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Editorial

Updates on gene editing and its applications

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Recent advancements in gene editing provide unprecedented tools for plant biology research and offer unlimited potential for improving existing crops and de novo domesticating new crops. This Focus Issue on Gene Editing and Its Applications presents the latest technological innovations in gene editing, addresses challenges in using the technology in both basic and applied plant biology research, and provides perspectives on future developments of the field. This Focus Issue consists of 9 UPDATES written in-depth by experts in a subfield of gene editing, 16 research papers that highlight either a recent technological breakthrough or an application of gene editing in solving a biological question and/or in improving a crop. In this editorial, we first summarize the progresses made in developing new gene editing reagents, delivering reagents into plant cells, analyzing editing events on large scales, and removing transgenes. We then discuss the recent developments in achieving various types of genetic modifications including point mutations, gene targeting (GT), chromosome engineering, and epigenetic modifications. Furthermore, we highlight studies that use CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-mediated gene editing in crop improvements and de novo domestication using the examples provided in this Focus Issue.

Programmable nucleases are at the center of the explosive growth of the genome editing field. CRISPR-associated protein 9 (Cas9) (Jinek et al., 2012) and Cas12a (Zetsche et al., 2015) nucleases and their derivatives have been the most widely used nucleases in gene editing. However, many diverse and unexplored CRISPR–Cas systems exist in bacteria and archaea and they offer a great potential to expand our plant gene editing tool box (Burstein et al., 2017). The new CRISPR–Cas systems may enable us to overcome the limitations of Protospacer Adjacent Motif sequences, target specificities, and the large protein size of Cas9. The new nucleases can also potentially reduce the licensing costs for breeding companies, further facilitating gene editing applications in crop improvement. Yuriko Osakabe and colleagues summarize the recent progress in this field (Wada et al., 2022). The recently expanded CRISPR–Cas editing systems of types I-D and I-E have been applied successfully to both plants and mammals (Osakabe et al., 2020, 2021). New types of mutation patterns can be generated by using the alternative Cas nucleases as demonstrated by the application of type I CRISPR–Cas10. In addition to small indels, type I CRISPR–Cas10 causes bi-directional long-range deletions up to 7.2 kb in tomatoes (Osakabe et al., 2021). Identifying novel nucleases and applying them in genome editing will continue to be an exciting research direction. Such nucleases can lead to many exciting and/or unexpected results in the near future.

Several approaches have been widely used to deliver gene editing reagents, which include preassembled nuclease-guide RNA (gRNA) complexes (ribonucleoprotein [RNP]) or plasmids that enable the production of a nuclease and gRNA(s). Both particle bombardment and *Agrobacterium*-mediated plant transformation have been effective in sending reagents into plant cells. Kumagai and colleagues have directly delivered CRISPR/Cas9 RNP complex into the shoot apical meristem of wheat using particle bombardment (Kumagai et al., 2022). They have generated the desired mutations in the "Green Revolution" genes in wheat. Their method does not require tissue culture, a time- and labor-intensive process. Moreover, their approach makes it feasible to edit genes in plants that are recalcitrant for transformation (Kumagai et al., 2022).

Once the gene editing reagents are in cells and have generated the genetic changes, the next step is to analyze the edited events to identify the desired outcomes. Kejian Wang and colleagues summarize the various methods for detecting both on- and off-target events and discuss the advantages and disadvantages of the methods (Huang et al., 2022).

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Traditional methods such as polymerase chain reaction (PCR)/enzyme digestion and direct Sanger sequencing have been used for detecting edited events. However, these methods are not suitable for large-scale analysis. On the other hand, next-generation sequencing-based methods for detecting gene editing outcomes enable simultaneous analyses of multiple target sites in multiple samples, greatly increasing the scale and efficiency (Huang et al., 2022). Analyzing editing events and selecting nonchimeric plants can also be facilitated by using a visible marker. Huawei Zhang and colleagues place two gRNA units in the same plasmid (Kong et al., 2022). One gRNA is designed to target the *GLABRA2* gene, which is required for trichrome development. The other gRNA targets the gene of interests. Disruption of the *GLABRA2* gene leads to the failure of trichrome development, providing a readily observable developmental marker that can serve as a proxy indicator for successful editing the targeted gene in the same plant. This strategy greatly increases the efficiency in isolating homozygous or bi-allelic T1 plants in Arabidopsis (Kong et al., 2022).

A major consideration in plant gene editing is that the end product should not contain any foreign DNA such as the *Cas9* gene and selection markers (He et al., 2017a, 2017b). If gene editing is achieved by introducing DNA encoding for a Cas nuclease and gRNAs into plant cells, the resultant mutant plants often carry the transgene fragments in their genomes. Transgenes in edited plants can cause undesired genetic changes, and generate regulatory and environmental concerns. Yunde Zhao and colleagues summarize in their UPDATE article the different strategies that have been developed to efficiently edit target genes without leaving any transgenes in plants (He et al., 2022). One solution is to bring in the Cas nuclease in a DNA-free form by using RNAs or RNPs for mutagenesis. DNA-based techniques can also be modified to ensure that the edited plants are transgene-free. For example, fluorescent markers (Gao et al., 2016), pigments (He et al., 2020), and chemical treatments (Lu et al., 2017) have all been employed as tools to distinguish transgene-containing plants from transgene-free edited plants. Moreover, "suicide" genes can be used to automatically eliminate transgenic plants that contain a transgene (He et al., 2017a, 2017b). For asexually reproduced plants, transgenes can be excised from the plant genomes using editing nucleases, site-specific recombination systems or transposition (He et al., 2022). For some species that are recalcitrant to transformation, haploid induction coupled with gene editing may be a feasible alternative (He et al., 2022). For the topic of DNA-free editing, the LETTER from Xiu-Feng Sun and colleagues is also of special interest as it reports on the application of the "suicide" gene approach with multiplex of gRNAs in rice (Liu et al., 2022a). They use the Transgene Killer CRISPR technology (He et al., 2018), which relies on the spatial–temporal expressions of the suicide cassettes consisting of *Cytoplasmic Male Sterility 2* (CMS) and the *BARNASE* genes to kill the cells carrying the transgenes. They have obtained transgene-free rice plants containing double to sextuple mutations, greatly reducing the generation time needed for achieving such a goal.

Desired genetic modification may differ significantly depending on the goals of gene editing. Fortunately, CRISPR systems can achieve virtually all types of genetic modifications such as deletions, insertions, and point mutations. Site-specific double-strand breaks (DSBs) generated by CRISPR systems enable the introduction of targeted mutations in genomes with high efficiency. However, because DSBs are mainly repaired by the nonhomologous end-joining (NHEJ) pathway in plant cells, the resulting mutations can vary and are not easy to predict (Puchta, 2005). This is a disadvantage of NHEJ-based mutagenesis, especially if a precise change is needed in the plant genome.

Homologous recombination (HR) is an alternative approach for introducing precise genetic modifications. Although HR is a minor pathway of DSB repair in somatic plant cells, in the presence of a single- or double-stranded DNA template, it enables the introduction of precise genomic changes from single base pair (bp) to kilo bps by GT. Lanqin Xia and colleagues (Chen et al., 2022) discuss the latest developments and breakthroughs of CRISPR/Cas-mediated gene targeting (GT) in plants. GT experiments have been mainly performed in Arabidopsis, rice, corn, tomato, and tobacco. A row of different strategies have been applied to improve GT efficiencies, such as increasing the copy number of the repair template (Baltes et al., 2014) or releasing the template from the transformed or integrated T-DNA (Fauser et al., 2012; Sun et al., 2016). Furthermore, the improvement of GT efficiencies by manipulating the DNA repair pathway is discussed along with an approach that uses NHEJ combined with single-stand annealing to obtain GT-like changes. The UPDATE article is complemented by two research papers that report on the improvements of GT methodology. Heriberto Cerutti and colleagues use Cas9 RNPs and single-stranded oligodeoxynucleotides for GT in *Chlamydomonas* (Akella et al., 2022). They report successful recoveries of precise edits in a gene of interest. They have improved the methodology by simultaneously targeting a second gene that provides a selectable marker. Using protoporphyrinogen IX oxidase or acetolactate synthase as selectable markers, they are able to detect scar-free GT of the gene of interest. The strategy may be promising to obtain GT events of any gene of interest beyond Chlamydomonas. Sandeep Kumar and his colleagues from Corteva Agriscience report the setup of a marker-free GT system in soybean (Kumar et al., 2022). Using a soil bacterium, *Ochrobactrum haywardense*, instead of the conventional *Agrobacterium*, they have introduced the Cas9 components and the donor template into the soybean embryonic axis. They regenerate edited T0 plants in less than 2 months and have obtained plants carrying precise heritable targeting events (Kumar et al., 2022). This is a significant improvement for achieving GT in the important crop soybean, in relation to frequencies but also about the fact that no selection is required.

Alternative to HR-based precision genetic modification, base- and prime editing can also lead to desired modifications with precision. Unlike HR that depends on the occurrence of DSBs and a repair template, base editors do not require the formation of DSBs and a repair template. In the UPDATE article, Jian-Kang Zhu and colleagues discuss the types of base editors that have been successfully applied in plants (Hua et al., 2022). In general, a base editor is composed of an inactivated nuclease such as de-activated Cas9 (dCas9) fused with an enzyme that can convert a nucleotide into a different one (Kumar et al., 2022). Currently, efficient editing from C to T, and A to G have been achieved in plants (Hua et al., 2022). Jian-Kang Zhu and colleagues also discuss various efforts to improve the efficiency and precision of the base editing and to reduce the target sequence restrictions.

One main concern for Cytosine Base Editors (CBEs), which converts a C to T, is their potential off-target effects. It is not only important to develop CBE for its high efficiency but also to test the specificity of the method. Yiping Qi and colleagues have tested a highly efficient A3A/Y130F-BE3 system for efficient C-to-T base editing in tomato (Randall et al., 2022). They perform whole-genome sequencing of four edited tomato plants. As controls, three transgenic plants carrying a green fluorescent protein gene and two wild-types plants are also sequenced. Interestingly, many single-nucleotide variations (SNVs) and indels are found in both green fluorescent protein and wild-type controls. They show that base editor only has a minor effect on off-target single nucleotide polymorphisms (SNPs), as the difference in SNVs between base-edited plants and controls is not statistically significant. However, the fact that indels are increased in the edited plants by a factor of three, which is statistically significant, indicates that the Cas9(D10A) nickase activity should be more of a concern in terms of off-target effects caused by the cytosine deaminase function of the A3A/Y130F-BE3 system (Randall et al., 2022).

Prime editing is based on a Cas9 nickase fused with a reverse transcriptase unit (Anzalone et al., 2019). The prime editing gRNA (pegRNA) has its 5′-sequence that matches the target region while its 3′-sequence contains the primer binding site sequence and reverse transcription template encoding the desired edit. Prime editing also does not rely on DSB and can precisely generate all possible base changes and small indels, providing a versatile technology for genome editing. Prime editing has been achieved in plants, but the reported efficacy has been relatively low. Hua et al. (2022) discuss various approaches scientists have employed to further improve prime editing in plants, but so far, the success has been very limited. Optimizing nuclease codon, NLS, and growth temperature, which are known factors important for other types of gene editing such as base editing, does not significantly improve prime editing. Altering pegRNA design by eliminating certain secondary structures has not been very helpful for improving prime editing efficiency either (Hua et al., 2022).

CRISPR/Cas system can also be used to generate epigenetic mutations (epi-mutations) using a catalytically inactive Cas protein (dCas) (Hilton et al., 2015). Specifically, the CRISPR/dCas constructs are designed to recruit a genetic or epigenetic effector protein to a target genomic region to manipulate the transcription of nearby genes or to modify histone or DNA. Gardiner et al., 2022 review the technologies used for constructing/engineering different genetic/epigenetic effectors (or effector domains) for targeted transcription manipulation. These constructs result in either transcriptional activation or repression, depending on the activities of the effectors. Gardiner et al., 2022 also point out that the development of CRISPR/dCas systems for targeted transcriptional control and epi-mutagenesis is still in its early stages. Further efficiency and functionality improvements are needed before such methodologies can be widely used in transcriptional or epigenetic manipulations in plants.

Besides generating modifications in target genes, CRISPR/Cas can also be used to restructure and engineer chromosomes. Introduction of duplications, inversions of large regions within a chromosome, or translocations between chromosomes, can lead to the breakage of linkages, providing useful genetic materials for crop breeding. Chromosome restructuring can also potentially cause a reproduction barrier, providing an effective means of protecting intellectual properties. In the UPDATE article, Holger Puchta and colleagues discuss using the NHEJ-based DSB repair pathway to achieve chromosomal engineering (Gehrke et al., 2022). They further provide reasons for why HR-based chromosome engineering is currently not a feasible technology for plants (Gehrke et al., 2022).

One of the goals of genome editing is to solve practical problems for breeding. Crops with gene-edited traits have already been approved for commercial production and marketing in some countries (Menz et al., 2020). Many years before the CRISPR systems were discovered, other genome engineering technologies have been explored. One of the successful approaches is the recombinase-based gene stacking. This approach allows effective insertion of transgenes into a specific, pre-characterized genomic location. David Ow, a pioneer of this method, together with his team, presents the success of using this strategy to stack different combinations of three candidate genes in cotton to combat a destructive fungus disease, verticillium wilt (Li et al., 2022). This "open source" genome engineering system can be an attractive alternative for circumventing intellectual property issues for some private laboratories.

Gene editing technology can be used to protect plants from diseases and to improve other important agronomic traits. Plant viruses are serious threats for crops as infections lead to massive yield losses. Ali and Mahfouz update us about recent approaches of engineering plant immunity to confer resistance against plant viruses (Ali and Mahfouz, 2022). Several approaches have been employed to boost immunity in plants by interfering with the transmission or life-cycles of viruses. Cas9 systems are mainly used to block the

replication of viral DNA, whereas Cas13 systems are used for combating RNA viruses. Another important aspect is the use of CRISPR/Cas systems as diagnostic tools for the detection of plant viruses in the field (Ali and Mahfouz, 2022). Draught is another major stress affecting crop yield. Nam-Chon Paek and colleagues use gene editing to generate loss-of-function mutations of the *WRKY5* gene in rice (Lim et al., 2022). They report that inactivation of *OsWRKY5* by gene editing leads to increased tolerance of draught, reduced water loss, and improved grain yield under drought stress (Lim et al., 2022).

Starch composition affects the quality and value of agricultural products. The starch composition can be changed by editing the starch branching enzyme (SBE). Michael Emes and colleagues utilize the Cas9 nuclease to edit a multigene, SBE family in the oil crop canola (Wang et al., 2022a, 2022b). They target all six SBE genes of *Brassica napus* in two consecutive rounds of transformation. They have obtained Cas9-free homozygous mutant plants that carry two to six mutations of the *SBE* members. Edited plants have altered starch branching frequency, higher starch-bound phosphate content, and different patterns of amylopectin chain length.

CRISPR/Cas-mediated mutagenesis has increasingly been used to reveal or validate functions of candidate genes identified in genetic studies of various plant species. Xiaolan Zhang and colleagues discovered that a basic helix—loop–helix gene *CsHEC2* plays a major role in wart development in cucumber (Wang et al., 2022a, 2022b). Mutation of *CsHEC2* generated using CRISPR/Cas9 result in reduced wart density, which is an important cucumber quality trait related to fruit appearance and market value. Bud dormancy is one of the most important developmental features for many perennial plants. Takahashi and colleagues demonstrate that CRISPR/Cas9-mediated mutations in an ortholog (*GtFT2*) of the Arabidopsis *FLOWERING LOCUS T* (*FT*) gene can cause a lower frequency and a delay of bud break in the herbaceous perennial gentian (*Gentiana triflora*) (Takahashi et al., 2022). This study added another evidence that the *FT* genes play a key role not only in flowering, but also in dormancy in perennial plant species.

One of the most impressive achievements of gene editing in plants is the de novo domestication of new crops from their wild relatives (Zsogon et al., 2018). Previous genetic and genomics studies have identified key genes responsible for the domestication of several crop species. The advancement of gene editing has made it feasible to simultaneously modify multiple genes. Agustin Zsogon and colleagues update us on the recent progress in crop domestication using CRISPR gene editing (Curtin et al., 2022). In this FI, a research article by Choun-Sea Lin and colleagues report a protoplast-based, foreign DNA-free CRISPR–Cas system for editing genes in *Solanum peruvianum*, a very important resource for tomato introgression breeding (Hsu et al., 2022). They generate mutants in *S. peruvianum* for 110 genes that are involved in small-interfering RNAs biogenesis and disease resistance (Hsu et al., 2022). They use both diploid and tetraploid protoplasts derived from in vitro-grown shoots for gene editing. They discover that the ploidy levels of the regenerated plants are not affected by polyethylene glycol (PEG)-Ca^{2+}-mediated transfection, and gene editing (Hsu et al., 2022). The reported transgene-free gene editing and protoplast regeneration technique will enable *S. peruvianum* domestication and pave the way for greatly increasing tomato polyploidization as well.

Gene editing makes the diversification of pathways and stacking multiple traits very efficient. Brassinosteroids (BRs) affect several agronomically important traits, including plant architecture and grain size. Hongning Tong and colleagues target several members of three gene families in the BR signaling pathway by multiplexing CRISPR/Cas9-based gene editing (Liu et al., 2022b). Some of the BR mutant combinations displayed altered developmental patterns, providing materials for BR research and clues for optimizing rice architecture.

Most reported gene editing events are in nuclear genes. However, plants have two other genomes (mitochondrial and chloroplast genomes) that are essential for normal plant growth and development. Editing the genes in the two organelle genomes also has great potentials in plant breeding. Some plant mitochondrial genomes carry *CMS*-associated genes that enable the production of high-yield F1 hybrid crop seeds. In rice, one of such genes is *orf352*. To confirm its role in *CMS*, Omukai et al. used mitochondrion-targeted transcription activator-like effector nucleases (mitoTALENs) to knockout *orf352* in the mitochondrial genome of the cytoplasmic male sterile rice line RT102A. They obtained a series of mutants that harbor HR repair events, generating different kinds of newly formed chimeric genes consisting of *orf352* fragments that lead to the determination that parts of the *orf352* is critical for CMS. Their results show that the *orf352* is critical for CMS, and the amino acids 179 to 210 from *orf352* may contribute to the establishment of pollen abortion (Omukai et al., 2022).

Armed with decades of experience, Pal Maliga and colleagues set the stage for the chloroplast transformation in *B. napus* (LaManna et al., 2022). In Arabidopsis, efficient chloroplast transformation requires plants that are sensitive to spectinomycin due to the absence of a chloroplast acetyl-CoA carboxylase (ACC), which is encoded by the nuclear *ACC2* gene. To obtain chloroplast transformation-competent *B. napus*, all *ACC2* copies of *B. napus* have to be deactivated. Because *B. napus* is an interspecific hybrid of *B. rapa* and *B. oleracea*, two orthologous *ACC2* copies exist in the genome. Using a multiplexing CRISPR system consisting of egg-cell-specific expression of Cas9 and four gRNAs, Pal Maliga and colleagues are able to obtain transgenic *B. napus* seedlings with nonfunctional *ACC2* genes. Because these mutant plants exhibit a spectinomycin hypersensitive phenotype, it is hopeful that they can serve as a useful resource for chloroplast transformation.

Genome editing continues to be one of the fastest-growing fields in biological science. This Focus Issue covers many aspects of gene editing ranging from technological breakthroughs to creative applications in fundamental research and crop improvement. The in-depth UPDATE articles and the accompanying research papers give our readership a clear picture of the current gene editing landscapes and future perspectives.

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