Present and future prospects for wheat improvement through genome editing and advanced technologies

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ABSTRACT

Wheat (Triticum aestivum, 2n = 6x = 42, AABBDD) is one of the most important staple food crops in the world. Despite the fact that wheat production has significantly increased over the past decades, future wheat production will face unprecedented challenges from global climate change, increasing world population, and water shortages in arid and semi-arid lands. Furthermore, excessive applications of diverse fertilizers and pesticides are exacerbating environmental pollution and ecological deterioration. To ensure global food and ecosystem security, it is essential to enhance the resilience of wheat production while minimizing environmental pollution through the use of cutting-edge technologies. However, the hexaploid genome and gene redundancy complicate advances in genetic research and precision gene modifications for wheat improvement, thus impeding the breeding of elite wheat cultivars. In this review, we first introduce state-of-the-art genome-editing technologies in crop plants, especially wheat, for both functional genomics and genetic improvement. We then outline applications of other technologies, such as GWAS, high-throughput genotyping and phenotyping, speed breeding, and synthetic biology, in wheat. Finally, we discuss existing challenges in wheat genome editing and future prospects for precision gene modifications using advanced genome-editing technologies. We conclude that the combination of genome editing and other molecular breeding strategies will greatly facilitate genetic improvement of wheat for sustainable global production.

Keywords: CRISPR/Cas, genome editing, GWAS, speed breeding, wheat (Triticum aestivum L.)

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INTRODUCTION

Common wheat (Triticum aestivum L., 2n = 6x = 42, AABBDD) is one of the most important crops in the world. It provides more than 30% of all calories consumed by the world population, and it is the main source of cereal-based processed products such as bread, cookies, and noodles (Petersen et al., 2006). Despite the fact that wheat production has significantly increased over the past decades following the “Green Revolution” in 1960 (Peng et al., 1999) and the advent of marker-assisted molecular breeding, it is still facing unprecedented challenges in the context of global climate change, increasing world population, and water shortages in arid and semi-arid lands (Hickey et al., 2019). Furthermore, intensive applications of diverse fertilizers and pesticides exacerbate environmental pollution and ecological deterioration (Yu et al., 2014; Hu et al., 2019; Tian et al., 2020; Wu et al., 2020; Liu et al., 2021). Moreover, the hexaploid nature and gene functional redundancy of wheat make advances in a genetics approach to selecting a desired phenotype very time-consuming and, in some cases, impossible because of gene linkage or gene drag (Li et al., 2020a). To ensure global food and ecosystem security, it is essential to enhance the resilience of wheat production and minimize environmental pollution through the use of cutting-edge technologies.

Genome-editing technologies have revolutionized the plant research field and have great potential for use in crop improvement. Clustered regularly interspaced short palindromic repeat
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(CRISPR)-CRISPR-associated protein (CRISPR-Cas), a versatile, simple, and inexpensive system for precise, sequence-specific modifications of DNA sequences, including targeted mutagenesis for gene knockout, single-base substitution, and gene/allele replacement in vivo, has dominated the genome-editing field over the past few years. The applications of CRISPR/Cas in both plant biological sciences and crop improvement are particularly important in the context of global climate change and in the face of current agricultural, environmental, and ecological challenges (Li and Xia, 2020; Ma et al., 2015; Zhan et al., 2021). Once double-strand DNA breaks (DSBs) are generated by CRISPR/Cas, the DSBs are repaired by the error-prone non-homologous end joining (NHEJ) pathway, accurate homology-directed repair (HDR), or both NHEJ and HDR (Cong et al., 2013; Danner et al., 2017; Jinek et al., 2012; Puchta, 1998; Zetsche et al., 2015) (Figure 1A). NHEJ is a predominant repair pathway for DSBs and mostly introduces random insertions and deletions (indels) at the junction of the newly rejoined chromosome (Hiom, 2010; Puchta and Fauser, 2014). Therefore, the majority of current genome-editing studies have used error-prone NHEJ to generate mutations and gene knockouts (, 2019b). The availability of a DNA or RNA donor repair template (DRT) with homologous sequences surrounding a DSB can trigger HDR, thereby allowing for precise gene replacement or insertions (, 2019b; Lu et al., 2020a). In addition, there are two alternative strategies for precise genome editing without a DSB and a DRT. One is base editing, which has emerged as an alternative and effective tool for HDR-mediated gene replacement that permits precise single-base substitution of an allele with a single-nucleotide polymorphism (SNP), facilitating precise plant genome editing through the replacement of one base with another in a programmable manner (Komor et al., 2016; Gaudelli et al., 2017). Another strategy is prime editing (PE), which enables all 12 types of base substitutions and small indels, substantially expanding the scope and capabilities of precise genome editing (Anzalone et al., 2019). To date, various CRISPR/Cas toolboxes have been developed and allow for targeted mutagenesis at specific genome loci, transcriptome regulation and epigenome editing, base editing, and precise targeted gene/allele replacement or tagging in plants (Zhan et al., 2021). In particular, precise replacement of an existing allele with an elite allele in a commercial variety through HDR is the holy grail of genome editing for crop improvement, as it has been very difficult, laborious, and time-consuming to introgress these elite alleles into commercial varieties without any linkage drag from parental lines within a few generations in crop-breeding practice (Li and Xia, 2020). In the near future, the development of diverse CRISPR toolboxes will greatly accelerate both biological research and genetic improvement in crop plants, especially for crop species with reference genome sequence datasets.

Wheat genome editing lags behind that of other food crops because of its complex genome and polyploid nature, as well as its relatively low transformation efficiency. The wheat reference genome RefSeq v.1.0 was recently released by the International Wheat Genome Sequencing Consortium (IWGSC) (McKenna et al., 2010; IWGSC, 2018). The recently developed WheatGmap platform contains more than 3500 next-generation sequencing datasets from hexaploid wheat, including whole-genome sequencing (WGS), whole-exome sequencing (WES), and transcriptome deep-sequencing (RNA-seq) datasets (Zhang et al., 2020), as well as a high-density genomic variation map constructed by re-sequencing 145 representative wheat cultivars in different periods (Hao et al., 2020). Together, these resources greatly facilitate gene discovery and trait dissection, providing a fundamental basis for target selection and off-target analysis in wheat genome editing. To facilitate wheat functional genomics and genetic improvement by cutting-edge genome-editing technology, we here present an informative summary of the application of state-of-the-art genome-editing technologies to crop plants, especially wheat. We then outline the applications of other technologies, such as integrative omics, synthetic biology, speed breeding, and high-throughput genotyping and phenotyping, to wheat biological research and improvement. Finally, we discuss current challenges in wheat genome editing and future prospects for the development of diverse strategies and advanced genome-editing tools to facilitate sustainable wheat production.

OVERVIEW OF CRISPR/Cas-MEDIATED GENOME EDITING IN WHEAT

CRISPR/Cas9-mediated knockout and its applications in wheat

To date, the majority of genome-editing studies have used NHEJ to generate loss-of-function mutations at specific gene loci in crop plants, including wheat (Figure 1A; Table 1). CRISPR/Cas9 has been widely used to improve wheat yields and quality. For example, lipoxigenase (LOX) has multiple functions in plant growth and development, defense against pathogens, and wound stress. Knockout of TaLOX2 altered grain size and weight and increased the storability of wheat (Shan et al., 2014; Zhang et al., 2016). Simultaneous targeting of all three gibberellin-regulated TaGASR7 genes, which have previously been reported to regulate grain size, significantly increased thousand-kernel weight (Zhang et al., 2018). Deletion of the phosphate 2 gene TaPHO2-A1 increased phosphorus uptake and grain yield under low-phosphorus conditions in wheat (Ouyang et al., 2016). Knockout of the RING-type E3 ligase-encoding gene TaGW2 increased the length and width of wheat grain, thereby increasing grain yield (Wang et al., 2018a; Zhang et al., 2018).

To improve wheat quality and meet the diverse demands of different populations, breeding a wheat variety with improved processing and nutritional quality and greater production without yield penalty is an important goal in wheat breeding programs. α-Gliadin is one group of gluten proteins in wheat flour that contributes to end-use properties for food processing; however, it contains major immunogenic epitopes that can cause severe health-related problems, such as celiac disease. Mutations of α-γ-gliadin, Waxy, and VIT2 resulted in decreased gliadin content (Jouanin et al., 2019) and increased branched starch content (Liu et al., 2020). Recently, high-amylose wheat was generated through targeted mutagenesis of TaSBEIIa in the modern winter wheat cultivar Zhengmai 7698 (ZM) and the spring wheat cultivar Bobwhite by CRISPR/Cas9, providing a new roadmap for breeding novel wheat varieties with increased nutritional values (Li et al., 2020b).

Successful introgression of useful genes/traits from wild relatives into wheat is limited by the low frequency of homoeologous crossover (CO). Complete deletion of a Ph1b (Tazip4-B2) gene...
in wheat increased the CO frequency during meiosis (Rey et al., 2018). Targeted knockout of an Ms1 (Male Sterility 1) gene, which contributes to pollen exine development and male fertility, produced complete male sterility in wheat cultivars (Okada et al., 2019). Similarly, three homologs of the glucose-methanol-choline oxidoreductase gene TaNP1 were disrupted in wheat, and the resulting Tanp1 triple mutants showed complete male sterility (Li et al., 2020c). TaCENH3α is a critical factor in zygotic centromere formation. Knockout of TaCENH3α homologs in the B and D subgenomes led to an increased haploid induction rate in wheat (Lv et al., 2020). This finding also paves the way for the deployment of CENH3 haploid induction technology in various crop plants.

Some laboratories have also improved wheat disease resistance using CRISPR/Cas9. Powdery mildew-resistant wheat plants were produced by integrating CRISPR/Cas9 into the genome using NHEJ and HDR pathways. In addition, CRISPR/Cas9 was used to disrupt the MDA (Methanol dehydrogenase alpha) gene, which is involved in the biosynthesis of cellular methanol. This resulted in a significant decrease in disease severity. These advances in wheat genome editing are providing new opportunities for improving crop productivity and sustainability.
were obtained by editing of a *TaMLO* gene in wheat (Shan et al., 2013; Wang et al., 2014). Similarly, *EDR1*, which plays a negative role in the defense response against powdery mildew, was knocked out by CRISPR/Cas9 to generate wheat plants with improved powdery mildew resistance (Zhang et al., 2017).

Fusarium head blight (FHB) is a severe disease that affects wheat quality and production. Recently, the random deletion of a sequence containing the start codon of a *TaHRC* gene by genome editing in the model wheat variety cultivar Bobwhite conferred resistance to FHB (Su et al., 2019). These results indicate that CRISPR/Cas9 is an efficient tool for improving wheat disease resistance.

Common wheat is a hexaploid crop species composed of A, B, and D subgenomes. It is challenging to simultaneously mutate multiple genes located at numerous genomic sites. Therefore, it is very useful to develop an efficient CRISPR/Cas9-mediated multiplex genome editing strategy to decipher complex traits and facilitate wheat improvement. *TaDA1*, *TaPDS*, and *TaNCED1* genes have been selected as targets for simultaneous editing of eight genomic loci in the model common wheat variety cv Bobwhite (Li et al., 2020d). However, because of the high homology of the three wheat subgenomes, the editing profile of each edited wheat plant could not be evaluated simply by DNA sequencing in hexaploid wheat. In addition, the inherent stability of these multiplex edited events and the off-target effects remain to be investigated. Recently, we successfully established an efficient CRISPR/Cas9 multiplex genome-editing system in an elite Chinese wheat variety using the polycistronic tRNA strategy (Luo et al., 2021a). Simultaneous editing of two,

| Target gene | Nucleases | Donor | Transformation | Stable lines | Improved agronomic traits | Reference | Edit |
|-------------|-----------|-------|----------------|--------------|---------------------------|-----------|------|
| *TaMLO*     | Cas9      | –     | bombardment    | +            | powdery mildew resistance | (Shan et al., 2013; Wang et al., 2014) | knockout |
| *TaLOX2*    | Cas9      | –     | bombardment    | +            | varied grain size, weight and increased storability | (Shan et al., 2014; Zhang et al., 2016) | knockout |
| *TaPHO2-A1* | Cas9      | –     | *Agrobacterium* | +            | increased phosphorus uptake | (Duyang et al., 2016) | knockout |
| *TaGASR7*   | Cas9      | –     | bombardment    | +            | increased yield           | (Zhang et al., 2016) | knockout |
| *TaEDR1*    | Cas9      | –     | *Agrobacterium* | +            | powdery mildew resistance | (Zhang et al., 2017) | knockout |
| *TaGW2*     | Cas9      | –     | bombardment    | +            | increased yield           | (Wang et al., 2018b) | knockout |
| *TaZIP4*    | Cas9      | –     | *Agrobacterium* | +            | increased homoeologous CO frequency | (Rey et al., 2018) | knockout |
| *TaHRC*     | Cas9      | –     | bombardment    | +            | Fhb resistance            | (Su et al., 2019) | knockout |
| *TaMs1*     | Cas9      | –     | bombardment    | +            | male sterility            | (Okada et al., 2019) | knockout |
| *TaSBElia*  | Cas9      | –     | bombardment    | +            | high amylase              | (Li et al., 2020a) | knockout |
| *TaDA1/TaPDS*/ *TaNCED1* | Cas9 | – | *Agrobacterium* | + – | | (Li et al., 2020d) | knockout |
| *TaCENH3*   | Cas9      | –     | *Agrobacterium* | +            | high haploid induction rate | (Lv et al., 2020) | knockout |
| *TaOsd1*, *TaARE1*, *TaNPT1*, *TaSBElia*, *TaSPDT* | Cas9 | – | bombardment    | + – | | (Luo et al., 2021a) | knockout and multiplexing |
| *TaLOX2*    | nCas9-D10A/ dCas9 | – | *Agrobacterium* | + | improved wheat quality | (Zong et al., 2017) | base editing |
| *TaALS*, *TaACC* | nCas9-D10A | – | bombardment | + | herbicide resistance | (Zhang et al., 2019a, 2019b) | base editing |
| *Ubi10*, *TaGW2*, *TaGASR7*, *TaDME1*, *TaLOX2*, *TaMLO*, *nCas9-H840A* | nCas9-D10A | – | protoplast transformation | – | – | (Lin et al., 2020) | primer editing |
| *Ubiquitin* | Cas9      | wheat dwarf virus replicons | bombardment | – | – | (Gil-Humanes et al., 2017) | HDR replicon |

Table 1. CRISPR/Cas-mediated genome editing in wheat.
Wheat genome editing and beyond

three, four, and five genes produced transgene-free plants with targeted mutagenesis at up to 15 genomic loci in one generation. The efficient multiplexing system established in our study can facilitate fundamental biological research, such as deciphering a complex trait conferred by multiple genes, and the translational breeding process in hexaploid wheat and other agriculturally important polyploid crop species for sustainable agriculture development (Luo et al., 2021a).

Base editing and its applications in wheat

Single-nucleotide point mutations are the genetic basis for variation in some important agronomic crop traits. Base editing can achieve the precise transition from one base pair to another at the target locus without inducing DSBs and without the need for a DRT. This process is independent of the HDR repair pathway. Currently available base editors, including cytosine base editors (CBEs) (Figure 1B) and adenine base editors (ABEs) (Figure 1C), were generated by fusing the Cas9 nickase (D10A) (nCas9) or dead Cas9 (dCas9) with either cytidine deaminase or adenine deaminase (Komor et al., 2016; Gaudelli et al., 2017). Single point mutations, such as C*G-to-T*A substitution or A*T-to-G*C substitution, can be generated in crop plants through CBEs or ABEs (Bharat et al., 2020). The deamination of substrate nucleotides in the editing window leads to the formation of uracil and hypoxanthine. Most base editors use Cas nickases such as nCas9 (D10A) to cut the gRNA target DNA strand, thus promoting DNA repair using the edited DNA strands as templates and ultimately resulting in precise base pair transitions at target sites. Although the application of nCas9 increases the editing activity of CBEs, the conversion from cytosine to uracil is inhibited by an endogenous uracil glycosylase that recognizes unnatural U-G pairing by catalyzing the removal of uracil. In the presence of uracil DNA glycosylase inhibitors (UGIs), the editing efficiency and accuracy of the CBE system can be improved.

Further optimization of ABEs was performed to enhance base editing efficiency, and eight amino acids of a deoxyadenosine deaminase TadA were mutated. This new type of ABE system, named TadA8e, deaminated nearly a thousand times faster than the previous wild-type TadA and greatly improved the efficiency of base editing (Richter et al., 2020). Moreover, the efficiency of CBEs or ABEs can be further improved by using a surrogate system to enrich the successful editing events (Xu et al., 2020a), optimizing the joint sequence between fusion proteins and increasing the number of UGI functional domains and nuclear localization sequences (NLSs) (Zeng et al., 2020). To date, base editing has been successfully applied to various crop plants, including rice (Li et al., 2017; Lu and Zhu, 2017; Ren et al., 2017; Shimatani et al., 2017; Zong et al., 2017), maize (Zong et al., 2017), soybean (Cai et al., 2020), cotton (Qiu et al., 2020), oilseeds (Kang et al., 2018), and others. Recently, by integrating the two existing types of base editor into one module, herbicide-resistant rice lines were generated through artificial evolution of the OsACC or OsALS gene (Li et al., 2020a; Kuang et al., 2020). The directed artificial evolution of an agriculturally important gene shows great potential to generate novel gene resources for crop improvement (Eshed and Lippman, 2019).

Currently, only two cases of base editing in wheat have been reported. An nCas9 (D10A) was fused to rat cytosine deaminase (rAPOBEC1) and UGI, and the resulting cytidine base editor, nCas9-PBE, was used in wheat base editing (Zong et al., 2017). Subsequently, wheat TaALS and TaACC genes were successfully edited by base editing, conferring herbicide resistance on the resulting wheat plants (Zhang et al., 2019a). However, the directed artificial evolution of agriculturally important genes by base editing has not yet been documented in wheat (Table 1).

Prime editing and its applications in wheat

A search-and-replace genome-editing approach, also referred to as PE (Figure 1D), has been developed to mediate precise small indels, single or multiple substitutions including transitions and transversions, and their combinations at specific gene loci in human cells without the requirement for DSBs or DRT (Anzalone et al., 2019). PE systems mainly conjugate a catalytically impaired Cas9 (H840A) (nCas9) with a reverse transcriptase M-MLV-RT (Moloney murine leukemia virus reverse transcriptase) and employ a prime editing guide RNA (pregRNA) to both specify the target site and encode the desired genetic information. Three components constitute a pregRNA: a single-guide RNA (sgRNA) that targets a specific site, a reverse transcription (RT) template that encodes the desired edit, and a primer-binding site (PBS) that initiates RT. In the PE system, the protein complex binds the target DNA and nicks the non-target strand. The resulting 3' DNA terminal hybridizes to the PBS in the pregRNA, then primes reverse transcription of new DNA that contains the desired edit using the RT template in the pregRNA. Subsequent automatic DNA repair incorporates the edited DNA flap in the non-target strand and further copies the edit into the complementary target strand, resulting in stably edited DNA. Protein engineering and elaborate guide RNA designs contributed to the advent of three PE systems, from PE1 to PE2 and then to PE3 and PE3b, with gradual improvements in editing efficiency and/or product purity. For example, PE1 was first generated by fusing an M-MLV-RT to the C terminus of nCas9. To enhance PE efficacy, PE2 was generated by replacing the original M-MLV-RT with an engineered M-MLV-RT with six mutations: H9Y + D200N + T306K + W313F + T330P + L603W. Then PE3 was further developed by using another nicking sgRNA at various distances from the nicks induced by pegRNA to direct a second cut in the non-edited strand to further increase the editing efficiency. However, as two single-strand breaks were induced near opposite DNA strands, the PE3 system-induced NHEJ exhibited higher indel frequencies. To suppress unwanted indels, PE3b uses a specific sgRNA to fit the edited DNA sequence, thereby ensuring that the second nicking occurs after the resolution of the edited strand flap. PE offers the advantages of improved precision genome-editing efficiency and product purity over conventional HDR strategies and overcomes the base editors’ weaknesses (Anzalone et al., 2019).

PE holds great promise for the introduction of genome modifications such as substitutions, insertions, and deletions that improve agronomic traits, and the feasibility and efficacy of prime editors for precise genome editing in cereal crops and tomato have therefore been investigated (Lu et al., 2020b; Butt et al., 2020; Xu et al., 2020b, 2020c; Li et al., 2020e; Hua et al., 2020; Lin et al., 2020; Tang et al., 2020). Although precise modification of both exogenous and endogenous genes in stable rice lines by PE has been implemented, the editing
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efficiencies at some target sites in rice are still lower, indicating that PE activity may not parallel Cas9 nuclease cleavage activity in plants, at least for the tested targets (Xu et al., 2020; Li et al., 2020; Lin et al., 2020). Also, prime editors only enable the replacement of short indels. In wheat proplasts, a series of plant prime editors were generated to achieve precise point mutations, including A-to-T, C-to-G, G-to-C, T-to-G, and C-to-A, at seven exogenous gene targets, and the frequencies of single nucleotide substitutions reached 1.4% (Lin et al., 2020). However, no stable, precisely edited wheat plants have yet been obtained, suggesting that significant improvements are required to develop more efficient plant prime editors, especially for polyploid species (Li et al., 2020).

CRISPR/Cas-mediated HDR and its applications in wheat

Compared with NHEJ, HDR is a precise repair pathway that is stimulated by the DRT surrounding a DSB and utilizes the DRT for DNA repair (Puchta, 1998) (Figure 1A). In general, single-strand annealing (SSA), synthesis-dependent strand annealing (SDSA), and the so-called double-strand break repair (DSBR) model are the main potential mechanisms for repairing DSBs by HDR (Puchta, 1998). SSA is activated when the complementary sequences at both ends of the DSB are more than 200 bp. During SSA, the complementary sequences anneal to one another, and the 3’ overhangs are trimmed. Therefore, SSA is a non-conservative DSB repair mechanism because of the loss of sequence between the complementary sequences (Puchta and Fauser, 2013). In DSBR and SDSA, the 3’ overhang at the DSB pairs with the homologous arms of the DRT, and replicative synthesis is initiated using DRT as a repair template (Symington, 2002). In DSBR, DNA synthesis occurs at both broken ends simultaneously, leading to a Holliday junction (Puchta and Fauser, 2013). Unlike DSBR, in SDSA, the new strands are synthesized at one broken end first and then withdraw from the DRT and anneal back to the locus (Puchta and Fauser, 2014; Paix et al., 2017). It has been reported that DSBR is an essential mechanism of meiotic recombination (Osman et al., 2011), whereas SDSA has been proposed as the primary repair mechanism upon the availability of a DRT in CRISPR/Cas-mediated HDR (Paquet et al., 2016; Richardson et al., 2016; Kan et al., 2017; Li et al., 2018a, 2020).

Precise replacement of an existing allele with an elite allele in a commercial variety through genome editing has been achieved in rice by CRISPR/Cas-mediated HDR (Li et al., 2018a, 2019a, 2019b, 2020). Allelic differences are usually derived from local landraces or related species, or even from orthologs in other plant species, and are often caused by differences of one or several SNPs or by defined indels of a gene fragment in either the promoter region or the coding region. These differences have been selected during domestication and subsequent improvement, and they account for significant differences in crop yield and other agriculturally important traits. Harnessing this genetic diversity and introducing elite alleles into commercial cultivars has been an important goal in crop breeding programs. However, breeders spend many years to introduce just one elite allele into commercial cultivars by crossing and back-crossing. Furthermore, it is difficult to remove any undesired genes/agronomic traits derived from the parental lines by crossing if they are closely linked to the target genes. Thus, it will greatly accelerate crop improvement if we can introduce elite alleles from landraces or related species into a commercialized crop variety without introducing unwanted gene or DNA fragments.

To date, CRISPR/Cas-mediated HDR has been documented in various plant species, including Arabidopsis, rice, tomato, and maize, albeit with varied efficiency (Bai et al., 2014; Butler et al., 2016; Čermák et al., 2015; Dahan-Meir et al., 2018; Gil-Humanes et al., 2017; Puchta and Fauser, 2013; Li et al., 2019a, 2019b; Lu et al., 2020a; Wang et al., 2017). In wheat, by using wheat dwarf virus replicons carrying CRISPR/Cas9 and DRT, HDR events were detected at an endogenous ubiquitin locus, and a promoter-less T2A:GFP sequence was inserted into the third exon of all three homoeologs in the A, B, and D subgenomes of the ubiquitin gene at a frequency of approximately 1% in wheat calli. Unfortunately, no stable, precisely edited wheat plants have been recovered, indicating that this system requires further optimization (Gil-Humanes et al., 2017) (Table 1). There are a number of reasons why it is difficult to perform HDR in wheat: (1) its large, polyploid, complex genome, the high homology among its three subgenomes, and its high content of repetitive DNA make it relatively difficult to manipulate wheat genes with CRISPR/Cas technology in comparison with rice, tomato, maize, and soybean; (2) wheat has a relatively low transformation efficiency; (3) NHEJ is the main pathway for DSB repair, and it also competes with the HDR pathway (Symington and Gautier, 2011; Puchta and Fauser, 2014; Sun et al., 2016a); (4) the HDR pathway mainly occurs in the late S and G2 phases of meiotic cells (Hiom, 2010; Puchta and Fauser, 2014); (5) limited DRT is delivered into the nucleus/cells for HDR (, 2019b); and (6) it is difficult to synchronize the induction of DSBs in the target gene with the delivery of sufficient DRT near the DSBs inside the nucleus (Altpeter et al., 2016; Endo et al., 2016; Li and Xia, 2020). Finally, an additional reason for lower HDR efficacy in wheat may be the presence of a Ph1 locus in chromosome 5B, which suppresses homoeologous recombination (Griffiths et al., 2006; Fan et al., 2021).

BEYOND GENOME EDITING

Genome sequencing

Genome sequencing provides fundamental genetic information for functional genomics, genome editing, and marker-assisted breeding in wheat (Figure 2). To date, a substantial amount of research has been conducted to accelerate wheat improvement through the genome sequencing of diverse wheat accessions or relatives (Caldwell et al., 2004; Paux et al., 2008; Brenchley et al., 2012; Hernandez et al., 2012; Jia et al., 2013; Ling et al., 2013; Jordan et al., 2015; Zhao et al., 2017; IWGSC, 2018; Ling et al., 2018; Bohra et al., 2020; Hao et al., 2020; Zhang et al., 2020; Zhou et al., 2020). In 2004, genome sequencing was used to investigate the origin of the D genome of the allopolyploid species Triticum aestivum and Aegilops cylindrica, providing an impetus to investigate the mode of polyploid formation in common wheat (Caldwell et al., 2004). The IWGSC aims to flow-sort and sequence the individual chromosomes of bread wheat, and significant progress has
been made with several chromosomes, such as 3B (Paux et al., 2008) and 4A (Hernandez et al., 2012). A whole-genome shotgun sequence analysis of bread wheat and its diploid relatives has allocated more than 60% of the genes to the A, B, and D genomes with more than 70% confidence (Brenchley et al., 2012). The sequences of diploid progenitor genomes will allow the complete and unambiguous assignment of their homoeologous relationships. Whole-genome analysis of the wheat D genome donor, _Aegilops tauschii_ (2n = 14; DD), revealed the expansion of agronomically relevant genes or gene families associated with disease resistance, abiotic stress tolerance, and grain quality. This draft genome sequence provides insight into the environmental adaptation of bread wheat and facilitates defining the large and complicated wheat genome (Jia et al., 2013).

Figure 2. Breeding of a green super wheat variety through CRISPR/Cas-mediated gene editing and other breeding technologies. (A) Genome sequencing. Wheat genome and pan-genome sequencing provide basic information for designing an sgRNA target and the evaluation of off-target effects in wheat genome editing. (B) GWAS analysis. GWAS enables the association of specific genes, SNPs, or markers on a chromosome with a specific trait. (C) A CRISPR/Cas-mediated multiplex system for multiple gene knockouts (KOs). (D) CRISPR/Cas-mediated HDR remains to be investigated as a means to improve HDR efficiency in wheat. (E) Development of a module for simultaneous HDR and/or base editing (BE) and knockout would greatly facilitate the translational breeding process for pyramiding favorable alleles in an elite wheat variety in a shorter time. (F) Development of diverse genotype-independent strategies. Genotype-independent strategies enable transformation of recalcitrant wheat varieties, thus facilitating the use of genome editing in diverse elite wheat germplasm. (G) Gene stacking by synthetic biology. Synthetic biology enables the accumulation of multiple transgenes of interest in the same plant genome to stack beneficial traits or generate a novel trait. (H) Speed breeding. Speed breeding enables a shortened generation time for seed harvesting in wheat. KO, knockout; BE, base editing; HDR, homology-directed repair.
presented an annotated reference genome with a detailed analysis of gene contents in all chromosomes. This annotated reference sequence greatly accelerated our understanding of the genetic basis of wheat (IWGSC, 2018). In 2018, a high-quality genome sequence of T. urartu was generated, providing a valuable resource for studying genetic variation in wheat and its wild relatives (Ling et al., 2018). A set of 145 elite cultivars selected from historical points of wheat breeding in China were re-sequenced. A high-density genomic variation map was constructed based on the sequencing data. The results indicated that haplotype block dynamics were a key driver promoting genomics-driven selection breeding. It was possible to breed high-yield varieties by taking advantage of haplotype block identification (Hao et al., 2020). WGS of populations from 25 subspecies in the genera Triticum and Aegilops was also performed. The sequencing results revealed that composite introgression from wild populations contributed to a substantial portion (4%-32%) of the bread wheat genome, which increased the genetic diversity of bread wheat and allowed its divergent adaptation (Zhou et al., 2020). Recently, a wheat gene mapping and genomic research platform, WheatGmap, was generated. It contains more than 3500 next-generation sequencing datasets of hexaploid wheat, including WGS, WES, and transcriptome deep-sequencing (RNA-seq) datasets, and it provides a convenient and powerful platform for wheat gene mapping and functional studies (Zhang et al., 2020). Nevertheless, the availability of a whole-genome sequence and pan-genome datasets from diverse wheat accessions and wild relatives will greatly facilitate genome editing in wheat for functional genomics and improvement.

Genome-wide association study

Genome-wide association study (GWAS) has become a common strategy for decoding genotype-phenotype associations in many species (Liu and Yan, 2019). GWAS is a statistical approach for mapping quantitative trait loci to link phenotypes of interest to genotypes by taking advantage of historic linkage disequilibrium (Figure 2). GWAS can identify more loci significantly associated with trait variations. For example, GWAS has been used to identify new alleles in wheat, including genes related to grain yield, crop domestication, and crop improvement (Li et al., 2019a, 2019b; Lujan Basile et al., 2019; Sehgal et al., 2019; Hao et al., 2020). Improvement of thousand-grain weight (TGW) is an important breeding target for improving grain yield (GY) in wheat. Based on a large set of germplasm, 15 haplotype blocks were found to be significantly associated with TGW, thereby expanding the opportunities for multi-gene pyramiding to derive new allele combinations for enhanced TGW and GY in wheat (Sehgal et al., 2019). The relationships among GY and related traits were analyzed using SNP-GWAS and Haplootype-GWAS methods in a diverse panel of 166 elite wheat varieties from the Yellow and Huai River Valleys Wheat Zone (YHRVWD) in China. In total, 120 loci were identified, 78 of which were novel loci, which can be used in breeding programs aimed at the accumulation of favorable alleles to develop high-yielding varieties (Li et al., 2019a, ). To better clarify the relationships between genomic structure and yield-related traits, SNP data and gene-related molecular markers of 102 Argentinean wheat cultivars were collected. GWAS was performed based on the SNP data and markers, and 97 regions associated with heading date, plant height, TGW, grain number per spike, and fruiting efficiency at harvest were identified, which will be beneficial for improvement of these traits (Lujan Basile et al., 2019). Recently, re-sequencing and GWAS analysis of a set of 145 elite wheat cultivars in China facilitated the identification of important genomic regions associated with crop domestication and improvement, providing a crucial gene resource for wheat improvement (Hao et al., 2020). Thus, GWAS has become an essential and reliable tool for identifying new haplotypes related to agriculturally important traits and for dissecting complex traits to facilitate marker-assisted breeding.

Speed breeding

To develop crops with higher yield and adaptability that can support 10 billion people by 2050, we must accelerate the crop genetic improvement process. In crop breeding, the development of novel elite crop varieties is time-consuming and laborious. It generally takes breeders 8–10 years to breed a novel variety. One possible solution for alleviating food shortages and increasing food security is the rapid development of elite crop varieties. Speed breeding, that is, the use of specific growing conditions such as optimal light intensity and temperature, prolonged photoperiod, and premature seed harvesting, can shorten the wheat generation time to 8–10 weeks (Watson et al., 2018; Chiurugwi et al., 2019) (Figure 2). In brief, this generally involves a longer light period of 22 h at 22°C, followed by a 2-h recovery in the dark at 17°C. The spikes are harvested ~2–3 weeks post-anthesis and dried at 28°C–30°C for 3–5 days before threshing (Zhan et al., 2021). Despite its importance and widespread use, large-scale mutation screening remains time-consuming, labor-intensive, and costly, especially in polyploid wheat plants. The Hi-TOM (high-throughput tracking of mutations) platform (available at http://www.hi-tom.net/hi-tom/) is an online tool that has high reliability and sensitivity in tracking various mutations. It is particularly suitable for high-throughput identification of all types of mutations induced by CRISPR/Cas systems, especially in polyploid crop species (Liu et al., 2019a). Hi-TOM is a simple tool-kit for genotyping wheat mutant lines. Target-specific and barcoding PCR are used to construct the library, which is sent for next-generation sequencing. The sequencing results can then be used to identify the mutations present in a cluster of wheat plants. Based on the Hi-TOM sequencing results, the editing profile (genotype) of each individual plant is further determined using A/B/D subgenome-specific primer sets. The PCR amplicons are used for Sanger sequencing, followed by decoding with CRISPR-GE (http://dsdecode.scgene.com/) to investigate the genotype and zygosity of the edited plants (Liu et al., 2019a). For edited plants that contain complex chimeric mutations, PCR products are cloned into a cloning vector. The genotype of each edited
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plant is finally determined based on the sequencing result. The development of automated high-throughput phenotyping systems has enabled phenotype evaluations of larger populations with increased selection intensity and accuracy (Araus et al., 2018). Therefore, combining high-throughput genotyping and phenotyping with diverse developed CRISPR toolboxes will greatly facilitate the application of genome editing to wheat improvement.

Synthetic biology

Synthetic biology refers to the design and construction of new biological parts, devices, and systems or the redesign of existing biological components and systems (Cook et al., 2014). It originated from bacterial systems and has now advanced to eukaryotes, including plants. Synthetic biology is expected to play an important role in meeting the challenge of food safety in the context of global climate change, increasing world population, and water shortages in arid and semi-arid lands, as it has excellent potential for redesigning a crop for a particular purpose (Wurtzel et al., 2019).

Gene stacking enables multiple transgenes of interest to be pyramided into the same plant genome, thereby improving several agronomic traits simultaneously (Liu et al., 2013) and producing novel germplasm that does not exist in nature (Figure 2). For example, wheat stripe rust, caused by Puccinia graminis f. sp. tritici (Pgt), is a significant disease that affects wheat production. Breeding wheat varieties resistant to Pgt is an alternative and effective sustainable solution. Two types of Pgt resistance genes have been isolated from wheat: all-stage resistance (ASR) genes and adult plant resistance (APR) genes (Periyannan et al., 2017). ASR genes are crucial for crop protection, but they usually show transient resistance when deployed alone because of the rapid evolution of pathogen effectors to avoid recognition. Although APR genes are remarkably durable in some cases, these genes generally provide only partial resistance, which is often insufficient for crop protection during severe pathogen epidemics. Recently, polygenic Pgt-resistant wheat plants have been generated by introducing a 37-kb transgene cassette of five Pgt resistance genes, including four ASR genes (TaSr22, TaSr35, TaSr45, and TaSr50) and an APR gene (TaSr55), into bread wheat at a single locus. These wheat lines showed very high resistance to Pgt (Luo et al., 2021b), suggesting that a novel and superior wheat germplasm could be obtained using synthetic biology. In rice, a highly efficient transgene stacking system was established to assemble six genes (the synthetic glycosyl-phosphate-tolerance gene I. variabilis-EPSPS*, the lepidopteran pest resistance gene Cry1C*, the brown planthopper resistance genes Bph14* and OsLecRK1*, the bacterial blight resistance gene Xa23*, and the rice blast resistance gene Pib9*) onto a transformable artificial chromosome vector, “multi-resistance rice” (Li et al., 2020g). Rice plants with improved pest and disease resistance were successfully generated. Genetically engineered crop species for biofortification of phytonutrients have also been developed by stacking up to five regulatory or structural genes of a given metabolic biosynthesis pathway. These crops include β-carotene-enriched “Golden Rice” (Ye et al., 2000; Paine et al., 2005), anthocyanin-enriched “Purple Tomatoes” (Butelli et al., 2006), folate-enriched rice (Blancquaert et al., 2015), anthocyanin-enriched “Purple Endosperm Rice” (Zhu et al., 2017), high-iron content wheat (Connorton et al., 2017), carotenoid-enriched rice (Tian et al., 2019), zeaxanthin-enriched rice, astaxanthin-enriched rice, and capsanthin-enriched rice (Ha et al., 2019). In years to come, it is expected that synthetic biology will be widely used to generate diverse novel gene resources and germplasm for wheat improvement.

CURRENT CHALLENGES AND FUTURE PERSPECTIVES

Precision genome editing through HDR in wheat

Precise replacement of an existing allele via HDR provides an attractive alternative to accelerate wheat improvement and facilitate sustainable wheat production (Figure 2). Although it is now feasible to achieve precise gene/allele replacement or gene targeting in Arabidopsis and some crop species, such as rice, maize, and tomato (Svitashev et al., 2015; Sun et al., 2016b, 2017; Endo et al., 2016; Begemann et al., 2017; Butt et al., 2017; Gil-Humanes et al., 2017; Shi et al., 2017; Wang et al., 2017; Li et al., 2018a, 2018b, 2019a, 2019b; Hummel et al., 2018), precision genome editing through HDR for targeted gene/allele replacement remains challenging in wheat, and no precisely edited stable lines have been recovered to date. Therefore, further development of diverse strategies to enhance HDR efficiency is required to facilitate wheat improvement through precision genome editing.

Considering the significance of HDR, many approaches that have been used to improve HDR efficiency in mammalian cells or other plant species can be evaluated in wheat to improve its HDR efficiency. First, a modular RNA aptamer-streptavidin strategy to enrich the availability of DRTs increased HDR efficiency in human cells to levels 18-fold higher than that of the conventional method (Carlson-Stevermer et al., 2017). Also, spatial and temporal co-localization of the DRTs and Cas9 protein via a SNAP-tag increased the efficiency of HDR 24-fold by increasing the concentration of DRTs in the nucleus (Savic et al., 2018). Second, HDR efficiency can be improved if the DRT is close to the DSB (Rolloos et al., 2015; Ma et al., 2017; Aird et al., 2018; Gu et al., 2018; Roy et al., 2018; Savic et al., 2018). For example, HDR efficiency was increased 30-fold by covalently tethering a single-stranded donor oligonucleotide (ssODN) to the Cas9/guide RNA ribonucleoprotein (RNP) complex via a fused HUH endonuclease 5 (Aird et al., 2018). Third, use of the egg cell- and early embryo-specific DD45 gene promoter to drive SpCas9 expression in Arabidopsis has the potential to increase the efficiency of HDR-mediated genome editing based on the fact that HDR occurs at a high frequency in egg cells (Miki et al., 2018; Wolter et al., 2018). Finally, by random insertion of a phosphorothioate-linkage and phosphorylation-modified DNA fragment, a tandem repeat-HDR (TR-HDR) strategy has recently been developed for fragment replacement and seamless in-locus tagging of either the N- or C-terminal end of proteins with a flag-tag of up to 130 bp in rice (Lu et al., 2020a). Technological improvements in mammalian cells and other plant species, as well as manipulations of diverse modules to enhance HDR efficacy, lead us to expect that in the long term, precise gene/allele replacement will be achieved in wheat using the CRISPR/Cas system and will be widely used to generate novel germplasm and breed elite wheat varieties.
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**Multiplex genome editing in wheat**

Polyploid species such as common wheat, with its large, complex genome and A, B, and D subgenomes, make it very challenging to create mutations at multiple genomic sites simultaneously. In addition, most agriculturally important traits are controlled by multiple genes or a complicated genetic network. For example, the yield per plant, one of the critical traits for breeding of high-yield wheat, is controlled by polygenes composed of major genes and genes with minor effects. To investigate the roles of these genes in yield per plant, knockout of single genes and combinations of genes or regions would be highly desirable for trait dissection, gene discovery, and marker-assisted breeding. Furthermore, pyramiding favorable alleles in an elite wheat variety by CRISPR/Cas9-mediated multiplex editing will significantly accelerate gene discovery and breeding. Therefore, establishing an efficient multiplex editing platform will be very valuable for wheat functional genomics and genetic improvement (Figure 2).

To this end, further optimization and systemic comparison of different vector components, such as strong constitutive promoters for Cas9 protein or gRNA expression and additional NLSs, will contribute to the development of a more efficient multiplex editing strategy in wheat. For example, we recently established an efficient multiplexing strategy in the elite winter wheat variety Zhangmai 7698 (ZM) using a polycistronic tRNA strategy to produce multiple mature single chimeric guide RNAs (sgRNAs) simultaneously. We used an expression cassette driven by a rice Actin promoter and terminated by a Poly A sequence to stabilize the transcripts and a Nos terminator to express tandem arrays of two, three, four, or five tRNA-sgRNAs in a single transcript unit. We demonstrated the simultaneous editing of two, three, four, or five genes at up to 15 genomic loci in this elite wheat variety by multiplex genome editing. Following embryo rescue and segregation, we successfully recovered transgene-free plants with targeted mutagenesis at up to 15 genomic loci in one generation (Luo et al., 2021a). To further expand the scope of multiplex editing in plants, diverse Cas nickases with various PAM requirements, such as SpCas9-NG, xCas9, SpG, SpRY, SaCas9, and Cas12a (Zhang et al., 2019b; Li et al., 2020h), could be employed to increase the flexibility of this technology in wheat. Moreover, apart from protein-coding genes, multiplexing systems can also cause targeted, sequence-specific mutagenesis in noncoding RNA regions and other genetic regulatory elements to facilitate the dissection of their functions and promote wheat improvement. The establishment of an efficient and flexible CRISPR/Cas multiplexing system would greatly facilitate fundamental biological research, enabling researchers to decipher a complex trait conferred by multiple genes and promoting the translational breeding process in hexaploid wheat and other agriculturally important polyploid crop species for sustainable agriculture development.

**Simultaneous HDR and/or base editing and knockout for wheat improvement**

In crops, allelic differences are responsible for significant differences in yield, grain quality, nutrient use efficiency, resistance to both abiotic and biotic stresses, and other agriculturally important traits (Ashikari and Matsuoka, 2006; Liu et al., 2014; Mai et al., 2014; Ashkani et al., 2015; Hori et al., 2016; Xu et al., 2016; Hao et al., 2020; Zhang et al., 2020). Most of the valuable alleles derived from local landraces or wheat relatives are caused by SNPs or by insertion/deletions of a gene fragment (Hao et al., 2020; Zhang et al., 2020). Introduction of elite alleles into commercial cultivars by crossing and back-crossing can take up to 10 years. Development of simultaneous HDR and/or base editing and knockout modules, or combinations thereof, will enable modifications of multiple loci simultaneously, thus pyramiding multiple beneficial alleles into a commercial variety in a short time period. This would be extremely valuable for improving the complex agronomic traits of wheat. With the development of next-generation DNA sequencing technologies, the availability of complete wheat genome sequences and pan-genome data (Wang et al., 2018b; Ling et al., 2018; Hao et al., 2020; Zhang et al., 2020), wheat trait dissection and gene discovery by GWAS, and diverse strategies for improving HDR efficiency, we expect that in years to come it will be feasible to design and breed “Green Super Wheat” with high yields, high nutritional values, resistance to both abiotic and biotic stresses, and high nutrient use efficiency, thereby meeting the growing food demand and ensuring global food and ecological security (Figure 2).

**De novo domestication of wild species and site-directed artificial evolution of agriculturally important genes**

Crop improvement by intensive artificial selection in traditional breeding practice often results in decreased stress resistance and loss of genetic diversity, which significantly threaten the stability of yield and food security under global climate change and the frequent occurrence of natural disasters (Hickey et al., 2019). Thus, it is urgent and imperative to enhance crop genetic diversity by exploiting novel gene resources through cutting-edge technologies. Through the comprehensive application of genomics, genome-editing, synthetic biology approaches, de novo domestication, and site-directed artificial evolution, species or crops could be quickly domesticated while maintaining their original excellent traits (Eshed and Lippman, 2019). For example, by introducing desirable traits into four stress-tolerant wild tomato accessions through multiplex genome editing, the progeny of edited plants exhibited the domesticated phenotype but retained parental disease resistance and salt tolerance ((Li et al., 2018c; Zsogon et al., 2018). Recently, a rational strategy for creating novel crops was proposed based on the de novo domestication of wild allotetraploid rice (Oryza alta, CCDD) (Yu et al., 2021). A series of allotetraploid rice lines with targeted improvements in various traits in O. alta were successfully produced through genome editing. The authors first established an efficient transformation and genome-editing system in allotetraploid rice. They then assembled a high-quality genome sequence of allotetraploid rice and identified O. alta homologs of agronomically important genes from diploid rice to serve as target genes for genome editing (Yu et al., 2021). The significance of this study is the demonstration that some wild crop species, such as wheat relatives or landraces, can be developed into a new variety by de novo domestication so as to ensure global food security (Figure 2). In addition, base editing can enable artificial evolution of agriculturally important genes in current crop varieties to develop novel gene resources and germplasm (Figure 2). For example, herbicide-tolerant rice germplasms have been produced by artificial evolution of OsALS1 and Wheat genome editing and beyond
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OsACC carboxyltransferase domains in rice by base editing (Li et al., 2020a; Kuang et al., 2020). Unfortunately, neither de novo domestication nor site-directed artificial evolution by genome editing have yet been documented in wheat.

Development of diverse genotype-independent and in planta strategies

CRISPR-mediated genome-editing technologies have revolutionized the plant research field and offer great potential for wheat biological research and genetic improvement. However, as in other plant species, genome editing in wheat relies on the delivery of exogenous DNA into plant cells. The main approaches to delivering exogenous DNA into plant cells are biolistic transformation or Agrobacterium-mediated transformation (Hayta et al., 2021; Smedley et al., 2021). The potential of genome editing to improve wheat’s agronomic performance is often limited by low plant regeneration efficiency and few transformable genotypes, and most modern wheat varieties are recalcitrant to genetic transformation and regeneration. To overcome this bottleneck, expression of a fusion protein combining wheat GROWTH-REGULATING FACTOR 4 (GRF4) and its cofactor GRF-INTERACTING FACTOR 1 (GIF1) substantially increases the efficiency and speed of regeneration in wheat, triticale, and rice, and increases the number of transformable wheat genotypes (Debernardi et al., 2020). In maize, a genotype-independent methodology has been established by transforming the Bbm expression cassette driven by the maize auxin-inducible promoter (Zm-Axig1 proc:Bbm) into immature maize embryos together with a nopaline synthase promoter to drive the expression of Wus2. These embryos were individually and uniformly transformed and could be directly germinated into plants without a callus phase (Lowe et al., 2018). In addition, appropriate ectopic expression of developmental regulators, including WUSCHEL2 (Wus2) and SHOOT MERISTEMLESS (STM) or MONOPTEROS (MP), could induce the formation of meristem-like structures in vivo in tobacco plants, sidestepping tissue culture-based transformation for plant genome editing (Maher et al., 2020). It is now essential to validate the utility of this strategy in important staple food crops (Li et al., 2020). Alternatively, a genome-editing procedure that did not require callus culture and regeneration was reported in wheat using in planta particle bombardment. The TaGASR7 gene was successfully edited by delivering plasmids expressing CRISPR/Cas9 components into the shoot apical meristems of imbibed seed embryos. With this method, it is now possible to transform cultivars that are recalcitrant to conventional transformation procedures (Hamada et al., 2018). However, this strategy remains technically challenging in other laboratories. Delivery of genome-editing machinery into the vast majority of wheat varieties is not possible because most elite wheat varieties are recalcitrant to traditional transformation methods such as bombardment and Agrobacterium-mediated transformation. A haploid induction (HI) editing technology, HI-Edit, was developed to enable direct editing of targeted genes not only in maize elite inbred lines but also in an elite wheat germplasm by a single cross (Kellher et al., 2019). Furthermore, an in planta and efficient genotype-independent strategy for genome editing in tobacco (N. benthamiana) was established using RNA viral vectors, sidestepping the tissue culture process in genome editing and helping to realize the promise of plant genome editing for the advancement of basic and applied plant research (Ellison et al., 2020). The development of RNA viral vector-mediated genome-editing methodology, which enables heritable mutations, will undoubtedly bypass the genotype-dependent bottleneck in wheat genome editing. With the development of diverse genotype-independent strategies for genome editing in other crop species, extension and validation of these strategies in wheat transformation will certainly expedite progress in wheat genome editing and enable its use in wheat improvement (Figure 2).

CONCLUDING REMARKS

Although impressive progress has been achieved in wheat genome editing during the past few years, the majority of successful genome editing in wheat has focused mainly on the generation of random mutations and knockouts through NHEJ. Precision genome editing in wheat remains very challenging. In years to come, the establishment of diverse, efficient platforms for multiplex genome editing, gene replacement through HDR, simultaneous HDR and/or base editing, knockout modules, site-directed artificial evolution, de novo domestication, and the development of diverse genotype-independent strategies for genome editing in wheat will greatly facilitate wheat functional genomics and improvement. By developing various genome-editing toolboxes, we will be able to pyramid several desired alleles in a commercial variety and improve wheat in a user-defined manner quickly, efficiently, and cost-effectively without compromising other elite agronomic traits and without linkage drag from deleterious genes. In addition, wheat whole-genome and pan-genome sequencing, GWAS, trait dissection, gene discovery, and speed breeding will provide massive amounts of genetic information and accelerate wheat improvement through genome editing. We believe that combining genome editing with other state-of-the-art breeding technologies will underpin efforts to breed green super wheat varieties for sustainable agriculture and a better environment to ensure global food security.

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