https://doi.org/10.1093/plphys/kiac037 PLANT PHYSIOLOGY 2022: 188: 1780–1794

Plant Physiology®

Update

# An update on precision genome editing by homology-directed repair in plants

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J.C., S.L., Y.H., and J.L. wrote the manuscript. L.X. revised the manuscript. All authors read and approved the final manuscript.
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## Abstract

Beneficial alleles derived from local landraces or related species, or even orthologs from other plant species, are often caused by differences of one or several single-nucleotide polymorphisms or indels in either the promoter region or the encoding region of a gene and often account for major differences in agriculturally important traits. Clustered regularly interspaced short palindromic repeats-associated endonuclease Cas9 system (CRISPR/Cas9)-mediated precision genome editing enables targeted allele replacement or insertion of flag or foreign genes at specific loci via homology-directed repair (HDR); however, HDR efficiency is low due to the intrinsic rare occurrence of HDR and insufficient DNA repair template in the proximity of a double-stranded break (DSB). Precise replacement of the targeted gene with elite alleles from landraces or relatives into a commercial variety through genome editing has been a holy grail in the crop genome editing field. In this update, we briefly summarize CRISPR/Cas-mediated HDR in plants. We describe diverse strategies to improve HDR efficiency by manipulating the DNA repair pathway, timing DSB induction, and donor delivery, and so on. Lastly, we outline open questions and challenges in HDR-mediated precision genome editing in both plant biological research and crop improvement.

## Introduction

Rapid population growth, unsustainable agricultural practices, and climate change lead to degradation of natural resources and necessitate the attempts to sustainably increase crop production for global food security. Allelic differences, which have been selected during domestication and subsequent improvements, account for major differences in crop yield and other agriculturally important traits. Most of the valuable alleles, which are usually derived from local landraces or wild relatives, are often caused by differences of one or several single-nucleotide polymorphisms (SNPs), or insertion/deletions (indels) of a gene fragment in either the promoter region or the gene's encoding region. Harnessing the genetic diversity and introducing the elite alleles into

Received November 12, 2021. Accepted January 20, 2022. Advance access publication February 2, 2022

## ADVANCES

- CRISPR/Cas-mediated HDR allows installation of all kinds of mutations without length limitation in a predefined manner, which enables introduction of a favorable allele from either landraces or wild relatives for crop improvement within two to three generations without introducing undesirable genes or traits. However, it remains challenging in plants, especially in crop plants.
- HDR efficiency in plants could be increased by using egg-specific promoters to drive the expression of sgRNA, tethering the DRT to the DSB, enriching the availability of DRT surrounded by replicon or using TR-HDR.
- CRISPR/Cas-mediated HDR expands our ability to tag genes of interest in locus in vivo, paving the way for characterizing localization and behavior of target genes/proteins in plant cells for fundamental biological research.

commercial cultivars has been a major goal in crop breeding programs. In crop breeding practice, introgression of the favorable alleles derived from either landraces or wild relatives into commercial varieties without any linkage drag from parental lines has been very difficult, laborious, and time-consuming. In general, it will take the breeders up to $>10$ years to introduce one beneficial allele into commercial cultivars by crossing and back-crossing. Nonetheless, it is very difficult or sometimes impossible 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 the elite alleles from landraces or the related species directly into the commercialized crop variety without introducing unwanted gene or DNA fragments in a short term.

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonuclease Cas9 system (CRISPR/Cas) has dominated the genome-editing field and demonstrated great potential either for plant functional genomics or crop improvement over the past few years. So far, three major CRISPR/Cas-mediated systems have been developed for precision genome editing in plants such as base editing (Komor et al., 2016; Gaudelli et al., 2017), prime editing (Anzalone et al., 2019), and homology-directed DNA repair (HDR)-mediated targeted gene replacement or insertion (Puchta and Fauser, 2013; Baltes et al., 2014; Čermák et al., 2015; Li et al., 2015; Shi et al., 2015; Butler et al., 2016; Endo et al., 2016; Sun et al., 2016a; Gil-Humanes et al., 2017; Shi et al., 2017; Wang et al., 2017a; Dahan-Meir et al., 2018; Li et al., 2018d, 2019b, 2020b; Miki et al., 2018; Peng et al., 2020; Lu et al., 2020b; Figure 1). Base editing enables single base transition within the editing window (Komor et al., 2016; Gaudelli et al., 2017; Figure 1A; Table 1). For precise single base substitution of an allele with single SNP, base editing, which is performed by engineered cytidine base editors (CBEs) and adenine base editors (ABEs), has emerged as an alternative tool to HDR-mediated gene replacement, facilitating precise editing of plant genome by transit single base to another without a double-stranded break (DSB) and DNA repair template (DRT) (Veillet et al., 2019). However, there are several limitations in the widespread use of base editors in plants, such as genome editing window, off-target effects, and so on (Table 1). Recently, a precision genome editing technology, prime editing, has been developed that directly mediates all 12 classes of point mutations and predefined indels of the target gene in human cells without requiring DSBs or DRT (Anzalone et al., 2019; Figure 1B;Table 1). Although prime editing could precisely install a wide range of sequences into DNA in plants, its precision editing efficiency remains low, at least for some target genes or plant species (Lin et al., 2020; Xu et al., 2020; Li et al., 2020a). Besides, due to the limited length of the reverse transcriptase (RT) template in the prime editing guide RNA (pegRNA), prime editing only enables small indels and base substitutions in mammalian cell lines as well as in plants (Anzalone et al., 2019; Li et al., 2020a; Wang et al., 2021; Table 1). In contrast, HDR or homologous recombination-based gene targeting enables the installation of all kinds of mutations or a fragment in a predefined manner, albeit at relatively lower efficiency (Puchta et al., 1996; Griffiths et al., 2006; Hiom, 2010; Symington and Gautier, 2011; Puchta and Fauser, 2013, 2014; Altpeter et al, 2016; Endo et al., 2016; Steinert et al., 2016; Sun et al., 2016b; Li and Xia, 2020; Fan et al., 2021; Figure 1C;Table 1). Targeted gene replacement is particularly important for introduction of a favorable allele from either landraces or wild relatives within two to three generations without introducing undesirable genes or traits. Thus, both limitations of base editing and prime editing and the advantages of HDR reiterate the necessities to further explore more efficient HDR technologies for precision genome editing in crop plants.

In this review, we provide an informative summary of the updated achievements of the CRISPR/Cas-mediated HDR in plants. We then outline diverse strategies to improve HDR efficiency by manipulating DNA repair pathway, timing the DSBs induction and donor delivery, and so on. At last, we discuss the existing challenges in precision genome editing by HDR and propose future perspectives of HDR in plant biological science as well as crop improvement.

## CRISPR/Cas-mediated HDR in plants

### CRISPR/Cas9-mediated HDR in plants

Once the DSB is produced by the CRISPR/Cas system, it can be repaired by nonhomologous end-joining (NHEJ) or HDR pathway. HDR is a precise repair pathway and is stimulated by the homologous DRT surrounding a DSB (Puchta, 1998; Puchta and Fauser, 2014; Baltes and Voytas, 2015; Li and Xia,

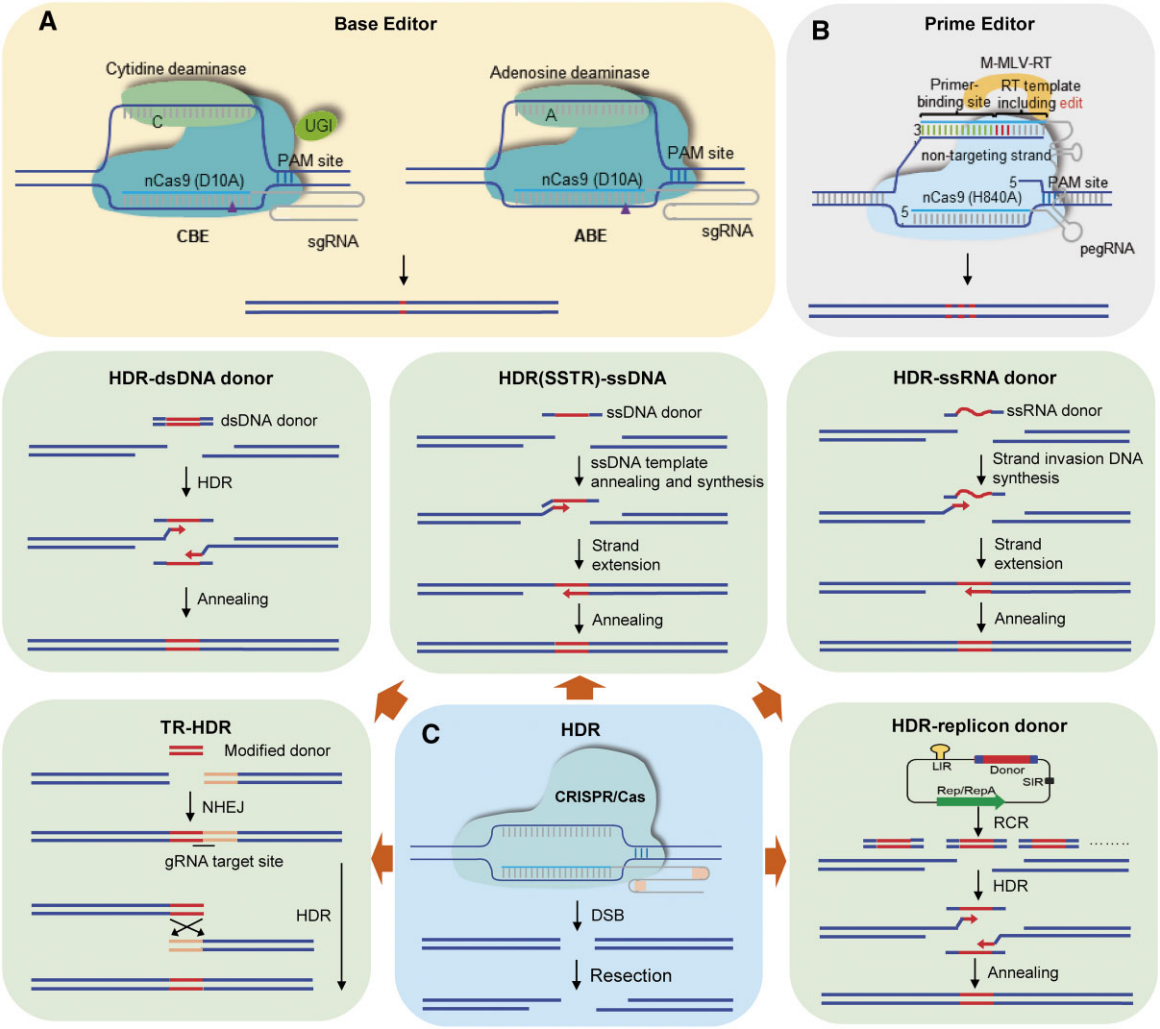


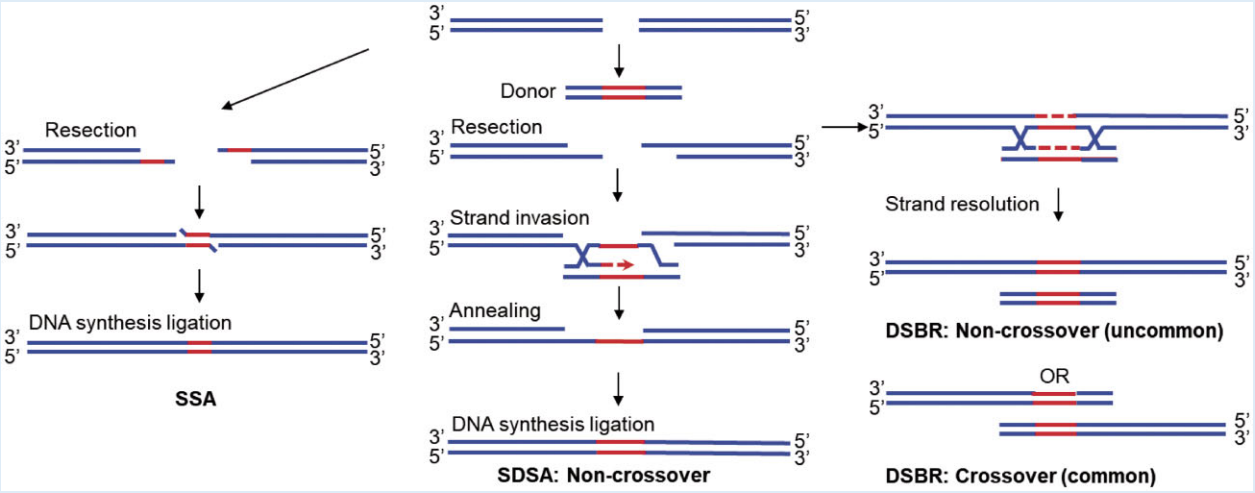

**Figure 1** Diverse strategies for precision genome editing by CRISPR/Cas system. A, Base editing mediated precise gene modification. ABE is fusions of adenosine deaminase and catalytically impaired Cas9 [nCas9 (D10A)], which catalyzes the deamination of adenine (A) within a narrow window of the nontargeting strand, and A·T-to-G·C conversion is achieved in the DNA strand. CBE is fusions of cytosine deaminase and catalytically impaired Cas9 [nCas9 (D10A)], which catalyzes the deamination of cytosine (C) within a narrow window of the nontargeting strand, and ultimately resulting in a C·G-to-T·A conversion. UGI, uracil DNA glycosylase inhibitor. B, Prime editing mediated precise gene modification. Prime editor contains a catalytically impaired Cas9 [nCas9 (H840A)] fused to an RT Moloney murine leukemia virus RT (M-MLV-RT) and an engineered pegRNA. First, the 3′ terminal of the nicked DNA strand hybridizes to the primer-binding site (PBS) after Cas9 (H840A) nicking, and then reverse transcription of the RT template is conducted by M-MLV-RT, resulting in desired edited DNA sequence in the genome. C, HDR mediated precise gene modification. DSB induced by Cas protein can be repaired by the HDR pathway, when donor repair template exists surrounding a DSB. When the donor repair template is dsDNA, ssDNA, or single strand RNA, the homologous arms of the donor repair template paired with the genome sequence, and are extended by DNA synthesis. Finally, newly synthesized strands withdraw from the donor templates and anneal back to the locus. For dsDNA repair template, both strands can act as repair template. For the TR-HDR strategy, a modified dsDNA donor with homology to the target sequence is inserted to the target site through CRISPR/Cas-mediated NHEJ, forming a tandem-repeat structure. Then, DSB induced by Cas protein at the newly formed gRNA target site trigger HDR between the tandem repeats, resulting in precise genome modification. For replicon donor, it can produce numerous copies of the donor which may serve as repair template during CRISPR/Cas-mediated HDR through rolling-circle replication. SSTR, Single-strand template repair.

2020; Figure 1C). In general, there are three main repair mechanisms of HDR: single-strand annealing (SSA) model, synthesis-dependent strand annealing (SDSA) model and so-called DSB repair (DSBR) model (see Box 1 Figure). In comparison with mammalian cells, it remains challenging to achieve efficient precise genome editing by CRISPR/Cas-mediated HDR in plants because of four reasons: (1) HDR is mainly active in the late S and G2 phase of the cell cycle (Puchta and Fauser, 2014); (2) As the predominant pathway for DSBs repair in somatic cells, NHEJ competes with the HDR pathway (Symington and Gautier, 2011; Puchta and Fauser, 2014; Sun et al., 2016a); (3) spatially and temporally co-localizing the CRISPR reagents and DRT is crucial for successful HDR events (Endo et al., 2016); and (4) limited DRT delivered into plant cells for HDR due to the presence of cell wall (Li et al., 2019b). In fact, when foreign DNA is

**Table 1** Advantage and disadvantage of different gene-editing system

| Gene Editing System | Advantages | Disadvantages |
|---|---|---|
| Base editing | • Precise single base substitution without DSBs and DRT.<br>• High efficiency | • Restricted gene editing window<br>• Cannot currently perform the transversion mutations<br>• Severe off-target |
| Prime editing | • It mediates all 12 classes of point mutations and predefined indels or deletion of the target gene | • Only enables small indels and base substitutions<br>• Low efficiency |
| HDR-mediated editing | • Enable the installation of all kinds of mutations or a fragment in a predefined manner | • Low efficiency |

**Box 1 Figure Proposed pathway for DSB repair by HDR.**




**Box 1. The potential mechanism of HDR**

There are three proposed main repair mechanisms of HDR: SSA, SDSA, and so-called DSBR (see Box 1 Figure). SSA is activated by the complementary sequences at both ends of the DSB and consequently results in the loss of the sequences between the complementary sequences. Therefore, SSA is classified as a nonconservative repair mechanism and occurs frequently in somatic cells (Puchta and Fauser, 2013). In DSBR and SDSA, following the DSB induction, the 3′ end of a single strand invades inside the homologous dsDNA and forms a D-loop. Reparative synthesis is then initiated using the DRT as a template. In DSBR, DNA synthesis occurs at both broken ends so that genetic information is copied from both strands of the DRT, which often leads to a crossover event (Puchta and Fauser, 2013). DSBR is a prominent mechanism during meiotic recombination (Osman et al., 2011). In SDSA, the DSB is resected and processed to generate 3′ overhangs on both sides of the DSB. The 3′ overhangs are then paired with the DRT and extended by DNA synthesis using the DRT as template. Then, the newly synthesized strands withdraw from the DRT and anneal back to the locus (Puchta and Fauser, 2014; Paix et al., 2017). SDSA has been proposed as a repair mechanism when the ssODNs are used as the DRT (Paquet et al., 2016; Richardson et al., 2016; Kan et al., 2017). Analyses of the sequence requirements for repairing DSBs generated by Cas9 in human cells indicated that the repair process is more consistent with SDSA upon the availability of the repair templates, in the forms of either ssODNs or dsDNAs (Paix et al., 2017; Li et al., 2020c). In mammalian cells, the most routine method of HDR is using long ssODNs as repair template (Hu et al., 2019; Gallagher and Haber, 2021). In plant cells, it is proposed that SDSA is involved in HDR of DSBs generated by CRISPR/Cas12a (also known as Cpf1) when the dsDNA is used as a DRT. The presence of the left homologous arm alone is sufficient for occurrence of HDR (Li et al., 2018d). Dissection of the potential mechanism underlying HDR-mediated gene replacement will certainly benefit increasing HDR efficiency in crop improvement.

introduced into plant cells, there are typically $10^5$ to $10^7$ illegitimate recombination events along with one homologous recombination event (Puchta, 1998; Shukla et al., 2009). Even so, because of its great importance in crop improvement, CRISPR/Cas-mediated HDR has been attempted in various plant species (Baltes et al., 2014; Čermák et al., 2015; Li et al., 2015; Svitashev et al., 2015; Butler et al., 2016; Endo et al., 2016; Sauer et al., 2016; Sun et al., 2016a; Butt et al., 2017; Gil-Humanes et al., 2017; Paix et al., 2017; Shi et al., 2017; Yu et al., 2017; Wang et al., 2017b; Dahan-Meir et al., 2018; De Pater et al., 2018; Hahn et al., 2018; Miki et al., 2018; Shan et al., 2018; Wolter et al., 2018; Barone et al., 2020; Beying et al., 2020; Peng et al., 2020; Schmidt et al., 2020; Lu et al., 2020b). For example, herbicide resistant plants have been generated by replacement of the wild-type (WT) acetolactate synthase (ALS) gene with a mutated *ALS* gene containing desired mutations through CRISPR/Cas9-mediated HDR in rice (*Oryza sativa* L.), maize (*Zea mays* L.), tomato (*Solanum lycopersicum*), and Arabidopsis (*Arabidopsis thaliana*) (Svitashev et al., 2015; Endo et al., 2016; Sun et al., 2016a; Wolter et al., 2018; Table 2). Besides, alcobaca (*ALC*) and the *crtiso* gene in tomato were successfully modified by CRISPR/Cas9-mediated HDR (Yu et al., 2017; Dahan-Meir et al., 2018). Moreover, replacement of an nitrate-transporter gene (*NRT1.1B*) allele in a *japonica* rice with an elite allele from *indica* rice, improved rice nitrogen use efficiency (Li et al., 2018b). In maize, the grain yield was increased by replacing the native *ARGOS8* promoter with the native *GOS2* promoter (Shi et al., 2017). In addition, using CRISPR/Cas9 system, a kanamycin (neomycine phosphotransferase II [*nptII*]) resistance cassette was successfully integrated into alcohol dehydrogenase 1 (*ADH1*) gene in Arabidopsis (Schiml et al., 2014), and green fluorescent protein (*GFP*) gene and *GFP* expression cassette were inserted into glutathione S-transferase gene and putative DNA demethylase (*ROS1*) gene in rice and Arabidopsis (Wang et al., 2017a; Miki et al., 2018), respectively.

One of the major reasons for low HDR efficiency is due to the limited DRT delivered into plant cells (Baltes et al., 2014). To enrich DRT template, an all-in-one vector containing Cas9, gRNAs and DRT, co-bombarded with a free DRT fragment, enabled efficient *ALS* gene replacement through CRISPR/Cas9-mediated HDR to generate herbicide-resistant rice plants (Sun et al., 2016a). Geminivirus replicons (GVRs) have been used to deliver sufficient DRTs into plant cells for CRISPR/Cas9-mediated HDR in tobacco (*Nicotiana tabacum* L.), tomato (cultivar MicroTom), rice, and wheat (*Triticum aestivum* L.), respectively (Baltes et al., 2014; Čermák et al., 2015; Butler et al., 2016; Gil-Humanes et al., 2017; Wang et al., 2017a; Dahan-Meir et al., 2018; Table 2). For example, in tobacco, bean yellow dwarf virus (BeYDV) replicon-based HDR strategy enhanced HDR frequencies (Baltes et al., 2014). In tomato cultivar MicroTom, GVR was also used to create heritable modifications (Čermák et al., 2015). In wheat, wheat dwarf virus (WDV) replicons enabled HDR events at an endogenous ubiquitin locus, albeit no stable wheat lines with precise modification were recovered (Gil-Humanes et al., 2017). By using WDV replicon, a GFP-2A-NPTII cassette was incorporated in a specific genomic locus in rice (Wang et al., 2017a). Furthermore, by taking advantage of the geminiviral replicon amplification, a fast-neutron-induced carotenoid isomerase (CRTISO) gene allele was repaired with the WT allele through HDR at an efficiency of 25% (Dahan-Meir et al., 2018). Besides, it was demonstrated that targeted recombination between homologous chromosomes can be achieved via CRISPR/Cas9-induced DSB repair in tomato (*Solanum pimpinellifolium*) and Arabidopsis, respectively, paving a way for the use of targeted gene conversion for precision breeding (Filler-Hayut et al., 2017; Ben Shlush et al., 2021; Filler-Hayut et al., 2021). Recently, Lu et al. reported a CRISPR/Cas9-mediated tandem repeat-HDR TR-HDR strategy by using a chemical modified donor with phosphorothioate linkages at the 5′- and 3′-ends of donor DNA strands for targeted gene replacement, and achieved replacement or precise modifications with a sequence of up to 130 bp at an efficiency of 6.1% (Lu et al., 2020b). However, it is worth mentioning that although the presence of free DRT itself may not be harmful, it may raise biosafety concerns in products commercialization because of the possible random integration of free DRT in the genome.

## CRISPR/Cas12a-mediated HDR in plants

CRISPR/Cas12a has recently been reported as an alternative genome editing system in eukaryotes (Zetsche et al., 2015; Wang et al., 2017b). Cas12a is a class II type V CRISPR/Cas DNA endonuclease that is smaller than Cas9, and functions as an endonuclease to generate DSB and also acting as an endoribonuclease to process Cas12a-associated CRISPR repeats into mature CRISPR RNA (crRNA) (Fonfara et al., 2016; Zetsche et al., 2017). Cas12a extends target recognition in T-rich protospacer adjacent motif (PAM) sequences "TTTN," and the cuts are located distal to the PAM site, generating staggered ends with overhangs of 4–5 nt upon cleavage (Zetsche et al., 2015). In addition, CRISPR/Cas12a has very low off-target effects in comparison with Cas9. Up to date, CRISPR/Cas12a has been exploited as an efficient and alternative genome editing tool in various organisms including plants such as rice, maize, tomato (Hongkwang cultivar, a local variety), cotton (*Gossypium hirsutum*), soybean (*Glycine max*), tobacco (*Nicotiana attenuata*), and so on (Begemann et al., 2017; Kim et al., 2017; Tang et al., 2017; Wang et al., 2017b; Xu et al., 2017; Bin Moon et al., 2018; Tang et al., 2018; Bernabé-Orts et al., 2019; Jia et al., 2019; Lee et al., 2019; Malzahn et al., 2019; Wolter and Puchta, 2019; Li et al., 2019a, 2020c; Merker et al., 2020; Schindele and Puchta, 2020; Vu et al., 2020; Huang et al., 2021). While the major applications of CRISPR/Cas12a in plants were to generate gene knock-out through NHEJ, successful HDR events by CRISPR/Cas12a system have been achieved in rice, tobacco, and Arabidopsis, respectively (Begemann et al., 2017; Li et al., 2018d, 2019b;

**Table 2** HDR in diverse plant species by CRISPR/Cas system

| Nuclease | Plant species | Target gene | Donor | Length of donor/left arm/right arm | Transformation methods | References | Note |
|---|---|---|---|---|---|---|---|
| Cas9 | Arabidopsis | *ADH1* | A kanamycin (*nptII*) resistance cassette | 3,167 bp/674 bp/673 bp | Agrobacterium | Schiml et al. (2014) | |
| Cas9 | Arabidopsis | *ALS* | *ALS* with mutant sites | 1,542 bp/800 bp/742 bp | Agrobacterium | Wolter et al. (2018) | EC1.1 promoter |
| Cas9 | Arabidopsis | *ROS1/DME* | GFP cassette/DME with mutant sites | 1,846 bp/801 bp/325 bp<br>2,274 bp/812 bp/742 bp<br>2,080 bp/609 bp/751 bp<br>2,779 bp/801 bp/325 bp<br>1,170 bp/530 bp/580 bp | Agrobacterium | Miki et al. (2018) | AtDD45 promoter |
| LbCas12a | Arabidopsis | *ALS* | *ALS* with mutant sites | 1,542 bp/800 bp/74 bp | Agrobacterium | Wolter and Puchta (2019) | EC1.1 promoter |
| ttLbCas12a | Arabidopsis | *ALS* | *ALS* with mutant sites | 1,542 bp/637 bp/905 bp | Agrobacterium | Merker et al. (2020) | EC1.1 promoter |
| Cas9 | Rice | *ALS* | *ALS* with mutant sites | –/-/- | Agrobacterium | Endo et al. (2016) | DNA ligase 4 knock out |
| Cas9 | Rice | *ALS* | *ALS* with mutant sites | 410 bp/100 bp/46 bp | Biolistic | Sun et al. (2016a) | – |
| FnCas12a/ LbCas12a | Rice | *CAO* | Chlorophyllide-a oxygenase (*CAO*) with mutant sites | 5,320 bp/∼1,000 bp/∼1,000 bp<br>5,250 bp/∼1,000 bp/∼1,000 bp | Biolistic | Begemann et al. (2017) | – |
| Cas9 | Rice | *NRT1.1B* | Nitrate transporter *NRT1.1B* with mutant sites | 451 bp/100 bp/100 bp | Biolistic | Li et al. (2018a) | – |
| LbCas12a | Rice | *ALS* | *ALS* with mutant sites | 635 bp/97 bp/121 bp | Biolistic | Li et al. (2018c) | – |
| LbCas12a | Rice | *ALS* | *ALS* with mutant sites | 635 bp/97 bp/121 bp | Biolistic | Li et al. (2019b) | – |
| Cas9 | Rice | *SLR1/TT1/ NRT1.1b/UBQ6* | Donor with mutant site/sequence with flag-tag fusion | –/-/- | Biolistic | Lu et al. (2020b) | – |
| Cas9 | Rice | Genomic safe harbors | Carotenoid biosynthesis cassette | ∼5.4 kb/794 bp/816 bp | Biolistic | Dong et al. (2020) | – |
| Cas9 | Rice | *ALS/CCD7/HDT* | *ALS* with mutant sites/ CAROTENOID *CCD7* with mutant site/C-terminal end with HA tag | 96 bp/25 bp/25 bp<br>89 bp/25 bp/25 bp | Agrobacterium | Ali et al. (2020) | – |
| Cas9 | Tomato | *ALC* | ALC with mutant site | 708 bp/235 bp/450 bp | Agrobacterium | Yu et al. (2017) | Replicon |
| Cas9 | Tomato | *Crtiso* | BeYDV replicons crtiso with mutant sites | 3,796 bp/1,778 bp/1,737 bp | Agrobacterium | Dahan-Meir et al. (2018) | Replicon |
| LbCas12a | Tomato | *ANT1* | BeYDV replicons anthocyanin mutant 1 (*ANT1*) and an *NPTII* expression cassette | 3,656 bp/988 bp/719 bp<br>4,156 bp/1,008 bp/719 bp | Agrobacterium | Vu et al. (2020) | Replicon |
| ttLbCas12a | Tomato | *ANT1/ SKT12/ EPSPS1* | BeYDV replicons *ANT/SKT1.2/ EPSPS1* expression cassette | 4,044 bp/866 bp/740 bp<br>4,141 bp/963 bp/740 bp<br>4,188 bp/989 bp/740 bp<br>3,742 bp/986 bp/839 bp | Agrobacterium | Vu et al. (2021) | Replicon |
| Cas9 | Maize | *ALS2* | | | Agrobacterium | | – |

(continued)

**Table 2** Continued

| Nuclease | Plant species | Target gene | Donor | Length of donor/left arm/right arm | Transformation methods | References | Note |
|---|---|---|---|---|---|---|---|
| | | | *ALS* gene with mutant sites/ dsRED expression cassette | 794 bp/508 bp/283 bp<br>5424 bp/1097 bp/1135 bp | | Svitashev et al. (2015) | |
| Cas9 | Maize | *ARGOS8* | *ARGOS8* promoter sequences | –/~400 bp/~400 bp | Agrobacterium | Shi et al. (2017) | – |
| SaCas9 | Tobacco | *ALS/SuRB* | *ALS* gene with mutant sites/ *SuRB* with mutant site | 1,854 bp/976 bp/546 bp<br>1,810 bp/610 bp/582 bp<br>1852 bp/976bp/582 bp | Agrobacterium | Huang et al. (2021) | – |
| Cas9 | Wheat | *Ubiquitin* | WDV replicons GFP/BFP/ GFP + dsRed cassette | 2,579 bp/747 bp/773 bp<br>2,348 bp/674 bp/647 bp<br>1,837 bp/210 bp/646 bp | Biolistic | Gil-Humanes et al. (2017) | Replicon |

Wolter and Puchta, 2019; Merker et al., 2020; Schindele and Puchta, 2020; Vu et al., 2020; Huang et al., 2021; Table 2).

Sufficient DRT and the temperature during tissue culture are two important factors affecting the efficacy of gene replacement by CRISPR/Cas12a (Begemann et al., 2017; Li et al., 2018d, 2019b; Malzahn et al., 2019; Huang et al., 2021). LbCas12a nucleases, delivered with a guide RNA and donor repair template (DRT), successfully generated precise gene insertions as well as indel mutations at the target site in the rice genome, and the targeting frequency was up to 8% (Begemann et al., 2017). Besides, co-delivery of all-in-one CRISPR/Cas12a vector with free DRT, enabled us to precisely replace the *ALS* gene with desired mutant version at relatively lower efficiency (Li et al., 2018d). Moreover, a transcript-templated HDR (TT-HDR) strategy has been demonstrated in yeast and human cells (Derr et al., 1991; Storici et al., 2007; Nowacki et al., 2008; Keskin et al., 2014). A chimeric single-guide RNA (sgRNA) might serve as both a guide RNA and a DRT (Butt et al., 2017). By coupling Cas12a to an array of crRNAs flanked with ribozymes, and a DRT flanked with ribozymes sequences, stable transgene-free lines with two desired mutations in *ALS* gene was successfully obtained in rice through TT-HDR, we successfully achieved targeted gene replacement in rice without free DRT, albeit at a relatively lower efficacy (Li et al., 2019b). In addition, Cas12a is sensitive to temperature treatment. High-temperature treatment during culture process can improve Cas12a-mediated genome editing in rice, maize, and Arabidopsis, respectively (Malzahn et al., 2019). In Arabidopsis, LbCas12a successfully mediated precise modification of *ALS* gene by HDR with a relatively higher efficiency, which greatly broadens the range of in planta HDR (Wolter and Puchta, 2019). Recently, an engineered temperature-insensitive LbCas12a (ttLbCas12a) with a D156R mutation was shown to be more efficient than the WT LbCas12a both for targeted mutagenesis and HDR in Arabidopsis, tobacco, and tomato (Hongkwang cultivar, a local variety), respectively (Merker et al., 2020; Schindele and Puchta, 2020; Huang et al., 2021; Vu et al., 2021; Table 2), demonstrating ttLbCas12a is an ideal tool in crop precision genome editing.

## Manipulating DNA repair pathway and taking advantages of cell cycle to improve HDR efficiency

Two main repair pathways have been evolved in cells for DSB repair: NHEJ and HDR. NHEJ is the more predominant DSB repair mechanism, and it competes with the HDR pathway in cells (Symington and Gautier, 2011; Puchta and Fauser, 2014). It is therefore reasonable to enhance HDR by inhibiting main components involved in NHEJ pathway or overexpressing HDR-related proteins (Li and Xia, 2020; Figure 2A). For example, HDR efficiency was significantly increased by knocking out *lig4* and *PolQ* and repressing *Ku70* and *Ku80* for inhibiting NHEJ pathway, respectively (Endo et al., 2016; Li et al., 2018a; Mara et al., 2019). Activation

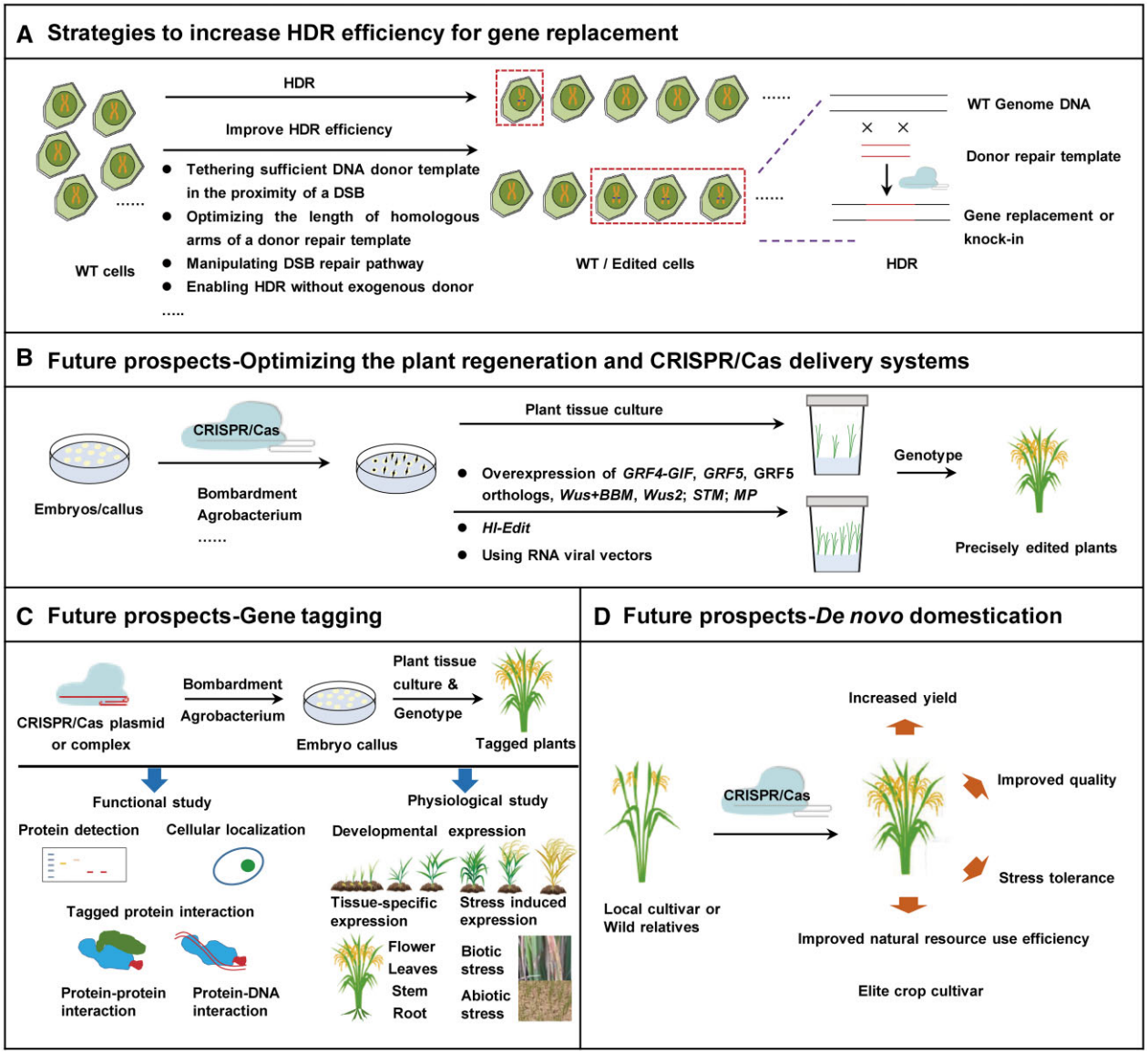



**Figure 2** Diverse strategies to improve HDR efficiency in plants as well as the future prospects. A, Diverse strategies to increase HDR efficiency. The efficiency of genome editing could be further increased by tethering sufficient DNA donor template in the proximity of a DSB, optimize the length of homologous arms of a donor repair template, manipulating DSB repair pathway, enabling HDR without exogenous donor, and so on. B, Future prospects-optimizing the plant regeneration and CRISPR/Cas delivery systems. Agrobacterium-mediated transformation and particle bombardment are two commonly used methods to deliver gene-editing reagents into the plant cells. However, genome editing in plants is generally limited by low regeneration efficiency and few transformable genotypes. It has been demonstrated that overexpression of GRF4-GIF1, GRF5, GRF5 orthologs, Wus, BBM, STM, and MP could increase the transformation and regeneration efficiency in plants. Also, a HI editing technology, HI-Edit, was developed to overcome the genotype dependence in many crop plants, such as wheat, maize, and so on. Recently, an efficient genotype-independent strategy for genome editing in tobacco and wheat has been established by using RNA viral. Following the optimized regeneration system and development of diverse CRISPR reagent delivery systems for genome editing in plants, extension and validation of these strategies in precision genome editing through HDR will certainly expedite its utility in crop improvement. C, Future prospects-gene tagging and applications. The development of CRISPR/Cas-based HDR profoundly pave a new way for characterizing localization and behavior of interested genes in cells by tagging them genes at their endogenous loci. First, Vectors containing CRISPR/Cas components and donor repaired template are prepared. Then, gene tagging components are introduced into donor materials. After that, tagged plants were obtained through genotyping. At last, tagged plants would be benefit functional analysis, dissecting the gene regulation network, and physiological study. D, Future prospects de novo domestication. Following the development of diverse strategies to increase HDR efficiency, it is possible to re-domesticate wild species or relatives through HDR to create Green Super Crop with improved yield, quality, stress tolerance, and resource use efficacy for sustainable agricultural development.

and interference of CRISPR (CRISPRa/i) by simultaneously activating *CDK1* and inhibiting *Ku80* significantly enhanced HDR occurrence (Ye et al., 2018). Furthermore, overexpression of RAD54, a protein involved in the HDR, led to an ~10-fold increase of HDR efficiency in Arabidopsis (Shaked et al., 2005; Even-Faitelson et al., 2011). It was also

demonstrated that overexpression of rice proteins RecQl4 (BLM counterpart) and/or Exo1 (Exo1 homolog) can enhance DSB processing, and thus could be applied in developing an efficient HDR system in rice (Kwon et al., 2012). In tobacco (*Nicotiana plumbaginifolia*), overexpression of a bacterial recombinant protein RecA in plants stimulates homologous recombination (Reiss et al., 2000). It has been demonstrated that overexpression of RAD51 can significantly increase CRISPR/Cas9-mediated homozygous knock-in efficiency in mouse embryos (Wilde et al., 2021). In addition, in human cell lines, SCR7 (the DNA ligase IV inhibitor) can improve efficiency of HDR-mediated genome editing (Chu et al., 2015; Maruyama et al., 2015). NU7441, a DNA-dependent protein kinase inhibitor, can improve the HDR efficiency in comparison to the control (Vu et al., 2021). These above strategies developed in various species including mammalian cells provide us some clues for performing HDR in crop plants, which can be evaluated and utilized in crop plants to further improve the HDR efficiency.

It has been documented that HDR is mainly active in the late S and G2 phases of the cell cycle (Hiom, 2010; Puchta and Fauser, 2014; Orthwein et al., 2015). Delivery of the CRISPR/Cas system into the cells in S phase can increase the HDR efficiency (Lin et al., 2014; Yang et al., 2016; Zhang et al., 2017). Fusion of Cas9 to the N-terminal region of human Geminin, Cas9-hGem (1/110), enabling this fusion protein as a substrate for the E3 ubiquitin ligase complex APC/Cdh1, and resulting in a cell-cycle-tailored expression with low levels in G1 but high expression in S/G2/M, increased the rate of HDR by up to 87% compared to WT Cas9 (Gutschner et al., 2016). In Arabidopsis, using the egg cell- and early embryo-specific promoter to drive the expression of Cas9 could improve the frequency of gene knock-ins and gene replacements via HDR (Miki et al., 2018; Wolter et al., 2018). It was therefore proposed that both the higher occurrence of HDR and tissue-specific Cas9 expression in late S and G2 phase of egg cells and early embryos, facilitated target gene replacement with improved HDR efficiency.

## Challenges and future perspectives

### Precise replacement of the target gene with the elite alleles for crop improvement

Precise replacement of the target gene with the elite alleles from landraces or relatives in commercial variety through HDR-mediated precision genome editing will greatly accelerate breeding process to breed for novel crop varieties which is otherwise time-consuming or impossible in conventional breeding practice (Li et al., 2021a). However, because of the intrinsic rare occurrence of HDR and insufficient DRT in the proximity of a DSB (Hiom, 2010; Symington and Gautier, 2011; Puchta and Fauser, 2014; Endo et al., 2016; Sun et al., 2016a; Li et al., 2019b), it still remains challenging to perform HDR in crop plants, especially in polyploid crop species such as wheat, which is a major staple food crop consumed by $>30\%$ of the world population (Petersen et al., 2006). To further improve the HDR efficiency, there are several open questions or challenges need to be addressed in the future. First, tethering sufficient exogenous DNA donor template in the proximity of a DSB will be a promising strategy to improve the occurrence of HDR in crop plants. For example, covalently tethering a single-stranded donor oligonucleotide (ssODN) to the Cas9/guide RNA ribonucleoprotein (RNP) complex via a fused HUH endonuclease 5, thus spatially and temporally co-localizing the CRISPR reagent and donor DNA, achieved improved HDR efficiency in human cells (Aird et al., 2018). In another study, the truncated Cas9 target sequences attached at terminal of the DRT, which could interact with Cas9 RNP and thus shuttle the templates to the nucleus, enhancing HDR efficiency approximately two- to four-fold in primary hematopoietic cells (Nguyen et al., 2020). Moreover, a chimeric protein Cas9-VirD2 which brings DRT in close proximity to the DSBs, facilitated the occurrence of HDR and enabled precise *ALS* and CLEAVAGE DIOXYGENASE 7 (*OsCCD7*) modification as well as in-frame fusions with the HA epitope at HISTONE DEACETYLASE (*HDT*) locus in rice (Ali et al., 2020). Second, the effect of the length of homologous arms on the efficacy of HDR need to be further investigated. For example, 600 bp homology arms in each side leads to high-level genome knock-in, with 97–100% of the donor insertion events being mediated by HDR in 293 T cells (Zhang et al., 2017). And linear DNAs (single and double-stranded DNA [dsDNA]) engage in a high-efficiency HDR mechanism that requires only ~35 bp of homologous arm with the targeted site to introduce edits ranging from 1 to 1,000 bp (Paix et al., 2017). Moreover, a 2.7 kilobase (kb) homozygous gene replacement was achieved in up to 11% of iPSC without selection when the homology arm length was around 2 kb (Byrne et al., 2015). In general, in mammalian cells, the most routine way to do HDR is using long ssODNs as repair template (Hu et al., 2019; Gallagher and Haber, 2021). However, this remains challenging in plants because only a few plant species such as tobacco could recover plantlets from protoplast. Given the presence of cell wall in plant cells, it is relatively difficult to deliver both CRISPR/Cas RNP complex and ssODNs into plant cells. Besides, massive plantlets could be regenerated if there is no selection during tissue culture and regeneration processes. So far, only a few examples have been documented in crop plants such as in maize (Svitashev et al., 2016). In plants, different lengths of DRT have been used to achieve gene replacement or knock-in at a range of varied efficiencies (Table 2). It remains to be investigated the effect of the length of homologous arms and the target fragment to be replaced on HDR efficiency systemically. Third, both suppression of the genes involved in the NHEJ pathway or overexpression of related proteins such as RAD54 involved in homologous recombination enhanced the occurrence of HDR; it would be helpful to compare which strategy has a better performance. At last, it would be valuable to establish a routine CRISPR/Cas-mediated HDR system by combining diverse strategies to achieve efficient gene/allele replacement or gene tagging in crop plants without free DRT. Therefore,

exploiting diverse strategies to enable the delivery of both CRISPR reagent and DNA repair donor template simultaneously inside the nuclei by an all-in-one vector would be a good choice. Retrons are bacterial phage-defense related operons composed of a specialized RT and a relevant noncoding RNA, which can be partially reverse transcribed by RT to produce a multicopy single-stranded DNA (ssDNA) (Yee et al., 1984; Millman et al., 2020). Recently, fusing retron RT to either the amino terminus or carboxy terminus of Cas9 with a XTEN linker to increase the spatial proximity between ssDNA and Cas9 and produce large amount of ssDNA in vivo for DSB repair, significantly increased the precision genome editing in human cells (Kong et al., 2021; Schubert et al., 2021). Retrons coupled with CRISPR can efficiently insert a GFP gene in yeast (saccharomyces) with efficiency up to 87% (Sharon et al., 2018). In comparison with conventional HDR, retron editing system enables precise gene editing without exogenous donor DNA, increases the abundance of donor msDNA in the vicinity of DSB stimulated by Cas9, thus improving the HDR efficacy, indicating it as an alternative, effective and promising tool in precision genome editing in crop plants. Therefore, in the years to come, it will be interesting to test the retron editing system for either target gene replacement or large fragment insertion in crop improvement.

## Optimizing the plant regeneration and CRISPR/Cas delivery systems

To utilize CRISPR/Cas technology for precision genome editing in plants, efficient delivery of the CRISPR/Cas reagent and DRT into a plant cell is an essential step. So far, *Agrobacterium tumefaciens*-mediated transformation and particle bombardment are two commonly used methods to deliver gene editing reagents into the plant cells (Ghogare et al., 2021; Hayta et al., 2021; Smedley et al., 2021). Precision genome editing in plants is generally limited by low regeneration efficiency and few transformable genotypes. For example, no precisely edited stable wheat lines have been recovered because of its complex hexaploidy genomic background and in fact, most of modern wheat varieties are recalcitrant to genetic transformation and regeneration. To overcome this bottleneck, overexpression of a fusion protein composed of a wheat GROWTH-REGULATING FACTOR 4 (GRF4) and its cofactor GRF-INTERACTING FACTOR 1 (GIF1) has increased the transformation and regeneration efficiency in wheat, triticale (*Triticum secale*) and rice (Debernardi et al., 2020). Meanwhile, introduction of GRF5 and GRF5 orthologs into canola (*Brassica napus*), soybean and sunflower (*Helianthus annuus* L.) significantly increased genetic transformation of the explant tissue (Kong et al., 2020). In addition, regulators that related to somatic embryogenesis have been used to boost plant regeneration. In maize, overexpressing two regulators, WUSCHEL (Wus) and BABY BOOM (BBM), improved regeneration frequencies in various transformation-recalcitrant genotypes (Lowe et al., 2016, 2018). Besides, appropriate ectopic expression of developmental regulators including Wus2 and SHOOT MERISTEMLESS (STM), or MONOPTEROS (MP), could induce the formation of meristem-like structures in vivo in *A. thaliana*, and sidesteps tissue culture-based transformation for plant genome editing (Maher et al., 2020). To overcome the genotype dependence in wheat, a haploid induction (HI) editing technology, HI-Edit, was developed to enable direct editing of targeted gene not only in maize elite inbred lines, but also in an elite wheat germplasm by a single cross (Kelliher et al., 2019). Recently, an efficient genotype-independent strategy for genome editing in tobacco and wheat have been established by using RNA viral vectors, which sidestep the tissue culture process in genome editing, and helped to realize the promise of plant genome editing for the advancement of basic and applied plant research (Ellison et al., 2020; Li et al., 2021b). The development of RNA viral vectors-mediated genome editing methodology will undoubtedly bypass the genotype-dependent bottleneck in precision genome editing. However, delivery of CRISPR reagent either for gene knock-out or simultaneous delivery of both CRISPR reagent and DRT for precision genome editing in wheat by using this RNA viral system has failed in our laboratory, indicating this strategy might be extremely technically challenging, especially in wheat. Nevertheless, following the optimized regeneration system and development of diverse CRISPR reagent delivery systems for genome editing in plants, extension and validation of these strategies in precision genome editing through HDR will certainly expedite its utility in crop improvement (Figure 2B).

## Gene tagging

Gene tagging is a powerful way to analyze the function of proteins in vivo. The development of CRISPR/Cas9-based HDR profoundly transformed our ability to directly tag interested genes at their endogenous loci, which pave a way for characterizing localization and behavior of target gene/protein in cells (Figure 2C). It has been demonstrated that overexpression or transient overexpression of fusion protein of a target protein with either a fluorescent protein or tag might not reflect the actual expression pattern of the endogenous gene (Beck et al., 2011). Similarly, an increased expression of a protein can break the balance between genes, resulting in severe impacts (Gibson et al., 2013). To overcome these drawbacks, inserting fluorescent genes or tags at endogenous genomic loci allows the preservation of a near-native cellular background. However, a slow progress has been made on endogenous genes tagging due to the less efficient HDR. So far, only a few successful cases have been reported, and in-locus tagging of proteins in plants has been challenging (Čermák et al., 2015; Shi et al., 2017; Dong et al., 2020; Lu et al., 2020b). In 2020, a rice protein tagging project (PRPT) was proposed, aiming to label all proteins in rice using a CRISPR/Cas9-based HDR (Lu et al., 2020a).

## *De novo* domestication

Crop improvement by intensive artificial selection of yield components in conventional breeding practice has resulted

in decreased stress resistances and loss of genetic diversity due to the linkage drag of deleterious genes/traits, which threatens the stability of yield and food security under the global climate change and frequent occurrences of natural disasters (Hickey et al., 2019). As a matter of fact, in breeding practice, breeders usually need to select the main yield traits while trade-off other agriculturally important traits to breed for a new variety. In contrast, wild relatives of crops could be rapidly re-domesticated using genome editing as proposed by Eshed and Lippman (2019). Also, precision genome editing can enable manipulation of a specific target gene for de novo domestication to facilitate the breeding process without affecting other genes (Figure 2D). Following the development of next-generation DNA sequencing technologies and omics, an immense amount of genomic data including full genome sequences, pan-genomic data, and transcriptomic and proteomic datasets have been generated for many crop species including the most important stable food species such as wheat and rice (Chen et al., 2011; Ikeda et al., 2013; IWGS, 2018; Ling et al., 2018; Li et al., 2018c; He et al., 2019). By combining the deep-sequenced genome data with the precision genome editing, it is possible to tailor crops or design future crops by precise editing of the key domestication genes alongside the development of the wild progenitors into current varieties for sustainable and climate-friendly agricultural development (Fernie and Yan, 2019). Although the successful domestication cases demonstrated so far are through gene knock-out (Zsögön et al., 2018; Li et al., 2018e; Yu et al., 2021), in the long run, it is possible to domesticate some wild relatives or landraces into modern crop varieties through HDR and thus to enrich the genetic diversity and facilitate crop improvement.

## Concluding remarks

Although impressive progresses have been made in genome editing during the past few years, it remains very challenging to achieve precision genome editing through HDR in crop plants. Manipulating the DNA repair pathways, timing the DSBs induction and donor delivery, and development of diverse HDR strategies will benefit the improvement of HDR efficiency in crop plants. Furthermore, optimization of tissue culture and development of various delivery systems may facilitate increasing the recovery of HDR events at a better efficacy in crop plants. Following technical improvements and optimization, it is reasonable to expect that CRISPR/Cas-mediated HDR will become a routine platform for precise gene/allele replacement, gene tagging and crop domestication, and thus be widely used for fundamental plant biological research and breeding of diverse crop elite varieties for sustainable agriculture to ensure global food and ecological security.

### OUTSTANDING QUESTIONS

- How can we improve the occurrence of HDR by providing sufficient exogenous DRT in the proximity of a DSB?
- Is suppressing the NHEJ pathway or overexpressing HDR-related proteins more effective for enhancing the occurrence of HDR?
- How does length of homologous arms affect the efficacy of HDR?
- How can we establish a routine CRISPR/Cas-mediated HDR system by combining diverse strategies to achieve efficient gene/allele replacement or gene tagging in crop plants without free donor repair template?
- How can we de novo domesticate wild relatives and landraces into cultivated crops by CRISPR/Cas-mediated HDR technologies rather than gene knock-out in the future?

## Acknowledgments

We apologize to those whose work we were unable to cite due to space and reference limitations. We thank Prof. Yunde Zhao, Section of Cell and Developmental Biology, University of California, San Diego, for his critical reading of this manuscript.

## Funding

Part of the work in our lab mentioned in this review is funded by the National Natural Science Foundation of China (32188102), Hainan Yazhou Bay Seed Lab (B21HJ0215), National Key Research and Development Program of China (2020YFE0202300), Innovative Talents Supporting Program of Postdoc (BX20200375), and National Engineering Laboratory of Crop Molecular Breeding.

*Conflict of interest statement.* The authors declare no conflicts of interests.

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