From Genetic Stock to Genome Editing: Gene Exploitation in Wheat

Article *in* Trends in Biotechnology · November 2017

DOI: 10.1016/j.tibtech.2017.10.002

6 authors, including:

Meng Wang
Chinese Academy of Sciences
18 PUBLICATIONS **398** CITATIONS
SEE PROFILE

Zhen Liang
Shanxi University
19 PUBLICATIONS **3,348** CITATIONS
SEE PROFILE

Weiming Shi
Institute of Soil Science Chinese Academy of Sciences Nanjing
206 PUBLICATIONS **5,988** CITATIONS
SEE PROFILE

Caixia Gao
Institute of Genetics and Developmental Biology, CAS
119 PUBLICATIONS **10,614** CITATIONS
SEE PROFILE

Trends in Biotechnology

CellPress REVIEWS

Review

From Genetic Stock to Genome Editing: Gene Exploitation in Wheat

Meng Wang,[1,2,3,4] Shubin Wang,[2,4] Zhen Liang,[3] Weiming Shi,[1] Caixia Gao,[3,*] and Guangmin Xia[2,*]

Bread wheat (*Triticum aestivum*) ranks as one of our most important staple crops. However, its hexaploid nature has complicated our understanding of the genetic bases underlying many of its traits. Historically, functional genetic studies in wheat have focused on identifying natural variations and have contributed to assembling and enriching its genetic stock. Recently, mold-breaking advances in whole genome sequencing, exome-capture based mutant libraries, and genome editing have revolutionized strategies for genetic research in wheat. We review new trends in wheat functional genetic studies along with germplasm conservation and innovation, including the relevance of genetic stocks, and the application of sequencing-based mutagenesis and genome editing. We also highlight the potential of multiplex genome editing toolkits in addressing species-specific challenges in wheat.

Trends

The natural variation represented in wheat genetic stocks, including collections of wild relatives and cultivated accessions, and artificially innovated introgression lines, has been and remains an important facilitator of genetic advance.

With the continuous release of wheat genome information, capturing agronomically important genes from the conventional wheat genetic stock is expedited and motivates future strategy.

The approaches of exome capture and next-generation sequencing helped to establish the first public mutant library in wheat.

Germplasm innovations through sequencing-based mutagenesis and genome editing will drive the elaboration of radical new strategies for wheat functional genetic studies.

The emergence of multiplex genome editing toolkits offers an alternative and efficient approach for addressing complex or species-specific challenges in wheat.

Strides in Wheat Germplasm Innovations Driving Functional Genetic Studies

Genetic resources (see Glossary) provide not just a foundation for crop breeding, but also a reservoir of agronomically important genes. For functional genetic studies in bread wheat (*Triticum aestivum*), a germplasm panel including both wild and cultivated types, with representatives of the various ploidy levels, has been assembled, forming a basic **genetic stock** to discover the crop's natural variations. Among those representatives, ancestral wheat relatives are good candidates to be used to improve modern **hexaploid** wheat, since they conserve considerable genetic variability of adaptive traits which can be transferred via direct hybridization. Then, along with advances in cytological techniques and chromosome engineering, artificial innovated breed stocks including **introgressed** wheat and **aneuploid** wheat were created, and thereafter more creative approaches to identifying genes were introduced [1]. Meanwhile, germplasm expressing the extremes of a given trait, in some sense acting as a mutant, has been widely exploited to reveal the genetic basis of important traits and to understand their molecular mechanistic bases in bread wheat.

The genetics of many traits in wheat have in the past been complicated by its polyploidy (Box 1). However, the ongoing release of large volumes of genome sequences [2] has greatly accelerated the identification of important genes [3]. Meanwhile, **exome capture** and **next-generation sequencing** approaches also helped to establish the first public mutant library in wheat [4]. Moreover, multiple optimized CRISPR-Cas9-mediated genome editing systems have been recently developed and applied in bread wheat, offering an even more targeted approach for germplasm innovations [5,6].

These mold-breaking advances should continue to elaborate radical new strategies for genetic research, especially at the gene level, in bread wheat. Furthermore, the roles of conventional wheat genetic stock are evolving instead of diminishing. This review will present current applications of wheat genetic stocks in exploiting and delivering genes for agronomically

[1]State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China
[2]The Key Laboratory of Plant Cell Engineering and Germplasm Innovation, Ministry of Education, School of Life Sciences, Shandong University, Jinan 250100, China
[3]State Key Laboratory of Plant Cell and Chromosome Engineering, and Center for Genome Editing, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

Box 1. The Hexaploid Nature of Bread Wheat

Bread wheat (2n = 6x = 42, genome AABBDD) is an allohexaploid species which emerged from two long-separated natural wide hybridization events. The first, occurring 0.5–3 million years ago, formed the tetraploid species wild emmer (*T. dicoccoides*, 2n = 4x = 28, genome AABB) as a result of a cross between the diploid *T. urartu* (2n = 2x = 14, genome AA) and an as yet unidentified and possibly no longer extant species of the genus *Aegilops* (2n = 2x = 14, genome SS); the second, much more recent (7000–9500 years ago) cross combined a domesticated form of wild emmer (*Triticum dicoccum*) with diploid goatgrass (*A. tauschii*, 2n = 2x = 14, genome DD), thereby adding the D genome.

important traits. New trends in wheat functional genetic studies, along with germplasm innovations through sequencing-based mutagenesis and genome editing, will be discussed. Moreover, the future hotspots of multiplex genome editing toolkits in coping with complex or species-specific challenges of wheat are emphasized.

Revealing Natural Genetic Variation in Genebank Collections

The current landscape of the genetic diversity of wheat has been formed by a series of natural and artificial events. A severe reduction in genetic diversity arose through the process of polyploidy speciation and domestication and continued over several decades of extensive selection and breeding [7]. However, as is the case for all genomes, the genome of the newly established polyploid wheat was not static, with novel variants arising as consequences of natural mutation. The **genomic buffering** allowed by polyploidy improved the chances of the crop retaining these variations [8]. The rapid geographical spread of bread wheat from its origin in West Asia, both westwards into North Africa and Europe (and later to the Americas) and eastwards through central Asia into China and India (and later to southern and central Africa, as well as to Australasia) exposed the gene pool to a diversity of environments, which had a profound effect on the species' heterogeneity.

In an effort to conserve as much as possible of wheat's genetic diversity, a number of large genebanks have been established over the past decades; the most extensive of these are curated at International Centre for the Improvement of Maize and Wheat in Mexico, the National Small Grains Collection in the USA, the Institute of Plant Genetics and Crop Plant Research in Germany, the Winter Cereal Collection in Australia, the Genetics Resources for Wheat Sciences in Japan, and the National Genebank in China [3] (Table 1). These genetic stocks, saturated with divergent phenotypes and physiologies, aid in exploiting genes of agronomic interest in wheat.

First, genebanks can offer alternatives to gene positional cloning. Currently in wheat, as in many crop species, the most feasible route to gene isolation has been via positional cloning [9]. For example, the boron-tolerant cultivar Halberd and intolerant cultivar Cranbrook were screened from the Australian wheat collection, laying the foundation for identifying the gene at the major boron tolerance locus [10]. Considering its polyploidy, its large genome, its high content of repetitive DNA and the problem of each **centimorgan (cM)** being represented by a very large number of base pairs (bps) in bread wheat, **forward genetics** is still time-consuming and tedious [3]. Accordingly, selecting the optimal germplasm that exhibits an extreme phenotype and a clear pedigree from the wheat genebank collections, the first step of positional cloning, is not trivial. Furthermore, acquiring an ever-more-accurate genome sequence has prompted the development of alternative approaches (Box 2), notably based on multiparent populations, which have also relied on genebanks as an enabling resource [11].

In the wheat genebanks, some gene resources are scarce or even unattainable through exhausted screenings in relative species or by mutagenesis. In extreme cases, some of these

[4]These authors contributed equally to this work

*Correspondence:
cxgao@genetics.ac.cn (C. Gao) and
xiagm@sdu.edu.cn (G. Xia).

Table 1. Online Resources for Wheat Genebanks and Wheat Genomic Information

Resource	URL
The International Maize and Wheat Improvement Center (CIMMYT)	www.cimmyt.org/
National Small Grains Collection in the USA	https://www.ars.usda.gov/pacific-west-area/aberdeen-id/small-grains-and-potato-germplasm-research/docs/barley-wheat-genetic-stocks-collections/
Institute of Plant Genetics and Crop Plant Research in Germany	www.ipk-gatersleben.de/en/gbisipk-gaterslebendegbis-i/
Winter Cereal Collection in Australia	www.dpi.nsw.gov.au/about-us/research-development/centres/tamworth/research-projects
Genetics Resources for Wheat Sciences in Japan	https://shigen.nig.ac.jp/wheat/komugi/
National Genebank in China	www.cgris.net/cgris_english.html
Wheat mutant libraries	www.wheat-tilling.com/
Genome of synthetic hexaploid wheat 'W7984′	www.cerealsdb.uk.net/cerealgenomics/CerealsDB/blast_WGS.php
RefSeq v1.0 by IWGSC	https://wheat-urgi.versailles.inra.fr/Seq-Repository/Assemblies
Wheat pangenome	https://wheatis.tgac.ac.uk/grassroots-portal/blast http://appliedbioinformatics.com.au/cgi-bin/gb2/gbrowse/WheatPan/

genes arose only from certain special germplasm in the collections, which in turn provides a unique solution to isolating these rare genes. An illustrative example is resistance to *Fusarium* head blight (FHB), where a rare gene was found to be present in the Chinese cultivar Sumai 3; the gene has since been isolated [12]. Similarly, in 2017, the *Ms2* gene was identified by positional cloning from a unique germplasm called 'Taigu genic male-sterile wheat', which was

Box 2. Current Status of Wheat Whole Genome Sequencing and Its Applications

The genome drafts of diploid wheat ancestors have been reported [30,31] and the reference sequences are being assembled. Meanwhile, a 10.1-gigabase genome of wheat tetraploid progenitor, wild emmer wheat, has been released recently [80]. As for hexaploid wheat, multiple assemblies of whole genome have been constructed via various approaches [33,50,81], and the available databases are shown in Table 1. Among those, the International Wheat Genome Sequencing Consortium (IWGSC) used an integration of chromosome-based sequencing (see the section 'Broad uses and new roles of aneuploid wheat stocks in gene identification') and other strategies leading to the first version reference sequence (RefSeq v1.0). More wheat accessions are being sequenced and a wheat 'pangenome' is being established [2,82] (Table 1). Coupled with the headway in building up genomic information, gene identification in wheat will be expedited [3] through the following approaches.

Firstly, the genomic information has greatly accelerated the discovery of single nucleotide polymorphisms (SNPs) and development of genotyping-by-sequencing (GBS) in wheat, thereby enriching the markers within high-density maps, which ensures faster gene positional cloning.

Using the genome sequencing information as a reference, transcriptomic data with a better resolution is available in wheat, allowing precise identification of homoeologs or alternative splicing variants [83].

Finally, along with the enrichment of genomic information, new approaches to gene discovery are evoked accordingly. For example, sequencing-based mutagenesis offers an informative resource for functional genetic study in wheat (see the section 'Mutant Libraries'). For another example, using the targeted chromosome-based cloning via long-range assembly strategy, specific chromosomes from certain cultivars of interest can be isolated and resequenced, resulting in the rapid cloning of leaf-rust resistance gene, *Lr22a* [84].

Glossary

Aneuploid: organisms or cells having an abnormal chromosome number compared with the wild-type counterpart of a chromosome set.
Centimorgan (cM): a measurement unit of genetic linkage between two given sites on the chromosome.
Diploid: organisms or cells containing a homologous pair of complete chromosome sets.
Exome capture: technique used to extract and sequence the exome in a genome.
Forward genetics: a methodology for determining the genetic architecture or underlying genes for a trait.
Genetic resource: the existing germplasm, which is of tremendous importance in sustaining the genetic diversity for a species.
Genetic stocks: plants or populations generated or selected for genetic studies and breeding programs, which can represent materials of broad types, such as germplasm collections, crossing populations, cytological materials and mutants.
Genomic buffering: a mutation in one of the functionally redundant genes has a smaller effect on the expressed phenotype(s) of these genes.
Hexaploid: organisms or cells containing three homologous pairs of complete chromosome sets.
Hybrid vigor: also known as heterosis, the increase in characteristics of any biological quality in a hybrid offspring over the parents.
Introgress: the movement of a chromosome segment from one species to another.
Next-generation sequencing: a generic term used to describe a number of different modern DNA sequencing technologies including Illumina Solexa sequencing, Roche 454 sequencing, and SOLiD sequencing, etc.
Reverse genetics: a research strategy used to find the effect on phenotypes of a DNA sequence.
Tetraploid: organisms or cells containing two homologous pairs of complete chromosome sets.

discovered in the 20th century in Taigu county, China [13,14]. Along with progress in genome sequencing, localized cloning of these rare genes in wheat will be expedited (Box 2).

Second, the accessions housed in genebank collections exhibit an abundance of not only phenotypic variation, but also haplotype variation, some of which may be functionally significant. Haplotypes can be informative in elucidating geographical provenance, the effect of anthropogenic selection and the genetic basis of environmental adaptation. Haplotypes of a given gene are particularly significant when they can be predicted to generate either an altered translation product or an altered expression profile. Based on a screen of over 1000 genebank accessions, seven new alleles of the gene *Pm3* (which encodes resistance against powdery mildew) have been identified [15]. Epigenetic variation, which can have major effects on gene expression [16], provides an additional target that is in principle accessible through the analysis of genebank collections. An example of such variation can be found in the gene *Ppd-B1*, which is an important determinant of photoperiodism: two methylation haplotypes have been associated with altered expression levels of the gene, and their presence correlated with photoperiod insensitivity [17].

Recently, high-throughput genotyping approaches and automated phenotyping technologies have been applied in the wheat genebanks, mainly for the core germplasm collections, the volume of which is more achievable and manageable but also enough to reflect most of the genetic diversity of whole collections [18]. With these advantages, the genetic architecture of one interesting trait could be achieved by approaches such as a genome-wide association study or genomic prediction [19,20]. Its major analytical advantage over the more conventional linkage-mapping approach is that more than two alternative alleles are considered simultaneously, while its practical advantage is that it avoids the time-consuming need to construct tailor-made mapping populations. Characterizing the diversity represented in a panel of accessions can guide gene isolation strategies by, for example, choosing a germplasm to capture extreme phenotypes or selecting contrasting genotypes at the targeted gene with less variation in the remaining regions of the genome.

Exploiting the Gene Pool of Diploid and Tetraploid Ancestral Species

In the evolutionary history of wheat, particularly in the process of polyploidization and domestication, only a limited representation of its diversity descended from its progenitors. Therefore, some favorable genes or gene alleles were locked in ancestral gene pools or flowed to the relative species, which evolved in different directions compared to bread wheat. The relevant species constituting the primary and secondary gene pools are *Triticum urartu*, *Aegilops speltoides* and another Sitopsis group *Aegilops* spp. (related to the B genome donor), *Aegilops tauschii*, and *Triticum dicoccoides* [21], but they also include the A genome carrier cultivated einkorn wheat (*Triticum monococcum*, A^mA^m) and the pedigree species of the G genome carrier branch, *Triticum timopheevii/Triticum araraticum* (AAGG) and *Triticum zhukovskyi* (AAAAGG) [7] (Figure 1A). These species have long been recognized as sources of useful genes for wheat improvement and are known to harbor considerable genetic variability that is relevant to adaptive traits [22]. Gene transfer from these species is moderately feasible via sexual crossing: their genomes are homologous to those of bread wheat, which is a congenital advantage when applying elite genes discovered from these relatives [1]. For example, a **diploid** ancestral wheat relative *T. monococcum* exhibited elite traits, including greater salinity tolerance and stem rust Ug99 resistance than wheat cultivars. Positional cloning determined that *TmHKT;15-A* [23] and *TmCNL9* [24], respectively, were responsible for these two traits; neither of the genes was present in bread wheat because *T. monococcum* was not the donor of the A genome to the polyploid wheat species. Through interspecific crossing, *TmHKT;15-A* and *TmCNL9* were transferred into a wheat cultivar, resulting in improved stress tolerance [23,25].

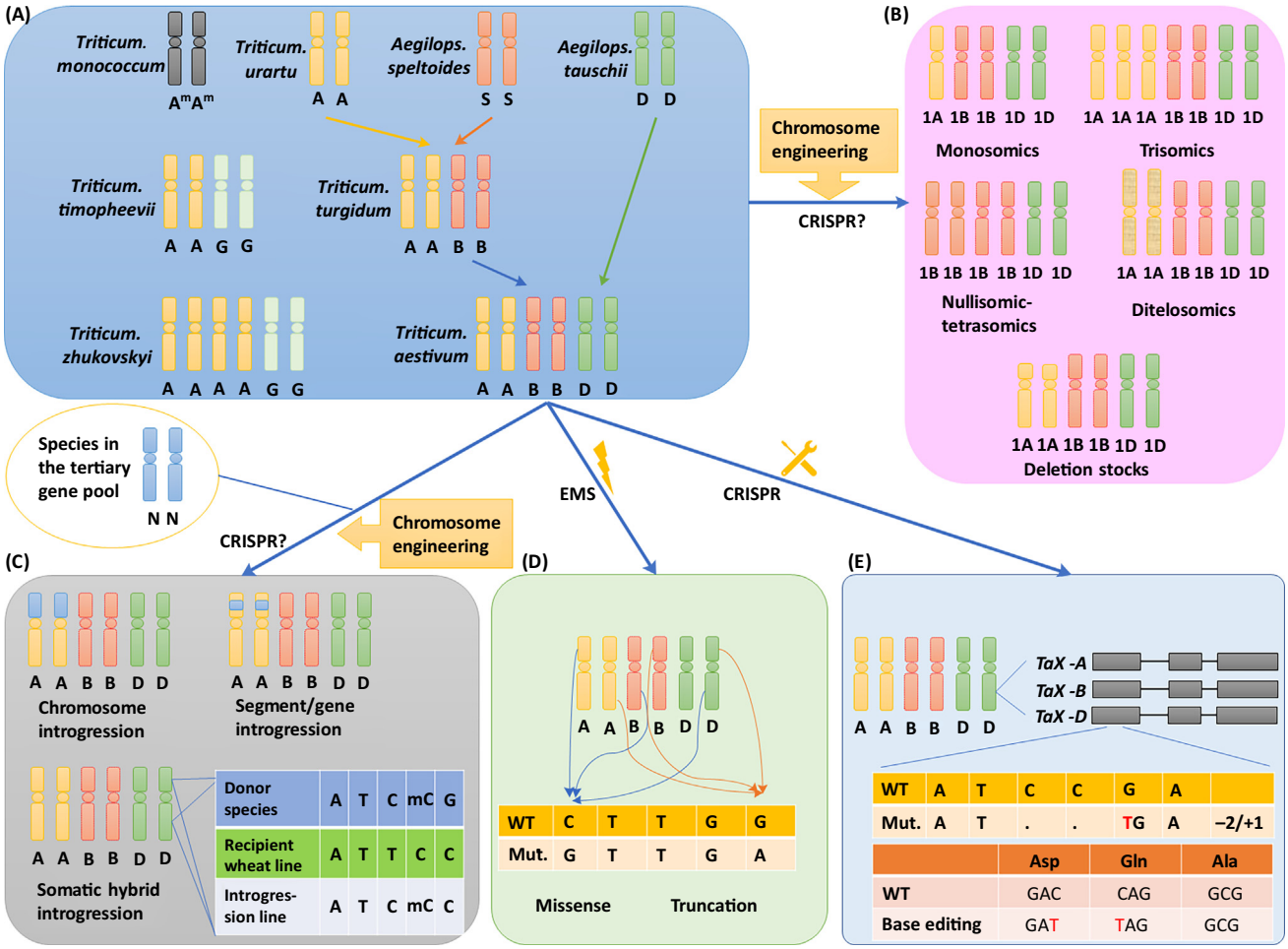

Figure 1. The Range of Genetic Resources Available in Wheat. (A) The evolution of bread wheat from its lower-ploidy relatives. (B) Aneuploid stocks of bread wheat, using the group 1 chromosomes as an example. (C) Introgression stocks. (D) An EMS mutant library. (E) CRISPR/Cas9 generated mutants. Abbreviations: EMS, ethyl methanesulphonate. In the tables: A, adenine; C, cytosine; EMS, ethyl methanesulphonate; G, guanine; mC, methylcytosine; mut, mutant; T, thymine; WT, wild-type. In the chromosome diagrams A, B, D, G, N, and S represent the type of the subgenome.

In the early stage, approaches described as subgenome chromosome walking strategy [26] and diploid–polyploid shuttle strategy [27] were successfully attempted based on the high colinearity between diploid wheat relatives and hexaploid wheat, which contributed significantly to positional cloning because they reduced the complexity of hexaploid wheat to a diploid background [28,29]. During the last five years, genome drafts of these ancestral wheat relatives have been released [30,31] and the reference sequences are currently being constructed (Box 2). Therefore, functional genetic studies of or relying on these wheat relatives will be more easily achieved.

Another avenue to take advantage of elite traits in these ancestral species is to create so-called 'synthetic wheats'. This process recreates hexaploid wheat by hybridizing AABB **tetraploids** (usually, but not exclusively, *Triticum durum*) with *Ae. tauschii*, followed by a whole-genome doubling by treating with the alkaloid colchicine; the process recapitulates the natural formation

of bread wheat [32]. Apart from its potential in breeding, nascent hexaploid wheat is a meaningful resource to establish a high-resolution genome profile [33], investigate the molecular mechanism of **hybrid vigor** [34,35] and determine the evolutionary fate of homoeoalleles (see more in [35] and in the 'Broad uses and new roles of aneuploid wheat stocks in gene identification' section later).

Extending Genetic and Epigenetic Diversity by Accessing the Tertiary Gene Pool

Species belonging to the tertiary gene pool comprise a number of grasses within the tribe Triticeae [36]. Although some of these species can be successfully hybridized with wheat, the *Ph1* system (which restricts chromosome pairing and recombination to homologs) prevents nonhomologous recombination [37,38]. The presence of *Ph1* forces a nonwheat chromosome to remain intact when present in a wheat nucleus, allowing as a result the addition of a single alien chromosome and the production of derived substitution lines. These lines have been used as a springboard to introgress subchromosomal segments by various techniques [36,39], most significantly by relaxing the strict homolog pairing through the inactivation or deletion of *Ph1*. Additionally, the genomic buffering ensured by polyploidy means that wheat can tolerate the presence of segments of chromatin derived from a nonhomologous genome (Figure 1C). Introgressions of exotic genes or segments will generate special wheat germplasms exhibiting the trait from the donor, and genomic shock during the intergeneric hybridization will cause extra genetic and epigenetic variations, resulting in multiple phenotypes in the progenies. Thus, novel genetic wheat materials derived from hybridizations with the species in the tertiary gene pool are a treasure trove for trait-associated functional gene analysis (Box 3).

Except for the exotic genes/segments and the extra genetic/epigenetic variations resulting in the divergent phenotype, the introgression line shares a common genetic background with its parental wheat line. Thus, functional omics approaches, such as genomics, epigenomics, transcriptomics, proteomics and metabolomics, have great potential to identify trait-associated genes in the introgression line, as the noises (meaningless discrepancies that disturb the following analysis) of the omics data between the introgression line and the parental line are much lower. Additional genetic manipulations like the backcross will further decrease the noises, leading to a more precise and direct identification of trait-associated gene [40]. Notably,

Box 3. Gene Identification from Wheat Introgression Lines

The conventional strategy to generate introgression lines is sexual hybridization supplemented with cytological techniques. The most successful of such transfers have involved alien segments harboring gene(s) conferring resistance to various diseases [85], such as a 6VS·6AL translocation (T6VS·6AL) wheat line, to which the chromosome 6V short arm of *Haynaldia villosa* carrying the powdery mildew resistance loci *Pm21* was transferred [86]. Based on this line, *TaStpk-V*, coding a putative serine and threonine protein kinase was cloned as the causal gene for *Pm21* through an integrative strategy of positional mapping, cytogenetic techniques, and transcriptomic analysis [87].

An alternative to the sexual hybrid route (and the only current known way forward where sexual hybrids are not possible) is to use (asymmetric) somatic hybridization, a technique in which protoplasts of the two donor species are fused *in vitro* and the fusions subsequently regenerated into whole plants (Figure 1C) [58]. The most fully characterized example of this approach involves tall wheatgrass (*Thinopyrum ponticum*) as a donor of genes conferring an improved response to abiotic stress. A practical outcome was the identification of a derivative that displayed a higher level of salinity tolerance than the recipient wheat cultivar, due to the introgression of a locus on chromosome arm 5AL. A detailed analysis suggested that the key gene introgressed encoded a poly (ADP ribose) polymerase (PARP) domain protein [88]. Moreover, a burst of epigenetic variation can also be triggered by a somatic hybridization [58]. For several stress-responsive genes, there were clear differences in transcript levels between stressed introgression line and the recipient wheat cultivar which could not be explained by differences in either the promoter or the coding sequence of these genes, instead, which were associated with the differences in DNA methylation level through epigenomic analysis [57].

as high-resolution omics data, especially transcriptomic data, is proliferating and an online database has been established for bread wheat [2,3], functional omics will be more effective for characterizing vital genes in these introgressions.

Broad Uses and New Roles of Aneuploid Wheat Stocks in Gene Identification

The buffered hexaploid nature of bread wheat makes it possible to generate the broadest range of aneuploid stocks in cv. Chinese Spring. These were initiated by first identifying monosomic (that is, lacking one member of a homologous pair) and trisomic (that is, carrying an additional copy of one chromosome) lines, followed by the development of nullisomic–tetrasomics (where one pair of chromosomes is replaced by an extra pair of one of its homeologues) and ditelosomics (where one pair of chromosomes is represented by a pair of telocentric rather than complete chromosomes) [41–43] (Figure 1B). Over many years, these cytogenetic stocks have been exploited to assign genes to a particular chromosome or chromosome arm [37]. Another major use of monosomic lines has been to generate single chromosome substitution and recombination lines, which allowed a number of quantitative effects to be assigned to a particular chromosome segment [44]. Furthermore, the demonstration that the presence in a cv. Chinese Spring background of a particular *Aegilops cylindrica* chromosome induced chromosomal breaks [45] was exploited to construct a set of deletion stock in wheat [46] (Figure 1B); the set involved representatives from each of the 21 wheat chromosomes, and has become a core resource for assigning the subchromosomal location of genes and markers [47,48].

Recently, these earlier constructed aneuploid stocks were rekindled in the context of genome sequencing (Box 2). The synteny between homeologs greatly complicates the assembly of sequences, but this problem can be avoided by using individual chromosomes (rather than the entirety of the nuclear DNA) as the sequencing template. Since some of the telocentrics are smaller than any of the intact chromosomes, flow cytometry can be used to isolate them from a preparation of mitotic chromosomes prepared from a ditelosomic line [49], thereby facilitating the chromosome-by-chromosome sequencing strategy adopted by the International Wheat Genome Sequencing Consortium [50].

The uses of aneuploid and deletion stocks has changed over the years, but especially by integrating with the diploid and tetraploid ancestral lines, they continue to offer interesting research options such as investigating relationship and divergence among the homoeologous genes. Utilizing the chromosomal deleted lines CS 5DL-5 and 5BL-14 and other genetic stocks encompassing *Q/q* homoeoalleles, which governs domestication characters in polyploidy wheat, different expression patterns and contributions to the domestication trait were studied [51]. As a hexaploid species with a clear parentage, bread wheat has become a model species to study polyploidization. Comprehensive comparisons of genomic sequence structures, expression levels, and epigenetic modifications among different ploidy levels of wheat lines will elucidate how polyploidization affects the gene dosage and why homoeologous genes have distinct evolutionary fates, giving us a deeper understanding of vital genes from the clues of natural selection [51–53].

Mutant Libraries

Mutations can be induced by exposing wheat grains (or plants) to a range of chemical or physical mutagens. Initially, once the DNA sequence of a gene target is known, it is possible to apply the TILLing (targeting induced local lesions in genomes) technique to select for lesions at

a specific target [54], which has contributed to the functional validations of genes isolated by positional mapping [12,29]. However, to identify the unknown mutant site, whole-genome resequencing, which has been an optimal alternative for species with small genomes, seems expensive and impractical in the background of bread wheat, which restricts the broad use of mutant populations.

The rapidly improving technical capacity of next-generation sequencing (NGS) and the genomic information enrichments of the bread wheat and its progenitors now offer a more direct means, sequence capture, of using mutant libraries for gene discovery in bread wheat. An extreme case was to rapidly identify disease resistance (R) genes in wheat using MutRenSeq method, which combined chemical mutagenesis with exome capture and sequencing [55]. Based on the abundance and the conservation of most R genes in plant genomes, a specific bait of R genes was designed, and the stem rust resistance genes *Sr22* and *Sr45* were successfully cloned from the wheat ethyl-methanesulphonate- (EMS)-derived mutant library through this method.

Recently, two comprehensive EMS mutant libraries of tetraploid wheat and hexaploid wheat respectively (Figure 1D), in which the mutant sites were deciphered and cataloged by exome capture, were developed [4]. For the hexaploid mutant stock, the high-density (an average of 5351 mutations per hexaploid line) and high-quality (an average of 23–24 missense and truncation alleles per gene) distribution of induced mutations along the coding regions helped to uncover previously masked variations, in particularly the recessive alleles (Figure 1D). Though additional operations, such as backcrossing (here, the library was generated from a germplasm with a spring growth habit and a relatively short generation time) for reducing the noises of background high-density mutations, are necessary to characterize specific mutant sites in the gene of interest, mutant libraries are profoundly useful resources to expedite gene function studies in bread wheat (Box 4).

The strides in NGS and the exome capture platform also give an opportunity to reassess older wheat stocks or mutant libraries. For example, compared to allopolyploidization and sexual hybridization, asymmetric somatic hybridization introgressed only a minimum of exogenous fragments into the progenies. Initial sequencing of large-scale ESTs (expressed sequence tags) based on cDNA libraries of the progeny and its parental line indicated that both point mutations and indels (insertions and deletions) were frequently induced by somatic hybridization in the coding sequences [56], which was similar to the mutant types and distributions of the EMS mutation library. Thus, through high-throughput and high-resolution sequencing based on NGS and the exome capture platform, seed stocks generated by somatic hybridization constitute another critical library to uncover both hidden variations and novel variations that

Box 4. How to Use a Wheat Mutant Library for Gene Exploitation

In terms of forward genetics, cumulative selections based on phenotyping per generation of backcrossing will dilute undesired background mutations. After a certain number of generations, using the predesigned SNP-based primers, the mutant gene or genes responsible for the phenotype can be rapidly captured. Otherwise, bulk segregant analysis of crossing progenies with opposite extreme phenotypes and exome capture resequencing may lead to an even faster identification of the mutant gene [89].

In terms of **reverse genetics**, to reduce the noise of background mutations, strategies including using multiple independent mutant lines all carrying the desired mutant gene, backcrossing with the nonmutagenized parent, and selecting for isogenic sibling lines that share background mutations make it possible to rapidly analyze the target mutation effect and then the targeted gene function. Further manipulations like genome editing can validate the results.

are produced by hybridization. Furthermore, as the genomic shock during this kind of hybridization can introduce epigenetic variations as well [57,58], epialleles may be identified from these libraries in combination with the recent developed epigenome in bread wheat [59].

Genome Editing in Bread Wheat

Optimizing the CRISPR/Cas9 System

Genome editing, mainly based on double-strand breaks (DSBs) which are created by sequence-specific nucleases, including zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats/associated nuclease Cas9 (CRISPR/Cas9) technology, has been successfully applied in a broad range of plant species. In recent years, it has emerged as a revolutionary tool to create genetic variation for crop improvement and functional genetic analysis [60,61]. For example, TALEN mediated mutagenesis in wheat was first demonstrated to edit *TaMLO* genes, resulting in a broad-spectrum resistance to powdery mildew [62]. Due to its unprecedented simplicity and efficiency, CRISPR/Cas9 has been quickly applied in many kinds of organisms [63]. The initial application of CRISPR/Cas9 technology in bread wheat introduced targeted mutations in the A genome homoeoallele of *TaMLO* [62]. However, because of the complex genome and transformation-recalcitrant nature of bread wheat, the efficiency of CRISPR/Cas9-mediated genome editing requires further improvement, especially to be able to create homoeologous triple mutants in a single step [2]. Moreover, standard CRISPR/Cas9 constructs can integrate into the wheat genome, generating unintended genomic changes and raising profound regulatory concerns about biosecurity. Therefore, there is a great need to develop editing systems that produce transgene-free plants. To eliminate such unwanted transgenic products, mutant plants have been generated after transient expression of CRISPR/Cas9 DNA, or by using *in vitro* transcripts of the Cas9-coding sequence and guide RNA, followed by growth of calli without herbicide selection [64]. Furthermore, a DNA-free CRISPR/Cas9 system using CRISPR/Cas9 ribonucleoproteins (RNPs) has also been developed [5]. Importantly, as RNPs are rapidly degraded by endogenous proteases in plant cells, CRISPR/Cas9 is only expressed for a short time, which should reduce the frequency of mosaicism and off-target effects in the regenerated plants. Completely transgene-free mutants were indeed obtained by using the CRISPR/Cas9 RNP-mediated genome editing system in bread wheat, and the frequency of off-target mutations was significantly reduced.

A variety of versatile CRISPR systems have been developed and applied recently, especially in mammals [65,66], and these various CRISPR systems will greatly expand the application of genome editing to gene exploitation and trait improvement in wheat. The most commonly used Cas9 enzyme is that of *Streptococcus pyogenes* (SpCas9), which recognizes the NGG protospacer adjacent motif (PAM). The limitation imposed by the need for the PAM sequence means that SpCas9 cannot cover the whole genome. Newly developed SpCas9 variants [67] (such as VQR, EQR and VRER) and other orthologues [68,69] (including SaCas9, StCas9 and NmeCas9) will greatly expand the choice of PAM sequences. Furthermore, the recently developed CRISPR/Cpf1 system promises to be a powerful tool for genome editing [70,71]. Some Cpf1 orthologues (FnCpf1, LbCpf1 and AsCpf1) recognize PAMs of TTN or TTTN and can be used to modify adenine–thymine (A–T) rich genomic regions. These newly developed CRISPR systems have greatly enriched the genome editing toolkit.

Applying CRISPR for Wheat-Specific Challenges

Recently, many multiplex genome editing toolkits have been developed based on CRISPR technology [66,72], and we expect that these various toolkits will be used to address more complex or species-specific challenges in bread wheat. For example, many major genetic loci

in bread wheat are composed of tandem gene clusters due to the high percentage of repetitive sequences [73]. These clusters could be edited simultaneously using a multiplex genome toolkit [74], or deleted by pairs of sgRNAs flanking the clusters. Meanwhile, Cpf1 functions as a dual nuclease that is specific to crRNA biogenesis and target DNA interference. The dual RNase and DNase activities makes Cpf1 feasible to multiplex genome editing using a single crRNA array [75]. It is worth noting that multiplex CRISPR/Cas9 systems also offer an alternative and efficient approach for obtaining chromosome deletion lines in plants [76] (Figure 1B). This is very beneficial for functional analysis of a gene cluster which frequently spans more than one million bases in wheat genome [77].

In addition to generating knockout or deletion mutants as repaired by the non-homologous end joining (NHEJ) pathway, double-strand breaks introduced by Cas9 can also be repaired by the homologous recombination (HR) pathway in a precise manner using homologous templates [66]. At chromosome level, given the common mechanism of HR, the sophisticated CRISPR/Cas9 technology is a promising method for the generation of introgression stocks by mitigating the linkage drag for efficiently introducing beneficial segments from wild relatives (Figure 1C). At gene level, gene targeting can produce gene replacements and insertions with high fidelity [78], which are useful to integrate elite alleles into certain genome sites and gene stacking. For example, wild relatives of wheat harbor abundant disease resistance (R) genes. Breeding these R genes into modern wheat requires a very long time to break the linkage between R genes and linked deleterious genes. By contrast, the CRISPR/Cas9 technology can be used to integrate R genes into the predetermined genome site in modern cultivar line, meanwhile avoiding the introducing of any linked deleterious alleles. Furthermore, genome editing also enables us to stack different R genes into the same locus, which will ensure more durable resistance. However, gene targeting relies on HR, which is very inefficient in plant cells. A replicon-based CRISPR/Cas9 system using a deconstructed version of the wheat dwarf virus (WDV) has been developed to improve the efficiency of gene-targeting in bread wheat [79]. Driven by the WDV, replicons of both CRISPR/Cas9 and a DNA repair template reached high copy numbers, which led to a 10-fold increase in gene-targeting frequency compared to standard methods of DNA delivery.

Moreover, the evolutionary and domestication processes in wheat appear to have tended to involve 'tinkering' with gene sequences inherited from progenitors rather than 'disassembling' or 'crippling' such sequences [8]. Therefore, a strategy based on inducing targeted point mutations should be more productive than one based on inducing knockout mutations. Currently, cytosines in targeted bread wheat genes can be efficiently converted into thymines using a CRISPR–Cas9 nickase–cytidine deaminase fusion (Figure 1E) [6]; this means that an optimized CRISPR/Cas9 system will not only cause knockouts, but should also be able to introduce elite variations into bread wheat. Together with the mutant library mentioned above, more hidden variations will be uncovered rapidly.

Concluding Remarks

According to the genetic property, every genetic resource plays a different and possibly unique role for different aspects of functional genetic studies in bread wheat (Figure 2). Utilizing these various genetic resources properly and comprehensively is the key solution to deciphering the genetic and molecular mechanisms underlying the agronomically important traits of wheat (see Outstanding Questions). Enriching the whole genome information and high-resolution omics data available for wheat should simplify the process of identifying genes from these genetic resources. Meanwhile, as the cost of sequencing continues to fall, it is becoming imaginable that current descriptions of genetic resources, based largely on phenotypic and some sample genotypic data, will be greatly enhanced by re-sequencing data.

Outstanding Questions

How can wheat genetic stocks be used efficiently for gene identification?

Along with enrichments in whole genome information and omics data, and advances in biotechnologies, what is the new role of conventional wheat genetic stock for gene discoveries? Are any new strategies emerging?

How can an informative wheat mutant library be utilized for forward genetic and reverse genetic studies?

How can an optimized genome editing system be applied to address more complex or species-specific challenges in wheat?

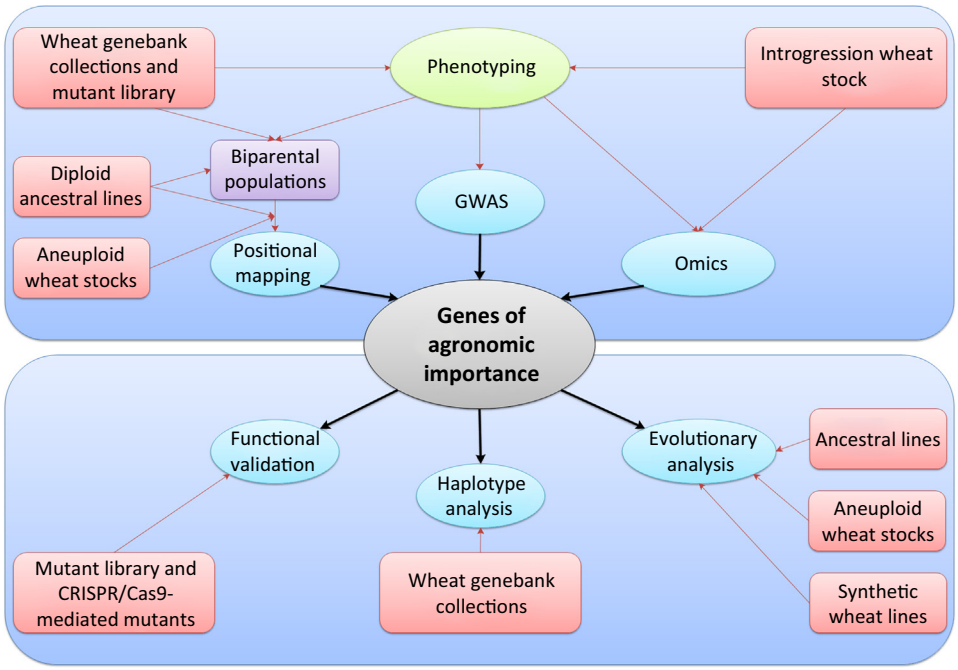

Figure 2. The Utilization of Genetic Resources for Functional Genetic Analysis in Bread Wheat. Wheat genebank collections, mutant libraries, and introgression stocks harboring abundant genetic variations are beneficial for identifications of vital genes through positional cloning, GWAS, or omics analysis. Diploid ancestral lines can be used to simplify the process of positional cloning in a diploid background. Aneuploid wheat stocks are a core resource for rapidly assigning the subchromosomal location of genes and markers. When a gene of agronomic importance is discovered, the gene function and the molecular mechanism can be analyzed in mutant wheat lines generated by EMS or CRISPR/Cas9. Haplotype analysis of a given gene in wheat genebank collections will reveal more elite alleles. Moreover, evolutionary analysis of an agronomically important gene based on ancestral lines, aneuploid wheat stocks and newly synthetic wheat lines will offer more information of natural selection. Abbreviations: EMS, ethyl methanesulphonate; GWAS: genome-wide association study.

Notably, optimizing the CRISPR/Cas9 system should allow for even more targeted germplasm innovations at both the chromosome and gene levels. At the chromosome level, using CRISPR/Cas9 can generate wheat deletion lines and introgression lines at certain genetic locus of interest, with a more precise and efficient manner. At the gene level, CRISPR/Cas9 now can be applied for targeted mutagenesis not only in a single copy of homoeologous gene, but also in homoeologous triple copies and even in tandem gene clusters. Moreover, the emergence of multiplex genome editing toolkits offers new opportunity to produce gene replacements, gene insertions and targeted point substitutions, which is promising to address more complex or species-specific challenges in bread wheat. Together with informative wheat mutant libraries developed recently, more agronomically important genes and hidden elite alleles will be exploited. Therefore, breeders, molecular biologists, and bioinformaticians need to cooperate more closely.

Acknowledgments

We apologize to authors whose relevant work we could not cite due to space limitations. This work is supported by the National Key Research and Development Project (2016YFD0101004, 2016YFD0102003), the National Natural Science Fund of China (No. 31601306), the Innovation Program of Chinese Academy of Sciences (No. ISSASIP1602), and the Natural Science Fund of Jiangsu Province, China (No. BK20161092).

References

1. Feuillet, C. *et al.* (2008) Cereal breeding takes a walk on the wild side. *Trends Genet.* 24, 24–32
2. Uauy, C. (2017) Wheat genomics comes of age. *Curr. Opin. Plant Biol.* 36, 142–148
3. Wang, M. *et al.* (2015) From genome to gene: a new epoch for wheat research? *Trends Plant Sci.* 20, 380–387
4. Krasileva, K.V. *et al.* (2017) Uncovering hidden variation in polyploid wheat. *Proc. Natl. Acad. Sci. U. S. A.* 114, E913–E921
5. Liang, Z. *et al.* (2017) Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nat. Commun.* 8, 14261
6. Zong, Y. *et al.* (2017) Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nat. Biotechnol.* 35, 438–440
7. Matsuoka, Y. (2011) Evolution of polyploid *Triticum* wheats under cultivation: the role of domestication, natural hybridization and allopolyploid speciation in their diversification. *Plant Cell Physiol.* 52, 750–764
8. Dubcovsky, J. and Dvorak, J. (2007) Genome plasticity a key factor in the success of polyploid wheat under domestication. *Science* 316, 1862–1866
9. Peters, J.L. *et al.* (2003) Forward genetics and map-based cloning approaches. *Trends Plant Sci.* 8, 484–491
10. Pallotta, M. *et al.* (2014) Molecular basis of adaptation to high soil boron in wheat landraces and elite cultivars. *Nature* 514, 88–91
11. Cavanagh, C. *et al.* (2008) From mutations to MAGIC: resources for gene discovery, validation and delivery in crop plants. *Curr. Opin. Plant Biol.* 11, 215–221
12. Rawat, N. *et al.* (2016) Wheat *Fhb1* encodes a chimeric lectin with agglutinin domains and a pore-forming toxin-like domain conferring resistance to *Fusarium* head blight. *Nat. Genet.* 48, 1576–1580
13. Ni, F. *et al.* (2017) Wheat *Ms2* encodes for an orphan protein that confers male sterility in grass species. *Nat. Commun.* 8, 15121
14. Xia, C. *et al.* (2017) A TRIM insertion in the promoter of *Ms2* causes male sterility in wheat. *Nat. Commun.* 8, 15407
15. Bhullar, N.K. *et al.* (2009) Unlocking wheat genetic resources for the molecular identification of previously undescribed functional alleles at the *Pm3* resistance locus. *Proc. Natl. Acad. Sci. U. S. A.* 106, 9519–9524
16. Shaked, H. *et al.* (2001) Sequence elimination and cytosine methylation are rapid and reproducible responses of the genome to wide hybridization and allopolyploidy in wheat. *Plant Cell* 13, 1749–1759
17. Sun, H. *et al.* (2014) DNA methylation pattern of *Photoperiod-B1* is associated with photoperiod insensitivity in wheat (*Triticum aestivum*). *New Phytol.* 204, 682–692
18. Blake, V.C. *et al.* (2016) The triticeae toolbox: combining phenotype and genotype data to advance small-grains breeding. *Plant Genome* Published online July 2016. http://dx.doi.org/10.3835/plantgenome2014.12.0099
19. Guo, Z. *et al.* (2017) Genome-wide association analyses of 54 traits identified multiple loci for the determination of floret fertility in wheat. *New Phytol.* 214, 257–270
20. Sun, C. *et al.* (2017) Genome-wide association study for 13 agronomic traits reveals distribution of superior alleles in bread wheat from the Yellow and Huai Valley of China. *Plant Biotechnol. J.* 15, 953–969
21. Marcussen, T. *et al.* (2014) Ancient hybridizations among the ancestral genomes of bread wheat. *Science* 345, 1250092
22. Cox, T. (1997) Deepening the wheat gene pool. *J. Crop Prod.* 1, 1–25
23. Munns, R. *et al.* (2012) Wheat grain yield on saline soils is improved by an ancestral Na^+ transporter gene. *Nat. Biotechnol.* 30, 360–364
24. Saintenac, C. *et al.* (2013) Identification of wheat gene *Sr35* that confers resistance to Ug99 stem rust race group. *Science* 341, 783–786
25. James, R.A. *et al.* (2011) Major genes for Na^+ exclusion, *Nax1* and *Nax2* (wheat *HKT1;4* and *HKT1;5*), decrease Na^+ accumulation in bread wheat leaves under saline and waterlogged conditions. *J. Exp. Bot.* 62, 2939–2947
26. Stein, N. *et al.* (2000) Subgenome chromosome walking in wheat: a 450-kb physical contig in *Triticum monococcum* L. spans the *Lr10* resistance locus in hexaploid wheat (*Triticum aestivum* L.). *Proc. Natl. Acad. Sci. U. S. A.* 97, 13436–13441
27. Huang, L. *et al.* (2003) Map-based cloning of leaf rust resistance gene *Lr21* from the large and polyploid genome of bread wheat. *Genetics* 164, 655–664
28. Feuillet, C. *et al.* (2003) Map-based isolation of the leaf rust disease resistance gene *Lr10* from the hexaploid wheat (*Triticum aestivum* L.) genome. *Proc. Natl. Acad. Sci. U. S. A.* 100, 15253–15258
29. Periyannan, S. *et al.* (2013) The gene *Sr33*, an ortholog of barley *Mla* genes, encodes resistance to wheat stem rust race Ug99. *Science* 341, 786–788
30. Jia, J. *et al.* (2013) *Aegilops tauschii* draft genome sequence reveals a gene repertoire for wheat adaptation. *Nature* 496, 91–95
31. Ling, H.Q. *et al.* (2013) Draft genome of the wheat A-genome progenitor *Triticum urartu*. *Nature* 496, 87–90
32. Ogbonnaya, F.C. *et al.* (2013) Synthetic hexaploids: harnessing species of the primary gene pool for wheat improvement. *Plant Breed. Rev.* 37, 35–122
33. Chapman, J.A. *et al.* (2015) A whole-genome shotgun approach for assembling and anchoring the hexaploid bread wheat genome. *Genome Biol.* 16, 26
34. Li, A. *et al.* (2014) mRNA and small RNA transcriptomes reveal insights into dynamic homoeolog regulation of allopolyploid heterosis in nascent hexaploid wheat. *Plant Cell* 26, 1878–1900
35. Li, A. *et al.* (2015) Making the bread: insights from newly synthesized allohexaploid wheat. *Mol. Plant* 8, 847–859
36. Jiang, J. *et al.* (1993) Recent advances in alien gene transfer in wheat. *Euphytica* 73, 199–212
37. Riley, R. (1958) Genetic control of the cytological diploid behaviour of hexaploid wheat. *Nature* 182, 713–715
38. Riley, R. *et al.* (1959) Genetic control of chromosome pairing in intergeneric hybrids with wheat. *Nature* 183, 1244
39. Qi, L. *et al.* (2007) Homoeologous recombination, chromosome engineering and crop improvement. *Chromosome Res.* 15, 3–19
40. He, X. *et al.* (2014) A genotypic difference in primary root length is associated with the inhibitory role of transforming growth factor-β receptor-interacting protein-1 on root meristem size in wheat. *Plant J.* 77, 931–943
41. Sears, E.R. (1954) Aneuploids of common wheat. *Mo. Agric. Exp. Stn. Res. Bull.* 572, 1–58
42. Sears, E.R. (1964) Nullisomic–tetrasomic combinations in hexaploid wheat. *Chromosome Manipulat. Plant Genet.* 29–45
43. Sears, E.R. and Sears, L.M. (1978) The telocentric chromosomes of common wheat. In *Proceedings of the 5th International Wheat Genetics Symposium*, pp. 389–407, Indian Society for Genetics and Plant Breeding, New Delhi
44. Yan, L. *et al.* (2003) Positional cloning of the wheat vernalization gene *VRN1*. *Proc. Natl. Acad. Sci. U. S. A.* 100, 6263–6268
45. Tsujimoto, H. and Tsunewaki, K. (1985) Gametocidal genes in wheat and its relatives. II. Suppressor of the chromosome 3C gametocidal gene of *Aegilops triuncialis*. *Can. J. Genet. Cytol.* 27, 178–185
46. Endo, T. and Gill, B. (1996) The deletion stocks of common wheat. *J. Hered.* 87, 295–307

47. Byrt, C.S. *et al.* (2007) HKT1; 5-like cation transporters linked to Na^+ exclusion loci in wheat, *Nax2* and *Kna1*. *Plant Physiol.* 143, 1918–1928
48. Wen, S. *et al.* (2012) Structural genes of wheat and barley 5-methylcytosine DNA glycosylases and their potential applications for human health. *Proc. Natl. Acad. Sci. U. S. A.* 109, 20543–20548
49. Vrána, J. *et al.* (2000) Flow sorting of mitotic chromosomes in common wheat (*Triticum aestivum* L.). *Genetics* 156, 2033–2041
50. International Wheat Genome Sequencing Consortium (2014) A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. *Science* 345, 1251788
51. Zhang, Z. *et al.* (2011) Duplication and partitioning in evolution and function of homoeologous Q loci governing domestication characters in polyploid wheat. *Proc. Natl. Acad. Sci. U. S. A.* 108, 18737–18742
52. Hu, Z. *et al.* (2013) Epigenetic modification contributes to the expression divergence of three *TaEXPA1* homoeologs in hexaploid wheat (*Triticum aestivum*). *New Phytol.* 197, 1344–1352
53. Yang, C. *et al.* (2014) Evolution of physiological responses to salt stress in hexaploid wheat. *Proc. Natl. Acad. Sci. U. S. A.* 111, 11882–11887
54. Slade, A.J. *et al.* (2005) A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. *Nat. Biotechnol.* 23, 75–81
55. Steuernagel, B. *et al.* (2016) Rapid cloning of disease-resistance genes in plants using mutagenesis and sequence capture. *Nat. Biotechnol.* 34, 652–655
56. Wang, M. *et al.* (2015) Asymmetric somatic hybridization induces point mutations and indels in wheat. *BMC Genomics* 16, 807
57. Wang, M. *et al.* (2014) Induced and constitutive DNA methylation in a salinity tolerant wheat introgression line. *Plant Cell Physiol.* 55, 1354–1365
58. Liu, S. *et al.* (2015) Genetic and epigenetic changes in somatic hybrid introgression lines between wheat and tall wheatgrass. *Genetics* 199, 1035–1045
59. Gardiner, L.J. *et al.* (2015) A genome-wide survey of DNA methylation in hexaploid wheat. *Genome Biol.* 16, 273
60. Voytas, D.F. and Gao, C. (2014) Precision genome engineering and agriculture: opportunities and regulatory challenges. *PLoS Biol.* 12, e1001877
61. Gao, C. (2015) Genome editing in crops: from bench to field. *Natl. Sci. Rev.* 2, 13–15
62. Wang, Y. *et al.* (2014) Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nat. Biotechnol.* 32, 947–951
63. Kanchiswamy, C.N. *et al.* (2016) Fine-tuning next-generation genome editing tools. *Trends Biotechnol.* 34, 562–574
64. Zhang, Y. *et al.* (2016) Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. *Nat. Commun.* 7, 12617
65. Mohanraju, P. *et al.* (2016) Diverse evolutionary roots and mechanistic variations of the CRISPR-Cas systems. *Science* 353, aad5147
66. Puchta, H. (2017) Applying CRISPR/Cas for genome engineering in plants: the best is yet to come. *Curr. Opin. Plant Biol.* 36, 1–8
67. Kleinstiver, B.P. *et al.* (2015) Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature* 523, 481–485
68. Steinert, J. *et al.* (2015) Highly efficient heritable plant genome engineering using Cas9 orthologues from *Streptococcus thermophilus* and *Staphylococcus aureus*. *Plant J.* 84, 1295–1305
69. Esvelt, K.M. *et al.* (2013) Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. *Nat. Methods* 10, 1116–1121
70. Zetsche, B. *et al.* (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163, 759–771
71. Tang, X. *et al.* (2017) A CRISPR-Cpf1 system for efficient genome editing and transcriptional repression in plants. *Nat. Plants* 3, 17018
72. Čermák, T. *et al.* (2017) A multipurpose toolkit to enable advanced genome engineering in plants. *Plant Cell* 29, 1196–1217
73. Greer, E. *et al.* (2012) The *Ph1* locus suppresses Cdk2-type activity during premeiosis and meiosis in wheat. *Plant Cell* 24, 152–162
74. Sánchez-León, S. *et al.* (2017) Low-gluten, non-transgenic wheat engineered with CRISPR/Cas9. *Plant Biotechnol. J.* Published online September 18, 2017. http://dx.doi.org/10.1111/pbi.12837
75. Zetsche, B. *et al.* (2017) Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. *Nat. Biotechnol.* 35, 31–34
76. Zhou, H. *et al.* (2014) Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. *Nucleic Acids Res.* 42, 10903–10914
77. Huo, N. *et al.* (2017) New insights into structural organization and gene duplication in a 1.75-Mb genomic region harboring the α-gliadin gene family in *Aegilops tauschii*, the source of wheat D genome. *Plant J.* Published online August 30, 2017. http://dx.doi.org/10.1111/tpj.13675
78. Li, J. *et al.* (2016) Gene replacements and insertions in rice by intron targeting using CRISPR-Cas9. *Nat. Plants* 2, 16139
79. Gil-Humanes, J. *et al.* (2017) High efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR/Cas9. *Plant J.* 89, 1251–1262
80. Avni, R. *et al.* (2017) Wild emmer genome architecture and diversity elucidate wheat evolution and domestication. *Science* 357, 93–97
81. Brenchley, R. *et al.* (2012) Analysis of the bread wheat genome using whole genome shotgun sequencing. *Nature* 491, 705
82. Montenegro, J.D. *et al.* (2017) The pangenome of hexaploid bread wheat. *Plant J.* 90, 1007–1013
83. Pfeifer, M. *et al.* (2014) Genome interplay in the grain transcriptome of hexaploid bread wheat. *Science* 345, 1250091
84. Thind, A.K. *et al.* (2017) Rapid cloning of genes in hexaploid wheat using cultivar-specific long-range chromosome assembly. *Nat. Biotechnol.* 35, 793–796
85. Friebe, B. *et al.* (1996) Characterization of wheat-alien translocations conferring resistance to diseases and pests: current status. *Euphytica* 91, 59–87
86. Chen, P.D. *et al.* (1995) Development and molecular cytogenetic analysis of wheat-*Haynaldia villosa* 6VS/6AL translocation lines specifying resistance to powdery mildew. *Theor. Appl. Genet.* 91, 1125–1128
87. Cao, A. *et al.* (2011) Serine/threonine kinase gene *Stpk-V*, a key member of powdery mildew resistance gene *Pm21*, confers powdery mildew resistance in wheat. *Proc. Natl. Acad. Sci. U. S. A.* 108, 7727–7732
88. Liu, S. *et al.* (2014) A wheat *SIMILAR TO RCD-ONE* gene enhances seedling growth and abiotic stress resistance by modulating redox homeostasis and maintaining genomic integrity. *Plant Cell* 26, 164–180
89. Wang, X. *et al.* (2017) Genomic analyses of primitive, wild and cultivated citrus provide insights into asexual reproduction. *Nat. Genet.* 49, 765–772