3BS*, and *Fhb1* indicates that it should be easy to maintain this linkage block in a breeding program.

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Chapter 37
Meta-analysis of Resistance to Fusarium Head Blight in Tetraploid Wheat: Implications for Durum Wheat Breeding

Noémie Prat, Maria Buerstmayr, Barbara Steiner, and Hermann Buerstmayr

Abstract Improvement of resistance to Fusarium head blight (FHB) is a continuous challenge for durum wheat (*Triticum durum*) breeding, where most germplasm are susceptible and low genetic variation is available for this trait. Research has focused on broadening the genetic basis by introducing alleles for FHB resistance from landraces and related species such as bread wheat (*Triticum aestivum*), cultivated emmer (*Triticum dicoccum*), wild emmer (*Triticum dicoccoides*) and Persian wheat (*Triticum carthlicum*) into durum wheat. We summarize and compare here QTL mapping studies carried out to date in tetraploid wheat. Thirteen QTL with small to moderate effects were repeatedly detected on 11 chromosomes with alleles improving FHB resistance deriving from relatives and from durum wheat itself. Comparison showed large overlaps of QTL positions with those identified in hexaploid wheat suggesting a common genetic basis for FHB resistance. FHB resistance breeding by allele introgression into durum wheat is feasible and QTL pyramiding in novel cultivars is a promising strategy for resistance breeding.

Keywords Durum wheat • Fusarium head blight • QTL • Resistance • Tetraploid wheat • *Triticum durum*

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Introduction

Durum wheat (*Triticum durum*) is the principal cultivated tetraploid wheat species. Its annual production accounts for ~5 % of the total wheat grown worldwide and it is used mainly for preparation of pasta and semolina (Taylor and Koo 2012). Durum wheat, as other small grain cereals, suffers from susceptibility to Fusarium head blight (FHB), a devastating disease that affects wheat growing regions throughout the world. FHB is caused by a broad range of fungi from the Fusarium genera (Xu and Nicholson 2009). FHB epidemics are a serious threat for wheat production as the disease leads not only to yield losses but also infests crops with potent mycotoxins hazardous for food safety (Pestka 2010). This is particularly alarming in durum wheat since it is predominantly intended for direct human consumption.

An increasing demand for pasta products has led to an expansion of durum wheat production zones from traditional warm and dry cropping areas to more humid regions with climatic conditions conducive to the disease. Solutions to prevent FHB damages are limited and the development of resistant cultivars is considered a sustainable and highly desired approach to reduce FHB damages (Bai and Shaner 2004).

FHB Resistance in Durum Wheat

Current durum cultivars are generally susceptible to FHB (Clarke et al. 2010; authors' unpublished results). Sources of resistance remain scarce despite efforts undertaken to discover FHB resistant lines: large collections of thousands of durum wheat accessions have been screened without identifying resistant lines (Elias et al. 2005). Surveys on material from CIMMYT and ICARDA identified only five lines from a Tunisian source with moderate resistance to FHB spread (Huhn et al. 2012) and four Syrian landraces with stable resistance (Talas et al. 2011). The lack of resistance found in durum wheat may be attributed to historically low exposure to FHB and to the limited breeding efforts put into this relatively modern crop, which led to a narrow genetic base compared to other wheat species (Ban et al. 2005; Oliver et al. 2008). It is also speculated that durum carries susceptibility factors and/or suppressor genes that compromise FHB resistance (Stack et al. 2002; Garvin et al. 2009; Ghavami et al. 2011).

Studies have thus been directed at evaluating relatives of durum wheat in order to broaden the genetic basis for breeding and efforts targeted at transferring FHB resistance into durum wheat.

In hexaploid wheat (*Triticum aestivum*) more than 100 QTL for FHB resistance have been mapped and some have been successfully integrated in breeding programs through marker assisted selection (Buerstmayr et al. 2009). Yet, attempts to transfer resistance into tetraploid wheat have met limited success (Oliver et al. 2007; authors' unpublished results). One hypothetical explanation for the often

disappointingly low effect of hexaploid wheat QTL alleles when transferred into durum wheat is that the D-genome, absent in tetraploid wheat, contributes resistance-inducing factors (Fakhfakh et al. 2011).

Consequently resistance has been sought in tetraploid close relatives of durum wheat, where transfer of resistance is not confounded by differences in ploidy levels. Moderate to good FHB resistant tetraploid accessions have been successfully identified (Oliver et al. 2008; Buerstmayr et al. 2003).

QTL Studies in Tetraploid Wheat

QTL mapping studies carried out to date in durum wheat have been based on resistance deriving from tetraploid sources including wild emmer *Triticum dicoccoides*, cultivated emmer *Triticum dicoccum*, Persian wheat *Triticum carthlicum* and durum wheat landraces. A list including information on the resistance source used in the mapping analysis, the inoculation methods performed and the type of resistance assessed for each study is given in Table 37.1.

We gather here QTL reported in tetraploid wheat which were repeatedly found in different years or in independent studies. A total of 13 small to moderately effective QTL were mapped to 11 chromosomes. Their positions are indicated with vertical bars in Fig. 37.1, and the names of the genotypes contributing to resistance allele and the applied inoculation methods are also specified.

Durum wheat itself contributed resistance-improving alleles for the QTL on 2B (Gladysz et al. 2007; Somers et al. 2006), 3B (Buerstmayr et al. 2012; Ghavami et al. 2011) and 5B (Ghavami et al. 2011). This backs up the idea that in current durum wheat a certain level of FHB resistance is already available. A potential susceptibility factor which increases durum wheat susceptibility was detected on 2A

Table 37.1 QTL studies carried out in durum wheat mentioning resistance source, inoculation method and type of resistance evaluated

Resistance source		Inoc.	Resistance	
T. dicoccoides	Israel A (2A; 3A)	SFI	FHB spread	Otto et al. (2002); Chen et al. (2007); Garvin et al. (2009)
T. dicoccoides	PI478742 (7A)	SFI	FHB spread	Kumar et al. (2007)
T. dicoccoides	Mt.Hermon#22	SFI	FHB spread	Gladysz et al. (2007)
T. dicoccoides	Mt.Gerizim#36	SFI	FHB spread	Buerstmayr et al. (2013)
T. carthlicum	Blackbird	SFI	FHB spread	Somers et al. (2006)
T. dicoccum	T. dic-161	spray	FHB severity	Buerstmayr et al. (2012)
T. dicoccum	BGRC3487	SFI	FHB spread	Ruan et al. (2012)
		spray	FHB severity	
T. durum	4 Tunisian lines	SFI	FHB spread	Ghavami et al. (2011)

SFI single floret inoculation, *spray* spray inoculation, *FHB spread* resistance to spread of the disease within the spike (type 2 resistance), *FHB severity* disease severity per plot after spray inoculation

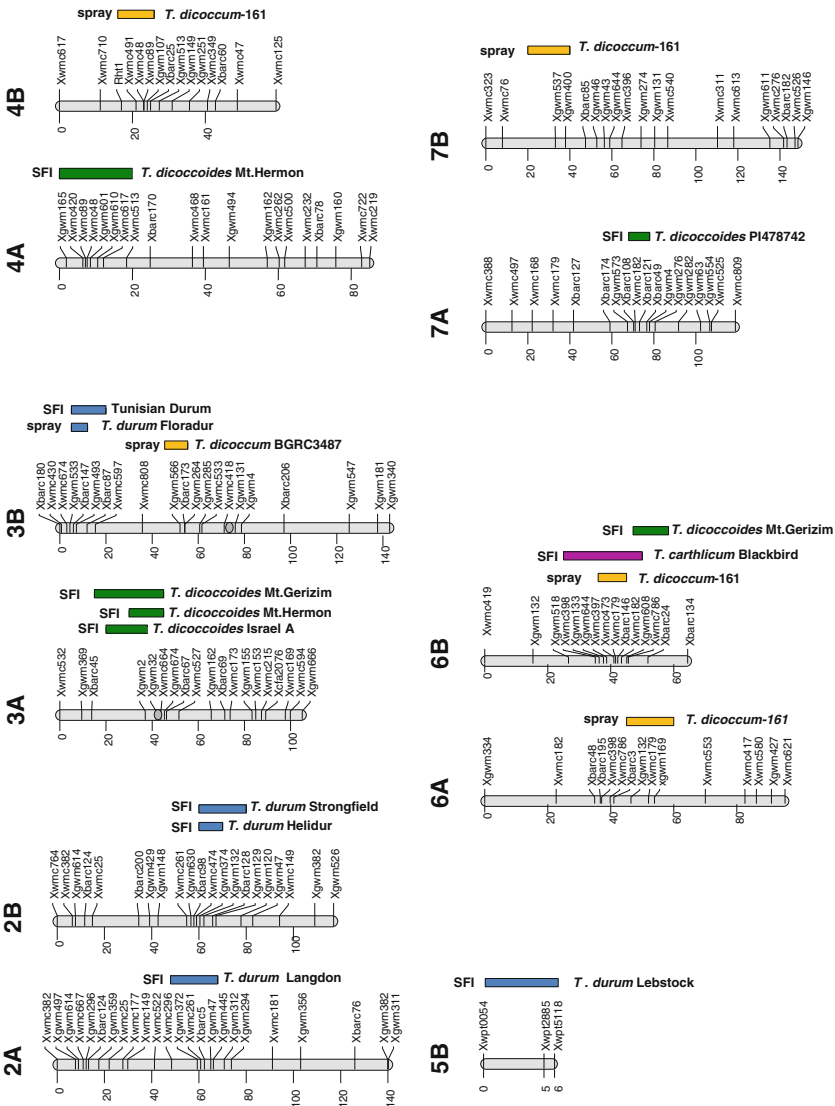

Fig. 37.1 Locations of QTL for FHB resistance on durum wheat chromosomes. QTL are identified with the names of the line contributing to the resistant alleles. The inoculation methods performed are also indicated: *SFI* single floret inoculation, *spray* spray inoculation

derived from *T. dicoccoides* Israel A (Garvin et al. 2009; Stack et al. 2002). Ghavami et al. (2011) also suspected the existence of a QTL influencing FHB resistance in the same chromosomal region in durum wheat.

Positions of many of the resistance QTL identified in tetraploid wheat coincided with QTL discovered in hexaploid wheat, suggesting common genes for resistance: e.g. QTL on 2B, 3A, 3B, 6B and 7B were found in the same regions where several QTL have been reported in hexaploid wheat (Buerstmayr et al. 2009). Positions of the QTL on 3B and 6B overlapped with those of the well documented genes *Fhb1* and *Fhb2*, respectively, which were first described in the hexaploid cultivar Sumai-3 (Buerstmayr et al. 2009). Allele survey at these loci by Buerstmayr et al. (2012) revealed different SSR marker haplotypes between tetraploid lines and Sumai-3. The existence of resistance improving alleles at these loci in tetraploid wheat may circumvent the need to transfer resistance from hexaploid Asian sources into durum wheat.

Developmental and morphological traits often correlate with FHB response both in hexaploid wheat (Buerstmayr et al. 2009) and in tetraploid wheat. For example, under field conditions with spray inoculation a large effect QTL for FHB resistance was mapped at the position of the major plant height gene *Rht-B1* on chromosome 4B and a FHB resistance QTL on 7B coincided with a QTL for heading date (Buerstmayr et al. 2012). It is not clear yet whether or not these genes have pleiotropic effects or rather an indirect influence on FHB resistance due to plant height and flowering date per se.

Conclusions and Perspectives

Only few accessions have been used as sources for FHB resistance in durum wheat to date, yet results are promising, yielding multiple QTL with small to medium effects. Common genetic basis for FHB resistance in tetraploid and hexaploid wheat is likely as the positions of their QTL overlap to a large extent. Introgression of positive alleles into durum wheat is feasible and markers located near the mapped QTL are amenable for marker-assisting backcrossing. Pyramiding multiple resistance improving QTL combined with selection against suspected susceptibility factors is a promising breeding strategy to improve FHB resistance in novel cultivars. Improvements in durum wheat breeding are underway. Recently, the evaluation of novel experimental lines descending from multiple crosses of *T. durum* with *T. aestivum*, *T. dicoccum* and *T. dicoccoides* in our field trials in Tulln (Austria) showed enhanced variation for FHB resistance including lines with improved and stable FHB resistance performance.

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Chapter 38
Interest of a Multiparental and Outcrossing Wheat Population for Fine Mapping

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Abstract The use of multiparental populations for QTL discovery has been recently highlighted by different theoretical and experimental developments. Here, we explored the interest of French populations using heterogeneous genetic stocks of cultivated wheat, maintained in situ over 12 sites since 1984 with an outcrossing mating system. We studied one of these populations (Le Moulon, 48.4°N, 21°E), derived from 12 cycles of random crosses between 60 founders, selected to maximize genetic diversity. Outcrossing was allowed by the integration of a nuclear male sterility allele (*ms1b*, Probus donor) in the population. We analyzed 1,000 Single Seed Descent lines (SSD) derived from the 12th generation of cultivation. This population was genotyped using the 9 K i-select SNPs (Single Nucleotide Polymorphisms) array, covering the whole genome. Polymorphism and quality checks resulted in the selection of around 6,500 SNPs. First, the evolution of genetic diversity was explored through the comparison of SSD lines and the inferred initial population. The low population structure and the strong decay in linkage disequilibrium between SSD lines and the inferred initial population confirmed the efficiency of the 12 cycles of the random outcrossing in producing a highly diverse and recombined population. Two years of observations of population earliness under different

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environments were used to show the complementarity of association genetics, which allowed the detection of already known *Vrn* major genes, and evolutionary approach, which, lead to the discovery of two new minor effect QTLs.

Keywords Dynamic management • Evolution approach • Recombinant population • Wheat

Introduction

Dynamic management (DM) aims at maintaining crop genetic diversity through in situ conservation of genetic resources. Genetically diverse populations are grown year after year, in various sites, differing for climate conditions, pathogen pressures and/or agricultural practices (Allard 1988; Henry et al. 1991; Porcher et al. 2004). In France, dynamic management has been experimented on bread wheat (*Triticum aestivum* L.) since 1984 (Henry et al. 1991), using three gene pools: two selfing populations (based on a pyramidal cross of 16 parents) and one outcrossing population. Samples of each of the three initial populations were sent to 7–12 sites in France and cultivated year after year in the same sites under the same conditions. Thus, these three "meta-populations" evolved over 10 to more than 20 generations without migration or conscious human selection (Enjalbert et al. 2011).

Studies on the selfing populations showed a good maintenance of global diversity at the network level (Raquin et al. 2008), both at phenotypic and molecular levels. A fast evolution of flowering time was observed, both over time and space (Rhoné et al. 2008): all populations flowered later than the initial population; and populations from Northern French sites flowered much later than Southern ones. Association genetics and spatio-temporal shifts in allelic frequencies revealed polymorphisms located in major genes controlling vernalization requirement or photoperiod sensitivity, partially explained climatic adaptation (Rhoné et al. 2008). Therefore, in addition to genetic resource preservation, DM populations can be an appropriate material to detect genes involved in local adaptation (Goldringer et al. 2001).

In the present study, we analyzed one outcrossing DM population, which is characterized by a high number of parents (60 lines), and numerous panmictic generations. In this population, wheat natural selfing habit was turned to an outcrossing mating system, using a recessive male sterility gene (*ms1b*), and harvesting solely open pollinated male-sterile plants. Taking the parental lines as reference, we studied an evolved population (12th generation) and tested for possible markers selection, trying to link detected markers to the observed evolution in vernalization requirement.

Materials and Methods

The population studied is derived from the cross of Probus, a mutant carrying a male sterile allele (*ms1b*), with 59 lines covering a large genetic diversity. Resulting F_1 progenies were alternatively selfed (F_2) or back-crossed (BC_1) with the 59 parents to reduce the Probus genome contribution to the population. These two progenies (F_2 & BC_1) were sown together in an isolated field surrounded by rye. Male sterile spikes were tagged during flowering and harvested at maturity. Then, and over 12 generations, a random sample of harvested seeds on male sterile plants was drawn each year, and resown in fall, in order to reach between 5,000 and 10,000 adult plants (2,500–5,000 male-sterile plants). After 12 generations we derived 1,000 SSD lines (F_5, Fig. 38.1).

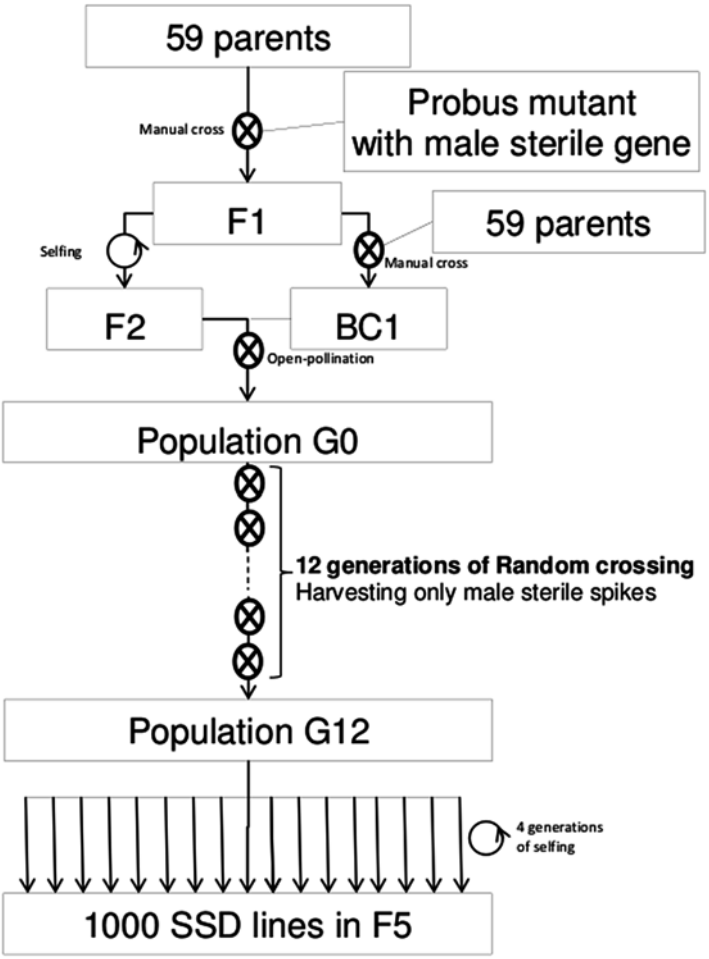

Fig. 38.1 Population creation scheme

First we inferred allelic frequencies in the initial population on the basis of the 56 parental lines (including Probus) still available in seed banks (four missing ones), estimating their contributions to the global pool using a Bayesian method (Thépot et al. 2015). The evolved population was studied through a subset of 380 SSD lines, representative of the phenotypic diversity of the 1,000 lines.

Vernalization requirement was assessed in field trial at Le Moulon over two seasons (2010–2011 and 2011–2012), with a spring sowing (April), on a single row of 20 seeds per genotype. For each row, the heading date was scored when half of the plants had half of the main ear emerged from the flag leaf. The heading date was transformed into sums of degree-days (dd) (sums of the mean temperature per day) from sowing to heading. On the basis of the bimodal distribution of the heading date (Fig. 38.2), SSD lines were classified as spring type (heading before 2,000dd), or winter type for the others. Genotypes with inconsistent behavior between both years were discarded (eight SSD lines).

Genotyping was performed using the 9 K i-select SNP array. Only SNPs unambiguously scored as biallelic after a visual inspection using Genome Studio software,

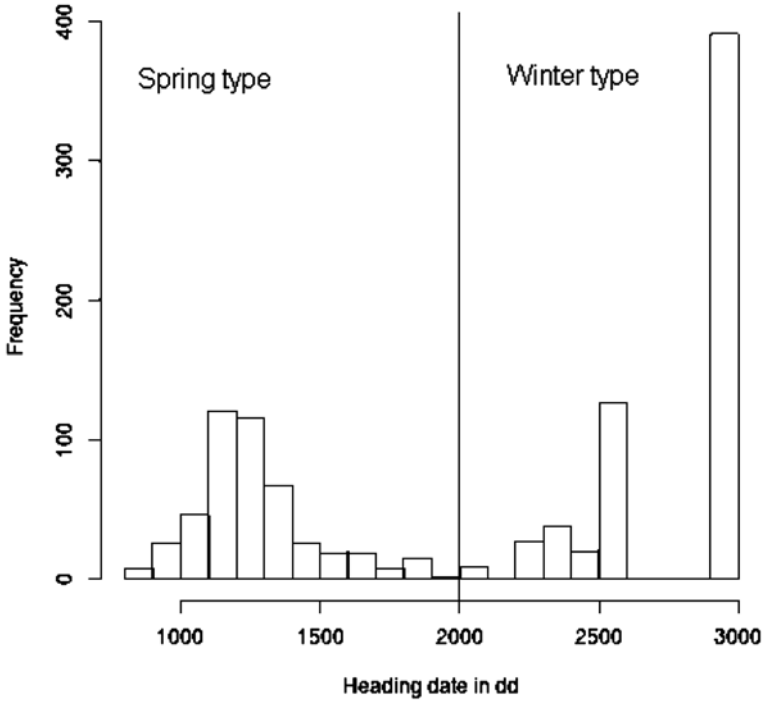

Fig. 38.2 Heading date distribution of all genotypes for spring sowing in 2011, the condition with the least vernalization to determine winter and spring type

were kept. Using KASPar SNP genotyping system (KBioscience), 14 additional polymorphisms located in candidate genes (earliness pathway such as PPD or VRN families) were genotyped.

The diversity detected by SNPs in both populations (parental lines and SSD lines) was compared using Minor Allele Frequency (MAF) and expected heterozygosity (He, Nei diversity). Evolution of growth habit was tested through a comparison of spring/winter ratio in the initial and the evolved populations (Chi square test). Strong shifts in allelic frequencies were used to detect markers under selection, using a new method (Thépot et al. 2015). Q-values were estimated to cope with the multiple tests (Storey and Tibshirani 2003). Each marker under selection was also tested for association with growth habit using a Logit model.

Results and Discussion

The genotyping of 436 lines (56 parents + 380 F_5 lines) with the 9 K i-select SNP assay resulted in 7,270 SNPs with high scoring quality. Among these SNPs, 88.4 % were polymorphic in the initial population and 85.8 % in the evolved population. This slightly higher diversity in the initial population was also observed on allelic frequencies of polymorphic SNPs (mean MAF: 0.18 vs. 0.17 and He: 0.25 vs. 0.24). The MAF distribution (Fig. 38.3) showed a globally high frequency of SNPs with a MAF inferior to 0.05, rare alleles being more frequent in the evolved population. This distribution contrasts with the one observed on a worldwide panel, using the same SNP array, which demonstrates a deficit of SNPs with a low MAF (Cavanagh et al. 2013). This deficit might be due to the fact that lines were chosen to maximize

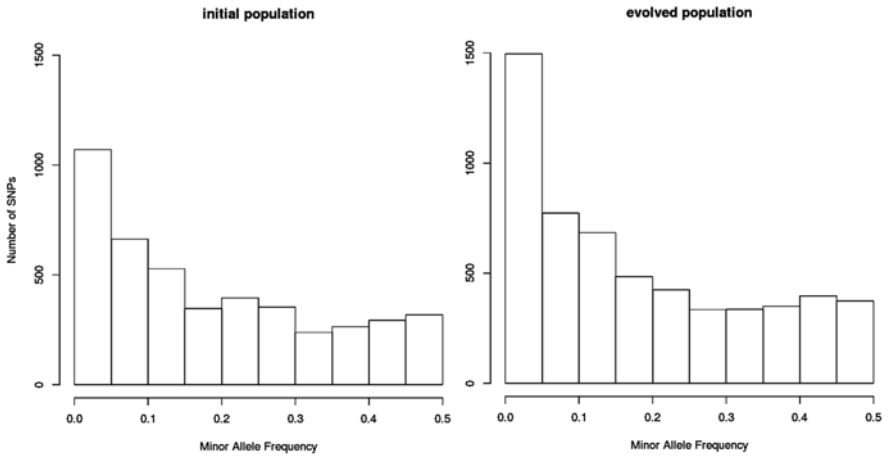

Fig. 38.3 Distribution of minor allele frequency in the initial and evolved populations

the genetic diversity, and SNPs were intentionally selected to favor common allele in a panel of 26 cultivars from mainly USA and Australia (Cavanagh et al. 2013).

Among the 6,476 polymorphic markers, 57 were detected under selection, representing 26 independent genomic areas. When assessing phenotypic evolution for flowering time, we observed a significant shift from 20 % of spring type in the initial population to 47 % in the evolved population. Among markers under selection, three were associated with the growth habit (p-value <0.05). These markers, located on the 5D and 4A chromosomes, only explained a rather limited part of the phenotypic variation (2.8 % with a global model included the three markers). Yet, they all have, experienced an increase of the spring allele frequency in the evolved population which explained a raise of spring type ranging from 3.5 % to 5.4 %. The 5D markers are located on the same chromosome as *Vrn-D1* but did not present linkage disequilibrium with the marker located in this gene (r^2 <0.009). Surprisingly polymorphisms in candidate genes like *Vrn* families (*Vrn-A1-Prom*, *Vrn-A1-ex7* and *Vrn-D1*), although strongly associated to the growth habit (p-value $<10^{-10}$, $r^2 = 28$ %) have not been detected as targeted by selection. To take into account the complexity of interaction between these three markers, we assumed that spring alleles are both dominant and epistatic (Rousset et al. 2011). Thus as soon as there is at least one spring allele, the haplotype was classified as spring type or winter type otherwise. For parental lines, these *Vrn* haplotypes are almost completely explaining phenotype (97.5 % of correspondence, $r^2 = 0.85$). However for the evolved population 30 % of SSD lines with winter *Vrn* haplotype exhibited a spring phenotype (Table 38.1) ($r^2 = 0.3$). This evolution might be explained by (i) high level of recombination that broke the initial full linkage disequilibrium between causal mutations and the three SNPs genotyped, and/or (ii) the increase of spring alleles at one (or several) non-genotyped *Vrn* genes, such as *Vrn-B1*. As SNPs, *Vrn* haplotypes did not present a significant shift (p-value = 0.29), although a 6 % increase of spring haplotypes was observed between the initial and the evolved population (Table 38.1). One hypothesis to explain this absence of significant shift at these candidate genes could be their strong effect: a little variation in frequency at these major genes may have a strong effect on growth habit.

Association genetics and evolutionary approach provided complementary results. The first method detected QTLs with major effects while the second detected QTLs with lower effect but contributing to the evolution of phenotypes. Joint study of phenotypic and genetic evolutions allowed to detect new markers involved in the control of the growth habit on the 5D and 4A chromosomes.

With its high diversity, absence of structure and low LD (Thépot et al. 2015), this population appears as a new QTL mapping resource, allowing the discovery of original genomic regions controlling traits of interest. Ongoing studies will better explore the potential of this population for detection, using the 1,000 SSD lines.

Table 38.1 Summary results of *Vrn* haplotypes evolution between the initial population and the evolved population and their association to the winter/spring phenotype

Genotypes			Haplotype type	Freq. initial population	Freq. SSD lines
Vrn-A1 promoter	*Vrn-A1* exon7	*Vrn-D1*			
S	S	W	Spring	19.6 %	25.3 %
S	H	W		(94 % S; 6 % W)	(93 % S; 7 % W)
S	H	NA			
W	S	S			
W	S	W			
W	S	NA			
W	H	S			
W	W	S			
NA	S	W			
W	W	W	Winter	79.3 %	69.7 %
				(0.8 % S; 99.2 % W)	(30 % S; 70 % W)
W	W	NA	NA	1.1 %	5 %
W	NA	W			
W	H	W			

Genotypes are coded with S, H, W, with S for the homozygote spring allele, H for heterozygote and W for homozygote winter allele, assuming that spring allele are dominant and epistatic

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Chapter 39
The Effect of Earliness per se (*Eps*) Genes on Flowering Time in Bread Wheat

Meluleki Zikhali and Simon Griffiths

Abstract Photoperiod (day-length) response, vernalization (response to extended periods of cold) and earliness per se (*Eps*) genes regulate flowering time in wheat. The vernalization and photoperiod response genes are relatively well studied. However, the role of *Eps* genes is yet to be fully understood but the current assumption is that *Eps* genes regulate flowering independent of vernalization and photoperiod. While some *Eps* genes have been cloned in both *Hordeum vulgare* and *Triticum monococcum*, none has been cloned in *Triticum aestivum* to date. The use of near isogenic lines (NILs) in both *T. monococcum* and *Triticum aestivum* has enabled *Eps* effects to be studied in more detail and candidate genes have been proposed for *Eps* effects in both species. *Eps* loci are reported to be involved in fine tuning flowering time and are also responsible for controlling spikelet number and size hence could be manipulated to increase wheat yield. This mini review summarises our current understanding of *Eps* and how manipulation of *Eps* genes can be used in predictive wheat breeding.

The world population demands more food, greater diversity of food, a balanced and healthy diet, produced on no more, and preferably less land, while conserving soil, water, and genetic resources. The major problem is that even though wheat yields are increasing (Lopes et al. 2012), the percentage increase is below the projected percentage demand with about 0.6 % deficit projected annually until 2050 (Dixon et al. 2009; Rosegrant and Agcaoili 2010). The challenge wheat breeders face is to bridge the gap between wheat demand and wheat production. It is therefore vital to direct wheat breeding efforts to the production of higher yielding varieties in order to ensure current and future food security (Reyolds et al. 2012). The part of the wheat plant that is important for direct consumption by humans is the grain and its production is dependent on flowering time. Manipulating flowering time is one avenue that can be exploited to increase wheat grain yield (Herndl et al. 2008; Greenup et al. 2009). However, in order to successfully increase grain yield, it is

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vital to thoroughly understand the genetic and physiological factors affecting wheat growth and development particularly flowering time genes (Gill et al. 2004).

There are three major classes of flowering time genes which are photoperiod (*Ppd*) response genes, vernalization (*Vrn*) response genes and earliness per se genes (*Eps*). Major photoperiod response genes enable wheat plants to perceive changes in day length with accelerated flowering occurring in long days while short days cause delayed flowering unless there are mutations in the *PHOTOPERIOD 1* (*Ppd-1*) genes (Beales et al. 2007; Wilhelm et al. 2009; Dı'az et al. 2012). Three main genes *VERNALIZATION 1*, *2* and *3* (*VRN1*, *VRN2* and *VRN3*) control the vernalization response in wheat (Yan et al. 2003; Trevaskis et al. 2007; Distelfeld et al. 2009a, b; Shimada et al. 2009; Distelfeld and Dubcovsky 2010; Dı'az et al. 2012). Wild type wheat require extended exposure to cold (known as winter wheat) before the transition from vegetative to reproductive growth while mutants do not require this exposure and are regarded as spring wheat (Fu et al. 2005).

The third class of genes controlling flowering time is earliness per se, also referred to as ear emergence per se, earliness in narrow sense, intrinsic earliness, and at times is called basic development rate (Laurie et al. 2004; Cockram et al. 2007; Shitsukawa et al. 2007; Lewis et al. 2008). A number of similar definitions have been proposed for *Eps*. *Eps* can be defined as the minimum number of days to reproductive growth, after vernalization and photoperiod requirements are satisfied (van Beem et al. 2005). Similarly, Appendino et al. (2003) defined *Eps* as the time to heading after both vernalization and photoperiod requirements are satisfied. Shitsukawa et al. (2007) defined narrow sense earliness or earliness per se as the earliness of fully vernalized plants grown under long days. Lewis et al. (2008) described *Eps* as all other genes controlling flowering time but not involved in either vernalization or photoperiod requirements. The *Eps* definitions suggest that these genes regulate flowering independent of both vernalization or photoperiod environmental cues (Bullrich et al. 2002).

The course and fine adjustment knobs of a light microscope can be used to visualise the role of the *Eps* genes in flowering time (Fig. 39.1). The *Ppd* and *Vrn* genes would be equivalent to the course adjustment knob and are responsible for adaptation to mega environments for example spring and winter wheat as well as short day and long day environments (Worland et al. 1994, 1998). The *Eps* genes are equivalent to the fine adjustment knob (Fig. 39.1) and are responsible for fine-tuning of wheat flowering time (Valarik et al. 2006) within mega-environments (Griffiths et al. 2009) and are responsible for wide adaptation of wheat to different environments (Lewis et al. 2008). Laurie et al. (2004) suggested that *Eps* factors may be largely responsible for the variation in flowering time in crosses within winter or spring types provided they have the same alleles at the major photoperiod and vernalization response loci.

Earliness per se causes differences of a few days in flowering time under field conditions (Valarik et al. 2006; Griffiths et al. 2009; Zikhali et al. 2014). In *Triticcum monococcum*, it has been shown that while the *Eps* effect on chromosome 1A designated *Eps-A^{m}1* causes flowering differences of only a few days at 23 °C, this difference increased to several weeks when the plants were fully vernalized and grown under long days at 16 °C (Appendino and Slafer 2003). In a recent study,

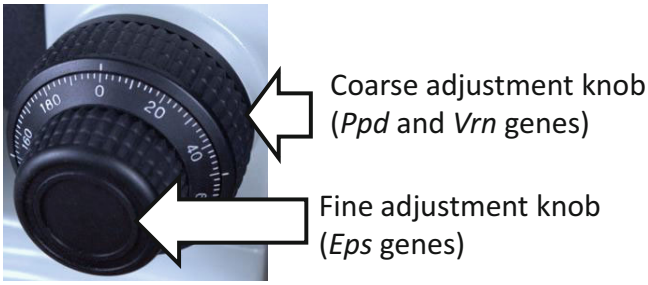

Fig. 39.1 Schematic presentation, using the fine and coarse adjustment knob of the light microscope, of the role of *Eps* genes in flowering time. The coarse adjustment knob represents the role of photoperiod (*Ppd*) and vernalization (*Vrn*) genes in influencing mega environment adaptation while the *Eps* genes adapt flowering within mega environments. Understanding *Eps* genes will enable their manipulation and fine-tuning of flowering time which may enable precision breeding in wheat

Zikhali et al. (2014) it was shown that cultivar Rialto flowers more than 12 days earlier than cultivar Spark in Short days but when grown for 8 weeks under short days and then moved to long days, the *Eps* effect on chromosome 1DL causes Spark to flower 5 days earlier. This result shows that while the overall difference in flowering time is 5 days, the *Eps* effect in Spark also overcomes the earliness conferred on Rialto by the short days prior to moving into the long days. Earliness per se is often considered polygenic (Rousset et al. 2011). Determining the role played by the individual *Eps* genes in each developmental phase may enable breeders to fine tune ear emergence in predictive wheat breeding (Griffiths et al. 2009) and increase wheat yield in different environments (Lewis et al. 2008). To determine the role of an individual *Eps* gene, on different wheat developmental phases requires knowing what the gene is and hence the need for accurate mapping of the gene responsible (Lewis et al. 2008).

Because of their relatively small effect, *Eps* genes were previously mapped only as QTLs in wheat (Miura et al. 1999). However, *Eps* genes have been defined more accurately in the recent years using near isogenic lines (NILs). One *Eps* gene that has been well defined after almost a decade of study is the *Eps-A^{m}1* reported to be on the distal region of *T. monococcum* chromosome 1A^{m}L (Bullrich et al. 2002; Valarik et al. 2006; Faricelli et al. 2010). The gene has been recently reported to be involved in determining the number of spikelets as well as the number of grains per spike in diploid wheat in addition to affecting heading time (Lewis et al. 2008). The genes *MOLYBDENUM TRANSPORTER 1* (*MOT1*) and *FILAMENTATION TEMPERATURE SENSITIVE H* (*FtsH4*) are the suggested candidates for the *Eps-Am1* (Faricelli et al. 2010) although work is in progress to definitively identify the gene responsible. The *Eps-3Am* locus has also been well defined (Mizuno et al. 2012; Gawroński et al. 2014). The *Eps-3Am* QTL interval in *T. monococcum* was fine mapped using high-density mapping (Gawrosnski and Schnurbusch 2012). A recent report suggested a *T. monococcum* ortholog of the *Arabidopsis thaliana LUX ARRHYTHMO/PHYTOCLOCK 1* (*LUX/PCL1*) as a potential candidate of the

Eps-3Am which was suggested to act by distorting the circadian clock (Gawroński et al. 2014).

There are some striking similarities between *Eps-Am1* and *Eps-3Am.* Both *Eps-Am1* and *Eps-3Am* loci were reported to determine the number of spikelets as well as the number of grains per spike in addition to affecting heading time (Lewis et al. 2008; Gawroński et al. 2014). Again both *Eps-Am1* and *Eps-3Am* have been reported to be thermosensitive (Bullrich et al. 2002; Gawroński et al. 2014). This means there is a possibility of manipulating *Eps* genes to increase yield and optimise adaptation. Grain quality can also be improved by manipulating *Eps* loci given that Herndl et al. (2008) showed that *Eps* together with the major genes that control vernalization and photoperiod flowering influence grain protein content.

However, presently there is scant information on the identity of *Eps* genes, and the mechanism of control that these *Eps* genes employ in hexaploid wheat. For instance, it is not certain whether *Eps* genes act independently of environmental cues (Cockram et al. 2007; Laurie et al. 2004; Bullrich et al. 2002), although many reports suggest that this is the case (Lewis et al. 2008; Cockram et al. 2007; Bullrich et al. 2002). Appendino and Slafer (2003) showed that *Eps* genes could respond to temperature. Laurie et al. (2004) underscored the need to study more about *Eps* genes given that little is known about them despite their immense potential in improving plant breeding. This was alluded to by Cockram et al. (2007) who suggested that *Eps* genes were a potential source of variation in targeted breeding given that they were present in both winter and spring crops. The *Hordeum vulgare EPS2* locus on chromosome 2H (Laurie et al. 1995) was also reported to be orthologous with the wheat group 2 loci (Laurie 1997). The candidate gene for this locus has only been recently shown in barley to be a homolog of the *Antirrrhunum* gene *CENRORADIALIS* (*CEN*) designated *HvCEN* (Comadran et al. 2012). Mutations at this gene were shown to cause the wild type indeterminate inflorescence of *Antirrhunum* to terminate into a flower (Bradley et al. 1996). Analysis of the *HvCEN* alleles led to the conclusion that *HvCEN* was important for geographic range extension as well as influencing the gradual separation between spring and winter barley (Comadran et al. 2012). The orthologue of this gene is yet to be identified in the economically important hexaploid wheat.

Following the work done by Griffiths et al. (2009), Zikhali et al. (2014) reported the validation of an *Eps* effect on 1DL in hexaploid wheat (Fig. 39.2). Near isogenic lines (NILs) of a cross between wheat varieties Spark and Rialto grown in the field and controlled environments enabled the QTL on 1DL to be defined as an *Eps* effect (Zikhali et al. 2014). The NILs segregated for heading date both in short and long days (Fig. 39.2) when fully vernalized (Zikhali et al. 2014). Zikhali et al. (2014) reported that *Triticum aestivum FLOWERING LOCUS T 3* (*TaFT3*) was not a candidate for the 1DL *Eps* effect. The 1DL *Eps* locus was reported to be most likely an orthologue of *Eps-Am1* and the genes *MOT1* and *FtsH4* were suggested as possible candidates for 1DL. In addition to *MOT1* and *FtsH4*, the gene *T. aestivum EARLY FLOWERING 3* (*TaELF3*), a circadian clock gene, was also suggested as a possible candidate for 1DL given that it also falls in the QTL interval of 1DL (Zikhali et al. 2014).

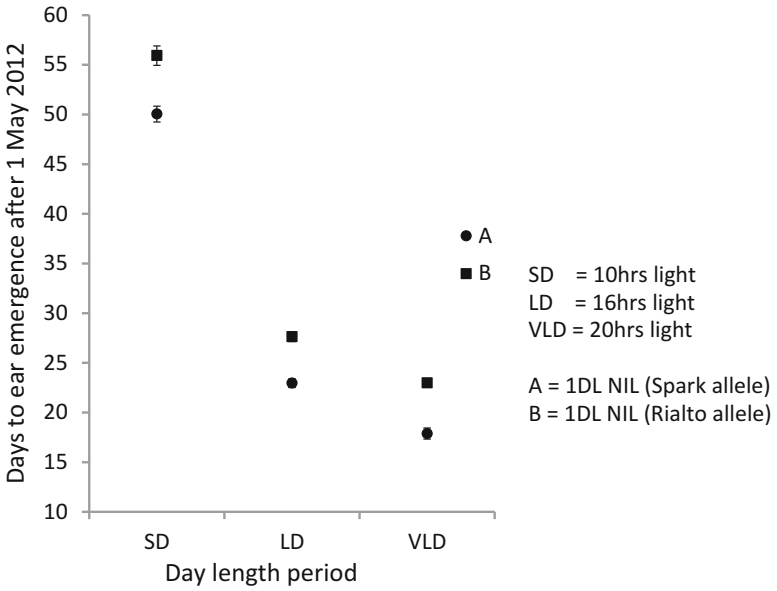

Fig. 39.2 Zadoks growth stage 55 for leading tillers of Spark X Rialto NILs grown under controlled environments. The heading days are the mean of 24 plants for the Spark (A) NIL and 30 plants for the Rialto (B) NIL. The additive effect is about five days in the three photoperiod treatments. Student's *t*-test was carried out for the mean heading days and all the four NILs pairs have a p value <0.0001, which is highly significant. The error bars are the Standard error of the mean (Adapted from Zikhali et al. (2014))

In a nutshell, *Eps* genes are gradually being understood with some QTL loci already cloned like the *Eps-3Am* locus in *T. monococum.* It is also becoming apparent that *Eps* genes may not be independent of environmentally cues as previously understood. For example the *Eps-3Am* locus has been found to have a circadian clock effect, which suggests that this gene responds to photoperiodic changes (Gawroński et al. 2014). Again the thermo sensitivity of both the *Eps-Am1* and *Eps-3A* loci (Gawroński et al. 2014; Bullrich et al. 2002) further suggests that *Eps* genes are not independent of environmental cues. A more accommodating definition of *Eps* would be the variation that is observed in flowering time when both vernalization and photoperiod requirements are fully met without being necessarily independent of environmental cues. The additive effect from multiple *Eps* loci maybe important for wheat adaptation and fine-tuning flowering time.

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Part XI
Toward Sustainable Wheat Production

The Special Session was sponsored by the OECD Co-operative Research Programme on Biological Resource Management for Sustainable Agricultural Systems, whose financial support made it possible for most of the invited speakers to participate in the Special Session.

Chapter 40
Recapitulating the OECD-CRP Session (Sponsored by the OECD's Co-operative Research Program on Biological Resource Management for Sustainable Agricultural Systems)

Masa Iwanaga and Tomohiro Ban

The Special Session was sponsored by the OECD Co-operative Research Programme on Biological Resource Management for Sustainable Agricultural Systems, whose financial support made it possible for most of the invited speakers to participate in the Special Session.

In the specially arranged OECD Co-operative Research Program (CRP) sponsored-session titled '*Sustainable food chain challenging for adaptation and mitigation to the climate change and global food security*', six distinguished scientists in their respective field presented their latest scientific progress. Relevant sectors to the International Wheat Genetics Symposium, the Japanese Flour Millers Association and the Australian Grain Research and Development Corporation (GRDC), presented the actual demands and requirements of the wheat industry in the plenary

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talks. Status and movement of the two international wheat science initiatives, the International Wheat Conference (IWC) and the Wheat Initiative (WI), were introduced and future interactions and compilation necessary to encourage the wheat science society in the area of wheat genomics/post-genomics were discussed. The section, 'Enhancing relevance and efficiency of wheat science for the society' recapitulated the scientific approaches to and the socio-economical relevance/efficiency of wheat science vital to drafting an action plan by the international wheat science society.

Aim of This Session and Its Relation with OECD-CRP

Wheat is the most widely grown staple food crop in the world. IWGS has been promoting the latest research outcomes and discussions on wheat genetics, breeding and biodiversity in order to contribute to the better understanding of the food chain issues. Worldwide food crisis and climate change, which adversely affect the food chain are common threats to all humankind. IWGS has endeavored to lead wheat research for development to save the world from starvation just like what the Green Revolution had done. The OECD members are on the verge of a phase to concentrate the wisdom of science in an attempt to challenge these global threats at the coming of age of wheat genomics. IWGS can open windows for sustaining links of international collaborative research covering the topics under the OECD-CRP research theme 'The Food Chain'.

Wheat is a major renewable resource for food, feed and industrial raw material, and is among the major crops of the OECD countries. It is the most widely cultivated cereal worldwide, covering over 200 million ha of agricultural lands and the second most abundant staple crop for humankind; it is also an important commodity crop for the OECD countries. We should address the challenge to double its yield in the coming half century to meet the food requirement as calorie intake of the growing global population. At the same time, we should preserve the earth environment conserving the biodiversity for sustainability, food hygiene and energy production. G20 Agriculture Ministers met in Paris on June 22nd–23rd, 2011 and agreed to formulate the Ministerial Declaration on 'Action Plan on Food Price Volatility and Agriculture'. It issued a statement to tackle the threats to increasing sustainable wheat production, and to promote the Wheat Initiative (WI) which reinforces synergies between national and international wheat research programs. IWGS will be a great opportunity to launch international discussions and actions towards recommending a direction for food chain policies.

Major Highlights of the Presentations

Part 1: Wheat Research for Sustainable Food Chain for Climate Change and Global Food Security

(Distinguished scientists in their respective field presented their latest scientific progress with perspectives for relevant wheat research and development)

1. Matthew P Reynolds (CIMMYT, Mexico), 'Exploring genetic resources to increase adaptation of wheat to climate change'

   - Seamless flow: Crop design to breeding
   - Whole plant (roots, spike etc.)
   - Genetic resources represent a vast and largely untapped opportunity
   - Innovative phenotyping (roots, air, etc.) for targeted environments is key to effective use of genetic resources
   - Transgenes look promising but need to be evaluated in elite backgrounds
   - Strategic trait-based use of well-characterized genetic resources achieves cumulative gene actions for yield potential (strategic, analytical crop improvement)

2. Beat Keller (Univ. of Zurich, Switzerland), 'Genomic approaches towards durable fungal disease resistance in wheat'

   - Durable resistance (host-pathogen dynamic interactions)
   - Characterization of the molecular basis of resistance gene function and specificity > develop a more durable type resistance based on major genes
   - Gene cloning and transgene research approach
   - Successful field trial (field tolerance)

3. Z. Li, presented by Xueyong Zhang (CAAS, China), 'New progress in wheat wide hybridization for improving the resistance to biotic and abiotic stresses'

   - Great Chinese history of wheat breeding, responding to changing needs such as yield, quality, salt tolerance, drought tolerance, Ug99 (Xiaoyan 6–81, Hexaploids)
   - Wisdom from the past experience for the future (addressing climate change; biotic and abiotic stresses)

     (i) Wide adaptation by germplasm with a wide genetic background (including alien spp. introgression)
     (ii) Extensive nurseries/trials in multi-environments

4. Michael Baum (ICARDA, Jordan), 'Global crop improvement networks to bridge technology gaps'

   - The International Wheat Improvement Network (IWIN) results in the development of both germplasm and human resources
   - It has sustained increase of wheat production & productivity > food security and farmer's livelihood

- Current contribution of IWIN > breeding, genetic gain & future role under difficult circumstances

  (i) New locations/shuttle breeding
  (ii) FIGS (for stem rust, septoria, etc.)
  (iii) Major progress for Ug99 in the region, etc.

5. Mark Sorrells (Cornell Univ., USA), 'Genomic selection in plants: Empirical results and implications for wheat breeding'

   - GS differs from MAS and Association Breeding in that the underlying genetic control and biological function is not necessarily known; complementing other breeding methods
   - GS preserves the creative nature of phenotypic selection to sometimes arrive at solutions outside the engineer's scope
   - Integrating environmental covariates and crop modeling into the genomic selection framework to predict G*E increases prediction accuracy and provides insight into the genetic architecture controlling G*E
   - Most important advantages are reductions in the length of the selection cycle resulting in greater genetic gain per year

6. Rowan Mitchell (Rothamsted Research, UK), 'Wheat genes or enhanced human nutrition'

   - Dietary fibre (DF) in wheat flour is derived from cell wall of the starch endosperm > composed (~70 %) of the polysaccharide arabinoxylan (AX) which is abundant in grasses such a wheat
   - Candidate genes were identified > Glycosyl transferase (GT43, 47, 61) for AX synthesis
   - QTLs for high DF from 'Yumai34' identified
   - Molecular understanding of the AX synthesis (including transgene research) will help to identify the casual alleles of the QTLs > accelerate the introduction of high fibre alleles into modern commercial cultivars.

Part 2: Enhancing Relevance and Efficiency of Wheat Science for the Society

7. Koji Murakami (Nisshin Flour Milling Inc., Japan), 'What does wheat flour industry expect from wheat science?'

   - The Evolution of Grain Quality from the Miller's Perspective
   - Japanese special requirement for wheat is 'Uniformity and Safety' for good product
   - Researchers' voice on supporting public research from the private sectors?

8. Francis Ogbonnaya (GRDC, Australia), 'What does industry seed sector expect from wheat science?'
   - Australian wheat industry > Export focused
   - Research outputs which support and grow market position = reliability of supply
     (i) Volume – yield potential and stability
     (ii) Quality – of the grain and in the grain
     (iii) Yield stability – disease resistance/ temperature tolerance/ Soil toxicity tolerance
     (iv) Reduced input costs > better use of fertilizers (NUE, PUE)
   - Government-Research-Industry-Growers-International linkages
9. Hans J. Braun (CIMMYT, Mexico), 'Improving wheat for world food security: Concerted approach of IWGS and International Wheat Conference (IWC)', and
10. Peter Lngridge (Univ. of Adelaide, Australia), 'Wheat Initiative (WI) for sustainable food chain'
    - Major importance and many challenges (still underinvested!!)
    - Status of the two international wheat science initiatives (IWGS, IWC)
    - Showing the opportunity for further international collaboration (WI) including Working Groups!

Outcomes/Conclusions in Terms of Policy Relevance

The 12th IWGS concluded with perspectives on wheat genetics/breeding including one stating that in-depth disciplinary research should combine with inter-disciplinary approaches and international collaborations for enhanced social impact. Research outputs that support and strengthen market position and increase reliability of supply will be recommended by policy makers who act on Government-Research-Industry-Growers-International linkages. Overall outcomes from the 12th IWGS included new approaches for production of valuable and safe materials and substances for the sustainable production of wheat as a staple food for the world. International researchers exchanged information on their latest researches on wheat genetics, genomics, gene function, evolution, genetic resources, and breeding for sustainable wheat production. Research outcomes on genome sequence information and transgenic plants were discussed. The major highlights distilled common threads, developed a coherent story, and added to the climax of the 12th IWGS focusing on wheat research for a sustainable food chain in the midst of challenging global climate change.

Chapter 41
Exploring Genetic Resources to Increase Adaptation of Wheat to Climate Change

Matthew Reynolds, Maria Tattaris, C. Mariano Cossani, Marc Ellis, Kazuko Yamaguchi-Shinozaki, and Carolina Saint Pierre

The opinions expressed and arguments employed in this publication are the sole responsibility of the authors and do not necessarily reflect those of the OECD or of the governments of its Member countries.The Special Session was sponsored by the OECD Co-operative Research Programme on Biological Resource Management for Sustainable Agricultural Systems, whose financial support made it possible for most of the invited speakers to participate in the Special Session.

Abstract The combined problems of climate change, population growth, and increased demands on a declining natural resource base force scientists to push crop performance to its limits. A powerful strategy is to explore genetic resources to identify promising material that can be used directly in breeding, for gene discovery, and to further understand the mechanisms of adaptation. Initially traits must be defined for stress targets using conceptual models, examples being better root systems to access subsoil deep water and the ability to store and remobilize water soluble carbohydrates from storage tissue. New sources of diversity for such traits can be found in collections such as the World Wheat Collection housed at CIMMYT; for example, Mexican landraces provide good sources of both of these traits. Being a

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polyploid, wheat has a useful secondary gene pool that can be used to re-synthesize hexaploid wheat, while transgenic approaches remove all taxonomic limits to plant improvement. To efficiently explore genetic resources, for crop improvement and to identify genetic and mechanistic bases, requires high throughput phenotyping approaches. For example, an airborne remote sensing platforms is used to determine spectral indices associated with temperature, water content, and pigment composition of leaves via thermal and multispectral imagery. Using the above approaches, best lines are used directly in pre-breeding to combine favorable combinations of traits and their alleles. These approaches have already delivered a new generation of drought adapted lines where cumulative gene action on yield is observed through strategic combination of stress adaptive traits many coming from landraces or products of wide crossing with wheat wild relatives.

Keywords Landraces • Physiological breeding • Phenotyping • Remote sensing • Synthetic wheat

Introduction

The CIMMYT coordinated International Wheat Improvement Network (IWIN) partners with hundreds of wheat breeders worldwide to provide new genotypes (~1,000 annually) to national programs as international public goods, through the following mechanisms (Braun et al. 2010):

- Free exchange of germplasm with all national public and private breeding programs worldwide, including accessions from genetic resource collections.
- Centralized breeding hubs that focus on generic needs – i.e., yield potential, yield stability, genetic resistance to range of biotic and abiotic stresses, consumer-oriented quality traits.
- Distribution of international nurseries specifically targeted to a number of major agro-ecosystems, via national wheat programs worldwide.
- Analysis of international yield trials and free access to all data collected.
- Global disease and pest monitoring to ensure relevance of current local, regional, and global breeding activities.
- Capacity building and training of research partners.
- Regular contact among research partners through consultation, workshops, etc., to help identify the latest technology needs.

Through the IWIN, wheat germplasm has spread continually since the Green Revolution and is now not only extremely well represented in farmers fields of the developing world, but is also commonly seen in the pedigrees of wheat lines in developed countries (Braun et al. 2010). The continued effectiveness of these wheat

breeding strategies have been demonstrated right up the present with yield data from hundreds of testing sites worldwide showing average genetic yield gains of 1 % every year in germplasm targeted to water limited environments (Manes et al. 2012), and 0.6 % per year in fully irrigated environments (Sharma et al. 2012) based on lines distributed since 1995. Germplasm distributed by IWIN is maintained resistant to the full range of diseases that commonly affect wheat (Braun et al. 2010). As a result, resource poor farmers in LDCs are buffered financially from having to apply expensive fungicide, while the environment is protected from the additional agrochemical burden. An important spin off of the IWIN are massive phenotypic datasets that have allowed breeders to identify germplasm with either specific adaptation to local challenges and diseases, or broad (spatial and/or temporal) adaptation to many locations and cropping systems (e.g., Gourdji et al. 2012).

In spite of this achievement, genetic gains still fall short of meeting the predicted demands by 2050 (Rosegrant and Agcaoili 2010), a mismatch that represents a serious challenge for future food security especially in the light of the challenges associated with climate change. One of the best ways to address this challenge from the point of view of genetic improvement is through a more systematic use of genetic resources in breeding. To achieve this requires a series of steps that are outlined in the rest of this paper.

Identify Crop Characteristics Conferring Stress Adaptation

Many crop characteristics have been reported as conferring improved stress adaptation (e.g., Rebetzke et al. 2009; Cossani and Reynolds 2012) and well controlled phenotypic studies can indicate whether these traits may be complementary in different combinations (Reynolds et al. 2007a). However, the only way to confirm their value definitively is to find sources with good expression of the traits and introgress into elite backgrounds. The approach has been successful within the IWIN for producing a new generation of drought adapted lines as will be discussed in the last section. Some of the traits that have proven to be most useful for both heat and drought adaption include (see Richards 2006; Rebetzke et al. 2009; Reynolds et al. 2010):

- Cooler canopy temperature that indicates access to subsoil water under drought and an adequate vascular system –including roots- to match evaporative demand at high vapour pressure deficit typical of heat stress in dry environments.
- Rapid early ground cover to avoid wasteful evaporation of water at the soil surface back to the atmosphere.
- Intrinsic transpiration efficiency that ensures conservative use of water when it is relatively abundant as a water budgeting strategy.

- Epicuticular leaf wax that reflects excess radiation and reduces evaporation from the leaf surface thereby reducing the risk of photo-inhibition and dehydration, respectively.
- Accumulation and remobilization of water soluble carbohydrates in storage organs like stems permitting grain-filling to continue even when post-anthesis stress is too severe to permit adequate carbon assimilation.
- Membrane thermo-stability that can be screened using electrolyte leakage, and chlorophyll fluorescence that, although it has not been used systematically in selecting parents, has become a recent focus for screening.

Exploration of Genetic Resources for Adaptive Traits: Landraces

CIMMYT and other wheat breeding programs have been using landraces to broaden the wheat gene pool for decades (Smale et al. 2002), mostly for disease resistance traits as these are relatively easy to detect in un-adapted material and select for in segregating progeny (Reynolds and Borlaug 2006). More recently Mexican landraces were collected -after approximately 500 years of essentially natural selection in some of Mexico's harshest rain-fed environments-. These were screened for heat and drought adaptive traits and a few of the lines show exceptional characteristics such as deep roots and ability to store water soluble carbohydrates in the stem (Reynolds et al. 2007b). Very recently, over 70,000 accessions of the World Wheat Collection have been screened for adaptation to heat and drought stress -mostly for the first time-, and diverse panels have been assembled for genetic analysis. These include elite durum and bread wheat lines, landraces from hot and dry regions worldwide, and lines derived from interspecific hybridization with wheat's relatives, including 're-synthesized' wheat (see next section).

Other more targeted approaches include characterization of panels identified using agro-ecological data also known as the Focused Identification of Germplasm Strategy or FIGS (http://www.figs.icarda.net/). The principle is that landraces from heat and drought stressed regions are more likely to contain stress adaptive-traits. In the case of drought, we obtained 204 landraces originating from drought-affected areas. These were grown in Cd Obregon, Sonora, under drought conditions (only one irrigation at sowing providing approximately 180 mm of available water to 1.2 m depth, and no significant rainfall during the growing season). CIMMYT varieties were included as checks. Total above ground biomass was estimated and grain yield after threshing (Fig. 41.1).

As shown in Fig. 41.1, lines capable of producing high biomass were identified. Over 45 % of the entries had greater biomass under drought than the CIMMYT check varieties, some with 40 % greater biomass. The harvest index of the lines was lower than that of CIMMYT checks and as a result only 5 % of the entries had

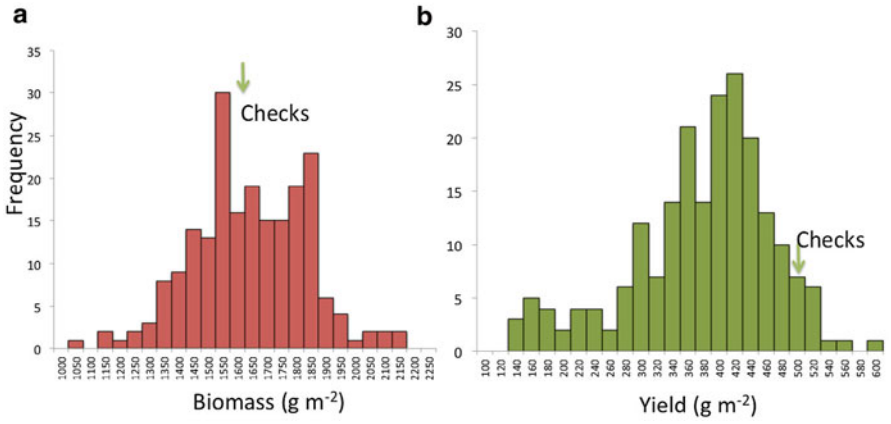

Fig. 41.1 Frequency distribution of 208 FIGS lines grown under drought for (**a**) total biomass (**b**) grain yield. The mean of three CIMMYT checks (Sokoll, Roelfs, Weebil) is indicated by the *green arrow*

greater yield than the CIMMYT varieties. This is not unexpected for landraces: most of the FIGS lines were tall, and some were late-flowering resulting in greater stress during grain filling. These traits would have contributed to the low harvest index of the landraces in addition to not necessarily being adapted to the photoperiod conditions of the experimental environment. The lines that demonstrated the capacity to produce high biomass are being crossed to CIMMYT elite lines as part of the Physiology Group's pre-breeding program. The progeny lines will be selected for semi-dwarf stature and appropriate maturity.

Inter-specific Hybridization to Broaden the Crop Gene Pool

Although the gene pools used in conventional breeding are relatively restricted (Hajjar and Hodgkin 2007), a vast pool of genetic resources is available for breeding. Different gene pools have been defined depending on the difficulty of employing them. The most easy to use are those from the *primary gene pool* represented by germplasm that share a common genome but which have become isolated from mainstream gene pools such as landraces (as discussed in the section above). The *secondary gene pool* is represented by closely-related genomes that can be utilized through inter-specific hybridization, and would include the development of so-called "synthetic" or "re-synthesized" wheat, where tetraploid durum wheat has been hybridized with *Aegilops tauschii*, the ancestral donor of the D-genome, to recreate hexaploid bread wheat (Mujeeb-Kazi et al. 1996). This approach has been successful in introducing disease resistance as well as drought adaptive traits

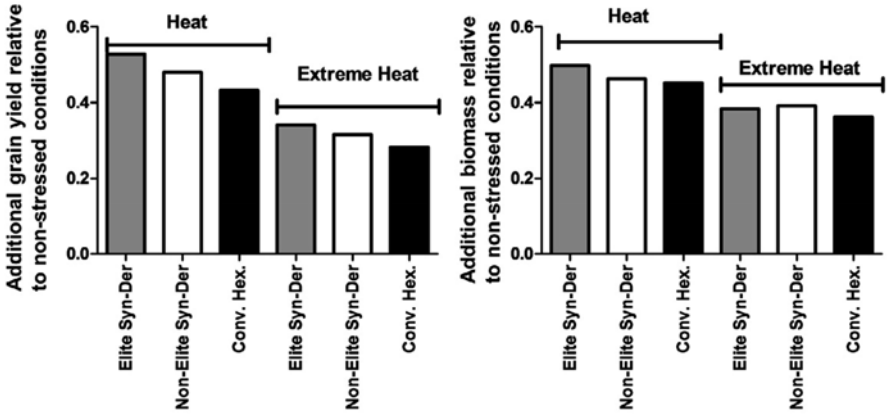

Fig. 41.2 Yield and biomass of synthetic hexaploid wheat derived lines under two different late sown environments expressed as additional yield (*left*) or biomass (*right*) relative to non-stressed conditions, NW Mexico, 2012–2013

(Reynolds et al. 2007b; Trethowan and Mujeeb-Kazi 2008). Many thousands of accessions of both ancestral genomes (AB and D) exist as candidates for interspecific hybridization. Physiological characterization is being used to help select the most promising accessions, as well as to select among the initial products (primary 'synthetics'), for pre-breeding and genetic studies. In parallel to this, genotyping of existing primary synthetics and potential progenitors (AB tetraploid, and *Ae. tauschii*) is being carried out to target under-utilized genetic diversity.

Analysis of impacts from using synthetic wheat in breeding have shown significant contributions to drought adaptation (Lopes and Reynolds 2011), and it appears that the introduction of alleles from the wild D genome have increased the capacity of roots to partition resources to lower soil profiles when experiencing water stress (Reynolds et al. 2007b). A recent analysis looked at the same lines under heat stress in the hot desert of NW Mexico. To generate moderate and more extreme heat stress in the field, fully irrigated crops were sown later than normal in the months of February and March, respectively (normal sowing occurring in December). Results showed highly significant impacts of synthetic derived (SYN-DER) lines in hot, irrigated environments on both yield and biomass (Fig. 41.2).

In general, and across all three environments (temperate, heat and extreme heat), the elite SYN-DER lines (primary synthetic lines backcrossed to a conventional wheat line) showed superiority in terms of performance over lines derived from single crosses to a conventional line or the conventional line itself. However, yield advantages of elite SYN-DER lines over conventional hexaploids were larger in both heat environments than under temperate environments. Biomass at maturity of the elite-SYN-DER lines was also higher than conventional hexaploid lines. The advantages in aboveground biomass were also observed at heading. The SYN-DER

lines also showed larger expression than conventional lines for water soluble carbohydrates in stems and tended to be earlier maturing. These results underline the value of exploring the secondary gene pool of wheat.

Transgenic Approaches

Transgenic approaches remove all taxonomic limits to plant improvement. The manipulation of genes through biotechnology has provided the opportunity of genetic engineering plant responses to abiotic stresses such as heat, drought, and salinity (Qin et al. 2011). The regulated expression of stress-induced transcription factors, for example, has been identified as an attractive tool for improving stress tolerance, since transcription factors can regulate the expression of a large number of relevant downstream genes associated to abiotic stress responses in genetically modified plants (Nakashima et al. 2009). Transcription factors such as DREB1/CBF, DREB2, AREB/ABF, and NAC, are used to improve stress tolerance to abiotic stresses in various grasses including wheat and rice.

Recent efforts to test the functionality of various transcription factors involved in complex physiological responses and to evaluate the effect of these under either constitutive or inducible promoters have been made in wheat. Transgenic wheat lines were developed at CIMMYT using new constructs generated at the Japan International Research Center for Agricultural Sciences (JIRCAS) and Rikagaku Kenkyūjo (RIKEN). The resulting lines were then characterized under glasshouse conditions and open field trials conducted at CIMMYT's subtropical experimental station in Tlaltizapán, Morelos, Mexico (18° 41′ N, 99° 10′ W, 940 m asl). In these open-field trials, physiological traits and grain yield performance of 116 homozygous low copy number GM wheat lines (14 gene-promoter combinations) were evaluated under different water regimes. The lines evaluated in the field showed no pleiotropic effect, nor unpredictable unwanted effects when compared to control lines (Saint Pierre et al. 2012). Based on a 3-year analysis, the most promising lines showed an average increase of yield under drought of 10 % when compared to non-transformed line (var. Fielder).

Even though promising results have been observed, several challenges have yet to be overcome before anticipating a high impact of genetically modified lines on wheat production. Efforts need to address the identification of appropriate gene and gene-promoter combinations, the insertion of the proper transgene in appropriate backgrounds, and field selection strategies. Regulation processes, commercialization, and marketing of genetically modified products are parallel challenges to face. From the technical side, a critical step in the case of genetically modified wheat is that more efficient transformation protocols as well as precise expression systems would need to be defined. The wheat variety Fielder is commonly used in transformation due to its good embryogenesis capacity and regeneration efficiency; however it has lower yield potential than modern elite wheat lines. Then, after insertion

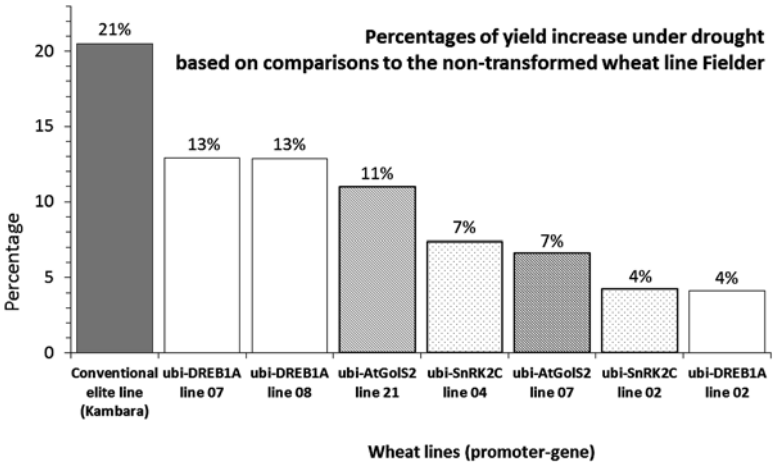

Fig. 41.3 Yield increases as percentage over non-transformed Fielder line, observed for the most promising lines from transformations using different gene-promoter combinations. Field trials were grown during 3-year, in open field trial in Mexico, 2010–2013

of the transgenes in wheat backgrounds (Fielder) it is necessary to transfer them to modern elite lines, which is currently achieved by backcrossing. Predictions based on extrapolating yield increases from the transformed line Fielder to new high performing germplasm (elite lines) should be made with caution. The challenge is, therefore, to break the yield barriers in modern elite wheat lines to finally achieve a significant improvement in wheat grain yield under stress. Collective and cooperative interventions from molecular biologists, physiologists, and breeders are required (Fig. 41.3).

High Throughput and Precision Phenotyping

The efficient exploration of genetic resources -for crop improvement and to identify genetic and mechanistic bases- requires a combination of precision and high throughput phenotyping approaches. Non-intrusive high spatial resolution spectral imagery can be applied to the monitoring of physiological characteristics such as canopy temperature, hydration status, and pigment composition, as well as permitting estimates of agronomic traits such as biomass and yield. Spaceborne remote sensing platforms have proven efficient at measuring some of these characteristics at a field scale, however their spatial resolution proves too low for accurate data retrieval at plot level in a plant breeding context. While ground-based remote sensing is used for predicting physiological and agronomic traits at a plot scale,

temporal variations of environmental variables, such as air temperature, can introduce confounding factors, especially when applied to large trials. Low-level airborne remote sensing platforms overcome these restrictions, allowing for fast, non-destructive screening of plant physiological properties over large areas, with enough resolution to obtain information at plot level while being able to measure thousands genetic resources in the field with one take.

Rapid advances in remote sensing technologies, data processing and availability of instruments has made it easier to implement remote sensing techniques to research numerous plant properties (e.g., Leinonen and Jones 2004; Jones et al. 2007; Berni et al. 2009). The increase in demand for large scale vegetation monitoring means that there is a move to such remote sensing applications in which simultaneous measurements of greater target areas can readily be made. In a recent study, Tattaris et al. (2013) applied a low level airborne remote sensing platform to derive indices relating to plant properties such as canopy temperature, water status, and pigment composition. Sampling was performed with a multispectral camera and thermal camera mounted on an eight rotor unmanned aerial vehicle (UAV) and helium filled tethered blimp. Airborne indices were validated by equivalent indices collected at ground level. These ground-based measurements have already been proven to be linked with yield and biomass (e.g., Reynolds et al. 1994; Aparicio et al. 2000). This strong agreement between methods acts to validate the use of the airborne indices. In addition, significant genetic correlations were found between the airborne indices derived using imagery and yield/biomass, larger than the equivalent correlations between the ground-based measurements and yield/biomass. This is probably because airborne approaches reduce error in two ways. Firstly, simultaneous measurement permitted through airborne platforms avoid the confounding effects associated with environmental drift when measuring plots one at a time with ground-based instruments. Secondly, aerial imaging techniques permit data smoothing through elimination of outlier pixels. These factors give additional confidence to the use of such methodologies in screening genetic resources and breeding progeny at a large scale where thousands of plots are involved.

Strategic Crossing to Achieve Cumulative Gene Action

Trait selection has made continual progress in wheat breeding through incorporating agronomic traits such as height and flowering time, resistance to a spectrum of prevalent diseases, quality parameters determined by end use, and yield based on multi-location trials (Braun et al. 2010). However, current genetic gains are not adequate to meet future demand while climate change threatens to erode these further. Breeding more specifically for heat and drought adaption can help to increase genetic gains (Reynolds et al. 2009). The main objective of strategic trait-based crossing is to accumulate traits that will be complementary for a given target environment (target-traits). Under water-limited situations, traits that improve water

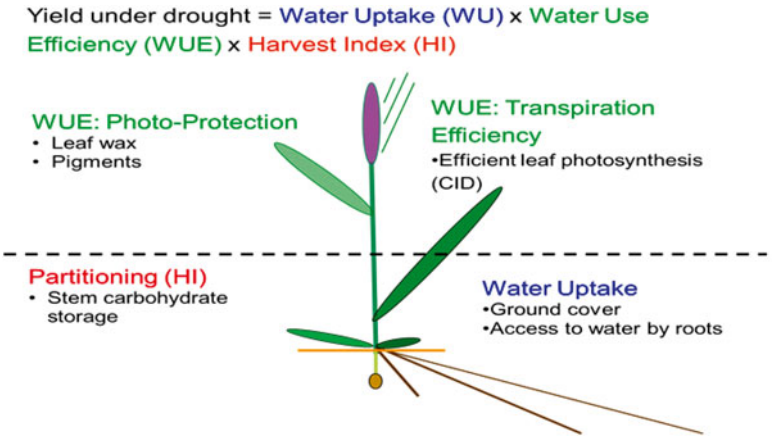

Fig. 41.4 Traits used in strategic crossing to combine complementary drought adaptive traits at CIMMYT since 2005

uptake, water use efficiency and partitioning to yield, respectively, are likely to work synergistically to maximize productivity (Richards 2006). Achieving this in a practical breeding context involves the following interventions, expressed chronologically:

(i) Target traits are identified using conceptual models as described above.
(ii) Genetic resources are screened to provide potential sources of target traits.
(iii) Candidate parents are characterized for traits that may contribute to genetic gains in target environments (Fig. 41.4).
(iv) Crosses are made such that traits expressed by respective parents encompass as many of the target-traits as possible. Top crosses may be used to facilitate the accumulation of traits.
(v) Early generation bulks are screened for integrative traits such as canopy temperature (CT) in relevant environments; families with warm canopies -compared to checks- are mainly discarded.

The focus of much physiological research in the past has been to identify traits for early generation selection (EGS) that can be used to screen progeny for favorable trait expression. There is consensus that the more feasible traits are either genetically relatively simple, or integrative traits that explain a reasonable degree of the variation in yield and are relatively quick and easy to measure (Richards 2006). In fact very few traits meet these criteria and have found application. A successful application in wheat was the use of carbon isotope discrimination (CID) to select progeny with high transpiration efficiency conferring a conservative rate of water use that is associated with increased yield when the soil water profile is restricted (Richards 2006). Another trait that has found application in EGS under a range of

environments is canopy temperature (CT). Under drought, selection for cooler CT permits genetic gains for yield (Olivares-Villegas et al. 2007) and genotypes with cooler canopies have been shown to extract more water from deeper soil profiles (Reynolds et al. 2007b). The trait is measured using infra-red thermometry and is ideal for high throughput screening for the following reasons (i) it is quick and easy to measure, (ii) the technology is inexpensive (currently US$200 for a hand held instrument) and is amenable to airborne approaches as described above, (iii) CT shows robust association with performance; trait expression shows low interaction with growth stage or time of day. The trait is used routinely by CIMMYT's wheat breeding program for rainfed environments to enrich for alleles associated with dehydration resistance. For example, F_3 and F_4 bulks are screened for CT under drought; a larger number of plants -also expressing favourable agronomic traits- are selected from cooler CT families while bulks with warmer CT (compared to checks) are discarded. The development of relatively easy to use spectral radiometers offers another high throughput screening approach for comparing spectral reflectance indices (SRIs) of genotypes.

The result of investment in physiological trait (PT) based crossing has generated advanced lines distributed by CIMMYT as part of the 23rd Semi Arid Wheat Screening Nursery –SAWSN- and the 17th. Semi Arid Wheat Yield Trial (17th SAWYT). Of the 205 candidate genotypes for SAWSN in 2009, 48 (23 %) were derived from PT crossing and a similar proportion were represented in the SAWYT. The PT lines performed well in 2010 with the average yield of the PT lines beating the group of conventionally bred lines at 75 % of the international sites. The most recent products of physiological breeding have shown spectacular results, with 70 % of newest PT (PT-SA) lines beating the drought adapted check Vorobey under drought in Mexico in 2012, some by as much as 30 %. The underlying assumption for the PT strategy is that crosses between parents with different but potentially complementary PT expression will realize cumulative gene action in selected progeny. This has been borne out in previous analyses (Reynolds et al. 2009) and was shown again in the newest PT-SA lines which have landraces, re-synthesized hexaploids, and early PT lines in their pedigrees (Fig. 41.5).

Conclusions

Phenotyping for expression of physiological traits is an efficient way to select among genetic resources for promising candidate parents that can be used to accumulate stress adaptive traits and alleles. High throughput phenotyping approaches can facilitate the process of screening and progeny selection as well as the characterization of mapping populations. Genetic analysis of the latter will ultimately lead to identification of molecular markers that can be used in breeding and mining genetic resources for allelic diversity.

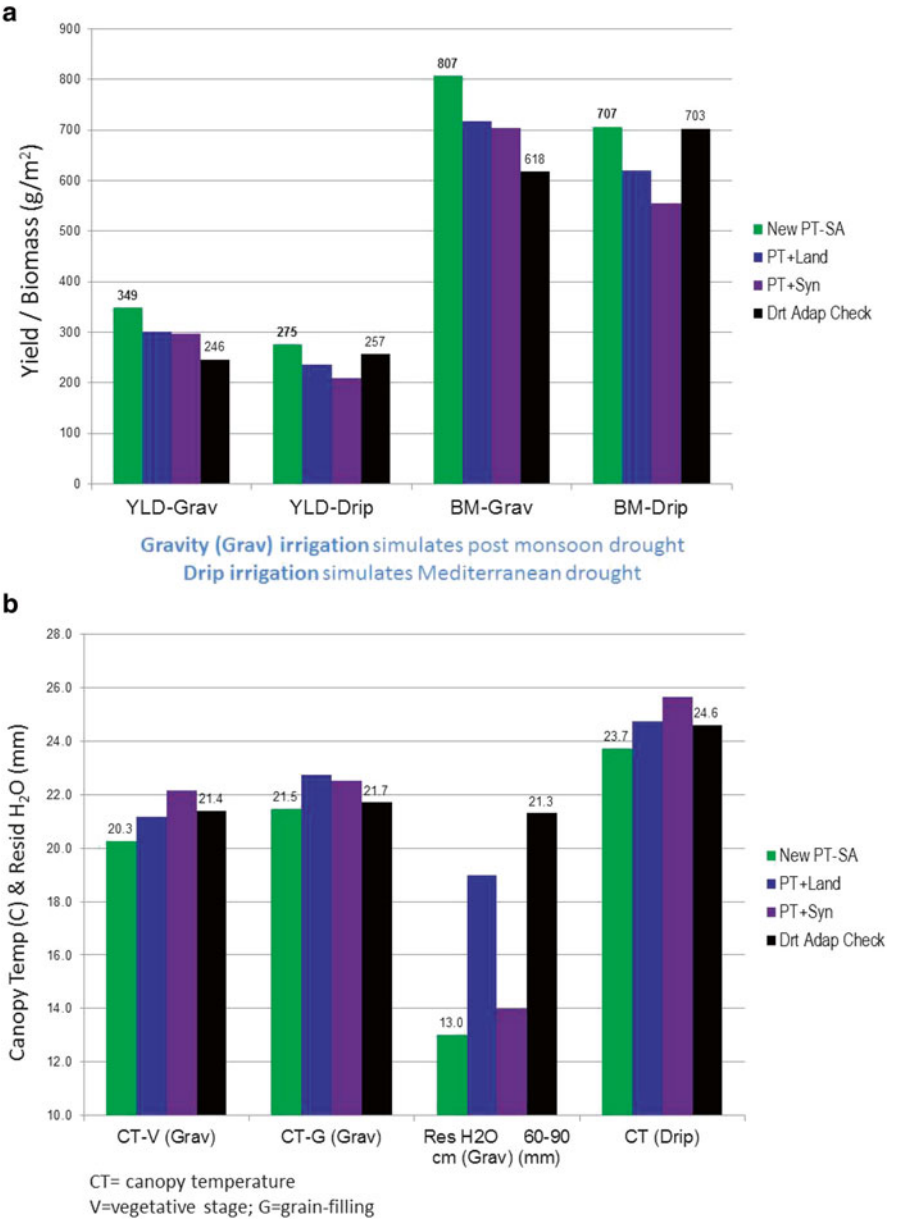

Fig. 41.5 New PT-SA line SOKOLL/3/PASTOR//HXL7573/2*BAU/4/WBLL4//OAX93.24.35/WBLL1 containing PT + synthetic (Sokoll) (PT + Syn) and PT + Mexican landrace (OAX93.24.35) (PT + Land) in its pedigree grown under two distinct drought environments: (**a**) yield and biomass under gravity and drip irrigation simulating post monsoon stored soil moisture, and Mediterranean drought environments, respectively. (**b**) Improved water relations in new PT line showing canopy temperature and residual soil moisture at harvest. Traits are compared with the drought adapted check Vorobey (also with synthetic in its background)

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Chapter 42
Genomic Approaches Towards Durable Fungal Disease Resistance in Wheat

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The opinions expressed and arguments employed in this publication are the sole responsibility of the authors and do not necessarily reflect those of the OECD or of the governments of its Member countries.

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Abstract In the last years there has been enormous progress in the molecular understanding of fungal disease resistance in plants. Research on effector-based immunity which is mediated by major resistance (*R*) genes has been greatly stimulated by the molecular isolation of plant resistance genes as well as the first fungal effectors. In addition, the first genes underlying QTLs or partial disease resistance have been cloned. However, much of this work is still in a phase of basic research and there is a need for translational approaches to realize the globally needed improvements of disease resistance in wheat. In particular, it is essential that future strategies are aiming at achieving durable resistance against pathogens. Durable resistance has been defined by Johnson (Genetic background of durable resistance.

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In: Lamberti F, Waller JM, Van der Graaff NA (eds) Durable resistance in crops. Plenum, New York, pp 5–24, 1983) as a resistance which remains effective in cultivars that are widely grown for long periods and in environments favorable to the disease. In this article we will discuss different molecular strategies towards achieving durable disease resistance in wheat. In particular, our group focuses on the *Pm3* allelic series of race-specific powdery mildew *R* genes and the *Lr34/Yr18/Pm38/Sr57* race non-specific multi-pathogen resistance gene.

Keywords Allele mining • Allele pyramiding • Durable disease resistance • Fungal pathogens • Multiline approach • Pathogen genomics

Genomics in Wheat: New Tools and Resources

New genomic tools have allowed scientists to develop approaches and strategies which are revolutionizing the way specific questions can be tackled in wheat. The availability of partial or complete genomic sequences, although mostly in a non-ordered form, allows for a more efficient characterization of agronomically important genes and to explore their use in classical or transgenic wheat breeding. Map-based isolation of genes relevant for agronomic traits and the study of their allelic diversity have become simpler and faster. In particular, the critical problem of insufficient marker coverage in a targeted region can now be approached more efficiently. In a recent study, we have shown that chromosome sorting performed in the group of J. Dolezel and H. Simkova at the Institute of Experimental Botany in Olomouc, combined with next generation sequencing can be successfully used to efficiently increase the number of single nucleotide polymorphism (SNP) markers in a specific chromosomal region (Shatalina et al. 2013). In this case, the targeted resistance QTL against *Stagonospora* glume blotch is located on chromosome 3B, the only chromosome that can be isolated in pure form because of its size. However, we have recently found that the same approach can also be used in fractions which are not pure but enriched for a specific chromosome, even if only 25 % of the sample consists of the target chromosome (Singla et al., unpublished data). Although we are only at the beginning of exploiting these new tools, there are already a number of applications which are of direct use and that increase the efficiency of resistance improvement in wheat.

Natural Diversity as a Rich Resource for Basic and Applied Wheat Genetics

The study of natural diversity at the molecular level in the form of molecular allele mining does not only allow for the identification of new functional disease resistance alleles, but also for the characterization of the molecular basis of resistance

gene function and specificity. By screening about 2,000 landraces from different geographical regions we were able to increase the number of known functional *Pm3* alleles from seven to 16 (Bhullar et al. 2009, 2010).

Based on the available large dataset of functional and non-functional *Pm3* alleles, we could derive a hypothesis on functionally important amino acids in this protein. In particular, we focused on the nucleotide-binding-site domain (NBS) of the PM3 protein. Alleles with a broad and a narrow resistance spectrum have been described. We found that a broad *Pm3*-spectrum range correlates with a fast and intense hypersensitive response (HR) in a *Nicotiana benthamiana* transient-expression system. This activity can be attributed to two particular amino acids in the ARC2 subdomain of the NBS. The combined substitution of these two amino acids in narrow-spectrum *Pm3* alleles enhances their capacity to induce a HR in *Nicotiana* and, very interestingly, we found that the same substitutions also broaden the resistance spectrum of the *Pm3f* allele in wheat, resulting in an improved version of this gene. These results demonstrate the possibility for improvement of the NBS-"molecular switch" acting in the conversion of initial pathogen perception by the LRR into resistance-protein activation. Thus, we have found a way to enhance the resistance spectrum of an existing gene via minimal targeted modifications in the NBS domain. Ultimately, this might also allow the design of synthetic genes with new specificities and, ideally, to develop a more durable type of resistance based on major genes. The application of such findings could be made with transgenic lines, but given that only very small changes are needed in the protein sequence, new technologies such as transcription activator-like effector nucleases (TALEN) and related approaches might be used for gene editing in wheat.

Natural diversity also reveals mechanism of resistance gene evolution. We have found that the *Pm8* gene derived from rye and present on the frequently used 1RS/1BL translocation in wheat is an ortholog of the wheat *Pm3* resistance gene (Hurni et al. 2013). The finding that orthologous genes have maintained their function against the mildew pathogen over an estimated seven million years revealed a surprising evolutionary stability of powdery mildew resistance gene activity. This is even more surprising given the fact that after the introduction of *Pm8* in cultivated wheat lines around 70 years ago, this resistance was rapidly overcome by the pathogen. Thus, we propose that the evolutionary events might have been quite different in the natural grassland ecosystems before agriculture compared to the modern agricultural environments.

Transgenic Strategies for a More Durable Use of Major Resistance Genes

We have recently explored the transgenic use of the *Pm3* resistance alleles in wheat resistance breeding. We have isolated the *Pm3a–g* alleles from different wheat lines and transformed them into the wheat genotype Bobwhite S26 under control of the maize ubiquitin promoter. A large field trial performed in Switzerland in the years

2008–2010 has shown that most of these alleles confer improved resistance to powdery mildew when overexpressed (Brunner et al. 2011). In particular, it is noteworthy that some transgenic lines had an improved resistance compared to the donor line with the same gene, indicating that overexpression can improve resistance activity.

Moreover, resistance was improved when transgenic lines with different functional *Pm3* alleles were mixed in the field (Brunner et al. 2012). This so-called multiline approach has been classically used in agro-ecosystems. However, the lines were never completely isogenic as the different resistance genes were introduced into the same genotypes by backcross breeding, resulting in relatively large chromosomal segments from the donors. Using a transgenic approach, we established true isogenic lines in the same genotype for a number of different *Pm3* alleles, with different race spectrum of resistance to wheat powdery mildew. We found a clear improvement of resistance in mixed stands containing lines carrying two different *Pm3* alleles, demonstrating the effectiveness of this approach.

The availability of many *Pm3* alleles in transgenic form also allowed us to pyramid two alleles in the same genotype. Whereas in classical breeding alleles can only be combined temporarily in F_1 hybrids, the transgenes have inserted at different locations in the genome and can therefore be combined in a stable, homozygous form after crosses and selection for the presence of two or more alleles in the same plant. We are currently studying the different double homozygous lines for several allelic combinations. We find situations of the expected additivity of gene function, but also interference. The molecular analysis of these lines is ongoing and promises to give fundamental new insight into resistance gene function.

Although the three approaches (overexpression, multilines, gene pyramidization) described above for the transgenic use of major *R* genes are still in an early phase in relation to applied wheat breeding, they show the potential of such new strategies. In particular it should be considered that wild relatives of crop plants hold great gene/allelic diversity for resistance. It is often difficult and very time-consuming to transfer genes from wild species into crops by classical breeding: it can take decades to derive agronomically useful material from such introgressions. Transgenic technology, together with a more rapid identification of the relevant genes in the wild germplasm, provide a promising way to use the natural diversity more efficiently and to rapidly develop pre-breeding material for further use.

Molecular Studies on the Durable Wheat Resistance Gene *Lr34/Yr18/Pm38/Sr57*

Lr34/Yr18/Pm38/Sr57 (subsequently referred to as *Lr34*) is a single wheat gene that confers durable and partial adult plant resistance against the four biotrophic diseases leaf rust, stripe rust, powdery mildew and stem rust. This phenotype is also referred to as slow-rusting or slow-mildewing and is linked to leaf tip necrosis, a

morphological marker associated with senescence-like processes. *Lr34* has been extensively used in wheat breeding for more than a century and no pathogen adaptation has been observed so far. Only a few genes with a similar phenotype have been identified in wheat, namely *Lr46/Yr29/Pm39* and *Lr67/Yr49/Pm46/Sr55*. *Lr34* encodes for an ATP-binding cassette (ABC) transporter protein (Krattinger et al. 2009). Members of this conserved protein family transport various substrates across biological membranes. The resistant *Lr34* allele, which differs by only two amino acid polymorphisms from the susceptible *Lr34* version, evolved after the domestication of hexaploid bread wheat 8,000 years ago through the acquisition of two gain-of-function mutations (Krattinger et al. 2013). An *Lr34* ortholog is absent in the closely related cereal barley. We therefore stably transformed the *Lr34* gene under its native promoter into barley cultivar Golden Promise (Risk et al. 2013). Interestingly, the gene conferred resistance against barley leaf rust (*Puccinia hordei*) and barley powdery mildew (*Blumeria graminis* f.sp. *hordei*), pathogens that are specific to barley and that do not infect wheat. In contrast to wheat, where *Lr34* confers resistance only in adult plants, resistance in barley was already observed at the seedling stage. We also observed a strong leaf tip necrosis phenotype that was already visible in seedlings and that had a negative impact on plant vigor and seed setting. These results demonstrate that *Lr34*-resistance is transferrable to other cereals. However, tight control of *Lr34* expression is necessary to avoid negative impact on yield. The use of different, tissue- or age-specific promoters might allow generating barley plants with adequate levels of resistance and no impact on yield.

New Tools for Resistance Breeding Based on Pathogen Genomics

It is known that resistance which is based on *R* genes or quantitatively acting genes can result in a yield penalty because of the associated costs of resistance. Therefore, new approaches based on completely different resistance mechanisms should also be considered and explored actively. One new strategy might be host-induced gene silencing (HIGS) which is based on RNAi and relies on a process where the presence of an RNAi construct in wheat would result in specific gene silencing in the pathogen. The success of this strategy has already been shown in transient assays (Nowara et al. 2010; Pliego et al. 2013). This approach is not based on endogenous resistance genes in the wheat host, but on sequence information from potentially relevant genes of the pathogen. Essential genes in the pathogen might be targeted by RNAi constructs expressed in wheat, resulting in down-regulation and ideally quantitative resistance against the specific pathogen species. In our group, we have recently developed the necessary genomic tools for such approaches in the wheat powdery mildew pathogen. The complete genomic sequence of the wheat powdery mildew genome is now available and can be used for such pathogen-based resistance strategies (Wicker et al. 2013).

Conclusions

The approaches described in this contribution are both based on classical as well as transgenic tools to improve disease resistance breeding in wheat. The transgenic approaches will critically depend on public acceptance and a predictable, efficient regulatory framework. Given the enormous challenges for wheat production in the next decades, it is essential that the wheat scientists globally promote and explain to the public the use of classical and novel tools in breeding.

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Chapter 43
Review and New Progress in Wheat Wide Hybridization for Improving the Resistance to Biotic and Abiotic Stresses

Zhen-Sheng Li, Bin Li, Qi Zheng, and Hongwei Li

The opinions expressed and arguments employed in this publication are the sole responsibility of the authors and do not necessarily reflect those of the OECD or of the governments of its Member countries.

The Special Session was sponsored by the OECD Co-operative Research Programme on Biological Resource Management for Sustainable Agricultural Systems, whose financial support made it possible for most of the invited speakers to participate in the Special Session.

Abstract Since 1956, our group has been working on the wide hybridization between wheat and tall wheatgrass (*Thinopyrum ponticum* Liu & Wang, 2n = 70). During the past 56 years, we developed a set of partial amphiploids (octoploids), addition lines, substitution lines, translocation lines. A series of wheat cultivars named as Xiaoyan, such as Xiaoyan 4, 5, 6, 54, 60 and 81 were released. They generally have multiple disease resistance and good adaptability to various environments. These traits might derive from tall wheatgrass, especially with respect of resistance to biotic and abiotic stresses. In this paper, we briefly review the history of this study focusing on research background, alien parental selection, establishment of breeding procedure, new germplasms and their application, as well as the newest varieties and their performance to biotic and abiotic stresses.

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Keywords Wheat breeding • Tall wheatgrass • Translocation

Research Background

In 1951–1955, our group worked in Beijing engaging in forage research. Because the national development need, we moved to the Northwest, Yangling, Shaanxi in 1956, where is one of the major wheat production area of Huanghuai winter wheat zone in China.

At that time, the local wheat production was just suffering a serious damage caused by stripe rust (*Puccinia striiformis* f. sp. *tritici*). Nearly all of the local varieties at that time lost their resistance because of appearance of race, CY1. In general, the disease caused about 20–30 % yield decline. The total loss in northern China was about 6 Mts each year (Zhuang 2003).

To search a novel way for wheat breeding with persistent resistance to disease, we began the research of wide hybridization between wheat and wild Triticeae species. Twelve grass species were crossed with common wheat. Three of them were successfully hybridized with wheat. They were *Agropyron elongatum* (*Thinopyrum ponticum* Liu & Wang, 2n = 70), *Agropyron intermedium* (*Th. intermedium* Dewey) and *Agropyron trichophorum* (*Th. trichophorum* Lōve). The best one was *Th. ponticum* based on the performance of their vigorous.

The Establishment of Breeding Procedure

Common wheat was used as female parent, tall wheatgrass as male parent in cross. The F_1 hybrids were very like the tall wheatgrass, but almost all of them were male sterile. Therefore, they were back-crossed with common wheat. BC_1F_1 were becoming wheat-like gradually, but it was not enough, hence they were continually back-crossed with common wheat. The BC_2F_1 individuals were self-crossed. Then, through selection and identification, four types of hybrids were obtained, including partial amphiploids, addition lines, substitution lines, translocation lines (Fig. 43.1; Li et al. 1960, 1962, 1977, 1990; Zhang et al. 1992, 1996).

It is well known that the partial amphiploids usually have middle characteristics between wheat and tall wheatgrass. It could not be used directly in wheat production. Alien addition lines are unstable. Alien substitution lines usually convey no favored characters besides the wanted ones. Therefore, the translocation lines were the final objects in wide hybridization breeding. The translocated small segments can be easily integrated into wheat genome. Hence, on second stage of research work, we focused on the application of translocation lines in wheat breeding. We developed three batches new wheat varieties. Their major characteristics, particularly resistance to biotic and abiotic stresses, planted regions, and the actual effects are introduced below.

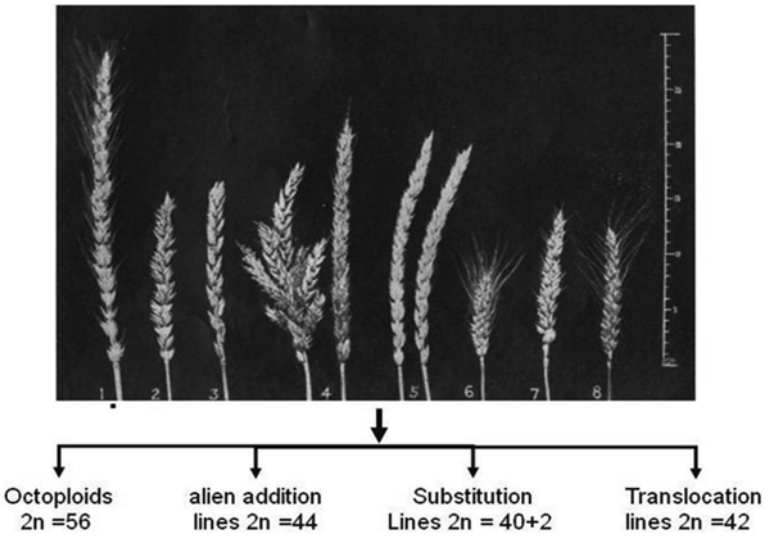

Fig. 43.1 Four types of hybrids derived from cross between wheat and tall wheatgrass

The First Released Variety, Xiaoyan 6

During early 1980s, we developed the first batch of new wheat varieties, named Xiaoyan 4, 5 and 6, respectively. Xiaoyan 4 and 5 passed the local variety registration and released in Shaanxi province while Xiaoyan 6 passed the national registration and released in 10 provinces (Li 1986).

Xiaoyan 6 has durable rust resistance and wide adaptability to various environments. So, it was released in all Huanghuai winter wheat area. Specially, as one of major wheat varieties, Xiaoyan 6 was cultivated for 16 years (1980–1995) in Shaanxi province, accumulatively cultivated about 10 Mha. Xiaoyan 6 was also used as a founder genotype in wheat breeding; more than 50 new varieties were created from its hybrids with other varieties in China. Its derivatives were cultivated more than 20 Mha accumulatively.

Favorable Characters Support the Sustainable Use of Xiaoyan 6 in Production and Breeding

Broad-Spectrum Resistance to Yellow Rust In 1950s, the yellow rust was frequently epidemic in winter wheat regions of northern China. New rust races created constantly. Up to the 1980s, eight physiological races of yellow rust had been identified. Because of their epidemic, nearly all of wheat varieties at that time lost their

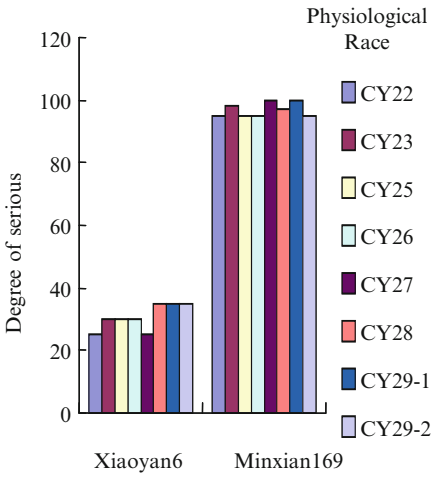

Fig. 43.2 Reaction of Xiaoyan6 to yellow rust races

resistance. In general, the disease caused about 20–30 % loss of yield. In the most serious year, the total loss in northern China was about 6 Mt (Wang et al. 1995).

Artificial infection of 8 yellow rust races (CY22, CY23, CY25, CY26, CY27, CY28, CY29-1 and CY29-2) on Xiaoyan 6 and Mingxian 169 (CK) showed that Xiaoyan 6 resisted to all of the tested epidemic races of yellow rust pathogens during 1980–1990s in North China. Xiaoyan 6 showed characters of a typical slow-rusting resistance variety, such as longer latent period, lower disease severity, and less loss of kernel weight (Fig. 43.2; Wang et al. 1996).

Higher and Stable Yield In 1979 trials test, grain yield of Xiaoyan 6 was 5.96 t/ha in average, boosting 15–31.9 % than the control Zhengyin 1, Fengchan 3, and Abodenza in Shaanxi province. In 1980 yield trail, it had 8.5–28 % more yield than the same controls. So, it easily passed the local and national registration of new cultivars.

Good End Use Quality Xiaoyan 6 has not only higher and stable yield, but also good flour quality. It is suitable for making both bread and Chinese favorable traditional foods, such as Chinese noodles, dumplings, steamed bread, and other traditional cakes (deeply fried dough cake) (Wei et al. 2000; Zhang et al. 2002).

The Second Released Variety, Xiaoyan 54

In the late 1980s, my group returned to Beijing. The winter temperature in Beijing was lower than Yangling, Shaanxi. In the new environment, new natural variations were found in Xiaoyan 6 population. Through systematical selections, Xiaoyan 54 was bred, which has two important characteristics. First, it was tolerant to drought

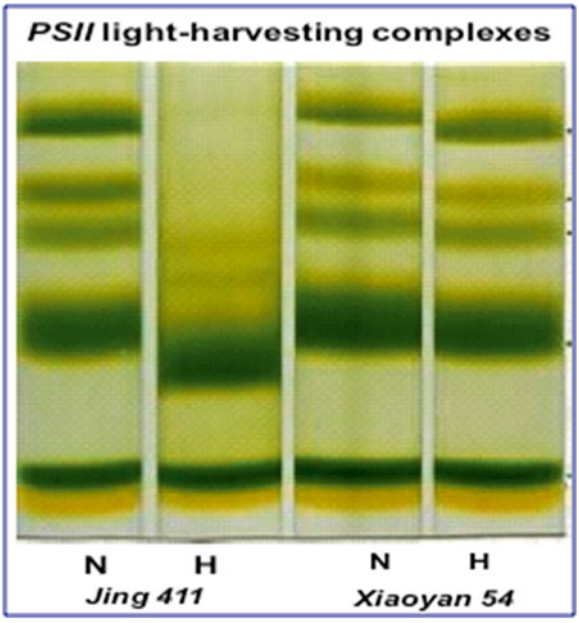

Fig. 43.3 After highlight treatment, the PSII light harvesting protein complexes keep intact in leaves of Xiaoyan 54, but it was damaged in Jing 411. *N* normal light, *H* high light

stress in rainfed farming. In 1998–1999, the annual rainfall declined about 40 %, only 317 mm in Guanzhong, Shaanxi province. The grain yield of Xiaoyan 54 was 3.9 t/ha, while the local variety, Shaan 354 was 1.18 t/ha. The WUE of Shaan354 was 0.25 kg/mm; however, Xiaoyan 54 was up to 0.8 kg/mm, which is 120 % of the control Shaan354 (provided by Huying Yi, Institute of Soil and Water Conservation, CAS & MWR).

Second, Xaioyan 54 is tolerant to high light stress (Fig. 43.3, Wang et al. 2000; Yu et al. 2001; Yang et al. 2006). After being stressed by high light, the PSII light-harvesting protein complexes in the leaves of "Xiaoyan 54" retain intact, but in Jing 411, they were partially damaged.

The Third Released Varieties, Xiaoyan 81 and 60

In order to meet the development of the Bohai Economic Zone, since 1990s we initiated a new breeding program, salt tolerant wheat breeding. Two new varieties, Xiaoyan 81 and Xiaoyan 60 were bred. The trail experiment showed that Xiaoyan 81 was more tolerant to salt than other varieties (Soil salt total content was almost as high as 0.3 %) in 2007. At CAS-Nanpi experimental station, Hebei province, the total soil salt content is about 0.2 %. Xiaoyan 60 grew much better than the local cultivar, Jimai 32. It also created much higher yield than the CKs in 2 years experiment on the salted land (Table 43.1).

Table 43.1 Yield performance of Xiaoyan 60 in salted field

Time	Test site	Yield (t/ha)			Increase yield (%)
		Xiaoyan 60	Jimai 32 CK1	Jinyin 32 CK2	
2012	Dry land (Haixing Country)	3.44	2.82		22
2013	Dry land (Haixing Country)	5.06	4.11		23
2013	Irrigated land (Jinghai Country)	8.52		6.47	27

The breeding success above mentioned made us confidence to further strengthen wild hybridization work. Ten years ago, our group repeated the original crossing work between wheat and tall wheatgrass that Li and his group did 50 years ago in Northwest Institute of Botany, CAS and used the available partial amphiploids developed in 1960–1980s to create a large number of new translocation lines. The following is some new results in the application of partial amphiploids.

The Application of Partial Amphiploids

In early 1980s, we developed some partial amphiploids, such as Xiaoyan (XY) 68, 693, 784, 7430, 7431 etc. (Fig. 43.4). Their genomes included three types which are AABBDDEE, AABBDDStSt and AABBDD + (E & St heterozygous) (Zhang et al. 1996). So, their characteristics are different. For example, in the spring of 2012, we had an opportunity to cooperate with Prof. Zakkie Pretorius, University of the Free, South Africa, to verify resistance of these partial amphiploids to stem rust Ug99. The XY68 and XY7430 showed immune, XY784 performed high resistant to Ug99. The local cultivar Fadkvz was used as control (Fig. 43.5; Table 43.2).

In addition, we also carried out the resistance identification of partial amphiploids to yellow rust, powdery mildew and tolerance to salt. So, my research group repeated the crossing work between wheat and partial amphiploids. Through the radiation treatment to their hybrid offspring, we have obtained more than 200 new translocation lines. Their cytological verification by GISH and FISH were carried out (Fig. 43.6). All of the translocation lines will be used in the multiple character identification and breeding in next stage.

Synthesis and Conclusion

Looking back 60 years work of wheat wide hybridization breeding, we believe that it has a huge potential and worth continuing, specifically in the aspect of improving wheat wide adaptability to biotic and abiotic stresses. Now climate change has been bringing various disasters on global wheat production. In China, wheat production

Fig. 43.4 The five partial amphiploids developed in 1980s at Yangling, Shaanxi

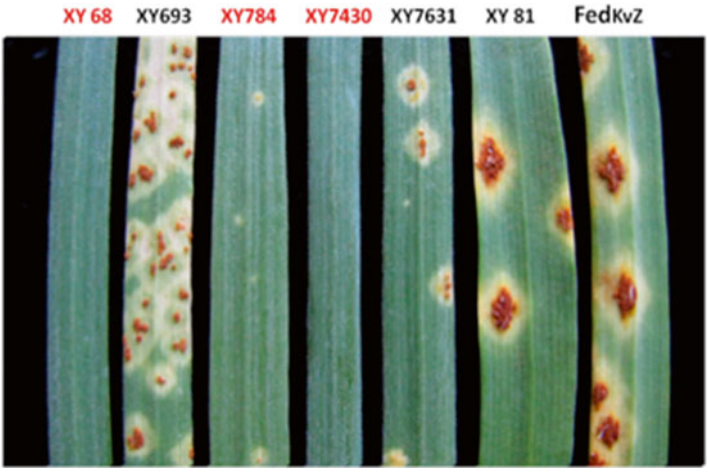

Fig. 43.5 Partial amphiploidsXY68 and XY7430 showed immune, XY784 high resistant to stem rust *Ug99*. The local variety Fadkvz was used as control

Table 43.2 Reaction of wheat-*Th. ponticum* partial amphiploids to *Ug99*

	XY68	XY693	XY7430	XY784	XY7631	Fed/Kvz	Satu	LCSr24
TTKSF	;	3p2,1p3	;	;1=	1	1	;	2
TTKSP	;	2p2,2p3	;	;1=	3p2+,1p3	1	;	3++
PTKST	;	3p1,2p2+	;	;1=	3p1,1p2	4	;	3=
TTKSF+	;	1	;	;1=	1p1,2p2	1	;	2

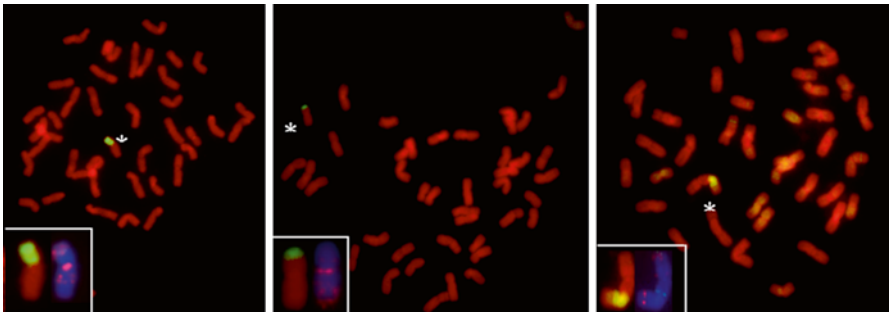

Fig. 43.6 New wheat-*Th. ponticum* translocation lines; whole arm translocation (*left*), small fragment translocation (*middle*) and insertion translocation (*right*)

is frequently threatened by drought, frost, logging, diseases and pests, which cause unpredictable losses. How to face these challenges?

Based on what we have experienced in the past years, it can be summarized into two strategies. The first is to develop more new germplasms with various genetic backgrounds, for example, using the alien species cross and backcross with common wheat to transfer useful traits to wheat to broaden its genetic basis. The second is to breed wide-adaptability and multi-resistant wheat varieties. The effective methods are establishing the multi-environment field trials and the multi-disease resistance verification facilities. To promote multi-environment verification of disease resistance and adaptability, national and international joint research is a very important strategy. Though the wheat varieties with wide-adaptability and multi-resistance are unable to overcome various disasters completely, they can reduce losses of yield and ensure relatively stable harvest.

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Chapter 44
Global Crop Improvement Networks to Bridge Technology Gaps

Michael Baum, Wuletaw Tadesse, Miloudi Nachit, Osman Abdalla, Sanjaya Rajaram, Ravi Singh, Tomas Payne, Karim Ammar, Alex Morgounov, and Hans Braun

The opinions expressed and arguments employed in this publication are the sole responsibility of the authors and do not necessarily reflect those of the OECD or of the governments of its Member countries.

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Abstract The International Wheat Improvement Network (IWIN), an alliance of national agricultural research systems (NARSs), International Maize and Wheat Improvement Center (CIMMYT), International Center for Agricultural Research in the Dry Areas (ICARDA), and advanced research institutes (ARIs), continues to deploy cutting-edge science alongside practical multi-disciplinary applications, resulting in the development of germplasm that has made major contributions during the Green Revolution. The continuous supply of improved germplasm for nearly half a century has also enabled developing countries to have a sustained increase of wheat production and productivity and thereby improving food security and farmers'

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livelihoods. Wheat production levels have increased from 235 million tons in 1961 to 691 million tons in 2012. Yet, global food consumption has exceeded production for 6 of the last 11 years (2004–2010), and food reserves are now 'dangerously low,' particularly for staple grains such as wheat and maize. Changing diets, urbanization, and other factors mean that demand for wheat is likely to only multiply further, and therefore wheat yields must increase from the current global average of 3 t per hectare. According to some estimates, the global wheat production must increase at least by 1.6 % annually to meet a projected yearly wheat demand of 760 million tons by 2020. In the year 2050, the world population is estimated to be nine billion and the demand for wheat reaches more than 900 million tons. Fulfilling this demand is very challenging with the current scenario of climate change, increasing drought/water shortage, soil degradation, reduced supply & increasing cost of fertilizers, increasing demand for bio-fuel, and emergence of new virulent diseases and pests. This paper presents a review and insight about the past and current contributions of IWIN, breeding progresses and genetic gains, and its future role in offsetting the major global challenges of wheat production.

Introduction

Wheat is one of the leading cereal crops which have provided daily sustenance for a large proportion of the world's population for millennia. According to FAO (2012), about 651 million tons of wheat was produced on average of 217 million ha with productivity level of 3.1 t ha^{-1}. The Central and West Asia and North Africa (CWANA) region produces more than 100 million tons of wheat in a total area of 55 million hectares at a productivity level of 2 t/ha which is less than the worlds average (3 t/ha) (Fig. 44.1).

Most of the wheat production in the developed world is rainfed while in the developing world, especially in the large producers India and China, more than half of the wheat area is irrigated. Wheat productivity varies not only between irrigated and rainfed production systems but also between countries applying similar agronomic practices. For example, among major rainfed wheat producers, the average national yield ranges from about 0.9 t ha^{-1} in Kazakhstan to 2.6 t ha^{-1} in Canada and up to 7.9 t ha^{-1} in the United Kingdom (FAO, 2005). Among major irrigated wheat

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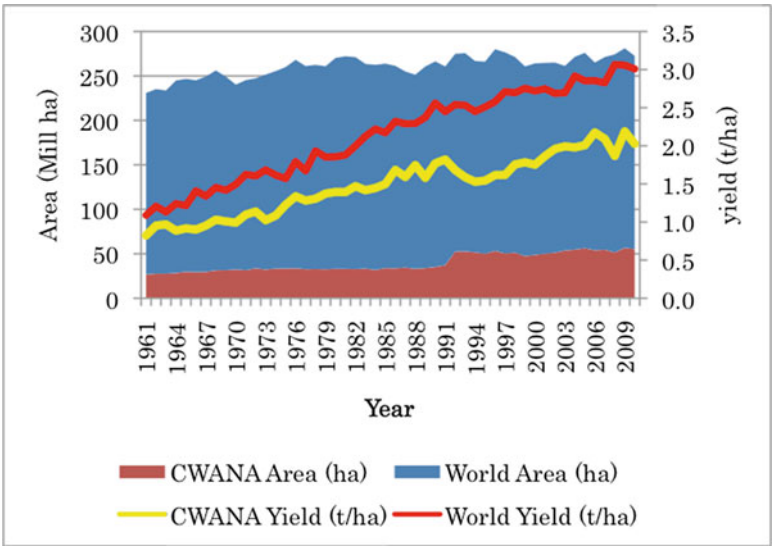

Fig. 44.1 Production area and yield of wheat in the world and CWANA, 1961–2010

producers, India has an average yield of 2.6 t ha^{-1} compared with 6.5 t ha^{-1} in Egypt. This clearly indicates the possibility of increasing wheat productivity in many countries by improving their respective wheat production packages.

The low productivity of wheat in the developing countries especially those in the CWANA region is due to abiotic stresses (drought, cold, heat, salinity) and biotic stresses (yellow rust, leaf rust, stem rust, root rots, Russian Wheat Aphid, Barley Yellow Dwarf Virus, Sunn pest, and Hessian Fly). Principally, drought and yellow rust are the most important wheat yield limiting factors. With the current climate change, it is anticipated that new pests and diseases will emerge as already exemplified in the recent epidemics of yellow rust across the CWANA region and Ug99 epidemic in East African countries. The effect of climate change is also evident on the quality of wheat as increased heat results in shriveled wheat grains.

The International Wheat Improvement Network (IWIN), which is an alliance of International Research Centers (CIMMYT & ICARDA), national agricultural research systems (NARSs) and ARIs, has contributed significantly to the development of germplasm that has made major contributions to the green revolution and to improving food security and farmers' livelihoods in many developing countries (Dixon et al. 2009). In this specific instance, it is noteworthy to mention Dr. Norman Borlaug, who has developed the semi-dwarf input responsive wheat cultivars for the Green Revolution in Mexico, India, Pakistan, and Turkey and many other developing countries.

Starting from 2011, CIMMYT and ICARDA are implementing the WHEAT Consortium Research Program (CRP WHEAT) which is part of a concerted effort of the Consultative Group on International Agricultural Research (CGIAR) to implement a new, results-oriented strategy through a series of CRPs that fully exploit the potential of international agricultural research for development to

enhance global food security and environmental sustainability. WHEAT draws on and potentiate the capacities and commitment of two leading international centers (CIMMYT and ICARDA), in partnership with farming communities, national research systems, advanced research institutes, private companies, policy makers, and diverse development organizations.

Major Objectives of IWIN

The general objective of IWIN is to enhance the productivity, yield stability, and end-use quality of wheat production at global level with major emphasis in the developing world.

Specific objectives:

- Develop high yielding and disease and pest resistant wheat genotypes with acceptable grain quality for irrigated and rain-fed production systems
- Identify germplasm for heat and salt tolerance
- Identify, map and pyramid major genes and QTLs for durable disease resistance and drought and heat tolerance
- Distribution of improved germplasm to NARS through the International wheat nursery and system
- Build capacity of NARS through long term and short term training in wheat breeding/genetics

Breeding Methods and Approaches

The wheat breeding program at CIMMYT and ICARDA applies both conventional and molecular breeding approaches and techniques in order to develop high yielding and widely adapted germplasm with resistance/tolerance to the major biotic and abiotic constraints prevailing in the developing world. Some of these strategies and techniques include classification of Mega-Environments (ME) and assembling of targeted crossing blocks, shuttle breeding, utilization of doubled haploids (DH) and marker assisted selection (MAS), key location yield trials, distribution of germplasm to NARS through international nurseries, and partnership & capacity building of NARS (Rajaram et al. 1995; Ferrara et al. 1987; van Ginkel et al. 2002; Ortiz et al. 2007; Tadesse et al. 2012a). As water is becoming scarce even in the irrigated areas, IWIN's germplasm development approach is to identify genotypes with disease resistance, high yield potential and water use efficiency so that wheat genotypes targeted for irrigated areas can cope with temporary drought periods. Similarly, this approach enables to minimize and maximize yield gains during drought and good seasons, respectively, for the rain fed production system.

Principally, high yielding and adapted hall mark wheat cultivars representing each MEs, synthetic wheats and elite lines from CIMMYT/ICARDA breeding

programs are used as parents. Physiological and molecular screening techniques in order to increase rates of genetic gains through (a) strategic trait-crossing to combine complementary traits in progeny, (b) high–throughput phenotyping to enrich for desirable alleles in intermediate generations and (c) exploration of genetic resources to broaden the genetic base for hybridization (Reynolds and Tuberosa 2008). Marker-assisted selection using recommended diagnostic markers is used in order to characterize new parental materials for disease resistance genes (yellow rust, leaf rust, stem rust, nematodes); insect resistance (Hessian fly and Russian Wheat Aphid), phenological traits such as photoperiodism (*Ppd*), vernalization requirement (*Vrn*); plant height (*Rht*), grain hardness and other genes (Gupta et al. 1999; Tadesse et al. 2012a). Diagnostic markers are also used for gene pyramiding in the F_2, F_1 top, and BC_1 F_1 populations (William et al. 2007). Segregating generations and fixed genotypes are evaluated in key locations using a shuttle breeding approach in order to develop disease resistant, high yielding, widely adapted and photoperiod insensitive genotypes within a short period of time.

Germplasm Distribution and Capacity Development

Based on request, different forms of germplasm such as genetic stocks for crossing bloc, segregating generations and finished (fixed) genotypes have been distributed globally on annual basis through the International Nursery system from CIMMYT and ICARDA. The genetic stocks and the segregating generations were sent with the objective of decentralizing the breeding program and creating genetic variability; while the fixed materials were sent with the objective of releasing adapted genotypes as varieties. The experience obtained so far is that most NARSs have released more varieties from directly introduced, semi-finished material than from early segregating populations (Byerlee and Moya 1993). Research infrastructure, budget availability, and overall strength of NARSs are the main factors accounting for these differences. As a matter of fact, unless specifically requested, most of the germplasm distribution to NARS from the CIMMYT/ICARDA wheat breeding program is semi-finished and finished materials.

All nurseries are distributed on annual basis based on the request from the national research programs across different regions of the world (Fig. 44.2). More than 620 co-operators requested the WHEAT nurseries during the 2013 season.

Germplasm distribution to the NARS by its own will not bring the expected result of releasing and adoption of improved varieties unless it is handled and managed by trained and qualified breeders. The wheat breeding programs at CIMMYT and ICARDA used to have both short and long term trainings in wheat breeding, and have trained hundreds of wheat breeders from all over the wheat growing NARSs. This had helped in the promotion of the ideals of international wheat breeding, and development and release of many wheat varieties. However, research and infrastructural support for public institutions that train plant breeders, and scientists in related disciplines, has steadily declined over the past three decades in most of

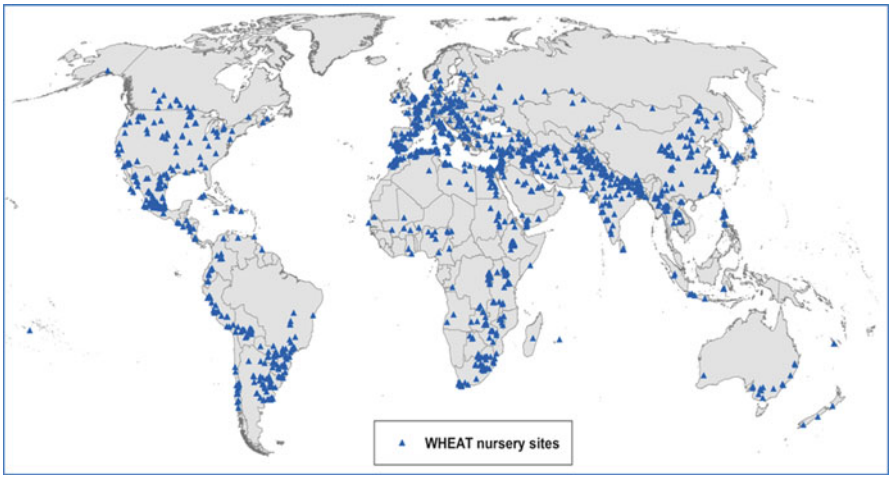

Fig. 44.2 Test sites of CIMMYT and ICARDA wheat international nurseries in 2012/2013

the developing countries NARS resulting in a great lack of trained agricultural researchers (FAO 2005). In the last decade many new technologies such as molecular markers have been rapidly developed and utilized in crop breeding institutes of the developed world. Such rapid advances in biotechnology and molecular genetics not only provide unprecedented opportunities to enhance breeding efficiency, but also create new challenges in training breeders with skills integrating both conventional and molecular breeding approaches and techniques. To this end, the wheat breeding programs at CIMMYT and ICARDA have established a training program on classical and molecular approaches for wheat improvement through which junior and mid-career scientists experience a comprehensive hands-on course on breeding for durable resistance, high yield potential and stability, drought tolerance, seed quality, and seed health issues using conventional and molecular tools.

Variety Release, Adoption and Impacts

The success of wheat improvement within the CGIAR has been remarkable, and today more than 70 % of all spring wheat cultivars grown in developing countries are CIMMYT- and ICARDA-derived, reaching 90 % in South Asia, parts of West Asia and North Africa (Byerlee and Moya 1993; Lantican et al. 2005) (Fig. 44.3). The impact of WHEAT has been witnessed not only by farmers, governments, policy makers and professionals but also by donors such as the World Bank. According to World Bank (2008), for no other major crop is the percentage of improved cultivars in farmers' fields in developing countries higher than for wheat. From the CIMMYT/ICARDA wheat breeding program, it has been reported that more than 1,500 wheat varieties have been released during the periods of 1966–1990 with

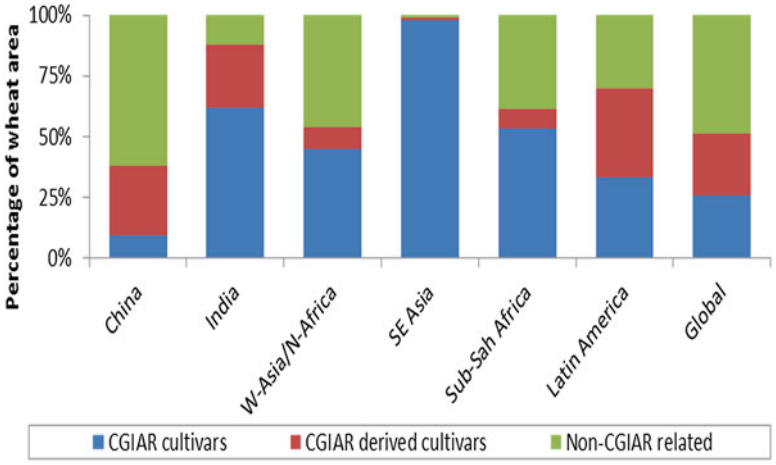

Fig. 44.3 Global and regional area coverage of CGIAR and non-CGIAR origin wheat cultivars

average of 65 varieties released annually. More than 80 % of the varieties released are spring bread wheat, which account for 77 % of the wheat area in the developing world outside of China (Byerlee and Moya 1993). Among the many crosses developed to-date at CIMMYT/ICARDA, the VEERY cross (KVZ/BUHO//KAL/BB) and its derivatives have been by far the most successful cross enabling in the release of at least 65 varieties in more than 30 countries. Among the VEERYs, Veery 5 (CM33027-F-15 M-500Y-0 M) was the most popular and released in many countries with different local names such as SERI 82 in Lebanon, Turkey, Mexico; Dashen in Ethiopia; Tilila in Morocco; Aziz in Yemen; Pirsabak 85 in Pakistan; Loerie in Zambia; MACS2496 in India; SCW101 in Zimbabwe; Rassol in Iran; GIZA 164 in Egypt; SASARAIB in Sudan and TAUSI in Tanzania.

After the VEERYs, Cham-6 (W3918A/JUP) also called Neser was released and grown in Syria, Lebanon, Jordan, Iraq and Algeria. Similarly, Kauz (JUP/BJY// URES) has been released in many countries with different local names such as Cham-8 in Syria, Tanur in Lebanon, Mehdia in Morocco, Atrak in Iran, Bacanora in Mexico, WH 542 in India and with other different names in many other countries. After Kauz, many successful varieties have been originated from the Attila cross (ND/VG9144//KAL/BB/3/YACO/4/VEE#5) and grown in many countries as mega-cultivars such as Kubsa in Ethiopia; Imam in Sudan; Utique 96 in Tunisia; PBW343 in India; Chamran, Gaher and Shiroodi in Iran; Ziyabey 98 in Jordan; MH-97 in Pakistan; and with different names many other countries (Tadesse et al. 2010). Recently, from the CIMMYT/ICARDA wheat breeding programs, Ug99 resistant wheat varieties have been released in many countries.

Adoption studies of the modern varieties (MVs) originated from the CIMMYT/ICARDA program have indicated that in the 1990s, MVs covered close to 50 million hectares, or 70 % of the wheat area in the developing world, excluding China (Byerlee and Moya 1993). According to their report, spring bread wheat varieties

have been the most successful with MVs occupying an estimated 85 % of spring bread wheat area and account for 93 % of production. Since spring bread wheat dominates developing country wheat production (about 70 % of the total), success in spring bread wheat accounts for the overall large area sown to semi-dwarfs. Though estimating the economic impact of the CIMMYT/ICARDA international wheat breeding program is very difficult given the diversity of environments and number of countries and research programs involved, Byerlee and Moya (1993), reported that the adoption of MVs of spring bread wheat over 1977–1990 resulted in about 15.5 million tons of additional wheat production in 1990, valued at about US$ three billion. For the spring wheat areas under consideration, this amounts to a production increase of 16 % (an increase of about 1.1 % annually over the period). Nearly 80 % of all durum wheat cultivars in developing countries are CIMMYT-ICARDA selections.

An adoption study conducted in Syria by the Farm Resource Management Program (FRMP) of ICARDA during 1991 showed that, modern high-yielding varieties (HYVs) such as Bohouth 2, 4, and 6; and Cham 2, 4, 6, 8, and 10 account for 87 % of the area planted and were grown by 86 % of the farmers surveyed (Tutwiler and Mazid 1991). The area planted by Mexipak 65 has dramatically reduced in the year 2000 and since 2005 Cham 8 covers more than 50 % of the spring bread wheat production area in Syria. Semi-dwarf durum wheat became available in the early 1970s, and now over half of the durum wheat area is sown to MVs. MVs of durum wheat such as Bohouth 1, Cham 1, Cham 3 and Cham 5, which were originated from the CIMMYT/ICARDA wheat breeding program, have been released and widely grown in Syria (Nachit 1992; Nachit et al. 1995). The utilization and adoption of such MVs of bread and durum wheat coupled by supplementary irrigation and other inputs such as fertilizers and herbicides by the Syrian farmers has increased the wheat production significantly without a change in the area of wheat production as indicated in Fig. 44.4.

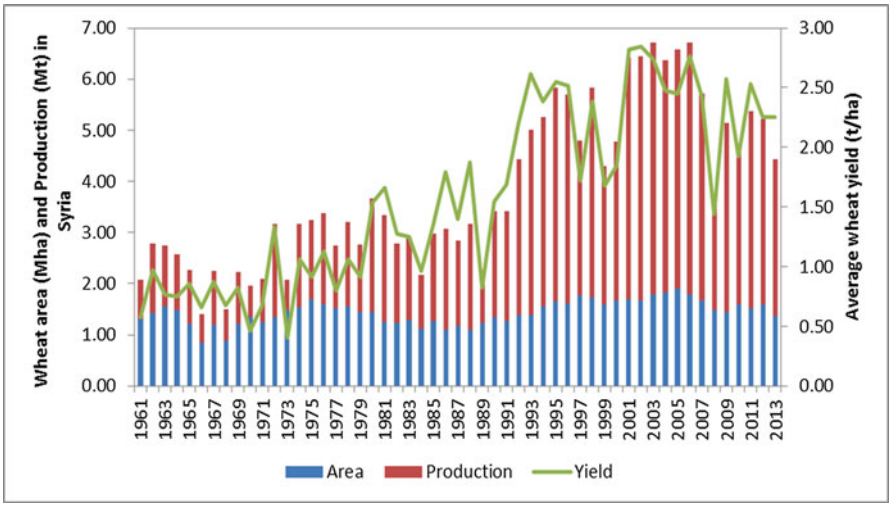

Fig. 44.4 Area, production and yield of wheat in Syria, 1961–2013

International breeding efforts focusing only on winter wheat started since 1986 through the establishment of International Winter Wheat Improvement Program (IWWIP). To-date more than 55 winter/facultative wheat varieties of IWWIP origin have been released in 10 countries of Central and West Asia including Afghanistan, Iran and Turkey (Tadesse et al. 2013). These new varieties have broad genetic diversity as they are developed from parents of diverse sources originated from ICARDA, CIMMYT, and from a wide range of genetically unrelated winter wheat from Turkey, Iran, Russia, Ukraine, Romania, Bulgaria, Hungary and US. Some lines such as OK82282//BOW/NKT and YMH/TOB//MCD/3/LIRA have been identified and released under different names in different countries indicating their broad adaptation. The former is released in Afghanistan and Kyrgyzstan while the later (Kinaci 97) has been released in Turkey, Afghanistan and Uzbekistan. However, MVs of winter wheat still cover a small area. Old cultivars such as Bezostaya and Gereek are dominantly cultivated in Turkey.

Breeding Progress

Development and identification of high yielding genotypes with wide adaptation and resistance to biotic and abiotic stresses remain the top priorities of the wheat breeding programs. There are different approaches of determining the breeding progress or the rate of genetic gain for grain yield and other traits. Some studies use yield of historical genotypes grown in the same environment while others have used mean yield to examine progress over time in highly productive environments. Trethowan et al. (2002) have used regression analysis using the mean of the five highest yielding entries expressed as per cent of the trial mean across years to determine the rate of breeding progress in elite spring wheat yield trial (ESWYT) and semi-arid wheat yield trial (SAWYT). Tadesse et al. (2010) have used success rate analysis of best lines for the high rain-fall wheat yield trials (HRWYT) of CIMMYT to demonstrate yield gains over years or trials. Genetic gain studies for the CIMMYT/ICARDA wheat breeding program have shown continuous progress in yield and other traits (Sayre et al. 1997; Trethowan et al. 2002; Sharma et al. 2012; Tadesse et al. 2010). Recently, Tadesse et al. (2013) have determined the breeding progress for IWWIP and reported that the grain yield of the best line (BL) increased at a rate of 110 kg/ha/year ($R^2 = 0.66$; $P = 0.001$), while the trial mean (TM) increased at a rate of 91.9 kg/ha/year ($R^2 = 0.53$; $P = 0.007$) indicating a continuous yield improvement at IWWIP.

In addition to grain yield, significant progress has been made by the IWIN in developing resistant wheat germplasm to diseases and pests ensuring that developing and deploying genetically resistant varieties adapted to target growing environments is the best economical and environmentally friendly strategy for controlling rust diseases of wheat particularly for resource poor farmers. However, because of the co-evolution of the host and pathogen, the deployment of individual

resistance genes leads to the emergence of new virulent pathogen mutants, and hence the 'boom and bust cycle' of cultivars performance continues. Recently, a new stem rust race Ug99 (TTKS) has been first detected in Uganda in 1999 and then spread to Kenya, Ethiopia, Yemen, Sudan and Iran, and became a global threat to the wheat industry of the world for the very fact that it over comes many of the known and most common stem rust resistance genes such as *Sr31, Sr24 and Sr36* (Singh et al. 2006; Jin et al. 2007; Haile et al. 2012). Similarly, the breakdown of yellow rust resistance genes *Yr9* in cultivars derived from "Veery" in the 1980s and *Yr27* in 2000s in major mega cultivars derived from "Attila" cross such as PBW343 (India), Inquilab-91 (Pakistan), Kubsa (Ethiopia) and others such as Achtar in Morocco, Hidab in Algeria and many other cultivars in the CWANA region (Solh et al. 2012) has caused significant wheat production loss. Through a coordinated international effort, many wheat varieties resistant to Ug99 and yellow rust have been released and replaced the susceptible cultivars.

In most developing countries, apart from grain yield and disease resistance, grain quality was not a strong criterion of variety selection. However, things have changed through time and some developing NARS are critically looking for better quality varieties suiting for preparation of different end products. Varieties such as Bezostaya, Achtar, Veery, HD1220, and Pavon-76 are known for their excellent grain quality. These varieties are still dominantly grown in some countries not only because of their wide adaptation, high yield potential and stability but also because of their high protein content and quality. With this understanding the wheat breeding programs at CIMMYT and ICARDA undertake evaluation of germplasm for quality traits following standard grain quality procedures. Most of the currently available elite genotypes for both irrigated and rain fed environments are excellent in quality with protein levels of 12–16 %. Most of these genotypes have the 5 + 10 (*Glu-D1*), 7 + 8 (*Glu-B1*) and 2* (*Glu-A1*) alleles. These alleles, especially the 5 + 10 *Glu-D1* allele, have been reported to be highly correlated with protein quality and are being used intensively as a selection criterion in wheat breeding for improving end-use quality.

Future Directions and Prospects

According to some estimates (Fischer 2009), the global wheat production must increase at least by 1.6 % annually during 2005–2020 to meet a projected wheat demand of 760 million tons by 2020. In the year 2050, the world population is estimated to be nine billion (Alexandratos 2009) and the demand for wheat reaches more than 900 million tons. Fulfilling this increasing demand for wheat is very challenging with the current scenario of climate change (IPCC 2007; Battisti and Naylor 2009), increasing drought/water shortage, soil degradation, reduced supply & increasing cost of fertilizers, increasing demand for bio-fuel, and emergence of new

virulent diseases and pests. Offsetting these challenges requires understanding of the drivers of past trends and future changes in wheat production, and designing an effective research strategy for gene mining, introgression and deployment with the application of new technologies and tools.

Located in the heart of the Fertile Crescent, ICARDA houses more than 41,000 wheat accessions of wheat including rich collections of landraces, primitive wheat, *Aegilops* and wild *Triticum* species. Synthetic hexaploid wheat (SHWs) produced by artificial resynthesis of bread wheat through hybridization between *Ae. tauschii* and *T. turgidum* are also available in the genebank. These wheat germplasm are novel sources of resistance genes against biotic and abiotic stresses for wheat production (Ogbonnaya et al. 2001; van Ginkel and Ogbonnaya 2007). However, despite the existence of this promising resource of new genes, there has been limited deployment and/or effective use in cultivated bread wheat mainly due to the high cost of screening of such huge number of accessions and the potential simultaneous transfer of deleterious genes. The development of Focused Identification of Germplasm Strategy (FIGS) and the availability of new molecular tools such as genotyping-by-sequencing (GBS) would enable to characterize and mine novel genes and alleles effectively and rapidly from such gene bank accessions. It is also important to apply modern tools including genome-wide selection, and advanced statistical analysis of multi-location evaluation data for wheat breeding in order to allow faster integration of desirable traits and improve breeding efficiency, especially for complex traits such as grain yield under optimum, drought, and heat conditions (Ferrara et al. 1987; Braun et al. 2010).

Major efforts are needed to break yield barrier in wheat to increase wheat yield potential by 50 % in order to cope the growing demand for wheat. Increasing the radiation use efficiency of wheat through modification of key enzymes (e.g., Rubisco) and biochemical pathways to increase photosynthesis, ear size and lodging resistance are key areas of wheat research through integration of physiological and molecular breeding methodologies to increase wheat yield potential. Further increase in yield potential would be achieved through the development of hybrid wheat systems based on native and transgenic interventions in collaborative approach, leveraging private sector technologies for the benefit of partners and stakeholders in the developing world.

The International Wheat Improvement Network (IWIN) coordinated by CIMMYT and ICARDA has been the most successful and efficient network for making available and widespread distribution of new wheat genotypes globally (Payne 2004; Reynolds and Borlaug 2006; Dixon et al 2009; Byerlee and Dubin 2010). Such a network need to be strengthened through the establishment of other net-works and collaborations in order to develop, disseminate, and market more productive, stress tolerant, and nutritive wheat varieties, and to perfect and promote production practices based on the principles of conservation agriculture that boost yields while conserving or enhancing critical resources like soil and water.

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Chapter 45
Genomic Selection in Plants: Empirical Results and Implications for Wheat Breeding

Mark E. Sorrells

The opinions expressed and arguments employed in this publication are the sole responsibility of the authors and do not necessarily reflect those of the OECD or of the governments of its Member countries.

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Abstract Genotyping-by-sequencing technology is rapidly reducing marker costs and increasing genome coverage allowing the widespread use of molecular markers and methods in plant breeding. Marker assisted selection (MAS) and recurrent selection are based on the selection of statistically significant, marker-trait associations. However, MAS strategies are not well suited for complex traits controlled by many genes. Genomic selection (GS) incorporates genome-wide marker information in a breeding value prediction model, thereby minimizing biased marker effect estimates and capturing more of the variation due to small effect QTL. In GS, a training population related to the breeding germplasm is genotyped with genome-wide markers and phenotyped in a target set of environments. That data is used to train a prediction model that is used to estimate the breeding values of lines in a population using only the marker scores. Prediction models can incorporate performance over multiple environments and assess G x E effects to identify a highly predictive subset of environments. Because of reduced selection cycle time, annual genetic gain for GS is predicted to be two to threefold greater than for a conven-

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tional phenotypic selection program. We have developed a new methodology for using genome-wide marker effects to group environments and identify outliers. In addition, environmental covariates can be identified that increase prediction accuracy and facilitate performance prediction in climate change scenarios. This new approach to crop improvement will facilitate a better understanding of the dynamic genome processes that generate and maintain new genetic variation.

Keywords Breeding methods • Genomic selection • Genotype by environment interaction • Marker-assisted selection • Molecular markers • Wheat

This chapter is a review of my presentation at the International Wheat Genetics Symposium held in Yokohama, Japan September 13, 2013.

Plant breeding is a predictive science. We are constantly trying to predict the performance of selected genotypes. Our evaluation methods are designed to predict traits such as grain yield, milling and baking quality, disease resistance. Prediction accuracy is important at every step in the breeding program and breeding methods are designed to improve accuracy of those predictions. Traditionally, breeding methods such as family-based selection, progeny testing, and more recently, the use of molecular markers has enabled marker-assisted selection, and genomic selection (GS). Novel breeding strategies are driven by technology and new knowledge.

Our molecular breeding goals include allele discovery, allele characterization and validation, and parent and progeny selection for superior alleles at multiple loci to generate transgressive segregation. We can increase the annual rate of genetic gain in several ways. The selection intensity can be increased or we can increase the heritability by improving the accuracy of selection or increasing the genetic variation. However the selection cycle time has the greatest impact on annual rate of gain so breeding methods that reduce the cycle time have the most impact.

Meuwissen, Hayes and Goddard first proposed genomic selection methodology in 2001 (Meuwissen et al. 2001). This methodology consists of two distinct populations, a training population that is genotyped with a large number of markers and phenotyped for important traits and a breeding population consisting of individuals that are genotyped but not phenotyped (Fig. 45.1). The training population consists of well-adapted breeding lines and varieties that are phenotyped in the target population of environments. Genome-wide markers are considered to be random effects and all marker effects on the phenotype are estimated simultaneously in a single model. One or more markers are assumed to be in linkage disequilibrium (LD) with each quantitative trait locus (QTL) affecting the trait of interest. We use a prediction model that attempts to capture the total additive genetic variance to estimate breeding value of individuals based on the sum of all marker effects. In the breeding population, genomic estimated breeding values (GEBVs) for each individual are obtained by summing the marker effects for that genotype. The prediction model can then be used to impose multiple generations of selection. The selected individuals can be recycled in the crossing program and/or evaluated in advanced replicated

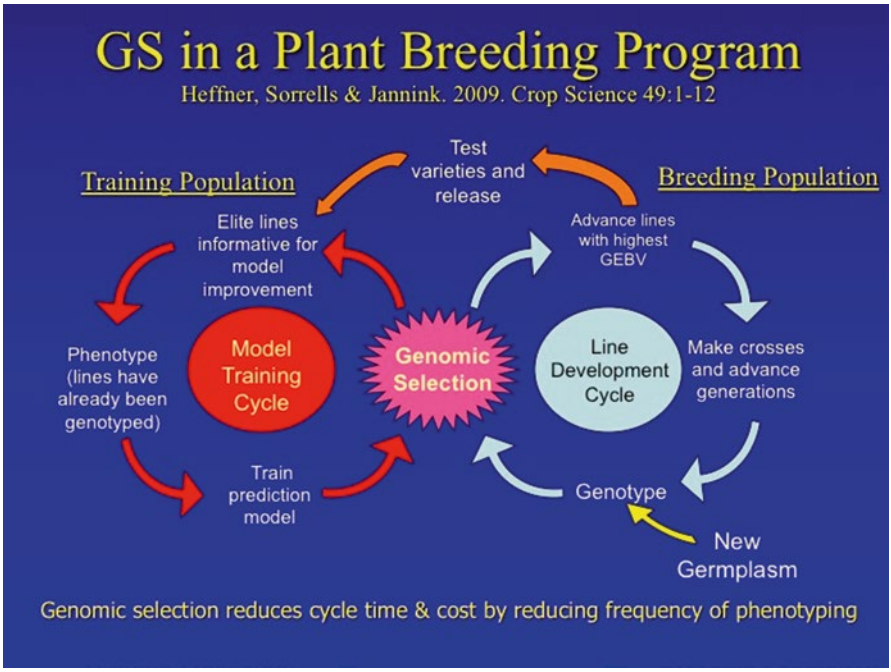

Fig. 45.1 Genomic selection training and breeding populations and their interactions in a plant breeding program

trials. Eventually the best lines are added to the training population and the prediction model is updated.

There are several factors that affect the accuracy of GEBVs. Because it is assumed that there is at least one marker in LD with each locus affecting the trait of interest, the level and distribution of LD between markers and QTL impact the accuracy of GEBVs. Using simulations, Meuwissen (2009) estimated that the minimum number of markers for across family predictions would be Ne*L where Ne is the effective population size and L is the genome size in Morgans. For example, wheat has a genome size of about 30 Morgans and if we assume an effective population size of 50, that would indicate that the minimum number of markers required would be 1,500. The size of the training population and its relationship to the breeding population are also important, and over time, re-training models is required. Meuwissen (2009) also estimated the minimum number of records for across family predictions would be 2*Ne*L. For wheat, that would be a population size of about 3,000. However, good accuracies have been reported for populations much smaller than that. The breeding population must be closely related to the training population for accuracy predictions. Population substructure in the training population can inflate accuracies and lead to inbreeding. Many small effect QTL or low LD favor Best Linear Unbiased prediction (BLUP) for capturing small effect QTL that may

not be in LD with a marker. More records are required for low heritability traits, just as for phenotypic selection.

Genomic selection was first developed and evaluated by the dairy industry. However plants have different constraints as well as some advantages. Obviously, mating schemes are quite different, even among different plant species. Animal parental values are mainly based on half-sib families with a sire in common among the progeny. Population size in animals is based on accumulating records over time for many families within a breed. For most crop species large populations can be easily generated either using biparental crosses or the progeny from multiple families. The ability to replicate is an important factor as well. Inbred lines, testcross hybrids, and clonally propagated crops can be replicated in time and space, while each animal is a unique genotype and heterozygous. Finally, genotype by environment interaction (GxE) is a more important issue in plant breeding than in animal breeding.

Next I would like to present some of the results of GS experiments from the Cornell wheat breeding program. Elliot Heffner was a Ph.D. student in my program when we initiated our GS research in 2007. He conducted experiments on GS in both biparental populations (Heffner et al. 2011b) and across multiple families in our breeding program (Heffner et al. 2011a). Jessica Rutkoski, a former Ph.D. student in our program has published a review on Genomic Selection for Adult Plant Stem Rust Resistance (Rutkoski et al. 2010), a study on GS for fusarium head blight resistance (Rutkoski et al. 2012), and methods for imputing missing data without ordered markers (Rutkoski et al. 2013).

Heffner et al. (2011b) used two doubled haploid biparental populations to evaluate GS for nine milling and baking quality traits tested over 3 years. The results averaged over both populations showed that the GS prediction models were 47 % more accurate than the multiple linear regression (MLR) model (Fig. 45.2). For the experiment involving multiple families, the training population consisted of 400 advanced breeding lines planted in an augmented field design in three locations over 3 years. It was genotyped using 1,500 polymorphic DArT markers and phenotyped for 13 agronomic traits. Prediction models included two multiple linear regression models, with or without the Kinship Matrix as a covariate, and four GS models, ridge regression, Bayes A, Bayes B, and Bayes C pi. The MLR model accuracy was similar with or without the Kinship matrix. GS accuracy was similar for all prediction models (~0.60). The GS prediction accuracy was 25 % greater than for MLR and phenotypic selection accuracy was 7 % greater than for GS. A comparison of eight of the individual traits reveals that the relative accuracy of GS compared to phenotypic selection is highest for the traits with the lowest heritability (Fig. 45.3). This highlights an important feature of GS. It is complementary to MAS because MAS is most effective for simply inherited, high heritability traits whereas GS is relatively more effective for low heritability traits. It is this complementarity that facilitates incorporation of GS into a molecular breeding program. To take advantage of the features of GS that increase annual genetic gain, a recurrent genomic selection program could be used to generate multiple cycles of selection per year (Fig. 45.4). In addition, once selections are inbred, whole-genome genotypes can be used to further select individuals with the highest GEBVs. For the

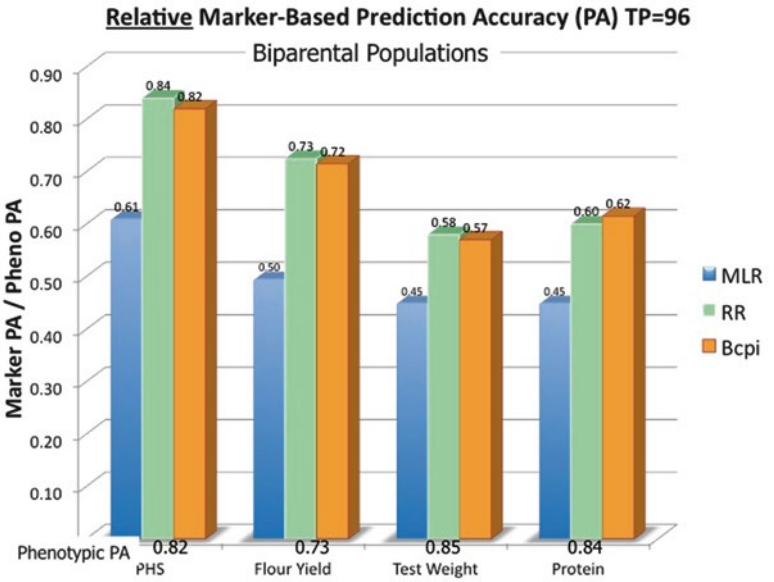

Fig. 45.2 Relative marker-based prediction accuracy (PA) for four quality traits in two biparental wheat populations. *MLR* multiple linear regression, *RR* ridge regression, *Bcpi* Bayes C pi

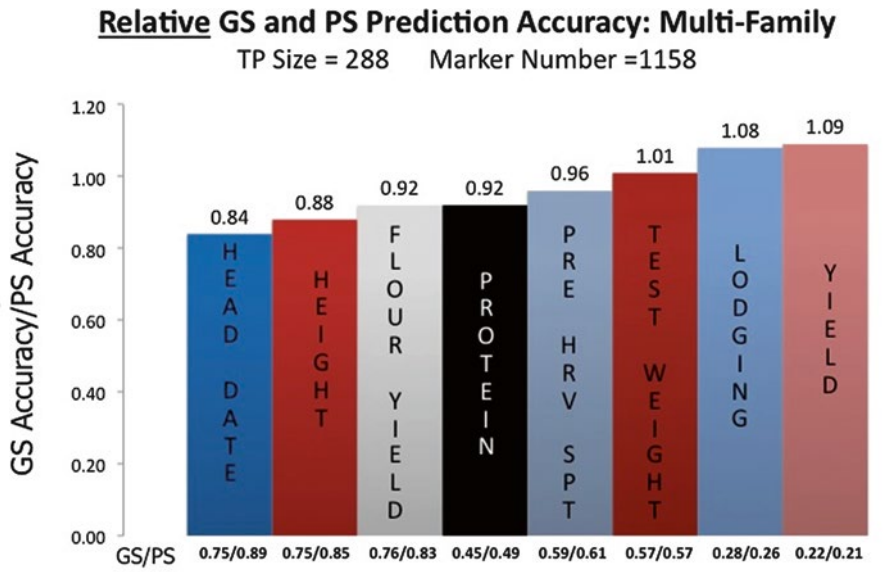

Fig. 45.3 Relative accuracy of GS compared to phenotypic selection (PS) for eight traits of varying heritability

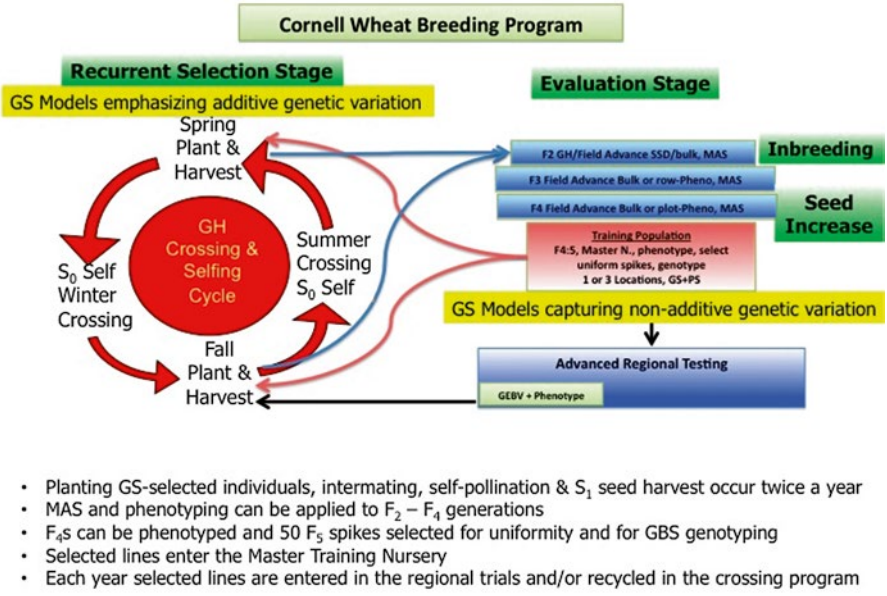

Fig. 45.4 Integration of GS in a wheat breeding program

recurrent genomic selection, GS models based on additive genetic effects would be appropriate, whereas for selecting purelines, models that incorporate non-additive effects may be more accurate.

There are several factors that need to be considered when initiating GS. Models for biparental populations are population specific but yield a higher accuracy. There is reduced epistasis and allele frequencies are balanced. Fewer markers and smaller training populations are required. Biparental populations are probably the only option for introgression of exotic germplasm. In contrast, multifamily GS allows prediction across a broader range of adapted germplasm, sampling of more environments, and larger training populations. In addition, cycle duration is reduced because model retraining is on-going.

Whole-genome genotyping opens up new opportunities for analyzing breeding trial data. For example, marker effects instead of genotypes can be used to increase GS prediction accuracy (Heslot et al. 2013). Advanced breeding trial data are typically unbalanced, i.e., all genotypes are not evaluated in all environments and this limits the kinds of analyses that can be used. However, if we use marker alleles instead, the data are balanced because all markers are evaluated in all environments. Heslot et al. (2013) used marker effects to identify outlier environments, classify relevant mega-environments, and to select an optimum subset of environments for GS prediction. Marker effects for each environment were calculated using the Bayesian LASSO GS model. Nearly 1,000 barley advanced lines were evaluated for grain yield in 58 European environments. The dataset was unbalanced with only 18

genotypes present in >50 % of the environments. Environment groups were based on Additive Main Effects & Multiplicative Interaction (AMMI) analysis, year. region, marker effects, and pairwise prediction accuracy between environments. Marker effects for all lines in each environment formed a balanced dataset for computing Euclidean distances between environments. Only clustering based on average reciprocal prediction accuracies significantly increased prediction accuracy. Clustering based on marker effects produced four clear subgroups that were not related to region or year, but also did not increase prediction accuracy. Reciprocal accuracies correlate with genetic correlations between environments based on a factor analytic model and were useful to measure genetic correlation without the numerical issues of factor analytic models. In a second experiment, Heslot et al. (2013) developed a protocol using the predictive ability of an environment for optimizing the composition of the training population. The procedure involves training a GS model in each environment and computing the mean accuracy for each training environment for predicting line performance in the other environments. They are then ranked and environments are removed one at a time starting from the least predictive.

The GS model is then trained and cross validated on the remaining training population and is referred to as the "Predictive set". The removed environments are referred to as the "Unpredictive set" and accuracy is predicted using the same GS model. Both accuracy measurements are used to decide the cut-off point for the optimum set. Using this procedure prediction accuracy rose from 0.54 to 0.61 with no change in heritability. Some outlier environments were included in the optimal model. Although it was not statistically significant, accuracy in the validation set increased from 0.279 to 0.292.

Probably the most important project we have ongoing is the merger of crop modeling methodologies integrating environmental covariates and crop modeling into the genomic selection framework to predict G*E (Heslot et al. 2014). Crop modeling has the goal of assessing the impacts of climate change on productivity and how crops adapt to climate change. Crop modelers calibrate crop models to give reliable predictions under baseline and future climate scenarios with the long-term goal of enhancing world food security and adaptation capacity. By combining crop modeling methods with whole-genome genotyping in a GS framework, we can predict G*E for unobserved environments, and thus, performance and stability based only on genotype. In addition, we can better understand the characteristics of the target population of environments and determine the genetic architecture controlling G*E. In this experiment, we used a dataset consisting of grain yield of 2,437 elite winter wheat lines grown in 44 environments over 6 years in France and genotyped with 1,287 SNP markers. Daily climatic weather data were used to characterize environments. In this study, we extended factorial regression to the GS context and developed a new machine learning approach to capture the non-parametric response of QTL to stresses. This approach was used along with a crop model to enable the use of daily weather data in prediction models. Those G*E predictions could be used to make breeding decisions for specific adaptation. Physiological integration of the environment data involved the use of a crop model (Sirius) to compute the

phenology and synchronize early, medium, and late maturing genotypes to the weather data. Stress covariates by developmental stage were derived by using knowledge about sensitivity of specific growth stages to abiotic stresses that were used as independent covariates in statistical genetic models for effect estimation and prediction.

Model description:

$$y_{ij} = \mu + \beta_j + u_i + \gamma_i f(x_j) + \varepsilon_{ij},$$

where Mu is the intercept, the Beta term is the environment effect, u is a genotype main effect, Gamma is the sensitivity of each genotype to a stress covariate x, that can be transformed by a function *f*(), and Epsilon is the model residual. Crop modeling allows us to leverage agronomy knowledge, reduce dimensionality and nonlinearity, and enables the use of existing breeding data. Performance is predicted as main effect plus G*E deviation and environment clustering based on predicted G*E. Because G*E can be predicted for any genotype in any environment, it is possible to use the table of G*E predictions to cluster environments and investigate the structure of the target population of environments. For this data set, environments were mainly grouped by year but also showed a North/South trend. If we plot the accuracy of the models with and without including G*E, we can get an overall view of the importance of this term (Fig. 45.5). Each dot represents an environment, and a dot above the line indicates higher accuracy with the model that included G*E. Overall, there was an 11.1 % increase in mean accuracy (P-value 0.02) and a 10.8 % decrease in the accuracy coefficient of variation. It is important to note that the largest gains occurred in environments where accuracies were low. These results are important because, if successful, we will be able to predict GxE for any genotype based only on genotype. This would allow the breeder to select genotypes that

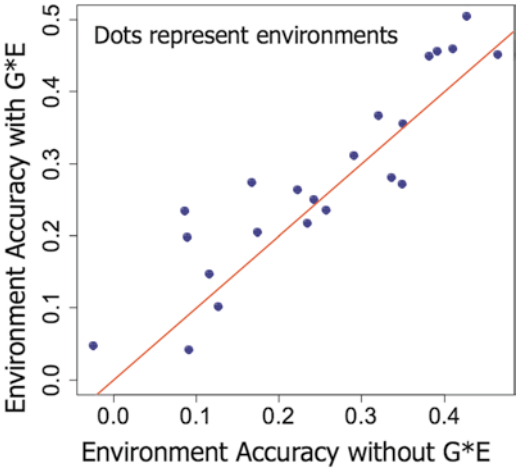

Fig. 45.5 Relative prediction accuracy of prediction models with and without modeling G*E

interact positively with a particular environment, genotypes that minimize GxE, or genotypes that interact positively with environmental factors that limit performance.

In summary, GS differs from MAS and Association Breeding in that the underlying genetic control and biological function is not necessarily known. GS preserves the creative nature of phenotypic selection to sometimes arrive at solutions outside the engineer's scope. Integrating environmental covariates and crop modeling into the genomic selection framework to predict G*E increases prediction accuracy and provides insight into the genetic architecture controlling G*E. The most important advantages are reductions in the length of the selection cycle resulting in greater genetic gain per year.

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Chapter 46
Dietary Fibre: Wheat Genes for Enhanced Human Health

Rowan A.C. Mitchell and Peter R. Shewry

The opinions expressed and arguments employed in this publication are the sole responsibility of the authors and do not necessarily reflect those of the OECD or of the governments of its Member countries.

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Abstract Dietary fibre (DF) has been shown to be a vital component of diet for human health, decreasing the risk of cardiovascular disease, type II diabetes and possibly bowel cancer. DF in wheat flour is derived from the cell walls of the starchy endosperm, which is principally composed (~70 %) of the polysaccharide arabinoxylan (AX). Diversity screens of elite wheat germplasm have established that variation in total and water-extractable AX within flour exists and has high heritability. Identification of genes which determine AX content will assist in introduction of high DF alleles into appropriate backgrounds. We identified candidate genes for the synthesis and feruloylation of AX from bioinformatics approaches. Using RNAi suppression of genes in wheat endosperm, we have shown that a glycosyl transferase (GT) family 61 gene is responsible for nearly all mono-substitution of xylose by arabinose on AX, and that genes in GT43 and GT47 families are responsible for the synthesis of the xylan backbone in AX. Using mapping populations derived from crosses of high AX x normal AX varieties, we are also seeking to identify QTLs for

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high AX. This combination of forward and reverse genetics will accelerate the introduction of the high fibre alleles into modern commercial wheat varieties.

Wheat as a Source of Dietary Fibre

In addition to providing calories and protein to much of the world's population, wheat is also an important source of dietary fibre (DF), with bread products alone contributing 20 % of the DF in the adult UK diet. There is also a significant shortfall in the intake of DF in many countries. For example, in the UK the daily intake of DF is only about 13 g per day (Buttress and Stokes 2008) compared with a recommended daily intake of 25–40 g a day in most other countries. Most of the DF consumed is non-starch polysaccharides, with resistant starch contributing about 20–25 % of the total in European diets (Cummings 1983). There is strong evidence that DF in wholegrain provides protection against the risk of a number of chronic diseases, including type 2 diabetes, obesity and cardiovascular disease, with both soluble and insoluble forms contributing to the beneficial effects (Slavin 2004; Topping 2007; Wood 2007; Lunn and Buttriss 2007; Buttress and Stokes 2008; Anderson et al. 2009; Fardet 2010). DF is not digested in the upper gastrointestinal (GI) tract and many of the beneficial effects result from fermentation in the colon. However, it also has effects throughout the GI tract, with insoluble fibre providing faecal bulk to speed up transit, while soluble fibre may increase the viscosity of the digesta to reduce the rates digestion and the uptake of nutrients in the small intestine, and hence lower the glycaemic load. The wide consumption and low cost of staple foods made from wheat mean that it is an excellent vehicle to deliver the health benefits conferred by DF to large populations at low cost. However, in order to achieve this it is necessary to increase the fibre content of wheat and, in particular, in the part of the grain that is most widely consumed: the starchy endosperm which forms white flour on milling.

Cell Wall Composition of Wheat

The wheat grain comprises three main parts which differ in their contents and composition of cell wall polysaccharides. The major storage tissue is the endosperm, which accounts for about 90 % of the dry weight of the mature grain (Barron et al. 2007). The outer layer of endosperm cells, called the aleurone layer, have thick cell walls which account for about 40 % of the dry weight. These surround the starchy endosperm cells, which have thin walls (about 2–3 % dry wt.) and contain mainly starch and storage proteins. The second major tissue is the embryo, which accounts for about 3 % of the mature grain (Barron et al. 2007). These tissues are sounded by several outer layers, the nucellar epidermis, testa and inner and outer pericarps.

Table 46.1 Compositions of cell wall types in wheat grain tissues (% dry weight)

	Cellulose	Lignin	Xylan	β-glucan	Glucomannan	Refs.
Starchy endosperm	2	0	70	20	7	A
Total bran	29	8	64	6	–	B
Aleurone	2–4	0	62–65	29–34	–	C
Outer pericarp (beeswing)	30	12	60	–	–	D

References: A, Mares and Stone 1973; B, Selvendran et al. 1980; C, Bacic and Stone 1981; Rhodes and Stone 2002; Antoine et al. 2003; D, Du Pont and Selvendran 1987

On milling the outer layers, embryo and aleurone form the bran fraction and the starchy endosperm cells the white flour.

The major cell wall polysaccharides in the cell walls of wheat grain are arabinoxylan (AX) and (1,3; 1,4)-β-D-glucan (β-glucan). AX comprises a backbone of β-D-xylopyranosyl residues linked through (1,4) glycosidic linkages, with some residues being substituted with α-L-arabinofuranosyl residues, while β-glucan comprises only glucose residues which are linked by (1,4) and (1,3) bonds. AX accounts for about 70 % of the total cell wall polysaccharides in white flour and 62–65 % in aleurone, while β-glucan accounts for about 20 % and 29–35 % in these tissues, respectively (Table 46.1). Cellulose is a minor component in both tissues (2–4 %) while the starchy endosperm cell walls also contain about 7 % glucomannan. By contrast, the composition of the outer bran layers has high contents of cellulose, lignin and complex glucuronarabinoxylans but no β-glucan (Table 46.1).

Genetic Variation and Heritability of AX Content

Analyses of wheat genotypes have shown extensive variation in the content and composition of AX in whole grain and white flour. A study carried out under the EU FP6 HEALTHGRAIN programme (Poutanen et al. 2008), showed that total DF varied from 11.5 % to 15.5 % dry wt (mean 13.4 %) in wholegrain of 129 wheat lines grown on the same site, while total AX (TOT-AX) ranged from 5.53 % to 7.42 % (mean 6.49 %) (Andersson et al. 2013). A more detailed study of 151 lines grown on the same site (including those studied by Andersson et al. 2013) compared the contents of TOT-AX and water-extractable AX (WE-AX) in flour and bran fractions (Gebruers et al. 2008; Ward et al. 2008). Although the bran fractions contained 13–22 % TOT-AX, WE-AX only ranged from 0.30 % to 0.85 %, corresponding to between 2 % and 5 % of the whole fraction. By contrast, white flour contained only 1.35–2.75 % TOT-AX but between 20 % and 50 % of this was water soluble (0.30–1.4 % dry wt. WE-AX). A small number of lines from this study were subsequently grown in an additional five environments (sites and/or years), allowing the broad sense heritability of the AX fractions to be determined. The ratios of genetic variance to total variance were 0.39 and 0.71 for TOT-AX in bran and flour,

respectively, and 0.47 and 0.59 for WE-AX in bran and flour (Gebruers et al. 2010; Shewry et al. 2010). These high heritabilities, particularly for the flour fractions, indicate that the contents of both TOT-AX and WE-AX should be amenable to manipulation by plant breeding.

Variation in AX Structure

AX comprises a backbone of β-D-xylopyranosyl residues linked through (1,4) glycosidic linkages, with some residues being substituted with α-L-arabinofuranosyl residues at either position 3 or positions 2 and 3. Another key attribute of AX structure is the degree of feruloylation of some of the three-linked arabinose. The solubility of AX is dictated by degree of arabinose substitution (increasing solubility), cross-linking of AX chains by intermolecular dimerization of ferulate (decreasing solubility) and the chain length of AX backbone (longer chains are less soluble) (Saulnier et al. 2007). Any of these attributes may influence the benefits that AX confers in the diet, either directly or by altering the solubility of AX. There is genetic variation in the proportions of monosubstituted and disubstituted xylose residues (Saulnier et al. 2007) but less is known about genetic variation of the other AX structural attributes. AX feruloylation, measured as total bound ester-linked ferulic acid, is highly variable with low heritability, but the extent of genetic variation within white flour (where levels are much lower) is unknown. More research is needed both of the structural attributes of AX which confer greatest benefit in human diet and on the wheat genes responsible for them to determine the importance and feasibility of genetic improvement of AX structure.

Genes Responsible for AX Synthesis in Wheat Endosperm

Identification of Candidate Genes

Xylan is more abundant in primary cell walls of grasses than those of dicots and arabinose substitution is far more common (the main substitution in dicot xylan being glucuronic acid). Feruloylation of AX occurs exclusively in grasses and other commelinid monocots. Since xylan is a highly abundant polymer within plant biomass, we expect transcripts encoding the synthetic enzymes to be highly abundant; exploiting this fact and the differences between grasses and dicots led us to identify candidate genes for the synthesis and feruloylation of AX (Mitchell et al. 2007). The results are summarized in Fig. 46.1; we identified clades within the glycosyl transferase (GT) gene families 43, 47 and 61 as likely involved in the synthesis of AX, and a clade from the BAHD acyl coA transferase superfamily as likely responsible for AX feruloylation.

Using RNA-Seq transcriptome analyses of developing wheat starchy endosperm, we found transcripts within all of these clades during the period of greatest AX synthesis (Pellny et al. 2012). In order to demonstrate their function, we transformed wheat to suppress their expression using an endosperm-specific promoter driving RNAi constructs.

Role of GT61 Genes in AX Synthesis

We compared five homozygous transgenic lines suppressing the most highly expressed GT61 gene in wheat endosperm with null segregant controls. Digestion of AX with xylanase and HPAEC quantification of resultant oligosaccharides showed that those oligosaccharides with single three-linked arabinose substitution were substantially decreased and ^{1}H-NMR showed almost complete abolition of this linkage in undigested soluble AX (Anders et al. 2012; Fig. 46.2). Furthermore our collaborators (Dupree group, University of Cambridge, UK) showed that expression of similar wheat and rice genes in Arabidopsis introduced arabinose linkages onto Arabidopsis xylan (Anders et al. 2012). Therefore these GT61 genes encode xylan arabinosyl transferases, consistent with their far greater expression in grasses than dicots (Fig. 46.1).

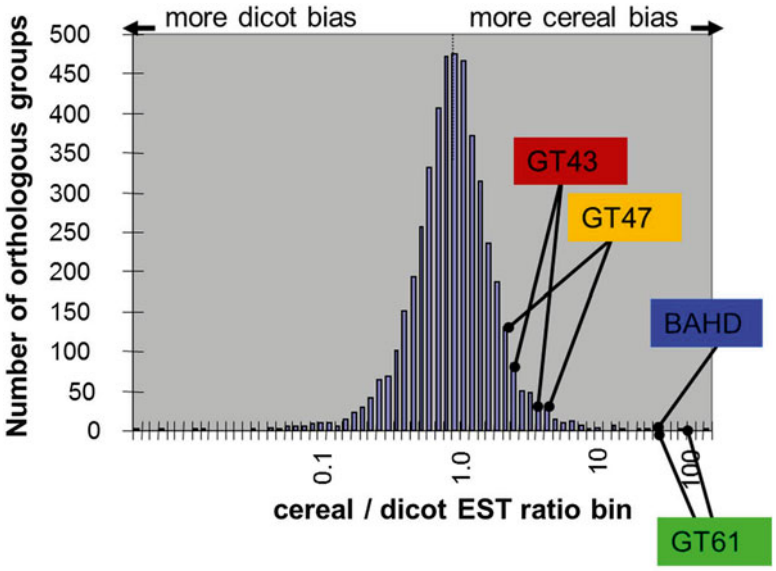

Fig. 46.1 Histogram of results of analyses in Mitchell et al. (2007). The number of orthologous groups are shown for each bin of log ratio of expression in cereals to that in dicots. Clades from families with greater expression in cereals and correct characteristics for AX synthesis (inverting GT families) and feruloylation (acyl transferases) are indicated

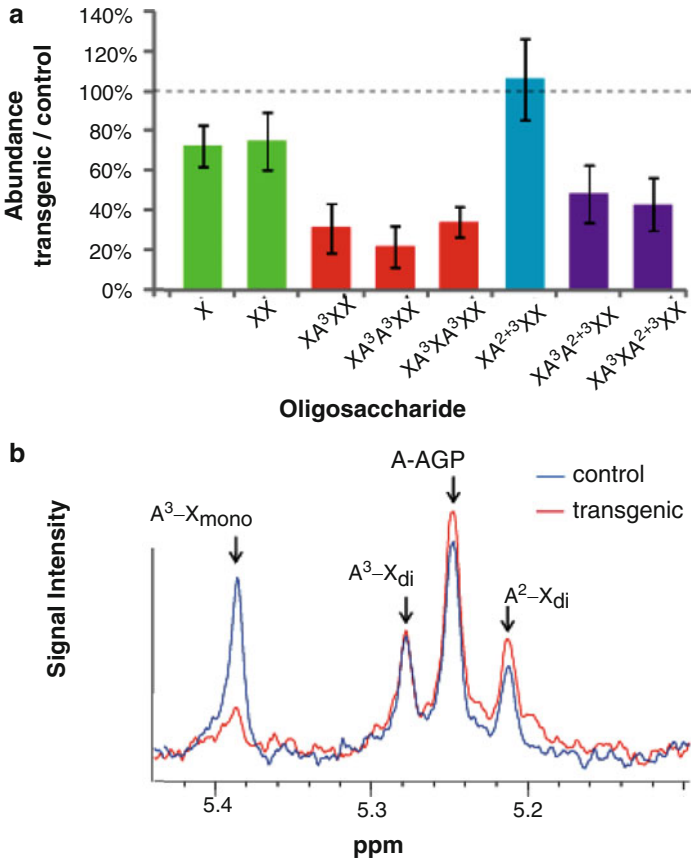

Fig. 46.2 Analysis of xylan structure in endosperm samples from homozygous TaGT61_1 RNAi transgenic wheat. (**a**) Oligosaccharide abundance from transgenic samples relative to corresponding azygous controls after xylanase digest; mean of five independent lines ±95 % confidence intervals. Columns are colored according to oligosaccharide substitution: unsubstituted (*green*), mono-substituted only (*red*), di-substituted only (*blue*), and mono- and di-substituted (*purple*). (**b**) ^{1}H-NMR spectra for transgenic (*red*) and azygous control (*blue*) samples showing H1 signals for arabinose in AX: A^{3}-X_{mono} denotes α-(1,3)–linked to mono-substituted xylose (Redrawn from Anders et al. 2012)

Role of GT43 and GT47 Genes in AX Synthesis

We compared homozygous transgenic lines suppressing either the most highly expressed GT43 (TaGT43_2) or GT47 (TaGT47_2) genes in wheat endosperm with their null segregant controls. Suppression of either gene had similar effects with AX content decreased to about 50 % of controls as determined by monosaccharide composition of non-starch polysaccharide (Fig. 46.3a; Lovegrove et al. 2013).

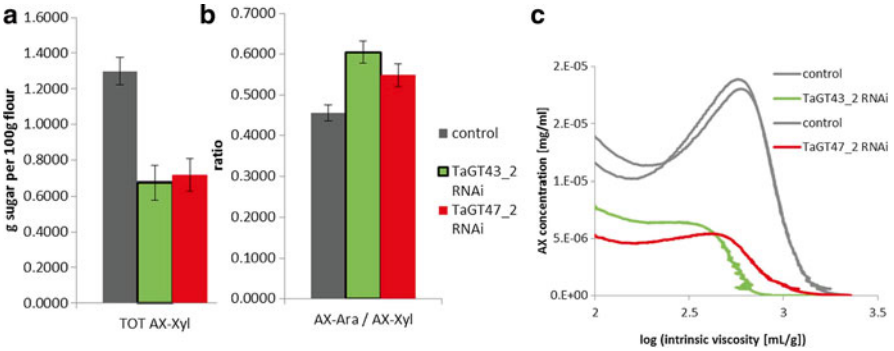

Fig. 46.3 Summary of results in Lovegrove et al. (2013) from RNAi suppression of TaGT43_2 and TaGT47_2 genes in wheat endosperm. (**a**, **b**) Total AX abundance (**a**) and arabinose to xylose ratio (**b**) from transgenic samples and null-segregant controls determined by monosaccharide analyses of non-starch polysaccharide; mean of three independent lines ±SE. (**c**) Log intrinsic viscosity of soluble AX fractions (which is proportional to log AX chain length; Dervilly-Pinel et al. 2001) separated by SE-HPLC versus concentration for samples from TaGT43_2 and a TaGT47_2 RNAi lines and corresponding controls

In contrast to suppression of TaGT61_1, the amount of arabinose substitution was increased (Fig. 46.3b). The TaGT43_2 and TaGT47_2 genes are orthologous to IRX9 and IRX10 in Arabidopsis, respectively. Mutations in IRX9 and IRX10 lead to decreased xylan and xylan chain length in Arabidopsis (Pena et al. 2007; Brown et al. 2009) and we obtained similar results by suppressing their orthologues in wheat endosperm. However, size-exclusion profiles of AX showed that suppressing TaGT43_2 had a greater effect on the longest chains of AX than suppressing TaGT47_2 (Fig. 46.3c).

Combining Forward and Reverse Genetics to Develop Wheat with Enhanced Health Benefits

Rapidly improving bioinformatics resources for wheat, e.g. the International Wheat Genome Sequencing Consortium (http://www.wheatgenome.org/) chromosome-sorted survey sequences, make it possible to start relating genes to traits *in silico*. In Table 46.2, the genes shown to be responsible for AX synthesis in wheat endosperm above are shown with their putative chromosome location, together with some published QTLs for DF or related traits in wheat. The causative alleles for these QTLs may be cis-elements controlling these genes or the genes themselves. As our understanding of which genes dictate DF content in wheat products improves, there are excellent prospects for efficient breeding of wheat varieties which deliver enhanced health benefits in the near future.

Table 46.2 Putative chromosomal location of AX synthetic genes (determined by blast against IWGSC chromosome survey sequences) and some published wheat QTLs; [A] meta-QTLs for DF (Quraishi et al. 2011) or [B] QTLs for AX (Nguyen et al. 2011)

Gene	Synthetic function	Location	Homeologue expression[a]	QTL(s) at this location
TaGT61_1	AX arabinosylation	Chr 6 L	B > A ~ D	Chr 6BL [A]
TaGT43_2	AX backbone	Chr 4S/L	A ~ D > B	Chr 4DL [B]
TaGT47_2	AX backbone	Chr 3 L	D > B ~ A	Chr 3DL [A, B]

[a]Determined by re-analyses of wheat endosperm RNA-Seq in Pellny et al. (2012)

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Appendices

Appendix 1: Program

Sunday, September 8, 2013	
13:00–20:00	Registration
18:00–20:00	Welcome cocktail
Monday, September 9, 2013	
Opening session	
Chairperson: **Takashi Endo**	
9:00–9:10	Opening remarks **Yasunari Ogihara**, Chairman, Symposium Organizing Committee
9:10–9:20	Welcome address **Takashi Suzuki**, Deputy Mayor of Yokohama City
9:20–10:05	Special lecture **Koichiro Tsunewaki** Prof. H. Kihara's genome concept and the succeeding wheat cytogenetics advancement in his school
10:05–10:25	Gathering for the memorial group picture of the 12th IWGS
10:25–10:40	Coffee & tea break
Chairperson: **Hirokazu Handa**	
10:40–11:25	Special lecture **Thomas Adam Lumpkin** How a gene from Japan revolutionized the world of wheat
11:25–12:10	Special lecture **Catherine Feuillet** The international effort to sequence the 17Gb wheat genome: yes, wheat can!
12:10–14:15	Lunch
Ernie Sears Memorial Lecture	
Chairperson: **Robert McIntosh**	

Y. Ogihara et al. (eds.), *Advances in Wheat Genetics: From Genome to Field*, DOI 10.1007/978-4-431-55675-6

14:15–15:00	Special lecture **Takashi R. Endo** New aneuploids of common wheat
	JSPS-ACORE sponsored session **Session 1: Germplasm and genetic diversity**
Chairperson: **Hans Braun**	
15:00–15:35	Plenary lecture **Helmut Knupffer** Genetic resources of Triticum
15:35–16:00	Keynote **Yehoshua Saranga** Genetic diversity in wild emmer wheat: a key for understanding past domestication and enhancing future production
16:00–16:20	Coffee & tea break
16:20–16:35	Oral presentation **Shahryar Kianian** Wheat nuclear and organelle genomics: where they intersect great things happen
16:35–16:50	Oral presentation **Kenji Kato** Genetic diversity in Asian wheat landraces, based on the analysis of heading time genes and non-adaptive DNA markers
16:50–17:05	Oral presentation **Manoranjan Dutta** Development of core set of wheat germplasm conserved in the National Genebank in India
Session 2: Cytogenetics and evolution	
Chairperson: **Hisashi Tsujimoto**	
17:05–17:40	Plenary lecture **Bikram S. Gill** Wheat chromosome analysis
17:40–17:55	Oral presentation **Pierre Sourdille** Evolutionary dynamics of genes controlling meiotic recombination in wheat: how are they retained expressed and variable?
18:00–20:00	Poster session (session 1, 2, 3, and 4)
Tuesday, September 10, 2013	
	NIAS-sponsored session **Session 3: Toward the whole genome sequence**
Chairperson: **Catherine Feuillet**	
9:00–9:35	Plenary lecture **Jizeng Jia** *Aegilops tauschii* draft genome sequence reveals a gene repertoire for wheat adaptation
9:35–10:00	Keynote **Yasunari Ogihara** Comprehensive functional analyses of expressed sequence tags in common wheat

10:00–10:15	Oral presentation **Kellye Eversole** The IWGSC bread wheat chromosome survey sequencing initiative
10:15–10:30	Oral presentation **Fuminori Kobayashi** Development of the BAC physical maps of wheat chromosome 6B for its genomic sequencing
10:30–10:50	Coffee & tea break
	NIAS-sponsored session **Session 4: Structural and functional genomics**
Chairperson: **Jizeng Jia**	
10:50–11:25	Plenary lecture **Jorge Dubcovsky** Functional genomic tools to understand wheat development
11:25–11:50	Keynote **Rohit Mago** Major haplotype divergence at the *Sr2* stem rust resistance locus
11:50–13:45	Lunch
13:45–14:00	Oral presentation **Tsuyoshi Tanaka** Sequencing of wheat chromosome 6B: toward functional genomics
14:00–14:15	Oral presentation **Jaroslav Dolezel** Chromosome genomics in wheat: are we finished?
14:15–14:30	Oral presentation **Liuling Yan** Genetic mechanism of vernalization requirement duration in winter wheat cultivars
14:30–14:50	Coffee & tea break
Session 5: Satellite workshops (with three parallel sessions)	
Session 5.1: Brachypodium research for wheat genetics and biomass research platform (sponsored by RIKEN Biomass Engineering Program)	
Coordinators: **Kazuo Shinozaki** and **Keiichi Mochida**	
14:50–15:00	Introduction **Keiichi Mochida**
15:00–15:30	Presentation 1 **David Garvin** Genome analysis of nonhost resistance to stem rust in *Brachypodium*
15:30–16:00	Presentation 2 **Matsuo Uemura** Cold-acclimation-induced changes of plasma membrane proteome in *Brachypodium distachyon*
16:00–16:30	Presentation 3 **Anthony Hall** Mutant hunting in hexaploid wheat

16:30–17:00	Presentation 4 **Shancen Zhao** Toward complex plant genome sequencing: retrospect and prospect
17:00–17:30	Presentation 5 **Daisuke Saisho** Bio- and information resources of *Brachypodium* toward genome-oriented breeding in Triticeae crops

Session 5.2: Genomic assessments of global wheat genetic resource collections (supported by NBRP-wheat and CIMMYT)

Coordinators: **Shuhei Nasuda** and **Thomas Payne**

14:50–15:00	Introduction **Hélène Lucas**
15:00–15:25	Presentation 1 **Benjamin Kilian** Major initiatives to improve wheat production in Europe
15:25–15:50	Presentation 2 **Kailash C. Bansal** Evaluation of 21,000 wheat accessions conserved in the National Genebank in India for tolerance to terminal heat stress
15:50–16:15	Presentation 3 **Singh Sukhwinder** Seeds of Discovery (SeeD), CIMMYT: unlocking the useful genetic variation from genebanks for wheat improvement
16:15–16:30	Break
16:30–16:55	Presentation 4 **Mark Humble** High-throughput phenotyping – a boost for genomics in the twenty-first century
16:55–17:20	Presentation 5 **Gina Lynn Brown-Guedira** TCAP: improving wheat germplasm for changing environments
17:20–17:45	Presentation 6 **Xueyong Zhang** Wheat core collection and its use in diversity and association research
17:45–18:10	Presentation 7 **Shuhei Nasuda** The core-collection of hexaploid wheat accessions conserved by the National BioResource Project-Wheat, Japan
18:10–18:40	Discussion and closing **Hans Braun**

Session 5.3: Application of genetic and genomic studies on disease resistance to wheat improvement (supported by KWS UK Ltd/Bayer CropScience/SATREPS Afghanistan Wheat Project, JST/JICA/BGRI)

Coordinators: **Tomohiro Ban**, **Hermann Bürtsmayr**, and **Matthew Rouse**

14:50–15:00	Introduction **Tomohiro Ban**

15:00–15:25	Presentation 1 **Hermann Bürtsmayr** Breeding wheat for resistance to Fusarium head blight: challenges and possibilities
15:25–15:50	Presentation 2 **Matthew Rouse** Rust diseases – review from BGRI achievement
15:50–16:15	Presentation 3 **Viktor Korzun** Marker-assisted selection for disease resistance in wheat breeding – promises and reality
16:15–16:40	Presentation 4 **Jeff Ellis** Genetic and genomic studies/tools of host-pathogen interaction to be applied for wheat resistance improvement
16:40–16:50	Break
16:50–17:15	Presentation 5 **Kostya Kanyuka** Modulation and manipulation of plant defences by *Mycosphaerella graminicola* during wheat infection
17:15–17:40	Presentation 6 **Jessica Elaine Rutkoski** Applying genomic selection to breed for stem rust resistance in wheat
17:40–18:05	Presentation 7 **Ravi Prakash Singh** Breeding for resistance to globally important wheat diseases – current status and future prospects
18:05–18:35	Discussion Panelists: **Beat Keller**, **Evans Lagudah**, and **Colin Hiebert**
18:35–18:40	Recap and closing **Francis Ogbonnaya**
18:30–20:00	Poster session (session 6 and 7)
Wednesday, September 11, 2013	
Session 6: Gene function and molecular biology	
Chairperson: **Beat Keller**	
9:00–9:35	Plenary lecture **Graham Moore** Exploiting comparative biology and genomics to understand a trait in wheat, Ph1
9:35–10:00	Keynote **Wolfgang Spielmeyer** Genetic control of plant architecture
10:00–10:20	Coffee & tea break
10:20–10:35	Oral presentation **Olesya Shoeva** The specific features of anthocyanin biosynthesis regulation in wheat
10:35–10:50	Oral presentation **Shigeo Takumi** miR156 is associated with the grass clump phenotype in hybrids between tetraploid wheat and *Aegilops tauschii*

10:50–11:05	Oral presentation **Yuji Ishida** High efficiency wheat transformation mediated by Agrobacterium tumefaciens
Symposium excursion	
Thursday, September 12, 2013	
Session 7: Biotic stress	
Chairperson: **Silvia German**	
9:00–9:35	Plenary lecture **Colin Hiebert** Stem rust resistance – two approaches
9:35–10:00	Keynote **Evans Lagudah** Wheat resistance genes to a subgroup of biotrophic pathogens: diversity and function
10:00–10:15	Oral presentation **Manisha Shankar** Germplasm enhancement for resistance to *Pyrenophra tritici-repentis* in wheat
10:15–10:35	Coffee & tea break
10:35–10:50	Oral presentation **Zhengqiang Ma** Precisely mapping scab resistance QTLs for breeding utilisation
10:50–11:05	Oral presentation **Sridhar Bhavani** KARI-CIMMYT-DRRW partnership in East Africa: working together to beat the threat of Ug99
Session 8: Abiotic stress (Supported by ALRC, TU) Arid Land Research Center Tottori University	
Chairperson: **Peter Langridge**	
11:05–11:40	Plenary lecture **Roberto Tuberosa** Genomics approaches to dissect the genetic basis of drought resistance in durum wheat
11:40–12:05	Keynote **Jochen C. Reif** Improving stress resistance switching from line to hybrid breeding
12:05–14:05	Lunch
12:45–14:00	IWGS IOC and LOC
14:05–14:20	Oral presentation **Hisashi Tsujimoto** Broadening the genetic diversity of common and durum wheats for screening abiotic stress tolerance
14:20–14:35	Oral presentation **Hollie Webster** Utilization of the IWGSC wheat survey sequence for RNASeq analysis of water stress effects in developing wheat heads

14:35–14:50	Oral presentation **Suchismita Mondal** Earliness in wheat: a key to adaptation under high temperature stress
14:50–15:10	Coffee & tea break
Session 9: Grain quality	
Chairperson: Tatyana Pshenichnikova	
15:10–15:45	Plenary lecture **Gerard Branlard** Coping with wheat quality in a changing environment: proteomics evidence of stresses caused by the changing environment
15:45–16:10	Keynote **Toshiki Nakamura** Starch modification: a model for wheat MAS breeding
16:10–16:25	Oral presentation **Craig F. Morris** Quality characteristics of soft kernel durum – a new cereal crop
16:25–16:40	Oral presentation **Tatsuya M. Ikeda** Proposal of international glutenin research group
16:40–16:55	Oral presentation **Tatyana Alexeevna Pshenichnikova** Enlargement of the genetic diversity for grain quality in bread wheat through alien introgression
16:55–18:40	Poster session (session 8, 9 and 10)
Business session	
18:40–19:20	IWGS organizing meeting for 'Plans for future IWGS and strategic perspectives' Chairperson: **Peter Langridge**, IOC Chairman
Friday, September 13, 2013	
NARO	**NARO sponsored session** **Session 10: Classical and molecular breeding**
Chairperson: **Mark Sorrells**	
9:00–9:35	Plenary lecture **Shunsuke Oda** Recent improvement of Japanese wheat
9:35–10:00	Keynote **Eduard D Akhunov** A haplotype map of wheat and its utility for wheat genetics and breeding
10:00–10:15	Oral presentation **Viktor Korzun** Molecular and classical wheat breeding in Europe: current status and perspectives
10:15–10:30	Oral presentation **Rima Thapa** Determining the order of resistance genes *Qsng-3BS*, *Fhb1* and *Sr2* and combining them in coupling on wheat chromosome 3BS

10:30–10:45	Oral presentation **Hermann Buerstmayr** Meta-analysis of resistance to FHB in tetraploid wheats – implications for durum wheat breeding
10:45–11:15	Coffee & tea break
	OECD-CRP session (sponsored by the OECD's Co-operative Research Programme on Biological Resource Management for Sustainable Agricultural Systems)
11:15–11:20	Opening remarks **Yasunari Ogihara**
11:20–11:30	Short address of OECD-CRP theme coordinator **Hitoshi Nakagawa**
Part 1: Wheat research for sustainable food chain challenging for adaptation and mitigation to the climate change and global food security	
Chairpersons: **Pedro Brajcich Gallegos** and **Hitoshi Nakagawa**	
11:30–12:00	Keynote **Matthew P Reynolds** Exploring genetic resources to increase adaptation of wheat to climate change
12:00–14:00	Lunch
14:00–14:30	Keynote **Beat Keller** Genomic approaches towards durable fungal disease resistance in wheat
14:30–15:00	Keynote **Zhen-Sheng Li** New progress in wheat wide hybridization for improving the resistance to biotic and abiotic stresses
15:00–15:20	Coffee & tea break
15:20–15:50	Keynote **Michael Baum** Global crop improvement networks to bridge technology gaps
15:50–16:20	Keynote **Mark E. Sorrells** Genomic selection in plants: empirical results and implications for wheat improvement
16:20–16:50	Keynote **Rowan Mitchell** Wheat genes for enhanced human nutrition
The 12th IWGS banquet	
19:00	Banquet
Saturday, September 14, 2013	
	OECD-CRP session **Part 2: Enhancing relevance and efficiency of wheat science for the society**
Chairperson: **Tomohiro Ban**	
9:00–9:30	Plenary lecture **Koji Murakami** What does wheat flour industry expect from wheat science?
9:30–10:00	Plenary lecture **Juan Juttner** What does seed sector expect from wheat science?

10:00–10:30	Keynote **Hans J. Braun** Improving wheat for world food security: concerted approach of IWGS and International Wheat Conference (IWC)
10:30–11:00	Keynote **Peter Langridge** Wheat Initiative (WI) for sustainable food chain
11:00–11:20	Coffee & tea break
11:20–11:50	Concluded recapitulation **Masa Iwanaga** International wheat genetics stewardship of green economy and sustainable food chain
Closing session	
11:50–12:00	IWGS closing remarks **Yasunari Ogihara**

Appendix 2: Poster Presentation List

P1-1 Abugalieva, Aigul. Kazakh Research Institute of Agriculture and Plant Growing, Kazakhstan
Wild germplasm wheat as resource, donors healthy food raw material

P1-5 Antonyuk, Maksym. National University of Kyiv-Mohyla Academy, Ukraine
Development of introgressive wheat lines with *Aegilops mutica* chromosomes

P1-6 BALFOURIER, Francois. INRA-Clermont-Ferrand, France
A worldwide bread wheat sample for the French BREEDWHEAT project

P1-7 Cattivelli, Luigi. Genomics Research Centre, CRA, Italy
Genetic diversity for CO_2 response in a panel of durum wheat genotypes grown in a free air CO_2 enrichment experiment

P1-8 Chatrath, Ravish. Directorate of Wheat Research, India
Indian Wheat Information System

P1-9 Distelfeld, Assaf. Tel Aviv University, Israel
Characterization of wheat *dicoccoides-aestivum* chromosome arm substitution lines

P1-11 Dundas, Ian. The University of Adelaide, Australia
Transfer to wheat of potentially new stem rust resistance genes derived from *Aegilops speltoides*

P1-12 Eliby, Serik. The University of Adelaide, Australia
Genetic polymorphism of SNP markers in Kazakh spring bread wheat

P1-16 Ji, Jun. Institute of Genetics and Developmental Biology, CAS, China
Development of wheat-rye-*Thinopyrum ponticum* trigeneric materials

P1-17 Kakeda, Katsuyuki. Mie University, Japan
Characterization of cleistogamous flowering and *TaAP2* homoeologs in wheat

P1-18 Karlov, Gennady. Russian State Agrarian University, Russia
Molecular characterization and DNA markers development of *thinopyrum*, *dasypyrum* and *pseudoroegneria* waxy genes

P1-20 Kawahara, Taihachi. Kyoto University, Japan
Genetic diversity in *Aegilops speltoides* - presence of two gene lineages.

P1-21 Keser, Mesut. International Center for Agricultural Research in Dry Areas (ICARDA)
Agronomic characterization and diversity of wheat landraces currently grown in Turkey based on nationwide inventory

P1-22 Knupffer, Helmut. Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany
English translation of the Russian "Flora of Cultivated Plants. Wheat" (Dorofeev et al. 1979): project progress report

P1-23 Kubo, Katashi. NARO Tohoku Agricultural Research Center, Japan
Suppressed-translocation and reduced-selectivity of cadmium (Cd) in wheat varieties with low grain Cd concentration

P1-24 Kudo, Eiji. Yamagata University, Japan
Reproductive diversification of Timopheevi wheat and reconsideration of its domestication

P1-25 Leonova, Irina. Institute of Cytology and Genetics SB RAS, Russia
Evaluation of genetic diversity of common wheat hybrid lines containing *T. durum* and *T. dicoccum* genetic material

P1-26 Li, Xingfeng. Shandong Agricultural University, China
Response to phosphorus insufficiency of *Trititrigia* germplasm Shannong303

P1-27 Lu, Yuqing(Lucy). Institute of Crop Science, CAAS, China
Identification of the translocation types and agronomic traits of wheat-*Agropyron cristatum* 6P translocation lines

P1-29 Nishijima, Ryo. Kobe University, Japan
Mapping of a dominant awnless gene in *Aegilops tauschii*

P1-31 Ohta, Shoji. Fukui Prefectural University, Japan
Geographical differentiation in *Aegilops caudata* L. : morphology, hybrid sterility and gametocidal genes

P1-32 Okada, Takashi. The University of Adelaide, Australia
Genetic variations among Triticeae for floral traits that enhance out-crossing in wheat for hybrid seed production

P1-33 Okamoto, Mamoru. The University of Adelaide, Australia
Evaluation of genetic diversity for nitrogen use efficiency and responsiveness in Australian wheat varieties

P1-36 Saito, Yoko. Kyushu University, Japan
Contribution of genetic diversity in Japanese wheat breeding research

P1-37 Sasanuma, Tsuneo. Yamagata University, Japan
Profiling of *Aegilops tauschii* collected in the North Caucasia

P1-38 Seki, Masako. Hokuriku Research Center, NARO Agricultural Research Center, Japan
Distribution of photoperiod-insensitive alleles *Ppd-A1a*, *Ppd-B1a* and *Ppd-D1a* in Japanese wheat cultivars

P1-40 Sharma, Ram. International Center for Agricultural Research in Dry Areas (ICARDA)
Improved winter and facultative wheat germplasm for medium saline soils in Central Asia

P1-41 Slafer, Gustavo. AGROTECNIO and University of Lleida, Spain
Selecting for improved fruiting efficiency to further rise wheat yield

P1-42 Turuspekov, Yerlan. Institute of Plant Biology and Biotechnology, Kazakhstan
DNA genotyping and phenotyping of spring wheat accessions from Kazakhstan

P1-45 Xu, Steven. Cereal Crops Research Unit, USDA-ARS, USA
Synthetic hexaploids derived from under-exploited tetraploids as a new resource for disease resistance in wheat

P1-46 Zhang, Jinpeng. Institute of Crop Science, CAAS, China
Genetic analysis and QTL mapping of high grain number per spike trait in two wheat novel germplasms

P1-47 ZHANG, LiYi. Institute of Upland Crops, Guizhou Academy of Agricultural Sciences, China
Investigating for haplotype diversity of stripe rust resistance loci in common wheat in southwest China

P1-48 Zhou, Yong-Hong. Sichuan Agricultural University, China
The taxonomic status of *Hystrix* Moench in Triticeae

P2-1 Badaeva, Ekaterina. Engelhardt Institute of Molecular Biology RAS, Russia
Chromosomal changes over the course of polyploid wheat evolution and domestication

P2-2 Chen, Wei. Nanjing Agricultural University, China
Preliminary study of *ZmCENH3* in suppression of chromosome elimination in wheat and maize hybrid

P2-3 Goncharov, Nikolay. Institute of Cytology and Genetics SB RAS, Russia
Major transcription factors *Vrn* and *Q* underlying the domestication of wheat

P2-4 Ishihara, Ayaka. Kyoto University, Japan
Dissection of barley chromosome 1H in common wheat

P2-5 Katkout, Mazen. Yokohama City University, Japan
Mapping quantitative trait loci for domestication related characters in hexaploid wheat

P2-6 Kishii, Masahiro. International Maize and Wheat Improvement Center (CIMMYT)
Production of *Leymus racemosus* chromosome translocation lines of bread wheat for wheat improvement

P2-7 Koba, Takato. Chiba University, Japan
Chromosomal stability in amphidiploids between tetraploid wheat cv. Langdon and *Aegilops uniaristata* and their progenies

P2-8 Kumar, Upendra. G. B. Pant University of Agriculture Technology, India
Physical mapping and expression analysis of *Ids3* (iron deficiency specific clones 3) gene in hexaploid wheat

P2-9 Kuraparthy, Vasu. North Carolina State University, USA
Genomic targeting and mapping of the breakpoint of ancestral translocation 5A-4A in wheat

P2-10 Li, Jianjian. Kyoto University, Japan
Assignment of a large volume of SSR markers to each rye chromosome by using wheat-rye addition and substitution lines

P2-11 Liu, Bao. Northeast Normal University, China
Rapid genome evolution in allotetra- and allohexaploid wheat

P2-12 Liu, Weihua. Institute of crop science, CAAS, China
Production and identification of wheat and *Agropyron cristatum* alien translocation lines

P2-13 Miraghazadeh, Asemeh. CSIRO Plant Industry, Australia
Deletion mutants of chromosome 4B identified in a dwarf mutant suppressor screen

P2-14 Mohammed, Yasir. Tottori University, Japan
An insertion in the promoter of the *Vrn-A1* of wheat-*Leymus* chromosome addition lines is responsible for early flowering

P2-15 Molnar-Lang, Marta. Agricultural Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Hungary
Development and characterization of new wheat/winter barley introgression lines

P2-16 Mori, Naoki. Kobe University, Japan
Genetic analysis of domestication traits in emmer wheat using an F_2 population

P2-17 Muramatsu, Mikio. Emeritus Professor, Okayama University, Japan
Duplication and the dosage effect of suppressing gene presumed in the formation of the branched spike type of wheat.

P2-18 Taketa, Shin. Okayama University, Japan
Physical mapping of the gene on the long arm of barley chromosome 1H that causes sterility in hybrids with wheat (*Shw*)

P2-19 Tomita, Motonori. Shizuoka University, Japan
Cluster-like chromosomal distribution of Revolver transposon and its transcription initiation site in *Secale* cereal

P3-2 Echenique, Viviana. Universidad Nacional del Sur and CONICET, Argentina
Analysis and characterization of the repetitive sequences of *T. aestivum* chromosome 4D

P3-3 Juhasz, Angela. Agricultural Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Hungary
The wheat allergome, allergen predictions in single cultivars using the tools of immunoinformatics

P3-4 Mayer, Klaus. Helmholtz Center Munich, Germany
Spatiotemporal transcriptome analysis of the wheat endosperm

P3-5 Poursarebani, Naser. Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany
Whole Genome Profiling™ for physical mapping of wheat chromosome 6A

P3-6 Raats, Dina. University of Haifa, Israel
The physical map of wheat chromosome 1BS provides an insight into its gene space organization and evolution

P3-7 Sergeeva, Ekaterina. Institute of Cytology and Genetics SB RAS, Russia
Comparative analysis of the long and short arms of bread wheat 5B chromosome by data of low coverage 454-sequencing

P3-8 Singh, Kuldeep. Punjab Agricultural University, India
Physical map and survey sequence of chromosome 2A of wheat

P3-9 Timonova, Ekaterina. Institute of Cytology and Genetics SB RAS, Russia
Development of a deletion map for 5B chromosome of bread wheat with the introgression lines involvement

P4-2 Boden, Scott. CSIRO Plant Industry, Australia
Flowering genes and yield potential in wheat

P4-3 Hao, Chenyang. Institute of Crop Science, CAAS, China
Association mapping and haplotype analysis of a 3.1-Mb genomic region involved in *Fusarium* head blight resistance on

P4-4 Iehisa, Julio. Kobe University, Japan
Application of SNPs derived from transcripts of two distinct *Aegilops tauschii* accessions to wheat genome analysis

P4-5 Jung, Yeonju. Yokohama City University, Japan
Transcriptome analysis of synthetic hexaploid wheats during the course of amphidiploidization.

P4-6 Kawaura, Kanako. Yokohama City University, Japan
Genomic structure and expression of α- and β-gliadin genes in hexaploid wheat

P4-7 Kimura, Tatsuro. Toyota Motor Corporation, Japan
Construction of a super high-resolution linkage map of common wheat by the array-based marker system

P4-8 Korol, Abraham. University of Haifa, Israel
Building ultra-dense genetic maps in the presence of genotyping errors and missing data

P4-10 Noma, Satoshi. Nisshin Flour Milling INC., Japan
Molecular characterization of the α- and β-gliadin multigene family in hexaploid wheat

P4-11 Onuki, Ritsuko. National Institute of Agrobiological Sciences, Japan
Construction of an SSR marker set in wheat chromosome 6B and physical positioning by comparison with barley genome data

P4-12 Pallotta, Margaret. The University of Adelaide, Australia
Allelic variation, dispersed gene duplication and tetraploid introgression determine boron toxicity tolerance in wheat

P4-13 Safar, Jan. Institute of Experimental Botany, Czech Republic
Fine mapping of flowering time gene *QFt.cri-3B.1* in bread wheat

P4-14 Shahinnia, Fahimeh. Australian Centre for Plant Functional Genomics, Australia
Identification of quantitative trait loci for leaf stomatal and epidermal cell traits in wheat (*Triticum aestivum* L.)

P4-15 Shcherban, Andrey. Institute of Cytology and Genetics SB RAS, Russia
The structure, expression and geographical distribution of different *VRN-B1* alleles of common wheat, *T. aestivum*

P4-17 Takamatsu, Kiyofumi. Kobe University, Japan
Comparison of gene expression profiles of hybrid necrosis in einkorn and common wheat

P4-18 Xia, Chuan. Institute of Crop Science, CAAS, China
Optimizing *de novo* common wheat transcriptome assembly using short-read RNA-Seq data

P4-19 Yao, Yingyin. China Agricultural University, China
Cloning and characterization of noncoding RNAs from wheat (*Triticum aestivum* L.)

P6-1 Bierman, Anandi. University of Stellenbosch, South Africa
Mapping and characterization of selected *Diuraphis noxia* resistance genes in *Triticum aestivum*

P6-2 Borrill, Philippa. John Innes Centre, UK
How does the *NAM-B1* transcription factor influence grain composition in wheat?

P6-3 Chandler, Peter. CSIRO Plant Industry, Australia
Overgrowth mutants in wheat: new alleles and phenotypes of the Green Revolution *Della* gene.

P6-4 Chono, Makiko. NARO Institute of Crop Science, Japan
Identification and characterization of novel mutations in wheat ABA catabolic gene

P6-5 Cseh, Andras. Agricultural Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Hungary
The transfer of barley *CslF* genes offers a new possibility to increase the β-glucan content of wheat

P6-6 Sonnante, Gabriella. National Research Council, Institute of Plant Genetics, Italy

Asparagine synthetase genes (*AsnS)* in durum wheat

P6-8 Eltayeb, Amin. Tottori University, Japan
Prospects of utilizing antioxidant capacities for improving wheat abiotic stress tolerance

P6-9 Eltayeb Habora, Mohamed. Tottori University, Japan
Identification of drought-stress responsive genes from the dune grass *Leymus mollis*, a wild relative of wheat

P6-10 Feng, Deshun. Shandong Agricultural University, China
Identification of powdery mildew induced differentially expressed genes from wheat-*Th. intermedium* alien addition lines

P6-11 Gonzalez, Oscar. John Innes Centre, UK
QTL: fine-grained phenotyping for phasic development in doubled haploid population of wheat (*Triticum aestivum*)

P6-12 Guo, Zi. Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK), Germany
The genetic analysis of floret fertility and related traits in wheat

P6-13 Himi, Eiko. Okayama University, Japan
Grain color is controlled by MYB transcription factors

P6-14 Kakei, Yusuke. Yokohama City University, Japan
Identification of genes responsive to hytohormones in *Brachypodium distachyon* using RNA-seq

P6-15 Khlestkina, Elena. Institute of Cytology and Genetics SB RAS, Russia
MYC-like family of transcription factors in wheat

P6-16 Kobayashi, Yurina. Yokohama City University, Japan
Gene expression analysis to clarify the bulb formation mechanism in *Hordeum bulbosum* L. for perennial crops

P6-17 Li, Tian. Institute of Crop Science, CAAS, China
Analysis of miRNA expression profiles in the development of wheat grains

P6-18 Li, Yingbo. Nanjing Agricultural University, China
HvFP3, a HMA domain protein interacted with the E3 ubiquitin ligase *HvCMPG*, is involved in wheat powdery mildew defense

P6-19 Liu, Dongcheng. Institute of Genetics and Developmental Biology, CAS, China
Cloning and genetic mapping of *TaGA2ox3* in common wheat

P6-20 Ma, Lingjie. Nanjing Agricultural University, China
Identification and analysis of a flower-specific promoter in bread wheat (*Triticum aestivum* L.)

P6-21 Makai, Szabolcs. Centre for Agricultural Research, Hungarian Academy of Science, Hungary
Evolutionary differences in the transcriptional regulation of HMW glutenin genes in Triticeae

P6-22 Matsunaka, Hitoshi. NARO Kyushu Okinawa Agricultural Research Center, Japan

Effect of marker-assisted selection for pre-harvest sprouting: Pyramiding *MFT-3A* and *Phs1*

P6-23 Miyazaki, Saori. National Institute of Genetics, Japan
RNA recognition motif of rice MEL2 regulating transition from mitosis to meiosis binds to U-rich RNA conserved sequence

P6-24 Ni, Zhongfu. China Agricultural University, China
Epigenetic modification contributes to the expression divergence of three *TaEXPA1* homoeologs in hexaploid wheat

P6-25 Nishida, Hidetaka. Okayama University, Japan
Photoperiod-insensitive alleles for *Ppd-1* affect expression pattern of *Ppd-1* homoeologs and their downstream genes

P6-26 Nishiura, Aiko. Fukui Prefectural University, Japan
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P6-27 Rikiishi, Kazuhide. Okayama University, Japan
Effects of seed maturation regulators on seed dormancy in wheat

P6-28 Schnurbusch, Thorsten. Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK), Germany
Genetic basis of spike-branching in wheat

P6-29 Sharp, Peter. University of Sydney, Australia
Serpins of multiple gene subfamilies in bread and durum wheat grain

P6-30 Shpylchyn, Vitaliy. National University of Kyiv-Mohyla Academy, Ukraine
Application of IRAP and RAMAP techniques for polymorphism studying of wheat genome with hybrid origin

P6-31 Takenouchi, Yu. HOKUREN Agricultural Research Institute, Japan
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P6-32 Tanaka, Miku. Fukui Prefectural University, Japan
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P6-34 WANG, JIRUI. Sichuan Agricultural University, China
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P6-37 Xu, Wenqi. Nanjing Agricultural University, China
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P6-38 Yessenbekova, Gulzat. Kazakh National Agrarian University, Kazakhstan
Identification of *Yr10* gene in wheat germplasm under application of molecular markers

P6-39 Zhang, Zengyan. Institute of Crop Science, CAAS, China
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P6-40 Zurn, Jason. North Dakota State University, USA
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P7-2 Bansal, Urmil. University of Sydney, Australia
Molecular mapping of a slow rusting resistance gene *Sr56* in winter wheat cultivar Arina

P7-5 Carter, Arron. Washington State University, USA
QTL for *Puccinia striiformis* f. sp. *tritici* resistance in a Coda by Brundage bread wheat RIL population

P7-6 Chen, Peidu. Nanjing Agricultural University, China
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P7-10 Hu, Zhaorong. China Agricultural University, China
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P7-11 Jaiswal, Jai. G. B. Pant University of Agriculture & Technology, India
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P7-12 Jayatilake, Dimanthi. The University of Adelaide, Australia
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P7-13 Kassa, Mulualem. Agriculture and Agri-Food Canada, Canada
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P7-14 Krattinger, Simon. University of Zurich, Switzerland
Lr34 multi-pathogen resistance gene; insights from haplotype studies and transgenic experiments

P7-15 Li, Na. Nanjing Agricultural University, China
Identification of a gene conferring powdery mildew resistance at later growth stages in a tetraploid wheat accession

P7-16 Lillemo, Morten. Norwegian University of Life Sciences, Norway
The impact of anther extrusion and plant height on *Fusarium* head blight and implications for resistance breeding

P7-18 McIntosh, Robert. University of Sydney, Australia
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P7-19 Naruoka, Yukiko. Washington State University, USA
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P7-21 Niwa, Sayaka. Yokohama City University, Japan

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P7-22 Orabi, Jihad. University of Copenhagen, Denmark
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P7-23 Ramirez-Gonzalez, Ricardo. The Genome Analysis Centre, UK
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P7-24 Rosewarne, Garry. International Maize and Wheat Improvement Center (CIMMYT)
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Gene cloning for the improvement and better understanding of the powdery mildew resistance in common wheat

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P7-38 Mergoum, Mohamed. North Dakota State University, USA
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Genetic and physiological analysis of traits related to salinity tolerance and improved end-use quality in wheat

P8-7 Fowler, David. University of Saskatchewan, Canada
A systems approach to understanding winter survival in wheat

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P8-13 Kawakami, Naoto. Meiji University, Japan
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P8-25 Peleg, Zvi. The Hebrew University of Jerusalem, Israel
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P8-27 Sasaki, Kentaro. NARO Hokkaido Agricultural Research Center, Japan
Identification of novel LEA-class protein involved in freezing tolerance in wheat

P8-28 Sehgal, Deepmala. International Maize and Wheat Improvement Center (CIMMYT)
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P8-32 Singh, Gyanendra. Directorate of Wheat Research, India
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P8-34 Yokota, Hirokazu. Kobe University, Japan
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P8-35 Zayed, Adel. Monsanto, USA
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P9-1 Abugalieva, Saule. Institute of Plant Biology and Biotechnology, Kazakhstan
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P9-6 Ishikawa, Goro. NARO Tohoku Agricultural Research Center, Japan
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P9-7 Jichun, Tian. Shandong Agricultural University, China
Effects of interaction between HMW-GS and waxy alleles on dough-mixing properties in common wheat

P9-8 Johansson, Eva. Plant Product Quality, Sweden
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Relationship between physicochemical characteristics of flour and sugar-snap cookie quality in Korean wheat cultivar

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The effect of heat, drought and cold stress on protein composition and some quality and Mixsmart characteristics

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Comparative analysis of wheat seed storage proteins in hexaploid wheat

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Mixograph based on farinograph water absorption to predict straight-dough bread-making quality

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Structural and physicochemical properties of endosperm starch from sweet wheat: a GBSSI and SSIIa double null mutant

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Additional alien chromosomes in common wheat effect on the protein profile in the endosperm and dough strength

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Use of high beta-glucan barley flour as wheat flour adjunct and substitute for useful source of functional dietary fiber

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Improvement of wheat quality through introduction of *Hordeum* species chromosomes into common wheat

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Allelic variation of the low-molecular-weight glutenin subunit genes in wheat

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Regulating the seed dormancy level by expressing a cereal *DOG1* homologous gene in transgenic wheat

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Cloning and function verification of a wheat Antiquitin gene promoter

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An integrated breeding programme for sustainable agriculture in France

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Marker assisted backcrossing for *Fusarium* head blight resistance and high grain protein content in winter wheat

P10-5 Da Silva, Paulo Roberto. Universidade Estadual do Centro Oeste, UNICENTRO, Brazil
Postulation of stem rust resistance genes in selected Brazilian bread wheat cultivars by molecular markers

P10-6 Degu, Tadesse. International Center for Agricultural Research in Dry Areas (ICARDA)
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P10-7 Fujita, Masaya. NARO Kyushu Okinawa Agricultural Research Center, Japan
Novel quantitative trait loci for cleistogamy in the bread wheat cultivar 'U24'

P10-8 Ganal, Martin. TraitGenetics GmbH, Germany
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P10-10 Ghanim, Abdelbagi. Joint FAO/IAEA Division, International Atomic Energy Agency, Austria
Mutation-induced variability for improved yield and rust resistance in wheat in hot irrigated environments

P10-11 Gils, Mario. Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany
A novel hybrid seed system for wheat

P10-12 Gosman, Nicolas. National Institute of Agricultural Botany, UK
Genome-wide association mapping and genomic prediction in northern European wheat

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Marker assisted breeding in bread wheat. Brief story and current status in Argentina

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Marker-assisted breeding for durable wheat rust and *Fusarium* head blight resistance

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Homozygous doubled haploid Australian and Kazakh wheat from isolated microspore cultures

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Common resistance QTL from five synthetic wheats against *Pratylenchus thornei* and *Pratylenchus neglectus*

P10-19 Kokhmetova, Alma. Institute of Plant Biology and Biotechnology, Kazakhstan
Identification of wheat germplasm resistant to tan spot using molecular markers

P10-20 Lado, Bettina. Facultad de Agronomia, UdelaR, Uruguay
Discovering novels QTL in Uruguayan Wheat germplasm using genome wide association analysis

P10-21 Maccaferri, Marco. University of Bologna, Italy
Tracking valuable QTLs for durum wheat breeding through integrated linkage and association mapping: from QTLs to SNPs

P10-22 Mahbubjon, Rahmatov. Swedish University of Agricultural Sciences, Sweden
Characterization and molecular cytogenetic of wheat-alien sources of resistance to stem rust and yellow rust

P10-23 Merchuk, Lianne. The Hebrew University of Jerusalem, Israel
Introgression of QTLs from wild emmer wheat shows a potential to improve drought resistance of modern wheat

P10-25 Nguyen, Anh. Kobe University, Japan
QTL analysis of flowering-related traits in the D-genome of synthetic hexaploid wheat

P10-28 Periyannan, Sambasivam. CSIRO Plant Industry, Australia
Rapid gene stacking and a robust assay to quantify fungal biomass associated with rust resistance in wheat

P10-29 Pozniak, Curtis. University of Saskatchewan, Canada
Utilization of the 3B reference sequence to localize *SSt1*, a gene conferring solid stem expression in wheat

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Wheat breeding using the *ph1* mutant to transfer agronomic desirable traits from *Hordeum chilense* into wheat

P10-31 Prins, Renee. CenGen Pty Ltd. And University of Free State, South Africa
The establishment of a molecular service laboratory for wheat breeding in South Africa

P10-32 Schondelmaier, Joerg. Saaten-Union Biotec GmbH, Germany
HYWHEAT: Hybrid wheat for reduced inputs and sustainable yield (CMS/Rf system)

P10-33 Seo, Yong Weon. Korea University, Korea
Molecular breeding approaches for improvement of wheat-rye translocations

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P10-38 Weschke, Winfriede. Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany
Increased grain yield and micronutrient content of winter wheat by ectopic expression of a barley sucrose transporter

P10-39 Wuerschum, Tobias. University of Hohenheim, Germany
Precision phenotyping of biomass accumulation in triticale reveals temporal genetic patterns of regulation

P10-40 Xia, Xianchun. Institute of Crop Science, CAAS, China
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