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INSIGHTS IN GENOME EDITING IN PLANTS: 2021

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Exploring C-To-G Base Editing in Rice, Tomato, and Poplar

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As a precise genome editing technology, base editing is broadly used in both basic and applied plant research. Cytosine base editors (CBEs) and adenine base editors (ABEs) represent the two commonly used base editor types that mediate C-to-T and A-to-G base transition changes at the target sites, respectively. To date, no transversion base editors have been described in plants. Here, we assessed three C-to-G base editors (CGBEs) for targeting sequences with SpCas9's canonical NGG protospacer adjacent motifs (PAMs) as well as three PAM-less SpRY-based CGBEs for targeting sequences with relaxed PAM requirements. The analyses in rice and tomato protoplasts showed that these CGBEs could make C-to-G conversions at the target sites, and they preferentially edited the C6 position in the 20-nucleotide target sequence. C-to-T edits, insertions and deletions (indels) were major byproducts induced by these CGBEs in the protoplast systems. Further assessment of these CGBEs in stably transformed rice and poplar plants revealed the preference for editing of non-GC sites, and C-to-T edits are major byproducts. Successful C-to-G editing in stably transgenic rice plants was achieved by rXRCC1-based CGBEs with monoallelic editing efficiencies up to 38% in T0 lines. The UNG-rAPOBEC1 (R33A)-based CGBE resulted in successful C-to-G editing in polar, with monoallelic editing efficiencies up to 6.25% in T0 lines. Overall, this study revealed that different CGBEs have different preference on preferred editing sequence context, which could be influenced by cell cycles, DNA repair pathways, and plant species.

Keywords: C-to-G base editors, PAM-less, SPRY, rice, tomato, poplar

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INTRODUCTION

Since 2016, numerous CRISPR-Cas9-derived base editors have been reported and were first used to edit mammalian genomes, and more recently for editing plant genomes (Molla and Yang, 2019; Zhang et al., 2019; Gurel et al., 2020). Currently, there are two major types of base editors used to edit plant genomes. The first type is cytosine base editors (CBEs) which direct C-to-T transition base changes (Komor et al., 2016; Nishida et al., 2016). Many CBEs based on different cytidine deaminases were reported for use in plants including rABOBEC1 (Li et al., 2017; Lu and Zhu, 2017; Zong et al., 2017), PmCDA1 (Shimatani et al., 2017; Tang et al., 2019; Zhong et al., 2019), hAID (Ren et al., 2018), human APOBEC3A (A3A) (Zong et al., 2018; Cheng et al., 2021), APOBEC3B (A3B) (Jin et al., 2020), and A3A/Y130F (Li et al., 2021a; Ren et al., 2021a; Randall et al., 2021). The second type is adenine base editors (ABEs) which confer A-to-G transition base changes (Gaudelli et al., 2017).

Unlike CBEs, ABEs utilize artificially evolved adenosine deaminases which showed high-efficiency and high-purity A-to-G base conversions in human cells (Gaudelli et al., 2017; Richter et al., 2020) and plants at both canonical NGG PAM sites and relaxed PAM sites (Hua et al., 2018; Li et al., 2018; Yan et al., 2018; Li et al., 2021b; Ren et al., 2021b; Xu et al., 2021; Yan et al., 2021). The development of plant CBEs and ABEs, while largely based on reagents first developed in human cells, has generated relatively high editing efficiency in many plant species and greatly boosted genome editing applications in agriculture (Molla and Yang, 2019; Zhang et al., 2019; Gao, 2021).

CBEs and ABEs can make C-to-T (G-to-A in the reverse complementary strand) and A-to-G (T-to-C in the reverse complementary strand) edits, respectively. They only induce base transition changes and collectively render 4 out of 12 possible base substitutions. It would be highly desirable to develop base editors that can perform transversion base changes (pyrimidine to purine or purine to pyrimidine). Although it is not uncommon to observe C-to-G editing events with CBEs, achieving C-to-G editing at higher efficiency requires dedicated C-to-G base editors. Excitingly, several C-to-G base editors were reported in human cells recently (Chen et al., 2021; Kurt et al., 2021; Zhao et al., 2021). These C-to-G base editors (CGBEs) are composed of a nCas9 nickase, a cytidine deaminase rAPOBEC1 (Chen et al., 2021; Zhao et al., 2021) or its engineered form rAPOBEC1 (R33A) (Kurt et al., 2021) that showed reduced off-target effects at the genome and transcriptome levels in human cells (Grunewald et al., 2019; Doman et al., 2020), and a base excision repair (BER) protein such as a uracil DNA glycosylase sourced from *E. coli* (UNG) (Kurt et al., 2021; Zhao et al., 2021) or rXRCC1 sourced from rat (Chen et al., 2021). The editing efficiency of these CGBEs is highly target-dependent and they all prefer a narrow editing window centered on the cytosine at the sixth position (C6) of the target sequences (Chen et al., 2021; Kurt et al., 2021; Zhao et al., 2021).

Such CGBEs hold great promise for C-to-G base editing in plants, further expanding the genome engineering revolution in agriculture (Molla et al., 2020). Since many of the CBEs and ABEs that showed promising editing performance in human cells were later found to be also highly efficient base editors in plants, we reasoned that development of plant CGBEs based on the human cell-tested or proven CGBEs would represent a straightforward approach to establish a first-generation plant C-to-G base editing tools. Therefore, in this study we set out to closely compare the three top CGBE platforms (Chen et al., 2021; Kurt et al., 2021; Zhao et al., 2021) with optimization for plant delivery and expression. To have a broad implication in tool development, we assessed the CGBEs in three distinct plant species, including rice (an annual monocot), tomato (an annual dicot), and poplar (a perennial dicot tree). By doing so, we hope to gain a better understanding of possible editing outcomes for these CGBEs among different plant species and cell types. As a result, the knowledge gained through this study could further guide future optimization toward achieving highly efficient C-to-G base editing in plants.

MATERIALS AND METHODS

Vector Construction

All the primers used in this study are listed in **Supplementary Table S1**. The pYPQ265 vector (Addgene # 164712) was reported in our recent publication (Ren et al., 2021a). To prepare Gateway compatible attL1-attR5 entry clone pYPQ265K (Addgene #173997), the backbone obtained from pYPQ166-D10A plasmid after restriction digestion with BsrGI-HF (NEB, catalog # R3575*) and NcoI-HF (NEB, catalog # R3193*) and CGBE1-gBk synthetic DNA (IDT gBlock) digested with BsrGI-HF and NcoI-HF were ligated together. Gateway compatible attL1-attR5 entry clone pYPQ265L2 (Addgene #174000) was prepared using NEBuilder® HiFi DNA Assembly kit (NEB, catalog # E5520) with primers 266E-INS_fwd and 266E-INS_rev to amplify zCas9-SpRY from pYPQ166-SpRY (Addgene # 161,520) and primers 266E-BB_fwd and 266E-BB_rev to amplify backbone from pYPQ265K. Gateway compatible attL1-attR5 entry clone pYPQ265N1 (Addgene #173998) was also prepared using NEBuilder® HiFi DNA Assembly kit with primers 265N1-BB_fwd and 265N1-BB_rev to amplify backbone from pYPQ265 and UNG-gBk synthetic DNA (IDT gBlock). Gateway compatible attL1-attR5 entry clone pYPQ265N2 (Addgene #174001) was prepared using NEBuilder® HiFi DNA Assembly kit with primers 266E-INS_fwd and 266E-INS_rev to amplify zCas9-SpRY from pYPQ166-SpRY and primers 266E-BB_fwd and 266E-BB_rev to amplify backbone from pYPQ265N1. Gateway compatible attL1-attR5 entry clone pYPQ265O1 (Addgene #173999) was prepared using NEBuilder® HiFi DNA Assembly kit with primers 265O1-BB_fwd and 265O1-BB_rev to amplify backbone from pYPQ265 and rXRCC1-gBk synthetic DNA (IDT gBlock). Gateway compatible attL1-attR5 entry clone pYPQ265O2 (Addgene #174002) was prepared using NEBuilder® HiFi DNA Assembly kit with primers 266E-INS_fwd and 266E-INS_rev to amplify zCas9-SpRY from pYPQ166-SpRY and primers 266E-BB_fwd and 266E-BB_rev to amplify backbone from pYPQ265O1.

All the T-DNA vectors used in this study are listed in **Supplementary Table S2** and were constructed using Gateway LR assembly reactions based on the protocols described previously (Lowder et al., 2015). To prepare sgRNA entry clones, forward and reverse primers (**Supplementary Table S1**) were phosphorylated with T4 polynucleotide kinase (NEB, catalogue #M0201*), annealed, and ligated with T4 DNA ligase (NEB, catalogue #M0202*) into pYPQ141C (Addgene # 69292) or pYPQ141D (Addgene # 69293) for rice base editing, and into pYPQ141B (Addgene #69291) for poplar and tomato base editing. Individual Gateway LR reactions consisted of an attL5-attL2 sgRNA entry clone, an attL1-attR5 base editor entry clone, and an attR1-attR2 destination vector. For rice base editing, the destination vector was pYPQ203 (Addgene # 86207) containing ZmUBI promotor for base editor expression.

For tomato base editing, the destination vector was pCGS710 containing 2x35S promoter. For poplar base editing, the destination vector was pYPQ202 (Addgene # 86198) containing AtUBQ10 promoter. The names of T-DNA vectors resulted from this LR Gateway assembly start with "pLR" (**Supplementary Table S2**). Both sgRNA and base editor entry clone recombination regions were confirmed by Sanger sequencing. Final T-DNA vectors were confirmed by restriction digestion with EcoRV-HF (NEB, catalog # R3195*) for T-DNAs used in tomato and with EcoR1-HF (NEB, catalog # R3101*) for T-DNAs used in rice and poplar.

Rice Protoplast Transformation and Stable Transformation

The Japonica cultivar Kitaake rice were used. The rice protoplast transformation was done by following our previously published protocols (Tang et al., 2017; Ren et al., 2019; Zhong et al., 2020). The rice stable transformation based on Agrobacterium was done by following a previously published protocol (Zhou et al., 2017; Zhou et al., 2019). Genomic DNA from protoplasts and transgenic seedlings were extracted using the CTAB method (Stewart and Via, 1993).

Tomato Protoplast Transformation

The Micro Tom Tomato cultivar was used. The tomato protoplast transformation was performed according to a recent publication (Randall et al., 2021). Transformed tomato protoplasts were directly mixed with Phire Plant Direct PCR Master Mix (ThermoFisher) for the downstream PCR based analysis. PCR products were pooled together for next-generation sequencing (Genewiz, United States).

Poplar Stable Transformation

Populus alba x tremula clone 717-1B4 was used for stable transformation as described (Leple et al., 1992). Transformed shoots were selected by regenerating on media containing hygromycin. The rooted plants were propagated and used for further genotyping. Two rounds Hi-Tom PCR were preceded to obtain amplicons using Phire Plant Direct PCR Master Mix (ThermoFisher).

Mutagenesis Analysis

For analysis of genome editing in rice and tomato protoplasts, barcoded PCR amplicons were subjected to NGS using an Illumina HiSeqX platform. The resulting data were analyzed by CRISPRMatch (You et al., 2018). For analysis of genome editing in stably transformed T0 lines in rice, PCR amplicons covering each target site were used for Sanger Sequencing followed by decoding. For analysis of genome editing in stably transformed T0 lines in poplar, barcoded PCR amplicons were sequenced by an Illumina HiSeqX platform (Genewiz, United States), followed by analysis using the HiTom tool (Liu et al., 2019) and CRISPRMatch (You et al., 2018).

RESULTS

Development and Comparison of Three CGBEs in Rice Protoplasts

To develop plant CGBEs, we decided to compare the best performing CGBEs from the three recent studies used to edit in human cells (Chen et al., 2021; Kurt et al., 2021; Zhao et al., 2021). Since these CGBEs were all based on rAPOBEC1, the rAPOBEC1-based CBE-BE3 (pYPQ265, BE3) (Ren et al., 2021a) was included as a control (**Figure 1A**). We used a maize codon optimized Cas9 (zCas9) which was previously shown to be very efficient for genome editing in Arabidopsis (Wang et al., 2015), maize (Lee et al., 2019), and wheat (Li et al., 2021c), and recently used for efficient base editing in rice (Ren et al., 2021a; Ren et al., 2021b), tomato (Randall et al., 2021), and poplar (Li et al., 2021a). We applied rice codon optimization for the other components of these CGBEs and generated three Gateway entry clones for them, which are pYPQ265K with UNG-rAPOBEC1 (R33A) fusion to the N-terminus of nCas9, pYPQ265N1 with rAPOBEC1 and UNG fusion to both ends of nCas9, and pYPQ265O1 with rAPOBEC1 and xRCC1 fusion to both ends of nCas9 (**Figure 1A**). These vectors are compatible with our multiplexed CRISPR-Cas9 toolbox which can generate T-DNA expression vectors in a single step three-way Gateway LR reaction (Lowder et al., 2015).

We first assessed these CGBEs in rice. Two target sites (OsALS-sgRNA32 and OsCGRS55-sgRNA) were chosen, with both containing multiple cytosines in the target sequences, allowing for assessment of editing efficiency at individual cytosines (**Figure 1B**). The single guide RNAs (sgRNAs) were expressed under an OsU3 or OsU6 promoter, while the CGBE protein fusions were expressed under a maize ubiquitin promoter (ZmUbi). We compared the three CGBEs with BE3 in rice protoplasts. The editing outcomes were analyzed by next-generation sequencing (NGS) of polymerase chain reaction (PCR) amplicons. The data showed no detectable C-to-G base editing by the canonical BE3 (pYPQ265) (**Figure 1C**), which rather generated high levels of C-to-T base editing at both sites, ~7% at the OsALS-sgRNA32 site and ~13% at the OsCGRS55-sgRNA site (**Supplementary Figure S1A**). The data indicate that BE3 only generates C-to-T base editing, not C-to-G base editing. By contrast, all three CGBEs showed detectable C-to-G base editing, with pYPQ265K outperforming pYPQ265N1 and pYPQ265O1 (**Figure 1C**). pYPQ265K generated ~1.75% C-to-G editing frequency at the OsALS-sgRNA32 site and ~0.70% editing frequency at the OsCGRS55-sgRNA site, while pYPQ265N1 and pYPQ265O1 generated 0.25–0.40% C-to-G editing frequencies (**Figure 1C**). All three CGBEs could edit multiple cytosines in the target sequences, with high C-to-G conversion activity for C6 in the target sequences (**Figures 1D,E**). Interestingly, while pYPQ265K showed relatively high C-to-G editing at both C6 and C9 positions at the OsCGRS55-sgRNA site, pYPQ265N1 and pYPQ265O1 showed a preference for editing the C9 position at this target site (**Figure 1E**).

We also examined other editing outcomes by the three CGBEs at the two target sites. Relatively high levels of C-to-T base editing

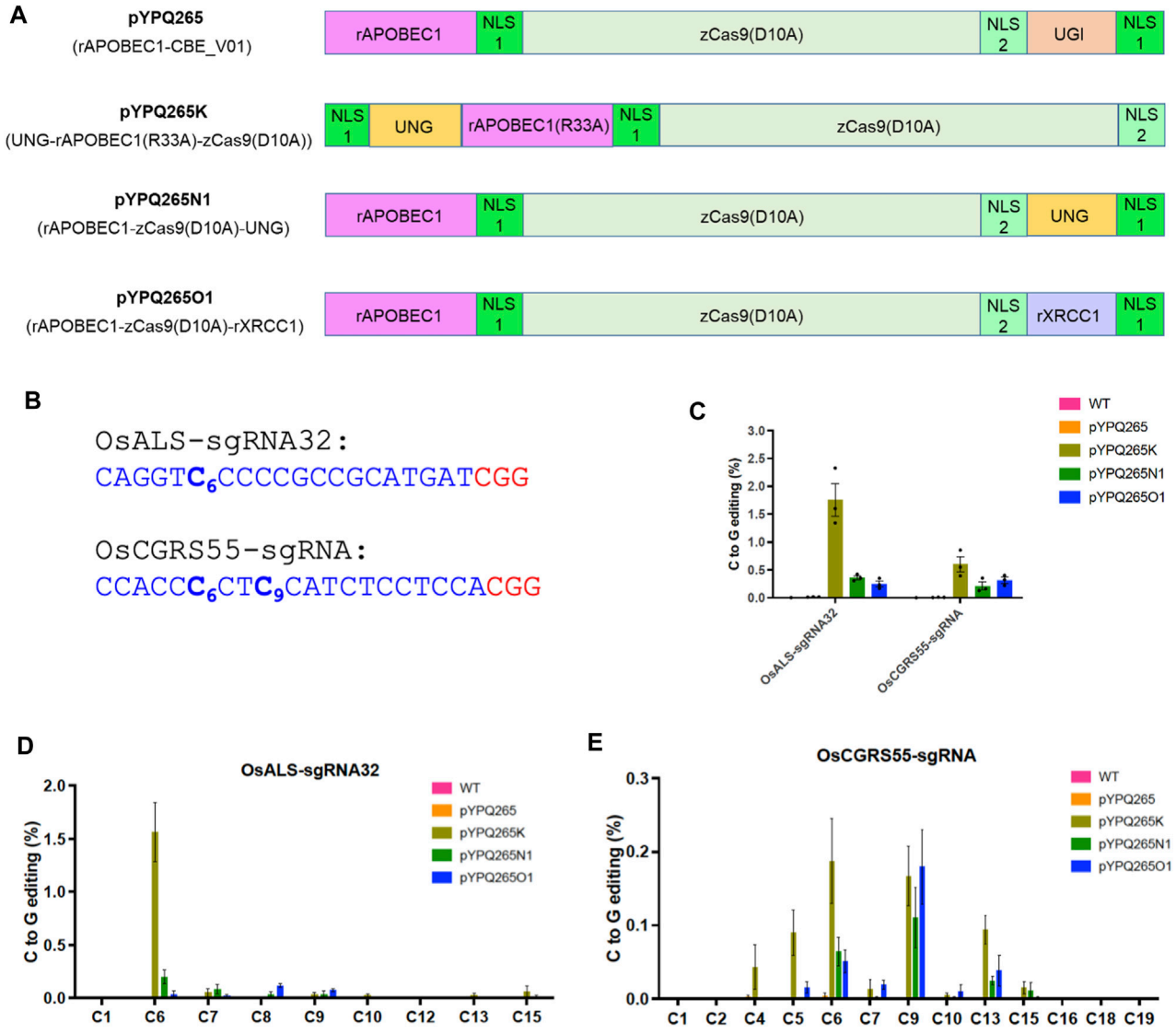

FIGURE 1 | Assessment of BE3 and three CGBEs in rice protoplasts. **(A)** Diagram of BE3 and three CGBEs. Note each nuclear localization single (NLS) is indicated by a green box. NLS 1 is a monopartite SV40 nuclear localization signal and NLS 2 is a bipartite nuclear localization signal of nucleoplasmin. Both NLS1 and NLS2 are recognized by importin α. **(B)** The target sites in the rice genome. The protospacer sequence is highlighted in blue and the PAM is highlighted in red. **(C)** NGS quantification of C-to-G editing by four base editors in rice protoplasts. For the wild type (WT) samples, sterile deionized water was used in protoplast transformation. **(D)** NGS analysis of C-to-G editing windows by different base editors at the OsALS-sgRNA32 site. **(E)** NGS analysis of the C-to-G editing windows by different base editors at the OsCGRS55-sgRNA site. The error bars represent standard errors of three biological replicates.

were observed for pYPQ265O1, ~4% at the OsALS-sgRNA32 site and ~3% at the OsCGRS55-sgRNA site, while pYPQ265N1 showed minimal C-to-T editing at these sites (**Supplementary Figure S1A**). The C-to-T editing window for BE3 is C4-C10 (**Supplementary Figures S1B,C**), consistent with previous reports (Komor et al., 2016). By contrast, rather low C-to-A editing frequencies were detected for all base editors (**Supplementary Figure S2**). Interestingly, high levels of insertions and deletions (indels) were generated by all three CGBEs, but not by BE3, with pYPQ265K showing the highest (~12% at both target sites) (**Supplementary Figure S3**), which could be attributed to the removal of UGI in these editors. Together, these data suggest C-to-T edits and indels are major byproducts of these CGBEs in rice protoplasts.

Comparison of Three CGBEs in Tomato Protoplasts

We next assessed these CGBEs in tomato protoplasts. Four target sites were chosen in the Solanum lycopersicum AGO7 (SAG O 7) gene (Husbands et al., 2009) (**Figure 2A**). We expressed the sgRNAs under the AtU3 promoter and CGBE protein fusions under the 2 × 35S promoter. These three CGBEs were also

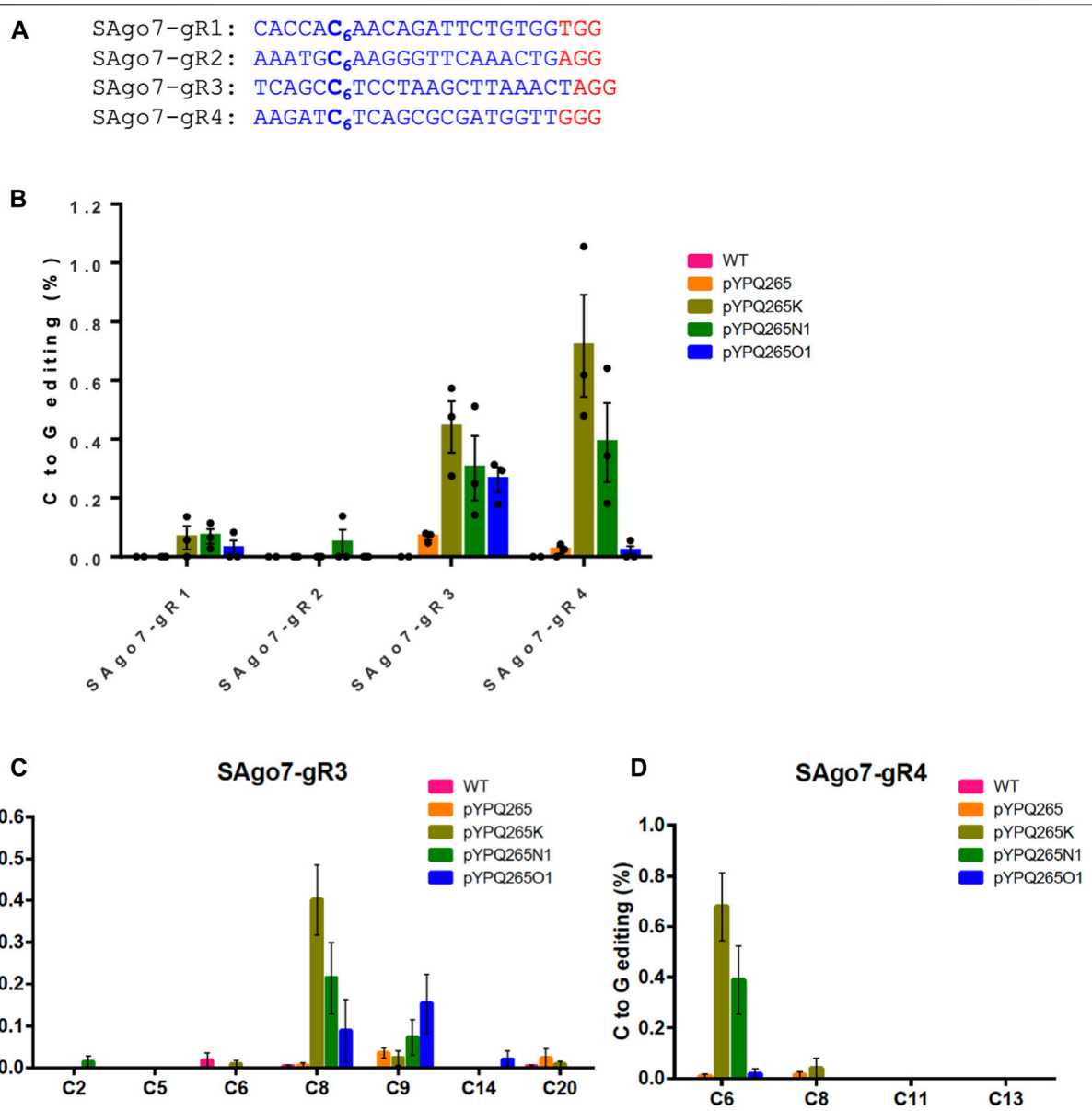

FIGURE 2 | Assessment of BE3 and three CGBEs in tomato protoplasts **(A)** Four target sites in the tomato genome. The PAM sequences are underlined and highlighted in red. **(B)** NGS quantification of C-to-G editing by four base editors in tomato protoplasts. For the WT samples, water was used in protoplast transformation. **(C, D)** NGS analysis of editing windows by different base editors at SAgo7-gR3 and SAgo7-gR4 target sites. The error bars represent standard errors of three biological replicates.

compared with BE3 in the tomato protoplasts. The editing outcomes were analyzed by NGS of PCR amplicons. While the CGBEs mostly failed at editing the SAgo7-gR1 and SAgo7-gR2 sites, they showed 0.3–0.7% C-to-G base editing frequencies at the SAgo7-gR3 and SAgo7-gR4 sites with pYPQ265K showing the overall higher C-to-G editing frequencies (**Figure 2B**). As expected, the BE3 pYPQ265 failed to covert C-to-G changes at all four target sites (**Figure 2B**). Analysis of editing windows showed different editing preference at the various sites. C8 was preferred by pYPQ265K and pYPQ265N1, and C9 was preferred by pYPQ265O1 at the SAgo7-gR3 site (**Figure 2C**), while C6 was preferred by pYPQ265K and pYPQ265N1 at the SAgo7-gR4 site (**Figure 2D**). These data suggest sequence context-dependent C-to-G editing by these CGBEs in tomato. Analysis of other editing outcomes showed that C-to-T editing and indels are major byproducts, ranging from ~2 to ~8% (**Supplementary Figures S4, S5**), while C-to-A editing was no more than 0.3% at all target sites (**Supplementary Figure S6**). These tomato protoplast data were generally consistent with the rice protoplast data.

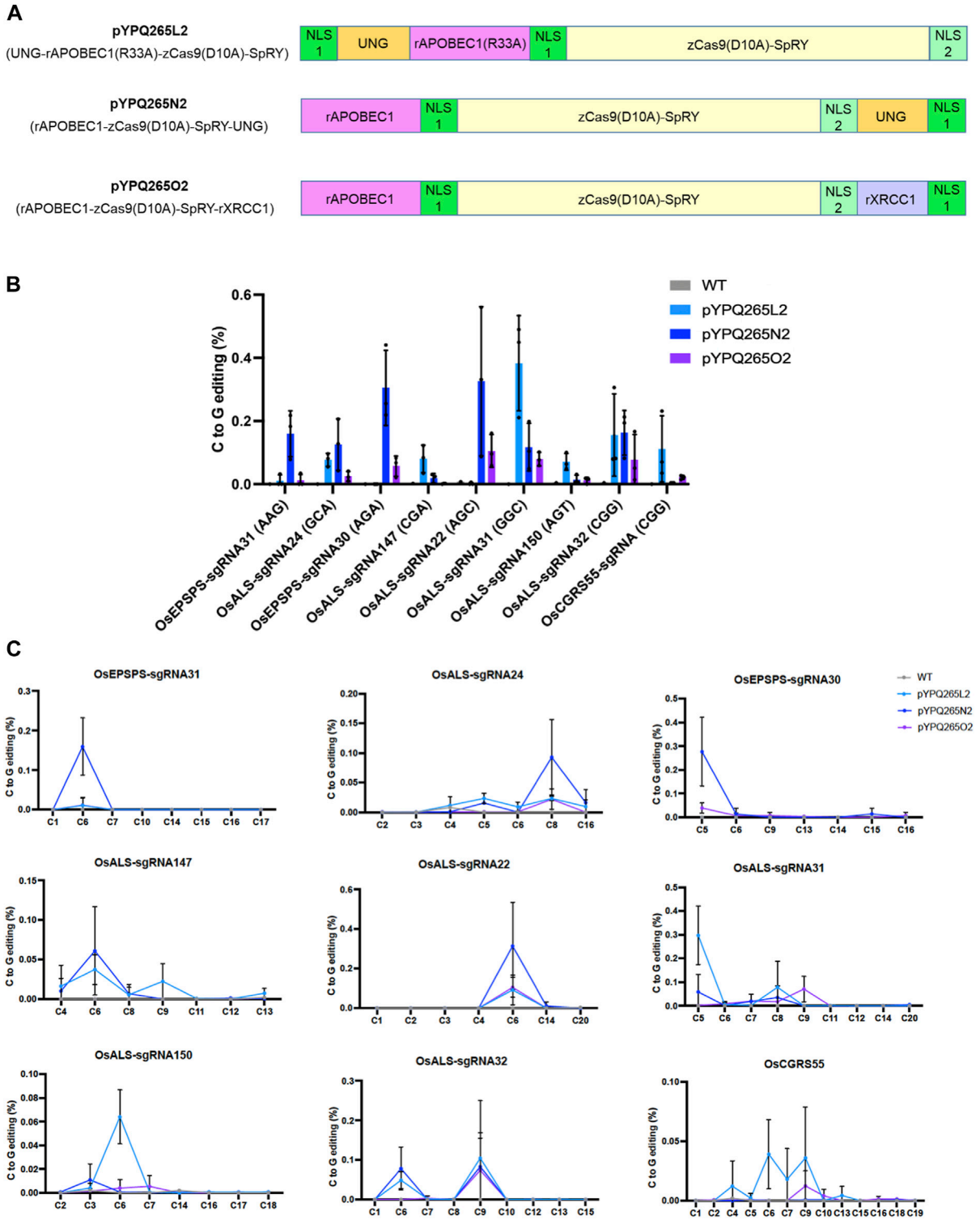

FIGURE 3 | Assessment of three SpRY-based CGBEs in rice protoplasts. **(A)** Diagram of three SpRY-based CGBEs. Note each NLS is indicated by a green box. NLS 1 is a monopartite SV40 nuclear localization signal and NLS 2 is a bipartite nuclear localization signal of nucleoplasmin. Both NLS1 and NLS2 are recognized by importin α. **(B)** NGS quantification of C-to-G editing at nine target sites in the rice genomes. **(C)** NGS analysis of editing windows by different SpRY-based CGBEs across different target sites. The error bars represent standard errors of three biological replicates.

Development and Assessment of Three SpRY-Based CGBEs in Rice Protoplasts

The C-to-G base editing data from rice and tomato protoplasts suggest that different CGBEs favor different cytosine positions in the target sites. To accommodate flexible editing at the possible favorable cytosines in the target sequences, we generated three corresponding CGBEs based on PAM-less SpRY (Walton et al., 2020; Li et al., 2021b; Ren et al., 2021b; Xu et al., 2021), namely pYPQ265L2, pYPQ265N2, and pYPQ265O2 (**Figure 3A**). We targeted seven relaxed NNN PAM sites as well as two NGG PAM sites that we targeted earlier with the wild type (WT) nCas9. Since these CGBEs prefer C6 in the 20-nucleotide targets in human cells (Chen et al., 2021; Kurt et al., 2021; Zhao et al., 2021), we made sure all these nine target sites contained a cytosine at the sixth position. Among the nine target sites, C-to-G editing was detectable (at ~0.1% or higher) at six sites (OsALS-sgRNA24, OsALS-sgRNA147, OsALS-sgRNA22, OsALS-sgRNA31, OsALS-sgRNA32, and OsCGRS55-sgRNA) by pYPQ265L2, at six sites (OsEPSPS-sgRNA31, OsALS-sgRNA24, OsEPSPS-sgRNA30, OsALS-sgRNA22, OsALS-sgRNA31, and OsALS-sgRNA32) by pYPQ265N2, and at three sites (OsALS-sgRNA22, OsALS-sgRNA31, and OsALS-sgRNA32) by pYPQ265O2 (**Figure 3B**). Analysis of editing windows regardless of the editor showed that the highest editing was observed at C6 at six target sites (OsEPSPS-sgRNA31, OsALS-sgRNA147, OsALS-sgRNA22, OsALS-sgRNA150, OsALS-sgRNA32, and OsCGRS55-sgRNA). Occasionally, C8 (e.g., at the OsALS-sgRNA24 site) was favored or C9 (e.g., at the OsALS-sgRNA32 and OsCGRS55-sgRNA) was co-favored with C6 for C-to-G editing (**Figure 3C**). The three SpRY-based CGBEs showed variable editing frequencies at these preferred editing positions, suggesting their different sequence preference for C-to-G editing.

We also assessed the byproduct editing outcomes by these SpRY-based CGBEs. Interestingly, pYPQ265O2 showed relatively higher levels of C-to-T editing (>1%) at three target sites (OsEPSPS-sgRNA30, OsALS-sgRNA22, and OsALS-sgRNA31), while pYPQ265L2 and pYPQ265N2 displayed low C-to-T editing frequencies (**Supplementary Figure S7A**). These C-to-T editing events appeared to have a larger editing window (C4-C8), even though peak editing frequencies were also often found to be centered around C6 (**Supplementary Figure S7B**). Indel frequencies with ~1–4% were generated by pYPQ265L2 and pYPQ265N2 at three target sites (OsALS-sgRNA22, OsALS-sgRNA31, and OsCGRS55-sgRNA) (**Supplementary Figure S8**). C-to-A base editing frequencies by these SpRY-based CGBEs were very low at all target sites, which were close to the background level of the negative controls (**Supplementary Figure S9**). These data showed that C-to-T editing and indels are also common byproducts of the three SpRY-based CGBEs in rice protoplasts.

Assessments of CGBEs in Stable Rice Lines

After development and assessment of these CGBEs in protoplasts, we sought to test them in stably transformed rice plants. We chose the OsALS-sgRNA32 site because it was targeted by all six CGBEs and the control BE3 in rice protoplasts. The seven T-DNA constructs corresponding to these seven base editors were used for Agrobacterium-mediated transformation of rice. We genotyped 16 to 21 individual T0 lines to reveal editing outcome at this target site for these constructs. High C-to-T base editing (47.6–94.1%) was observed for canonical BE3 (pYPQ265) and three CGBEs recognizing the canonical NGG PAMs (pYPQ265K, pYPQ265N1, and pYPQ265O1) (**Figure 4A**). Only pYPQ265O1 generated one monoallelic C-to-G editing at the C6 position (**Figures 4A,B**). The SpRY-based CGBEs failed to generate any editing events at the OsALS-sgRNA32 site among the 16–21 T0 transgenic lines examined (**Figure 4A**). We decided to test PAM-less C-to-G editing at the OsALS-sgRNA22 site with a relaxed AGC PAM. Our earlier rice protoplast data showed that C-to-G editing was observed for pYPQ265N2 and pYPQ265O2 (**Figure 3B**). Analysis of transformed rice lines showed quite high frequency C-to-T editing, 75.0% for pYPQ265N2 and 47.6% for pYPQ265O2 (**Figure 4A**). Importantly, four T0 lines (pLR3793-3, 4, 16, and 21) carried monoallelic C-to-G editing at the C6 position and four additional T0 lines (pLR3793-10, 11, 14, and 19) carried biallelic editing events each containing one C-to-G editing allele at the C6 position with the other allele being 10bp deletion (**Figure 4C**). Altogether, these data suggest that the rXRCC1-based CGBEs (pYPQ265O1 and pYPQ265O2) could generate pure C-to-G editing at the C6 position of the target sequences in rice stable lines. Since OsALS encodes an essential enzyme, complete knockout of OsALS would be lethal. Hence, it is likely the editing frequencies that we observed at OsALS were underestimated.

Assessment of CGBEs in Stable Poplar Lines

We also wanted to assess the CGBEs in a dicot plant species using stable transformation. We chose a Populus hybrid (Populus tremula × P. alba hybrid clone INRA 717-1B4) in which efficient C-to-T and A-to-G base editing was recently demonstrated (Li et al., 2021a). Two sgRNAs with canonical NGG PAMs were designed, with sgRNA8 targeting *PtPDS1* and *PtPDS2*, and with sgRNA9 targeting *PtPDS1*. In all cases, both *P. alba* and *P. tremula* genomes were targeted due to the presence of identical target sequences (**Figure 5A**). All three CGBE fusion proteins (pYPQ265K, pYPQ265N1, and pYPQ265O1) were expressed under an Arabidopsis Ubiquitin 10 (AtUbi10) promoter and the sgRNAs were expressed under an AtU3 promoter. For each construct, 32 T0 lines were generated and analyzed with the Hi-Tom NGS platform (Liu et al., 2019). Interestingly, among all 192 T0 lines assessed, only four lines contained base edits and they were all derived from the pYPQ265K CGBE with sgRNA8 (**Figure 5B**). Among them, two lines (4023-7 and 4023-22) contained C-to-G editing at sixth and eighth positions, respectively (**Figure 5C**). The two other lines (4023-4 and 4023-25) contained C-to-T editing at the sixth and seventh positions, respectively (**Figure 5C**). Based on the percentages of NGS reads, the 023-22 line was a monoallelic line with C8-to-G8 base change (**Figure 5C**). Interestingly, although sgRNA8 could also target PtPDS2 (**Figure 5A**), no base edits could be found in this gene, suggesting PtPDS1 was more accessible than PtPDS2 for base editing in this poplar hybrid.

A

LR #	gRNA Cloning	Cas9 entry clone	T0 lines	WT (number; percentage)	C to T (number; percentage)	C to G without indels (number; percentage)	C to G with indels (number; percentage)
3741	OsALS-sgRNA32	pYPQ265	20	0; 0%	17; 85.0%	0; 0%	0; 0%
3744	OsALS-sgRNA32	pYPQ265K	18	9; 50.0%	9; 50.0%	0; 0%	0; 0%
3747	OsALS-sgRNA32	pYPQ265N1	17	0; 0%	16; 94.1%	0; 0%	0; 0%
3750	OsALS-sgRNA32	pYPQ265O1	21	9; 42.9%	10; 47.6%	1; 4.8%	0; 0%
3755	OsALS-sgRNA32	pYPQ265L2	16	16; 100%	0; 0%	0; 0%	0; 0%
3775	OsALS-sgRNA32	pYPQ265N2	21	21; 100%	0; 0%	0; 0%	0; 0%
3795	OsALS-sgRNA32	pYPQ265O2	17	17; 100%	0; 0%	0; 0%	0; 0%
3773	OsALS-sgRNA22	pYPQ265N2	20	5; 25.0%	15; 75.0%	0; 0%	0; 0%
3793	OsALS-sgRNA22	pYPQ265O2	21	3; 14.3%	10; 47.6%	4; 19.0%	4; 19.0%

B

```
OsALS-sgRNA32 target:     catcacgggccaggtcccccgccgcatgatcggcaccgacgcc

pLR3750-1 :
                Allele 1: catcacgggccaggtgccccgccgcatgatcggcaccgacgcc  C-to-G
                Allele 2: catcacgggccaggtcccccgccgcatgatcggcaccgacgcc  WT
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C

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OsALS-sgRNA22 target:     ttgaaTGCGcCCCCACTTGGGATCATAGGCAGCACATGCtcct

pLR3793-3:
                Allele 1: ttgaaTGCGcCCCCAgTTGGGATCATAGGCAGCACATGCtcct     C-to-G
                Allele 2: ttgaaTGCGcCCCCACTTGGGATCATAGGCAGCACATGCtcct     WT
pLR3793-4:
                Allele 1: ttgaaTGCGcCCCCAgTTGGGATCATAGGCAGCACATGCtcct     C-to-G
                Allele 2: ttgaaTGCGcCCCCACTTGGGATCATAGGCAGCACATGCtcct     WT
pLR3793-10:
                Allele 1: ttgaaTGCGcCCCCAgTTGGGATCATAGGCAGCACATGCtcct     C-to-G
                Allele 2: ttgaaTGCGcCCCCAC----------AGGCAGCACATGCtcct     -10bp
pLR3793-11:
                Allele 1: ttgaaTGCGcCCCCAgTTGGGATCATAGGCAGCACATGCtcct     C-to-G
                Allele 2: ttgaaTGCGcCCCCAC----------AGGCAGCACATGCtcct     -10bp
pLR3793-14:
                Allele 1: ttgaaTGCGcCCCCAgTTGGGATCATAGGCAGCACATGCtcct     C-to-G
                Allele 2: ttgaaTGCGcCCCCAC----------AGGCAGCACATGCtcct     -10bp
pLR3793-16:
                Allele 1: ttgaaTGCGcCCCCAgTTGGGATCATAGGCAGCACATGCtcct     C-to-G
                Allele 2: ttgaaTGCGcCCCCACTTGGGATCATAGGCAGCACATGCtcct     WT
pLR3793-19:
                Allele 1: ttgaaTGCGcCCCCAgTTGGGATCATAGGCAGCACATGCtcct     C-to-G
                Allele 2: ttgaaTGCGcCCCCAC----------AGGCAGCACATGCtcct     -10bp
pLR3793-21:
                Allele 1: ttgaaTGCGcCCCCAgTTGGGATCATAGGCAGCACATGCtcct     C-to-G
                Allele 2: ttgaaTGCGcCCCCACTTGGGATCATAGGCAGCACATGCtcct     WT
```

FIGURE 4 | C-to-G base editing in stable rice lines **(A)** Summary of editing outcomes in transgenic T0 lines by different base editors. In brackets, number corresponds to the number of T0 lines having defined editing outcomes. **(B)** An example T0 line with a pure (i.e., monoallelic and non-chimeric). C-to-G editing allele **(C)** Example T0 lines with pure (i.e., monoallelic and non-chimeric). C-to-G editing alleles. The target sequences are highlighted in blue. The PAM sequences and the C-to-G changes are highlighted in red.

Furthermore, no indels were found among all the T0 lines analyzed. Taken together, the data suggest the UNG-rAPOBEC1 (R333A)-based CGBE (pYPQ265K) can generated C-to-G editing with undetectable indel byproduct formation in poplar.

DISCUSSION

Despite the great progress in achieving highly efficient C-to-T and A-to-G base transition editing in plants, plant transversion editors have not been previously reported. Here we compared three CGBEs toward targeted C-to-G editing in plants. Our assessment in rice and tomato protoplasts showed that these CGBEs, not the BE3, could induce C-to-G editing at the target sites. pYPQ265K, which is based on UNG-rAPOBEC1 (R33A), appeared to be the best among the three CGBEs for generating the overall higher C-to-G conversion rates. However, C-to-T edits still predominated among the editing outcomes, suggesting room for improvement in achieving high C-to-G base editing purity by minimizing byproduct formation. Consistent with reports in

A

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sgRNA8   ACGCACCCGCAAAGGAGGG PAM (for both P. alba and P. tremula)
PtPDS1   ACGCACCCGCAAAGGAGGG CGG
PtPDS2   ACGCACCCGCAAAGGAGGG TGG

sgRNA9   GACTACACAAAACAAAAGTACT PAM (for both P. alba and P. tremula)
PtPDS1   GACTACACAAAACAAAAGTACT TGG
```

B

Constructs	Reagents	T0 lines	Genotype of T0 lines (number; percentage)			
			Base edited without indels	C-T without indels	C-G without indels	indels
pLR4023	pYPQ265K-*PDS1*-sgRNA8	32	4; 12.5%	2; 6.25%	2; 6.25%	0; 0%
pLR4024	pYPQ265N1-*PDS1*-sgRNA8	32	0; 0%	0; 0%	0; 0%	0; 0%
pLR4025	pYPQ265O1-*PDS1*-sgRNA8	32	0; 0%	0; 0%	0; 0%	0; 0%
pLR4026	pYPQ265K-*PDS1*-sgRNA9	32	0; 0%	0; 0%	0; 0%	0; 0%
pLR4027	pYPQ265N1-*PDS1*-sgRNA9	32	0; 0%	0; 0%	0; 0%	0; 0%
pLR4028	pYPQ265O1-*PDS1*-sgRNA9	32	0; 0%	0; 0%	0; 0%	0; 0%

C

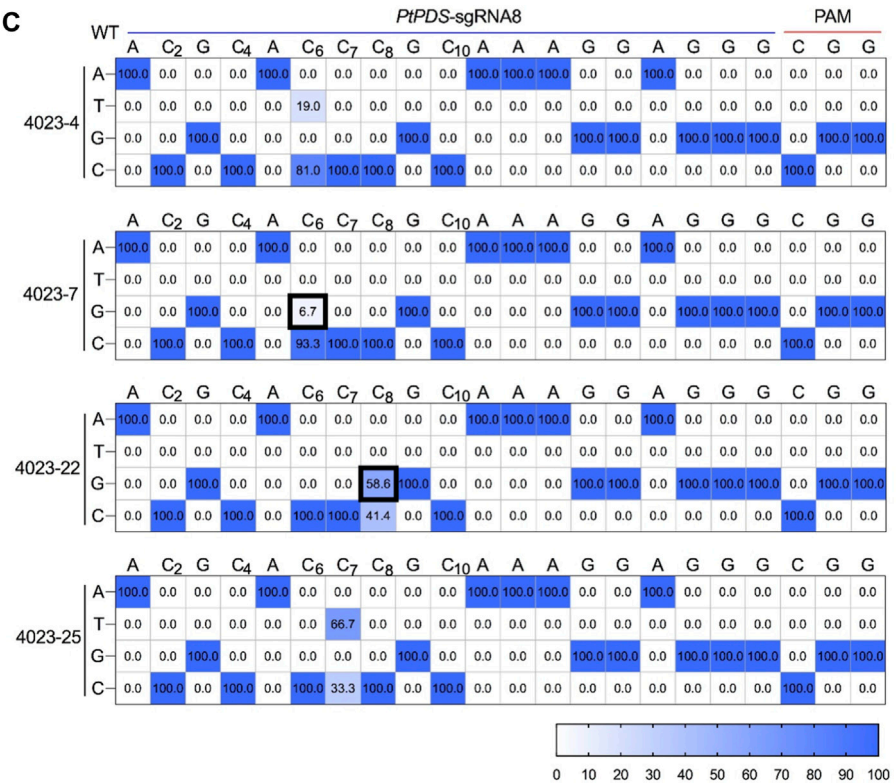

FIGURE 5 | C-to-G base editing in stable poplar lines **(A)** Target sequences in the poplar hybrid. Note both *P. alba* and *P. tremula* genomes are targetable by the sgRNAs due to having identical protospacers. **(B)** Summary of editing outcomes in transgenic T0 lines (i.e., monoallelic and non-chimeric) by different CGBEs in poplar **(C)** Base-edited T0 lines (i.e., monoallelic and non-chimeric). at the PtPDS-sgRNA8 site with editing frequencies quantified by NGS and Hi-Tom analysis.

human cell lines (Chen et al., 2021; Kurt et al., 2021; Zhao et al., 2021), these three CGBEs all greatly improved the ratios of C-to-G editing over C-to-T editing, as the control BE3 barely generated any C-to-G editing events in rice protoplasts (**Figure 1**) and tomato protoplasts (**Figure 2**). Such effects could be partly explained by the removal of UGI and addition of UNG or rXRCC1 (Chen et al., 2021; Kurt et al., 2021; Zhao et al., 2021). Evaluation of editing windows for these three CGBEs in rice and tomato protoplasts showed editing preference for C6 in the 20-nucleotide target sequence, which is a general feature reported for CGBEs (Chen et al., 2021; Kurt et al., 2021; Zhao et al., 2021). rAPOBEC1 used in these CGBEs are known to have poor editing activity at GC context when a targeting C is proceeded by a G (Komor et al., 2016). By contrast, when the target C is flanked by A and/or T, it is highly likely to be edited by CGBEs, according to data in human cells (Kurt et al., 2021). Interestingly, all the C-to-G edited stable lines in rice and poplar seemed to obey this rule, showing editing in the TC, AC, and CC

context (**Figures 4**, **5**). In addition, these CGBEs induced very low levels of C-to-A transversion editing in rice and tomato protoplasts (**Supplementary Figures S2, S6**), consistent with the observations in human cells (Chen et al., 2021; Kurt et al., 2021; Zhao et al., 2021).

While these general rules seem to hold true in human and plant cells, we also discovered major differences for the CGBEs in plants compared to in human cells. First, the overall C-to-G editing frequencies in rice and tomato cells (0.4–1.8%) were nearly one magnitude lower than those reported in human cells (Chen et al., 2021; Kurt et al., 2021; Zhao et al., 2021). Furthermore, these CGBEs still produced much more C-to-T editing events than C-to-G editing events in the protoplasts of rice and tomato, as well as in stable transgenic rice lines. While this could be partly explained by protoplasts cells in our experiments being mostly non-dividing, our data in stably transformed rice and poplar plants also showed overall low C-to-G editing frequencies. In rice, only the rXRCC1-based CGBEs (pYPQ265O1 and pYPQ265O2) generated pure C-to-G editing events (**Figure 4**). In poplar, only the UNG-rAPOBEC1 (R33A)-based CGBE (pYPQ265K) produced pure C-to-G editing events (**Figure 5**). Second, although these CGBEs all generated relatively high levels of indel frequencies in the protoplasts (**Supplementary Figures S3, S5**), indel mutations were undetectable for most CGBE constructs in stably transformed plants (**Figures 4**, **5**). These observations suggest that the performance of CGBEs is highly dependent on the cell cycles and DNA repair pathways in plants.

To expand the targeting ranges, we developed CGBEs based on PAM-less SpRY (Walton et al., 2020; Ren et al., 2021b). These SpRY CGBEs were able to edit PAM-relaxed target sites, albeit with low efficiency in rice protoplasts (**Figure 3**), which could be partly due to vector self-editing, a feature of PAM-less SpRY systems (Ren et al., 2021b). Remarkably, one SpRY CGBE, pYPQ265O2, generated 38.0% C-to-G editing (8 out of 21 lines) at the OsALS-sgRNA22 site in the T0 lines (**Figures 4A,C**). Interestingly, C-to-G editing by the same construct only generated 0.1% frequency in rice protoplasts (**Figure 3B**). Interestingly, the UNG-rAPOBEC1 (R33A)-based pYPQ265K generated equivalent C-to-G editing frequency to C-to-T editing frequency (6.25 vs. 6.25% at one target site in poplar (**Figure 5B**). It is of note that germline transmission of these observed C-to-G editing events need to be further investigated, especially in rice. The discrepancy for C-to-G editing frequencies and outcomes between protoplasts and stable plants further supports that differential DNA repair activities in different cell types and plant species play an important role in the base editing process. Therefore, it would be very important to understand DNA repair, especially the BER pathway, in different plant species, tissue types, and at different cell cycle stages. We envision that harnessing plant-sourced BER pathway genes, in a similar approach to the development of rXRCC1-based CGBE (Chen et al., 2021), may aid the future development of CGBEs with improved C-to-G base editing efficiency in plants.

Here, we closely compared three CGBE platforms, which are top-performing CGBEs in human cells (Chen et al., 2021; Kurt et al., 2021; Zhao et al., 2021), hoping to identify the best performer for C-to-G base editing in plants. However, our data do not indicate there is a clear winner among the tested CGBEs. For example, the UNG-rAPOBEC1 (R33A)-based pYPQ265K appeared to have the highest C-to-G editing frequencies in rice and tomato protoplasts at the canonical NGG PAMs (**Figures 1**, **2**). However, it is the rXRCC1-based pYPQ265O1 and pYPQ265O2 that generated pure C-to-G base editing lines in rice (**Figure 4**). Though, pYPQ265K was successful in producing pure C-to-G base editing lines in poplar (**Figure 5**). When we compared the three CGBE platforms with PAM-less SpRY, the rAPOBEC1-nSpRY-UNG (pYPQ265N2) appeared to be very robust, editing six out of nine target sites in rice protoplasts (**Figure 3B**), suggesting possible differential compatibility of these CGBE systems with the Cas protein. Yet, pYPQ265N2 did not generate stably edited lines in rice. Furthermore, the fact that SpRY-based pYPQ265O2 could generate 38.0% C-to-G editing frequency at one target site in rice suggests there is potentially a strong context dependency for editing outcomes. It might be possible to resolve the mechanism through mining a large editing data set. While we were preparing this manuscript, a recent study reported a similar phenomenon in human cells (Koblan et al., 2021). The authors only observed moderately improved C-to-G editing efficiency after replacing the *E. coli* UNG with a UNG ortholog from *Mycobacterium smegmatis* (UdgX). After establishing an APO-UdgX-Cas9n (AXC) CGBE platform, the authors used CRISPRi to screen a library of 476 DNA repair genes to uncover determinants of base editing outcomes in human cells. The resulting gene candidates were then used for enhancing C-to-G editing as protein fusions. Interestingly, no single CGBE outperformed other CGBEs at all target sites, echoing our findings in plants. The authors ended up using machine learning to develop a program termed CGBE-Hive for predicting the performance of individual CGBEs based on a large amount of editing data generated in human cells (Koblan et al., 2021). Thus, it is envisioned that a similar approach in plants may be needed for understanding the editing preference of CGBEs in plants to advance the use of C-to-G editing and improve reliability to aid basic and applied plant research. With more advances in guide RNA library based CRISPR screens in plants, it could be realized in the future.

CONCLUSION

In this study, we assessed a total of six CGBEs for editing NGG PAM sites as well as PAM-less target sites in plants. Albeit low efficiencies, C-to-G editing was achieved in stable transformed lines of rice and poplar. This work represents a first step toward achieving efficient C-to-G base editing in plants. Future research is warranted for the development of improved CGBEs with high editing activity and purity in plants.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA747640 https://www.ncbi.nlm.nih.gov/, PRJNA747683.

AUTHOR CONTRIBUTIONS

YQ, YZ, and SS designed the experiments. SS generated all the constructs. SL and TF did rice protoplast transformation and data analysis. YC did the tomato protoplast transformation and data analysis. SL, TF, YX, JZ, and XZ conducted rice stable transformation and analysis. GL did poplar stable transformation and analysis. GC provided resources for poplar transformation. YQ and YZ wrote the paper with input from other authors. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Transgene-free Genome Editing in Plants

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Genome editing is widely used across plant species to generate and study the impact of functional mutations in crop improvement. However, transgene integration in plant genomes raises important legislative concerns regarding genetically modified organisms. Several strategies have been developed to remove or prevent the integration of gene editor constructs, which can be divided into three major categories: 1) elimination of transgenic sequences via genetic segregation; 2) transient editor expression from DNA vectors; and 3) DNA-independent editor delivery, including RNA or preassembled Cas9 protein-gRNA ribonucleoproteins (RNPs). Here, we summarize the main strategies employed to date and discuss the advantages and disadvantages of using these different tools. We hope that our work can provide important information concerning the value of alternative genome editing strategies to advance crop breeding.

Keywords: genome editing, CRISPR/Cas9, transgene-free, transgene integration, editor delivery

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INTRODUCTION

Genome editing is a revolutionary technology for the advancement of plant science and crop breeding (Chen et al., 2019). The technique is based on site directed nucleases (SDNs), including meganucleases, Zinc-finger nucleases (ZFN), transcription activator like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system (Gaj et al., 2013; Puchta and Fauser, 2014). Due to its simplicity and easy manipulation, the CRISPR/Cas system is ubiquitously used in the development of genome editing tools (Kantor et al., 2020). The basic CRISPR/Cas system requires two components: a Cas nuclease, such as Cas9, Cpf1 and a guide RNA (gRNA) (Zetsche et al., 2015; Jiang and Doudna, 2017). The gRNA can be programmed to bind to target DNA, and direct the Cas nuclease to perform a double-strand break (DSB) within the target site. DSB repair in plants is majorly achieved through an error prone non-homologous end joining (NHEJ) pathway, which usually leads to some base insertions/deletions (indels) and generates mutations at the target site (Jiang and Doudna, 2017). To date, several base and prime editor tools were developed based on CRISPR/Cas in order to perform more precise editing (Zhu et al., 2020). These editing tools are helping breeders modifying target genes to the desired sequence for improving crop yield and quality, and increase biotic/abiotic stress tolerance and herbicide resistance in crops (Chen et al., 2019). Genome editing is therefore considered designated the next generation breeding strategy.

Legislation and regulation are critical for marketing approval of edited crops (Jones, 2015). Genome editing generates small indels, base-pair changes and specific short sequence changes through HDR (homologous recombination) that are indistinguishable from natural genome variants. Accordingly, in several countries and geographical regions, these types of mutants are not categorized genetically modified organisms (GMO), and are thus exempted from GMO

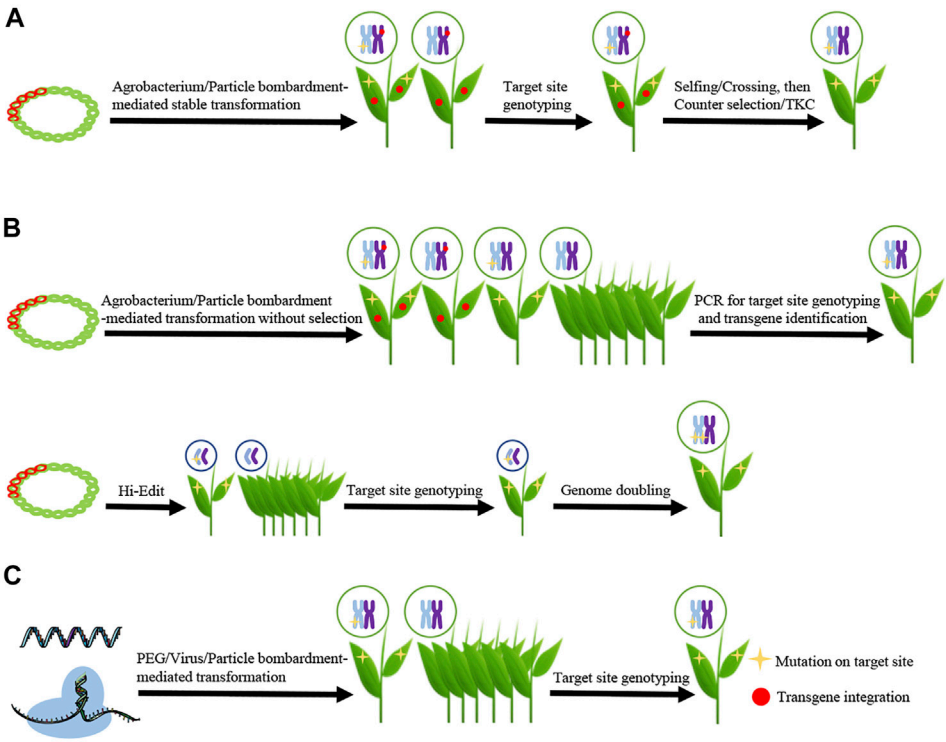

FIGURE 1 | Schematics showing the main strategies for isolating transgene-free and genome-edited plants **(A)** Eliminating transgenic sequence through genetic segregation. CRISPR/Cas DNA (represented by red double helix) is delivered into plant cells using *Agrobacterium tumefaciens* or particle bombardment. The transgenic plants are isolated, and then genome edited plants are selected through target site genotyping. The transgene-free and genome edited plants are isolated from progenies of transgenic genome edited plants which is facilitated by counter-selection or transgene killer CRISPR (TKC) **(B)** Transiently expressing the editor from DNA vectors. CRISPR/Cas DNA could be delivered into plant cells using *Agrobacterium tumefaciens*/particle bombardment (upper schematic) or Hi-Edit (lower schematic). For the strategy using agrobacterium/particle bombardment-mediated transformation, transgene-free and genome edited plants are isolated from all the regenerated seedlings by PCR for target site genotyping and transgene identification. For the strategy using HI-Edit, the haploid progenies are selected and genotyped. The genome edited haploid plants are genome doubled to produce the transgene-free and genome edited doubled haploid line **(C)** Delivering editors in a DNA-independent manner. CRISPR/Cas9 RNA or Ribonucleoproteins (RNPs) are delivered into plant cells by polyethylene glycol (PEG)-, virus- or particle bombardment-mediated transformation, and then transgene-free and genome edited plants are isolated from all the regenerated seedlings by target site genotyping. Mutation on target site is represented by yellow star and transgene integration by red dot.

regulation (Kim and Kim, 2016; Turnbull et al., 2021). Obviously, a major challenge for the application of genome editing in crop breeding is generating transgene-free edited plants.

Conventionally, editor genes are placed in DNA constructs and then delivered to various plant cells using *Agrobacterium tumefaciens* or particle bombardment-mediated transformation (Altpeter et al., 2016). With selection markers, such as antibiotic or herbicide-resistant genes, the first generation (T0) transgenic plants are isolated, and genome edited plants distinguished from transgenic plants through DNA sequencing (Yin K. et al., 2017). In order to obtain transgene-free edited plants, it is necessary for the integrated foreign DNA to segregate out via selfing or crossing with wild-type plants (Gao, 2021). This is a labor intensive and time-consuming process, and thus not suitable for several plant species. Here, we summarize the current strategies used to remove or avoid the integration of foreign transgene DNA in edited plants (**Figure 1**), discuss the advantages and disadvantages of each strategy, and evaluate the forthcoming challenges for the widely application of these strategies in crop improvement.

Eliminating Transgenic Sequences Through Genetic Segregation

Stable transformation-mediated genome editing is suitable for most plants. Despite being a very simple and efficient strategy, isolating second generation (T1) non-transgenic edited plants is labor and time intensive. Hence, a handful of methods were developed to facilitate this process (**Figure 1A**).

Transgene Counter-selection Strategies

To select the transgene-free plants from the progenies of transgenic genome edited plants, some visible selection markers were introduced. In *Arabidopsis*, Gao et al. inserted an mCherry expressing cassette into the CRISPR/Cas9 construct, driven by the seed-specific promoter *At2S3* (Xi et al., 2010; Gao et al., 2016). The transgenic seeds exhibit red fluorescence and can be visually detected in T1. This fluorescence marker-assisted system saves time for plant growth, genomic DNA extraction and genotyping. This strategy is suitable for establishing high-throughput and automated sorting systems.

Lu et al. developed an RNA interference (RNAi)-based strategy, termed CRISPR-S, in order to select transgene-free progenies in rice (Lu et al., 2017). The rice P450 cytochrome protein CYP81A6 confers plant resistance to bentazon, a commonly used herbicide (Pan et al., 2006). The addition of a CYP81A6-hpRNAi expression cassette to a CRISPR/Cas9 construct resulted in bentazon-hypersensitive transgenic plant. After spraying 1,000 mg/L of bentazon to four-leaf-stage seedlings of T1 edited lines, the transgenic plants started dehydrating and/or died. In contrast, while transgene-free seedlings were able to grow normally (Lu et al., 2017). The authors demonstrated all bentazon-resistant plants are transgene-free in their report.

Stuttmann et al. employed three transgene counter-selection markers in tobacco (*Nicotiana. benthamiana*) (Stuttmann et al., 2021). The fluorescence-based marker used the promoter of oleosin-coding genes from tomato to drive the expression of the fluorescence protein. All plants grown from non-fluorescent seeds of genome edited plants were transgene-free. The remaining two transgene counter-selection markers used were pepper's *Bs3* gene (*Ca-Bs3*), which operated under the control of its own promoter (Romer et al., 2007); and a fusion protein comprised by the yeast cytosine deaminase coding gene (*ScFCY*) and the *E. coli* phosphoribosyl transferase-coding gene (*EcUPP*), which was driven by the *Arabidopsis* ubiquitin promoter. The results showed that *Ca-Bs3* caused cell death when induced by the effector *AvrBs3* (Boch et al., 2014). Hence, transgene-free plants could be detected by infiltration with a *Pseudomonas fluorescens* strain expressing *AvrBs3*. FCY converts nontoxic 5-fluorocytosine (5-FC) into the toxic antipyrimidine 5-fluorouracil (5-FU), which blocks thymidine synthetic processes and is incorporated into DNA and RNA (Mullen et al., 1992; Longley et al., 2003). In addition, EcUPP enhances the toxicity of 5-FU (Tiraby et al., 1998). These results demonstrate that the use of this marker allows for an easy screening of transgene-free progenies by adding 5-FC into the medium.

Transgene Killer CRISPR (TKC) System

He et al. developed an TKC system in rice to enable active and automatic self-elimination of the transgene in edited progenies (He et al., 2018; He et al., 2019). The TKC technology works by adding two cassettes expressing the suicide genes *barnase* and *CMS2* into the CRISPR/Cas9 construct. *Barnase* is a bacterial gene encoding for a toxic protein with nuclease activity that is able kill plant cells (Mariani et al., 1990). The expression of *barnase* is driven by the promoter of rice's early embryo specific gene *REG2*, which ensures the gene is solely expressed during early embryonic stages (Sun et al., 1996). In addition, CMS2 specifically disrupts mitochondrial functions during the development of the male gametophyte and causes male sterility (Wang et al., 2006; Hu et al., 2012). The expression of this gene is under the control of the *35S* promoter. This strategy allows Cas9 to edit target genes during transformation, and during callus and vegetative growth stages of T0 plants. When T0 plants undergo reproductive growth, these suicide genes are either expressed (*barnase*) or begin to work (CMS2). The pollen and embryos containing the transgenes are therefore killed, which ensures that all harvested seeds are transgene-free. In total, 203 T1 plants were analyzed by He et al., none of which were transgenic (He et al., 2018).

Transiently Expressing the Editor From DNA Vectors

Although eliminating transgenic sequences through genetic segregation has been successfully performed in the majority of edited plant species, this strategy needs sexual segregation and thus takes an extra generation to be effective. This makes it time consuming and not suitable for plants with long juvenile stages, such as pear, or vegetatively propagated plants, including potato and strawberry.

Transient expression of CRISPR/Cas9 DNA through particle bombardment-mediated transformation.

The ability of particle bombardment to mediate transient transgene expression is well-recognized (Takemoto and Jones, 2014; Ozyigit and Yucebilgili Kurtoglu, 2020). Hence, Zhang et al. developed a transiently expressed CRISPR/Cas9 DNA (TECCDNA)-based genome editing system to avoid transgene integration (Zhang et al., 2016). Specifically, using the TECCDNA system, the authors successfully introduced constructs expressing gRNA and Cas9 into immature wheat embryos using particle bombardment. After this, the seedlings were regenerated without any selection pressure and sequenced (**Figure 1B**). The frequency of mutagenesis, estimated by dividing the number of regenerated mutants by the total number embryos used in the bombardment experiment, was estimated between 2.6 and 5.0%. The frequency of transgene-free genome edited plants was determined by PCR and estimated between 43.8 and 86.8% of the T0 mutants (Zhang et al., 2016).

Transient expression of CRISPR/Cas9 DNA through A. tumefaciens-mediated transformation.

A. tumefaciens is also able to mediate transient transgene expression. Accordingly, Chen et al. established a method similar to TECCDNA in tobacco (Chen et al., 2018). Specifically, tobacco leaf-disc explants co-incubated for 3 days with *Agrobacterium* harboring the Cas9 and sgRNA *PHYTOENE desaturase (PDS)* construct were used for callus induction and seedling regeneration without any selection (Wang et al., 2009). Among the regenerated seedlings obtained from 415 explants, a total of 197 exhibited an albino phenotype with a mutagenesis frequency of 47.5% (calculated as the number of mutants over the total number of explants used for infection) or 2.57% (calculated as the number of mutants over the total number of regenerated seedlings) (Chen et al., 2018). Among all *pds* plants, 17.2% were transgene-free.

Haploid Induction (HI) Editing Technology (Hi-Edit)

Since most crop varieties are recalcitrant to *A. tumefaciens* - and/or particle bombardment-mediated CRISPR/Cas9 delivery, Kelliher et al. established the Hi-Edit method to directly edit elite inbred lines by crossing in maize (Kelliher et al., 2019)

(**Figure 1B**). In the Hi-Edit method, the CRISPR/Cas9 construct was firstly transformed to NP2222, which is a common line used for transformation. The $Cas9^+$ progenies from regenerated plants were crossed with a native haploid-inducer line, RWKS, to select F2 individuals that are homozygote for both the haploid inducing gene and the Cas9 insertion. The pollens from these F2 individuals were used to fertilize the egg cells of the elite inbred lines. Finally, the transgene-free mutant of interest could be identified in the descendant haploid progenies. Genome editing was achieved in five out of six maize elite inbred lines with >3% editing ratio in haploid progenies (Kelliher et al., 2019). These mutants were transgene-free, since they lacked the Cas9-containing DNA from the haploid inducer parent. Hi-Edit can also be applied to dicotyledons, such as *Arabidopsis*.

Delivering Editors in a DNA-INDEPENDENT Manner

Editors can be also delivered in a DNA-independent manner, including *in vitro* transcribed RNA or preassembled Cas9 protein-gRNA ribonucleoproteins (RNPs) (**Figure 1C**). Because no transgene is involved in this process, all edited plants are transgene free.

Transient Expression of CRISPR/Cas9 RNA (TECCRNA)-Based Genome Editing Method

In the TECCDNA system, it is possible that some small degraded vector fragments are integrated into the plant genome and difficult to detect by PCR. In order to avoid this possibility, the TECCDNA method was optimized to the TECCRNA system (Zhang et al., 2016). In this improved method, RNA is used as a vector (instead of DNA) to deliver the Cas9/sgRNA editor. The *in vitro Cas9* and sgRNA transcripts were introduced in immature wheat embryos using particle bombardment, and the seedlings regenerated without any selection pressure. A 1.1% mutagenesis frequency was detected in the TECCRNA system (corresponding to 17 T0 mutants over 1,600 bombarded immature embryos) with *TaGW2* sgRNA (Yang et al., 2012). Among these, 35.3% (6/17) contained a mutation in all six *TaGW2* alleles (Zhang et al., 2016). Since RNA molecules are unlikely to integrate into the plant genome, all of the TECCRNA mutants should be transgene-free.

RNA Virus-Mediated CRISPR/Cas9 Delivery

Engineered virus vectors are used in biomedicine to deliver the CRISPR/Cas9 reagents into human cells (Yin H. et al., 2017). In plants, the *sonchus yellow net rhabdovirus* (SYNV), which is a negative-stranded RNA virus, was used by Ma et al. to deliver the Cas9 and the sgRNA encoding RNA sequence into tobacco leaves (Wang et al., 2015; Ma et al., 2020). The Cas9 and sgRNA sequence were inserted into the SYNV genome and their expression driven by native viral promoters. Two pre-$tRNA_{Gly}$ were applied to the flanking regions of the sgRNA sequence to ensure sgRNA activity (Xie et al., 2015). The engineered SYNV was transformed into agrobacteria and then infiltrated into tobacco leaves. Systemic leaves were analyzed for mutagenesis efficiency (instead of infiltrated leaves), which ranged from 40 to 91% (Ma et al., 2020). The systemic leaves were further used for plant regeneration without selection, with >90% of the regenerated plants harboring mutations on the target locus (57% of which were inheritable) (Ma et al., 2020). Importantly, the progenies of the regenerated mutants were all virus free.

Preassembled CRISPR/Cas9 Ribonucleoproteins (RNPs)-Mediated Genome Editing

Ribonucleoproteins (RNPs) composed of Cas9 protein and *in vitro* transcribed sgRNA have also been delivered into diverse plant cells for transgene-free genome editing (Woo et al., 2015; Svitashev et al., 2016; Liang et al., 2017; Park and Choe, 2019). RNPs were successfully delivered into the protoplasts of tobacco, Arabidopsis, lettuce, and rice, as well as to rice zygotes using polyethylene glycol–calcium (PEG–Ca^{2+})-mediated transfection (Woo et al., 2015; Toda et al., 2019). RNPs were also introduced into embryonic maize and wheat cells by particle bombardment. After RNP induction, the plants were regenerated from these cells without any selection. The mutagenesis efficiency of RNPs varied considerably. For example, up to 46% of the induced lettuce calli from RNP-transfected protoplasts were mutated, and the mutation was transmitted to the progenies (Woo et al., 2015). The proportion of mutants ranged from 14 to 64% of the total regenerated rice plants from RNP-transfected zygotes, and 1.3–4.4% of RNPs delivered by particle bombardment in wheat (Liang et al., 2017; Toda et al., 2019). Because no foreign DNA was introduced during CRISPR/Cas9 RNP mediated genome editing, the mutants obtained were completely transgene-free.

DISCUSSION

CRISPR/Cas9 system-mediated genome editing leads to efficient target modification in plants, including the model plant Arabidopsis and several crop species (Chen et al., 2019; Kong et al., 2021). This technology thus promises to accelerate basic research and crop improvement. Importantly, the elimination of CRISPR/Cas9 integration is highly desirable for gene functional studies and public acceptance of genome edited crops. The several strategies designed to avoid transgene incorporation were summarized in this mini-review (**Figure 1**).

Plant genome editing generally relies on *Agrobacterium*- and/or particle bombardment-mediated delivery of DNA carrying CRISPR/Cas9 reagents (Altpeter et al., 2016). All seedlings should be transgenic if the plant regeneration procedure is achieved under selection, with transgene-free plants being screened out from their progenies. The transgene-counter selection and TKC strategies were developed to facilitate this process (Gao et al., 2016; Lu et al., 2017; He et al., 2018; Stuttmann et al., 2021). Plants can also be regenerated without selection, although the transgene-

free edited plants are often detected with lower efficiency, as a significant number of unmutated plants also regenerate (Zhang et al., 2016; Chen et al., 2018). There is a need to overcome a variety of persisting problems to facilitate the future application of *Agrobacterium*- and particle bombardment-mediated DNA delivery of CRISPR/Cas9. For example, not all crop varieties can be transformed or regenerated after transformation (Anjanappa and Gruissem, 2021); part of the CRISPR/Cas9 construct might integrate into the plant genome, and avoid detection by PCR (Zhang et al., 2016); particle bombardment causes genomic damage (Ozyigit and Yucebilgili Kurtoglu, 2020); most importantly, the identification of transgene-free genome edited plants using *Agrobacterium*- and particle bombardment-mediated DNA delivery is laborious and time consuming, independently of whether selection was applied or not during the regeneration process (Zhang et al., 2016; Chen et al., 2018; He et al., 2018).

To completely avoid DNA integration, RNA and RNPs are used to express CRISPR/Cas9 reagents in plant cells (Zhang et al., 2016; Park and Choe, 2019). These methods also decrease the off-target mutations, which remains a major concern of CRISPR/Cas9 integration (Zhang et al., 2015; Zhang et al., 2016; Zhang et al., 2018), and thus have a good prospect of commercialization. However, the difficulty to deliver RNPs limits their readily implementation by most labs (Woo et al., 2015; Subburaj et al., 2016). In addition, it is necessary to solve problems associated with the use of different types of plant cells as the target of CRISPR/Cas9 expressed from *in vitro* transcribed RNA or RNPs. In cases where embryonic cells are used, the mutagenesis efficiency is relatively low since the vast majority of regenerated plants are unmutated (Liang et al., 2017). The mutagenesis efficiency increases when protoplasts are used (Woo et al., 2015). However, it remains technically challenging to isolate, culture and regenerate plants from protoplasts across several important crops (Lin et al., 2018). The use of RNA viruses to deliver CRISPR/Cas9 expressing RNA into plant cells, likely constitutes the most convenient and efficient strategy to generate transgene-free genome edited plants at present (Ma et al., 2020). However, constraints regarding the host range associated with specific viruses remains an important limiting factor to the implementation of this strategy (Dawson and Hilf, 1992). To date, RNA virus-mediated CRISPR/Cas9 delivery is only applicable in tobacco. We highlight the need for the development of new delivery strategies for CRISPR/Cas9 RNA and RNPs in order to improve delivery efficiency, and build more robust screening systems to distinguish transgene-free mutants from unmutated samples. These advances are urgently needed to promote the application of CRISPR/Cas9 technology in agriculture.

AUTHOR CONTRIBUTIONS

HZ and LL conceived the review. XG and LL wrote the draft and prepared the figure. HZ edited and finalized the manuscript. All authors read and approved the final manuscript.

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Non-GM Genome Editing Approaches in Crops

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CRISPR/Cas-based genome editing technologies have the potential to fast-track large-scale crop breeding programs. However, the rigid cell wall limits the delivery of CRISPR/Cas components into plant cells, decreasing genome editing efficiency. Established methods, such as *Agrobacterium tumefaciens*-mediated or biolistic transformation have been used to integrate genetic cassettes containing CRISPR components into the plant genome. Although efficient, these methods pose several problems, including 1) The transformation process requires laborious and time-consuming tissue culture and regeneration steps; 2) many crop species and elite varieties are recalcitrant to transformation; 3) The segregation of transgenes in vegetatively propagated or highly heterozygous crops, such as pineapple, is either difficult or impossible; and 4) The production of a genetically modified first generation can lead to public controversy and onerous government regulations. The development of transgene-free genome editing technologies can address many problems associated with transgenic-based approaches. Transgene-free genome editing have been achieved through the delivery of preassembled CRISPR/Cas ribonucleoproteins, although its application is limited. The use of viral vectors for delivery of CRISPR/Cas components has recently emerged as a powerful alternative but it requires further exploration. In this review, we discuss the different strategies, principles, applications, and future directions of transgene-free genome editing methods.

Keywords: transgene-free, genome editing, virus induced genome editing, CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 (CRISPR associated protein 9)-mediated genome editing, non-GM approach, crops, RNPs

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1 INTRODUCTION

Plant breeding aims to produce improved crop varieties with enhanced agronomic traits and better nutrition qualities for a growing human population. However, traditional breeding methods are often slow, and the production of new traits is restricted by the species' existing genetic variation pool (Voytas and Gao, 2014; Baltes et al., 2017; Mao et al., 2019; Wang et al., 2019; Nasti and Voytas, 2021).

Genome editing allow plant breeders to manipulate crop genomes at the nucleotide level with high precision. In particular, the advent of prokaryotic-derived Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated protein (Cas) systems and its use in plant genome editing has been a crucial turning point towards a new era of crop breeding. Cas9 and Cas12a, are two popular RNA guided engineered nucleases (RGENs) which mediate genome editing, directed by the sequence-specific pairing of a guide RNA (gRNA) to the target DNA (Jinek et al., 2012). CRISPR/Cas systems have been widely adopted for a variety of applications, including gene disruption by the production of insertion-deletion mutations (indels) (Mao et al., 2013), site-specific sequence

integration (Čermák et al., 2015; Wang et al., 2017), transcriptional control (Lowder et al., 2015; Pan et al., 2021), and base editing (Zong et al., 2017) among others (Zhan et al., 2021). Precise genome editing tools like CRISPR/Cas gives plant breeders unprecedented control over the breeding process at the molecular level. Combined with our current knowledge and the rapid progress in plant genomics, the versatile CRISPR/Cas systems can efficiently introduce genetic variations into the plant genome for crop improvement.

The efficient introduction of genome editing reagents into plants remains one of the grand challenges for this technology (Zhang et al., 2018; Mao et al., 2019; Yang, 2020). Indeed, the minimal CRISPR/Cas complex, a large Cas protein (>140 kDa for Cas9 and Cas12a) and gRNA needs to be delivered across the rigid cell wall, into the nucleus of plant cells. Currently, delivery mostly relies on *Agrobacterium*-mediated or biolistic genetic transformation methods (Baltes et al., 2017). However, the stable integration of transgenes in both methods lead to an array of issues, such as the integration of transgenes at random sites in the plant genome which can disrupt essential genes or result in variable transgene expression (Sun et al., 2016; van Kregten et al., 2016; Liu et al., 2019). In addition, many plant species and elite crop varieties are recalcitrant to genetic transformation and/or plant regeneration (Sun et al., 2016). Removal of transgenes through segregation is also difficult or even impossible in asexually propagated or highly heterozygous crops. Most importantly, many jurisdictions impose heavy regulations on Genetically Modified Organisms (GMOs) which restrict their development, commercialization and use in agriculture (Turnbull et al., 2021). The general public's perception of GMO plants is likewise negative, leading to a shift away from GMO products.

CRISPR/Cas genome editing tools enable precise and traceable modifications that are no different from naturally occurring genetic variations selected during conventional breeding (Voytas and Gao, 2014; Pacher and Puchta, 2017; Zhang et al., 2018). Many countries such as the USA, Japan and Australia exclude some or all kinds of genome-edited crops from GMO regulation if they are free of transgenes or foreign DNA (Pacher and Puchta, 2017; Tsuda et al., 2019; Entine et al., 2021). Even the European Union, which regulates all gene-edited plants as GMOs, released a study that recognized its regulations as "not fit for purpose for some new genomics techniques" (European Commission, 2021). Thus, plant genome editing approaches that avoid transgenesis have recently gained considerable attention. Protocols using Cas9 ribonucleoproteins or transient gene expression with viral vectors have emerged as promising tools for genome editing, whilst avoiding foreign DNA integration. These methods do not involve GM and are collectively named as transgene-free genome editing. Here, we will briefly discuss major advances in transgene-free plant genome editing.

2 RIBONUCLEOPROTEINS

The direct use of CRISPR/Cas ribonucleoproteins (RNPs) is the most obvious approach to achieve transgene-free genome editing. RNPs can be easily assembled by combining purified Cas protein and *in vitro* transcribed or chemically synthesized gRNA before being introduced into cells using chemical or physical delivery methods (**Figure 1**). As such, the use of RNPs circumvents the design and multi-step construction of recombinant vectors, as well as promoter and codon optimisation issues. Most importantly, the CRISPR/Cas RNPs are only transiently present in plant cells prior to degradation by proteases and nucleases (Woo et al., 2015; Zhang et al., 2016; Liang et al., 2017; Banakar et al., 2019). This significantly reduces mosaicism and off-target effects caused by extended exposure of genomic DNA to CRISPR reagents in the conventional DNA delivery systems (Woo et al., 2015; Zhang et al., 2016). The use of RNPs completely avoids transgenesis and should not fall under regulatory oversight (Wolter and Puchta, 2017). Furthermore, RNPs mediate genome editing shortly after cell transfection, as gene transcription and translation is not necessary, enabling the development of approaches for rapid evaluation of multiple CRISPR/Cas systems efficiency in plant tissues (Banakar et al., 2020; Kim et al., 2020).

2.1 Protoplast Transformation

The use of CRISPR/Cas9 RNPs was first reported in 2014, for human cell mutagenesis (Kim et al., 2014), and have since been extensively adopted for plant genome editing in a variety of plant species including *Arabidopsis thaliana*, rice, lettuce, tobacco (Woo et al., 2015; Kim et al., 2017), petunia (Subburaj et al., 2016; Yu et al., 2021), grapevine, apple (Malnoy et al., 2016), maize (Svitashev et al., 2016), wheat (Liang et al., 2017; Liang et al., 2018), soybean (Kim et al., 2017), potato (Andersson et al., 2018; González et al., 2020; Nicolia et al., 2021b), cabbage (Murovec et al., 2018; Park et al., 2019; Lee et al., 2020), banana (Wu et al., 2020), pepper (Kim et al., 2020), witloof (De Bruyn et al., 2020), carrot (Klimek-Chodacka et al., 2021), and tomato (Nicolia et al., 2021a). In most cases, polyethylene glycol-calcium (PEG-Ca^{2+})-mediated cell transfection was the method used to deliver the RNPs into plant protoplasts. However, PEG-mediated transformation may cause cell cytotoxicity leading to limited reproducibility. Surprisingly, Andersson et al. (2018) reported that a large proportion of the regenerated plants contained inserts at the target site, containing either random fragments of potato chromosomal DNA or originating from the DNA template used to synthesize the gRNA. Despite the success of PEG-mediated delivery of RNPs in certain transformation-recalcitrant species, few plant species have been satisfactorily regenerated from protoplasts (Yue et al., 2021). Moreover, genome instability caused by protoplast regeneration is not infrequent (Fossi et al., 2019). Due to a lack of well-established and species-specific protoplast isolation and regeneration techniques, especially for monocotyledonous plants, the adoption of PEG-mediated RNP genome editing has been limited thus far (Yue et al., 2021). Other useful strategies for the delivery of genes or proteins to mammalian cells, such as electroporation and lipofection, have also been tested in plants. Electroporation of *Chlamydomonas Reinhardtii* cells with CRISPR/Cas9 RNPs resulted in approximately 1% editing efficiency (Baek et al., 2016). Electro-transfection of CRISPR/Cas9 RNPs into cabbage protoplasts provided a 1.6% increase in editing efficiency compared to PEG-mediated transfection (Lee

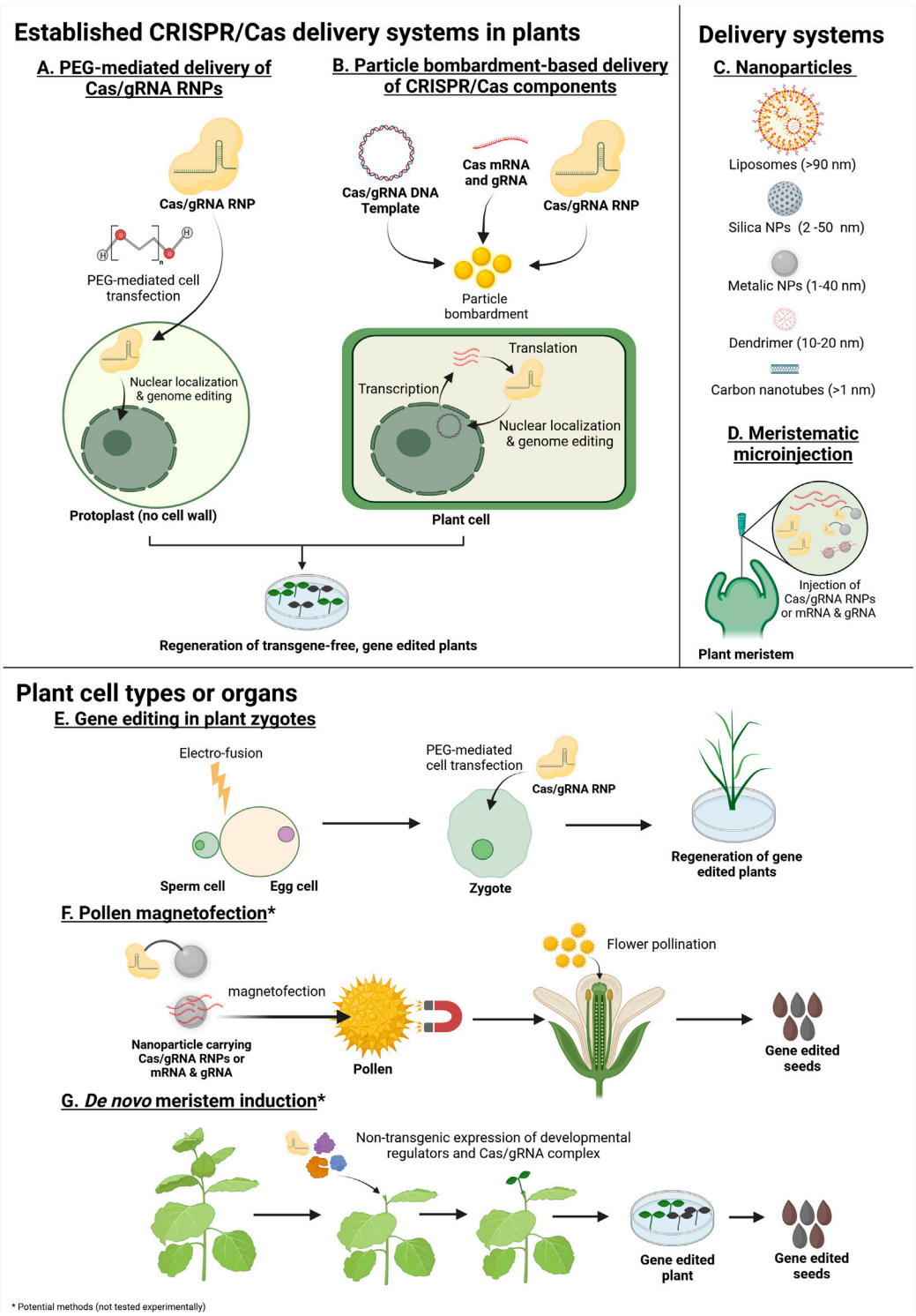

FIGURE 1 | Methods with potential for transgene-free genome editing in plants using *in vitro*/chemically synthesized Cas mRNA and gRNA, DNA templates or Cas/gRNA ribonucleoproteins (RNPs) (Created with BioRender.com). The asterisk (*) indicates methods which have not been experimentally tested. **(A,B)** Established, GM-free systems for the delivery of CRISPR/Cas/gRNA into plants. **(A)** Polyethylene glycol (PEG)-mediated delivery of preassembled Cas/gRNA RNPs into plant protoplasts. PEG mediates the uptake of Cas/gRNA RNPs into protoplast cells. The Cas/gRNA complex enters the nucleus and induces genome editing. The protoplasts are then regenerated to produce transgene-free gene edited plants. **(B)** Particle bombardment-based delivery of preassembled Cas/gRNA RNPs, Cas mRNA plus gRNA or Cas/gRNA DNA expression cassettes. The CRISPR components are loaded onto particles and introduced into plant cells using a gene gun or biolistic device. The Cas/gRNA complexes are localized to the nucleus and induce genome editing. Regeneration from bombarded plant tissue without selection produces gene edited plants

(*Continued*)

FIGURE 1 | whilst avoiding transgenesis (in a large proportion of the regenerated plants). **(C,D)** Potential delivery systems for the application of transgene-free genome editing in plants. **(C)** Common types of nanoparticles currently used in biotechnology. Nanoparticles, such as carbon nanotubes, have been explored as delivery systems for DNA/RNA and protein into mesophyll through stomata pores (around 10 nm). Other nanoparticles, larger than 10 nm, can be introduced into plants by chemical, or physical methods. Therefore, nanoparticles could act as a carrier for the delivery of genome editing reagents into plant cells. Note that nanoparticles are not restricted to spherical forms. **(D)** Plant meristematic microinjection using phytoinjectors. Phytoinjectors could potentially be adopted for the injection of Cas mRNA and gRNA, RNPs or nanoparticle-bound genome editing reagents. **(E–G)** Potential target cell types and organs for transgene-free genome editing. **(E)** Gene editing in plant zygotes. The isolated sperm and egg cells are electro-fused to form zygotes. Early zygotes lack cell walls and the Cas/gRNA RNPs are transfected with PEG. Zygotes are then regenerated to obtain gene edited plants. **(F)** Gene editing using pollen. Nanoparticles carrying Cas mRNA and gRNAs or Cas/gRNA RNPs could be transfected into pollen through magnetofection. The transfected pollen is used to pollinate a flower to produce transgene-free gene edited seeds. **(G)** Non-transgenic delivery/transient expression of developmental regulators and CRISPR/Cas reagents in plant organs may produce gene edited, *de novo* meristems which can be cultured to obtain gene edited seeds.

et al., 2020). Lipofection was demonstrated to transport RNPs into negatively charged tobacco BY2 protoplasts by mixing the CRISPR/Cas9 RNPs with positively charged cationic lipids, resulting in a 6% editing efficiency (Liu et al., 2020).

2.2 Particle Bombardment

Particle bombardment can be used to deliver CRISPR/Cas RNPs into multiple tissues such as immature embryos, leaf discs and calli and is not limited by plant-host range (Altpeter et al., 2005). Major cereal crops, such as rice (Banakar et al., 2020), wheat (Liang et al., 2017; Liang et al., 2019), *Brassica* (Murovec et al., 2018) and maize (Svitashev et al., 2016) have been successfully edited by bombardment with 0.6 μm gold particles coated with CRISPR/Cas RNPs using a helium gene gun. Mutated plants were generated from bombarded embryogenic wheat calli in 6–8 weeks without selection (Liang et al., 2017). In general, the mutagenesis efficiency using particle bombardment of CRISPR/Cas RNPs is modest or low, requiring large-scale mutant screening (Banakar et al., 2019). The addition of a selectable marker plasmid increases the editing efficiency of CRISPR/Cas RNPs, but this approach can result in DNA integration into the plant genome (Svitashev et al., 2016; Banakar et al., 2019). Biolistic bombardment may also result in genome damage, which could lead to phenotypic changes or reduced fitness (Liu et al., 2019).

2.3 Future Directions

2.3.1 Zygotes and Pollen as Delivery Targets

Other plant material, such as zygotes and pollen have the potential to avoid protoplast regeneration. Rice zygotes are created by uniting isolated egg and sperm cells, a process known as gamete fusion. Cell walls are immature during the early stages of gamete fusion, allowing Toda et al. (2019) to perform PEG-mediated transfection of preassembled CRISPR/Cas9 RNPs. After 30–40 days of culture, 14–64% of the generated plants from the zygotes contained CRISPR-induced mutations (Toda et al., 2019). This approach is promising and could be applied to other species with available gamete fusion and regeneration protocols. Pollen manipulation could also circumvent many of the tissue culture and regeneration problems. Pollen grains in many plant species are permeable through apertures of 5–10 μm in diameter and thus are theoretically amenable to the delivery of preassembled RNPs using nanotechnological approaches and are discussed in the next section (Zhao et al., 2017).

2.3.2 Nanoparticles for Cargo Delivery

Nanoparticles (<100 nm) have been successfully used to deliver DNA, RNA and proteins into plant cells (Martin-Ortigosa et al., 2013; Zhao et al., 2017; Demirer et al., 2019; Demirer et al., 2020). Polyethyleneimine (PEI)-coated Fe_3O_4 magnetic nanoparticles were used to carry exogenous DNA plasmids into the pollen grains of several dicot plants, including cotton, pepper, pumpkin and cocozelle (Zhao et al., 2017). The DNA-loaded nanoparticles were combined with pollen in solution and subjected to a magnetic field to enhance the movement of the particles to the bottom of the recipient and into the pollen grains in a process known as magnetofection (Zhao et al., 2017). Artificial pollination using magnetofected pollen produced genetically modified seeds (Zhao et al., 2017). This approach could theoretically be used to introduce preassembled RNPs into pollen instead of DNA but unfortunately attempts to use magnetofection by several research groups have failed, casting some doubts about the efficiency of the method (Vejlupkova et al., 2020).

In mammalian cells, nanoparticle delivery of CRISPR/Cas9 RNPs have been accomplished (Lee et al., 2017), however no nanomaterial-mediated transgene-free CRISPR/Cas genome editing has been reported in plants so far (Ranjan et al., 2017; Sanzari et al., 2019; Demirer et al., 2021). The use of conjugated nanomaterials/RNPs as delivery method and subsequent release methods by enzymatic or light-mediated cleavage is an attractive possibility for future research (Ahmar et al., 2021; Demirer et al., 2021; Nadakuduti and Enciso-Rodríguez, 2021; Wang et al., 2021).

3 VIRUS INDUCED GENOME EDITING

Viral vectors are an efficient tool for gene expression in plants (Scholthof et al., 1996) and have been extensively used for foreign and endogenous gene expression as well as targeted gene silencing (Brisson et al., 1984; French et al., 1986; Chapman et al., 1992; Lu, 2003; Giritch et al., 2006; Gao et al., 2013; Torti et al., 2021). The use of viruses offers multiple advantages including 1) Transient and systemic gene expression without the need for transgenesis (Ellison et al., 2021); 2) high gene expression levels (Pogue and Holzberg, 2012); and 3) the availability of a broad range of viruses that can be engineered for gene expression infecting different plant species (**Supplementary Table S1** and **Supplementary Table S2**). Thus,

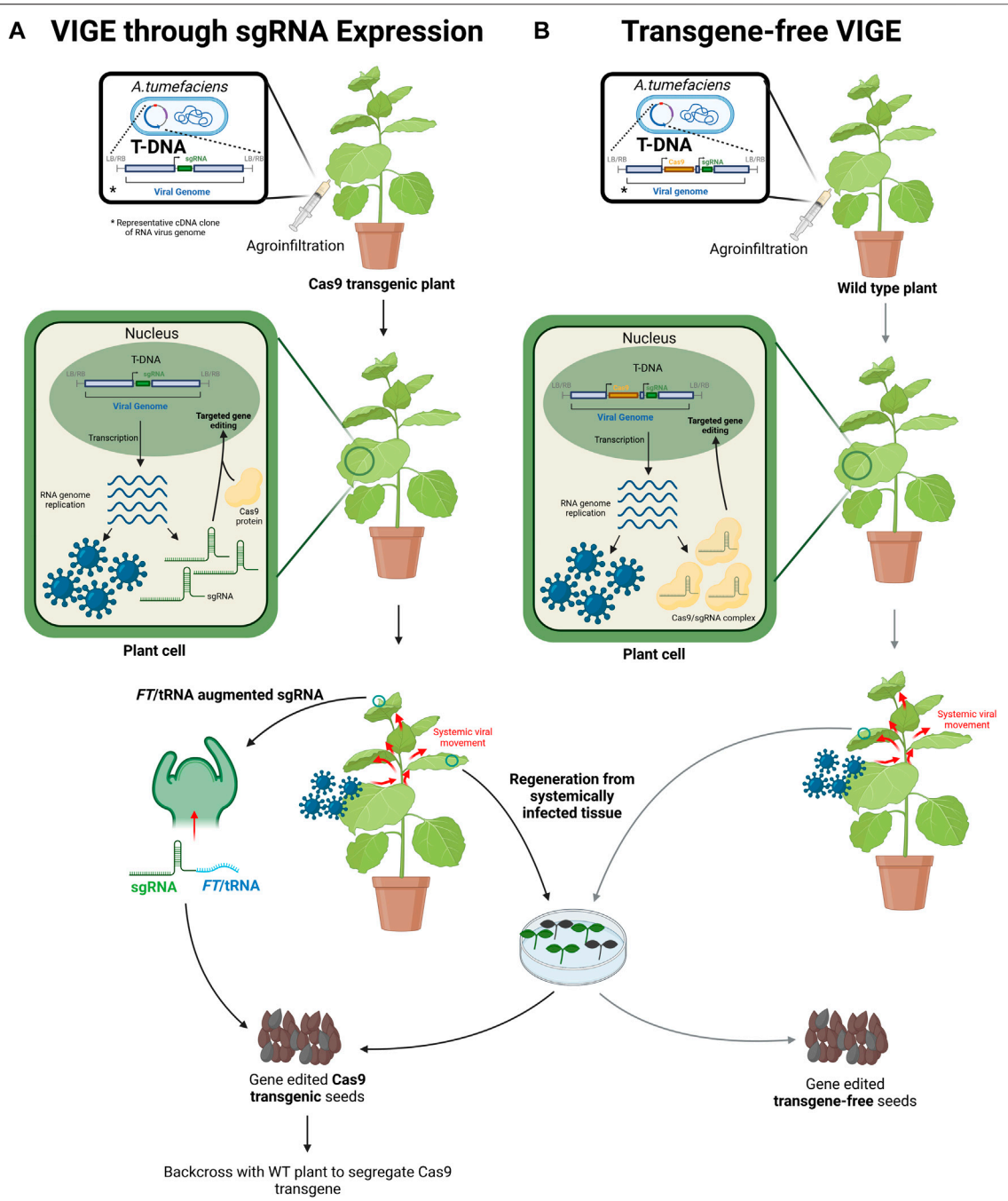

FIGURE 2 | Virus induced genome editing (VIGE) systems in plants (Created with BioRender.com). **(A)** VIGE through gRNA expression. The gRNA is cloned into the complementary DNA (cDNA) of an RNA virus genome in a binary vector. *A. tumefaciens* carrying the binary vector with the recombinant viral cDNA is agroinfiltrated into a leaf on Cas9 transgenic plants. The viral cDNA is expressed to produce viral RNA which self-replicates. gRNAs transcribed from the viral vector are bound by the Cas9 protein expressed from the plant integrated transgene. The Cas9/gRNA complex is localized in the nucleus where it induces targeted gene editing. Viral genomes are encapsidated into recombinant virions which exit the plant cell inducing systemic infection. When gRNAs are fused to *FT* or tRNAs, the augmented gRNA may travel and enter meristematic cells. Gene editing in meristematic cells can produce edited Cas9 transgenic seeds. Alternatively, systemically infected leaf tissue can be used for plant regeneration. Genome edited Cas9 transgenic seeds are obtained from regenerated plants. The gene edited transgenic Cas9 progenies can be backcrossed with wild type (WT) plants to segregate the Cas9 transgene. **(B)** Transgene-free VIGE system. Certain viruses are capable of carrying and expressing cassettes containing Cas9 and gRNAs. The Cas9 gene and gRNA are introduced into the viral genome cDNA in a binary vector and agroinfiltrated into WT plants. The viral cDNA is expressed to produce viral RNA which self-replicates. Cas9 protein and gRNA are transiently expressed from the viral genome to form a complex. The Cas9/gRNA complex is localized to the nucleus for targeted genome editing. Viral genomes are encapsidated into recombinant virions exiting the plant cell and induces systemic infection. Systemically infected leaf tissue can be used for plant regeneration. The regenerated plants produce transgene-free gene-edited seeds.

viruses provide an attractive platform for transgene-free delivery of genome editing reagents, providing a promising solution to the delivery bottleneck. In this section, we explore the recent advances in virus induced genome editing (VIGE) (**Figure 2**). Strategies to insert foreign genes, such as genome editing tools, into viral genomes are well established for many plant viruses (Scholthof et al., 1996; Mortimer et al., 2015) (**Supplementary Table S2**). The gene-of-interest is inserted into viral genomes, sometimes replacing non-essential viral genes (Chapman et al., 1992). Infection with recombinant viral genomes into plant tissues is predominantly achieved through agroinfiltration (Marillonnet et al., 2004; Marillonnet et al., 2005). Alternatively, infection can be accomplished by mechanical inoculation of the viral genome or the use of previously infected tissue (Takamatsu et al., 1987). Once inside a cell, the viral genome undergoes its replication lifecycle, inducing local gene expression in the inoculated area before producing complete virions and colonising the plant (Lico et al., 2008; Mortimer et al., 2015). Concurrently, the inserted gene-of-interest is expressed systemically alongside the viral infection. Transgene-free expression of foreign genes, such as genome editing reagents, can be achieved if genome integration is not involved in the viral lifecycle.

3.1 Genome Editing Using Positive-Strand RNA Viruses

RNA viruses have a strong potential for transgene-free genome editing as they multiply through RNA replication and are not usually reverse transcribed into DNA throughout their lifecycle (Ellison et al., 2021).

3.1.1 Delivery of Zinc Finger Nucleases and Meganucleases

Tobacco rattle virus (TRV) is a bipartite positive-strand RNA virus (PSV) infecting many dicotyledonous plant species (MacFarlane, 2008; Marton et al., 2010; Ali et al., 2015a). TRV has been modified to express a zinc finger nuclease (ZFN), targeting a loss-of-function *GUS* transgene in *Nicotiana benthamiana* and *Petunia hybrida* transgenic lines (Marton et al., 2010). Zinc finger nuclease-mediated editing of *GUS* restored GUS activity producing a visible phenotype upon staining in systemic leaves. In addition, TRV infected tissues from these experiments were used for regeneration. Virus-free and zinc finger nuclease-free seedlings containing edits in the *GUS* gene were identified from T0 seeds of the regenerated plants (Marton et al., 2010). TRV was also used to express a meganuclease targeting *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*) in *Nicotiana alata* (Honig et al., 2015). DFR is involved in the synthesis of anthocyanins, simplifying the visual identification of mutations by the presence of reduced pigmentation in the *N. alata* purple petals. Analysis of the progeny of three infected plants revealed the presence of two plants containing mutations in one of the two DFR genes present in the genome (Honig et al., 2015).

3.1.2 Delivery of CRISPR/Cas gRNAs

In contrast with zinc finger nucleases and meganucleases, the delivery of CRISPR/Cas reagents with PSVs is notably more difficult. The size of the Cas9 coding region (>4 kb), significantly larger than ZFNs (~1, 2 kb), create problems for delivery using PSVs due to their limited cargo size. Large insertions also cause genome instability from selective pressure towards viral particles lacking the insert (Walker et al., 2015; Kujur et al., 2021; Tsanova et al., 2021). Initial studies overcame the size problem by using the PSV system to express small gRNAs into transgenic plant lines constitutively expressing Cas9 as a proof-of-concept. PSVs such as TRV, Beet necrotic yellow vein virus (BNYVV), Potato virus X (PVX) and the legume-infecting, Pea early browning virus (PEBV) have been used to express gRNAs in Cas9-positive *N. benthamiana* lines (Ali et al., 2015a; Ali et al., 2015b; Ali et al., 2018; Jiang et al., 2019; Uranga et al., 2021a). Efficient VIGE was detected in systemically infected leaves with the four PSVs, ranging from ~30 to ~85% editing efficiency. Furthermore, when PVX infected tissues were used for regeneration of plants by tissue culture, edited seedlings were recovered (Uranga et al., 2021a). It is to note that gRNA delivery and highly efficient gene editing in Cas9 transgenic *N. benthamiana* was also achieved with a DNA virus, Cabbage leaf curl virus (Yin et al., 2015).

For monocotyledonous plants, the tripartite PSV, barley stripe mosaic virus (BSMV) was used to express gRNAs targeting endogenous genes in the agriculturally important crops, wheat and maize using a similar approach to the ones mentioned above (Hu et al., 2019). BSMV showed very high editing efficiency in systemic leaves of Cas9 transgenic wheat (~62–78%) and maize (~48%) lines. Expression of gRNAs using the foxtail mosaic virus (FoMV) mediated efficient VIGE in Cas9 transgenic *Setaria viridis* (~60% in systemic leaves) but efficiency significantly dropped in Cas9 transgenic maize (~3–6%) (Mei et al., 2019). In both VIGE systems, wheat, maize and *S. viridis* plants were easily infected by rub-inoculation with *N. benthamiana* leaves from plants previously infected by agroinfiltration (Hu et al., 2019; Mei et al., 2019). The possibility of efficient *trans*-species rub-inoculation is especially important for monocots where the introduction of viral genomes into mature plants is difficult, demonstrating the versatility of viral vectors.

Despite the high editing efficiency demonstrated by the above-mentioned PSV vectors in systemic tissues, an important consideration is whether this approach produces gene edits in the progeny of infected plants. Unfortunately, these studies either 1) failed to obtain gene edits in the progeny (FoMV infected *S. viridis*), 2) obtained an extremely low frequency (2/1,320 seedlings from TRV infected *N. benthamiana*) or 3) heritability was not determined (BNYVV, PEBV and BSMV infected *N. benthamiana*, FoMV infected maize).

3.1.3 Heritable Genome Editing Through gRNA Augmentation

To optimize heritable VIGE in CRISPR/Cas systems, Ellison et al. (2020) fused the *A. thaliana FLOWERING LOCUS T* (*FT*) mRNA to the 3' end of the gRNA in an approach called gRNA augmentation. Substantial evidence suggests that the *FT* mRNA moves systemically in the plant and enters the meristem to induce flowering (Li et al., 2009; Li et al., 2011). The idea behind gRNA augmentation is that the addition of *FT* or

other mobility sequences such as tRNAs to the gRNA will confer systemic mobility and access to meristematic cells to produce heritable editing. When transgenic *N. benthamiana* plants overexpressing Cas9 were infected with TRV vectors containing *FT* augmented gRNAs, up to 65% of the progeny contained CRISPR-generated mutations (Ellison et al., 2020). Since the initial report, gRNA *FT* augmentation has been used with other dicotyledon viruses such as PVX and a DNA virus, Cotton leaf crumple virus, generating heritable edits of 22% in *N. benthamiana* and >4% in *A. thaliana* seedlings, respectively (Lei et al., 2021; Uranga et al., 2021a). This strategy has also been implemented in monocot crops such as wheat with surprising results. Cas9 transgenic wheat lines were infected with BSMV expressing either unaugmented gRNAs or augmented gRNAs with wheat *FT* (Li et al., 2021). Unexpectedly, gene edits were present in almost all M1 progeny from plants infected with unaugmented gRNA whereas, the progeny of plants infected with BSMV-*FT* augmented gRNA were rarely edited. To quickly remove the Cas9 transgene, the authors used anthers from plants infected with BSMV-gRNA for pollination of wild type plants. The progeny was self-pollinated to obtain edited plants lacking the Cas9 transgene (Li et al., 2021). This BSMV VIGE system has the potential to circumvent the need for tissue culture in genome editing pipelines involving monocots such as maize and barley which BSMV infects.

3.1.4 Transgene-Free Genome Editing Using Positive-Strand RNA Virus

Despite achieving high gene editing efficiency and sometimes heritable editing, the above discussed approaches are not truly transgene-free as they need to use a Cas9 expressing line as starting plant material for infection. PVX is a monopartite PSV with a filamentous flexible architecture which may allow to incorporate the large Cas9 gene into its RNA genome (Ariga et al., 2020). Recently, PVX was used to transiently express Cas9 and gRNA in *N. benthamiana*. No systemic editing was discussed in this work, suggesting that none was found possibly because the incorporation of the large Cas9 cassette led to defective viral movement. Nevertheless, regeneration of plants from tissues agroinfiltrated with the viral cDNA yielded >50% plants containing mutations with 18% also containing a T-DNA integration. The same strategy was attempted using a nickase Cas9-base editor fusion with >60% of regenerated plants containing base edits while ~30% contained T-DNA integration (Ariga et al., 2020). The progeny from regenerated plants retained genomic edits but were free of PVX RNA. To avoid T-DNA integration, the authors performed agroinfiltration to establish infection in a source plant and used mechanical inoculation from the source plant into recipient plants. In this way, the recipient plants were never in contact with *Agrobacterium,* eliminating the risk of T-DNA incorporation, thus providing a DNA free method for editing. Unfortunately, this approach proved much less efficient than the direct agroinfiltration with only 2–4% of regenerated pants containing mutations. A non-systemic PSV expression vector based on the tobacco mosaic virus was also developed which expressed both Cas9 and gRNA for gene editing in the presence of *p19*, a viral suppressor of RNA silencing. However, no attempt was made to regenerate gene edited plants (Chiong et al., 2021).

Foxtail mosaic virus (FoMV), from the same *Potexvirus* genus as PVX, has been used to mediate systemic gene editing in a transgene-free fashion (Zhang et al., 2020). *N. benthamiana* leaves were simultaneously agroinfiltrated with a FoMV vector containing a Cas9 expression cassette and a second FoMV vector containing a gRNA cassette targeting the *PHYTOENE DESATURASE* (*PDS*) gene. Sequencing of the targeted genomic region detected no edits but addition of a cassette containing the viral RNA silencing suppressor, *p19,* to the gRNA vector produced gene editing in systemic tissues (Zhang et al., 2020). These results are in line with recent research indicating that RNA silencing suppressors can increase genome editing efficiency (Mao et al., 2018; Zhang et al., 2020; Chiong et al., 2021; Mao et al., 2021). Unfortunately, the authors did not attempt to regenerate plants from systemically infected tissue or test heritable editing in the progeny of infected plants and previous studies have failed to obtain edited seeds (Zhang et al., 2020).

Similarly, Uranga et al. (2021b) achieved transgene-free genome editing using two compatible viruses that can co-infect the same cells. The PSV, Tobacco etch virus (TEV) was used to express CRISPR/Cas12a by replacing the *NIb* gene in the TEV genome. Another PVX virus expressing both the gRNA and the *NIb* gene to supplement the recombinant TEV was constructed. Both recombinant viral genomes were co-agroinfiltrated into wild type *N. benthamiana*, mediating around 20% gene editing efficiency in systemic leaves. Although the authors did not investigate the heritability of gene editing, we believe that regeneration from systemically infected tissue is likely to produce gene-edited progenies (Uranga et al., 2021b).

3.2 Genome Editing Using Negative-Strand Viruses

Rhabdoviruses are a group of negative-strand RNA viruses containing a large genome (>10 kb) (Jackson et al., 2005). Rhabdoviruses have large cargo capacities and high gene stability, making them a suitable candidate for transgene-free genome editing (Walker et al., 2015; Dietzgen et al., 2017). The Barley yellow striate mosaic virus (BYSMV) was the first monocot-infecting rhabdovirus developed into an expression system (Gao et al., 2019). When a BYSMV-based vector was used to express Cas9 and gRNA in *N. benthamiana* plants, Sanger sequencing successfully detected different indels in the infiltrated area, but systemic gene editing was not discussed. The authors also explored the use of BYSMV as a gene expression platform for planthoppers and monocots such as barley, wheat and *Setaria italica*; however, genome editing was not investigated in these systems (Gao et al., 2019).

The *Sonchus* yellow net virus (SYNV), a rhabdovirus infecting dicotyledonous species, has been also used to express Cas9 and gRNA in *N. benthamiana*. SYNV-mediated gene editing generated mutations in systemic tissues with high efficiency for a *GFP* transgene (77–91%), as well as three endogenous genes (40–79% for *PDS*, 53%–91% for *RDR6*, and 79–91% for *SGS3*) (Ma et al., 2020). Up to 93% of plants regenerated from systemically infected leaves contained some form of gene editing which also produced

edited seeds in the next generation. Unfortunately, heritable editing was not detected in seeds from the initially infected plants (Ma et al., 2020). Although the host range of SYNV is extremely limited, there are multiple rhabdoviruses infecting a diverse range of plant species, thus, a suite of rhabdovirus expression platforms can be developed for different plant species.

3.3 Future Directions

Viruses have the potential to become an efficient and versatile vector for the delivery of CRISPR genome editing reagents, however, several important limitations still need to be addressed, the most important being the large size of Cas9. Although some of the available methods can achieve seed heritability using mobility sequences, they still require the use of transgenic Cas9 lines while transgene-free genome editing using viral vectors involves tissue culture for plant regeneration (**Figure 2**) (Ellison et al., 2020; Ma et al., 2020; Zhang et al., 2020). It will be interesting to investigate whether heritable editing can be achieved by combining mobility sequences in a rhabdovirus, FoMV or TEV-PVX based VIGE platform (Uranga et al., 2021b). Several recent studies have also discovered new RGENs with dramatically reduced size compared to Cas9 (~1,000–1,400 amino acids). For example, the phage derived CasΦ is a compact RGEN (~700–800 amino acids) capable of generating gene edits in *A. thaliana* protoplasts, albeit at low efficiency (Pausch et al., 2020). Obligated mobile element-guided activity (OMEGA) is another class of transposon-encoded RGENs with a reduced size (~400 amino acids) (Altae-Tran et al., 2021). Miniature CRISPR associated RGENs such as Cas12f are also being explored for gene editing capability and engineered for improved efficiency (Bigelyte et al., 2021; Xu et al., 2021). The discovery and optimization of these smaller genome editing tools may facilitate delivery with germline infecting PSVs such as TRV or BSMV.

4 DISCUSSION

Transgene-free genome editing can be an ideal technology to breed sustainable and more nutritious crops; however, several plant-specific challenges need to be overcome before it can achieve its full potential. One of the most significant hurdles is the delivery of CRISPR/Cas components into plant cells, whilst avoiding transgenesis. The use of preassembled CRISPR/Cas RNPs is arguably the most direct option, but the available methods utilise specific plant cells or organs such as protoplasts, immature embryos, or zygotes followed by regeneration of whole plants. These processes are technically difficult, inefficient, and are not available for many economically important crops. Viral vectors have also emerged as promising tools for transgene-free genome editing, especially because they can be used in full plants and there is a large number of virus vectors for gene silencing which are available to be implemented for VIGE (**Supplementary Table S2**). Multiple VIGE systems have been developed for transient and systemic expression of Cas9 and/or gRNAs with efficient editing in dicotyledonous and monocotyledonous species (**Supplementary Table S1**). But efficient heritable mutations were only achieved through plant regeneration. Some viral systems and innovative approaches such as gRNA augmentation with mobility sequences can achieve heritable gene editing through seeds but they still need to use transgenic Cas9 plants. The ideal VIGE system should combine transgene-free and heritability at acceptable efficiency to alleviate the genome editing delivery bottleneck. The use of nanomaterials as delivery systems for plants is only starting but we expect to see rapid advances with this approach. Alternative strategies such as the use of mRNA-based genome editing (Zhang et al., 2016) or the *de novo* induction of meristems (Maher et al., 2020) have been reported but have not yet being been adopted by the wider research community. Even though steady and continuous progress is being made, the field is in need of completely new approaches and the ideal solution might involve development of new and disruptive technologies.

AUTHOR CONTRIBUTIONS

ZG and MC have contributed equally to this work and share first authorship. JB, ZG, and MC conceived the idea. ZG and MC wrote the manuscript. All authors contributed to the discussion and revision of the final manuscript.

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SUPPLEMENTARY MATERIAL

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Advances in Delivery Mechanisms of CRISPR Gene-Editing Reagents in Plants

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Gene-editing by CRISPR/Cas systems has revolutionized plant biology by serving as a functional genomics tool. It has tremendously advanced plant breeding and crop improvement by accelerating the development of improved cultivars, creating genetic variability, and aiding in domestication of wild and orphan crops. Gene-editing is a rapidly evolving field. Several advancements include development of different Cas effectors with increased target range, efficacy, and enhanced capacity for precise DNA modifications with base editing and prime editing. The existing toolbox of various CRISPR reagents facilitate gene knockouts, targeted gene insertions, precise base substitutions, and multiplexing. However, the major challenge in plant genome-editing remains the efficient delivery of these reagents into plant cells. Plants have larger and more complex genome structures compared to other living systems due to the common occurrence of polyploidy and other genome re-arrangements. Further, rigid cell walls surrounding plant cells deter the entry of any foreign biomolecules. Unfortunately, genetic transformation to deliver gene-editing reagents has been established only in a limited number of plant species. Recently, there has been significant progress in CRISPR reagents delivery in plants. This review focuses on exploring these delivery mechanisms categorized into *Agrobacterium*-mediated delivery and breakthroughs, particle bombardment-based delivery of biomolecules and recent improvements, and protoplasts, a versatile system for gene-editing and regeneration in plants. The ultimate goal in plant gene-editing is to establish highly efficient and genotype-independent reagent delivery mechanisms for editing multiple targets simultaneously and achieve DNA-free gene-edited plants at scale.

Keywords: gene-editing, CRISPR-Cas9, gene targeting, agrobacterium-mediated transformation, biolistics, protoplasts, nanoparticles

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INTRODUCTION

CRISPR/Cas9 derived from *Streptococcus pyogenes* (SpCas9) is the most used gene-editing reagent in plants. Unlike its predecessors, zinc finger nucleases (Gao et al., 2010; Osakabe et al., 2010; Zhang et al., 2010) and Transcription Activator-like Effector Nucleases (TALENs) (Cermak et al., 2011; Li et al., 2012), which rely on protein-based DNA recognition mechanisms, CRISPR/Cas systems are RNA-guided endonucleases. The resulting versatility, simplicity, and cost-effectiveness brought about by CRISPR led to significant advances in plant genome engineering. In the CRISPR/Cas9 system, a chimeric single guide RNA (sgRNA), formed by fusion of CRISPR RNA (crRNA) and a *trans*-activating crRNA (tracrRNA), directs the SpCas9 nuclease to generate blunt double-strand

breaks (DSBs) at the genomic DNA target site three bases upstream of Protospacer Adjacent Motif (PAM) sequence of 'NGG' (Jinek et al., 2012). The DSBs are repaired either by error-prone non-homologous end joining (NHEJ) resulting in insertion-deletion mutations (InDels) leading to gene knock-out or by precise, albeit inefficient, homology-directed repair (HDR) through which DNA insertions are achieved by providing an external donor repair template (DRT) (Atkins and Voytas, 2020; Dong and Ronald, 2021). In addition to Cas9, multiple other Cas variants with alternative PAM requirements have been identified and successfully utilized in plants expanding the range of DNA recognition (Kaya et al., 2016; Jia et al., 2017; Steinert et al., 2017; Zhang Y. et al., 2019; Veillet et al., 2020). Furthermore, base editors (BEs), including cytosine, adenine, and glycosylase BEs can precisely convert one target DNA base to another without a DSB. BEs rely on base excision repair, facilitating both transition and transversion mutations, and are increasingly being used in plant systems (Shimatani et al., 2017; Zong et al., 2017; Shan and Voytas, 2018; Zhang R. et al., 2019; Li et al., 2020; Zhao et al., 2020). In addition, prime editing (PE), a versatile "search-and-replace" strategy, was also developed (Anzalone et al., 2019) and optimized in plants (Butt et al., 2020; Lin et al., 2020; Tang et al., 2020; Xu et al., 2020). PEs copy desired edits incorporated into the PE gRNA (PegRNA) directly into the genomic DNA by target primed reverse transcription. With this existing toolbox of various CRISPR reagents, the biggest challenge in plant genome-editing remains to be the efficient delivery of these reagents into plant cells.

Several plant species have larger and more complex genome structures compared to other living systems. Polyploidy and genomic rearrangements are common in plants, and rigid cell walls surrounding the plant cells deter the entry of any foreign biomolecules. Furthermore, genetic transformation to deliver transgenes has only been established in a limited number of plant species and genotypes within each species. This is currently considered the biggest bottleneck in plant genome engineering. Gene-editing reagents are delivered into plants, most commonly as plasmid DNA constructs and predominantly by *Agrobacterium*-mediated transformation or particle bombardment are summarized in tables recently (Sandhya et al., 2020; Ghogare et al., 2021). In both methods, the plasmid DNA with CRISPR/Cas expression cassette is likely to get integrated into a random genomic site(s), leading to continued expression in host genomes. With the revision of the regulatory landscape of gene-edited lines in the US (USDA press release[1]) and across the world (Nadakuduti et al., 2018; Lassoued et al., 2021), developing gene-edited lines without integrating foreign genomic DNA into the host plant is gaining prominence. DNA-free delivery of *in vitro* transcripts (IVTs), pre-assembled ribonucleoprotein complexes (RNPs), or transient expression of plasmid DNA constructs delivered into protoplasts, and subsequent regeneration of gene-edited plants have been successful in several plant species (Liang et al., 2017; Andersson et al., 2018; González et al., 2020, 2021; Sidorov et al., 2021; Zhang et al., 2021). This review will focus on various advances in CRISPR delivery mechanisms in plants categorized into *Agrobacterium*-mediated delivery and breakthroughs for efficient and heritable mutagenesis and gene targeting (GT) in plants; particle bombardment mediated delivery of DNA, RNA, and protein biomolecules for plant gene-editing, and protoplast transfection and regeneration of transgene-free gene-edited plants. The ultimate goal in plant gene-editing is to establish highly efficient and species non-specific reagent delivery mechanisms for editing multiple targets simultaneously and achieve DNA-free gene-edited plants at scale.

Breakthroughs in *Agrobacterium*-Mediated Delivery of CRISPR Reagents for Efficient and Heritable Mutagenesis and Gene Targeting

Agrobacterium-mediated genetic transformation remains the principal means of delivering gene-editing reagents including CRISPR/Cas variants, base editing and prime editing reagents, into plants (Lin et al., 2020). This method typically involves inoculating the explants with *Agrobacterium* expressing gene-editing cassettes integrated into its T-DNA (**Figure 1A**). Upon infection of plant cells, the T-DNA containing the CRISPR cassette likely gets integrated into the host plant genome leading to stable genetic transformation. Transgene-free gene-editing has been achieved by transient expression of CRISPR reagents by regenerating events without employing selection (Chen et al., 2018). This is important for generating edited plants with no foreign DNA to avoid regulatory oversight and for vegetatively propagated plants, where segregating out the integrated transgene by making crosses is not feasible. *Agrobacterium* has a limited host range, and several plant species are recalcitrant to *Agrobacterium*-mediated transformation. Furthermore, the regeneration process involving tissue culture leads to undesirable somaclonal variations in edited lines. Floral dip method of transformation, only amenable to *Arabidopsis thaliana* and some related species (Clough and Bent, 1998; Lu and Kang, 2008) can generate transformed seeds, bypassing the need for regeneration. Other means of avoiding regeneration process include, the use of *A. rhizogenes*, which can drastically reduce time between reagent delivery and mutation evaluation, as well as widening the range of species transformed (Yoshida et al., 2015; Triozzi et al., 2021).

Co-delivery of Developmental Regulators with CRISPR Reagents via *Agrobacterium* to Expedite and Improve Gene-editing Efficiency in Plants

Developmental regulators (DRs) are genes involved in dictating meristem identity in plants. Ectopic expression of DRs in plants has resulted in somatic embryogenesis, formation of embryos from somatic tissues (Lowe et al., 2016). Overexpression of DRs such as *Baby Boom* (*Bbm*) and *Wuschel2* (*Wus2*) enhanced regeneration and transformation frequency in both dicot and monocot plants (Srinivasan et al., 2007; Deng et al., 2009; Lowe

[1]https://www.usda.gov/media/press-releases/2018/03/28/secretary-perdue-issues-usda-statement-plant-breeding-innovation.

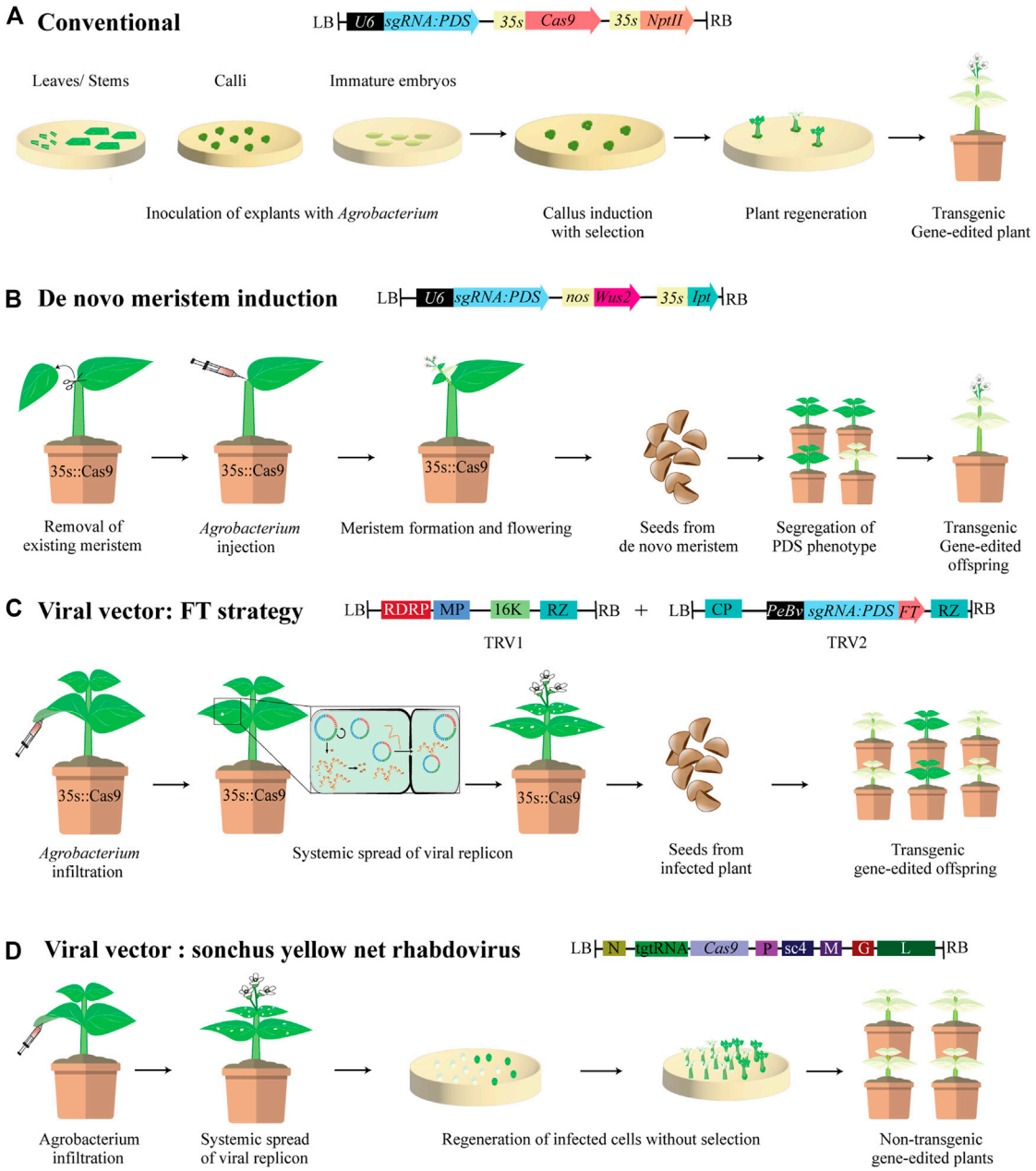

FIGURE 1 | Agrobacterium mediated delivery of CRISPR gene-editing reagents in plants. **(A)** Conventional *Agrobacterium*-mediated transformation consisting of T-DNA carrying expression cassette for *Streptococcus pyogenes* Cas9 and kanamycin resistance gene *NptII,* both driven by cauliflower mosaic virus 35S promoter (CaMV 35S), and a single guide RNA (sgRNA) driven by the U6 promoter targeting the *phytoene desaturase* (*PDS*) gene. Explants are infected and co-cultivated with agrobacterium cultures, then placed on selective media for callus induction and regeneration. The resulting gene-edited lines are transgenic and have photobleaching phenotype. **(B)** *A. tumefaciens* T-DNA harboring sgRNA targeting *PDS* along with plant developmental regulators (DRs) *Wuschel2* (*Wus2*) driven by nopaline synthase (*nos*) promoter, and *isopentenyl synthase (ipt)* driven by 35S promoter are injected in Cas9 expressing soil grown plants after meristem removal. DRs induce new meristems at the wounded site and *pds* phenotype is visible in edited meristems. Offspring from seeds produced on *de novo* meristems show segregation for photobleaching phenotype. Maher et al. (2020) found that *de novo* meristems with bi-allelic mutations did not set viable seeds, and edited offspring are only recovered from meristems exhibiting mosaicism. **(C)** Tobacco rattle virus (TRV) is a bipartite RNA virus: TRV1 encodes replicases RNA-dependent RNA polymerase (RDRP), a movement protein (MP), a 16 KDa cysteine rich protein, and a ribozyme (RZ) and can independently replicate itself and move within the plant during infection. TRV2, encodes a coat protein (CP) and, a sgRNA targeting *PDS* fused to *Flowering locus T* (*FT*) driven by a pea early browning virus promoter (PeBv). FT is a mobile RNA which increases infection spread by reaching the shoot apical meristem (SAM). TRV1 and TRV2 are introduced into T-DNA regions of agrobacterium and infiltrated into 35S: Cas9 transgenic plants. Systemic infection of the plant leads to editing of somatic and germline cells thereby increasing heritability. Infected plants exhibit photobleaching and *pds* phenotype segregates in progeny. **(D)** Sonchus yellow net rhabdovirus (SYNV) is a negative-strand RNA virus encoding the core structural proteins nucleoprotein (N), phosphoprotein (P), and the large RNA polymerase (L), and Sc4 protein, matrix protein (M), glycoprotein (G) which are involved in cell-to-cell

(*Continued*)

FIGURE 1 | movement. The viral cassette is manipulated to express a Cas9 nuclease and a tRNA-gRNA-tRNA (tgtRNA) which is processed to release the sgRNA targeting the *PDS* gene by tRNA processing enzymes. Soil grown plants are infiltrated with agrobacterium harboring the SYNV plasmid. Explants from systemically infected leaves are prepared and placed on non-selective regeneration medium. Regenerants are then transferred to soil. Since Cas9 is delivered virally and SYNV does not integrate into the host genome nor have a DNA-phase, the resulting plants are non-transgenic.

et al., 2016). This phenomenon was leveraged to induce *de novo* meristems in somatic tissues by injecting *Agrobacterium* cultures co-delivering DRs and gene-editing cassettes directly into soil-grown plants (**Figure 1B**). *Wus2* and *Isopentenyl transferase* (*Ipt*), when co-delivered with gene-editing reagents by *Agrobacterium* injections into dicot plants generated meristems in somatic tissues with edits, enabling tissue culture free gene-editing (Maher et al., 2020). This can potentially be a high throughput and less tedious approach when Cas9 expressing plants are generated. Alternatively, Growth Regulating Factor (GRF) and GRF-interacting Factor (GIF) cofactor when expressed as GRF4-GIF chimera increased the speed and efficiency of regeneration (Debernardi et al., 2020). Co-delivery of GRF4-GIF chimera and CRISPR-Cas9 on the same T-DNA increased the regeneration efficiency in both monocots and dicots and produced fertile edited plants (Debernardi et al., 2020). The expression of DRs is extremely beneficial in plant species that are recalcitrant to regeneration or ones with long regeneration periods to reduce the time and cost of plant gene-editing.

Viral Vectors and Mobile RNAs for Systemic Delivery of CRISPR Reagents for Heritable Gene-Editing

Recently, viral vectors showed promise for efficient delivery of CRISPR reagents into germline cells to achieve heritable and DNA-free gene-editing (Ali Z. et al., 2015; Ellison et al., 2020; Ma et al., 2020; Kujur et al., 2021; Li et al., 2021). Traditionally, heritable modifications are accomplished by stable expression of the CRISPR cassettes and generating transgenic lines through regeneration. Autonomously replicating viral vectors delivered into plants via *Agrobacterium* offer an alternative for heritable gene-editing in plants. RNA viruses don't integrate into the plant genome but have lower cargo capacity impeding their use for Cas9 delivery. Tobacco rattle virus (TRV), a bipartite positive-strand RNA virus is widely used in plants. TRV mediated sgRNA delivery into Cas9 overexpressing lines by agroinfiltration has been optimized in dicots, albeit with low heritability of edits (Ali et al., 2015; Cody et al., 2017). To improve heritability, the endogenous mobile RNA *Flowering Locus T* (*FT*) has been fused to sgRNA to enhance mobility and facilitate systemic distribution within plant to reach germline cells (**Figure 1C**) (Ellison et al., 2020). Barley stripe mosaic virus (BMSV) has been engineered to deliver sgRNAs into wheat to achieve heritable genome editing. Furthermore, by co-infiltration of a pool of BMSV vectors harboring different sgRNAs resulted in multiplexed mutagenesis in the progeny (Li et al., 2021). Sonchus yellow net rhabdovirus (SYNV), a negative-strand RNA virus with higher cargo capacity, has been engineered to carry both Cas9 and sgRNA for DNA-free in planta editing (**Figure 1D**) (Ma et al., 2020).

Enhancing Gene Targeting by *Agrobacterium*-Mediated Delivery of CRISPR Reagents

GT includes precise DNA modifications based on HDR using a DRT with homology to the host target DNA on both ends. DSBs generated by CRISPR/Cas reagents initiate the cell repair process. However, NHEJ is the predominant repair mechanism in plants cells to repair these DSBs as HDR is not active throughout the cell cycle. This, in combination with inefficient delivery of DRT to facilitate HDR, make GT very inefficient in plants. To increase GT frequencies, viral replicons including Bean Yellow Dwarf Virus (BeYDV) (Baltes et al., 2014; Butler et al., 2015; Čermák et al., 2015; Cermak et al., 2017; Wang et al., 2017; Vu et al., 2020) or wheat Dwarf virus (WDV) (Gil-Humanes et al., 2017) have been successfully used in several dicot and monocot plants. These viral replicons carrying the CRISPR expression cassette and DRT undergo rolling-circle replication in the host cells thereby increasing the abundance of nuclease and availability of DRT for HDR (Baltes et al., 2014). The GT event is not heritable if it doesn't occur in the germline cells. To increase the heritability of GT, germline-specific promoters including the egg-cell, early embryo-specific promoter and pollen-specific promoters or promoters active in the shoot apical meristems (SAM) have been employed to drive Cas9 expression (Wang et al., 2015; Yan et al., 2015; Mao et al., 2016). Furthermore, to improve the efficiency of heritable in-frame gene insertions and amino acid substitutions by HDR, plants expressing Cas9 from germline-specific promoters are used for sequential transformation with HDR constructs containing DRT and sgRNA targeting the gene of interest. This led to an increase in GT efficiency of up to 9% (Miki et al., 2018). Since GT is a rare phenomenon, even with all the advances to improve efficiency, selection must still be employed to detect positive GT events. A *piggyBac* transposition system from T-DNA has been used to eliminate the GT selection marker from host plant genome. In this method, a transposon integrates into the host genome at TTAA element and excises without a footprint (Nishizawa-Yokoi et al., 2015). Recently, a novel marker elimination system was developed wherein the excision is based on I-SceI recognition site. By overlapping this recognition site on 5′ and 3′ homology arms of the DRT, seamless marker elimination and precise GT have been achieved (Endo et al., 2021). To this end, the same research group also developed a *piggyBac*-mediated transgenesis system to temporarily express CRISPR and selection marker cassettes from T-DNA with subsequent excision of *piggyBac* via transposase after successful editing and selection had occurred (Nishizawa-Yokoi and Toki, 2021).

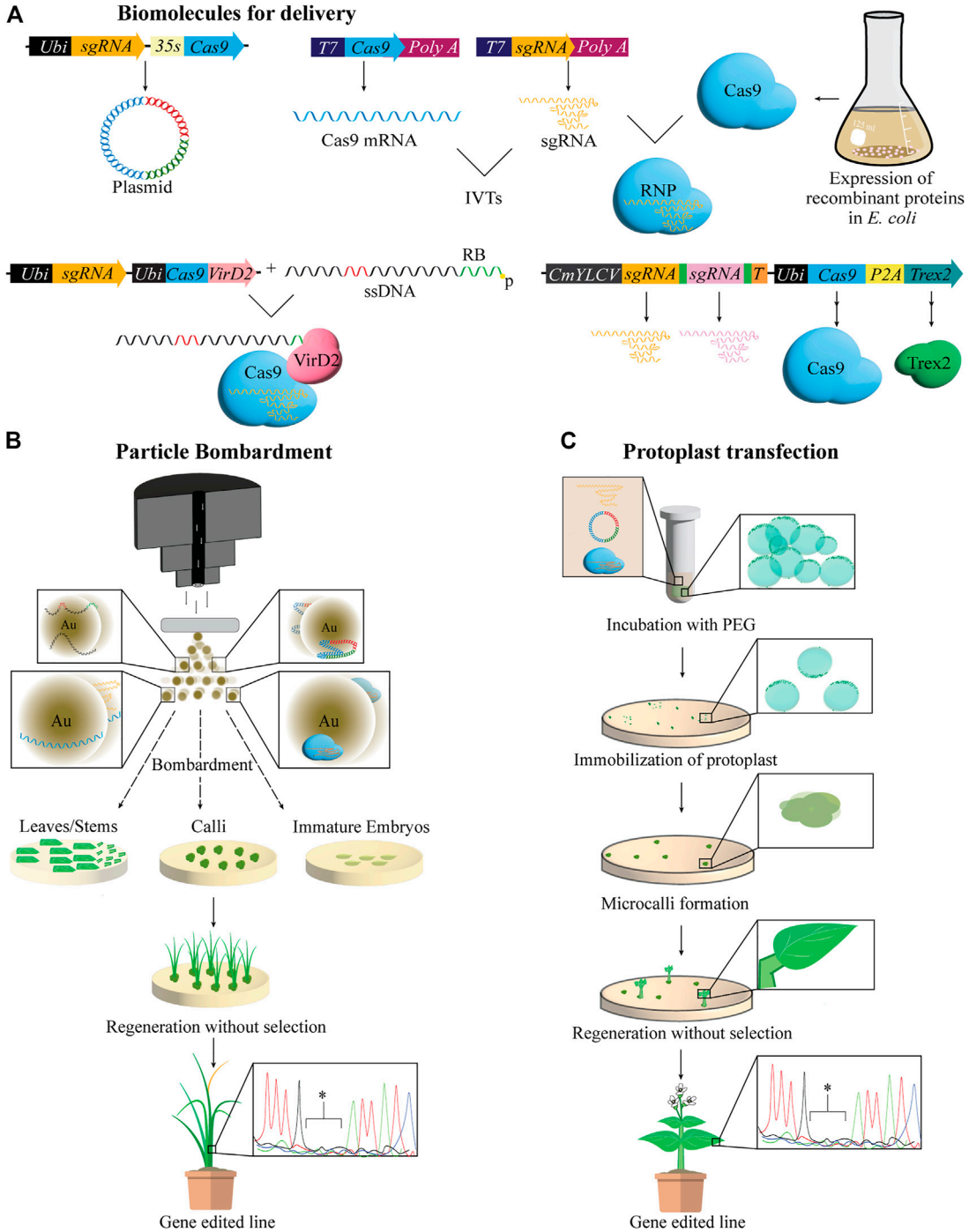

FIGURE 2 | Biomolecules delivered via biolistics and protoplast transfections for regenerating gene-edited plants. **(A)** Biomolecules used for gene-editing are delivered into plants cells in a variety of forms including plasmid DNA, ssDNA, mRNA or ssRNA, prepared via *in vitro* transcription (IVT), and preassembled ribonucleic proteins (RNPs) using IVTs and recombinant proteins. Targeted mutagenesis and gene targeting (GT) can be enhanced by various mechanisms. For example, fusion of Cas9 to VirD2, one component of the agrobacterium relaxosome complex integral to the cleavage of T-DNA from the Ti plasmid, as well as its localization and integration in the plant genome, has been shown to increase homology-directed repair (HDR) mediated GT using a donor repair template (DRT). DRT in this case is a single stranded DNA (ssDNA) harboring the desired edits (red) and the canonical 25 bp right border (RB) sequence (green), and is delivered to the plant cell along with the Cas-VirD2 fusion protein. VirD2 will covalently bind the template, thus bringing it in close proximity to the DSB induced by Cas9. Delivering Trex2 exonuclease has also been shown to increase HDR as well as the efficiency of multiplex editing when sgRNA are co-delivered and processed by t-RNA system, illustrated by green boxes between sgRNA. p = phosphorylation. **(B)** Particle bombardment or biolistics, rely on the physical disruption of plant cell walls by metal particles, often gold, coated with ssDNA or dsDNA, IVTs or RNPs, which are introduced to the cell. Bombarded explants can be regenerated in tissue culture with or without selection to recover gene

(*Continued*)

FIGURE 2 | edited plants. Au = gold particles. **(C)** Protoplast transfection and regeneration is shown. polyethylene glycol (PEG) mediated transfection is the most common way to deliver biomolecules for gene-editing to protoplasts. Post transfection, protoplasts are immobilized on culture media where protoplasts undergo cell divisions to form microcalli, followed by shoot and root formation and finally resulting in regeneration of entire gene-edited plants. Editing at the target site is confirmed by sequencing represented in the chromatogram * = deletions.

Biolistics for Delivery of CRISPR Reagents Into Plants as DNA, RNA, or Proteins

Biolistics or particle-bombardment, is a common alternative for transforming plants recalcitrant to *Agrobacterium*-infection. It relies on physically breaching the plant cell wall and membrane with gold or tungsten microprojectiles coated with biomolecules accelerated to very high velocities. Biolistics offers the possibility of delivering a variety of cargo including plasmid DNA, ssDNA, RNA, or ribonucleic proteins (RNPs) assembled from IVTs and recombinant proteins (**Figure 2A**). Major drawbacks of biolistic delivery include random integration of cargo at multiple genomic sites when delivered as DNA and labor-intensive preparation of explants such as calli or immature embryos with the capability to regenerate.

Advances in Biolistic Delivery for DNA-free Gene-Editing and Chromosome Engineering

Instead of plasmid DNA, bombarding RNPs was successfully demonstrated to produce transgene-free gene-edited lines in cereal crops (Svitashev et al., 2016; Liang et al., 2017; Banakar et al., 2019, 2020; Zhang et al., 2021) (**Figure 2B**). In addition, when a single base pair mismatch was present in the protospacer of sgRNA targeting homeologs, a dramatic decrease in off-target editing was observed with RNPs as compared to plasmid DNA delivery indicating high specificity of RNPs (Liang et al., 2017). Furthermore, RNPs also facilitated large heritable inversion of 75.5 Mb in maize chromosome 2, when assembled with guide RNAs flanking the junctions of the desired inversion (Schwartz et al., 2020). Such precise chromosomal engineering in invaluable to crop breeding. To avoid labor-intensive preparation of explants, *in planta* biolistic delivery using SAM as a target tissue (Hamada et al., 2017) for germline transmission was employed as an alternative (Hamada et al., 2018; Imai et al., 2020). Embryonic SAM exposed mature wheat seeds were bombarded with plasmid DNA expressing CRISPR cassettes to generate gene-edited lines (Hamada et al., 2018; Imai et al., 2020). Alternatively, when pollen was used as a target tissue to bombard plasmid DNA for gene-editing in *Nicotiana benthamiana*, the bombarded pollen retained fertility and delivered the cargo into the ovules (Nagahara et al., 2021). Furthermore, technical improvements have also been made to overcome variability between bombardments. A double-barreled gene gun in combination with cell counting software was developed to scale bombardment experiments with an internal standard, thereby reducing standard deviation between bombardments by half (Miller et al., 2021).

Gene Insertion or Replacement by Intron Targeting and Determining Genomic Safe Harbors

To leverage the relatively more efficient NHEJ compared to HDR for targeted insertions, DNA fragments were inserted in selected introns such that any mutations by NHEJ would not affect protein-coding sequences of either endogenous or inserted genes. By bombarding calli with plasmids expressing pairs of sgRNA targeting adjacent introns of target genes and DRT harboring desired mutations flanked by the same intronic sgRNA sites, replacement of endogenous gene has been achieved at 2% frequency. Additionally, the gene replacement events were heritable (Li et al., 2016). Enhancers and promoters up to 2 Kb were introduced into the target site using these modified DRTs (Lu et al., 2020). Another strategy for targeted insertion by NHEJ is to determine the genomic safe harbors (GSH) in the host plant genome, within which integrations of transgenes do not cause any genic disruptions or adverse morphological effects. A 5.2 Kb carotenoid biosynthesis cassette was inserted at targeted GSH to generate marker-free rice with high carotenoid containing seeds and no-off target mutations observed (Dong et al., 2020).

Enhancing HDR by Delivery of Transcript-Donor Templates or by VirD2 Relaxase-Cas9 Fusion

Recent advances in HDR by particle bombardment include delivery of ssDNA, including a canonical 25 bp right border (RB) sequence of T-DNA, as DRT co-delivered with a plasmid expressing Cas9-VirD2 fusion protein (Ali et al., 2020) (**Figure 2A**). Achieving a 20.8% HDR efficiency, this method relies on the ability of the VirD2 protein, an *Agrobacterium* virulence factor, to covalently bind the RB of DRT, thus bringing it in close proximity to the DSB induced by Cas9 (**Figure 2A**) (Ali et al., 2020). Other attempts to improve HDR include the delivery of DRT as transcripts. RNA-DRT was shown to result in higher HDR efficiency than DNA-DRT when delivered to rice calli, possibly due to the high stability of RNA:DNA complexes, resulting in edited rice with two desired point mutations in the *ALS* gene conferring herbicide tolerance (Li et al., 2019). This transcript-templated HDR (TT-HDR), approach improves not only HDR efficiency but also creates a DNA-free path to HDR-mediated gene-editing, which may avoid regulatory hurdles.

Protoplasts Provide a Versatile System for DNA-free Genome Editing in Plants

Protoplasts are plant cells devoid of cell walls, which offer a versatile platform for DNA-free GE and a good transient system to evaluate the activity of gene-editing reagents before moving into a more-labor intensive transformation pipeline (Nadakuduti et al., 2019; Lin et al., 2020). Polyethylene glycol (PEG)-mediated transfection and electro-transfection are two common methods to deliver plasmid DNA, IVTs, or RNPs into protoplasts for transient expression of CRISPR cassettes. Subsequently, edited plants can be regenerated from transfected protoplasts by tissue culture procedures (**Figure 2C**). Plasmid DNA may integrate into the host genome randomly as filler DNA during protoplast transfection (Gorbunova and Levy, 1997; Kim and Kim, 2016). However, IVTs or RNPs offer DNA-free gene-editing by immediately editing the target site, bypassing transcription and translational machinery respectively in the cell and rapidly degrade (Liang et al., 2017, 2018; Andersson et al., 2018; González et al., 2020, 2020, 2020; Lee et al., 2020; Sidorov et al., 2021; Zhang et al., 2021). However, plant regeneration from protoplast remains unestablished in many plant species. In addition, somaclonal variations and genome instability is reported in regenerated lines (Fossi et al., 2019). Once efficient protoplast isolation, transfection, and regeneration have been established in a plant species, it could be a high throughput platform by combining with flow cytometry and omic analyses for optimizing gene-editing. Furthermore, multiplexing, editing multiple genes at a time has been achieved using protoplasts (Klimek-Chodacka et al., 2021; Nicolia et al., 2021; Yu et al., 2021; Zhang et al., 2021). By co-delivering Three Prime Repair exonuclease 2 (TREX2) and CRISPR/Cas9 into protoplasts, targeted mutagenesis using a multiplexing strategy was further improved (Weiss et al., 2020) (**Figure 2A**).

Nanocarrier-Mediated Delivery of CRISPR/Cas Reagents in Plants

Nanotechnology has evolved in the past decade in the field of plant genetic engineering. Nanomaterials including carbon nanotubes (CNTs), carbondots, mesosporous silicon nanoparticles (MSNs) etc have been used to deliver biomolecules such as DNA, RNA, RNPs and proteins etc., discussed in recent reviews (Kumari and Singh, 2021; Mujtaba et al., 2021). Nanoparticle-mediated delivery of DNA and proteins into both nuclear and chloroplast genomes has been achieved in plants (Demirer et al., 2019, 2020; Kwak et al., 2019). Furthermore, Cre protein was previously delivered via MSNs for maize GE via *loxP* site demonstrating the feasibility of gene-editing (Martin-Ortigosa et al., 2014). Gene-editing using RNPs delivered by nanoparticles has been achieved in human cells (Wang et al., 2016; Lee et al., 2017; Mout et al., 2017). However, it has yet to be achieved in plants mainly due to high delivery efficiencies required for GE.

Future Aspects of Delivering Plant-Gene Editing Reagents

Relying on tissue culture-based plant genetic transformation methods and inefficient reagent delivery mechanisms are the major bottle necks to overcome before we realize the full potential of gene-editing in plants. Current advancements in delivery mechanisms, including *de novo* meristem induction or use of viral vectors to circumvent tissue culture, rely on *Agrobacterium* for delivery and have been demonstrated only in dicots and need to be expanded to monocots. Delivering repair templates for HDR through these innovative methods is also a future possibility. Furthermore, smaller sized Cas9 alternatives would overcome the cargo capacity of some of these viral vectors. *Agrobacterium,* however, has a narrow host range for infection and several species are recalcitrant to *Agrobacterium* transformation. Particle bombardment has been shown to be better equipped for co-delivery of cargo for simultaneous editing than *Agrobacterium* and is universally applicable to all plant species and cell types (Kuang et al., 2020). Chromosomal inversions achieved via bombardment could revolutionize breeding by unlocking regions for chromosomal cross overs, creating novel linkage groups and facilitating targeted recombination to maximize genetic gain in crops. However, complex segregation patterns of DNA integrated in bombarded plant genomes might complicate downstream uses of transformed plants. *Agrobacterium* and biolistic transformation of pollen also bypasses regeneration but often results in pollen with lower viability (Wang et al., 2008; Zhao et al., 2017). In addition, pollen-tube transformations may result in chimerism (Ali A. et al., 2015). While pollen magnetofection has improved on these drawbacks (Zhao et al., 2017), its application remains constrained to dicots (Vejlupkova et al., 2020). The prospects of nanoparticles as delivery engines for plant genome editing are also encouraging (Demirer et al., 2021) and further advances are essential to facilitate plant gene-editing.

AUTHOR CONTRIBUTIONS

SN conceived the idea; SN and LL wrote the manuscript. All authors read and approved the manuscript.

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Crop Quality Improvement Through Genome Editing Strategy

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Good quality of crops has always been the most concerning aspect for breeders and consumers. However, crop quality is a complex trait affected by both the genetic systems and environmental factors, thus, it is difficult to improve through traditional breeding strategies. Recently, the CRISPR/Cas9 genome editing system, enabling efficiently targeted modification, has revolutionized the field of quality improvement in most crops. In this review, we briefly review the various genome editing ability of the CRISPR/Cas9 system, such as gene knockout, knock-in or replacement, base editing, prime editing, and gene expression regulation. In addition, we highlight the advances in crop quality improvement applying the CRISPR/Cas9 system in four main aspects: macronutrients, micronutrients, anti-nutritional factors and others. Finally, the potential challenges and future perspectives of genome editing in crop quality improvement is also discussed.

Keywords: crop, quality, improvement, gene editing, CRISPR/Cas9

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1 INTRODUCTION

Quality is the most important economic character of crops, determining products' application value and market competitiveness. With the continuous improvement of people's living standards, higher requirements are put forward for crop quality. Cultivating more nutritious, more delicious and healthier crop varieties is of great significance to improving people's living standards and the sustainable development of social health. However, the pace of crop quality improvement has been relatively slow in the world due to the over-emphasis on demand for crop yield. The authors think that three main reasons are hindering the pace of crop quality improvement:1) The genetic control network of quality traits is extremely complex, and the available gene resources are still not abundant; 2) Crop quality traits are susceptible to environmental factors, such as fertilizer, climate and biological stress; 3) Traditional breeding methods (cross-breeding, mutation breeding and transgenic breeding) are time-consuming, random and introduce foreign genes.

A new generation of genome editing techniques, represented by regularly clustered interspaced short palindromic repeats/CRISPR-associated endonuclease 9 (CRISPR/Cas 9), is a revolutionary technology developed in the field of life sciences in recent years, which can produce predictable and heritable changes in specific locations of plant genomes. It includes deletion, insertion and replacement of base sequences to achieve precise improvement of plant traits. At the same time, compared with the traditional transgenic technology, the insertion site of the CRISPR/Cas9 expression vector is different from that of the gene-editing site. After the endogenous gene editing, the exogenous inserted plasmid can be removed by separating chromosomes during the generation of offspring gametes, thus eliminating the need to introduce exogenous genes. There is no

transgenic controversy, and the application prospect is very broad. Providing a new turning point for the agricultural technology revolution.

At present, there are more and more cases of crop quality improvement using the CRISPR/Cas9 system. Here, we briefly review the various genome editing ability of the CRISPR/Cas9 system, summarized the recent progress in CRISPR/Cas9-mediated crop quality improvement, and further discussed potential challenges and future perspectives of genome editing in crop quality improvement.

2 ESTABLISHMENT OF THE CRISPR/CAS9 SYSTEM

The CRISPR/Cas system is widely present in bacterial and archaebacterial genomes and is part of the adaptive immune system of microorganisms (Mojica et al., 2005; Grissa et al., 2007; Jinek et al., 2012). It consists of two core components: the clustered regularly interspaced short palindromic repeats (CRISPR) and Cas protein. The CRISPR constitutes 20–50 bp palindromic repeat sequence (Repeat), non-repeating 20–58 bp spacer sequence (Spacer) and AT-rich leading sequences (Leader). Cas protein acting as DNA endonuclease helps bacteria acquire new space sequences, essential for the bacterial immune system. According to the number and function of Cas proteins, CRISPR/Cas system has been divided into two classes and five types (I–V) (Makarova et al., 2011; Makarova and Koonin, 2015). Type I, III and IV belong to class I requiring multiple Cas proteins to form complex to work cooperatively. At the same time, type II and V belongs to class II interfering with target genes using only one single Cas protein. The immune process of the CRISPR/Cas system can be divided into three stages in bacteria (Makarova et al., 2011): 1) When foreign DNA infects bacteria, short DNA homologous fragments from protospacer sequences on bacteriophages or plasmids are integrated into the downstream of CRISPR leading sequence to form new space sequences; 2) CRISPR is induced to be transcribed into long RNA precursors (pre-crRNA), which are then truncated into short mature crRNAs, then the crRNAs precisely bind to trans-activating RNAs (tracrRNA) to fuses into tracrRNA/crRNA complexes; 3) The complexes regulate and guide Cas protein to precisely destroy the foreign DNA sequence, and produce DNA double-strand breaks (DSBs).

With a better understanding of the bacterial CRISPR/Cas immune system and its operational principle, scientists began to modify and apply this system to plant and animal genome editing (Cho et al., 2013; Cong et al., 2013; Feng et al., 2013; Mali et al., 2013; Ma et al., 2015; Yin et al., 2017). CRISPR/Cas9 system is the only class II type system reported for gene editing (Hsu et al., 2014). By artificial design, the tracrRNA/crRNA complex was simplified to a short guide RNA (sgRNA), which contains a ~20 nt fragment complemented to a specific site of target genes and followed by a protospacer adjacent motif (PAM) in the target genes of interest. Under the guidance of sgRNA, DSBs are created by Cas9 nuclease at ~3 bp upstream of the PAM motif and then repaired through the error-prone non-homologous end-joining (NHEJ) or the error-free homology-directed repair (HDR) pathways. The NHEJ repaired way usually results in gene knockout to lose protein function (Liu et al., 2019). Alternatively, the HDR pathway can be triggered when an exogenous DNA repair template is provided, resulting in the introduction of the repair template into a target genomic region (Chapman et al., 2012).

3 CRISPR/CAS9 SYSTEM IN PLANT FUNCTIONAL GENOMICS RESEARCH

At present, the applications of the CRISPR/Cas9 system in plant genome editing mainly focus on gene function research and genetic improvement of crops. It has shown various genome-editing abilities, such as gene knockout, knock-in or replacement, base editing, prime editing, and expression regulation (**Figure 1**).

3.1 Gene Knockout

Gene knockout is the most extensive application of the CRISPR/Cas9 system in plant functional genome research, which can be divided into single and multiplex gene knockout (**Figure 1A**). Under the guidance of sgRNA, the Cas9 nuclease cleaves the target DNA segment in the exon region of the gene to cause DSBs. The preferred NHEJ repair pathway is prone to produce insertions/deletions of one or several bases near the cutting site (Feng et al., 2014; Ma et al., 2015). When the number of inserted or missing bases is not multiple 3, the frameshift mutation will occur, and the target protein cannot be correctly encoded. It is worth noting that small insertions or deletions in edited cell lines may produce abnormal transcripts or proteins, causing unexpected effects that complicate functional analysis (Tuladhar et al., 2019). Therefore, many studies tend to produce two DSBs within a distance to delete larger genomic fragments to make true null alleles of coding and non-coding genes by NHEJ or microhomology-mediated end joining (MMEJ) repair (Owens et al., 2019; Tan et al., 2020).

Based on the high efficiency of CRISPR/Cas9 system-mediated gene knockout, multiplex gene knockout technology provided great convenience for functional analyzing the gene families, elucidating the regulation of multiple genes for complex agronomic traits, and analyzing the signal pathway in plants. Multiple sgRNAs with different targets for different genes can be designed and assembled into an expression cassette for transformation (**Figure 1A**). For example, our group constructed a CRISPR/Cas9 vector for targeting eight agronomic genes simultaneously based on the isocaudamer-based method. All editing genes have high mutation efficiencies in the T_0 generation, and both heterozygous and homozygous genotypes at eight genes were obtained (Wang et al., 2015; Shen et al., 2017). In wheat, using the polycistronic tRNA strategy, Xia's group established an efficient CRISPR-Cas9 multiplex system which can edit multiplex genes simultaneously. They succeeded in targeting mutagenesis at up to 15 genomic loci, restoring transgenic free plants and pyramiding favorable alleles in an elite wheat variety within 1 year (Luo et al., 2021). In the case of homologous genes

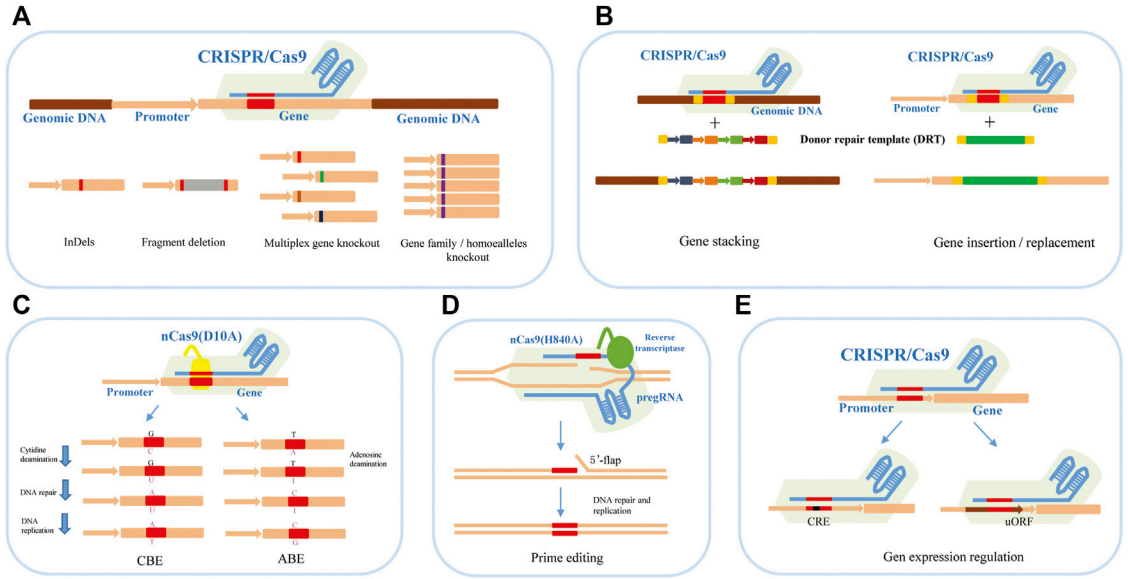

FIGURE 1 | The applications of the CRISPR/Cas9 system in plant genome editing. **(A)** CRISPR/Cas9-mediated gene knockout can achieve InDels, fragment deletion, and multiplex gene knockout. **(B)** CRISPR/Cas9-mediated gene knock-in and replacement mediated by HDR can achieve gene stacking and gene insertion or replacement to produce new traits in breeding. **(C)** CRISPR/Cas9-mediated base editing for crop trait improvement including CBE-mediated C-to-T base-editing strategy and ABE-mediated A-to-G base-editing strategy. **(D)** CRISPR/Cas9-mediated prime editing for crop trait improvement. **(E)** CRISPR/Cas9-mediated gene expression regulation by editing the cis-regulatory elements and upstream open reading frames (uORFs).

or gene families, one sgRNA targeting the conserved sequence can also achieve good results (Li A. et al., 2018; Sánchez-León et al., 2018).

3.2 Gene Knock-In or Replacement

During plant domestication, some alleles are consistent and fixed within elite germplasm, such as the wheat *Q* allele (Zhang et al., 2011), rice *qSH1* (Konishi et al., 2006), maize *teosinte branched 1* (Doebley, 2004) and tomato *fw2.2* (Frary et al., 2000). Importantly, there are still many beneficial and favourable alleles in some local germplasm or related species. The replacement of endogenous genes or DNA fragments by the HDR pathway plays a useful role in crop breeding and trait improvement. HDR-mediated editing is a powerful genome-precise editing tool that enables targeted gene replacement and direct introduction of elite alleles from local or related species into commercial varieties within a few generations without any linkage drag (**Figure 1B**). At present, precise knock-in or replacement of gene fragments has been achieved in many plants (Svitashev et al., 2015; Luo et al., 2016; Sun et al., 2016; Begemann et al., 2017; Shi et al., 2017; Wang et al., 2017; Li J. et al., 2018; Li S. et al., 2018; Hummel et al., 2018; Li et al., 2019). However, the genes of previous reported HDR cases are often resistant genes, which rely on selection pressure, or visible makers to enrich the HDR events. The editing efficiency is very low. There are four main challenges in performing HDR in plants: 1) HDR is in constant competition with NHEJ for the repair of DSBs, but the latter is the main pathway of DSB repair (Puchta 2005; Fauser et al., 2014; Shi et al., 2017; Fan et al., 2021); 2) HDR is only active during the late S and G2 phases, while the NHEJ is active during the overall cell cycle except for mitosis (Heyer et al., 2010; Karanam et al., 2012; Truong et al., 2013; Orthwein et al., 2014); 3) The donor repair template (DRT) is limited to deliver into the nucleus/cells for HDR; 4) The timing of DSBs induction and DRT transmission in target genes is difficult to coordinate (Gil-Humanes et al., 2017; Wang et al., 2017; Li and Xia, 2020).

To make editing more efficient, various strategies have been attempted in plants. There are mainly the following aspects: 1) Increasing the amount of DRT by using the bombardment deliver method or geminivirus replicons (GVR) (Baltes et al., 2014; Svitashev et al., 2015; Sun et al., 2016; Dong et al., 2020); 2) Improving the Cas9 frequency by using the specific promotors, such as egg cell- or early embryo-specific gene promoter; 3) Coordinating the delivery of Cas protein, two single-guide RNAs (sgRNAs) and DRT by using the all-in-one vector which includes all components (Peng et al., 2019). Despite these efforts, further improving HDR frequency and delivering sufficient DRT into plant cells remains very challenging.

3.3 Base Editing

Single-nucleotide point (SNP) mutations are the genetic basis for sharping phenotypic diversity among individuals. Base editing refers to the transformation of a single base at a specific position in a target DNA fragment. This technology does not depend on the generation of DNA DSBs so as to avoid both the randomness of NHEJ and the low efficiency of HDR. Base editors are created by fusing an engineered base modification enzyme, such as deaminase, with the catalytically dead version of the *Streptococcus pyogenes* Cas9 (dCas9) or nickase version of Cas9 (nCas9) (D10A) and currently include cytosine base

editors (CBEs) and adenine base editors (ABEs) (**Figure 1C**). CBEs use a naturally occurring cytidine deaminase to convert target cytosines to uracil, which catalyzes C•G to T•A conversion (Komor et al., 2016). Drawing inspiration from CBEs, adenosine deaminase would result in inosine, which is read as guanine by replication and transcription machinery. Therefore, ABEs would correct A•T to G•C mutation theoretically (Gaudelli et al., 2017). The establishment of CBEs and ABEs system enables single base editing to realize four types of base conversion and has been successfully used in crop plants (Bharat et al., 2020). Recently, some progress has been made in the study of crop gene-directed evolution using base editing technology. For example, Li et al. (2020b) developed saturated targeted endogenous mutagenesis editors (STEMES) fusing cytidine deaminase with adenosine deaminase to achieve C: G > T: A and A: T > G: C substitutions simultaneously, which facilitate directed evolution of plant genes by generating *de novo* mutations. A series of highly efficient BE toolkits were developed to achieve C-to-T mutation almost without PAM restriction, and the A-to-G mutation scope was largely expanded in rice (Zhang et al., 2021). Kuang et al. (2020) presented a base-editor-mediated gene evolution method (BEMGE), which is a powerful tool that can accurately identify functional genetic variations and develop specific traits in crop breeding programs soon.

3.4 Prime Editing

Although the base editing technique has been able to achieve precise C•G–T•A and A•T–G•C base conversion, it is difficult to achieve precise transversion between pyrimidine and purine bases and precise insertion or deletion of small fragments. In 2019, David R. Liu's lab developed a revolutionary new tool for precise gene editing, the prime editor (PE), which is a search-and-replace genome-editing method and has realized all 12 kinds of base substitutions, precise insertions of up to 44 bp, deletions of up to 80 bp and combinations of these edits in human cells (Anzalone et al., 2019). PE is composed of three components: nCas9(H840A), reverse transcriptase (RT), and prime editing guide RNA (pegRNA). Compared with sgRNA of CRISPR/Cas9 system, pegRNA has an additional RNA sequence at the 3′ end, including prime binding site (PBS) that pairs with the nCas9 (H840A)-nicked ssDNA strand sequence and initiates RT, and reverse transcriptase template (RT template) that contains the genetic information for the desired mutations. Under the guidance of pegRNA, nCas9(H840A) cleaves and releases the non-target DNA strand to hybridize to the PBS in the pegRNA. Reverse transcriptase synthesizes new DNA using the RT template, and the newly synthesized DNA containing the target editing is introduced into the genome to replace the original DNA sequence through DNA repair (**Figure 1D**). At present, this technology has been established and optimized in plants (Lin et al., 2020). However, the editing efficiency of PE is very low and varies greatly at different loci, which cannot meet the needs of basic research and practical application (Li J. et al., 2020; Gao et al., 2021). Scientists have optimized it from different angles to improve the editing efficiency of PE in plants. For example, Lin et al. (2020) optimized prime editors for plants through codon, promoter, and editing-condition optimization. They successfully obtained the regenerated prime-edited rice plants at frequencies of up to 21.8%. Lin et al. (2021) evaluated the melting temperature of PBS and showed that 30°C leads to maximal efficiency. They also presented a dual-pegRNA approach, which uses two pegRNAs *in trans* encoding the same edits, substantially enhances PE efficiency. A web application called PlantPegDesigner was provided to simplify the design of optimal pegRNAs or pegRNA pairs.

3.5 Expression Regulation

Although the most frequent application of CRISPR/Cas9 is to create null alleles by targeting the coding sequences, loss-of-function mutations in coding regions may result in pleiotropic or deleterious effects (Li et al., 2020a; Hendelman et al., 2021). Numerous researches in both animals and plants have revealed that many genetic changes driving evolution, domestication, and breeding occurred in *cis*-regulatory regions, including upstream, introns, and downstream regions of genes (Wang et al., 2014; Ding et al., 2021). Compared with coding region mutations, *cis*-regulated region editing is more likely to induce small phenotypic changes by modifying the level, timing, or space of gene expression, which benefit crop improvement.

To date, the alteration of gene expression in plants genome editing has focused mainly on editing or directly deleting the *cis*-regulatory elements (CRE) in the promoter region of the target genes (**Figure 1E**). For example, Rodríguez-Leal et al. (2017) edited the promoters of genes that control fruit size, inflorescence branching, and plant architecture by using a multiplexed CRISPR/Cas9 promoter targeting approach. This approach obtained the artificial QTL variation and precisely regulated fruit size and other important agronomic traits in tomatoes. Hendelman et al. (2021) used a *cis*-regulatory editing system to generate a comprehensive allelic series for tomato *WUSCHEL HOMEOBOX9* (*WOX9*), which plays a species-specific role in embryo and inflorescence development. This research showed that tomato *WOX9* has a pleiotropic function, which is regulated by *cis*-sequence elements at different locations of the gene promoter region. A similar strategy, Liu et al. (2021) achieved quantitative variation of maize yield-related traits by making weak promoter alleles of CLE genes associated with increased meristem size through CRISPR-based promoter fine-tuning.

In many cases, many genes that regulate important traits require a high rate of translation to achieve crop improvement, rather than loss or reduction of function (Xu et al., 2017; Reis et al., 2020). Upstream open reading frames (uORFs) are important *cis*-regulatory elements in the 5′ leading sequence of eukaryotic mRNAs, and usually inhibit the translation initiation of downstream ORFs through ribosome stalling (Zhang et al., 2020; Kurihara, 2020). Fine-tuning the translation by regulating uORFs can effectively improve the translation efficiency of target genes for the improvement of crop traits (**Figure 1E**). For example, editing the uORF of *LsGGP2* increased oxidative stress tolerance and the ascorbate content of lettuce by ~150% (Zhang et al., 2018). In *Arabidopsis thaliana*, deleting the uORF of *PHO1* increased shoot Pi content and improved shoot growth under low external Pi supply (Reis et al., 2020).

TABLE 1 | List of research on crop quality improvement by using CRISPR/Cas9 system.

Traits	Target traits	Crop	Target gene	Type of edit	References
Macronutrient improvement	Starch	Rice	*GBSSI*	Gene knockout	Ma et al. (2015) Zhang et al. (2017) Fei et al. (2019)
			GBSSI	Expression regulation	Huang L. et al. (2020) Zeng et al. (2020b)
			GBSSI	Base editing	Li H. et al. (2020) Xu Y. et al. (2021)
		Barley	*GBSSI*	Gene knockout	Zhong et al. (2018)
		Cassava	*GBSSI*	Gene knockout	Bull et al. (2018)
		Maize	*GBSSI*	Gene knockout	Gao et al., 2020; Dong et al. (2019)
		Rice	*SBEIIb*	Gene knockout	Sun et al. (2017)
		Wheat	*SBEIIa*	Gene knockout	Li et al. (2021)
	Proteins	Barley	*D-hordein*	Gene knockout	Li Y. et al. (2020)
		Wheat	*a-gliadin*	Gene knockout	Sánchez-León et al. (2018)
		Sorghum	*a-kafirins*	Gene knockout	Li A. et al. (2018)
		Soybean	*Gly m Bd 28 K* *Gly m Bd 30 K*	Gene knockout	Sugano et al. (2020)
		Rice	*OsAAP6, OsAAP10*	Gene knockout	Wang et al. (2020)
	Oils	Rapeseed	*BnaA.FAD2.a*	Gene knockout	Okuzaki et al. (2018)
		Rapeseed	*BnaFAD2*	Gene knockout	Huang H. et al., 2020
		Rapeseed	*BnTT8*	Gene knockout	Zhai et al., 2020
		Camelina	*CsFAD2*	Gene knockout	Lee et al. (2021)
		Soybean	*FAD2-1*	Gene knockout	Al Amin et al. (2019)
		Soybean	*FAD2-2*	Gene knockout	Do et al. (2019)
		Rice	*OsFAD2-1*	Gene knockout	Abe et al. (2018)
Micronutrient improvement	Anthocyanins	Rice	*Rc*	Gene knockout	Zhu et al. (2019)
		Tomato	*ANT1*	Gene knock-in	Čermak et al. (2015)
		Tomato	*SlMYB12*	Gene knockout	Deng et al., 2018; Yang T. et al. (2019)
		Carrot	*DcMYB7*	Gene knockout	Xu et al. (2019)
	Vitamins	Rice	*SSU-crtI, ZmPsy*	Gene knock-in	Dong et al. (2020)
		Lettuce	*LsGGP2*	Expression regulation	Zhang et al. (2018)
	γ-aminobutyric acid	Tomato	*SlGAD2, SlGAD3*	Gene knockout	Nonaka et al. (2017)
		Rice	*GAD3*	Gene knockout	Akama et al. (2020)
		Tomato	*GABA-TP1, GABA-TP2, GABA-TP3, CAT9, SSADH.*	Gene knockout	Li R. et al. (2018)
Elimination of Anti-nutritional factors	Phytic acid	Rice	*OsPLDα1*	Gene knockout	Khan et al. (2019)
		Rapeseed	*BnITPK*	Gene knockout	Sashidhar et al. (2020)
	Heavy metals	Rice	*OsNramp5*	Gene knockout	Tang et al. (2017)
		Rice	*OsHAK1*	Gene knockout	Nieves-Cordones et al. (2017)
	Enzymatic browning	Potato	*StPPO2*	Gene knockout	González et al. (2020)
	Steroidal glycoalkaloids	Potato	*St16DOX*	Gene knockout	Nakayasu et al. (2018)
	Acrylamide	Wheat	*TaASN2*	Gene knockout	Raffan et al. (2021)
Other improvements	Fragrant aromas	Rice	*OsBADH2*	Gene knockout	Ashokkumar et al. (2020); Hui et al. (2021)
		Rice	*OsBADH2*	Gene knockout	Tang et al. (2021)
		Maize	*BADH2*	Gene knockout	Wang et al. (2021)
	Shelf-life	Banana	*MaACO1*	Gene knockout	Hu et al. (2021)
		Petunias	*PhAC 1*	Gene knockout	Xu et al. (2019)
		Tomato	*RIN*	Gene knockout	Ito et al. (2015)
		Tomato	*Cnr*	Gene knockout	Gao et al. (2019)
		Tomato	*Nor*	Gene knockout	Gao et al. (2019)
		Tomato	*ALC*	Gene replacement	Yu et al. (2017)
		Tomato	*PL*	Gene knockout	Wang D. et al. (2019)

4 CRISPR/CAS9 SYSTEM FOR CROP QUALITY IMPROVEMENT

A balanced, varied, and appropriate healthy diet ensures a person's needs for macronutrients and essential micronutrients. It protects against undernutrition, micronutrient deficiencies, and non-communicable diseases such as obesity, diabetes, heart disease, stroke, and cancer. This is the foundation of a good life. The edible part of crops is mainly composed of macronutrients (starch, protein and oil) and micronutrients (anthocyanins, vitamins, γ -aminobutyric acid *etc.*). In the past 5 years, scientists have used CRISPR/Cas9 technology to study the nutritional components of crops and flavour and storage characteristics, and achieved great success (**Table 1**).

4.1 Macronutrient Improvement

4.1.1 Starch

Starch, a high molecular carbohydrate, is a major component of harvestable crop organs and a major energy source in the human diet and an industrial feedstock. It widely exists in maize (*Zea Mays*), rice (*Oryza sativa*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), potato (*Solanum tuberosum*), cassava (*Manihot Esculenta*), and other starch crops. Starch consists of amylopectin and amylose and is stored as semi-crystalline granules in the chloroplasts of leaves and amyloplasts of storage organs. Amylopectin has a dendritic structure, which determines the crystallinity of starch granules. Amylose is a linear glucose polymer, accounting for only about 20% of the granules. Still, it strongly determines the physicochemical properties of starch, such as stickiness, palatability, and digestibility during cooking and processing (Jobling, 2004; Sonnewald and Kossmann, 2013; Emmambux and Taylor, 2016). At present, the genetic mechanism of starch has been well analyzed. With glucose-1-phosphoric acid (Glc-1-P) as raw material, ADPG is formed under the action of glucose pyrophosphorylase (AGPase). Amylose is synthesized under the action of grain binding starch synthase GBSSI; Amylopectin is synthesized by soluble starch synthase (SSS), branching enzyme (SBE) and debranching enzyme (DBE). At the molecular level, all the genes involved in starch synthesis and regulation can affect starch quality. Knocking out *Wx* (GBSSI) leads to the rapid reduction of amylose, amylopectin content up to nearly 100% in starch granules, referred to as waxy or glutinous starch. For example, Ma et al. (2015), Zhang et al. (2017) and Fei et al. (2019) successfully reduced the amylose content of the mutant to less than 5% by knocking out the exon region of rice waxy gene *Wx* to obtain waxy rice. The same knockout strategy is also used for barley and cassava (Bull et al., 2018; Zhong et al., 2018). Gao et al. (2020) created waxy corn hybrids by deleting large segments of a *waxy* allele using CRISPR–Cas9 in 12 elite inbred maize lines. Field trials at 25 locations showed that CRISPR-waxy hybrids were agronomically superior to introgressed hybrids. Specially, this CRISPR-Cas9 waxy corn is considered unregulated by the relevant regulations of APHIS and has been conducted a pre-commercial launch in the Midwestern United States in 2019. 'Sweet-waxy compound corn' is now widely favoured for its chewiness and sweetness. Dong et al. (2019) edited *SH2* and *Wx* and identified single or double mutations that can be used to produce super-sweet, waxy or sweet and waxy compound corns (SWCs) that can be used in specialty corn breeding. In addition to occurring frameshift of *Wx*, scientists are trying to fine-tune AC by manipulating *Wx* at expression, post-transcriptional, and translational levels. Rice *Wx* variants including Wx^{lv}, Wx^{a}, Wx^{b}, Wx^{in}, Wx^{mw}, $Wx^{op/hp}$, Wx^{mp}, Wx^{mq}, and *wx* determine the eating and cooking quality (ECQ) of rice. Huang L. et al. (2020) generated six novel *Wx* alleles by editing the region near the TATA box of the Wx^{b} promoter, which downregulated *Wx* expression and fine-tuned grain AC. Zeng et al. (2020b) targeted the 5′UTR intronic splicing site (5′UISS) of Wx^{a} to alter the intron-splicing efficiency and generate new quantitative trait alleles. Li et al. (2020c) and Xu Y. et al. (2021) used the CRISPR/Cas9-mediated base editing system to target regions closed to the mentioned 'soft rice' allele responsible sites for mild reduction of rice AC.

Foods with high amylose content and resistant starch (RS) contribute to improving human health and reducing the risk of serious noninfectious diseases, while cereal crops high in RS are not widely available (Zhu et al., 2012). Researches showed that the starch branching enzyme (*SBE*) gene controls amylopectin synthesis, and the contents of amylose and resistant starch (RS) would increase in SBE mutated crops (Shimada et al.,2006). Sun et al. (2017) conducted targeted mutagenesis of rice *SBEIIb* using CRISPR/Cas9 technology, and the AC and RS contents were significantly increased to 25.0 and 9.8%, respectively. Li et al. (2021) also conducted directed mutagenesis of *TaSBEIIa* of winter wheat and spring wheat varieties through CRISPR/Cas9, and obtained transgenic high-straight wheat with improved starch composition, structure and properties.

4.1.2 Proteins

Plant seed storage proteins (SSPs) are important sources of human dietary protein, mainly from cereals and legumes. According to the solubility-linked physical properties, SSPs are classified as four fractions: water-soluble albumins, salt-soluble globulins, alcohol-soluble prolamins, and alkaline-soluble glutelins. The proportions of these four kinds of proteins in different crop seeds are different. For example, glutelin is the most abundant protein fraction in rice and comprises about 60–80% of the total SSPs, while prolamin is the dominant one in maize, wheat and barley (Yang Y. et al., 2019). Many studies have shown that the level and proportion of protein fraction content greatly impact crop quality. Manipulating seed storage protein content by CRISPR/Cas9 gene editing is an effective way to regulate seed nutritional value. In barley, D hordein is one of the storage proteins in the grain, which has a negative effect on malting quality. Li Y. et al. (2020) used CRISPR/Cas9 technology to edit the *D hordein* gene in a spring barley cultivar and obtained two mutated lines. Transcriptomic analysis and protein SDS-PAGE showed that the transcription level of *the D hordein* gene and D hordein content in the mutant was lower than that of the wild type, which provided a basis for breeding high malt quality varieties. In wheat, the α-gliadin family is the main protein

group associated with celiac disease. This genetic autoimmune disorder damages the small intestine and interferes with the absorption of nutrients from food. The gliadin contains a 33-amino acid polypeptide, called 33-mer, the main immunodominant peptide in celiac patients. Sánchez-León et al. (2018) targeted a conserved region adjacent to the coding sequence for the 33-mer in the gliadin genes with two sgRNAs and obtained low gluten hexaploid and durum wheat, of which immunoreactivity was reduced by 85%. A similar knockout strategy was carried out in sorghum. A single sgRNA was designed to mutate conserved region encoding the α-kafirins endoplasmic reticulum signal peptide. Edited plants' grain protein digestibility and lysine content significantly increased (Li A. et al., 2018). In soybean, many allergens have been identified, resulting in 5–8% of babies and 2% of adults being allergic to soybean in the United States and Europe (Heppell et al., 1987). Sugano et al. (2020) designed two sgRNAs simultaneously site-directed mutate two genes encoding the major allergens Gly m Bd 28 K and Gly m Bd 30 K in two Japanese soybean varieties and successfully obtained Cas9-free plants with no Gly m Bd 28 Kor Gly m Bd 30 K protein. In rice, grain storage protein seriously affects the quality of rice, especially the eating and cooking quality (ECQ) (Yang et al., 2015; Yang et al., 2020). Generally, the higher rice grain protein content (GPC) will lead to the worse ECQ; thus, the cultivars with good ECQ always are required to have relatively lower GPC, usually less than 7%. Wang et al. (2020) used CRISPR/Cas9 system to knock out *OsAAP6* and *OsAAP10* in three high-yielding *japonica* varieties and one *japonica* line, respectively. The protein content of the mutants was decreased, and the ECQ was increased significantly.

4.1.3 Oils

Seed oils are primarily used as edible oils, and their industrial application has also been gradually increasing (Biermann et al., 2011). The most seed oil contains high content of polyunsaturated fatty acids (PUFAs) such as linoleic acid but low content of monounsaturated acids (MUFAs) such as oleic acid. Notably, PUFAs oil tends to oxidize, resulting in rancidity, off-flavours, and short shelf-life. In contrast, high MUFAs oil is 10-fold higher auto-oxidizing stable than linoleic acid, which is not only beneficial for lowering cholesterol and reducing systolic blood pressure, but also preferred for industrial uses, for example as biodiesel duels and biolubricants (O'Keefe et al., 1993; Davis et al., 2008; Terés et al., 2008). Therefore, industry and food products prefer to use high-oleic vegetable oil, and many studies have begun to alter the fatty acid composition of oilseed crops artificially. Fatty acid desaturase 2 (FAD2) catalyzes the conversion of oleic acid to linoleic acid in plants, and many studies reported that suppressing *FAD2* gene expression can develop the high-oleic oilseed crops (Okuley et al., 1994; Sivaraman et al., 2004; Jung et al., 2011; Nguyen et al., 2013; Chen et al., 2015; Lee et al., 2016; Lee et al., 2017; Wood et al., 2018). Recently, scientists have done a lot of work to improve the oil quality using CRISPR/Cas9 technology, mainly focusing on some oil crops, such as rapeseed, soybean, camelina, *etc*. Okuzaki et al. (2018) targeted the *BnaA.FAD2.a* (*FAD2_Aa*) in *B. napus* to increase the oleic acid content. Huang H. et al. (2020) designed two sgRNAs, one of which targets four copies of *BnaFAD2*. The oleic acid content in seeds of mutant increased significantly, with a maximum of more than 80% compared with wild type of 66.43%, and with a decrease in linoleic and linolenic acid content. Compared with black-seeded rape, yellow-seeded *B. napus* has the characteristics of the thinner seed coat, low lignin and polyphenol content, high oil content and high protein content, so it is widely accepted as a good-quality trait. Zhai et al. (2020) targeted *BnTT8,* which controls flavonoid accumulation in crops, to successfully obtain yellow-seeded lines with elevated oil and protein content and altered fatty acid (FA) composition using the CRISPR/Cas9 system. In hexaploid *Camelina sativa*, Lee et al. (2021) used a single guide RNA covering the common region of the three *CsFAD2* homologs. When all three homologous genes were knocked out, seed MUFA levels increased by nearly 80%, but with a stunted bushy phenotype. However, transformants with two pairs of *CsFAD2* homologs mutated but the other pair with wild-type heterozygous showed normal growth, and a seed MUFAs production increased up to 60%. In soybean, Al Amin et al. (2019) and Do et al. (2019) respectively mutated *FAD2-1* and *FAD2-2* loci using the CRISPR/Cas9 system to increase the oleic acid content in edited soybean plants. Besides oil crops, rice bran oil (RBO) contains many valuable healthy constituents, including oleic acid. In rice, three functional *FAD2* genes were found, and only *OsFAD2-1* is the highest expressed in rice seeds. Abe et al. (2018) disrupted the *OsFAD2-1* gene by CRISPR/Cas9 system, and the oleic acid content of homozygous knockout plants was increased to more than twice that of wild type.

4.2 Micronutrient Improvement

4.2.1 Anthocyanins

Anthocyanins are water-soluble flavonoid compounds widely distributed in plants and confer pigmentation to plants. They are all recognized antioxidants with human health benefits, such as reducing the risk of diabetes, obesity, cardiovascular disease (CVD), and certain cancers (Wang and Stoner 2008; Tsuda 2012; Vinayagam and Xu 2015; Wallace et al., 2016). Wild rice species (*Oryza rufipogon L.*)are rich in proanthocyanidins and anthocyanidins and show red pericarp, which is regulated by two complementary genes *Rc* and *Rd*. *Rc* encodes a basic helix-loop-helix (bHLH) transcription factor, and *Rd* encodes a dihydroflavonol-4-reductase (DFR) protein (Sweeney et al., 2006; Furukawa et al., 2007). At present, most cultivated rice varieties produce white grain due to the frameshift mutation in the exon of the *Rc* gene. Zhu et al. (2019) successfully reverted frameshift mutation into in-frame mutations by using CRISPR/Cas9-mediated method, restoring the function of *Rc* allele and converting three elite white pericarp rice varieties into red ones with high content of proanthocyanidins and anthocyanidins. Čermák et al. (2015) inserted a 1938 bp donor template into the promoter region of tomato *ANT1* gene controlling anthocyanin biosynthesis, resulting in overexpression and ectopic accumulation of pigments in tomato tissues. In addition, consumers in different regions have different fruit colour preferences; scientists also use CRISPR/Cas9 technology to regulate genes related to anthocyanin synthesis to achieve the

effect of changing fruit colour. For example, European and the American consumers prefer red fruit tomatoes, while pink fruit tomatoes are more popular in Asian countries, especially in China and Japan (Lin et al., 2014). However, most tomato breeding materials are red fruit materials. Using the CRISPR/Cas9 system, Li's group accelerated the breeding process by disrupting the *SlMYB12* gene, a key determinant for flavonoid accumulation, of a superior red tomato inbred line to produce tomato plants with pink fruit (Deng et al., 2018; Yang T. et al., 2019). Xu et al. (2019) knocked the *DcMYB7,* which activates the expression of its DcbHLH3 partner, a structural gene in the anthocyanin biosynthetic pathway, in a solid purple carrot using CRISPR/Cas9 system resulted in carrots with yellow roots.

4.2.2 Vitamins

Vitamins are a small part of the organic compounds that are needed in the human diet. We have known that vitamin deficiency is directly linked to human disease. For example, vitamin A deficiency causes visual problems, such as night blindness and even blindness in severe deficiency. However, as rice lack provitamin A (mainly β-carotene), the poor populations in the developing countries of South and Southeast Asia, where white rice is a staple food, cannot meet vitamin A intake dependency criteria. Dong et al. (2020) inserted a 5.2 Kb carotenoid biosynthesis cassette consisting of the coding sequences of *SSU-crtI* and *ZmPsy* at two genomic safe harbors in rice using CRISPR-Cas9 technology and successfully obtained marker-free rice plants with high carotenoid content in seeds. Numerous epidemiological studies have shown a positive association between dietary or plasma levels of vitamin C content and health benefits. The major source of vitamin C in the human diet is ascorbic acid (ASA) from fruit and vegetables. Zhang et al. (2018) targeted the uORF initiation codon region of *LsGGP2*, a key enzyme in vitamin C biosynthesis in lettuce, it not only increased the antioxidant stress ability of lettuce, but also increased ascorbate content by ~150%.

4.2.3 γ-aminobutyric Acid (GABA)

The γ-aminobutyric acid (GABA), a four-carbon nonprotein amino acid widely presenting in plants, functions as an inhibitory neurotransmitter in the central nervous system for animals to alleviate hypertension ((Bachtiar et al., 2015). In plants, GABA is first synthesized from its precursor glutamate by glutamate decarboxylase (GAD), and then catabolized to succinate by GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH) in a subsequent reaction. Applying the CRISPR/Cas9 system to regulate the related genes in the GABA synthesis pathway can rapidly increase the GABA content plants and improve crops nutritional quality. Previous reports indicated that GAD has a C-terminal autoinhibitory domain that regulates enzymatic function, and deletion of this domain increases GAD activity. Nonaka et al. (2017) deleted the autoinhibitory domain of *SlGAD2* and *SlGAD3*, expressed during tomato fruit development using the CRISPR/Cas9 system, and the premature termination before the autoinhibitory domain increased GABA accumulation by 7–15 fold. Similarly, Akama et al. (2020) knocked the GAD3, which is predominantly expressed in rice seeds and obtained the edited lines with seven-fold higher levels of GABA. Li R. et al. (2018) manipulated the GABA shunt in tomatoes by targeting five key genes, namely *GABA-TP1*, *GABA-TP2*, *GABA-TP3*, *CAT9* and *SSADH*. The accumulation of GABA in the leaves and fruits of the edited lines was significantly increased, and the GABA content in the leaves of quadruple mutants was 19-fold higher than that of wild type.

4.3 Elimination of Anti-nutritional Factors

Anti-nutritional factors (ANFs) refer to substances in the feed that adversely affect digestion, absorption and utilization of nutrients and cause adverse physiological reactions in humans and animals, such as phytic acid (PA) and heavy metal quinones, steroidal glycoalkaloids, and free asparagine. The use of gene-editing techniques to eliminate the ANFs in crops edible parts could benefit human health.

4.3.1 Phytic Acid

PA acts as a major reservoir of phosphorus in seeds from cereals to oilseeds but strongly chelates essential minerals in human and monogastric animals, leading to so-called "hidden hunger." The lipid-dependent and lipid-independent pathways are two known phytic acid biosynthesis pathways (Bhati et al., 2014; Kuo et al., 2018). The lipid-dependent pathway involves the inositol lipid phosphatidylinositol (PI), which produces phytic acid through a continuous reaction processes. Khan et al. (2019) used the CRISPR/Cas9 system to generate mutants of a phospholipase D gene (*OsPLDα1*) to disrupt the production of phosphatidic acid and reduce the phytic acid in rice seeds. Compared with the wild type, the expression of key genes related to phytic acid biosynthesis was changed and the phytic acid content was significantly reduced in *osplda1* mutants. In *Brassica napus. L.*, the key enzyme ITPK (inositol tetrakisphosphate kinase) catalyzes the penultimate step for synthesising PA in the lipid-independent pathways (Raboy, 2009). Knocking out three functional paralogs of *BnITPK* resulted in low PA and high free phosphorus using CRISPR-Cas9 system (Sashidhar et al., 2020).

4.3.2 Heavy Metals

Heavy metals, which can be taken up by crops and transported to their edible parts, is widely known to be harmful to health. Cadmium (Cd) is a highly toxic heavy metal that causes osteoporosis, kidney failure, cancer, and cardiovascular diseases for humans (Bertin and Averbeck, 2006). Rice with excessive cadmium is the main source of dietary cadmium intake. Previous researches reported that the natural resistance-associated macrophage proteins 5 (NRAMP5) mediate the root uptake of Cd (Ishikawa et al., 2012; Sasaki et al., 2012). Tang et al. (2017) designed two sequence-specific single guide RNA (sgRNA) to target exon IX of *OsNramp5* in two rice cultivars. Hydroponic culture and Cd-contaminated paddy field trials showed that Cd concentrations were dramatically decreased in shoots and roots of *osnramp5* mutants.

As a result of the Fukushima nuclear accident, massive releases of radioactive cesium (Cs) isotopes ^{134}Cs (2-year half-life) and ^{137}Cs (30-year half-life) are expected to have contaminated about half of Japan's soil (Yasunari et al., 2011). Cesium (Cs) is a group I alkali metal with chemical properties similar to potassium (K). Several cloned K^+ transporters, like HAK/KUP/KT family, can also transport Cs^+ in the plants (Véry et al., 2014; Scherzer et al., 2015). Nieves-Cordones et al. (2017) used the CRISPR-Cas system to knock out the *OsHAK1*, resulting in a strong reduction of radioactive cesium contents in mutated plants when grown in Fukushima soil highly contaminated with $^{137}Cs^+$.

4.3.3 Enzymatic Browning

Enzymatic browning refers to the process in which polyphenol oxidases (PPOs) catalyze the formation of phenolic substances into quinones in the presence of oxygen, resulting in the formation of dark precipitate in fruits and vegetables and loss of nutritional quality. A lower PPO activity in plants would reduce the enzymatic browning phenotype. González et al. (2020) induced *StPPO2* gene mutations in tetraploid potato using CRISPR/Cas9 system. Compared to the control, mutations in the four alleles of the *StPPO2* gene resulted in a reduction of PPO activity by up to 69% and a 73% reduction in enzymatic browning in tubers.

4.3.4 Steroidal Glycoalkaloids

Also, in potatoes, there are high levels of toxic compounds of steroidal glycoalkaloids (SGAs), α-solanine and α-chaconine, in the flowers and the tuber sprouts. Nakayasu et al. (2018) edited *St16DOX* encoding a steroid 16α-hydroxylase in SGA biosynthesis, to generate two SGA-free *St16DOX*-disrupted potato hairy root lines.

4.3.5 Acrylamide

Acrylamide in food is a processing contaminant that forms from free asparagine and potentially increases the risk of developing cancer for humans. In wheat, Raffan et al. (2021) knocked out the asparagine synthetase gene *TaASN2* using four guide RNAs targeting all three homologues of *TaASN2*. Compared with the wild type, the concentration of free asparagine in seeds of the plants with all six *TaASN2* alleles edited was significantly decreased, up to 90%.

4.4 Other Improvements

4.4.1 Fragrant Aromas

The fragrant aromas of dishes or staple food keep the mind at ease and improve appetite. The most famous example is the aromatic rice varieties basmati and jasmine rice, with a popcorn-like scent, which are popular worldwide. 2-acetyl-1-pyrroline (2AP) is the key flavour compound in rice aroma volatiles. Rice flavour is mainly controlled by recessive genes *OsBadh2/fgr* (betaine aldehyde dehydrogenase 2). It is reported that *OsBadh2* converts γ-aminobutyraldehyde (GABald) to gamma-aminobutyric acid (GABA), and the reduced or loss of BADH2 activity promotes the GABald to be converted into 2AP (Bradbury et al., 2005). Comparative sequencing revealed an 8bp deletion in the 7th exon of *OsBadh2* in most fragrant rice varieties, which resulted in the loss of the original function of *Badh2*, thus producing fragrance in rice leaves and grains. Based on it, Ashokkumar et al. (2020) employed the CRISPR/Cas9 tool to target the 7th exon of *OsBADH2* and created novel alleles to introduce aromas into an elite non-aromatic rice variety. Similary, Hui et al. (2021) targeted the 7th exon of *OsBADH2* in no-fragrant *japonica* and *indica* varieties and provided important genetic resources for grain aroma improvement in three-line hybrid rice. Tang et al. (2021) first used CRISPR/Cas9 to delete the exon nucleotide at the exon-intron junction of *OsBADH2*, which induces the exon skipping of *OsBADH2*, resulting in high 2AP production and grain fragrance. As rice, naturally fragrant germplasm has been observed in other plants, such as soybean (Juwattanasomran et al., 2011), cucumber (Yundaeng et al., 2015), coconut (Vongvanrungruang et al., 2016), sorghum (Yundaeng et al., 2013), and mung bean (Attar et al., 2017). The 2AP accumulation all results from a loss of function, a weak allele, or lower expression of *BADH2*. However, no such germplasm was found in maize. Wang et al. (2021) generated the word's first aromatic maize by simultaneous genome editing of the two *BADH2* genes.

4.4.2 Long Shelf-Life

Crop shelf life is a key quality trait in the modern supply chain, especially for fruit and ornamental crops. The short shelf life greatly limits crops' transportation, marketing, and storage, resulting in huge postharvest losses. Ethylene is the natural plant hormone that makes fruits ripen and flowers to senescence quickly. Therefore, genetic modification to reduce endogenous ethylene or impair the ethylene biosynthetic pathway might be an effective method to prolong the shelf life of crops (Elitzur et al., 2016). Ethylene derived from methionine is converted to S adenosylmethionine (SAM) by SAM synthase, then to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase and finally to ethylene by ACC oxidase (ACO) (Yang and Hoffman, 1984). Many researches have shown that *ACO*s are involved in fruit ripening and flower senescence, and the knockout of *ACOs* can effectively increase the shelf life of crops (Do et al., 2005; Inaba et al., 2007; Huang et al.,2007). Recently, Hu et al. (2021) conducted an RNA-seq analysis on mature green bananas and identified a banana *ACO* gene *Ma07_t19730.1*. This gene can be strongly induced by ethephon and inhibited by 1-MCP to a greater extent in the pulp and peel tissues. Under the natural ripening conditions, the CRISPR/Cas9-based *MaAC O 1* (*Ma07_t19730.1*)-disrupted mutants exhibited reduced ethylene production and longer shelf life than the WT. Petunias are favoured by the floricultural industry for their different flower shapes and colours and are used as a bedding plant. However, newly produced individual flowers show rapid senescence in the mother plant. Xu et al. (2020) designed two specific sgRNAs to target *PhACO1* of petunias and successfully obtained edited lines with significantly reduced ethylene production and enhanced flower longevity. Some transcription factors (TFs) operating upstream of ethylene biosynthesis pathways also play important roles in regulating the shelf life of crops. As in other climacteric fruits, for example, tomatoes produce much

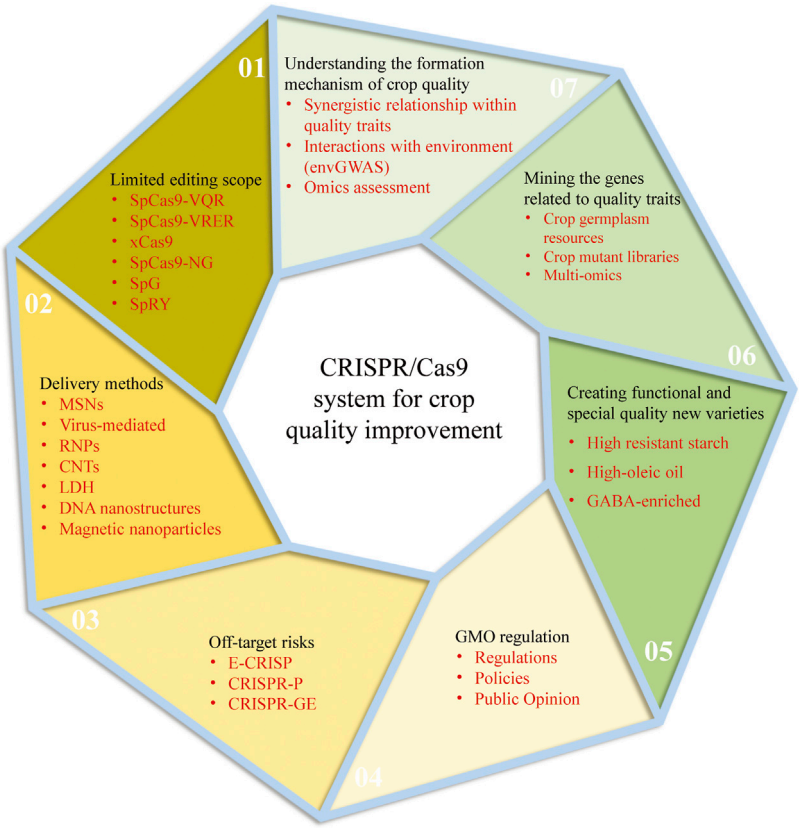

FIGURE 2 | The current challenges and perspectives of the CRISPR/Cas9 system and the future development trend of crop quality improvement.

ethylene during ripening. The use of naturally occurring ripening mutants increases shelf life with a delay in the ripening process, such as *Nr* (Never ripe), *alc* (alcobaca), *rin* (ripening inhibitor), *nor* (non-ripening), and *Cnr* (colorless non-ripening) (Robinson and Tomes, 1968; Tigchelaar et al., 1973; Thompson et al., 1999; Garg et al., 2008). Ito et al. (2015) knocked out the *RIN* using three sgRNAs to produce incomplete-ripening fruits in which red colour pigmentation was significantly lower than that of the wild type. Similarly, CRISPR-*Cnr* mutant lines showed delayed fruit ripening phenotype, CRISPR-*Nor* mutant lines showed partially immature fruit (Gao et al., 2019). The *alc* mutants were found to have good fruit colour, flavour and resistance to bacterial diseases (Casals et al., 2011). Using the HDR-mediated gene replacement, Yu et al. (2017) successfully replaced ^{317}T of *the ALC* gene with ^{317}A and created a tomato line, significantly prolonged tomato storage time and shelf life. In addition to regulating crop endogenous ethylene content, shelf life is also related to alterations in cuticle properties and remodelling of the fruit cell walls (Keegstra, 2010). Pectin, which is abundant in the primary cell wall (PCWs) and mesenchymal layer (ML) of fruits, has long been known to undergo degradation during ripening (Brummell, 2006). Uluisik et al. (2016) reported a tomato pectate lyase (*PL*) gene, which is crucial for fruit softening, and the silencing of this *PL* altered texture without affecting other aspects of ripening. Wang D. et al. (2019) used CRISPR/Cas9 technology to knock out this gene and obtained similar results.

5 CHALLENGES AND PERSPECTIVES

CRISPR/Cas9 system has been rapidly developed and applied since its birth in 2013 with the characteristics of simplicity, high accuracy, short cycle and low cost. However, there are still some unsolved problems in using the CRISPR/Cas9 system for genetic improvement of crop quality. The following will be analyzed from two aspects: the current challenges and perspectives of the CRISPR/Cas9 system and the future development trend of crop quality (**Figure 2**):

5.1 Challenges and Perspectives of CRISPR/Cas9 System

5.1.1 Limited Editing Scope

As we all know, the targeting specificity of the CRISPR-Cas9 system is determined by two conditions: one is the specific binding of sgRNA sequence to genomic DNA sequence; another is that Cas9 protein specifically recognizes the protospacer adjacent motif (PAM) on genomic DNA. The Cas9 protein from *Streptococcus Pyogenes* (SpCas9) is the

current universal Cas9 protein and specifically recognizes the NGG sequence on DNA as the PAM sequence, limiting the range of DNA sites that Cas9 protein can target. To overcome this limitation, a series of SpCas9 variants derived through protein directed evolution method has greatly expanded the editing range of the CRISPR/Cas system, Such as SpCas9-VQR, SpCas9-VRER, xCas9, SpCas9-NG, and SpG, *etc* (Hu et al., 2016; Hu et al., 2018; Wang J. et al., 2019; Zhong et al., 2019; Zeng et al., 2020a; Qin et al., 2020; Ren et al., 2021). There is hardly even a restriction on PAM sequences, such as the SpRY variant (Xu Z. et al., 2021; Ren et al., 2021). However, all the above modifications have low editing activity, and further optimization is needed to improve editing efficiency at target sites.

5.1.2 Off-Target Risks

Compared with ZFNs and TALENs genome editing techniques, CRISPR/Cas9 system based on gRNA has more advantages in specific recognition. However, due to a large number of genome bases of the edited objects, similar fragments are also widely available. If these similar fragments are recognized, they will cause an off-target effect. This kind of non-specific genome editing is easy to cause uncertainty to the biological response of the edited object, which affects the reliability of this technique in research and application. With the development of high-throughput sequencing technology, many crop genome data are readily available. Based on sequence database, many software or online tools have been developed to aid in designing target sites or evaluating the outcome of genome/gene editing, such as E-CRISP (Heigwer et al., 2014), CRISPR-P (Lei et al., 2014), and CRISPR-GE (Xie et al., 2017), which will enable researchers to examine the specificity of the target sequence further and thus reduce the risk of off-target.

5.1.3 Delivery Methods

The robust delivery of CRISPR-Cas9 reagent into plant cells is the basis for the effective application of CRISPR-Cas9 in plants. At present, there are two main methods for plant transformation: biological bombardment and Agrobacterium-mediated transport, but both of them have certain limitations. Agrobacterium-mediated delivery system is the most commonly used tool for plant genetic transformation, but it can only be applied to a small range of plant species or tissues due to the limitations of host genotypes; Biological bombardment can deliver biomolecules to a wide range of plant species or tissue cells, but it is inefficient and risks genome sequence destruction and tissue damage. It should be noted that these traditional methods cannot avoid the lengthy tissue culture process, and the foreign DNA fragments are needed to be integrated into the host genome, thus producing transgenic plants. Therefore, novel delivery strategies are urgently needed. The Cas9 protein-gRNA ribonucleoproteins (RNPs) is one of the most important genome-editing techniques without foreign DNA integration into plant cells. The purified Cas protein and gRNA are preassembled into a CRISPR/Cas RNP complex with complete activity *in vitro*, which is then directly introduced into plant cells through physical or chemical methods (Woo et al., 2015). This delivery method could avoid transgene integration and off-target mutations. Plant virus systems have also been modified to introduce CRISPR/Cas reagents into plant cell, which are especially helpful for homologous directed recombination mediated gene targeting (Ali et al., 2015; Gil-Humanes et al., 2017; Ellison et al., 2020). In addition, there are studies showed that nano-materials, such as Mesoporous Silica nanoparticles (MSNs), Carbon nanotubes (CNTs), layered double hydroxide (LDH) clay nanosheets, DNA nanostructures, and magnetic nanoparticles, are potential vectors for delivering various forms of CRISPR/Cas reagents (Wang P. et al., 2019; Demirer et al., 2019; Kwak et al., 2019; Zhang et al., 2019). Nano-materials can be diffused through plant cell walls without mechanical assistance and without causing tissue damage. These new genetic transformation technologies are expected to become the most important transformation methods in the future.

5.1.4 GMO Regulation

At present, the safety of gene editing products is still controversial. The European Union has approved about 118 genetically modified organisms, but most of them are fed to animals, only a handful of is for human consumption directly. There is almost no genetically modified (GM) food market in Europe; gene-edited crops are considered GM products and regulated (Bruetschy, 2019). While some countries, like the United States, Canada, Australia, Japan, Argentina, and Brazil, have treated gene-edited crops (without foreign genes) as non-GMOs, which are already on the market. For example, browning resistant mushrooms created by gene editing at the University of Pennsylvania in 2016 are not regulated in the United States (Waltz, 2016). Therefore, the commercial application of gene-edited crops still needs the support and improvement of relevant regulations, policies and public opinion environment.

5.2 Developing Trends for Crop Quality Improvement

5.2.1 Systematic Understanding Formation Mechanism of Crop Quality

Crop quality is a comprehensive and complex character manifested in the interaction with environmental factors, quality, and yield characters. Therefore, many characters often restrict each other during crop quality improvement. For example, nitrogen fertilizer as an environmental factor can promote the increase of rice yield and grain protein content. Although the yield and nutritional quality of rice were improved, the increase of grain protein content significantly decreased the rice eating and cooking quality (Yang et al., 2015; Yang et al., 2020). In the breeding process, rice yield and grain quality are often difficult to balance. In the future studies, besides focusing on a certain quality trait, attentions should also be paid to studying the synergistic relationship within different quality or yield characters and their interactions with environment. The genetic basis, molecular network and metabolic regulation mechanism of quality traits should be studied from multiple dimensions such as transcriptome, proteome and metabolome. It is worth mentioning that the envGWAS, which uses environmental or non-genetic variables as traits in GWAS to

map loci associated with those variables, is an effective and popular way in such studies. This approach has been used to analysis impact of many environmental factors on crop traits, such as geographical location, climate, soil, even age and so on (Li J. et al., 2019; Millet et al., 2019; Sharma et al., 2020).

5.2.2 Multi-Strategy Mining of Genes Related to Quality Traits

With the development of functional genomics and molecular biology, many genes controlling important quality traits have been successfully cloned, such as *Wx, BADH2, FAD2, etc.* However, compared with yield traits, the study of crop quality traits started late and was even blank in some crops with complex genetic backgrounds. Therefore, further exploration of key genes controlling quality is the precursor and basis of quality genetic improvement. We can conduct gene mining through the following strategies: 1) Crop germplasm resources are a treasure-house of abundant genetic variation. By extensively collecting crop germplasm resources, systematically evaluating quality traits and screening excellent germplasm resources, it can provide a guarantee for digging quality genes and identifying excellent alleles; 2) Using chemical, radial mutagenesis or gene-editing techniques to create crop mutant libraries to screen the mutants with changed quality traits and clone related genes; 3) Using the multi-omics method to explore quality-related genes, especially to identify key genes that respond to environmental variation, which is helpful to reveal the interaction between genetic variation and environmental variation.

5.2.3 Creation of Functional and Special Quality New Varieties

At present, people are faced with the dual challenges of nutritional deficiency and overnutrition. Taking in too much sugar or lipid due to unreasonable diet structure leads to overnutrition and induces obesity, cardiovascular disease, diabetes, and kidney disease. In addition, due to unbalanced regional economic development and natural conditions, the "lack of nutrition" and "hidden hunger" problems are also more prominent, such as nutritional anaemia and vitamin A deficiency. Chronic diseases related to diet and nutrition are increasingly threatening people's health. Based on this, the concepts of "nutrition-oriented agriculture" and "functional agriculture" have attracted more attention, and people are gradually accepting the preventive and therapeutic effects of nutrition-healthy food and functional food. At present, biofortification crops with the significant increase of one or more nutrients can be obtained by molecular design breeding or gene-editing methods, such as high resistant starch rice, giant embryo rice, golden rice, *etc*., but the progress is still slow. In the future, we need to further search for functional germplasm resources, analyze the synthesis and metabolic pathways of relevant bioactive compounds and their regulatory mechanisms, and create more new crop germplasm with high nutrition or special functions.

AUTHOR CONTRIBUTIONS

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Hemp Genome Editing—Challenges and Opportunities

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Hemp (*Cannabis sativa* L.) is a multipurpose crop with many important uses including medicine, fibre, food and biocomposites. This plant is currently gaining prominence and acceptance for its valuable applications. Hemp is grown as a cash crop for its novel cannabinoids which are estimated to be a multibillion-dollar downstream market. Hemp cultivation can play a major role in carbon sequestration with good CO_2 to biomass conversion in low input systems and can also improve soil health and promote phytoremediation. The recent advent of genome editing tools to produce non-transgenic genome-edited crops with no trace of foreign genetic material has the potential to overcome regulatory hurdles faced by genetically modified crops. The use of Artificial Intelligence - mediated trait discovery platforms are revolutionizing the agricultural industry to produce desirable crops with unprecedented accuracy and speed. However, genome editing tools to improve the beneficial properties of hemp have not yet been deployed. Recent availability of high-quality *Cannabis* genome sequences from several strains (cannabidiol and tetrahydrocannabinol balanced and CBD/THC rich strains) have paved the way for improving the production of valuable bioactive molecules for the welfare of humankind and the environment. In this context, the article focuses on exploiting advanced genome editing tools to produce non-transgenic hemp to improve the most industrially desirable traits. The challenges, opportunities and interdisciplinary approaches that can be adopted from existing technologies in other plant species are highlighted.

Keywords: HEMP, non-transgenic, genome editing, cannabinoids, tissue culture, next generation technologies, artificial intelligence for crop improvement

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INTRODUCTION

Cultivation of hemp (*Cannabis sativa*) has increased globally in recent years and is a profitable enterprise that generates a range of useful products such as bioactive cannabinoids, seed, seed oil, fibre, textiles, construction materials and biocomposites. Archaeological evidence from Western China dating from 500 BCE suggests *Cannabis* was used for ceremonial purposes by ancient Chinese cultures during burial ceremonies (Ren et al., 2019). The medicinal properties of cannabinoids are extensively documented and renewed interest in these compounds in recent decades has driven growth in the health product and medical markets. The classification of *Cannabis* is typically determined by plant chemistry. In Europe, hemp was defined as *Cannabis sativa* plants containing less than 0.2% of the intoxicating cannabinoid Δ9-tetrahydrocannabinol (THC), but recent changes to laws and the adoption of the new Common Agricultural Policy have increased this to less than

0.3%. This figure is less than 0.3% in North America and Asia (Russo, 2017; Hammami et al., 2021). Drug-type *Cannabis* plants are grown for their high levels of the intoxicating THC and are commonly referred to as marijuana. *Cannabis* is a reservoir for a range of valuable secondary metabolites including cannabinoids and terpenes. Cannabinoids that have documented medical properties include cannabidiol (CBD), cannabigerol (CBG), cannabinol (CBN), cannabichromene (CBC), cannabidiolic acid (CBDA), cannabidivarin (CBDV), cannabicyclol (CBL), cannabivarin (CBV), cannabichromevarin (CBCV) and cannabielsoin (CBE). Cannabinoids accumulate in the secretory cavity of the hair-like glandular trichomes which are found in greatest abundance on the female inflorescences (Livingston et al., 2020). CBD is one of the most prominent non-intoxicating cannabinoids that has potential in treatment of various medical conditions including epilepsy, chronic pain, autism and post-traumatic stress disorder. Currently, medical *Cannabis* is legal in more than 50 countries including China, Australia, Germany, Israel, Canada and most of the U.S. The medical *Cannabis* market is rapidly growing from $3.5 billion at retail prices in 2019 to an estimated $20.2 billion during 2020–2025 (Aliekperova et al., 2020). Hemp is one of the earliest documented fibre crops used by humans with claims of domestication as early as 12,000 years ago (Ren et al., 2021). Hemp fibre is a strong, durable material with good insulative properties. It is used to make clothing, textiles, building materials and polymers. Hemp-based bioplastics have shown potential and could be superior in some respects to traditional polymers. They also offer a more sustainable, greener alternative to petroleum-based plastics (Fike, 2016). Materials such as Hempcrete® offer a means of carbon-negative building methods which can reduce net greenhouse gas emissions (Ip and Miller, 2012). Industrial hemp is an excellent carbon sink. Finnan and Styles (2013) found that hemp is comparable to the energy crops miscanthus and short-rotation coppice willow in net greenhouse gas emission abatement, and superior to sugar beet and oil seed rape. A comparative study carried out in Sweden demonstrated how hemp had similar biomass energy yield to maize and sugar beet (Prade et al., 2011). Hemp also has potential as a break crop between the planting of two food crop cycles and could play an important role in sustainable farming. This strategy can reduce soil pathogens, improve soil structure, and enrich soils if crop residues are ploughed in. Studies have shown how food crops such as wheat (Gorchs et al., 2017) and soybean (Liu et al., 2012) benefit from increased yield after hemp breaks crops over continuous systems. This accounted for yield increases of 37–48% in wheat monocultures and 9.1–10.8% in soybean monocultures. Hemp has documented nematicidal properties also and some of these yield gains can be attributed to suppression of these parasites (Adesina et al., 2020). Demand for hemp seed, oil and press-cake (remains of seed once pressed for oil) has contributed to the increased cultivation of hemp in the US (Adesina et al., 2020). As a food source hemp-derived protein has high nutritional value and excellent digestibility. The seed contains all the essential amino acids required by humans. There are also reported health benefits from consumption of hemp-derived protein including decreasing hypersensitivity and cholesterol (Shen et al., 2021). Overall, the cultivation of hemp has clear benefits and there is a growing market for hemp-derived products. Maximising the potential of this plant calls for more high-performing cultivars. Developing new cultivars through traditional breeding can often take a lot of time and labour. However, molecular breeding strategies such as marker assisted selection have refined the process of breeding, but these strategies are not well developed in this species. The recent development and adoption of genome editing technologies such as CRISPR (clustered regularly interspaced short palindromic repeats) offers a means to improve hemp varieties in a more precise and less time-consuming way. Many hemp varieties don't self-pollinate, and this prevents using this strategy to obtain homozygous plants. Gene-editing technology allows breeders to modify genes on both alleles to achieve homozygous lines in one generation (Deguchi et al., 2020). Potential targets for gene-editing in hemp include genes controlling cannabinoid production and accumulation, fibre deposition, disease susceptibility and seed oil quality. This review discusses the opportunities for improving hemp with gene editing technology, and the potential challenges and opportunities in adopting these technologies.

TARGETS FOR HEMP CROP IMPROVEMENT

The many different uses of *Cannabis* motivate the development of high performing cultivars with improved cannabinoid production, fibre accumulation, disease resistance and food quality. The growing demand for cannabinoids means there is an opportunity to develop high-yielding cultivars using novel methods. However, more research is needed to understand potential trade-offs when applying this technology. A knockout of the THC acid synthase gene via genome editing is a way to derive THC-free, high-CBD plants which would have huge value in countries with strict laws on THC levels. A patent filed by Canopy Growth Corporation details overexpression of genes regulating trichome development (e.g. GLABROUS INFLORESCENCE STEMS (GIS)) produced trichomes in greater density and abundance, and had a ten-fold increase in THC production over unmodified plants (Roscow, 2019). Using non-transgenic genome editing technology, the target gene(s) can be overexpressed by editing the respective regulatory (enhancer/promoter) elements upstream of a gene. Genome editing has been successfully applied to other important medicinal plant species such as *Dendrobium officinale*, *Papaver somniferum*, *Dioscorea zingiberensis* and *Salvia miltiorrhiza* (Alagoz et al., 2016; Kui et al., 2017; Feng et al., 2018; Zhou et al., 2018) and there is huge scope to modulate metabolite production via CRISPR/Cas9. Fibre quality of cultivated hemp plants can be improved by upregulating the expression of genes involved in the formation of bast fibres (phloem fibres). Several well-known transcription factors including NST1, MYB46 and WILM1 control secondary cell wall deposition and bast fibre development in hemp hypocotyls. The genes SND2, VND1 and NST1 are master regulators of secondary cell wall development (Behr et al., 2016). Hemp is susceptible to a range of diseases that can lead to loss in yield and decrease the overall value

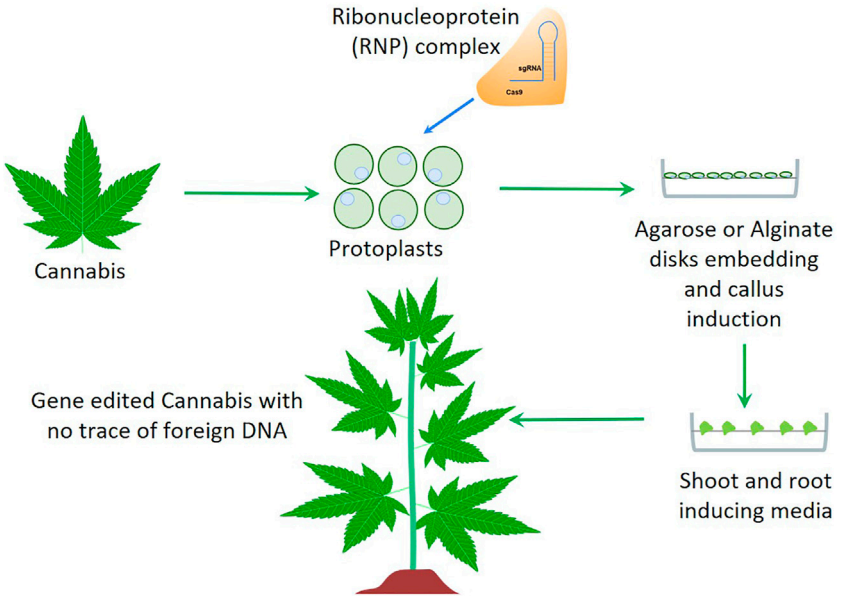

FIGURE 1 | A schematic overview of non-transgenic genome editing technology applicable for *Cannabis* improvement. *Cannabis* protoplasts isolated from *in-vitro* grown plants are transfected with a mixture of Cas9 and sgRNA followed by agarose or alginate embedding and plant regeneration.

of the crop. Common pathogens of hemp include fungi, oomycetes, viruses, nematodes, and bacteria. Genome editing technologies offer a way to generate disease resistant varieties with greater precision and in a faster time frame than traditional breeding methods. Targeting resistance (R) and susceptibility (S) genes are one way to increase a plant's resistance. A recent study has identified a powdery mildew resistance (R) gene in a *Cannabis sativa* cultivar, designated PM1, that confers resistance to the pathogen *Golovinomyces ambrosiae* (Mihalyov and Garfinkel, 2021). Improving food quality of seed and seed oil is also possible. Targeting *FAD2* genes which are involved in converting oleic acid to linoleic acid and linolenic acid offer a reliable target to upregulate oleic acid production. Mutagenesis studies on the hemp cultivar Finola have shown increased oil quality (high oleic content) and shelf life through targeted mutations of fatty desaturase genes *CsFAD2* and *CsFAD3* (Bielecka et al., 2014). Genome editing of *Brassica napus* fatty acid desaturase gene 2 (*FAD2*) using CRISPR/Cas9 has been successfully demonstrated, producing high oleic acid content seed. Back-crossing of the progeny of one transformed line showed the mutation was inheritable and no transgenic DNA was inherited (Okuzaki et al., 2018).

OPPORTUNITIES AND CHALLENGES FOR HEMP IMPROVEMENT USING NEXT GENERATION TOOLS

DNA-free/footprint-free Genome Editing

Genome Editing by CRISPR/Cas is revolutionizing plant biology and agriculture in developing improved crops with novel traits. CRISPR/Cas technology allows for sequence specific editing of the target genome, thereby allowing for precise control over gene modifications and associated traits, in a low cost and straightforward manner. This level of control over DNA sequence change is unprecedented. It is a vast improvement over previous genome modification tools and opens new doors for exciting developments in the fields of medicine and agriculture. Agrobacterium-mediated CRISPR transformation is being widely used for targeted crop improvement to develop gene knockouts, knock-ins, transcriptional regulation, and epigenetic changes in the genome to achieve novel traits. However, agrobacterium-mediated transformation may pose a bottleneck for regulatory approval because of the introduction of external plasmid DNA into the plant genome. The newest next-generation genome editing technology encompasses modifying/editing the DNA bases by direct delivery of CRISPR/Cas ribonucleoprotein (RNP) complexes into plant tissue, such as protoplasts, embryos or *in-vitro* grown calli (Woo et al., 2015; Malnoy et al., 2016; Osakabe et al., 2018). The transformed plant tissue is grown in a suitable media to regenerate entire plants followed by screening for the genome edited plant lines (**Figure 1**). This approach eliminates the opportunity for plasmid encoded DNA elements to integrate into the plant genome, thereby mimicking natural mutations. In addition to introducing mutations and deleting entire fragments of DNA elements, CRISPR/Cas technology is also being used to introduce a specific DNA fragment to a precise location in the genome. A specific donor DNA is included together with Cas9 and sgRNA which spans the flanking regions of the target site with the donor DNA element in between. The presence of this single-stranded DNA triggers the Homology Directed Repair (HDR) mechanism wherein the donor DNA is used as a template by the DNA repair machinery to repair the cut target site, and consequently the target DNA sequence gets introduced to the target genome (Chen

et al., 2019). This new generation of precision methods has several applications in hemp breeding such as gene knockout/knock-in, base editing, gene- and genome-wide screening, modifying gene regulation, and developing virus resistant plants, as demonstrated in different recalcitrant species such as wheat, maize and grape. These strategies have been clearly detailed in the review article by Chen et al. (2019) by providing specific examples.

Interdisciplinary Approaches for Hemp Biology

The availability of cannabis genome sequences (Braich et al., 2020; Gao et al., 2020) and growing number of RNA-sequencing datasets (Massimino., 2017; Braich et al., 2019; Braich et al., 2019, 2019; Zager et al., 2019; Livingston et al., 2020; Livingston et al., 2020, 2020) allows for the use of big data analysis methods for greater understanding of *Cannabis* biology beyond the expression levels of genes. A systems-biology approach uses information-rich complex datasets to provide meaningful results by extrapolating the relationship between individual biomolecules. Biomolecules (e.g. genes, transcription factors, metabolites, promoters) are represented as nodes and the connections between them as edges in this in-silico molecular network. Two molecules (nodes) connected by an edge would mean a possible interaction in terms of physical interaction, biosynthesis, regulation and/or co-expression between them. Once the networks are developed, the dynamics of the interactions can be studied with a focus on the hubs that can be central to a biological function of interest (Breitling, 2010). This integrated approach is quite useful to make sense of the vast amounts of datasets produced by holistic studies and will provide a combined biological insight (emergent behaviour) that isolated experiments simply cannot. New genes participating in defense response pathways are predicted and validated using a systems biology approach in *Arabidopsis thaliana* (Windram et al., 2012). This emphasizes the potential of interdisciplinarity in biological research. Furthermore, combining the metabolomics datasets to develop network models using machine learning has been successful in predicting metabolic pathways in tomato (Toubiana et al., 2019). Applying these established methods in hemp will speed-up the understanding of molecular processes and metabolite accumulations in the context of improving desirable traits in hemp such as higher CBD production.

Another application of computational methods is the use of Artificial Intelligence (AI) to identify single nucleotide polymorphisms (SNPs) associated with important agricultural traits in Genome Wide Association Studies (GWAS). By using the available genomic sequences from different varieties of a certain crop species, these deep learning-based prediction methods can identify SNPs associated with the trait of interest. The machine learning algorithms are first trained with a combination of data including genotypic, phenotypic, agronomic practices and environmental data before it is used on a test dataset for predicting SNPs (Wang et al., 2020; Mieth et al., 2021). This is just one of the applications of AI and deep learning to accelerate knowledge discovery. The review article by Wang et al. (2020) provides a good overview of its various applications in plant research and agriculture. GWAS studies have been carried out on hemp with respect to fibre quality (Petit et al., 2020a) and flowering time and sex determination (Petit et al., 2020b). Hesami et al. (2021a) applied machine learning algorithms *in silico* to predict off-target gRNA activity in modifying centromeric histone H3 (CENH3) genes in *Cannabis*. Of the three machine learning algorithms used, the Random Forest (RF) had the highest precision. These predictive models offer a powerful tool in designing effective genome-editing protocols in *Cannabis*. Interdisciplinary approaches will accelerate the knowledge-discovery and will be valuable to understand cannabinoid biology and genetics, given imperfect genome sequence and annotations, recalcitrance for transformation and the lack of standard protocols/procedures for Cannabis.

Micropropagation and Plant Regeneration

Micropropagation of *Cannabis* tissues in a disease-free aseptic environment is an important step towards a successful transformation protocol. Some varieties of *Cannabis* are recalcitrant to *in vitro* culture and transformation. An optimal strategy may be to transform more amenable varieties and backcross these into elite lines, which is still time and labor intensive. Adhikary et al. (2021) mentions that the *Cannabis* industries have been developing tissue culture and micropropagation techniques over the last 2 decades and are held as a trade secret to preserve competitive advantage with other commercial entities. Optimizing micropropagation protocols for non-meristematic tissues is important for genome editing applications. Factors including plant growth regulators (PGRs), type of light, carbohydrate sources, additives, temperature and genotype influence micropropagation success (Hesami et al., 2021b). Zhang et al. (2021) found that *Cannabis* embryo hypocotyls of immature grains collected 15 days after anthesis exhibited the greatest regeneration rate and were also more amenable to agrobacterium transformation. The authors used G41sg vector to deliver sgRNA targeting phytoene desaturase gene (CsPDS1) generating albino plants. This is the first published report of successful gene editing in *Cannabis sativa*, which paves the way for further developments in non-transgenic genome editing technology. Regenerating transfected protoplast cells into complete plants is also challenging. The first report of DNA-free (or non-transgenic) genome editing described the successful regeneration of genome edited lettuce protoplasts into complete plants (Woo et al., 2015). The authors incubated preassembled complexes of purified Cas9 protein and guide RNA with plant protoplasts in the presence of polyethylene glycol (PEG), a standard and widely used transfection method. Interestingly, RNA-guided mutations were detected as early as 24 h, suggesting the quick Cas9 activity even before the cell cycle was completed. The transfected protoplasts were mixed with a 1:1 solution of 50% B5 medium and 2.4% agarose to make agarose embeddings plated on 6-well plates (Woo et al., 2015). For regenerating plants from genome edited grapevine protoplasts the authors embedded the protoplasts in alginate disks and stimulated the formation of mini-calli in NN-based cultivation medium (Nitsch and Nitsch, 1969) optimized for regeneration

TABLE 1 | Protoplast transformation and regeneration technologies in different species applicable for *Cannabis* improvement.

DNA-free GE technology	Crop/Tissue	Method overview	Reference
Transformation and Regeneration	Wheat Immature Embryos	CRISPR/Cas9 is delivered as DNA (plasmid constructs) or RNA (*in vitro* synthesized transcripts) into immature wheat embryos by particle bombardment, transferred onto callusing media from which seedlings are regenerated	Zhang et al. (2016)
Transformation and Regeneration	Maize Immature Embryos	Guide RNA–Cas9 ribonucleoprotein (RNP) complexes are delivered into maize embryo cells, cultured, selected on appropriate antibiotics and the plants regenerated	Svitashev et al. (2016)
Transfection and Regeneration	Grapevine protoplasts	Protoplasts immobilized in alginate disks were stimulated for mini-calli formation followed by embryo formation and plant regeneration	Scintilla et al. (2021)
Transfection in all four and Regeneration only in lettuce	Arabidopsis, tobacco, rice and lettuce protoplasts	PEG mediated transfection of sgRNA-Cas9 RNP complexes into protoplasts and mixed with a 1:1 solution of 0.5× B5 medium and 2.4% agarose to make agarose embeddings, which were cultured onto callus inducing medium and subsequently transferred to shoot inducing and root inducing media	Woo et al. (2015)
Transfection only	Apple and Grapevine protoplasts	PEG mediated transfection of sgRNA-Cas9 RNP complexes into protoplasts	Malnoy et al. (2016)
Transfection only	*Petunia hybrida* protoplasts	PEG mediated transfection of sgRNA-Cas9 RNP complexes into protoplasts	Subburaj et al. (2016)
Regeneration only	Potato Protoplasts	Protoplasts immobilized in alginate lens are transferred onto callus induction media, and the resulting calli to proliferation media and then to greening media	Moon et al. (2021)
Callus formation	Arabidopsis shoot and root protoplasts	Detailed molecular methods to confirm every stage of protoplast regeneration, special medium designed for Totipotent cell formation, protoplasts immobilized in alginate beds for colony formation	Pasternak et al. (2021)
Regeneration only	Strawberry protoplasts	Isolated protoplasts are embedded in 0.6% agarose and transferred onto regeneration media	Barcelo et al. (2019)

(Scintilla et al., 2021). Beard et al. (2021) demonstrated PEG mediated transient transformation of *Cannabis* sativa protoplasts with a p35S:GFP expression cassette and achieved a transformation efficiency of up to 31%, thus demonstrating the viability of protoplast transformation in this species. **Table 1** lists the published transformation and regeneration technologies for non-transgenic genome editing in different plants. Effective protoplast culture protocols provide a platform for whole plant regeneration, and a platform to test sgRNAs in optimizing CRISPR protocols.

In another approach, researchers used immature embryos from wheat and maize to bombard the mixture of either CRISPR/Cas RNPs or DNA/RNA elements encoding Cas proteins with sgRNAs coated on microparticles (Svitashev et al., 2016; Zhang et al., 2016; Liang et al., 2017, 2018). The embryos were transferred to callusing media, and then to shoot and root regeneration media for complete plant development. This procedure can be applied to develop non-transgenic *Cannabis* plants by using an embryo extraction protocol (Soler et al., 2016). The following biolistic transformation and whole plant regeneration method needs optimization in *Cannabis*.

Co-transformation of developmental regulator genes in combination with the target genes of interest have proven to increase or induce callus formation in recalcitrant varieties of sorghum, maize and wheat (Che et al., 2021; Hoerster et al., 2020; Nalapalli et al., 2021). In *Cannabis*, co-transformation of native homologs of developmental regulators in combinations increased shoot regeneration efficiency up to 1.7-fold with CsGRF3–CsGIF1 chimera and all chimeras containing CsWUS4 (Zhang et al., 2021). WUSCHEL (WUS) is essential for *de novo* establishment of the shoot stem cell niche (Zhang et al., 2017) and co-transfecting WUS into protoplasts could induce the formation of calli and subsequently shoots.

DISCUSSION

The current legal status of gene-edited crops in the European Union as genetically modified organisms (GMOs) prevents the full deployment of these technologies in *C. sativa*. A move toward social acceptance of gene-edited crops requires a raising of public awareness and a clear distinction between transgenic and non-transgenic plants. Highlighting the use of random mutagenesis by chemical and radiological means for the last century in developing new crop varieties offers utility in advocating the use of targeted mutagenesis technologies. Strictly speaking, DNA-free, gene-edited crops are equivalent to crop varieties derived through random mutagenesis, which include many important food crop species (e.g. bananas, barley). The caveat being that targeted mutagenesis is not random and offers greater control and specificity and reduces the incidence of deleterious mutations and the impact of mutation load (Jung and Till, 2021). The ruling of the Court of Justice of the European Union in 2018 on genome editing groups this new technology with GMOs as outlined in directive 2001/18. The distinction of gene-edited crops as genetically modified organisms (GMOs) within the EU also excludes them from organic certification. This may serve as a hurdle in the public acceptance of these crops as healthy, safe and nutritious. This contentious decision has been challenged by the European Federation of Biotechnology (EFB). They argue that the

Site Directed nuclease 1 (SDN1) format of gene editing is fundamentally different from the genetic engineering outlined in directive 2001/18. Safety concerns of introducing foreign DNA and causing off-target mutations are avoided in SDN1 as no foreign DNA is introduced and whole genome sequencing of the transformed organism can investigate any potentially dangerous mutations (Hjort et al., 2021). The recent refinement of CRISPR methods circumvents the issue of introducing transgenes into gene-edited crops, where *Agrobacterium* plasmid DNA is not used, and nucleases are delivered directly into the cells (Ishii, 2018). Even though hemp has large genetic diversity and traditional breeding still offers utility to improve varieties, application of these new plant breeding technologies allows highly specific changes in markedly shorter timeframes. In producing new allelic variation in crop species, CRISPR is the most powerful tool available to breeders, and should be exploited for its full potential.

AUTHOR CONTRIBUTIONS

DS, BP, CK, OK, RH and RB conceptualized the article. DS, RH and RB wrote the manuscript. BP, CK and OK revised the manuscript, provided critical insights and improved the manuscript. All authors approved the manuscript for publication.

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frontiers in Genome Editing

MINI REVIEW
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Mini-Review: Transgenerational CRISPR/Cas9 Gene Editing in Plants

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CRISPR/Cas9 genome editing has been used extensively in a wide variety of plant species. Creation of loss-of-function alleles, promoter variants and mutant collections are a few of the many uses of genome editing. In a typical workflow for sexually reproducing species, plants are generated that contain an integrated CRISPR/Cas9 transgene. After editing of the gene of interest, T-DNA null segregants can be identified in the next generation that contain only the desired edit. However, maintained presence of the CRISPR/Cas9 transgene and continued editing in the subsequent generations offer a range of applications for model plants and crops. In this review, we define transgenerational gene editing (TGE) as the continued editing of CRISPR/Cas9 after a genetic cross. We discuss the concept of TGE, summarize the current main applications, and highlight special cases to illustrate the importance of TGE for plant genome editing research and breeding.

Keywords: CRISPR/Cas9, gene editing, egg cell, pollen, HI-Edit, floral dip

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INTRODUCTION

CRISPR/Cas9 has rapidly become the predominant tool for plant genome editing (Chen et al., 2019). An important reason is that the CRISPR/Cas9 system only requires co-expression of a generic Cas9 endonuclease and one or more specific single guide RNAs (sgRNA) (Cong et al., 2013). The pairing of the Cas9 ribonucleoprotein complex with target DNA triggers Cas9-mediated DNA cleavage which results in a double stranded break (DSB) (Jinek et al., 2012). The system can easily be engineered to target a DNA region of choice as the specificity is only determined by a ~20 bp sgRNA spacer complementary to the targeted sequence and a 2–3 bp sequence directly downstream of the target, the protospacer adjacent motif (PAM), which is NGG for *Streptococcus pyogenes* Cas9 (Jinek et al., 2012). DSBs are recognized by endogenous DNA repair mechanisms, of which non-homologous end joining (NHEJ) plays the predominant role in plant cells (Puchta, 2004). When DSBs are repaired perfectly, they are prone to additional rounds of Cas9 cutting. An imperfect repair leads to the creation of an insertion or deletion (indel) at the targeted site, also ending recognition by the sgRNA-guided Cas9 protein. A variety of repair outcomes is possible at each site, although the most often observed edit is a single base pair insertion, often A or T (Bortesi et al., 2016). Alternatively, microhomology-mediated end-joining (MMEJ) may result in larger deletions (>2 bp) through microhomology sites flanking the DSB (van Overbeek et al., 2016). Combinations of insertions and deletions have been reported in *Arabidopsis* through synthesis-dependent MMEJ (Pauwels et al., 2018).

Researchers typically use CRISPR/Cas9 to target exonic open reading frames to generate loss-of-function mutants for functional analysis (Feng et al., 2013; Nekrasov et al., 2013; Fauser et al., 2014;

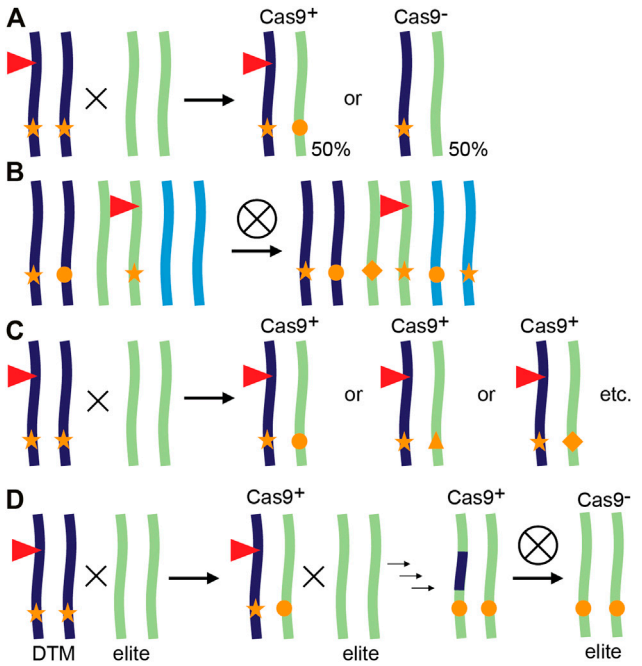

FIGURE 1 | Transgenerational gene editing and applications in plants. **(A)** Principle of transgenerational gene editing (TGE). A transgenic plant represented as a chromosome pair is hemizygous for a CRISPR/Cas9 containing T-DNA locus (red triangle) and edited in both alleles (stars). When crossed with a WT, the resulting progeny either lacks the T-DNA and inherits a single edited allele or inherits the T-DNA, resulting in (transgenerational) editing of the inherited WT allele. **(B–D)** Examples of TGE. **(B)** TGE for continued editing of homoeoalleles in wheat. A transgenic line may have edits only in a subset of homoeoalleles at the homologous chromosomes. After self-crossing and selecting plants that inherited the T-DNA, all homoeoalleles may now be edited (Wang et al., 2018b). **(C)** TGE for allelic variation. In tomato, a loss-of-function mutant (stars) also contains a CRISPR/Cas9 containing T-DNA targeting the promoter of the mutant gene. After a cross with WT, resulting T-DNA containing plants have one loss-of-function allele (star), and an allele with a promoter edit (other symbols). Every individual F1 plant has potentially a different promoter edit and phenotype as the phenotype is not determined by the inherited loss-of-function allele (Rodríguez-Leal et al., 2017). **(D)** Desired-target mutator (DTM) strategy. A maize plant hemizygous for a CRISPR/Cas9 containing T-DNA locus (red triangle) is crossed with an elite inbred line, resulting in TGE and editing of the elite allele. Additional rounds of TGE and backcrossing result in a new edited variety with no linkage drag (Li et al., 2017a).

Pauwels et al., 2018). Alternatively, promoter elements or other *cis*-regulatory elements are targeted to disrupt regulation of genes and avoid pleiotropic effects associated with complete loss-of-function (Swinnen et al., 2016). For delivery to plant cells, *Agrobacterium*-mediated transformation is most often used. After transfer of a CRISPR/Cas9 encoding T-DNA molecule to the plant cell, Cas9 and the sgRNA are expressed and are able to edit the target sequence of interest. The T-DNA also harbors a selection marker, allowing selection of plants in which the T-DNA has integrated in the genome and is transcriptionally active. The method for stable transformation by *Agrobacterium* differs from species to species. In most plant transformation protocols, explants such as leaves, roots or immature embryos are infected with *Agrobacterium*, after which callus formation is induced in tissue culture. This allows selection of transgenic cells and subsequent regeneration of primary transformants (T0 generation) either by organogenesis or somatic embryogenesis. The model plant *Arabidopsis thaliana* is an exception and allows for the use of *in planta* transformation. In the floral dip method, *Arabidopsis* flowers are brought into contact with *Agrobacterium*, resulting in transformation of haploid female gametophyte cells, before fertilization by self-pollination (Desfeux et al., 2000). Therefore, the primary transformant is the female gametophyte in *Arabidopsis* and the first generation analyzed for gene editing is the T1 generation.

T0 primary transformants in crops, or T1 plants in the case of *Arabidopsis*, are most commonly analyzed by examining the genotype in leaf samples. For diploid plants, often more than two alleles can be found, indicating that the plants are genetic mosaics. These are individuals that have developed from a single cell, and have subsequently acquired mutations during development resulting in the presence of two or more populations of cells with different genotypes (Frank and Chitwood, 2016). Such plants are often referred to as chimeric in the literature, but chimerism denotes the presence of two or more genotypes in a single individual arisen from the conglomeration of cells of more than one genotype in the early stages of development (Frank and Chitwood, 2016). In light of these definitions, the occurrence of multiple different alleles in one plant, caused by incomplete or late CRISPR/Cas9 activity, should be considered mosaicism. Somatic mosaic mutational patterns may indicate that the CRISPR/Cas9 machinery is not always active immediately after *Agrobacterium*-mediated delivery and that different cell lineages already were established. Moreover, leaf samples do not always reveal the genotype of the cells making up the germline and hence the mutations that will be transmitted. For example, a study in *Arabidopsis* found that more than half of mutations in T2 were not present in T1 (Feng et al., 2014).

TRANSGENERATIONAL EDITING

In a typical workflow for CRISPR/Cas9 gene editing in plants such as maize (*Zea mays*), T0 plants are identified that contain a single CRISPR/Cas9 T-DNA locus and show some degree of editing at the site(s) of interest. After a backcross to wild-type (WT), the T-DNA locus will likely show Mendelian segregation in the progeny and T1 Cas9 null-segregants can be identified. These do not contain the CRISPR/Cas9 transgene but may have inherited a mutant allele from the T0 parent. If so, the mutation is now heterozygous and cannot be mosaic as it went through a single-cell stage, the fertilized egg cell. However, one can also continue with the progeny that still contains a CRISPR/Cas9 transgene. If still active, the Cas9 nuclease will now encounter a novel WT allele introduced by the cross, which can be edited and yield independent alleles (**Figure 1A**). This continued editing of CRISPR/Cas9 after a genetic cross is referred to as transgenerational gene editing (TGE) (Wang et al., 2018b).

TGE has been used for several applications, although not always named TGE (**Figures 1B–D**). We provide three

examples: editing of additional alleles in polyploid crops, creation of allelic variation and editing of target genes in recalcitrant genetic backgrounds.

Editing of Homoeoalleles in Polyploid Crops

In the case of polyploid crops such as hexaploid common wheat (*Triticum aestivum*) and tetraploid cotton (*Gossypium hirsutum*), mutations are often only present in a subset of the homoeoalleles targeted by the same sgRNA (Wang et al., 2018a; Wang et al., 2018b; Zhang et al., 2019). Furthermore, efficient transmission and stacking of first-generation mutations becomes increasingly harder, or almost impossible with polyploidy due to Mendelian genetics. By selecting T1 plants that contain the Cas9 transgene, plants can be identified with edits in additional homoeoalleles (**Figure 1B**, Wang et al., 2018a; Wang et al., 2018b; Zhang et al., 2019). CRISPR/Cas9 can lead to off-target editing when Cas9 makes a DSB at a site with high sequence similarity to the target site and contains a PAM (Hahn and Nekrasov, 2019). Although expressing CRISPR/Cas9 for more than one generation during TGE increases on-target homoeoallele editing, off-targeting is not necessarily increased. Extensive analysis of off-targeting for two sgRNAs in maize T1 plants that contained an active CRISPR/Cas9 module failed to detect any off-targets at sites predicted by genome-wide CIRCLE-seq analysis (Lee et al., 2019). In addition, a multitude of tools are available that allow careful design of spacer sequences to limit sequence similarities, provided a reference genome is available (Haeussler et al., 2016; Hahn and Nekrasov, 2019).

Creating Novel Genetic Variation

The variety of GE repair outcomes can be exploited to create an array of alleles with potentially different molecular functions, resulting in different phenotypes. As an example, we recently reported independent alleles in the coding region of the maize gene *SAMBA*. Although obtained with the same sgRNA, different phenotypic outcomes were observed and related to translation re-initiation and formation of a truncated protein (Gong et al., 2021). This can be combined with TGE as exemplified in tomato by use of a multiplex mutagenesis drive system to create genetic variation at promoter regions (**Figures 1C**, Rodríguez-Leal et al., 2017; Wang et al., 2021b). In this system, variation caused by TGE was expanded by combining up to eight sgRNAs targeting the same promoter region (Wang et al., 2021b). Using TGE to create novel genetic variation is also interesting for species or genotypes that are difficult to transform. Obtaining a single CRISPR/Cas9-expressing T0 plant can then be sufficient to create a variety of different alleles in subsequent generations. An example of a difficult-to-transform crop is soybean (*Glycine max*) for which TGE was used to create novel alleles in T1 and T2 generations (Zheng et al., 2020).

Editing of Recalcitrant Genetic Backgrounds

TGE can also be exploited to introduce mutations in genetic backgrounds that cannot be transformed (**Figure 1D**). In maize, an *in vivo* desired-target mutator (DTM) strategy was designed to accelerate the breeding process and simultaneously avoid linkage drag compared to introgression of an allele from another variety (Li et al., 2017a). T0 transgenic plants were generated targeting *LIGULELESS1* (*LG1*) and crossed with a WT recalcitrant elite maize inbred line. This resulted in approximately 20% mutation frequency in T1 caused by TGE based on the recessive *lg1* phenotype. One to three additional rounds of TGE and marker assisted backcrossing can subsequently be used to select individuals that are transgene-free and have the desired mutation in the recovered elite background (Li et al., 2017a).

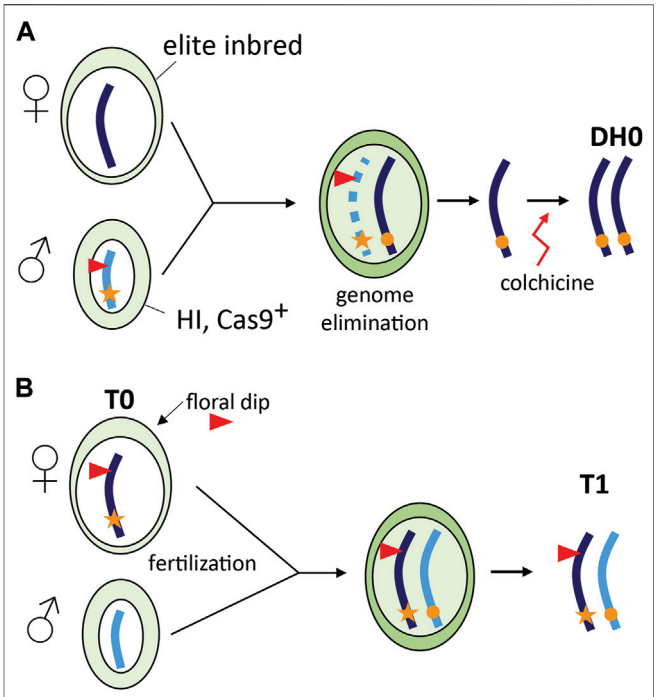

FIGURE 2 | Special cases of TGE. **(A)** Combining haploid induction and gene editing (HI-Edit) in maize. A WT elite maize inbred line is pollinated using a haploid inducer line that contains a CRISPR/Cas9 containing T-DNA locus (red triangle). After fertilization, the male genome is gradually eliminated, but the temporary presence of CRISPR/Cas9 may still edit the elite allele. After doubling of the haploid plant genome using colchicine, a homozygous edited elite DH0 line is obtained (Kelliher et al., 2019; Wang et al., 2019). **(B)** CRISPR/Cas9 gene editing in *Arabidopsis thaliana* using floral dip. The female gametophyte (T0) is transformed using *Agrobacterium tumefaciens* leading to a transformed egg cell that may already be edited. Self-pollination with WT pollen leads to a fertilized egg cell and subsequent zygote in which TGE may take place.

Combining Haploid Induction and Gene Editing

A special case of TGE is the combination of *in vivo* haploid induction and CRISPR/Cas9 gene editing in grasses (**Figure 2A**). This concept has been first demonstrated in maize and is referred to as haploid inducer (HI)-Edit (Kelliher et al., 2019) or haploid-inducer mediated genome editing (IMGE) (Wang et al., 2019).

The technology was developed for editing of elite maize inbreds that are recalcitrant to genetic transformation. In maize, HI lines derived from "stock 6" (Coe, 1959) generate a fraction (~3%) haploid offspring when used as a pollen donor by an incompletely understood mechanism of paternal genome elimination (Li et al., 2017b). In the HI-Edit strategy, the T-DNA containing the CRISPR/Cas9 construct is transformed or introgressed in a HI line. The resulting line is subsequently used as a pollen donor and crossed with an elite inbred line. As paternal genome elimination likely progresses gradually during the first cell divisions (Jacquier et al., 2020), the temporary expression of the CRISPR/Cas9 machinery from the paternal genome can induce targeted mutations in the remaining maternal genome (Kelliher et al., 2019). Modern maize HI lines produce up to 16% haploids (Kalinowska et al., 2019), while editing of the maternal genome in the maize HI-Edit system currently occurs in only 2–4% of haploids (Kelliher et al., 2019; Wang et al., 2019). As a result, less than 1% of the progeny are edited using this strategy, which underlines the importance of continued research and development in this area. A major difference with TGE in HI-Edit and the other applications is the transient presence of the CRISPR/Cas9 transgene and the resulting transgene-free progeny (Kelliher et al., 2019; Wang et al., 2019; Jacquier et al., 2020).

CRISPR/Cas9 Editing in *Arabidopsis* Using Floral Dip

In CRISPR/Cas9 editing of *Arabidopsis*, T1 genotypes can be viewed as the result of TGE as a novel WT allele is introduced after transformation of the haploid female gametophyte. Transformation using floral dip results in stable T-DNA insertion in female gametophytes (T0) resulting in seeds (T1) hemizygous for the T-DNA locus (Clough and Bent, 1998; Desfeux et al., 2000). For CRISPR/Cas9-induced mutagenesis, we envisage that if the editing machinery is expressed in the female gametophyte, the haploid cells could already be edited and after fertilization and zygote formation the paternal allele can be edited as well (**Figure 2B**).

Role of the Promoter Driving Cas9 in TGE

Early experiments with Cas9 driven by the cauliflower mosaic virus 35S promoter yielded mostly genetic mosaic T1 *Arabidopsis* plants with edits that often could not be inherited (Feng et al., 2014). Hence, edits in the paternal and/or maternal genome were introduced only after the first cell divisions of the early zygote, and additionally not present in the germline. These observations are often attributed to an insufficient activity of the 35S promoter in the germline and very early in development (Kong et al., 2021). Many research groups have investigated the use of alternative promoters to drive Cas9, circumvent mosaicism and achieve germline editing (Wang et al., 2015; Yan et al., 2015). A recent publication describes a strategy for the successful generation of non-mosaic mutants in the T1 generation in *Arabidopsis* (Kong et al., 2021). In this setup, Cas9 is driven by a fusion of the egg cell-specific EC1 promoter with the EC1.2 enhancer and combined with a sgRNA targeting the gene of interest and a sgRNA targeting an endogenous marker gene. Loss-of-function of the latter results in a visual phenotype, on which candidate edited plants are then preselected. It was reported that this system produces plants that are mostly non-mosaic homozygous, transheterozygous (with hetero-allelic mutations) or heterozygous, and only up to 7% mosaic, suggesting that the promoter is highly active in the egg cell, but also in early development stages to allow TGE.

Besides egg cell-specific promoters, pollen-specific promoters have been evaluated for Cas9 expression (Mao et al., 2016; Lei et al., 2020; Jing et al., 2021). In *Arabidopsis*, the promoter of *SPOROCYTELESS,* expressed in sporogenous cells and microsporocytes, was used to drive Cas9. As expected, T1 plants did not show abundant editing while 12–56% of T2 plants showed mutagenesis with up to 88% heterozygous non-mosaic, indicating mutations were inherited from T1-edited germ line cells (Mao et al., 2016). A cross of wheat with maize pollen can result in production of haploid wheat embryos (Laurie and Bennett, 1988) and such an intergeneric wide cross has been used for HI-Edit with CRISPR/Cas9 expressing maize as the pollen donor and a recalcitrant wheat variety as acceptor (Kelliher et al., 2019; Budhagatapalli et al., 2020). In one such use of HI-Edit, the pollen-specific regulatory region of *PROFILIN3* was used to drive Cas9 expression in maize pollen. It was found that several wheat haploids showed large deletions in the target gene (Kelliher et al., 2019). This might imply that also for HI-Edit, cell type-specific expression may be a promising strategy.

CONCLUSIONS AND PERSPECTIVES

CRISPR/Cas9-based genome editing already is an indispensable tool in plant genetics and breeding and many new technologies are being developed to expand the CRISPR toolbox such as base and prime editing. Many of these new tools could also benefit from TGE-based approaches, especially when editing efficiency is low. A particularly interesting application of TGE is HI-Edit and the research field of haploid induction has seen a number of recent breakthroughs that will impact successful use of HI-Edit in crops. For example, alternative haploid inducers based on CENH3 have now been developed for maize (Wang et al., 2021a) and wheat (Lv et al., 2020). First developed in *Arabidopsis*, haploid inducers based on CENH3 result in maternal genome elimination to produce paternal haploid progeny after pollination with wild-type pollen (Ravi and Chan, 2010). Due to the postzygotic gradual loss of maternal chromosomes, CENH3 systems are compatible with HI-Edit as shown for *Arabidopsis* (Kelliher et al., 2019). A CENH3-based approach in grasses would expand the HI-Edit strategy to both maternal and paternal haploids and may potentially improve overall efficiency of recovering edited plants compared to the stock 6-based system.

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Genome Editing for Improving Crop Nutrition

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Genome editing technologies, including CRISPR/Cas9 and TALEN, are excellent genetic modification techniques and are being proven to be powerful tools not only in the field of basic science but also in the field of crop breeding. Recently, two genome-edited crops targeted for nutritional improvement, high GABA tomatoes and high oleic acid soybeans, have been released to the market. Nutritional improvement in cultivated crops has been a major target of conventional genetic modification technologies as well as classical breeding methods. Mutations created by genome editing are considered to be almost identical to spontaneous genetic mutations because the mutation inducer, the transformed foreign gene, can be completely eliminated from the final genome-edited hosts after causing the mutation. Therefore, genome-edited crops are expected to be relatively easy to supply to the market, unlike GMO crops. On the other hand, due to their technical feature, the main goal of current genome-edited crop creation is often the total or partial disruption of genes rather than gene delivery. Therefore, to obtain the desired trait using genome editing technology, in some cases, a different approach from that of genetic recombination technology may be required. In this mini-review, we will review several nutritional traits in crops that have been considered suitable targets for genome editing, including the two examples mentioned above, and discuss how genome editing technology can be an effective breeding technology for improving nutritional traits in crops.

Keywords: genome editing, nutritional improvement, crops, CRISPR/Cas9, TALEN, high GABA tomato, high oleic soybean oil

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INTRODUCTION

The demand for food is increasing due to global population growth; the worldwide population is projected to require a 1.7-fold increase in cereals and a 1.8-fold increase in livestock products by 2050 (MAFF, Japan, 2019). Therefore, efforts should be made to increase food production using all possible methods. In addition, changing global dietary habits (from a starch-based diet to a high-fat, high-protein diet) and the aging of the population have led to an increase in lifestyle-related diseases, resulting in ever-increasing health concerns worldwide. Medical solutions are direct, but they are costly and not widely applicable. Therefore, it is important to address these lifestyle-related diseases with comprehensive nutritional intake through food. For these reasons, improving crop nutrition has become an important national policy in many countries. However, with conventional breeding techniques, it usually takes more than 10 years to produce a commercial cultivar that includes the desired traits. This is because many generations of crosses and backcrosses are generally required to accumulate a set of QTLs for the target traits. Some crop species have accumulated useful genetic resources that provide an important basis for creating new superior varieties with excellent genetic

tools. However, it is difficult to overcome the time constraint, and time is one of the major hurdles in responding to ever-changing international needs.

The advent of genome editing technologies (ZFN, TALEN, CRISPR/Cas9, etc.) has attracted a great deal of attention because the remove the limitations of conventional breeding methods (for more information on each technology in crops, we recommend reading the following reviews: Rojas-Vásquez and Gatica-Arias, 2019; Matres et al., 2021). These technologies are capable of creating precise mutations in targeted genes, and their use in the field of crop breeding is on the rise. Some of the crops that are currently being produced include disease- and stress-tolerant crops, high oleic acid soybeans, and high GABA-accumulating tomatoes. If the advantages of genome editing are utilized to the fullest, it will be possible to modify the accumulation of target functional components while retaining 100% of the host's other useful traits by controlling the expression and modifying the functions of genes involved in the pre- and postmetabolism of the target functional components. Therefore, genome editing technology is expected to be an effective breeding method to modify the metabolism of nutritional functional components, especially for crops whose biosynthetic systems are known.

In this mini-review, we discuss the current status of developmental research on the improvement of functional components in crops using genome editing technology and provide an outlook for the future.

IMPROVING NUTRIENT COMPOSITION BY CONTROLLING GENE EXPRESSION

One of the targets of genome editing is to regulate the expression level of genes involved in the metabolism of the target nutrient or undesirable substance itself. In this case, there are two targets: one is as far upstream of the CDS as possible, and the other is the untranslated region involved in regulating expression, including the 5′UTR. The former target induces a frameshift as far upstream as possible of the CDS and results in a termination codon that is not normally present, thereby achieving incomplete translation by premature termination of translation. The latter target aims to regulate the expression level of target genes and proteins by mutating the untranslated regions of genes involved in the regulation of transcription and expression levels.

Development of High Oleic Soybean Oil

Soybean oil contains high amounts of polyunsaturated fatty acids such as linoleic acid and linolenic acid and low amounts of monounsaturated fatty acids such as oleic acid. Soybean oil is hydrogenated to improve its fatty acid composition, but this process produces trans-fatty acids that are rather harmful to human health (Ascherio et al., 2008). In 2019, Calyxst (Minnesota, United States) developed a new soybean with more than 80% oleic acid (similar to olive oil) using TALEN and launched Calyno^{TM}, the world's first genome-edited soybean with improved oleic acid content. They achieved the goal of more than 80% oleic acid and less than 3% linolenic acid by knocking out the fatty acid desaturase *FAD2-1A, FAD2-1B* and *FAD3A* exons, which metabolize oleic acid to linoleic acid, using TALEN (Haun et al., 2014; Demorest et al., 2016). This strategy involves knocking out genes involved in the downstream metabolism of oleic acid synthesis in the soybean fatty acid metabolic pathway that essentially leads to linolenic acid, thereby increasing the accumulation of the intermediate product oleic acid and inhibiting the downstream synthesis of linolenic acid and linoleic acid.

OTHER EXAMPLES

Similar genome editing has been carried out in many crops and many genes, including knockout of the vacuolar invertase gene *VInv*, which produces reducing sugars responsible for acrylamide production in potatoes (Clasen et al., 2016), and knockout of the *PPO* gene in mushrooms (Waltz, 2016a) (**Table 1**). The strategy of targeting the CDS to induce frameshifting often results in a simple disruption of the target gene, and even if the structure and function of the protein encoded by the target gene are not clear, it is easy to obtain the desired phenotype. The ease of application is one of the reasons why this strategy has been used in many cases. On the other hand, the traits that can be altered by this strategy are limited to those that occur when the molecular biological events that the target gene is responsible for are restricted, such as the repression of the synthesis or degradation of specific substances, repression of the conversion to downstream metabolites, repression of the transport to specific compartments, and so on. However, for the nutritional improvement of crops, when the goal is to increase the synthesis and accumulation of the target product, sometimes the "suppressive" modification described above is not sufficient to meet market needs.

Possibility of Regulating Expression by Improving the Untranslated Region

The transcriptional efficiency of genes is mainly controlled by the promoter region in the 5′UTR and the terminator region in the 3′UTR. In addition, there are transcriptional control regions called enhancers in the upstream and intergenic regions of genes. In the introns of some genes, there are also miRNAs that control or inhibit transcription. Furthermore, some transcribed mRNAs contain translation control regions (uORFs) that inhibit the translation of mRNAs, and in fact, there are some successful cases where vitamin C contents have been improved (Li et al., 2018c; Zhang et al., 2018). By targeting these elements and causing genomic mutations, it is possible to suppress or overexpress their expression levels without disrupting the CDS of the target gene. To date, there are few reports of genome editing targeting these elements, but the **Supplementary Table S1** summarizes the most likely examples. Although not within the scope of this review because crop nutrition improvement was not the target, a study in rice demonstrated that precise multiple-base editing at miRNA target sites is possible (Ohtsuki et al., 2018).

TABLE 1 | Applications of CRISPR/Cas9 in major crop species to improve nutritional contents.

Common name	Phenotype	Target gene	Target region	GE result on target expression or activity	GE technique	Molecular function of the target gene	References
Rice	increased content of resistant starch	*SBEI and SBEIIb*	CDS	DOWN	CRISPR/Cas9	Regulate amylose contents	Sun et al. (2017)
Rice	low cesium accumulation	*OsHAK-1*	CDS	DOWN	CRISPR/Cas9	Cs + uptake in roots	Nieves-Cordones et al. (2017)
Sorghum	increased digestibility and protein quality	*k1C gene family*	n.i.	DOWN	CRISPR/Cas9	α-kafirins (major storage proteins)	Li et al. (2018a)
Bread wheat	low gluten content	*sgAlpha-1 sgAlpha-2*	CDS	DOWN	CRISPR/Cas9	the immunoreactive α-gliadin	Sánchez-León et al. (2018)
Soybean	altered fatty acids levels	*FAD2-1A* and *FAD2-1B*	CDS	DOWN	TALEN	Fatty acid desaturase 2	Haun et al. (2014); Demorest et al. (2016)
Peanut	increased oleic acid content	*FAD2A* and *FAD2B*	CDS	DOWN	CRISPR/Cas9	Converts oleic acid to linoleic acid gene coding sequences	Yuan et al. (2019)
Peanut	0.5–twofold increase in the oleic acid content	*FAD2*	CDS	DOWN	TALEN	Converts oleic acid to linoleic acid gene coding sequences	Wen et al. (2018)
Sweet potato	decreased amylose content	*GBSSI*	CDS	DOWN	CRISPR/Cas9	Granule-bound starch biosynthesis	Wang et al. (2019)
Sweet potato	decreased amylopectin content; increased amylose content	*SBEII*	CDS	DOWN	CRISPR/Cas9	Starch branching for amylopectin	Wang et al. (2019)
Potato	decreased browning	*PP02*	CDS	DOWN	CRISPR/Cas9	Converts phenolic substrates to quinones	González et al. (2020)
Potato	decreased steroidal glycoalkaloid content	*16DOX*	CDS	DOWN	CRISPR/Cas9	Steroidal glycoalkaloid biosynthesis	Nakayasu et al. (2018)
Potato	reduced levels of acrylamide	*Vinv*	CDS	DOWN	TALEN	Accumulation of reducing sugars which cause acrylamide accumulation.	Clasen et al. (2016)
Tomato	reduced concentration of γ-aminobutyric acid	*GABA-TP1, GABA-TP2, GABA-TP3, CAT9 and SSADH*	CDS	DOWN	CRISPR/Cas9	Essential genes for the γ-aminobutyric acid (GABA) pathway	Li et al. (2018b)
Tomato	Pink tomatoes	*MYB12*	CDS	DOWN	CRISPR/Cas9	Flavonoids Metabolic Pathways	Deng et al. (2018); Zhu et al. (2019)
Tomato	oprange tomatoes and yellow tomatoes, respectively	*CRTISO or PSY1*	CDS	DOWN	CRISPR/Cas9	Carotenoids Metabolic Pathways	Dahan-Meir et al. (2018)
Tomato	purple tomatoes	*SlANT1*	Promo-ter	UP	TALEN and CRISPR/Cas9	Anthocyanin biosynthesis	Čermák et al. (2015)
Tomato	5.1-fold increase in the lycopene content	*SGR1, LCY-E, Blc, LCY-B1, and LCY-B2*	CDS	DOWN	CRISPR/Cas9	Carotenoids Metabolic Pathways	Li et al. (2018d)
* Tomato	increased carotenoid, lycopene, and β-carotene	*SlDDB1, SlDET1, SlCYC-B*	CDS	DOWN	Target-AID	Carotenoids Metabolic Pathways	Hunziker et al. (2020)
* Tomato	sevenfold to 15-fold increase in GABA accumulation	*SlGAD2 and SlGAD3*	CDS (AID)	UP	CRISPR/Cas9	Aminobutiric acid Metabolic Pathways	Nonaka et al. (2017)
Wild tomato	increased vitamin C content	*GGPI*	uORF	UP	CRISPR/Cas9	Vitamin C metabolism	Li et al. (2018b)
Tomato	decreased anthocyanin content	*SlANT2, SlAN2-like*	CDS	DOWN	CRISPR/Cas9	Anthocyanin biosynthesis	Yan et al. (2020); Zhi et al. (2020)
Tomato	decreased anthocyanin content	*HYS*	CDS	DOWN	CRISPR/Cas9	Anthocyanin biosynthesis in response to light	Qiu et al. (2019)
Tomato	increased phenylalanine-derived volatile content	*FLORAL4*	CDS		CRISPR/Cas9	Regulates phenylalanine-derived volatiles in fruit	Tikunov et al. (2020)
Tomato	decreased volatile organic compounds	*RIN*	CDS	DOWN	CRISPR/Cas9	Ripening control via ethylene	Ito et al. (2017); Zhi et al. (2020)
Tomato	SSC, fiber, fructose, ascorbic acid, total	*L1L4*	CDS	DOWN	ZFN	Metabolite pathway	Gago et al. (2017)

(Continued on following page)

TABLE 1 | (*Continued*) Applications of CRISPR/Cas9 in major crop species to improve nutritional contents.

Common name	Phenotype	Target gene	Target region	GE result on target expression or activity	GE technique	Molecular function of the target gene	References
	phenol, carotene, oxalic acid						
Tomato/ wild tomato	high lycopene content	*cycB*	CDS	DOWN	CRISPR/ Cas9	Metabolite pathway	Zsögön et al. (2018)
Eggplant	decreased browning	*PP04, PPOS, and PP06*	CDS	DOWN	CRISPR/ Cas9	Converts phenolic substrates to quinones	Maioli et al. (2020)
Grape	decreased tartaric acid content	*IdnDH*	CDS	DOWN	CRISPR/ Cas9	Tartaric acid biosynthesis Vegetables	Ren et al. (2016)
Carrot	decreased anthocyanin content	*F3H*	CDS	DOWN	CRISPR/ Cas9	Anthocyanin biosynthesis	Klimek-Chodacka et al. (2018)
Brassica rapa	decreased fructose, glucose, and increase sucrose contents	*BrOG1A and BrOG1B*	CDS	DOWN	CRISPR/ Cas9	Primary metabolism	Jiang et al. (2020)
Rapeseed	increased seed oil content	*SFAR4 and SEARS*	CDS	DOWN	CRISPR/ Cas9	Oil degradation	Karunarathna et al. (2020)
Rapeseed	increased oleic acid content; decreased linoleic and linolenic acid contents	*FAD2*	CDS	DOWN	CRISPR/ Cas9	Fatty acid biosynthesis	Okuzaki et al. (2018)
Chinese kale	yellow color of Chinese kale with improved market prospects	*BoaCRTISO*	CDS	DOWN	CRISPR/ Cas9	Carotenoid biosynthesis	Sun et al. (2020)
Lettuce	increased oxidation stress tolerance and ascorbate content	*LsGGP2*	uORF	UP	CRISPR/ Cas9	Deleted uORFs of LsGGP2 to increase the translation of mRNAs	Zhang et al. (2018)
Banana	increased F-carotene content	*LCYe*	CDS	DOWN	CRISPR/ Cas9	ß-carotene metabolism	Kaur et al. (2020)
Mush-room	decreased browning	*PPO*	CDS	DOWN	CRISPR/ Cas9	Converts phenolic substrates to quinones	Waltz, (2016b), *review
Pome-granate	unique accumulation of gallic acid 3-0- and 4-0-glucosides	PgUGT84A23 and PgUGT84A24	CDS	DOWN	CRISPR/ Cas9	UDP-dependent glycosyltransferases (UGTs) enzymes with overlapping activities in ß-glucogallin biosynthesis	Chang et al. (2019)

IMPROVING NUTRIENT COMPOSITION BY REGULATING GENE FUNCTION

Another goal of genome editing is to regulate the functions of proteins encoded by genes involved in the metabolism of a nutrient of interest or an unfavorable substance. In this case, the target may be the active center of the enzyme, the binding region of a ligand, or the activity control domain. Various patterns are possible depending on the combination of the physiological and structural properties of the target protein and the target trait.

Development of Tomatoes With High Accumulation of GABA

Tomatoes have been an excellent source of GABA among crops (Briguglio et al., 2018; Gramazio et al., 2020), and they are also a major crop commonly eaten around the world.

Sanatech Seed Co., Ltd. (Tsukuba, Japan) launched the first genome-edited tomato in Japan, "Sicilian Rouge High GABA", in 2021. This high-GABA tomato contains approximately four to five times the amount of GABA found in ordinary tomatoes. Considering that the high GABA tomatoes (without genome editing technology) available on the market until now contained approximately 1.5 times more GABA, this increase in the GABA content is a revolutionary improvement. CRISPR/Cas9 genome editing, which is responsible for this increase, targets the autoinhibitory domain (AID) on the C-terminal side of *GAD3*, an enzyme involved in the biosynthesis of GABA (Nonaka et al., 2017). By inducing a frameshift in this autoinhibitory domain, early termination of translation occurred, and the autoinhibitory domain of *GAD3* was excised (Nonaka et al., 2017). This strategy increases the enzymatic activity per molecule involved in GABA biosynthesis by eliminating inhibitors of *GAD3*, whose activity is normally suppressed, without modifying the expression level of *GAD3* itself.

Other Examples

As mentioned earlier, when modifying the function of a gene by genome editing, it is necessary to know the function of the protein or

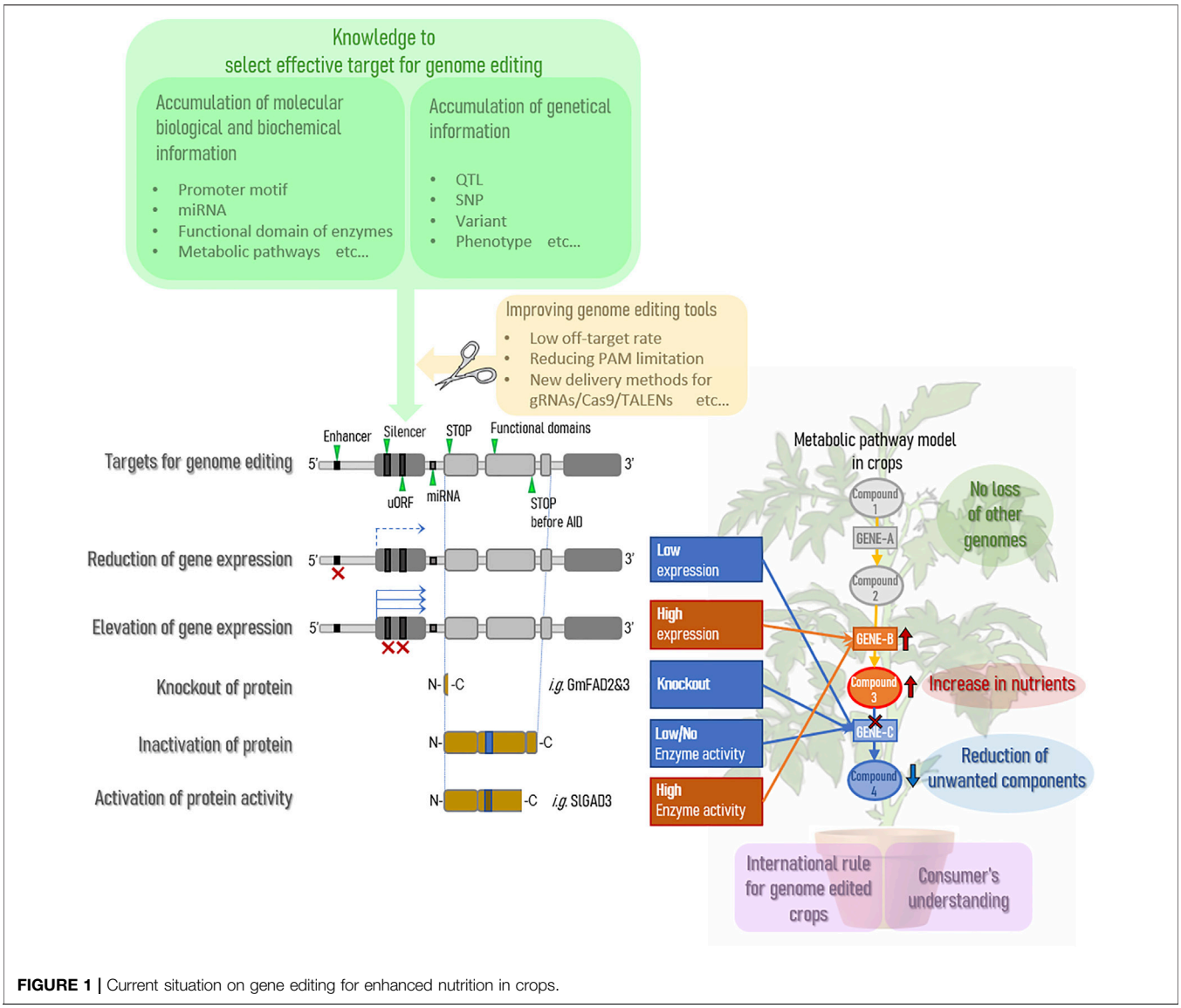

FIGURE 1 | Current situation on gene editing for enhanced nutrition in crops.

peptide encoded by the gene at the molecular level. The fact that there are far fewer examples of genome editing that have actually been implemented and have been effective compared to genome editing for knockout purposes (**Table 1**, asterisks) makes it easy to imagine the many challenges that need to be overcome. However, TARGET-AID has proven that it is possible to reproduce amino acid substitution mutations and obtain phenotypes by genome editing technology when the mutation is known (Hunziker et al., 2020).

Understanding the Molecular Mechanism of Protein Function Regulation is Necessary for Controlling Gene Function by Genome Editing

In many cases, the normal function of a protein is achieved by a complex interplay of various factors, including the regulatory conditions of the active/inactive form. Therefore, the selection of targets for genome editing requires both molecular biological and biochemical knowledge of molecular mechanisms such as protein domain structure, protein–protein interactions, and activation control by feedback/feed-forward regulation.

On the other hand, genome editing can be useful for basic research to obtain such knowledge. Compared to conventional gene transfer methods (e.g., overexpression and RNA interference), genome editing has the advantage of removing the effects of foreign genes and finely modifying the targets at the domain and base levels, which is the same advantage that genome editing brings to crop breeding.

FUTURE PROSPECTS AND CHALLENGES

Genome editing technology is expected to expand as a way to improve the nutritional status of agricultural crops. Currently,

many QTLs have accumulated in crops on the market through breeding, and genome editing technology is expected to improve the nutritional status of crops without compromising almost 100% of these useful QTLs. In other words, it can dramatically improve the effort to maintain QTLs by backcrossing and greatly reduce the time and cost of new breeding endeavors. However, there are several points that need to be improved before stable practical applications can be achieved. A summary is provided in **Figure 1**.

It is Necessary to Improve the Technology to Modify the Target Genes More Precisely

Current genome editing technologies cannot completely eliminate the risk of off-target effects. To overcome this weakness, various challenges are being addressed to improve the off-target rate (Manghwar et al., 2020), including the identification of factors affecting the occurrence of off-target effects (Modrzejewski et al., 2020) and the development of CRISPR/Cas type I-D (Osakabe et al., 2020). When specific motifs are already known in the promoter region, the need to substitute arbitrary bases is expected to increase and improvements in nickase and base editing technologies (Nishida et al., 2016; Sakata et al., 2019) will be increasingly required; these improvements will be accelerated as our understanding of the molecular mechanisms of key trait expression increases. In addition, when using the CRISPR/Cas9 system, a PAM sequence is currently required in the vicinity of the region of the target gene for which genome editing is desired; however, this is a major barrier to target selection. Currently, the challenge is to improve and eliminate this limitation of PAM sequences (Collias and Beisel, 2021).

Understanding the Molecular Mechanism of Expression for Important Traits in Target Crops is Necessary

In the future, as the accuracy of the technology for modifying target genes improves, the effect of genome editing can be maximized by controlling point (SNP)-level mutations for efficient expression of traits. For this purpose, it is more important to understand the underlying molecular mechanisms. Thus, it will become increasingly important to collect and store diverse genetic resources and analyze them to accumulate more basic research knowledge on the target crop and more SNP information related to phenotypes.

There is a Need to Establish Rules for Genome Editing Crops in Each Country

As we have discussed, since the discovery of the CRISPR/Cas9 mechanism in 2012, the progress of genome editing and its introduction into basic and applied science has accelerated worldwide. For the legality of this technology to persist, international rules must be generated quickly and appropriately.

Genome editing can be broadly classified into three categories depending on the type of mutation being introduced: SDN-1 uses nonhomologous end joining (NHEJ) to make relatively small deletions, insertions, and base substitutions; SDN-2 uses a species own homologous region as a template for homologous recombination repair (HDR); and SDN-3 introduces genes from outside of the species for repair. For each of these three types, there is currently an international debate on whether genome-edited crops should be treated as recombinant (GM) or nonrecombinant (non-GM). In fact, the treatment of genome-edited crops varies from country to country, with many European countries treating genome-edited crops in a more restrictive manner. In this context, in 2020, EFSA reported its view that the SDN-1 and SDN-2 types are not subject to risk assessment if they do not contain exogenous DNA (Naegeli et al., 2020).

In addition, on april 29, 2021, the European Commission published the results of its review of the place of "new genomic technologies (NGTs)" in EU law, which strongly suggests that there are limits to the ability of existing legislation to apply to NGTs and their products and that legislation needs to be adapted to scientific and technological advances (https://ec.europa.eu/food/plants/genetically-modified-organisms/new-techniques-biotechnology/ec-study-new-genomic-techniques_en). Furthermore, on 29 September 2021, a statement was issued by the UK government on its plans to lift GMO-like restrictions on genome editing (https://www.gov.uk/government/consultations/genetic-technologies-regulation/outcome/genetic-technologies-regulation-government-response).

While we should continue to monitor the views of other countries, the fact that the high oleic soybeans and high GABA tomatoes, which are categorized as SDN-1, have finally reached the market and are now available to the general public is a large step in the history of genome-edited crops and is certainly a major benchmark for countries to formulate future directions and appropriate rules.

There is a Need to Improve Consumer Understanding of Genome-Edited Crops

Needless to say, the ultimate recipients of the developed genome-edited crops are consumers. When ordinary consumers purchase genome-edited crops at supermarkets, they are most likely to be concerned about safety. For consumers to understand the safety of genome-edited crops, it is necessary to communicate as correctly and clearly as possible how genome-edited crops were developed, why they are safe, what makes them different from conventional crops, and what makes them different from GM crops. Sanatech Seed Co., Ltd., the company that developed the high GABA tomato, has established two websites, one in Japanese and one in English, with Q&A pages for general questions, thus creating a platform of information that ordinary consumers can refer to when they are curious (https://sanatech-seed.com/en/). Web tools such as social networking services (SNSs), which are currently undergoing remarkable technological innovation, can be used as tools that provide opportunities for multidirectional communication, unlike traditional one-way mass media. Therefore, by using these new tools, we may be able to accomplish the task of information dissemination more effectively. On the other

hand, until the new products created by this new technology are widely accepted by the public, consumers may be need to select and examine more appropriate information without getting caught up in sensational topics. Scientists and the international community should remain equally (or even more) loyal to the consumer's motivation to understand these technologies.

DISCUSSION

Just 8 years after the publication of the first paper on CRISPR/Cas9 by Charpentier and Doudna in 2012 (Jinek et al., 2012), the Nobel Prize in Chemistry was awarded to CRISPR/Cas9 in 2020. The launch of the first CRISPR/Cas9 genome-edited crop in 2021 is a testament to the superiority of CRISPR/Cas9 as a crop breeding technology and reflects the current pressure on the breeding field and the international community to solve food supply problems. This Nobel Prize-winning genome editing technology is anticipated to help improve global nutrition.

AUTHOR CONTRIBUTIONS

AN and HE conceived this mini review. AN and HE wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Predictable NHEJ Insertion and Assessment of HDR Editing Strategies in Plants

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Canonical CRISPR-Cas9 genome editing technique has profoundly impacted the fields of plant biology, biotechnology, and crop improvement. Since non-homologous end joining (NHEJ) is usually considered to generate random indels, its high efficiency mutation is generally not pertinent to precise editing. Homology-directed repair (HDR) can mediate precise editing with supplied donor DNA, but it suffers from extreme low efficiency in higher plants. Therefore, precision editing in plants will be facilitated by the ability to predict NHEJ repair outcome and to improve HDR efficiency. Here, we report that NHEJ-mediated single nucleotide insertion at different rice genes is predictable based on DNA sequences at the target loci. Three mutation prediction tools (inDelphi, FORECasT, and SPROUT) have been validated in the rice plant system. We also evaluated the chimeric guide RNA (cgRNA) and Cas9-Retron precISe Parallel Editing via homologY (CRISPEY) strategies to facilitate donor template supply for improving HDR efficiency in *Nicotiana benthamiana* and rice. However, neither cgRNA nor CRISPEY improved plant HDR editing efficiency in this study. Interestingly, our data indicate that tethering of 200–250 nucleotides long sequence to either 5′ or 3′ ends of guide RNA did not significantly affect Cas9 cleavage activity.

Keywords: CRISPR-Cas9, genome editing, guide RNA engineering, homology-directed repair, non-homologous end joining, NHEJ prediction

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INTRODUCTION

Crop improvement greatly relies on exploiting existing- and creating new-genetic variations. Conventional CRISPR-Cas tools have greatly facilitated the generation of targeted genetic variations in plants by producing random indels through the non-homologous end joining (NHEJ) repair pathway (Xie and Yang, 2013; Molla et al., 2020a). Base editing, an emerging technology, can precisely install four transition and two transversion point mutations (Molla and Yang, 2019; Molla and Yang, 2020a; Molla et al., 2020b; Molla et al., 2020c). However, neither CRISPR-Cas nor base editing can generate precise indels, which are also important for plant trait improvement. To generate precise indels, we mainly depend on utilizing the homology-directed repair (HDR) pathway. Unfortunately, HDR is template-directed and inefficient, limiting its application in crop improvement. Cas9-induced double-strand break (DSB) in DNA is repaired predominantly through the NHEJ pathway in higher plants. Therefore, unlike HDR, NHEJ-mediated mutagenesis is highly efficient in plants. If we could predict the DSB repair outcome, it would facilitate generating precise indels.

Although Cas9 was believed to trigger random repair outcomes, a growing body of evidence indicates that the repair outcomes are non-random and depend on target DNA sequence (van Overbeek et al., 2016; Chakrabarti et al., 2019; Taheri-Ghahfarokhi et al., 2018; Molla and Yang, 2020b; Li et al., 2021). Large datasets were utilized to develop models (inDelphi, FORECAST, SPROUT, and CROTON) for predicting Cas9 repair outcome in mammalian cells (Shen et al., 2018; Allen et al., 2019; Leenay et al., 2019; Molla and Yang, 2020b; Li et al., 2021). Those computational tools predict repair outcomes, mainly the frequency of 1 bp insertions and small deletions, with high efficiency (Molla and Yang, 2020b). Although the models have been developed exploiting Cas9 repair data from mammalian cells, we hypothesize that they could also be used to predict repair outcomes in plant cells since the mutations largely depend on the local DNA sequence near the DSB (Molla and Yang, 2020b). However, no single study reports Cas9 repair outcome prediction in plants or validates those predictor models in plants.

HDR is highly valuable in precise gene replacements, knock-in, and installing complex modifications. However, achieving a decent efficiency in higher plants is a major hurdle to using HDR regularly in crop improvements. For HDR to be successful, adequate donor repair templates are needed to be available near the DSB. Temporal and spatial coordination between DSB creation and supplying adequate donor templates are considered the major bottlenecks in HDR (Li et al., 2018; Huang and Puchta, 2019). Several strategies such as using geminivirus replicons (Čermák et al., 2015), chimeric guide RNA (cgRNA) (Butt et al., 2017), chemically modified donor DNA, tandem repeat HDR (TR-HDR) (Lu et al., 2020), and transcript-templated HDR (TT-HDR) (Li et al., 2019) have been used to overcome these bottlenecks. To make the donor templates available onsite of DSB, one attractive approach is to fuse them with the guide RNA (gRNA) sequence. For example, the use of chimeric guide RNA (cgRNA) molecule, containing gRNA fused with donor template, has been demonstrated to induce HDR in rice (Butt et al., 2017). cgRNA strategy was based on donor template fusion at the 3' end of sgRNA and RNA-templated DNA repair (Butt et al., 2017). Recently, an interesting strategy, Cas9-Retron precISe Parallel Editing via homologY (CRISPEY), described the utilization of bacterial retron to produce single-stranded donor DNA that is tethered with sgRNA (Sharon et al., 2018). CRISPEY strategy has been shown to improve HDR efficiency up to 96% in yeast (Sharon et al., 2018). In human cells, CRISPEY achieved HDR rates of up to 11.3% (Kong et al., 2021; Zhao et al., 2021). However, there is no report of plant HDR improvement utilizing a bacterial retron system.

In this study, we explored to achieve HDR-mediated editing using cgRNA and CRISPEY strategies in tobacco and rice for six different target genes. We also investigated predicting Cas9 repair outcome in plants utilizing the predictor models generated for mammalian systems. The findings could be helpful for precise genome editing in plants.

MATERIALS AND METHODS

Vector Construction

We have designed modified versions of the CRISPEY construct described earlier (Sharon et al., 2018). For GFP to BFP conversion in *Nicotiana benthamiana*, we fused tobacco codon optimized *E. coli* Ec86-reverse transcriptase (Ec86-RT) with P2A-Cas9 for co-translational expression of both Ec86-RT and Cas9 by CaMV35S promoter. P2A is a self-cleaving peptide. A chimeric RNA of Ec86 retron sequence with a gRNA was expressed by AtU6 promoter. It was designed in such a way that a portion of retron sequence was replaced with a donor template sequence harboring the mutations necessary for GFP to BFP conversion (**Supplementary Table S1** and **Supplementary Sequence**).

For rice, we first constructed a basic vector pK-CRISPEY, which contains three distinct cassettes. The first one is a rice codon-optimized Ec86-RT expression cassette. Ec86-RT was driven by OsUbi10 promoter and terminated with Agrobacterium gene seven terminator. The second one was to express a chimeric RNA of Ec86 retron sequence with a gRNA. A portion of retron sequence was replaced with specific donor template sequences. This cassette was driven by CaMV 35S promoter and terminated by Arabidopsis HSP terminator. The 5′ and 3′ end of the chimeric retron-guide sequence was flanked by the hammerhead (HH) ribozyme and the hepatitis delta virus (HDV) ribozyme, respectively. Two Aar1 sites were incorporated upstream of the gRNA scaffold sequence for easy cloning of donor template plus protospacer sequence. The donor template was at the 5' end of the protospacer. The third cassette was to express SpCas9. We synthesized the first two cassettes and cloned them at the HindIII/BsaI sites of pRGEB32 vector replacing 402 bp to construct pK-CRISPEY. The original hygromycin phosphotransferase (HPT) gene cassette of pRGEB32 was intact (Xie et al., 2015). We separately synthesized donor plus protospacer for each of the three targets, *OsALS*, *OsCC,* and *OsActin,* and cloned at the AarI sites of pK-CRISPEY. Three vectors were named as pK-CRISPEY-ALS, pK-CRISPEY-CC, and pK-CRISPEY-Actin (**Supplementary Table S1** and **Supplementary Sequence**).

For the cgRNA approach, a polycistronic-tRNA-gRNA (PTG) multiplexing cassette was designed to repair three targets simultaneously (Xie et al., 2015). It comprises three gRNAs, *OsALS, OsPita*, and *OsPtr*, each with their specific repair template at the 3′ ends of the scaffolds. *ALS* and *Pita* required only a few base pairs modification, so the repair template was designed with 100 bp homology flanks from the DSB (**Supplementary Table S1**). Since the *Ptr* needed a 12 bp deletion and various base-pair changes over a larger region, we used 125 bp homology arms. Synonymous mutations were introduced in repair templates to prevent Cas9 from re-cutting after successful HDR repair. The PTG fragment with repair templates was synthesized (GenScript, NJ, United States) and then cloned downstream of the OsU3 promoter into the binary vector pRGEB32 using the compatible overhangs generated by BsaI digestion. Guide RNAs with repair templates fused at their 3' ends are termed as chimeric guide RNA (cgRNA) following an

earlier report (Butt et al., 2017). This new construct is termed pCgAPP (**Supplementary Sequence**).

pK-CRISPEY-ALS, pK-CRISPEY-CC, pK-CRISPEY-Actin, and pCgAPP were introduced into *Agrobacterium tumefaciens* strain EHA105 *via* electroporation for subsequent agroinfiltration and/or stable transformation in tobacco and rice.

Agroinfiltration and Generation of Stable Transgenics in *Nicotiana benthamiana*

Nicotiana benthamiana 16c, a transgenic line highly expressing mGFP, was used in this study (Ruiz et al., 1998). 16c line was a generous gift from Prof. David Baulcombe (United Kingdom). Agroinfiltration was performed using four to 6 week old plants grown at 25°C and 75% humidity (75%) under the 16 h light (100 μmol photons m^{-2} s^{-1}) according to a previously described protocol with few modifications (Yang et al., 2000). Briefly, MMA solution (10 mM MES, 10 mM MgCl2, 150 μM acetosyringone) was used as infiltration solution to resuspend *Agrobacterium* cells (EHA105) to an OD_{600} = 1. The abaxial leaf regions to be infiltrated were punctured with a small needle. A 1-ml syringe (without needle) was used to infiltrate *Agrobacterium* suspension.

To develop stable transgenic plants, fully expanded fresh leaves were collected and sterilized by immersing in 70% ethanol for 60 s, washing in 7.5% bleach plus one drop Tween-20 solution for 20 min, and repeatedly washing in sterile distilled water. Round leaf discs were prepared by pressing a cork borer against an *N. benthamiana* leaf on a Petri dish base. Leaf discs were incubated in *Agrobacterium* suspension (OD_{600} = 1, 100 μM acetosyringone) for 30 min. The discs were blot dried and incubated in cocultivation media (4.3 g/L MS salts, 30 g/L sucrose, 1 mg/L 6-benzylaminopurine, 0.1 mg/L 1-naphthaleneacetic acid, and 100 μM Acetosyringone) for 2 days in the dark. Leaf discs were washed for removing extra bacterial cells, blot dried, and transferred to regeneration selection media (4.3 g/L MS salts, 30 g/L sucrose, 1 mg/L 6-benzylaminopurine, 0.1 mg/L 1-naphthaleneacetic acid, 400 mg/L Timentin, and 10 mg/L hygromycin). The plates were incubated at 28°C with an 18 h light regime. Leaf discs were moved to fresh media plates every 14–15 days. After 2 weeks of selection, callus tissue starts appearing from the cut ends of the disk. Shoots growing from the selected calli were dissected and placed in rooting media (MS salts, 30 gm/L sucrose, 25 mg/L hygromycin) to produce plantlets. Shoots were individually excised from the calli once they reached a height of >3 mm. After 2 weeks in rooting media, roots were adequately developed. Plantlets with well-developed roots were transferred to soil pot in greenhouse.

Rice Transformation and Regeneration

Kitaake and Jupiter (*Oryza sativa* subsp. *japonica*) rice genotypes was used for genetic transformation. Mature embryo-derived calli were transformed with all four constructs using the *Agrobacterium*-mediated method following an earlier described protocol (Molla et al., 2020a). Briefly, transformed calli were selected in hygromycin (50 mg/L) containing media. Selected and proliferating calli were either transferred to regeneration media or collected for DNA isolation. Regenerated shoots were transferred to rooting media. Well-rooted plantlets were transferred to soil and grown in a greenhouse.

Microscopical Analysis

Segments of leaf tissue (1–2 cm) were excised, and the pieces were mounted in water on glass microscope slides with a coverslip. The leaves were imaged using an Observer SD spinning disc confocal microscope (Zeiss, Germany). Samples were visualized and photographed using 405 nm (Blue) and 488 nm (Green) filters.

Genotyping of Editing Outcomes

For the three CRISPEY constructs, hygromycin-resistant calli were used for DNA isolation after two rounds of selection. Isolated DNAs from calli samples were used to amplify the target regions. PCR products were pooled and subjected to deep amplicon sequencing by using Genewiz amplicon EZ sequencing service (Genewiz, United States). After adapter ligation and library preparation, the samples were sequenced using a 2 × 250 paired-end configuration. Image analysis and base calling were conducted by the Illumina Control Software on the Illumina instrument. Raw sequence data were demultiplexed using bcl2fastq version 2.17.1.14. Read pairs were trimmed for adapter sequences and low-quality basecalls using Trimmomatic version 0.36. Each read pair was then merged using the bbmerge tool from the BBtools software toolkit. The target sequence between conserved flanking primers was extracted from each merged pair. For each sample, one excel file was generated to contain the unique nucleotide sequences and their abundances, and one excel file was generated to contain the unique amino acid sequences and their abundance for each sample. The QIIME data analysis package was used to generate OTU sequences. OTU clusters are defined by a 97% identity threshold.

We also regenerated plants (Jupiter variety) for pCgAPP construct. Total DNA was isolated from leaf samples collected from each individual plant following an earlier described protocol (Molla et al., 2020c). Target regions of *ALS*, *Pita*, and *Ptr* loci were amplified by PCR using specific pair of primers. Purified PCR products were sequenced and decoded using TIDE for editing outcomes (Brinkman et al., 2014). All primers used for genotyping are listed in **Supplementary Table S1**.

Prediction of NHEJ Outcome

We employed three models, inDelphi, FORECast and SPROUT, for predicting NHEJ mutation outcome (Molla and Yang, 2020b). The inDelphi is available with the link: https://indelphi.giffordlab.mit.edu/. Protospacer with 50 bp flanking sequence on each side was provided as input in the inDelphi user interface. FORECasT was accessed with the link: https://partslab.sanger.ac.uk/FORECasT, while SPROUT was accessible by following the link: https://zou-group.github.io/SPROUT. For FORECasT, around 40–50 bp target genomic sequence, including protospacer, is required. SPROUT requires 20 bp protospacer plus 3 bp PAM sequence for prediction. The prediction outputs from each model were

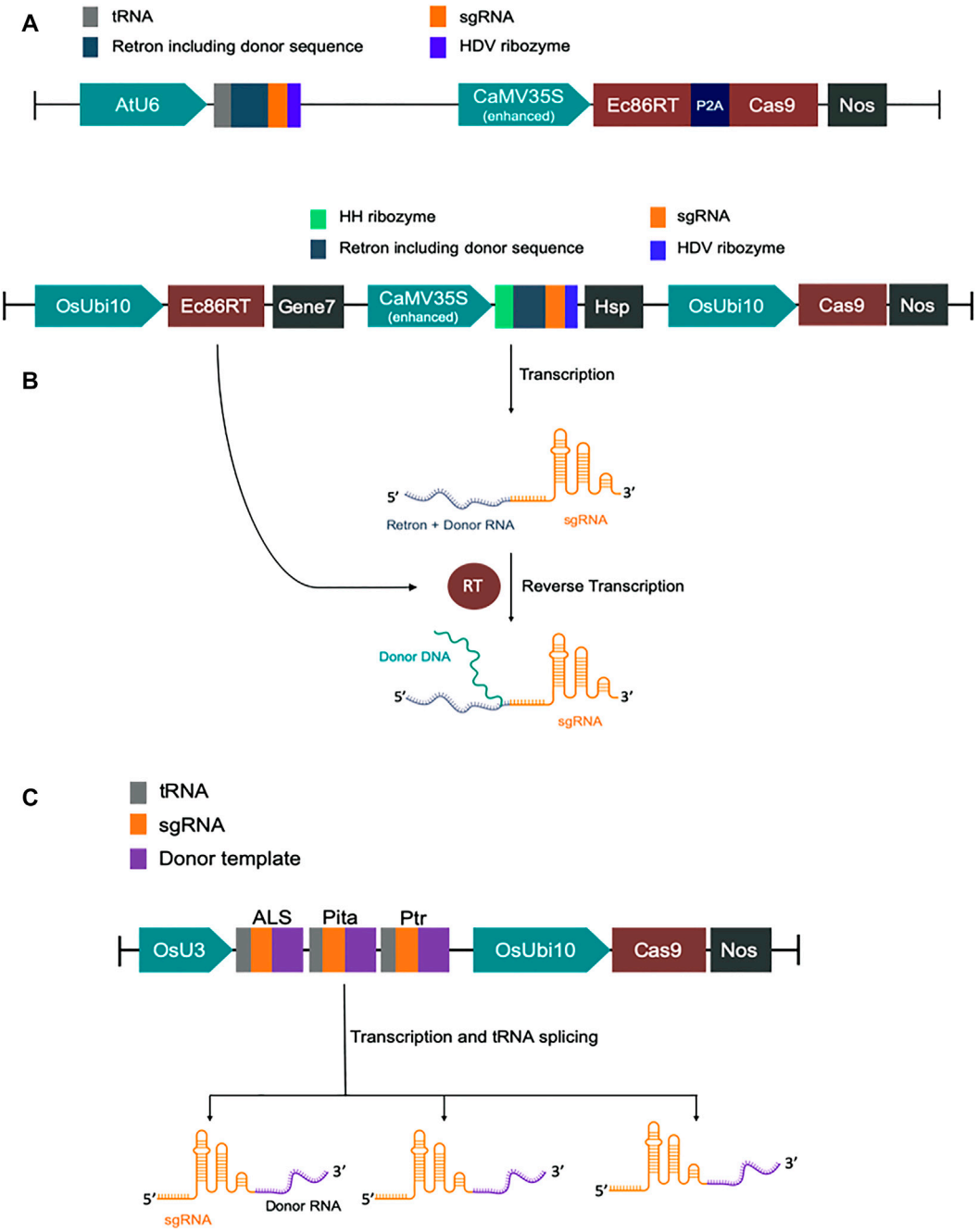

FIGURE 1 | Schematic diagram of DNA constructs. **(A)** Map of CRISPEY constructs used in *Nicotiana benthamiana*. **(B)** Map of pK-CRISPEY with donor used in rice. **(C)** Schematic diagram of pCgAPP construct.

compared with the observed data from deep sequencing (*ALS, CC,* and *Actin* targets) and Sanger sequencing (*Pita* target). The data from retron and cgRNA experiments were reanalyzed for NHEJ outcomes. We have considered the single base pair insertion and different deletion types and their frequency in our analysis.

Statistical Analysis

The data were analysed using Graphpad prism nine software (GraphPad Software, La Jolla, CA, United States). One-way analyses of variance (ANOVAs) and Dunnett's multiple comparisons test were used to compare the differences between different groups.

RESULTS AND DISCUSSION

Utilizing Retron for HDR in *Nicotiana benthamiana* and Rice

Retrons are prokaryotic retroelements that can produce multicopy single-stranded DNA (msDNA). Bacterial retrons have recently been shown to function in antiphage defense (Millman et al., 2020). Retron Ec86, from *E. coli*, contains a cassette that encodes a unique RNA (msd-msr) and a reverse transcriptase (Ec86-RT) (Inouye et al., 1999). Ec86-RT can reverse transcribe the msd portion into single-stranded DNA that remains tethered to its template RNA (Inouye et al., 1999). By altering a part of msd-msr sequence, single-stranded DNA (ssDNA) containing desired mutations flanked by homology to a targeted genomic region could be produced *in vivo*. Retron-derived ssDNA has been demonstrated to facilitate template-mediated genome editing in yeast (Sharon et al., 2018; Gallagher et al., 2020), mammalian cells (Kong et al., 2021; Zhao et al., 2021), and bacteria (Schubert et al., 2021). We fused the retron seq (msd-msr altered with donor sequence of interest) at the 5′ end of the gRNA to generate a chimeric transcript. After reverse transcription by Ec86RT, the donor ssDNA template would be tethered to the transcript and be available near the DSB for template-dependent repair. Envisaging retron could be harnessed for HDR-editing in plant cells, we set out to validate first in a GFP expressing *N. benthamiana* 16c line. To test if retron can promote HDR, we used a reporter system that results in GFP to BFP conversion. We designed a binary construct to express Cas9, Ec86RT, and retron sequence harboring information to produce donor ssDNA templates (**Figure 1A**). We introduced a single nucleotide change (TAT > CAT) for mGFP to BFP conversion (Tyr > His) and an additional three synonymous mutations in the protospacer seed region to prevent re-cutting the donor template by Cas9. We could not detect blue fluorescence when we analyzed leaf samples by confocal microscopy after several rounds of agroinfiltration. Similar results were obtained for samples collected after 3, 7, and 10 days of infiltration. Assuming that transient expression might not be sufficient to induce HDR, we performed leaf disc transformation with the construct and regenerated >50 stably transformed plants (**Supplementary Figures S1A,B**). Plants were analyzed for change in fluorescence from green to blue. We could not find a single plant with altered fluorescence (**Supplementary Figure S1C**). Sanger sequencing of 30 randomly chosen plants also did not reveal successful editing. We reasoned that the failure might be due to the inefficiency of the guide RNA used for the experiment. The guide RNA contains 5′-CTTA-3′ immediately adjacent to PAM sequence (**Supplementary Figure 1D**). The 5′-CTTA-3′ motif has been shown to be inefficient in genome editing in an earlier systematic study (Graf et al., 2019).

Then we attempted to test the same strategy in rice by targeting three genes, *OsALS* (OsKitaake02g183100), *OsCC* (OsKitaake05g165200), and *OsActin* (OsKitaake03g316400), separately (**Figure 1B**). For *ALS*, one nucleotide change (TGG > TTG) causing W > L for herbicide tolerance and another nucleotide change for PAM destruction were included in the donor template. We attempted to knock-in a 12 bp sequence harboring EcoRI and HindIII recognition sites in *CC* target and 6 bp EcoRI recognition sequence in *Actin* target. Three constructs were independently transformed to rice calli via the *Agrobacterium*-mediated transformation. After two consecutive selections in hygromycin, proliferating calli were collected for DNA isolation. For initial verification, we amplified the *ALS* target region and digested it with Mfe1 since successful editing events should generate a recognition site for the enzyme. We observed many samples exhibited Mfe1 positive results. It should be noted here that Mfe1 could also be generated if there is a single T insertion (NHEJ) at the cut point. Then, we sequenced randomly chosen 12 samples and analyzed them using Synthego ICE tool. Excitingly, the ICE analysis revealed a knock-in efficiency of up to 23% in the tested calli for the *ALS* target (**Supplementary Figure S2**).

For further validating the result and obtaining a clear idea of editing efficiency, we performed amplicon deep sequencing. For each construct, DNA was isolated from 30 independent calli samples. We amplified target regions using PCR and pooled 10 samples into one for deep amplicon sequencing. On an average, we obtained >50 K reads for each sample. Strikingly, results revealed no HDR events occurred in the case of *ALS* and *Actin* targets. This observation indicated that, for one or two nucleotide replacements, as in the case of *ALS*, the ICE tool is not highly sensitive and may mislead on the editing output. Amplicon deep sequence is recommended to get a clear picture of the editing events. However, for the *CC* target, we observed only one read with a perfect 12 bp knock-in. We have observed high NHEJ efficiency in all three cases. Overall, retron-mediated template editing has not been successful in tobacco and rice. Around 16 bacterial retron systems have been experimentally validated and have their fully annotated components available in the public database (Simon et al., 2019). Recently, modifications in the retron non-coding RNA that increases production of reverse transcribed DNA have been identified (Lopez et al., 2021). Systematic studies are required to find suitable retron systems for plant genome editing.

Chimeric gRNA Approach for HDR in Rice

Another approach to make the repair template available at the vicinity of DSB is to fuse the template sequence with gRNA sequence. After successful transcription, a chimeric guide RNA (cgRNA) would be produced containing gRNA for Cas9-mediated targeted DSB generation and RNA repair template for HDR editing (**Figure 1C**). An earlier study demonstrated 2.14% HDR efficiency of cgRNA approach in regenerated rice plants (Butt et al., 2017). Similarly, Cas12a mediated DSB coupled with RNA donor template was reported to achieve successful HDR in rice (Li et al., 2019). Encouraged by these studies, we tested the strategy at three rice loci, *ALS*, *Pita*, and *Ptr*. The pCgAPP construct was transformed in rice calli and 196 hygromycin resistant T_0 rice lines were obtained through regeneration. For the *ALS* and *Pita* targets, successful HDR event would generate the recognition sites for MfeI and NcoI,

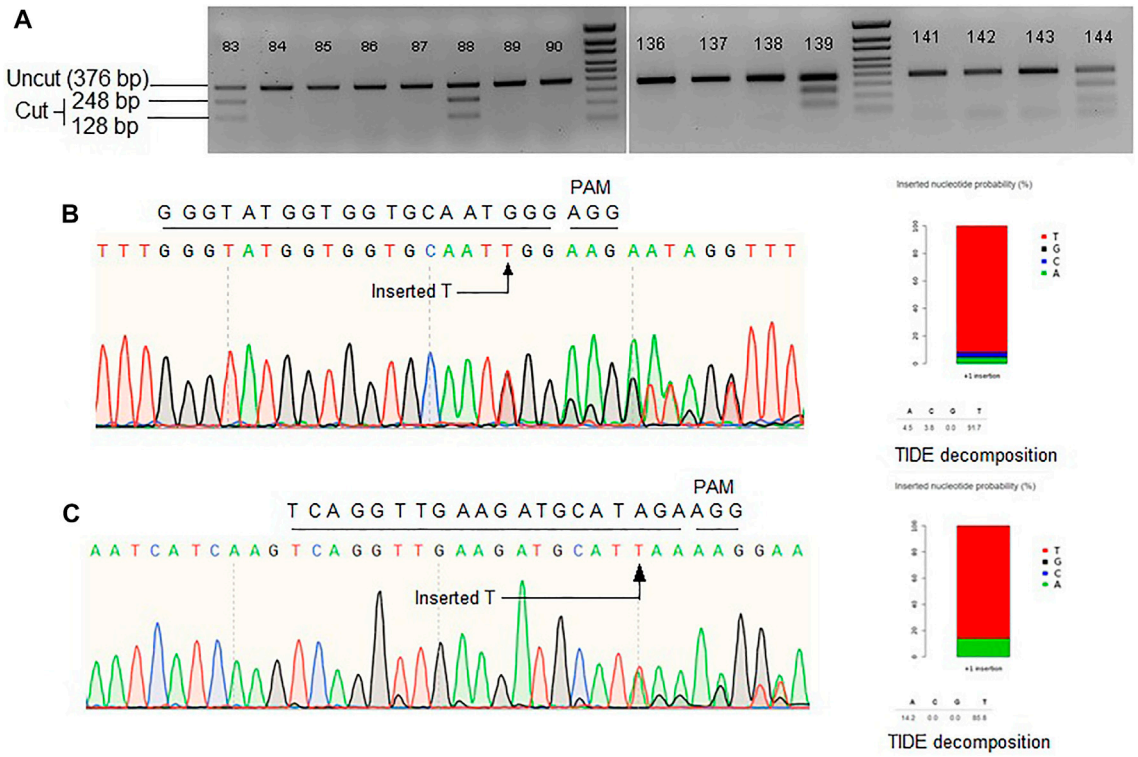

FIGURE 2 | (A) Representative gel images showing Mfe1 digestion of *ALS* product. Single T insertion at the cut point generates Mfe1 recognition site. **(B)** Representative Sanger chromatogram of *ALS* locus. **(C)** Representative Sanger chromatogram of *Pita* locus. TIDE decomposition of the chromatogram in **(B,C)** showing single T insertion. Wild-type protospacer and PAM sequences are shown above each of the chromatograms.

respectively. To screen successful HDR events at the *Ptr* locus, PCR positive/negative assay was employed by using one primer for the genomic region outside the homology included in repair template and a primer for the region over the deleted 12 bp polymorphism only found in the repair template (**Supplementary Figure S3A**). For *ALS* target, 25 out of the 196 lines indicated success of the MfeI site generation (**Figure 2A**). However, no *Nco*I positive lines were generated for *Pita* and no amplification was observed for *Ptr*. These results indicated unsuccessful HDR events at *Pita* and *Ptr* loci.

To further investigate editing outcomes, the lines in which successful MfeI sites generated were sequenced for all three target loci. Sanger chromatograms were decoded, aligned and compared to the WT and the repair template to analyze editing outcomes (**Figures 2B**,C). While for *ALS* there were many positive MfeI digestion results, sequencing revealed these occurred by single-T insertions (NHEJ indel) and not by HDR. If HDR created the desired changes, no indel should be observed, and all the substitutions from the repair template would be included. It is also interesting that some alleles contain a correct substitution but at one side of the DSB point, suggesting one-sided HDR events (**Supplementary Figure S3B**). Overall, we observed 12% (3/26) of the alleles to have a possible one-sided HDR in *ALS* by evidence of base substitution. For the *Pita* gene, randomly chosen 17 lines were sequenced. We observed 76% of the lines with indels. We could not detect any instances of HDR in *Pita* locus. Lastly, we sequenced *Ptr* locus in 20 random lines and have not found any evidence of editing either by NHEJ or HDR. The *Ptr* protospacer contained a GCC motif in the first four nucleotides proximal to PAM, which was reported to be inefficient in cleaving by Cas9 (Graf et al., 2019). Based on the results we obtained for the three loci in rice, it seems cgRNA approach is not efficient in mediating HDR editing.

Prediction of NHEJ-Mediated Precise Insertion in Rice Genes

Cas9 induced DSB generation and subsequent NHEJ-mediated genome editing is highly efficient in plants. The NHEJ repair outcome is considered random and, therefore, not useful in precise genome editing applications. However, several recent studies in animal systems showed that the Cas9-mediated editing outcome is reproducible and predictable depending on the features of target DNA sequences (Molla and Yang, 2020b). The ability to predict the spectrum of DSB repair outcomes would facilitate us in performing more efficient gene knock-out and precision genome editing applications without HDR. Using the DSB repair products of thousands of target DNA loci in mammalian cells, several machine learning models have been generated to predict the spectrum of CRISPR-Cas9 editing products (Shen et al., 2018; Allen et al., 2019; Leenay et al., 2019; Li et al., 2021). To the best of our knowledge, no study has

A

Target Gene	Protospacer sequence (5′-3′)	Nucleobase at -4 of the protospacer
OsALS (cgRNA)	GGGTATGGTGGTGCAA**T**GGG	T
OsALS (CRISPEY)	GGGTATGGTTGTGCAA**T**GGG	T
OsPita	TCAGGTTGAAGATGCA**T**AGA	T
OsPtr	AAAAACCAAAACCAGC**T**GCC	T
OsCC	CTGCTTGATGTGGAGA**C**GGG	C
OsActin	CTGGCCCATCCATTGT**G**CAC	G

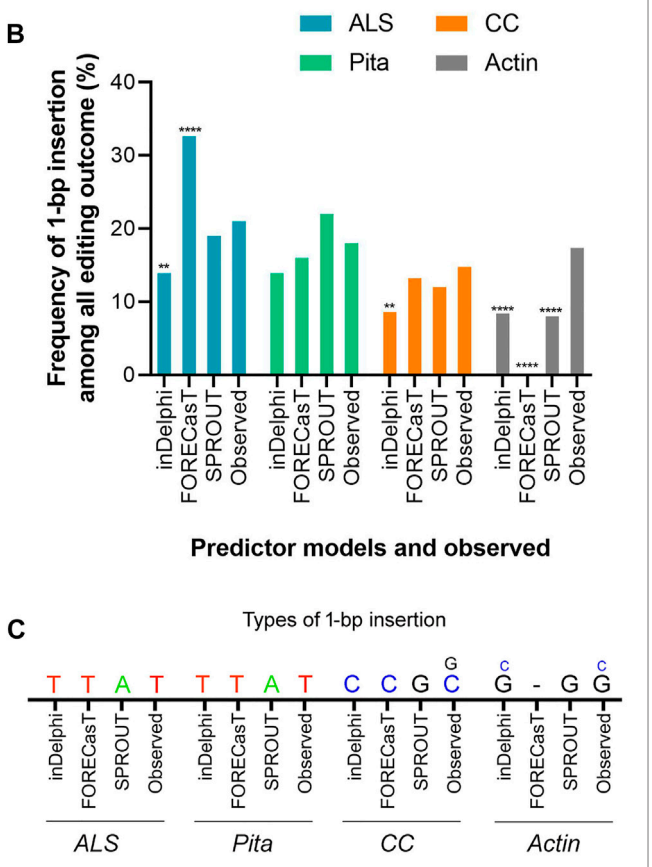

FIGURE 3 | Prediction of 1-bp insertion using models. **(A)** Protospacer sequences used in the study. ALS protospacers for Jupiter (used in cgRNA) and Kitaake (used in CRISPEY) are differed by a single nucleotide. **(B)** Frequency of 1-bp insertion predicted by inDelphi, FORECasT, and SPROUT versus observed frequency. Each prediction data was compared with the observed data. For *ALS*, *CC*, and *Actin* loci, deep sequencing data was treated as observed data. Observed data for *Pita* derived from Sanger sequencing. (**) denotes $p \leq 0.01$; (****) denotes $p \leq 0.0001$. **(C)** Types of 1-bp insertion predicted and observed across four loci. Smaller letters indicate second most prevalent insertion.

reported predictability of Cas9-induced DSB repair outcome in plants. Therefore, we set out to analyze our dataset to examine if the same prediction rules are applicable in plants and if the existing machine learning models could be applicable to foresee the Cas9-induced mutation outcome in plants.

The editing outcome that could be most reliably predicted is single nucleotide insertion (Molla and Yang, 2020b). Earlier studies reported that the inserted nucleotide is identical to the nucleotide at –4 from the PAM sequence (Chakrabarti et al., 2019; Lemos et al., 2018). Therefore, if a T nucleotide is present at –4 of the protospacer sequence, another T nucleotide is highly likely to be inserted (Chakrabarti et al., 2019). However, the predictability decreases in the order T > A > C > G at –4 position (Molla and Yang, 2020b). For a preliminary investigation, we have carefully chosen three protospacers, for targeting *ALS*, *Pita* and *Ptr*, having a T at –4 position (**Figure 3A**). The inDelphi model predicted single T insertion for 13.9% of all products in the *ALS* target locus (**Figure 3B**; **Supplementary Figure S4**). Interestingly, we observed 25 lines (12.75%) showed positive Mfe1 digestion out of 196 plants tested from our cgRNA experiment, indicating a single T insertion at the cut point. Sanger sequencing of those lines validated the result (**Figure 2B**). Similarly, deep sequencing data from our retron experiment showed an average of 21.13% single T insertion with the same *ALS* guide (**Figure 3B**). The FORECasT has also predicted single T insertion as the most dominant class of mutations (**Supplementary Figure S4**). Although SPROUT predicted 19% of the total reads with insertion, it failed to accurately predict the most likely inserted base (**Supplementary Figure S4**). For the *Pita* guide, inDelphi and FORECasT computed 13.9% and 16% single T insertion, respectively (**Supplementary Figure S5**). Our experimental data showed 18% of the *Pita* alleles were with a single T inserted. This time also, SPROUT was inaccurate in predicting the inserted base.

FORECasT, SPROUT, and inDelphi predicted 13.2%, 12%, and 8.6% 1 bp insertion in the *OsCC* target locus, respectively. Our deep sequencing data showed 14.77% single C insertion (**Figure 3C**). inDelphi and FORECasT accurately predicted a C insertion (**Supplementary Figure S6**). For the *OsActin* target, our result showed most abundant insertion type is single G (13.76%) followed by single C (3.62%) (**Figure 3C**). Surprisingly, inDelphi predicted the insertion types accurately. SPROUT displayed that the most likely inserted base is G (**Supplementary Figure S7**). However, FORECasT was not able to predict the insertions.

The above result indicates that the models are pretty good in predicting the insertion types, especially 1 bp insertion and their fraction in the plant system. We found that the inDelphi outperformed the FORECasT and SPROUT in anticipating the mutation outcome, especially the insertion class and frequency. In contrast to the notion that the NHEJ outcome is random, our data suggest that the Cas9-induced double-strand break repair outcome is non-random and could be predicted (Molla and Yang, 2020b). Single nucleotide insertion is the most predictable class of repair genotype. We found that the inserted nucleotide is identical to the nucleotide at –4 from the PAM sequence in accordance with earlier studies in mammalian cells (Allen et al., 2019; Chakrabarti et al., 2019). The insertion of a single base identical to –4 nucleotide in the protospacer indicates the occurrence of the following sequential events: Cas9-induced 5' single-base overhang generation, filling in by DNA polymerase, and ligation by ligase 4 (Zuo and Liu, 2016; Lemos et al., 2018; Molla and Yang, 2020b). These events

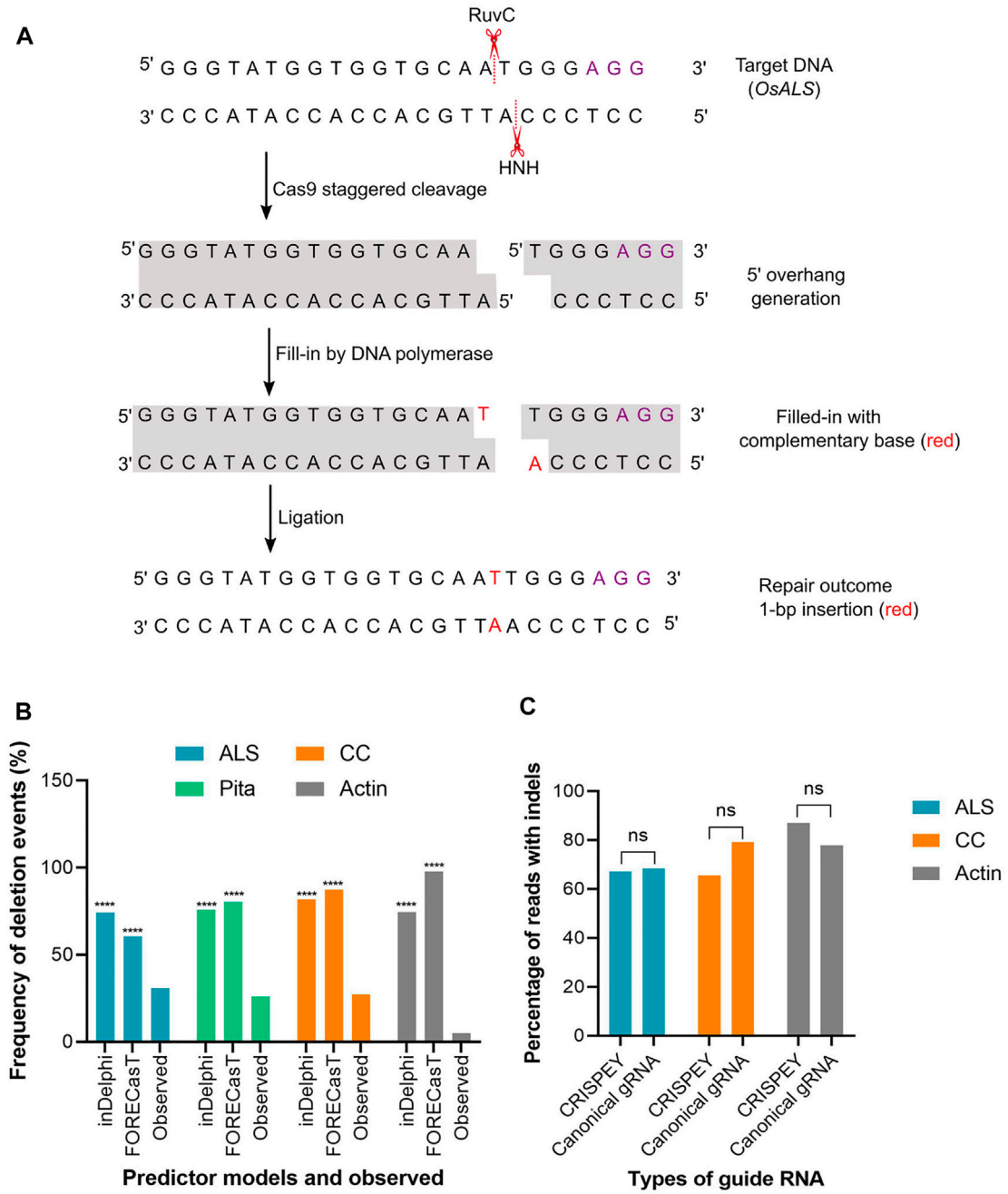

FIGURE 4 | (A) A hypothetical model to explain the generation of 1-bp insertion. Os*ALS* target sequence is used as an example. Violet font depicts PAM sequence. Model was redrawn from Molla and Yang (2020b). **(B)** Percentage of deletion events predicted by inDelphi or FORECasT and experimentally observed. Observed value was compared separately with inDelphi and FORECasT predicted values. **(C)** Indels generated with canonical and 5′ extended guide RNA. A 228 bp long sequence was fused at the 5′ end of the gRNA. (****) denotes $p < 0.0001$. ns, non-significant.

logically explain the generation of 1 bp insertion identical to the −4 base in the noncomplementary strand since the base at −4 acts as a template (**Figure 4A**). A recent study in mammalian cells showed that the fraction of 1 bp insertion relative to other repair genotypes can be increased by the exogenous application of the ATM kinase inhibitor KU-60019 (Bermudez-Cabrera et al., 2021).

Prediction of Deletion and Base Substitution

Unlike insertion, the models tested here performed poorly in predicting the frequency and types of deletion and substitution events. Microhomology-mediated end joining (MMEJ) pathway (also known as alternative-NHEJ) is often associated with

deletions events generated from Cas9-induced DSB repair. MMEJ deletes intervening bases between short tracts of local matching sequences (Her and Bunting, 2018) and, hence, the repair outcome is predictable by analyzing microhomologous sequences. Since SPROUT does not display deletion types and their respective frequencies, we considered analyzing only inDelphi and FORECasT in this section. Both inDelphi and FORECasT displayed prediction of MMEJ deletion with a high percentage. The difference between the predicted frequency and the observed frequency of deletion events was highly significant ($p < 0.0001$) (**Figure 4B**). We have also noticed that the models predicted deletions deviated from the observed deletion types in the tested genomic sites (**Supplementary Tables S2–4**). For example, a AA deletion was found to be the most frequent (average 6.7%) deletion type in the *ALS* locus (**Supplementary Table S2**). However, inDelphi and FORECasT failed to predict this deletion in their top nine deletion types (**Supplementary Figure S4**). For *Actin*, both inDelphi and FOREcasT projected an identical 7 bp deletion with >10% frequency (top deletion class) (**Supplementary Figure S7**). Surprisingly, we could not find a single read with the −7 bp deletion in a total of 179 K reads (**Supplementary Table S3**). Similarly, observed deletion types at the *CC* locus did not match the predicted deletions (**Supplementary Figure S6** and **Supplementary Table S4**). It is notable that while types of insertion were common across different pooled samples for the same target, deletion types were found rarely common (**Supplementary Tables S2–4**). In our deep sequencing data, we found a significant amount of reads with combined insertion and deletion. Base substitutions were also common. However, the models (inDelphi, FORECasT, and SPROUT) cannot foresee these kinds of mutation classes. Our results indicate that these models are not good at predicting deletions and base changes in rice plant.

5′ and 3’ Modifications of Guide RNA Do Not Seem to Impact CRISPR-Cas9 Editing Efficiency

The commonly used single guide RNA (gRNA) is a fusion of the crispr RNA (crRNA) and transactivating crRNA (tracrRNA) through a short RNA loop (Jinek et al., 2012). Engineering and manipulation of gRNA has been one of the important areas of research for broadening the applications of the CRISPR-Cas system. The introduction of extra nucleotides at either of the gRNA ends could be useful for many genome editing applications, such as adding RNA aptamers to recruit different effector proteins and fusing donor templates for HDR. The degree of gRNA modifications that can be tolerated without affecting its binding with Cas9 and subsequent genome targeting is crucial to define (Nowak et al., 2016). Whether fusion of long sequence at either of the 5′ and 3’ ends of gRNA has any impact in Cas9 cleavage efficiency is not well established in the plant system.

In the retron approach, donor template coding sequences were fused at the 5′ end of the gRNA (**Figure 1B**). The length of the sequence fused was 228 nucleotides, including the chimeric retron-donor. The 5′ end is crucial for gRNA function as the 20 bp protospacer that determines the genomic target site is located at this end. From our deep sequencing data at all three target sites, it is clear that the DSB formation ability of Cas9 was not hampered at all by the fusion at 5′ end of gRNA. For example, in *ALS*, the CRISPEY construct yielded an average of 67.24% mutation, while the control construct with canonical gRNA showed 68.30% of the population with mutation (**Supplementary Table S2**). Similarly, for the *Actin* target, 77.86% mutant population was obtained with canonical gRNA, whereas 87% mutant population was generated by 5′ extended gRNA (**Supplementary Table S3**). *CC* target was mutated with an average efficiency of 79.15% with 5′ extended gRNA (**Supplementary Table S4**). The results of this study indicate that 5′ extension of gRNA is probably not an inhibitory factor for Cas9 cleavage in plants (**Figure 4C**). However, a recent *in vitro* study showed that 5′ addition of only two to three unpaired nucleotides in SpCas9 gRNA has a significant effect on the cleavage activity of the RuvC domain (Mullally et al., 2020). Another study reported 5’ end modifications of gRNA retain cleavage activity in mammalian cells, although found some length effect (Kocak et al., 2019).

On the contrary to the retron approach, we fused 200–250 bp donor template sequence at the 3′ end of the respective gRNA sequence (**Figure 1C**). As evidenced by our result at *ALS* and *Pita* loci, DSB induction efficiency was very high with the gRNA extended at 3′ end. In terms of the overall NHEJ editing efficiencies of the randomly chosen *ALS* lines tested, 92% of the lines had editing, with 84.6% as monoallelic and 7.7% as biallelic. On the other hand, 76.4% of the tested lines for *Pita* had editing, with 47% as monoallelic and 29.4% as biallelic. Of the nine lines where both *ALS* and *Pita* were sequenced, 55.5% (5) displayed editing in both loci, confirming multiplex editing (**Supplementary Table S5**). These results indicate a fusion of ~200 nt long sequence at the 3′ end of gRNA did not significantly impact cleavage efficiency in rice *ALS* and *Pita* loci. cgRNAs were also found to be fully functional in generating DSB in rice in an earlier study (Butt et al., 2017). The recently developed prime editing technique depends on a 3′ extended guide RNA (Anzalone et al., 2019). Similarly, modification of the 3′ end of gRNA was well tolerated by SpCas9 in a previous study (Palumbo et al., 2020). Taken together, our data suggest that both 5′ and 3’ ends of gRNA are amenable for modification without significantly affecting the Cas9 cleavage activity in rice.

CONCLUSION

We demonstrated that the Cas9 repair outcome, specifically the type and fraction of 1 bp insertion, is predictable in the plant system by employing machine learning models. Among the models tested, inDelphi outperformed the other two models, FORECasT and SPROUT. Applicability of those models to the plant system greatly enhances the ability of plant researchers to better design their experiments for knockout as well as precise genome editing. However, the models failed to accurately predict deletions. We also presented data showing ineffectiveness of retron- or cgRNA-

mediated approaches to achieve HDR in rice. Moreover, we showed 5′ and 3′ extension of gRNA with 200–230 nt long sequences did not impact high cleavage activity of Cas9. Although more genomic sites need to be tested for getting a comprehensive idea about the impact of this fusion on DSB generation efficiency, our data would encourage researchers to explore new enhancements to CRISPR-Cas tools.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Original raw paired-end sequence data are available in NCBI data base with BioProject accession number PRJNA795336.

AUTHOR CONTRIBUTIONS

YY, KM, and JS designed the experiments. KM and JS made constructs, performed experiments and analyzed data. MW provided suggestions and assisted experiments and data analysis. KM, JS, and YY wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgeed.2022.825236/full#supplementary-material

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