Trends in Plant Science

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Spotlight

PrimeRoot for targeted large DNA insertion in plants

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Genome editing technologies such as clustered regularly interspaced short palindromic repeats (CRISPR) have revolutionized plant breeding through targeted genome and transcriptome modifications. However, accurate insertion of large DNA cargoes remains challenging. Recently, Sun and colleagues introduced PrimeRoot, a groundbreaking technology that enables precise and targeted integration of large DNA cargoes into plant genomes with remarkable efficiency and accuracy.

Plant breeding has been a foundation for agriculture, driving the development of crops with desired agronomic traits. Conventional breeding methods are hampered by limitations in speed, precision, and versatility. Recently, the CRISPR system has become a revolutionary genome editing technology in plant breeding [1], offering unparalleled precision in selectively modifying specific genes. However, achieving true precision and predictability in CRISPR-based modifications is fraught with challenges. The reliance on a Cas nuclease for inducing double-stranded breaks (DSBs), along with error-prone non-homologous end joining (NHEJ), can inadvertently introduce random insertions or deletions (indels) at the target site, posing significant hurdles to precise editing [2]. While the basic CRISPR system can enable large deletions, innovation is required to develop new tools for achieving large insertions at defined sites.

In recent years, precision genome editing technologies such as base editing and prime editing have emerged as new CRISPR-derived methods without requiring DSBs [2]. However, these tools are limited to only nucleotide edits, short deletions, or insertions (up to 50 bp) [2]. Twin prime editing (twinPE), utilizing a prime editor protein and two prime editing guide RNAs (pegRNAs), allows for insertion of larger DNA sequences but is limited to around 100 bp [3]. The challenges of achieving precise insertion of large DNA cargoes underscore the urgent need for innovative approaches to overcome limitations and advance genome editing technologies.

Site-specific recombinases (SSRs)

Natural transposable elements offer a promising avenue for efficient genome integration, bypassing the need for DSBs and providing precise routes for DNA incorporation into host genomes [4]. SSRs such as serine and tyrosine recombinases sourced from transposable elements stand out as a promising class of genome editing tools, as they catalyze the insertion of large DNA cargos into sequence-defined landing sites without exposed DSBs or reliance on DNA repair pathways [5]. However, a major limitation of SSRs is their lack of programmability.

To address this limitation, recent breakthroughs in mammalian systems by Anzalone *et al*. [3] and Yarnall *et al*. [6] have shown the successful combination of serine recombinase with existing prime editing technology, utilizing modified pegRNAs to install specific recombination sites. These advancements have demonstrated impressive editing efficiencies of up to 20%, along with the insertion of DNA fragments as large as 36 kb, opening up new possibilities for precise large DNA insertions in eukaryotic cells [6].

PrimeRoot for precise large DNA insertion

To achieve a similar feat in plants, Sun and colleagues developed PrimeRoot (prime editing-mediated recombination of opportune targets) (Figure 1A) [7], a cutting-edge technology that enables targeted and precise integration of large DNA cargos into plant genomes. The system relies on an enhanced prime editor [8], optimized guide RNA designs, along with a superior tyrosine recombinase (Figure 1A). The dual-enhanced plant prime editor (ePPE) system [8], consisting of two adjacent pegRNAs each containing a reverse transcription template that exhibits homology to the template of the other pegRNA, is employed to efficiently target insertion of recombination sites (Figure 1A) [7]. The recombinase then accurately excises two identical recombination sites on the donor vector, generating an intermediate donor that exclusively contains the desired DNA fragment with one corresponding recombination site for targeted insertion (Figure 1A). PrimeRoot achieves precise integration of up to 11.1 kb of donor DNA with remarkable accuracy [7].

To further optimize the system, the recombinase is directly fused to the C terminus of the ePPE system, resulting in PrimeRoot.v2 (Figure 1A) [7]. PrimeRoot.v2 proved to be superior to PrimeRoot.v1 and achieves up to 4% GFP integration in maize protoplasts [7], highlighting its wide applicability in plants. Then, Sun and colleagues further advanced PrimeRoot by developing PrimeRoot.v3, which utilizes a sequential transformation of PrimeRoot and donor components. The PrimeRoot.v3 employs *Agrobacterium*-mediated T-DNA insertion to transform PrimeRoot reagents into calli. Calli enriched with the desired recombination site insertion are used for donor transformation. Remarkably, the PrimeRoot.v3 system achieves highly efficient and accurate insertion of large DNA cargos, ranging from 1.4 to 4.9 kb, with up to 8.3% efficiency using particle

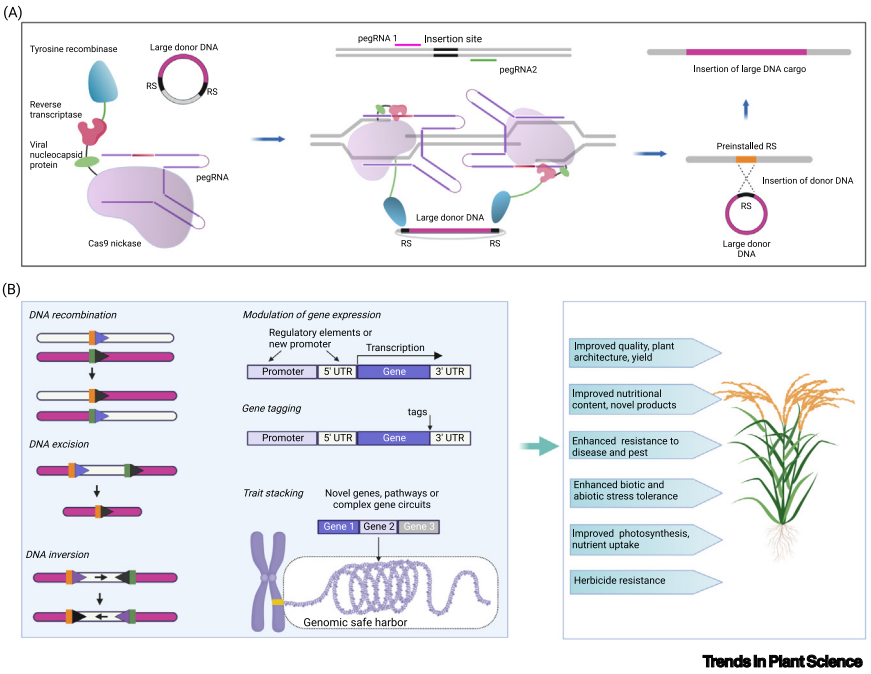

Figure 1. Overview of PrimeRoot and its potential applications in plant breeding. (A) Schematic of programmable large DNA insertion with PrimeRoot. The PrimeRoot system consists of an enhanced plant prime editor, an optimized prime editing guide RNA (pegRNA), and a superior tyrosine recombinase. To achieve targeted DNA insertion at the desired locus, two adjacent pegRNAs are utilized, each containing reverse transcription (RT) templates of recombination site (RS) sequence that share exclusive homology with one another. Concurrently, the recombinase recognizes and excises two identical RS sites on the donor vector, leading to an intermediate donor that only contains the desired DNA fragment with one corresponding RS. Finally, the desired DNA fragment is integrated into the newly established RS, leading to a precise large DNA insert at the target site. (B) The potential applications of PrimeRoot in plant breeding. The potential applications of PrimeRoot in plant breeding are vast, as it enables targeted DNA cargo insertion, as well as accurate large DNA excision, DNA replacement, DNA inversion, and even chromosome rearrangements. PrimeRoot has promising prospects for various plant breeding applications, including modulation of gene expression, trait stacking, tagging endogenous genes, enhancing disease resistance and stress tolerance, and more.

bombardment. However, when using the *Agrobacterium*-mediated approach, the efficiency of precise insertion is slightly lower at 3.9% [7]. These findings highlight the versatility and efficiency of the PrimeRoot.v3 system in achieving precise DNA integration into plant genomes.

Advantages of PrimeRoot

Agrobacterium-mediated delivery is widely adopted in plant transformation. However, its random T-DNA insertions can disrupt coding regions and functional elements [7]. While site-specific T-DNA integration can be mediated by using recombinases such as Cre [9], it is challenging to install the recombination sites like Lox-P in the first place. In addition, the linking of selectable markers and genes of interest in the same T-DNA leads to the retention of antibiotic resistance genes in modified plants. Targeted DNA insertion can be achieved by NHEJ or homology-directed repair of CRISPR-induced DSBs [10]. However, DSB-based methods have limitations, as they can induce undesired effects like insertions, deletions, and translocations. In comparison, the advantages of PrimeRoot include its ability to install recombination sites at predefined genomic loci and its independence of DSBs.

Applications of PrimeRoot for plant breedings

In their study, Sun *et al*. showcased PrimeRoot for precise DNA insertions in rice [7]. By utilizing PrimeRoot, they inserted the rice *Actin1* promoter into the 5′ untranslated region of *OsHPPD*, which presumably could enhance *OsHPPD* expression. Additionally, by identifying suitable genomic safe harbor (GSH) regions for targeted gene insertions, they successfully integrated a 4.9-kb donor cassette containing *pigmR* into a GSH region, resulting in disease-resistant plants [7].

Beyond these proof-of-concept demonstrations, PrimeRoot offers promise for a range of plant breeding applications (Figure 1B), including modulation of gene expression, trait stacking, tagging endogenous genes, as well as introducing novel genes and pathways to improve crop varieties' yield, disease resistance, and stress tolerance. PrimeRoot can also be used to create complex gene circuits for synthetic biology applications, including the introduction of regulatory elements, and producing crops with improved nutritional content.

Future perspectives of PrimeRoot

Site-specific recombination is known to be dependent on the nature of DNA substrates and site polarity, which can yield various outcomes [5]. Given this premise, we envisage that PrimeRoot not only enables targeted DNA cargo insertion but also accurate large DNA excision, DNA replacement, DNA inversion, and even chromosome rearrangements, contingent on the pre-installed recombination site arrangement and orientation (Figure 1B). These capabilities would enhance the versatility of plant genome editing.

As with any technology in the early stage, PrimeRoot has its own limitations. One limitation is that it requires the pre-installation of a recombination site using the prime

editor. The prime editing frequency in plants has exhibited a considerable paucity, with numerous sites remaining unedited or edited with low efficiency, particularly in dicotyledonous plant species [11]. Second, the low integration efficiency of existing SSRs could limit the practical application of the PrimeRoot system. Exploiting more promising SSR candidates from microbial and viral (meta) genomes is crucial to further optimize the PrimeRoot system [12]. Finally, improving the efficiency of delivering donor templates into plant cells is another hurdle for the widespread adoption of PrimeRoot in other plants. Further optimization of delivery methods is necessary.

Nevertheless, despite these challenges, the potential benefits of the PrimeRoot system in plant breeding and synthetic biology are significant and continued efforts to overcome these limitations could help revolutionize the field.

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Declaration of interests

The authors declare no competing interests.

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