Grinding Prime Editors into Powerful Tools for Plant Genome Editing

By YAN Fusheng (Staff Reporter)

Prime editing, a "search-and-replace" CRISPR-based genome editing technique developed by Dr. David R. Liu and his colleagues in 2019, has brought genome editing to a new level.

It can introduce all mutation types to the target DNA, including 12 nucleotide substitutions, as well as short insertions and deletions without causing double-strand breaks or requiring donor DNA, providing a safer way to rewrite genes.

The workhorse of primer editors (Pes) consists of a fusion of Moloney-murine leukemia virus reverse transcriptase (MMLV-RT) with the nCas9 (H840A) nickase and a prime editing guide RNA (pegRNA). The pegRNA contains a spacer sequence that hybridizes to the target DNA site, a primer binding site (PBS) sequence, and an RT template sequence that encodes the desired edit.

As illustrated in Figure 1, a typical prime editor consists of a Cas9 nickase domain fused to a reverse transcriptase domain. The spacer sequence of an engineered prime editing guide RNA (pegRNA) guides PE to its genomic DNA target and also encodes the desired edit within an extension. After nicking the PAM-containing strand, the newly released genomic DNA 3' end hybridizes to the pegRNA extension to form a primer-template complex. The reverse transcriptase domain then directly copies the template from the pegRNA extension into the genomic DNA, adding the edited sequence to the target locus. The product of reverse transcription, an edited 3' flap, can then incorporate into the DNA duplex by competing with the original and redundant 5' flap sequence. After 5' flap excision and ligation of the edited strand, the non-edited complementary strand is replaced by DNA repair using the edited strand as a template.

Since its development, Dr. GAO Caixia of the CAS Institute of Genetics and Developmental Biology (IGDB) and her team have been actively applying this new editing technique for plant genome editing. GAO highly expected to use this new editing tool for next-generation crop breeding. To make this tool practically applicable, they need to ensure that prime editors precisely rewrite

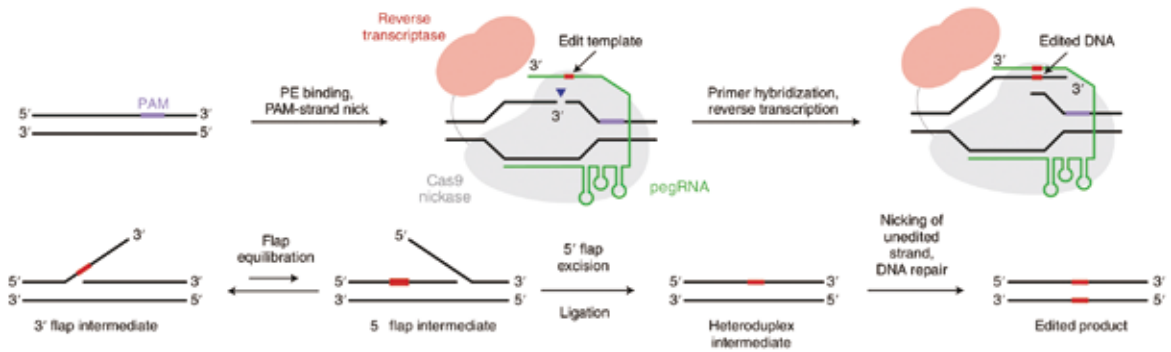

Figure 1. A schematic model shows how prime editing rewrites target DNA. (Credit: David R. Liu's lab/*Nature*)

the target gene, not other undesired genes, with an acceptable editing efficiency.

Barely Lose the Bull's Eye

To thoroughly evaluate the off-target concern, GAO and her research team performed a genome-wide analysis of the off-target events of prime editing in rice plants. They reported this study in *Nature Biotechnology* in 2021.

The off-target effects are, in principle, of two types: guide RNA (gRNA)-dependent and gRNA-independent; the former results from similarities between target and off-target sequences, and the latter mainly from the activity of nCas9 nickase at non-target sites.

Using pegRNAs with primer binding sites (perfectly matched) or spacers containing mismatches to the chosen target sequence, they found that mismatches located in seed sequence regions of the spacer (near the PAM) and near the nicking site greatly reduced the frequency of editing, implying high editing specificity. They assessed the activity of 12 pegRNAs at 179 predicted off-target sites and detected extremely low frequencies of off-target edits (0.00~0.23%). Thus, designing pegRNAs with homology to fewer off-target sites is demanded to achieve highly specific editings.

The gRNA-independent off-target effects generally have no sequence preference. They are analog to the incidences that a string can get cut into pieces when you put it together with a scissor into a box and shake the box long enough.

To assess how often these undesired incidences could occur to the strings of genomic DNA inside a cell, Gao and her co-workers investigated whether overexpression of PEs could cause undesired edits at the genome-wide level. They delivered five PE constructs targeting different genes with or without pegRNA expression cassettes into rice calli via *Agrobacterium*-mediated transformation, a widely used technique for introducing exogenous genes into a plant. As a result, they obtained regenerated T0 plants with desired edits (the PE group).

They found the number of SNVs (single nucleotide variant) and indels (small insertions/deletions) in the PE group was not significantly higher than in the control group (expressing only the Cas9 nickase). Moreover, mutation type and distribution analysis further demonstrated that the PE and control groups did not differ significantly. All these results agree that the PE system did not induce significant numbers of genome-wide pegRNA-independent off-target edits in plants.

To thoroughly release the off-target concern, GAO and her team also interrogated the fused reverse transcriptase that may interfere with the natural reverse transcription events in cells but detected no such events. So, they ruled out the possibility of M-MLV reverse transcriptase causing nonspecific effects in plant cells.

All these results agree upon PE's high specificity in modifying plant genomes. The reported superior genome-wide specificity of PE in plants intrigued more enthusiasm in applying prime editing in gene therapy and agriculture.

Griding the Cutter Shaper

After relieving the off-target concern, its relatively low efficiency is the remaining obstacle.

In a recent study published in *Nature Biotechnology*, GAO and her team reported that the removal of ribonuclease H domain of the MMLV-RT

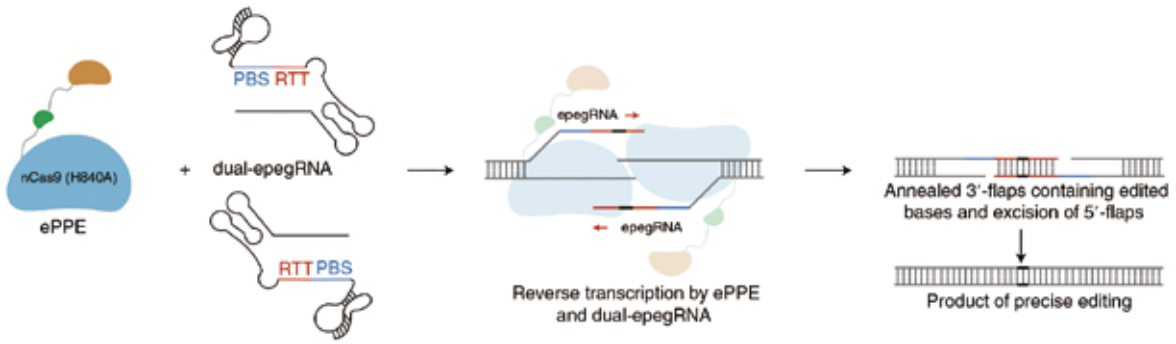

Figure 2. ePPE using the dual-pegRNA strategy results in the same edit on both DNA strands. (Image by GAO's Team/*Nature Biotechnology*)

and insertion of a viral nucleocapsid protein with nucleic acid chaperone activity can each improve the editing efficiency by several folds.

The removal of the RNase H domain stabilizes the heteroduplex between the sgRNA-DNA. The nucleocapsid protein serves as a chaperone during the reverse transcription process via its nucleic acid-annealing activities.

They found that the engineered plant prime editor (ePPE) with both modifications stimulated much higher prime editing efficiency in plants by, on average, 5.8-fold compared with the original PPE. They also precautiously confirmed that this tweak does not cause additional off-target effects.

As a proof of concept, they created herbicide-resistant rice by an amino-acid substitution, observing an editing frequency of 11.3% using ePPE compared with 2.1% using the original PPE.

Additionally, they demonstrated that the appliance of engineered dual-prime editing guide (epeg) RNAs further improved the editing efficiency by more than two folds, as shown in Figure 2.

"We anticipate that the engineered prime editors described in the present study will propel the field of plant genome editing and provide a new and improved tool for use across a wide range of research and agricultural applications," said the authors.

A Role to Play in Crop Improvement

Four techniques have been used during different periods of plant breeding based on biotechnological developments, as shown in Figure 3. Notably, genome editing technologies can efficiently modify plant genomes to improve traits without integrating foreign DNA into the genome and half the time required for crop improvement. These precise breeding techniques, such as prime editing, are coming to define next-generation plant breeding.

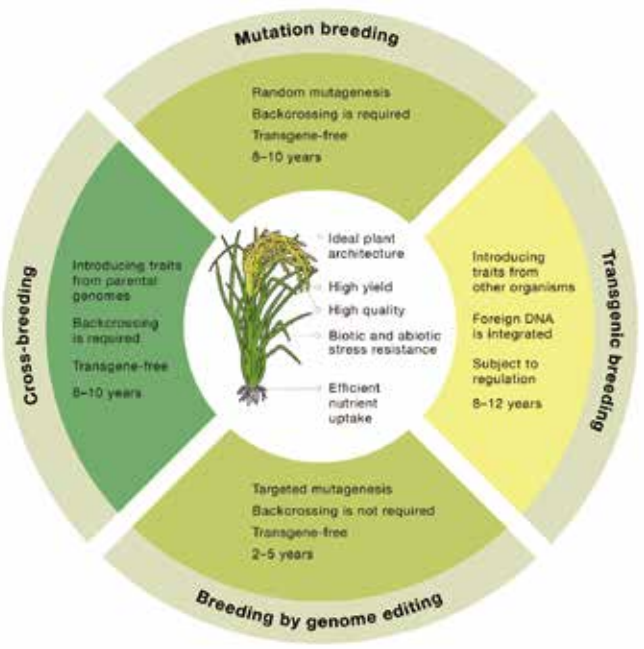

Figure 3. Plant breeding techniques commonly used to introduce new traits into an elite crop variety. (Image by GAO Caixia/*Cell*)

As a game-changer in modifying plant genomes, Gao and her team rapidly applied prime genome editing in plants, including rice, wheat and maize, since its first appearance in 2019.

"Genome editing opens a new toolkit for plant breeding to be performed at an unprecedented pace and in an efficient and cost-effective way, which will propel plant breeding to go beyond its current limit and move to the next generation." writes Gao in a review article recently published in *Cell*, "Efficient, precise, and targeted mutagenesis via genome editing has laid the foundation for many next-generation breeding strategies that will revolutionize the future of agriculture."

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