Genome Editing and Generation of Transgene-Free Lines

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Abstract

Genome editing, particularly using CRISPR-Cas9 technology, has revolutionized plant biotechnology by enabling precise genetic modifications without leaving foreign DNA in the final product. This article explores the process of generating transgene-free genome-edited plants, covering the design of CRISPR-Cas9 vectors, delivery mechanisms, and the activation of genome editing through non-homologous end joining (NHEJ) or homology-directed repair (HDR). It also discusses methods for eliminating transgenes, such as transient expression and the Cre-LoxP system, and highlights the importance of selfing and backcrossing in generating stable, transgene-free lines. These strategies are critical for crop improvement and meet the growing demand for sustainable and regulatory-compliant agricultural practices.

Introduction

The advent of genome editing technologies, particularly CRISPR-Cas9, has transformed plant biotechnology by enabling precise genetic modifications. Unlike traditional breeding methods, which rely on random genetic variations, genome editing allows for targeted changes in specific genes, providing a more efficient approach to enhance traits like disease resistance, stress tolerance, and nutritional value in plants. CRISPR-Cas9 works by introducing a double-strand break at a specific genomic location, which is then repaired through non-homologous end joining (NHEJ) or homology-directed repair (HDR), depending on the repair mechanism activated.

While genome editing holds immense potential, one of the main challenges is the presence of foreign DNA in the edited plants. These foreign sequences, including the CRISPR-Cas9 components, can present regulatory challenges and consumer resistance. To address these concerns, generating transgene-free plants is essential. This involves ensuring that the CRISPR-Cas9 system does not leave behind any foreign genetic material in the final plant product. The development of transgene-free plants is crucial for regulatory compliance and consumer acceptance. This article outlines the key steps in genome editing and strategies for generating transgene-free lines, with a focus on their transformative potential in crop development.

Genome Editing Process and Generation of Transgene-Free Lines

Genome editing using CRISPR-Cas9 technology represents a significant advancement in plant biotechnology, enabling precise modifications to plant genomes. The process involves several critical steps, from vector design to the final generation of transgene-free plants.

Step 1: Design and Construction of Editing Vectors

The first step in the genome editing process is the design of the CRISPR-Cas9 editing vector, which includes several key components:

Guide RNA (gRNA)

Designed to specifically target the gene of interest, the gRNA consists of 20 nucleotides complementary to the target DNA sequence. It directs the Cas9 protein to the precise location in the genome for cleavage. To design gRNA, bioinformatics tools such as CRISPR-Cas Designer, CHOPCHOP, and CRISPOR are commonly used to identify specific genomic targets and assess potential off-target effects. It is synthesized as single-stranded RNA or through in vitro transcription from a DNA template comprising the target-specific spacer sequence and a conserved scaffold region for Cas protein binding.

Cas9 Protein

The Cas9 protein is an RNA-guided endonuclease that creates a double-stranded break at the target site. The repair of this break is carried out by the plant's DNA repair machinery, leading to gene knockout through NHEJ or specific modifications via HDR if a donor template is provided.

Selectable Marker

A selectable marker, such as an antibiotic resistance gene, is included in the vector for identification during the transformation process. Selectable markers, such as kanamycin resistance (nptII), hygromycin resistance (hph), and glyphosate resistance (EPSPS), are used during plant genetic transformation to identify successfully transformed

cells. These markers help select cells that have incorporated foreign DNA by allowing them to survive in the presence of antibiotics or herbicides. Importantly, the selectable marker is not intended to remain in the final plant but is used only during the selection of transformed cells.

Once designed, these components are incorporated into a plasmid vector that facilitates the transfer of the CRISPR-Cas9 system into plant cells.

Step 2: Delivery of CRISPR-Cas9 Components into Plant Cells

The CRISPR-Cas9 system must be efficiently delivered into plant cells for successful genome editing. Several delivery methods are employed:

Agrobacterium-Mediated Transformation

Commonly used for dicotyledonous plants, this method utilizes *Agrobacterium tumefaciens*, which naturally transfers T-DNA into plant cells, to introduce the CRISPR-Cas9 plasmid along with the editing components. This method ensures stable integration of the editing components, enabling efficient genome editing. It is highly efficient for callus or explant tissue and often combined with tissue culture techniques for plant regeneration.

Particle Bombardment (Gene Gun)

DNA-coated microprojectiles are used to physically deliver the CRISPR-Cas9 system into plant cells. This method is particularly useful for monocots. It uses high-velocity DNA-coated microprojectiles (commonly gold or tungsten particles) to penetrate plant cells and deliver the CRISPR-Cas9 system. This technique bypasses the requirement of a biological vectors, making it ideal for species resistant to *Agrobacterium*-mediated transformation. The DNA integrates into the plant genome upon entering the nucleus, enabling stable expression of CRISPR components. This technique is versatile and effective for transient expression studies or stable genome modifications in recalcitrant plants.

Protoplast Transformation

Protoplast transformation with CRISPR-Cas starts by enzymatically removing cell walls to isolate protoplasts. CRISPR elements (gRNA and Cas protein) are introduced through PEG-mediated transfer or electroporation. Transformed protoplasts are cultured in recovery media to enable the expression of genetic edits. Edited protoplasts can either regenerate into plants or undergo direct analysis.

Viral Vectors

Modified plant viruses can also be used to deliver CRISPR-Cas9 components, offering high transformation efficiency across a variety of plant species. Commonly used modified viral vectors for delivering CRISPR-Cas9 components into plant cells include Tobacco mosaic virus (TMV) and barley stripe mosaic virus (BSMV). These vectors are engineered to carry the guide RNA and Cas9 sequences, allowing efficient and systemic delivery throughout the plant. Viral vectors are particularly advantageous for species or tissues that are difficult to transform using traditional methods. They enable transient expression of CRISPR components without integrating into the genome, reducing the risk of off-target effects and regulatory concerns.

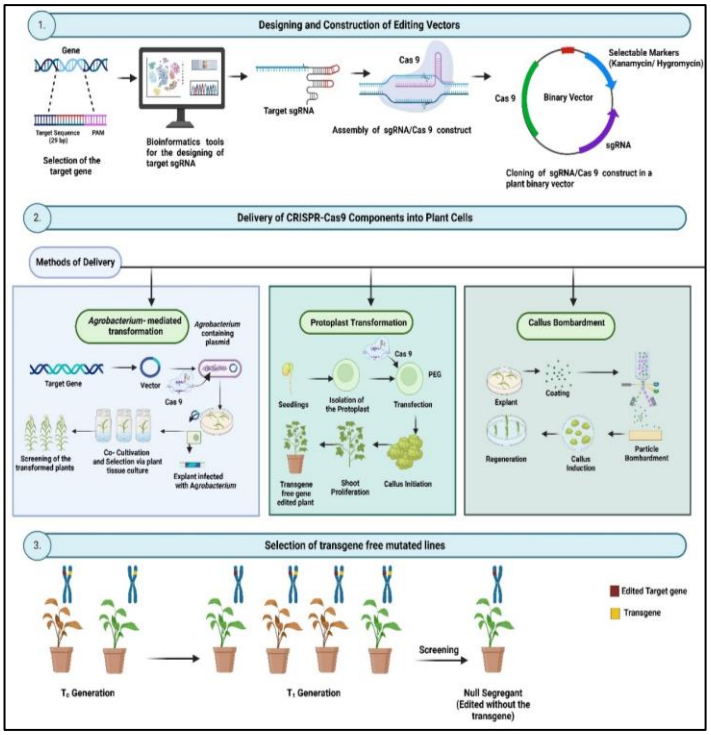

Fig 1. Generation of Transgene free edited plants via genome editing (1.) Designing and Construction of Editing Vectors (2.) Delivery of CRISPR- Cas9 components into plant cells (3.) Selection of transgene free mutated lines

Step 3: Inducing Genome Editing

Once the CRISPR-Cas9 system is delivered into plant cells, the Cas9 protein creates a double-strand break at the target genomic location. The break is repaired via one of two main pathways:

Non-Homologous End Joining (NHEJ)

This error-prone repair pathway results in insertions or deletions (indels) at the break site, which can disrupt the targeted gene's reading frame. This can knock out the gene, making NHEJ particularly useful for functional gene studies, creating loss-of-function mutants, or disabling harmful genes.

Homology-Directed Repair (HDR)

In the presence of a donor DNA template, HDR can facilitate precise modifications, such as the insertion of a new gene. Though more accurate, HDR is less efficient than NHEJ and requires the introduction of a donor template. At this stage, the edited plant may still contain the CRISPR-Cas9 plasmid and selectable marker, necessitating the next step of removing these foreign elements.

Step 4: Removal of the Editing Vector

To generate transgene-free plants, it is crucial to remove the CRISPR-Cas9 vector from the plant cells after genome editing. Various strategies are employed to ensure that foreign DNA is not retained in the final plant product. Selfing is a method where genome-edited plants are allowed to self-pollinate over several generations. Initially, in the first generation (F1), the edited plants carry both the desired genetic modification and transgenic components, such as the Cas9 gene and selectable markers. As self-pollination continues through subsequent generations (F2, F3, etc.), the transgenic components gradually segregate and are diluted. This process ensures that the unwanted foreign DNA is eliminated, leaving only the desired genetic edits. Over time, the edited plants will express the traits of interest without the presence of foreign DNA. Molecular techniques, such as PCR or qRT-PCR, are typically used to verify the absence of transgenic sequences in the final transgene-free offspring.

Conclusion

The generation of transgene-free genome-edited plants involves several key steps: from the design of the CRISPR-Cas9 vectors and their delivery into plant cells to the removal of the vector and the generation of stable, transgene-free lines through selfing or backcrossing. These methods enable the development of plants with enhanced traits, free from foreign DNA, addressing both regulatory concerns and consumer acceptance. The successful application of these strategies aligns with the demand for sustainable agriculture and crop improvement, and as genome editing technologies continue to evolve, they hold great potential for addressing global challenges in food security and climate resilience.

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