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TRANSGENE-FREE GENOME EDITING IN PLANTS: A BRIEF OVERVIEW

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ABSTRACT

The presence of transgene residues in genome-edited plants presents several challenges, including interference with genetic analysis, increased risk of off-target effects, and strict regulatory scrutiny that limits the commercialization of such crops. As a result, there has been a significant shift in plant biotechnology toward developing transgene-free genome editing techniques that retain the precision and efficiency of CRISPR/ Cas systems while avoiding the integration of foreign DNA. This review summarizes the major strategies currently used to produce transgene-free genome-edited plants. These include: (1) genetic segregation of transgenes in sexually reproducing crops through selfing or backcrossing; (2) transient expression systems where CRISPR components are temporarily expressed without stable integration; and (3) DNA-free approaches involving the direct delivery of CRISPR reagents in the form of RNA molecules or Cas9/gRNA ribonucleoprotein (RNP) complexes. Each approach is discussed in terms of its underlying mechanism, efficiency, limitations, and suitability for different plant species particularly contrasting annual versus perennial and vegetatively propagated crops. Special emphasis is given to emerging technologies such as virus-based delivery vectors, graft-mobile genome editing systems, nanoparticle-mediated delivery, and base editors that allow precise nucleotide changes without introducing double-strand breaks or transgenes. Furthermore, recent innovations are evaluated for their potential to minimize off-target mutations and increase editing precision, which is crucial for regulatory compliance and public acceptance. By eliminating the need for transgene removal in subsequent generations, these approaches offer time-saving, cost-effective, and sustainable alternatives to conventional transgenic breeding. Overall, transgene-free genome editing represents a transformative leap in plant science, offering practical solutions to enhance crop improvement, meet food security challenges, and align modern biotechnology with evolving global biosafety standards and societal expectations.

Keywords: CRISPR, Transgene free, RNP complex, GMOs, Genome Editing, cas12a, DNA free · Base editing

Introduction

Genome editing has emerged as one of the most powerful tools in modern plant science and crop improvement. It enables precise modifications in the plant genome to identify the gene functions and enhance key agronomic traits such as yield, stress tolerance, and resistance to diseases and herbicides (Chen *et al.,* 2019). This technology relies on site-directed nucleases (SDNs), which create double-stranded breaks (DSBs) at targeted genomic loci. Major types of SDNs include mega nucleases, zinc

finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the widely adopted CRISPR/Cas system (Puchta and Fauser, 2014). Among these, the CRISPR/Cas system has gained prominence due to its simplicity, cost-effectiveness, and versatility. It operates through two main components: a CRISPR-associated (Cas) protein, such as Cas9 or Cpf1, which induces the DNA break, and a guide RNA (gRNA) that directs the Cas protein to the specific genomic target (Jiang and Doudna, 2017). Following the DSB, the cell predominantly employs the error-prone non-homologous end joining (NHEJ) pathway to repair the break, often resulting in small insertions or deletions (indels) that can disrupt gene function. These mutations are useful for functional genomics and trait improvement. To enhance editing precision and minimize unintended mutations, advanced CRISPR-based tools such as base editors and prime editors have been developed. These systems enable single-nucleotide changes without generating DSBs, offering safer and more refined genome modifications (Zhu *et al.,* 2020). Originally discovered as part of the bacterial immune defence against viruses (Ishino *et al.,* 1987), CRISPR technology has since been adapted for plant systems. In plant genome editing, outcomes are typically classified into three categories: SDN1, involving small indels through NHEJ; SDN2, enabling precise substitutions using a repair template; and SDN3, allowing the insertion of larger DNA sequences (Hilscher *et al.,* 2017). These classifications help define the scope and regulatory status of genome-edited crops across various jurisdictions.

Why Transgene free genome editing?

The need for transgene-free genome editing in plants arises from the regulatory, public perception, and biosafety challenges associated with genetically modified organisms (GMOs). Conventional CRISPR-based editing often involves the integration of foreign DNA constructs, such as Cas9 and selectable marker genes, which subjects the resulting plants to strict GMO regulations (Sprink *et al*., 2016). This limits their acceptance and commercialization, especially in regions with restrictive biotech policies. In contrast, genome edits such as small insertions/deletions or base substitutions indistinguishable from natural mutations are exempt from GMO regulations in several countries (Turnbull *et al*., 2021). Therefore, generating foreign DNA-free edited plants is essential for regulatory approval, public trust, and global trade. Traditional approaches like segregation of the transgene through selfing or crossing are laborious and unsuitable for many crops (Gao, 2021). Hence, developing efficient, non-transgenic editing methods is crucial for accelerating crop improvement and ensuring broader adoption of genome editing technologies. The continued presence of gene editing elements will also increase the risk of off-target effects (Zhang *et al*., 2018). Furthermore, the removal of transgenes is likely a prerequisite for gaining government approval for commercial applications of gene-edited plants (Turnbull *et al*., 2021).

Three general strategies have been employed to achieve gene editing without leaving any transgene residuals in edited plants: (1) avoid using foreign DNA components or foreign DNA integration in gene editing experiments; (2) eliminate transgenes after target genes have been edited; and (3) cross a gene editing donor plant to edit a transgene-free acceptor plant and induce haploid formation to remove the donor genome in the F1 generation. All three strategies have been successful with a wide range of effectiveness.

Methods of Transgene free Editing

Eliminating transgene through genetic segregation

The CRISPR/Cas9 system comprising Cas9, guide RNA (gRNA), and selectable markers like the kanamycin-resistance gene is typically assembled on a single plasmid and introduced into plant cells via Agrobacterium-mediated transformation or gene gun. Once integrated into the plant genome, these transgenes enable targeted genome editing. Most T0 plants carry the CRISPR construct and are often heterozygous at the insertion site. In the T1 generation, both the edited gene and the transgene segregate according to Mendelian inheritance. If a single transgene insertion occurs, approximately 25% of T1 plants may be transgene-free. However, multiple insertions or linkage between the transgene and the edited locus can hinder segregation-based elimination. To obtain edited, transgene-free plants, additional generations of selfing or backcrossing to wild-type are required making the process laborious and time-consuming. Alternatively, recombinase-based systems (e.g., Cre/lox) offer a strategy for transgene removal (Anand *et al*., 2019). (Fig. 1)

A rapidly emerging strategy for achieving transgene-free genome editing in plants is the direct delivery of preassembled Cas9 protein–gRNA ribonucleoproteins (RNPs) into plant cells. Unlike plasmid-based methods that often result in the integration of foreign DNA into the host genome raising regulatory and biosafety concerns the RNP approach introduces no recombinant DNA, making it inherently DNA-free and more acceptable under

various regulatory frameworks (Toda *et al.,* 2019; Park and Choe, 2019).

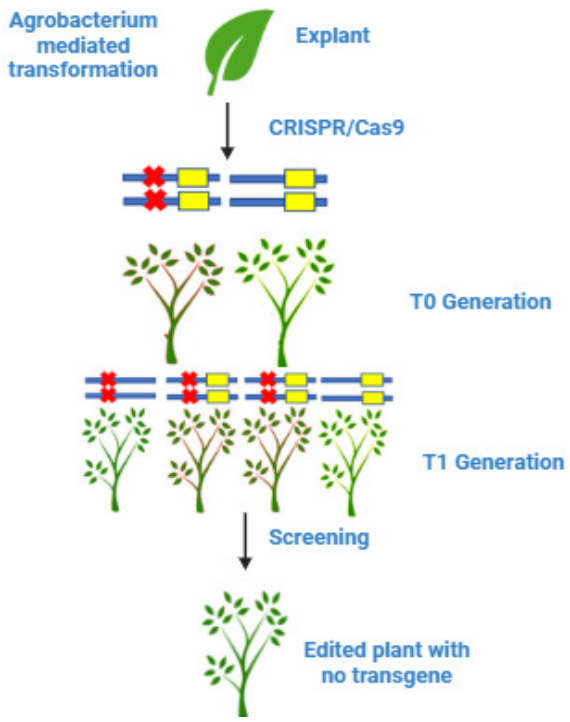

Fig. 1: Eliminating transgene through genetic segregation (Image created by BioRender)

DNA-Free Genome Editing Using Ribonucleoproteins (RNPs)

In this approach, RNP complexes, consisting of the Cas9 nuclease and synthetic guide RNAs (gRNAs), are assembled in vitro prior to delivery. Alternatively, Cas9 mRNA and gRNAs, produced in vitro, may be co-delivered into plant cells, where Cas9 mRNA is translated to generate the active nuclease. Upon delivery, these RNPs quickly induce site-specific double-strand breaks (DSBs) at the target locus. Due to their transient nature, they are rapidly degraded by endogenous proteases. Additionally, as no foreign DNA is integrated, RNP-mediated genome-edited plants are more likely to be exempt from stringent GMO regulations in several countries. Various physical and chemical methods are employed for delivering RNPs and mRNA into plant cells; Particle Bombardment (Svitashev *et al.,* 2016; Liang *et al.,* 2017), PEG-Mediated Transfection of protoplasts (Toda *et al.,* 2019), Liposome-Based Transformation (Liu *et al.,* 2020). These methods facilitate DNA-free genome editing, avoiding the variabilities in Cas9 expression that often arise from promoter strength and tissue specificity in DNA-based systems.

RNP-mediated editing faces limitations: (1) **Lack of Selectable Markers:** DNA-free systems do not allow the use of antibiotic or herbicide resistance genes for selection. Consequently, many regenerated plants are not edited, requiring extensive screening to identify successful edits especially in the absence of visible phenotypic changes. (2) **Unsuitability for HDR-Based Editing:** Some gene editing strategies, such as homology-directed repair (HDR), require a repair template typically provided as a DNA molecule. As DNA is not introduced in RNP systems, this poses a challenge. However, the use of RNA transcripts as HDR templates has been proposed as a potential solution. (3) **Species-Specific Regeneration Protocols:** Most RNP-based transformations are conducted in protoplasts, immature embryos, or callus tissue. Unfortunately, efficient regeneration protocols for protoplasts and embryos are not well established in many crop species, which limits the broader applicability of this method (Fig. 2)

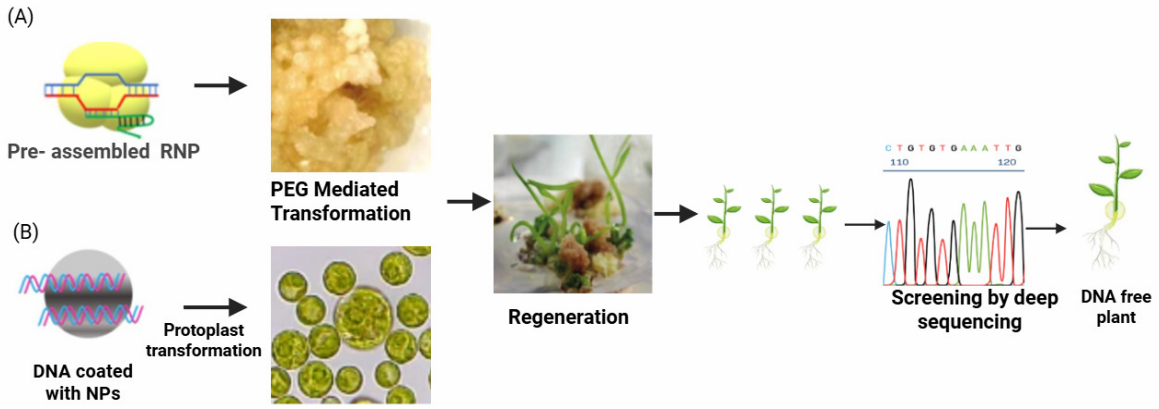

Fig. 2 : (A) RNP based transgene free editing (B) Nanoparticle-based transgene free editing (Image created by BioRender)

Nanoparticle-based system

Nanoparticle-based delivery systems have emerged as a promising approach to overcome the recalcitrance of many economically important plant species to traditional transformation methods. Nanoparticles (NPs), including inorganic NPs (gold, silver, iron oxide), carbon-based NPs, silicon-based NPs, and polymeric NPs, can efficiently deliver biomolecules for genome editing. Among these, inorganic NPs are primarily used to transport DNA into plant cells. CRISPR/Cas9 has been combined with a variety of nanoparticles such as nanocapsules, gold nanoparticles, hydrogels, peptide-based NPs, DNA nanoclew, polymeric NPs, unilamellar and multilamellar liposomes, and magnetic NPs, which serve as carriers to facilitate gene incorporation into host cells. These combined nanoparticles are taken up by plant cells to induce desired alterations in the target gene sequence.

Zhao *et al.* (2017) demonstrated pollen magnetofection using positively charged magnetic nanoparticles (MNPs) that bind to negatively charged DNA to form MNP–DNA complexes. These complexes are mixed with pollen and subjected to a magnetic field, enabling DNA entry through pollen apertures before pollination. This method preserves pollen viability and functionality, is simple to perform, equipment-free, supports multiple gene delivery, and resulted in insect-resistant transgenic cotton seeds. This technology is genotype-independent, culture-free, regeneration-free, fast, and capable of multi-gene transformation.

Other studies have reported the direct uptake of nanoparticles like quantum dots, metal/metal oxide NPs, silica NPs, and carbon nanotubes across various crops, indicating their efficiency in generating transgene-free plants. Among these, gold nanoparticles (AuNPs) are especially valued for their physicochemical stability and biocompatibility. CRISPR-Cas9 reagents can be delivered using gold-based nanomaterials and lipid NPs, such as AuNP and AuNC (Chen *et al.*, 2019; Vats *et al.*, 2022). Furthermore, nanoparticles can be targeted to meristematic cells (Sanzari *et al.*, 2019; Khan *et al.*, 2019), and editing these cells can lead to chimeric edits from which edited, transgene-free plants can be regenerated via tissue culture or cuttings.

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

Haploid induction (HI) technology can fix the genotype of crops in a short period of time and has long been used in crop breeding (Chang and Coe, 2009). Studies have found that mutations in specific genes in plants can induce haploid production, or the generation of offspring with only one set of chromosomes. The disruption of MATL/PLA1/NLD locus, which encodes a sperm cell-specific phospholipase, resulted in the production of defective male gametophytes in maize (Dong *et al.,* 2018), rice (Wang *et al.,* 2019), wheat (Liu *et al.,* 2020).

In addition, CRISPR–Cas9-mediated deletion of the N-terminal a-helix of centromere-specific histone 3 (CENH3) resulted in the generation of Arabidopsis HI lines (Kuppu *et al.,* 2020). Genome editing of wheat TaCENH3a resulted in a HI rate of 7%. Editing the restored frameshift alleles of heterozygous genotypes triggered a higher paternal HI rate (Lv *et al.*, 2020) than homozygous combinations. (Fig 3)

Similarly, it was also found that maize plants heterozygous for the cenh3 null allele could efficiently induce haploid production as either the male or the female parent of a cross (the effective HI rate reached 20%; (Wang *et al.,* 2021). Maize plant is recalcitrant to A. *tumefaciens* 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). In the Hi-Edit method, the CRISPR/Cas9 construct was firstly transformed to NP2222 (inbred line), which is a common line used for transformation. The Cas9 and 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.

Developing transgene-free plants through distance hybridization

Earlier studies have shown that haploid wheat can be created by crossing maize with wheat via distant hybridization (Laurie and Bennett, 1988). By combining the HI-Edit system and intergeneric hybridization, scientists successfully edited the target genes in wheat using maize pollen produced by a transgenic maize plant carrying the Cas9 and gRNA cassettes (Kelliher *et al.,* 2019). In the process of embryonic cell division of the hybrid offspring of

wheat and maize, the maize chromosomes are eliminated due to the asynchronous process of DNA replication, aggregation, and centromere formation, resulting in transgene-free haploid wheat. Wheat is especially suitable for editing by HI because wheat is difficult to transform and has a strong ability to accept pollen from many plants in the same family. (Fig 3)

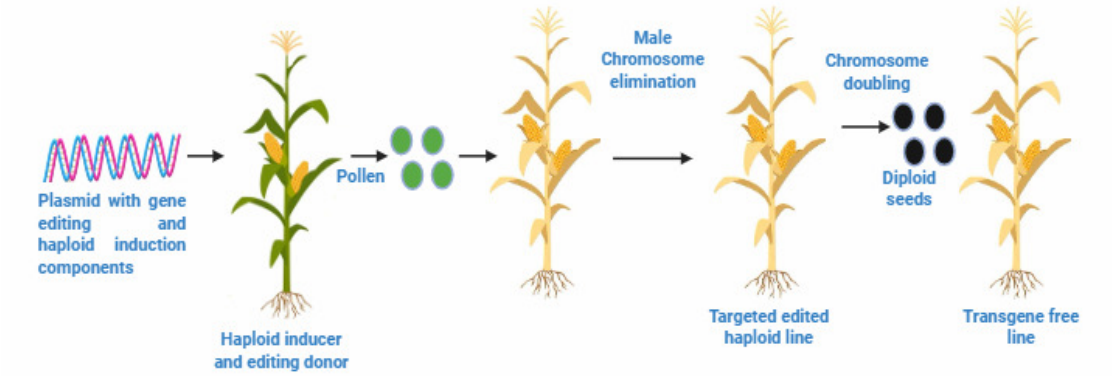

Fig. 3 : Haploid Induction (HI) Transgene free Editing Technology in maize (Image created by BioRender)

CRISPR-Cpf1 System (Cas12a)

The CRISPR-Cpf1 system, also referred to as Cas12a, is emerging as a powerful tool for DNA-free genome editing in plants. Unlike the widely known Cas9, Cpf1 recognizes a T-rich PAM sequence (5′-TTTN-3′), which is located at the 5′ end of the target DNA, providing a unique advantage in targeting specific genomic regions. It requires only a single crRNA for guidance, eliminating the need for a trans-activating crRNA (tracrRNA) as required in the Cas9 system. Variants like LbCpf1 (from *Lachnospiraceae bacterium*) and AsCpf1 (from *Acidaminococcus sp.*) are commonly used, with AsCpf1 often showing superior editing efficiency. The crRNAs used in this system are notably short around 43 nucleotides which makes them easier to synthesize chemically.

One of the advantages of Cpf1 over Cas9 is its ability to make staggered cuts and induce larger deletions, which facilitates efficient editing via non-homologous end joining (NHEJ) especially useful in SDN1 and SDN2-type edits. This characteristic makes Cpf1 particularly suitable for precision breeding and functional genomics in crops. Cpf1 can be delivered as a ribonucleoprotein (RNP) complex, typically using a 1:6 molar ratio of Cpf1 to crRNA, and introduced into plant protoplasts using PEG-mediated transfection. This method has shown high efficiency while avoiding the integration of foreign DNA. In fact, studies like those by Kim *et al*. (2017) have demonstrated minimal off-target activity and no detectable foreign DNA insertion, which is crucial for developing non-GMO edited plants. However, it is important to design crRNAs carefully. The Cpf1-crRNA complex can tolerate one to two base mismatches, which could potentially lead to unintended modifications. It generally does not tolerate mismatches of four bases or more. Therefore, avoiding target sites with possible three-base mismatches elsewhere in the genome is critical to minimize off-target effects (Dong *et al.*, 2016).

Transgene Killer CRISPR (TKC)

The development of *Transgene Killer CRISPR (TKC)* technology marks a significant advancement in achieving transgene-free genome-edited plants, particularly in rice. The TKC system strategically utilizes spatio-temporal expression of suicide gene cassettes to eliminate transgene-containing male gametes and embryos, thereby ensuring that only transgene-free progeny are produced. The TKC system integrates two key toxic genes within a CRISPR/Cas9 plasmid: 1) CMS2 (Cytoplasmic Male Sterility 2): Also known as ORFH79, this gene disrupts mitochondrial function during pollen development, leading to male sterility. It is typically driven by the Cauliflower Mosaic Virus 35S (CaMV35S) promoter, though its weak activity in monocots, especially in microspore cells, has prompted efforts to replace it with stronger monocot promoters.2) BARNASE: A toxic ribonuclease from *Bacillus subtilis*, expressed under the Rice Embryo Globulin-2 (REG2) promoter, which is active during early embryogenesis. BARNASE effectively eliminates transgene-containing embryos. (Fig 4)

These suicide genes are temporally activated during the reproductive stage of T_0 plants, following the initial CRISPR-Cas9-mediated genome editing during tissue culture and regeneration. The outcome is

the selective self-elimination of transgenic sperm, egg cells, and embryos, resulting in the exclusive formation of transgene-free seeds (He *et al.,* 2018).

Fig. : 4 TKC construct for transgene free editing

CASE Toolkit: An Advancement of TKC Technology

The Customized Assembly and Simplified Editing (CASE) toolkit, developed for rice (Oryza sativa), further streamlines TKC-based editing (Chen *et al.*, 2018; Liu *et al.*, 2022). This toolkit integrates TKC with a multiplex editing platform for efficient generation of transgene-free plants in the T_1 generation. The CASE toolkit comprises: Four gRNA cloning vectors with U3, U6a, U6b, or U6c small non-coding RNA (snoRNA) promoters. A TKC-MCS-U3 gene-editing backbone vector that allows for the modular assembly of gRNA cassettes via compatible restriction sites. The flexibility to incorporate synthetic gRNA cassettes spaced with self-splicing tRNA for multiplex editing.

To enhance TKC performance in monocots, Yubing *et al.* (2019) replaced the CaMV35S promoter with the rice ACTIN1 promoter, which is more active in monocots and particularly in floral organs. Additionally, the OsGEX2 pollen-specific promoter was used to drive CMS2 expression, resulting in more efficient elimination of transgenic pollen.

Promoter editing

Promoter editing offers a precise, transgene-free strategy to alter gene expression without modifying the coding sequence. Li *et al.* (2020) used CRISPR–Cas9 with two guide RNAs to delete a specific region of the *xa13* gene promoter in rice. Since *xa13* controls both bacterial blight susceptibility and pollen fertility, targeting the CDS would have caused sterility. Instead, deleting its promoter prevented induction by the pathogen while maintaining pollen function, resulting in disease-resistant yet fertile rice. The edited lines were considered nontransgenic as no foreign DNA was introduced, and the promoter change only altered expression levels, unlike CDS edits that produce aberrant proteins. The double-sgRNA design allowed precise, stable, and predictable deletions, and PCR was sufficient to identify mutation types, reducing the need for sequencing. This method simplifies mutant selection and enhances reliability for developing transgene-free edited crops (Li *et al.* 2020).

Transient Genome Editing Using Agrobacterium

Agrobacterium-mediated transient genome editing offers a viable method for producing transgene-free plants, particularly in perennial species where other methods like particle bombardment or RNP delivery face limitations. In this approach, Cas9 and sgRNA genes are transiently expressed without integrating foreign DNA, as demonstrated in tobacco (Chen *et al.,* 2018). The absence of chemical selection markers like kanamycin makes regeneration more efficient, increasing the number of mutant calli and shoots. However, due to the lack of visible selection, a large number of regenerants must be screened to identify successful edits.

Although this method results in relatively few T_0 plants that are both transgene-free and edited, it generally produces more edited lines than RNP-mediated methods, which yield fully transgene-free T_0 plants but with low editing frequency. Agrobacterium-mediated infiltration is already widely used for transient protein expression in *Nicotiana benthamiana*, including promoter analysis, protein–protein interaction, and subcellular localization (Krenek *et al.*, 2015). Moreover, transient expression of Cas9 and gRNAs using Agrobacterium without stable T-DNA integration has been reported in various studies (Chen *et al.,* 2018; Danilo *et al.,* 2019; Bánfalvi *et al.,* 2020).

This method is accessible, cost-effective, and does not require specialized equipment, making it suitable for most plant molecular biology labs. However, a major drawback is the frequent occurrence of mosaicism, requiring subsequent generations to obtain stable, heritable edits. Furthermore, the lack of selection pressure complicates the identification of edited individuals, and some plants may still carry T-DNA insertions, necessitating extensive genotyping. Modifying the T-DNA integration machinery in Agrobacterium may help enhance editing precision and reduce unwanted insertions.

RNA Virus-Mediated Transgene Free Editing

RNA virus-mediated genome editing offers a transgene-free alternative for plant gene editing. In a key study, Ma *et al.* (2020) used the negative-stranded RNA virus Sonchus yellow net rhabdovirus (SYNV) to deliver both Cas9 and sgRNA directly into tobacco plants. The Cas9 and sgRNA, flanked by pre-tRNAGly sequences for proper processing (Xie *et al.,* 2015), were inserted into the SYNV genome and expressed using native viral promoters. After transforming SYNV into Agrobacterium and infiltrating it into leaves, systemic leaves showed high editing efficiency (40–91%). Regeneration from these tissues produced over

90% edited plants, and 57% of mutations were heritable—all without any virus remaining in the next generation.

Unlike many positive-strand RNA viruses, which can only deliver sgRNAs due to limited cargo size, SYNV can carry the full CRISPR–Cas9 system. This makes it suitable for multiplex editing and large deletions in a DNA-free manner. A tRNA–SYNV–Cas9 construct can even target multiple genes at once. Because SYNV does not integrate into the plant genome, the resulting plants are truly non-transgenic. This method avoids complex tissue culture steps and allows direct delivery into intact plants, saving time and cost. SYNV can also be passed from plant to plant mechanically, bypassing Agrobacterium use. However, challenges remain, such as off-target effects from small mismatches and limited virus host ranges. Still, with tools like reverse genetics and improved viral vectors (Zhou *et al.,* 2019), this approach holds strong promise for safe, efficient, and transgene-free genome editing in crops.

Grafting and Mobile RNA-Mediated Genome Editing

A novel approach for achieving transgene-free genome editing in plants using grafting was demonstrated by Yang *et al.* (2023) in *Arabidopsis thaliana* and *Brassica rapa*. The method relies on engineering mobile CRISPR/Cas9 transcripts by fusing tRNA-like sequences (TLS) to Cas9 mRNA and guide RNAs, which enables their root-to-shoot movement across graft junctions. These Cas9-TLS and gRNA-TLS constructs were delivered into *A. thaliana* using Agrobacterium-mediated transformation, generating transgenic rootstocks that were grafted with wild-type scions of both *Arabidopsis* and *B. rapa*. The TLS elements facilitated the long-distance movement of the editing components from the rootstock to the scion, where genome edits were introduced, and the resulting scions produced seeds containing heritable, transgene-free mutations (Yang *et al.,* 2023). The technique effectively bypasses the need for direct transformation of the scion or regeneration from edited tissue. Editing efficiency was reported to be ~0.1% for homozygous and ~1.6% for heterozygous edits, and importantly, the seeds from the scions were free of any transgene. Although the method requires the generation of a transgenic rootstock, it eliminates the need for further generations to remove transgenic elements, as required in other transgene-free methods, and avoids the regeneration of plants from protoplasts. The full process, including rootstock generation and seed selection, takes approximately 6–7 months. However, this system currently faces limitations in monocot crops like sugarcane due to the absence of vascular cambium and the presence of scattered vascular bundles, which are essential for successful graft union formation (Melnyk *et al.,* 2015). This grafting-based mobile RNA system provides a promising platform for non-transgenic genome editing, especially in dicotyledonous crops where vascular connectivity can be exploited.

CBE-mediated editing

Transgene-free genome editing in the T_0 generation is highly desirable but challenging, especially for perennials and vegetatively propagated crops. While gene-edited, transgene-free plants can be obtained through segregation in annual crops like rice via backcrossing, selfing (He *et al.,* 2020), or transgene-killer strategies (He *et al.*, 2018), such approaches are not feasible in crops like citrus and apple due to genetic heterogeneity and apomictic reproduction (Wang *et al.,* 2017; Huang *et al.,* 2022). A promising alternative is the co-editing strategy using CBE (cytosine base editor)-mediated editing, which offers multiple advantages including transient expression of Cas/gRNA that degrades over time, mimicking protoplast transformation (Woo *et al.,* 2015), avoidance of T-DNA insertion from Agrobacterium-mediated transformation, and generation of edited plants directly in the T_0 generation without further backcrossing. This approach is particularly useful for crops like grape, citrus, banana, and potato that are highly heterozygous, vegetatively propagated, or have long juvenility. For instance, using this strategy, transgene-free citrus was developed within 6 months, a significant improvement over the ~20 years needed in traditional breeding (Omura *et al.,* 2016). Initial success in CBE-mediated editing was reported by Alquézar *et al.* (2022) and Huang *et al.* (2022), who independently developed transgene-free citrus.

The improved co-editing method enables cost-effective, time-efficient, one-step production of transgene-free edited plants in the T_0 generation and holds great potential for other crops requiring vegetative propagation. Compared to transgenic plants, transgene-free genome-edited plants enjoy regulatory ease as per USDA-APHIS and EPA guidelines (Su *et al.*, 2023), eliminate risks associated with stable T-DNA integration and reduce off-target effects through transient Cas/gRNA activity (Liang *et al.,* 2017). The limited functional time of the editing complex contributes to higher precision, as supported by studies on off-target minimization via transient editing (Randall *et al.,* 2021). Thus, CBE-based co-editing

represents a robust platform for rapid, precise, and transgene-free crop improvement.

Base Editing

Naturally occurring single nucleotide variations (SNVs) in promoter regions can significantly influence key agronomic traits. For example, an SNV in the promoter of the rice *OsPAO5* gene enhances mesocotyl elongation, facilitating direct-seeded cultivation (Lv *et al*., 2021). Similarly, a single nucleotide change in the promoter of the *bsr-d1* gene reduces peroxidase production, conferring broad-spectrum resistance to rice blast (Li *et al*., 2017). These cases illustrate the potential of targeted single-base edits to create elite alleles directly in high-performing crop cultivars.

CRISPR/Cas-derived base editors enable precise base substitutions at specific genomic loci without introducing double-strand breaks (DSBs) or requiring donor DNA (Rees and Liu, 2018). These editors consist of a deaminase fused to a catalytically impaired Cas9 (or Cas variant), guided by a single-guide RNA (sgRNA), which modifies target bases within a small editing window (Komor *et al*., 2016; Li *et al.*, 2023). The two most commonly used systems are: (1) Cytosine base editors (CBEs): Convert C·G to T·A (2) Adenine base editors (ABEs): Convert A·T to G·C (Gaudelli *et al*., 2017).

Recent innovations include: **C·G to G·C base editors** (CGBEs) (Chen *et al*., 2021; Zhao *et al*., 2021), **A-to-C/T base editors (AYBEs)**: (Tong *et al*., 2023). **Dual base editors**: Combine CBEs and ABEs to achieve multiple base conversions simultaneously (Xu *et al*., 2021). The editing window size is crucial for precision. A broader window increases the number of targetable sites but may introduce off-target edits or "bystander" effects (Porto *et al*., 2020). To enhance specificity or broaden scope, the editing window has been fine-tuned through various strategies (Jiang *et al*., 2018; Anzalone *et al*., 2020; Xiong *et al*., 2022).

Prime Editing

Introducing a transcription factor-binding motif into a promoter often requires editing multiple adjacent bases something that typically exceeds the capabilities of base editors. Prime editing, an advanced CRISPR/Cas-based technology, enables precise insertion, deletion, and substitution of short DNA sequences at specific genomic loci in a flexible “search-and-replace” manner (Anzalone *et al.,* 2019). The core system consists of a fusion between Cas9 nickase (H840A) and a reverse transcriptase (RT), guided by a specially designed prime editing guide RNA (pegRNA). After the Cas9 nickase introduces a single-strand break at the target site, the pegRNA binds to the nicked strand via its primer-binding site (PBS), and the RT synthesizes the desired sequence using the RT template region of the pegRNA (Anzalone *et al.,* 2019). Prime editing has been successfully applied in plant genome editing (Xu *et al*., 2020), but its broader application has been limited by low editing efficiency and constraints on the length of insertable sequences (Gao, 2021). To address these challenges, various improvements have been explored, such as: Optimizing pegRNA stability and structure, Adjusting the length of the PBS and RT template (Zong *et al.*, 2017), Increasing pegRNA expression (Jiang *et al.*, 2020). Recent innovations, such as using paired pegRNAs targeting opposite DNA strands, have enabled higher editing efficiencies and longer sequence insertions (Anzalone *et al.*, 2022; Choi *et al*., 2022; Sun *et al*., 2023).

Conclusion

Traditional genome editing often involves integrating editing components such as gRNA and Cas constructs into the host genome. Even when the construct is later degraded, DNA fragments may integrate illegitimately at double-strand break (DSB) sites, potentially causing unintended mutations. Moreover, any existing homologous sequences in the genome can lead to off-target effects. Persistent expression of genome-editing components further increases this risk. In contrast, DNA-free genome editing, which avoids the permanent insertion of foreign DNA, represents a cutting-edge approach. It minimizes off-target effects and aligns with both scientific and regulatory requirements for safer, genetically edited crops. Two prominent DNA-free delivery methods protoplast-mediated transformation and particle bombardment have shown success in certain crop species. However, the lack of efficient regeneration protocols remains a major limitation for broader adoption across many crops. To realize the full potential of DNA-free genome editing in sustainable agriculture, future research must focus on improving regeneration systems and expanding the applicability of this technology to diverse crop species.

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