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CRISPR-based gene editing in plants: Focus on reagents and their delivery tools

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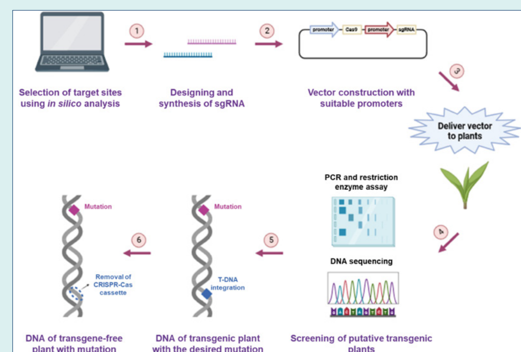
Abstract

Introduction: CRISPR-Cas9 technology has revolutionized plant genome editing, providing precise and efficient methods for genetic modification. This study focuses on the advancements and delivery of CRISPR-Cas9 in plant gene editing.

Methods: A comprehensive search in scientific databases, including PubMed, ScienceDirect, and Google Scholar, was conducted to gather information on CRISPR-Cas9 gene editing and its delivery in precise gene modification in plants.

Results: The evolving landscape of CRISPR nucleases has led to the development of innovative technologies, enhancing plant research. However, successful editing is contingent on efficient delivery of genome engineering reagents. CRISPR-based gene editing in plants utilizes diverse delivery methods: *Agrobacterium*-mediated transformation for bacterial transfer, biolistic transformation for physical gene insertion, electroporation for direct gene entry, expression of developmental regulators for gene expression modulation, and tobacco rattle virus as a viral vector, each offering distinct advantages for precise and efficient genetic modification in plants.

Conclusion: CRISPR-Cas9 gene editing stands as a pivotal advancement in plant genetics, offering precise gene manipulation with applications in agriculture and biotechnology. The continuous refinement of reagent delivery tools reinforces CRISPR-Cas9's transformative role in plant genome editing, with significant implications for broader scientific applications.



Introduction

The challenge of improving crop productivity stems from the limited genetic variation within elite breeding materials. In the 1950s, attempts to induce new alleles through random mutagenesis using chemicals or irradiation were both ineffective and time-consuming, resulting in the introduction of numerous mutations simultaneously and causing adverse effects.[1] The advent of site-specific nucleases marked a significant advancement, enabling precise DNA break insertions that were previously unattainable. Reorganizations requiring a precise cut are triggered by the repair machinery of cellular DNA, leading to innovations such as base editing and directed transcriptional regulation.[2]

As sequencing technologies rapidly advance, genomic data from an increasing number of plant species become accessible. Genome editing tools, in turn, offer the promise of accurate gene editing, presenting new opportunities for crop improvement.[3] In 2023, it has been thirteen years since the creation of the first genetically modified plants. These plants were originally developed through a traditional transformation process, facilitated by Agrobacterium. This method has now advanced to incorporate techniques involving zinc finger nucleases and homing endonucleases.[4-6] TALENs (Transcription Activator Like Effector Nucleases) were later successfully introduced for plant genome editing.[7,8] While early sequence-specific nucleases like transcription activator-like effector (TAL effector) nucleases, zinc finger nucleases, and mega-nuclease have demonstrated

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practicality in plant genome editing, their dependence on complex protein engineering limits their applications. The emergence of guide RNA-based CRISPR-Cas9 has revolutionized gene editing due to its ease of use and versatility, gradually replacing previous platforms.[9]

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) constitutes a bacterial immune system that employs RNA-guided nucleases to cleave and eliminate invasive DNA sequences from bacteriophages or plasmids. Researchers have capitalized on this natural process to develop the potent gene editing tool known as CRISPR-Cas9. In 2020, less than a decade after the introduction of the crispr-cas9 tool, Dr. Emmanuelle Charpentier and Dr. Jennifer a. Doudna were jointly awarded the Nobel Prize in chemistry (Nobel Prize press release).[10] The power of CRISPR-Cas9 and other CRISPR-Cas complexes lies in their ability to achieve sequence-specific cleavage of nucleic acids after DNA-RNA recognition and binding. This feature allows for the efficient movement of double-strand breaks (DSBs) to any target sequence of interest at a low cost.[11] Presently, two classes of CRISPR are categorized into six types. The defining characteristic of these classes is the nature of the effector and how it breaks the target site. Class 1 systems (type I, III, and IV) form multi-subunit complexes using numerous Cas proteins and crRNAs, while Class 2 CRISPR-Cas systems (type II, V, and VI) employ a single effector module, represented by a large multi-domain protein (Fig. 1).[12,13]

Both Archaea and Bacteria exhibit abundant Class 1 systems, while Class 2 systems are predominantly limited to bacteria.[14] In the natural CRISPR system, a complex composed of Cas9, crRNA, and tracrRNA is capable of cleaving foreign DNA. The cleavage is contingent upon the presence of PAM (Protospacer Adjacent Motif), an adjacent short sequence motif to the target region (Fig. 2).

Within Cas9, two nuclease domains (HNH and RuvC) are located three base pairs upstream of the PAM.[15] The DSBs induced by CRISPR-Cas9 are repaired through two main DNA repair pathways: homology-directed repair (HDR) and nonhomologous end-joining (NHEJ). HDR, an error-prone pathway, can result in random insertions and deletions, while the high-fidelity repair method is suitable for gene replacement or insertion (Fig. 3).[16]

CRISPR genome-editing systems applied across a wide variety of plant species have made substantial progress and continue to rapidly advance.[17] The initial plants edited by CRISPR emerged in 2013,[18,19] and since then, the technique has successfully applied in 24 plant families across 45 genera leading to imparting valuable agricultural traits.[20] Particularly, the recently developed precise CRISPR-Cas technologies have significantly impacted agriculture by enabling specific base changes. Beyond improving crops, this technology has given rise to new plant biotechnologies with the potential to advance protein engineering and gene regulation. These innovations have not only influenced basic biological research but also expanded the possibilities for widespread adoption.[3]

The initial step in CRISPR-based gene editing involves designing and synthesizing guide RNAs that are complementary to the target DNA sequence. These guide RNAs consist of a 20-nucleotide binding sequence recognizing the target, followed by a scaffold sequence interacting with Cas9. Ensuring the specificity of the binding sequence to the target DNA, free from off-target effects, is crucial. Various online tools like CRISPR-plant and crispor aid in designing guide RNAs for plant genomes.[21] Once the guide RNA sequence is designed, it can be synthesized using in vitro transcription methods. Commonly employed is the T7 polymerase-based transcription system, utilizing a DNA template containing the T7 promoter and the guide RNA sequence. The synthesized guide RNA can then be purified and annealed to the Cas9 nuclease, facilitating efficient genome editing.[22]

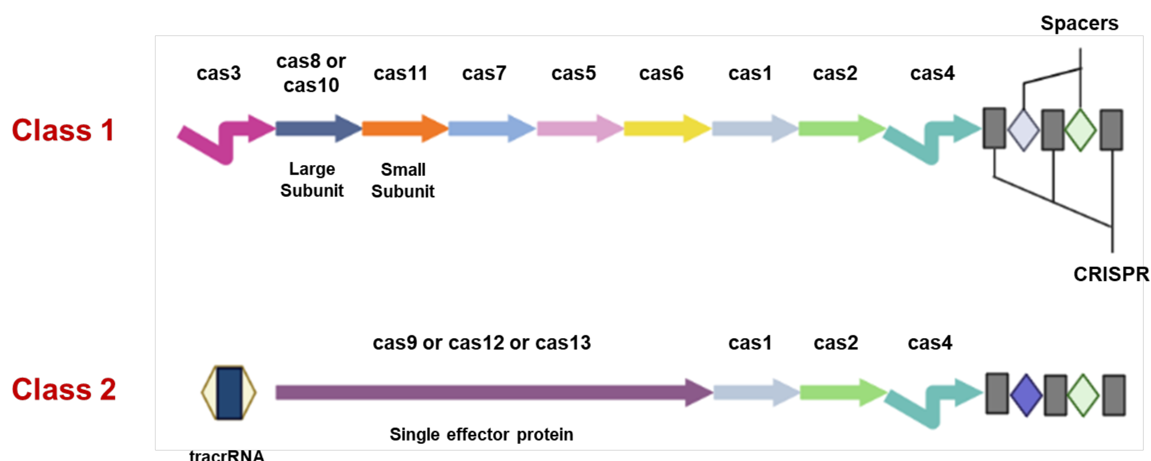

Fig. 1. The generic organization of class 1 and class 2 CRISPR/Cas loci. The Class 2 systems consist of a single multidomain effector protein, whereas the class 1 systems are composed of several Cas proteins.

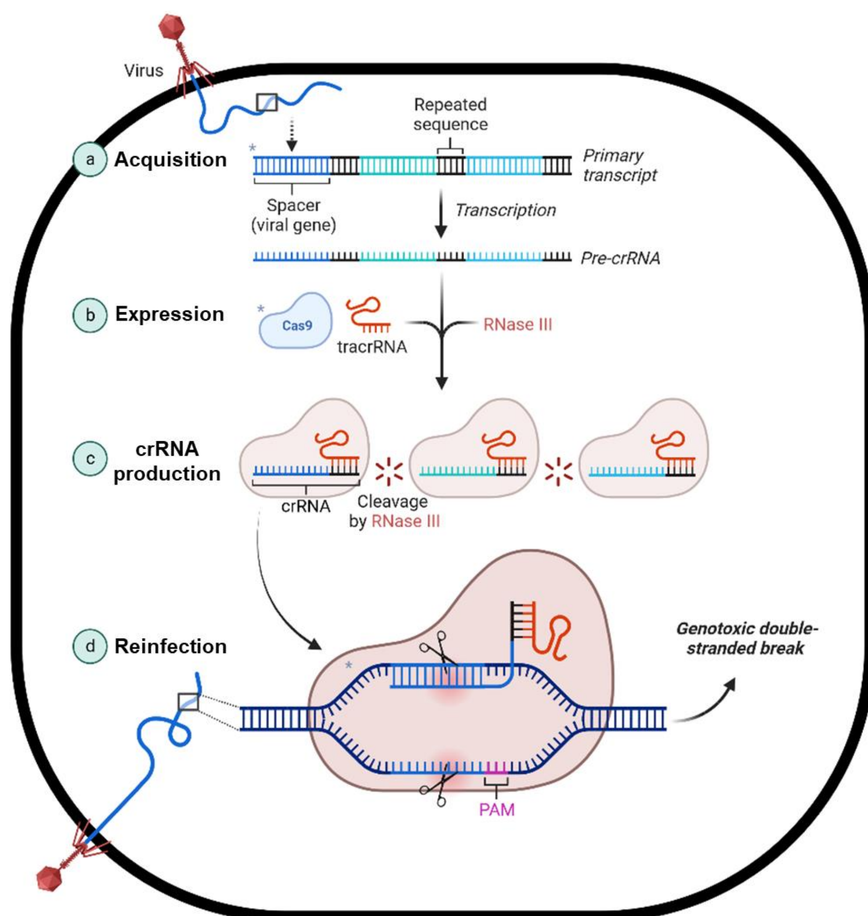

Fig. 2. Streptococcus' adaptive immune system against invading genetic element.

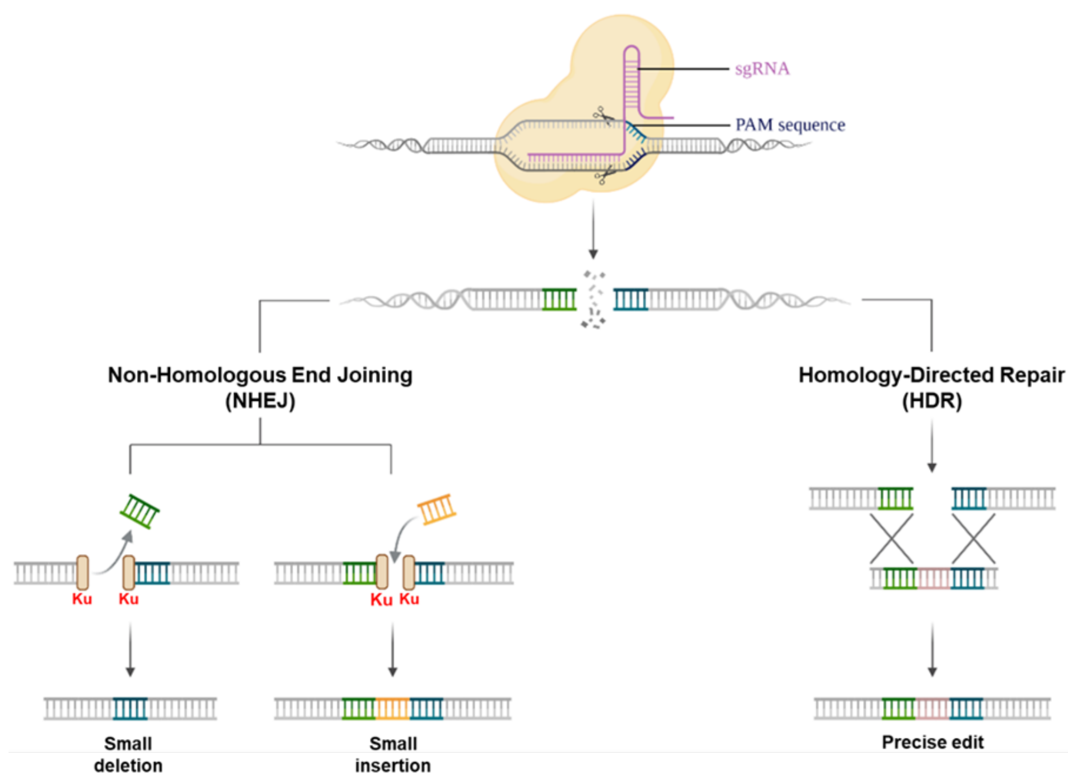

Fig. 3. CRISPR-based gene editing repair pathways in plants: NHEJ (Ku-dependent) directly ligates DSB ends without templates, while HDR uses homologous sequences. Crucial for optimizing CRISPR tool in plant gene editing.

This review focuses on different classes of gene editing reagents derived from the CRISPR-Cas system, recently added to the CRISPR toolbox. These include: (I) Cas effectors and multiple Cas variants expanding the range of identifiable target regions and increasing tissue precision, (II) base editing to specifically install all 12 possible base pair modifications without double-strand breaks or donor templates, and (III) prime editing, capable of precisely copying guide RNA information to the target site on DNA. Together, these advancements offer numerous applications in genome editing and beyond, encompassing epigenetic modulation, live cell chromatin imaging, targeted gene regulation, and chromatin manipulation.

Despite its effectiveness, the challenge lies in the delivery of CRISPR-Cas9 reagents to plant cells, posing a significant hurdle to the efficacy of these techniques.[15] Plant cells present unique challenges in delivering gene editing components due to factors such as a rigid cell wall, frequent polyploidy, resistant species, and the integration of Cas9 cassettes into host genomes. Alongside CRISPR-Cas reagents, this review explores recent innovations in delivering these reagents to plants, current gaps in knowledge, and future prospects.

To conduct this review, a literature survey approach was employed by investigating various online databases, including PubMed, Web of Science, Scopus, Cochrane Library, Science Direct, ProQuest, Embase, and Google Scholar, up until July 25th, 2023. Initially, a search was conducted in the Scopus database using the keywords "Genome editing," "CRISPR/Cas," "CRISPR reagents," "CRISPR delivery," and "Plants genome editing." The search was limited to research and review articles, focusing on the historical background of relevant research and articles. Subsequently, a search was performed in the PubMed database using the specified keywords. Filters were applied to restrict the results to research and review articles. Articles specifically concentrating on plant genetics using CRISPR were scrutinized. Further, in the ProQuest database, a search was executed with the phrases "Genome editing," "CRISPR/Cas," "CRISPR reagents," "CRISPR delivery," and "Plants genome editing," reviewing relevant articles, books, and theses. In the Web of Science database, a search was conducted using keyword titles, adding filters related to article type and time. Selected articles were examined, and evaluations by reviewers regarding these articles were reviewed. Within the Science Direct database, articles were investigated using the provided keywords, focusing on original articles and reviews in the field of plant genome editing. The Embase database was utilized for a search with titles related to the specified keywords, and articles were evaluated for accuracy and importance in the field of genome editing. Additionally, in the Cochrane library, systematic references and critiques of genome editing tools were accessed. Finally, a comprehensive evaluation of results, including articles, books, and references, was conducted using the Google Scholar search engine with the specified keywords. Entry and exit criteria included selecting suitable articles based on title, abstract, and relevant keywords, evaluating criticisms and potential critiques of the articles, and choosing credible sources considering the publication date and accuracy of the presented information. This strategy enables us to leverage the latest research and studies in the field of genome editing and CRISPR-Cas technology, facilitating the compilation of a comprehensive review article.

Classes of CRISPR-Cas derived genome-editing reagents

Currently, three different types of CRISPR technology can be used to edit plant genomes[3,23]: the base editors, the prime editors, and the CRISPR-Cas nucleases. The former requires DSB to induce, while the others do not. Due to the progress that has been made in the expansion of these technologies, the development of new genome editing tools is expected to continue. Due to the rapid development and discovery of new tools for genome editing, it can be challenging to choose the right one for a particular application. This is especially true for researchers who are new to the technology.[24]

CRISPR-Cas effectors and multiple CRISPR-Cas variants

The simple process of targeted mutagenesis is now routine with the help of the CRISPR-Cas system. This is mainly used to analyze the function of genes, but it can also be utilized to improve crop traits.[25,26] This system is versatile.[1] The Cas9 nuclease is a key component of the CRISPR-Cas system and is responsible for cutting the target DNA. The conventional Cas9 nuclease from Streptococcus pyogenes (spCas9) has been widely used in genome editing applications in plants. However, there are several limitations associated with the use of spCas9, including the large size of the protein, off-target effects, and the potential for unintended mutations. To overcome these limitations, several Cas9 variants have been developed, including the smaller size Cas9 (saCas9) and the double-nicked Cas9 (d-Cas9). The d-Cas9 is a variant of the Cas9 nuclease in which both nuclease domains have been mutated to create a "dead" enzyme that is unable to cut the target DNA. Instead, the d-Cas9 can be programmed to bind to the target DNA without cleaving it, which reduces the potential for off-target effects and unintended mutations. The Cas9 nuclease can be transformed into dCas9 by removing its two domains. Although dCas9 has DNA-binding potential, this process deactivates its cleavage activity. By binding to an effector domain, the protein can guide various enzymatic functions at a targeted genome site.[27,28] The ability to perform site-

targeted modifications, such as gene regulation, is a major advantage of the CRISPR-Cas system.[29,30] It can also be used for various other applications, such as imaging of genomic loci[31] and base editing without DSB induction.[32,33]

Due to the diversity of the natural CRISPR-Cas platforms, they remain largely untapped for biotechnological tools so far.[13,34] Currently, various methods are being used to identify and characterize these systems, such as data mining and bioinformatic prediction. The ability to create a practical genome engineering tool requires that the newly identified CRISPR-Cas systems have activity in mammalian cells. Unfortunately, this is not the case for all types of systems. To find further Class 2 type II systems, with Cas9 as its main protein, and other variants extensive screening was conducted. The first effector, categorized as class 2, that was identified outside of Cas9 was Cas12 (formerly known as Cpf1).[35] The Cas12a showed high activity in mammalian cells. Its particular properties, such as its staggered breaks and the requirement for completely different PAMs, made it an incredibly useful tool for genome engineering. Shmakov *et al*[34] identified three new effectors belonging to Class 2. However, two of them, containing C2c1 and C2c3, are alike to Cas12. Instead, the C2c2 is completely irrelevant. Owing to their unique features, the system was then assigned to a new type (Class 2 type VI) termed CRISPR-Cas13a. The presence of HEPN (higher eukaryotes and prokaryotes nucleotide-binding) domains entirely linked to RNase activity, suggested that the Cas13 system is exclusively focused on RNA. This could be a good hypothesis as it means that the system is not only capable of cleaving single-stranded RNA, but it also doesn't utilize double-stranded RNA.[36] After the activation of the target RNA, the other RNAs had been cleaved in an unspecific manner. It is suggested that Cas13 is involved in dormancy induction or programmed cell death. In addition, the high activity of Cas13 orthologue has been proven in eukaryotic cells.[37,38] The researchers found that the unspecific degradation of RNA in prokaryotic systems was not present in eukaryotic cells. This discovery opens up a broad range of new opportunities for the development of targeted RNA therapeutics.[39]

The CRISPR-Cas9 system's versatility is exemplified by the development of Cas9 variants, addressing limitations associated with the conventional Streptococcus pyogenes Cas9 (spCas9). Notably, the smaller size Cas9 (saCas9) and double-nicked Cas9 (d-Cas9) offer solutions to issues like off-target effects. This innovation allows for precise gene editing in plants.[40]

Base editing

Base editing is a powerful and efficient tool for single-base substitution in plants. It can be used in combination with other tools to vastly enhance the scope and efficiencies of genetic editing.[41,42] The two main types of base editors are the adenine (ABE) and cytidine (CBE) (Fig. 4).

Base editing has several advantages over other gene editing techniques. First, it enables single-nucleotide changes to be made in the genome, which can be crucial in fine-tuning gene expression and protein function. Second, base editing does not require a donor template, which simplifies the editing process and reduces the possibility of introducing unintended mutations. Third, since base editing does not generate double-stranded breaks, there is a reduced risk of off-target effects and chromosomal rearrangements.[43]

For CBE, the combination of the cytidine deaminase and the Cas9 nickase (nCas9) or dCas9 (Fig. 5) creates a narrow window of targeting site for the deamination of the cytosine in the non-targeted strand of DNA. A single-base substitution occurs by deamination of the original cytosine (C) and uracil (U) formation. This process is carried out through the recognition of U as the thymine, which is a component of the DNA.[32] In mammals, a distinct version of the Lachnospiraceae Cpf1 has been identified as a potential target for single-base substitutions.[44]

Adenine deaminase, which is fused with nCas9 or dCas9, is responsible for the deamination of adenine and converting it into inosine (I). This process results in the recognition of inosine, as guanine. ABE allows base substitutions (A.T to G.C).[45] Based on previous studies, many important traits in plants are conferred through target alleles that have one or several single nucleotide polymorphisms (SNPs). It can take breeders several years to introduce these favorable alleles to commercial cultivars. Due to the difficulties in performing gene editing using the CRISPR-Cas-mediated method in plants, base editing has become an applicable technique for inducing nucleotide substitution. The efficiency and simplicity of the ABE and CBE base editors have been broadly used in various organisms for genome editing. These tools are commonly used in plant gene functional annotation and correction.[46]

Prime editing

Prime editing is a further development of the CRISPR-Cas9 system that combines the features of gRNA and a modified Cas9 (nickase) enzyme fused to reverse transcriptase (RT). The RT activity allows the prime editor to write a new DNA sequence into the genome at the target site, rather than relying on the DSB for repair. Prime editing uses a prime gRNA (pegRNA) that contains a template sequence, complementary to the target DNA, and guides the nickase to make a nick in the non-target strand. The RT generates a new DNA sequence that pairs with the opposite strand of the nicked DNA, resulting in a precise edit at the target site.[47] A prime editor (PE) is a type of editing tool that can be created without the need for donor DNA or DSBs. The main component of this process is a combination of the Cas9 nickase and

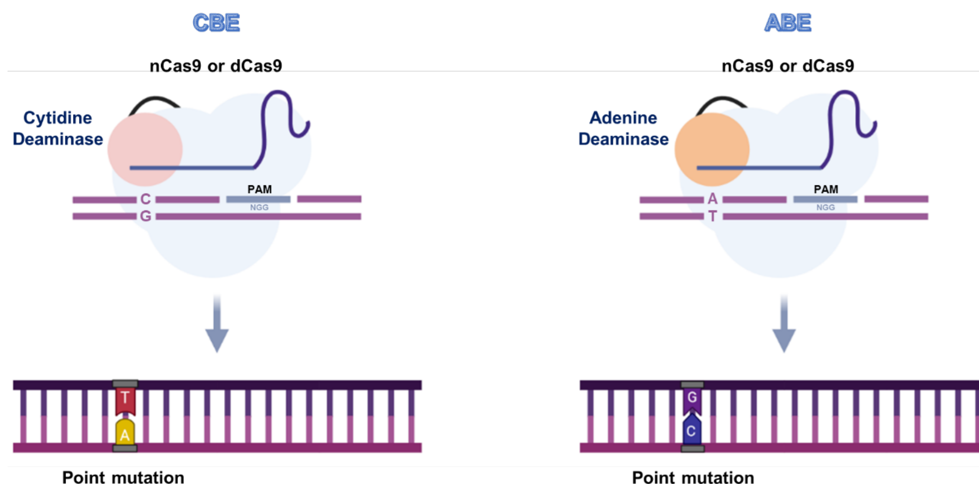

Fig. 4. Base editors. When either catalytically dead Cas9 (dCas9) or Cas9 nickase (nCas9) is fused to a nucleobase deaminase, guide RNA (gRNA) directs the base editor to the chosen target sequence. Bases are swapped, and cells repair the DNA.

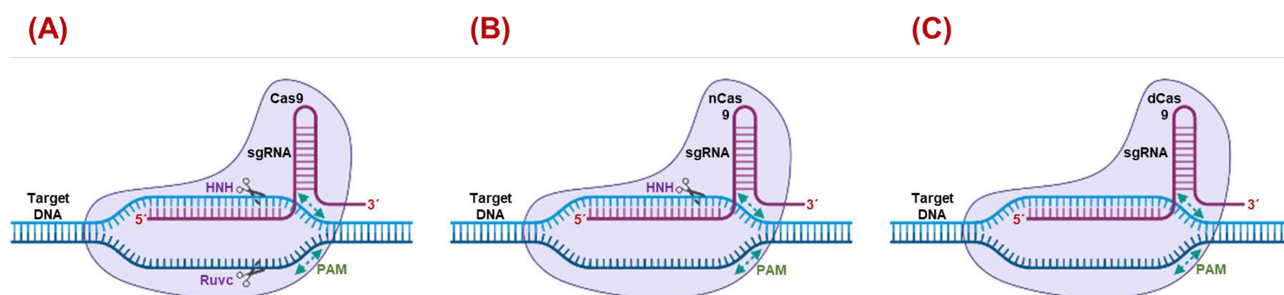

Fig. 5. Mechanisms of Cas9, nCas9 and dCas9. **(A)** Targeted double-stranded breaks introduced by Cas9. **(B)** Single-strand breaks introduced by nCas9. **(C)** Target recognition by dCas9.

the RT. A guide RNA, known as prime-editing guide RNA (pegRNA) can mediate the nicking of a specific site by the nCas9 compartment, which is then served as a template for the RT, directing to the customization of the mutations. PEs can efficiently produce various small insertions and base conversions in human cells. They can also expand the targeting range.[48]

Delivery of CRISPR-Cas gene-editing reagents to plants

Besides selecting the right tools, it is also important to deliver the correct CRISPR reagents to the plant cells. In certain systems, such as human cells, the purified mRNA or protein of a Cas system can be delivered to a zygotic cell concurrently. This method can improve the targeting possibility by controlling the concentration of gRNAs and Cas proteins. Although this approach has been shown to work in plants, it still has a long way to go before it can be used in other organisms. The design of a CRISPR construct can affect the outcome of the editing procedure.[49-54] This is why the various elements of the technology must be considered when it comes to optimizing the expression of gRNAs and Cas proteins. Besides these, other factors such as the presence of certain gene regulatory elements (GREs) are also taken into account to ensure that the process is performed efficiently.

Usually, the reagents for genome editing are delivered to plants through a construct that contains a Cas gene and at least one gRNA. This construct can be used in combination with other plant-specific constructs to experiment. Several delivery tools have been developed for efficient and effective CRISPR-Cas9 gene editing in plants. These methods are summarized in Table 1 and are illustrated in Fig. 6.

Agrobacterium-mediated transformation (AMT)

Agrobacterium tumefaciens is a soil bacterium that is widely used for plant genetic transformation.[56] It can naturally infect plants by transferring a small piece of DNA called T-DNA.[57] Researchers have exploited this natural trait by transforming Agrobacterium to carry modified T-DNA sequences that encode desired traits. By infecting plant cells with these modified Agrobacterium strains, the T-DNA is delivered to the target genome and integrated into the plant DNA, leading to the desired genetic modification.[58] AMT-mediated CRISPR-Cas9 editing is a promising method for genetically manipulating plant species to enhance their agronomic traits.[59] This delivery system has been used for gene editing in plants such as Arabidopsis,[60] tobacco,[61] lettuce,[62] and wheat.[63]

Table 1. Summary of CRISPR delivery methods in plant genome editing

Method	Description	Applications and Advantages	Limitations and Challenges	Ref.
Agrobacterium-Mediated Transformation (AMT)	Agrobacterium tumefaciens delivers CRISPR components through T-DNA transfer, allowing gene editing in various plants.	- AMT effectively modifies wheat, tomato, tobacco, etc. - CRISPR-Cas9 via AMT enhances grain weight, yields, and provides resistance to powdery mildew.	- Limited cargo capacity. - Off-target effects can occur.	55
Biolistic Transformation	Particles coated with CRISPR components are bombarded into plant cells, introducing DNA without direct cell contact.	- Utilized in maize, wheat, barley, rice, etc. - Overcomes limitations of other methods in introducing foreign DNA into plant tissues.	- Tissue culture is often required for regeneration. - Limited efficiency compared to other methods.	55
Electroporation	Electrical current creates temporary pores in cell membranes, facilitating efficient entry of CRISPR components.	- High efficiency and minimal off-target effects. - Used in tobacco and switchgrass for CRISPR-Cas9 delivery.	- Dependency on electric field application.	55
Expression of Developmental Regulators	WUS and BBM induce new embryogenesis, transforming rejected lines. Ectopic expression of regulators (IPT, STM, BBM, GRF4, GIF1) enhances transformation frequencies.	- Successful transformation of previously rejected lines. - Increased genome-edited plant numbers with CRISPR-Cas9 and developmental regulators.	- Poor performance in DNA delivery and plant regeneration.	55
RNA Viruses and Mobile Guide RNAs	TRV, a positive-strand RNA virus, delivers sgRNAs into Cas9-overexpressing plants through Agrobacterium infiltration. Sonchus yellow net rhabdovirus delivers sgRNA and SpCas9.	- TRV achieves systemic, heritable gene editing. - Sonchus yellow net rhabdovirus induces heritable mutations.	- Low cargo capacity of viruses. - TRV can't transmit to progeny.	55

The AMT was applied to Arabidopsis plants to create stable mutations in the *GGAT1* gene using CRISPR/Cas9, resulting in homozygous mutants by the T2 generation. These mutations, which were stable through subsequent generations and exhibited Mendelian segregation, led to specific photorespiration phenotypes and reduced GGAT enzyme activity, demonstrating the effectiveness of AMT in precise genetic modifications.[60] Using AMT in tobacco, the CRISPR/Cas9 system effectively targeted and repaired non-functional GFP genes. This led to restored GFP function, visible as green fluorescence in cell nuclei, demonstrating the system's capability for precise genetic modifications in tobacco via AMT.[61] Using AMT, lettuce cotyledon explants were transformed with constructs targeting the *LsNCED4* gene, crucial for seed germination temperature sensitivity. This resulted in kanamycin-resistant callus and high-temperature germination in knockout *NCED4* lines, serving as a selectable marker for germline editing. Specific mutations were inherited through the germline, indicating that AMT can effectively induce desired genetic changes in lettuce.[62] Using AMT, another study introduced CRISPR/Cas9 constructs targeting the *TaGW2* gene in wheat. This led to the generation of *TaGW2* mutant lines with reduced grain size compared to wild-type wheat plants. These edited lines exhibited stable inheritance of the mutations across generations, demonstrating the efficacy of AMT for precise gene editing in wheat.[63] Moreover, in tomatoes, AMT-mediated delivery of CRISPR-Cas9 was used to target the *MLO* gene, resulting in resistance to powdery mildew.[64]

The CRISPR-Cas9 editing system has revolutionized plant genetics, and its combination with Agrobacterium-mediated transformation offers a highly effective method for introducing precise genetic modifications into plant genomes.[65] The ability to customize crop plants to meet specific demands for yield, disease resistance, and environmental stress make this technology highly attractive to plant breeders.[66] Futuristic applications include the development of drought-resistant plants, improving photosynthetic efficiency, and facilitating the growth of crops in harsh environments.[67]

Biolistic Transformation

One of the critical ways of introducing foreign DNA into a plant's genome is biolistic transformation. Biolistic transformation is a technique for introducing DNA into plant cells or tissues directly through a bombardment of microscopic particles. The particles are coated with the DNA of interest and then accelerated toward the target cells or tissues with a small explosion. Afterward, the DNA can integrate into the plant's genome through processes such as homologous recombination, illegitimate recombination, or through insertion into random sites in the genome. This method has gained popularity due to its ability to introduce foreign DNA into plant tissues without the need for direct contact with the cells, including tissues that are difficult to transfect using traditional techniques such as Agrobacterium-mediated transformation.[68] Biolistic transformation, also known as particle bombardment, can use small gold particles coated with plasmids carrying the CRISPR-Cas9 system to deliver it into the plant cells. This technique has been utilized for gene editing in a variety of plant species, such as maize,[69] wheat,[70] barley,[71] and rice.[72] CRISPR/Cas9 genome editing in maize utilizes transformation methods

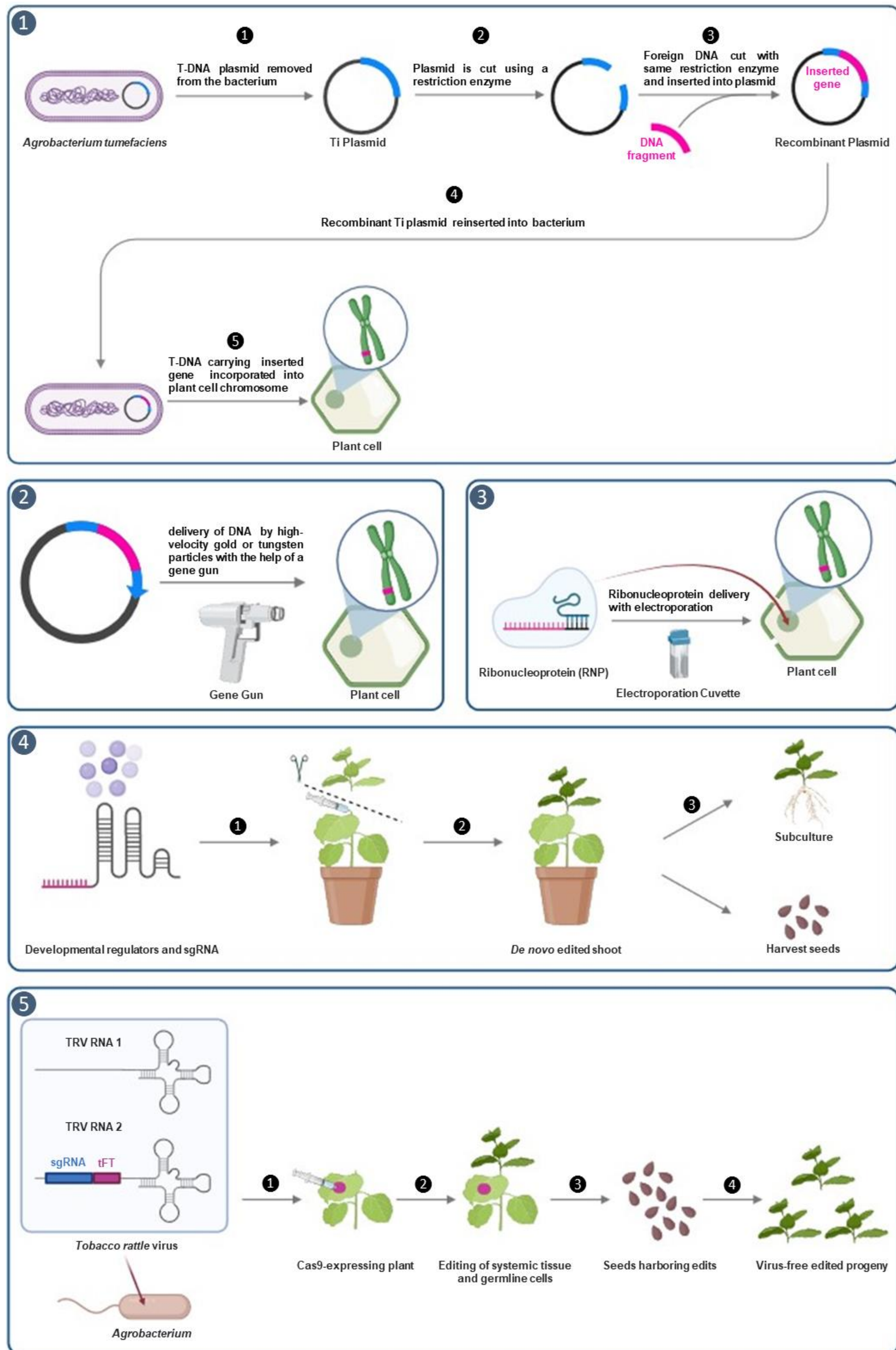

Fig. 6. Exploring the Frontiers of Gene Editing: Diverse CRISPR Reagent Delivery Tools - **(1)** Agrobacterium-Mediated Transformation (AMT): Harnessing Bacterial Mechanisms; **(2)** Biolistic Transformation: Gene Transfer via Particle Bombardment; **(3)** Electroporation of RNP (ribonucleoprotein): Direct Entry by Electric Pulses; **(4)** Expression of Developmental Regulators: Modulating Gene Expression; **(5)** Tobacco Rattle Virus (TRV) through Agrobacterium Infiltration: Viral Vector Integration.

like Agrobacterium-mediated and biolistic delivery, along with protoplast transformation for precise gene editing. Advancements include improved transformation efficiency and the use of transient assays for vector validation. Multiplex gene editing strategies address the challenges of polyploidization, focusing on gene knockouts and editing multiple gene copies for desired traits. Despite maize having fewer published reports than other crops, significant research and practical applications are emerging in the field.[69] The refined CRISPR/Cas9 protocol for wheat genome editing integrates particle bombardment delivery of IVTs (*in vitro* transcribed) or RNPs (ribonucleoprotein) with a selection-free tissue culture method and mixed-pool screening. This approach, successful in two wheat varieties, enhances efficiency by allowing transient expression of CRISPR/Cas9, simplifying plant regeneration, and reducing foreign DNA integration and off-target effects, especially with RNP-mediated editing.[70] The optimized synthetic Cas9 gene for barley genome editing, delivered through biolistic transformation, achieved significant mutation rates in targeted genes. This method was used for both simplex and multiplex editing, showing high efficiency in inducing mutations, particularly in the *HvCKX1* gene. These mutations were successfully inherited in the T1 generation, highlighting the effectiveness of biolistic transformation in CRISPR/Cas9 mediated genome editing in barley.[71] Biolistic transformation was utilized for co-delivery of CRISPR-Cas9 ribonucleoprotein and a selectable marker plasmid in rice, targeting the *OsPDS1* gene to induce albino phenotypes as successful edit markers. While all delivery platforms achieved targeted mutations, biolistic methods particularly showed high random DNA insertion rates. Of the transformed events, 24.6% were albino and 75.4% were green, and mutations were confirmed to be inheritable to subsequent generations.[72]

Electroporation

Contrary to the obtained successes, agrobacterium-mediated transformation and particle bombardment methods face a series of limitations in terms of efficiency and scalability.[73] Moreover, they often result in off-target effects, which can be detrimental to plant growth and development.[74] Electroporation emerges as an alternative method, utilizing electrical currents to create temporary pores in cell membranes. Based on this method, DNA, RNA, and proteins have been successfully delivered into plant cells. Recent studies have shown that electroporation can also be used to deliver CRISPR components into plant cells, with high efficiency and minimal off-target effects.[75] For example, CRISPR-Cas9 system was delivered via electroporation in tobacco LJ911 to edit the *Va* gene, resulting in transgene-free homozygous edited plants with increased resistance to Potato virus Y (PVY). Moreover, electroporation was used to introduce the CRISPR/Cas9 expression vector into *Agrobacterium tumefaciens* for genetic transformation of tobacco. Pathology tests confirmed the immunity of the edited tobacco lines to PVY. The research provided valuable genetic resources for the breeding of PVY-resistant tobacco and highlighted the regulatory framework for gene-edited products in different countries, emphasizing the potential agricultural applications of this technology.[40,76]

Expression of developmental regulators

CRISPR/Cas9 technology has significantly revolutionized plant biotechnology by enabling the expression of developmental regulators (DRs) that are crucial in directing plant growth, development, and environmental response. Developmental regulators are genes that determine meristem identity and orchestrate growth patterns and developmental pathways. By employing CRISPR, these regulators can be modified to induce desired plant characteristics. Examples include WUSCHEL (WUS) and LEAFY (LFY), essential for plant regeneration and flower development, respectively. CRISPR modifications in these genes can lead to improved tissue culture techniques and altered flowering patterns, which are vital for crop breeding.[77] Furthermore, the ectopic expression of DRs has led to significant morphological and physiological changes in plants.[78] These examples showcase the immense potential of CRISPR in manipulating plant growth and development to meet agricultural needs and adapt to changing environmental conditions. The utilization of developmental regulators as a CRISPR delivery method represents a groundbreaking strategy in plant genome editing. This innovative approach addresses challenges associated with traditional methods by enhancing precision and efficiency. Researchers have achieved transgene-free gene editing by transiently expressing CRISPR reagents through regenerating events without employing selection, demonstrating the versatility of this technique.[78] Another effective method involves Agrobacterium-mediated delivery, where Cas9 expression cassettes, sgRNA, and growth regulators expressing cassettes are co-delivered into wild-type plants. This not only simplifies the process but also improves the overall efficacy of genome editing.[79] Furthermore, an improved gene-editing efficiency method includes co-delivering developmental regulators with CRISPR components, showcasing advancements in the field.[80] Additionally, non-GM (non-genetically modified) editing approaches, such as non-transgenic delivery and transient expression of developmental regulators and CRISPR/Cas reagents in plant organs, offer a promising avenue for gene editing in crops. These multifaceted approaches collectively highlight the evolving landscape of CRISPR-based plant genome editing, providing researchers with a diverse toolkit to tailor interventions based on specific

plant species and desired outcomes.[81] As a successful example of a plant modified using WUS through CRISPR/Cas9 technology, a study focused on the application of CRISPR/Cas9 genome-editing system in *Brassica rapa* var. *rapa* (turnip), emphasizing the role of the *BrrWUSa* gene in improving plant regeneration. The gene, when activated, notably enhanced transformation frequencies in turnip. Additionally, the research successfully edited the *BrrTCP4b* gene in turnip, leading to increased leaf trichome numbers. This illustrates the potential of CRISPR/Cas9 in advancing plant biotechnology, particularly in species with low transformation efficiency. The findings highlight the significant impact of developmental regulators on plant regeneration and genome editing.[77] Another study demonstrates the efficacy of CRISPR-mediated cytosine base editing in *Arabidopsis thaliana*. It specifically targets the LFY gene, a key factor in flowering. The CRISPR system induced loss-of-function mutations in LFY, leading to observable mutant phenotypes like altered floral structures. This result highlights CRISPR's potential for precise genetic manipulation in plants, particularly for functional studies and trait development in crop research.[82]

RNA viruses and mobile guide RNAs

One of the most promising gene-editing methods that could be used for high-throughput production is by employing the tobacco rattle virus (TRV) which is a positive-strand RNA virus. This method involves delivering the sgRNAs into the plants in which Cas9 is overexpressed, through Agrobacterium infiltration. Fusion of sgRNAs with RNA mobile elements, (such as the Flowering locus T) leads to achieve systemic gene-editing with heritable mutations. Flowering locus T promotes the mobility of reagents in apical meristems. The TRV vector, carrying the modified sgRNAs, is then used by Agrobacterium to infiltrate plants. This method can generate bi-allelic mutations without the presence of virus transmission to the progeny. For example, TRV has been successfully utilized for gene editing in plants like *Nicotiana benthamiana* and *Arabidopsis*. It effectively delivered sgRNAs into plants overexpressing Cas9, resulting in targeted mutagenesis at specific genomic locations. Moreover, Sonchus yellow net rhabdovirus, a negative-strand DNA virus, was engineered to contain both sgRNA and SpCas9 sequences and could be delivered to wild plants through Agrobacterium infiltration. Heritable mutations successfully resulted from this method.[83] Unfortunately, the negative effects of using DNA or positive-strand RNA viruses are their low cargo capacity, which prevents the complete CRISPR-Cas9 expression cassettes from being delivered to the plants. However, this approach allows for efficient genome editing across different plant species, showcasing virus as a versatile tool in plant biotechnology and gene function studies.[84]

Plant-based CRISPR-Cas9 gene editing

CRISPR/Cas9 applications in beneficial crops

The advancements in CRISPR/Cas9-mediated gene editing have revolutionized agriculture, particularly in enhancing crops like rice and soybean. This cutting-edge technology enables the simultaneous editing of multiple targets, creating a diverse cell population with various gene modifications. The precision and efficiency of CRISPR/Cas9 have opened new possibilities for crop improvement, allowing scientists to tailor plants for desired traits such as increased yield, resistance to pests, and improved nutritional content. As researchers delve deeper into the potential applications of this technology, the future holds promise for addressing global challenges in food security and sustainable agriculture through the development of genetically optimized crops.[40] For example, in apple (*Malus domestica*), genes *DIPM-1*, *DIPM-2*, *DIPM-4* were targeted through PEG-mediated protoplast transfection to confer resistance to fire blight disease, while the *PDS* gene was edited using Agrobacterium-mediated leaf discs transformation to induce albino phenotypes. In banana (*Musa spp.*), the *TFL1* gene was edited for early flowering, and *MaGA200x2* for albino phenotypes and semi-dwarfing size, both through Agrobacterium-mediated transformations. Soybean (*Glycine max*) saw the editing of *GmFT2a* via PEG-mediated protoplast transfection for controlling flowering time. Cacao (*Theobroma cacao*) used *TcNPR3* edited through Agrobacterium-mediated transient leaf transformation for resistance against Phytophthora tropicalis. Rice (*Oryza sativa*) utilized *OsSWEET13* edited via CRISPR/Cas9 ribonucleoprotein delivery for bacterial blight resistance. Citrus species like Carrizo Citrange and Grapefruit (*Citrus × paradisi*) used *PDS* and *CSLOB1* genes, respectively, for inducing albino phenotypes and canker disease resistance through Agrobacterium-mediated transformations. Wheat (*Triticum aestivum*) targeted *TaGW2* through biolistic particle delivery to increase grain size and weight. Sweet Orange (*Citrus sinensis*) edited *DMR6* for Huanglongbing resistance, and Maize (*Zea mays*) targeted *ZmIPK* to study inositol phosphate metabolism, both through particle bombardment. Kiwifruit (*Actinidia deliciosa*) and Barley (*Hordeum vulgare*) used *PDS* and *HvPM19* genes for albino phenotype and drought tolerance enhancement, respectively. Strawberry (*Fragaria × ananassa*) targeted *APETALA3* (*AP3*) for flowering control. Potato (*Solanum tuberosum*) and Tomato (*Solanum lycopersicum*) used *StALS* and multiple genes like *BoPDS*, *CENH3*, *DcMYB113-like*, and *CiPDS* for traits such as herbicide resistance, haploid line induction, anthocyanin biosynthesis, and albino phenotypes through various transformation methods. These examples demonstrate the wide-ranging potential of CRISPR in enhancing desired traits across different plant species (Table 2).

Table 2. Diverse applications of CRISPR-based genome editing in plant species

Plant species (Scientific name)	Gene of interest	Transformation method	Aim (target trait)	Ref.
Apple (*Malus domestica*)	*DIPM-1, DIPM-2, DIPM-4*	PEG-mediated protoplast transfection	Fire blight disease resistance	85
	PDS	Agrobacterium-mediated leaf discs transformation	Albino phenotypes	86
Banana (*Musa* spp.)	*TFL1*	Agrobacterium-mediated embryogenic cell suspension	Early flowering	87
	MaGA200x2	Agrobacterium-mediated suspension cells transformation	Albino phenotypes, semi-dwarfing size	88
Soybean (*Glycine max*)	*GmFT2a*	PEG-mediated protoplast transfection	Flowering time control	89
Cacao (*Theobroma cacao*)	*TcNPR3*	Agrobacterium-mediated transient leaf transformation	Phytophthora tropicalis resistance	55
Rice (*Oryza sativa*)	*OsSWEET13*	CRISPR/Cas9 ribonucleoprotein (RNP) complex delivery	Bacterial blight resistance	90
Citrus (*Carrizo Citrange*)	*PDS*	Agrobacterium-mediated epicotyl transformation	Albino phenotypes	91
Grapefruit (*Citrus × paradisi*)	*CSLOB1*	Agrobacterium-mediated epicotyl transformation	Canker disease resistance	92
Wheat *(Triticum aestivum)*	*TaGW2*	Biolistic particle delivery	Increasing grain size and weight	93
Sweet Orange (*Citrus sinensis*)	*DMR6*	Agrobacterium-mediated epicotyl transformation	Huanglongbing resistance	94
Maize (*Zea mays*)	*ZmIPK*	Particle bombardment	To study inositol phosphate metabolism	95
Kiwifruit (*Actinidia deliciosa*)	*PDS*	Agrobacterium-mediated transformation	Albino phenotype	96
Barley (*Hordeum vulgare*)	*HvPM19*	Biolistic transformation	drought tolerance enhancement	97
Strawberry (*Fragaria × ananassa*)	*APETALA3 (AP3)*	Agrobacterium-mediated leaf disk	Flowering control	98
Potato (*Solanum tuberosum*)	*StALS*	PEG-mediated protoplast transfection	Herbicide resistance	99
Tomato (*Solanum lycopersicum*)	*BoPDS*	Agrobacterium-mediated hypocotyl transformation	Albino phenotypes	100
	CENH3	Protoplast transformation and Agro infiltration	Haploid lines induction	101
	DcMYB113-like	Agrobacterium-mediated transformation	Anthocyanin biosynthesis	102
	CiPDS	Agrobacterium-mediated leaf sections and protoplast transfection	Albino phenotype	103

Challenges facing CRISPR/Cas9 delivery in plants

In the realm of plant genetics, CRISPR/Cas9 technology has introduced groundbreaking possibilities, yet it faces significant challenges, particularly in the context of various delivery methods. *Agrobacterium*-mediated transformation utilizes the natural ability of *Agrobacterium* to transfer DNA into plant cells. However, its efficiency can be limited by the species and tissue type of the plant, and there's a risk of random DNA integration, which might disrupt essential genes or regulatory regions in the plant genome.[104] Biolistic transformation, known for its physical gene insertion, uses high-velocity microprojectiles to deliver DNA into cells. While versatile across various species, challenges include potential damage to the target cells and the random integration of the transgene, leading to variable expression levels.[68] Expression of developmental regulators requires precise control over gene expression timing and level. The challenge is achieving this control without disrupting normal plant development and ensuring stable gene expression.[105] Overcoming these challenges is crucial for the optimal utilization of CRISPR/Cas9 in plant genetics, ensuring the integrity of targeted plants and enhancing gene delivery methods' efficiency and precision.[106]

In overcoming these challenges, innovative delivery methods like electroporation have been explored, showing promise in enhancing efficiency. Moreover, by utilizing this method, researchers aim to achieve greater precision in gene delivery and minimize off-target effects.[107] Additionally, the integration of viral vectors and guide RNAs presents a versatile strategy, contributing to the scalability and rapid production of genetically modified plants, thus paving the way for transformative breakthroughs in agricultural biotechnology.[108] These cutting-edge approaches capitalize on advancements in genetic engineering, offering a more precise and streamlined means of introducing genetic materials.

Future prospects and limitations

CRISPR/Cas9 technology has significantly advanced plant genome editing, offering new possibilities for crop improvement. However, it faces challenges such as plant regeneration inefficiencies and resistance to genetic transformation processes, which hinder its full integration into agriculture.[109,110] To address these issues, researchers are exploring developmental regulators, especially growth-regulation factors. Merging these insights with CRISPR-Cas9's precision, they aim to boost plant regeneration efficiency. This interdisciplinary approach could greatly increase genome-edited plants, expanding CRISPR/

Cas9's use in agriculture. Integrating developmental regulators with CRISPR/Cas9 is not only a solution to current challenges but also marks a shift towards a deeper understanding of plant biology. Continuous research on plant genomes and refining genome-editing techniques will likely transform agriculture, positioning CRISPR/Cas9 as a key innovator. This effort foresees a future where genetically modified crops are crucial for global food security and sustainability.[110]

Conclusion

CRISPR-Cas9 technology has significantly transformed plant genetics by providing a precise and efficient way to modify genes. This method is favored over traditional techniques due to its accuracy, ease of use, and versatility, enabling advancements in crop yield, disease resistance, and stress tolerance. However, challenges exist, including difficulties in delivering CRISPR-Cas9 reagents to plant cells and the risk of off-target effects, which can result in unintended genetic modifications. Future developments in CRISPR-Cas9 for plant genetics should concentrate on improving reagent delivery and overcoming plant regeneration and genetic transformation obstacles. By integrating developmental regulators, CRISPR-Cas9 can have broader applications in agriculture, potentially revolutionizing crop development and aiding in global food security and sustainable agriculture.

In summary, CRISPR-Cas9 is a pivotal innovation in plant genetics. Addressing its limitations and leveraging its capabilities will likely lead to significant contributions in agriculture and plant biotechnology, enhancing global food security and sustainability.

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Authors' Contribution

Conceptualization: Vida Ebrahimi and Atieh Hashemi
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Writing–review & editing: Atieh Hashemi

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

Ethical Statement

There is no involvement of human or animal in this study.

Research Highlights

What is the current knowledge?

- Meganucleases, TAL effectors, and CRISPR nuclease revolutionize plant genome editing, offering precise methods for genetic modification.
- Comprehensive search in PubMed, ScienceDirect, and Google Scholar to gather information on CRISPR-Cas9 gene editing in precise gene modification, particularly in plants.
- Extensively studied for accurate gene modification in various organisms, with a focus on plants.
- CRISPR-Cas9 enables precise gene manipulation with promising applications in agriculture and biotechnology.

What is new here?

- Emphasis on CRISPR-Cas9 gene editing's momentum and applications across diverse scientific disciplines.
- In-depth exploration of various reagent delivery tools for CRISPR-mediated gene editing in plants.
- Recognition of the importance of efficient gene editing reagent delivery and the impact of rapid CRISPR nuclease evolution.

Potential for transformative advancements in plant genetics and related fields due to accurate genome modification.

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