Genome editing – a technology in time for plants

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A tool for safe and site-specific mutagenesis has long been sought by plant biochemists. The recent emergence of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) genome-editing technology addresses this need. Using this technology, the lettuce genome was recently edited without the use of conventional Agrobacterium-mediated DNA delivery. As this method does not leave a trace of foreign DNA in the plant genome, it promises to advance the field of plant biotechnology for genetically modified organisms (GMOs) without the burden of costly de-regulation processes.

The importance of mutants in gene discovery

The visible phenotypes of loss-of-function or gain-of-function mutants provide valuable clues as to the functions of genes of interest. For instance, an analysis of a set of growth-retarded dwarf mutants of the model plant Arabidopsis thaliana revealed both the metabolic and signal transduction pathways by which the plant steroid hormones, brassinosteroids (BRs), promote growth. However, mutants with defects in some enzymatic steps are elusive. In such cases, sequence-specific mutagenesis would be a useful approach for analysing gene function; however, in contrast to the situation in mice, yeast and Escherichia coli, homologous recombination-based mutagenesis techniques are not available for Arabidopsis. Thus, genetic studies in Arabidopsis involve random mutagenesis, followed by the identification of mutants with defects in a specific gene of interest.

Agrobacterium-mediated gene-tagging mutagenesis

Feldmann and colleagues used Agrobacterium-mediated Transfer (T)-DNA insertional mutagenesis to randomly tag genes in Arabidopsis. The initial collection of Feldmann's T-DNA mutants led to the discovery of a host of genes involved in various physiological processes in Arabidopsis such as AGAMOUS (which functions in floral organ determination), GLABRA1 (hair development), COPI (light signalling), AUX1 (auxin transport) and HYPOCOTYL3 (phytochrome B signalling). To date, hundreds of thousands of T-DNA mutants have been generated in Arabidopsis, and the genomic DNA sequences flanking the T-DNA tags have been sequenced in efforts to map individual insertional events in the genome. Multi-million dollar projects yielded T-DNA insertion mutants for over 80% of the ~28,000 genes present in Arabidopsis.

Despite the community-wide availability of an Arabidopsis T-DNA mutant population and extensive genetic analysis, more than 20,000 genes have no associated visible phenotype. Many Arabidopsis genes exist as multiple and functionally redundant copies, and thus loss-of-function of any one of these genes does not result in a visible phenotype. For instance, 244 cytochrome P450 (CYP) genes and 694 F-box protein genes have been reported in Arabidopsis, the majority of which await functional characterization. One approach to generate visible phenotypes for functionally redundant genes involves creating higher order mutants. However, this is time-consuming and is not always possible, especially when the genes of interest are closely linked on the same chromosome. Targeted mutagenesis for one or multiple genes is an elegant strategy to generate mutants for the thousands of genes with no associated T-DNA insertions, and higher order mutants for functionally redundant genes. Whilst this can sometimes be achieved by RNA interference (RNAi), this technology has limitations; genome editing offers a promising alternative.
Site-specific genome editing in plants

Three main types of site-specific genome-editing techniques are available for plants, including the Zinc Finger Nuclease (ZFN), Transcription Activator-Like Effector Nuclease (TALEN) and CRISPR-Cas9 nuclease systems. ZFN, TALEN and CRISPR-Cas9 each consist of two functional parts, one that directs the enzyme to a specific DNA sequence in the genome, and the other that functions as a DNA endonuclease.

These genome-editing enzymes cleave target DNA to yield a double strand break (DSB). When enzymatic non-homologous end joining (NHEJ) ligates the DSBs, a small deletion or insertion of DNA occurs, which often disrupts genetic information. On the other hand, if homologous DNA spanning the DSB is present at the time of NHEJ, this DNA can be inserted into the genome.

Because the part of ZFN and TALEN that directs the enzyme to a specific DNA sequence is located within the protein, both of these enzymes require prior engineering of protein sequences so that they can be delivered to specific DNA sequences. ZFN can be engineered to contain modular Zn-finger domains that bind to specific DNA sequences of interest. The chimeric DNA-binding component of the enzyme is linked to the FokI DNA endonuclease domain. The synthetic DNA encoding the engineered enzyme is delivered into plant cells through conventional gene delivery methods, such as Agrobacterium- or biolistic bombardment-mediated transformation.

Similarly, TALEN uses a set of modular DNA recognition domains derived from plant pathogenic bacteria of the genus Xanthomonas, and each of the 34 amino acid repeat domains recognizes one base pair of DNA. Sets of domains linked together to identify a desired sequence can be fused in-frame with FokI endonuclease to form genome-specific restriction enzymes. Thus, ZFN and TALEN technologies involve a mandatory protein-engineering step and empirical validation that the recombinant protein successfully cleaves only the target sequence, rendering these approaches time-consuming.

By contrast, CRISPR-Cas9 RNA-guided endonucleases (RGENs) are directed to specific DNA sequences not by
protein but by RNA, specifically, a single-molecule guide RNA (gRNA) that is approximately 100 nucleotides long. Furthermore, the Cas9 protein harbours two endogenous nuclease subdomains, HNH and RuvC, thus abolishing the need for artificial linking to FokI.

Because CRISPR-Cas9 RGEN is targeted to DNA in a mechanism that involves RNA rather than protein, it is easier to design, synthesize and incorporate the targeting molecule into the Cas9 nuclease apoprotein. The emergence of CRISPR-Cas9 RGEN has revolutionized the field of genome editing; indeed, a Google search using "CRISPR-Cas9" as a keyword resulted in 48,000 hits as of April 2016. Given that the technology first appeared in 2012, it is fast becoming a routine technique. While CRISPR-Cas9 was first used to edit the genomes of viruses and prokaryotic cells, it is now being widely used in eukaryotic cells, including humans and plants.

Genes encoding CRISPR-Cas9 components have successfully been expressed both stably and transiently in plants. Multiple targets can be edited simultaneously when several gRNAs are expressed in one cell. Considering that redundant genes are common in plant genomes, CRISPR-Cas9-mediated multiplexed genome editing could yield higher order mutants with relative ease compared with conventional crossing methods.

**Are plants engineered with CRISPR-Cas9 GMOs?**

Conventional genetically modified plants are generated by introducing DNA into a cell or group of cells that can give rise to or be regenerated into an intact plant. However, each of the RGEN components can be separately prepared and assembled in vitro and subsequently introduced into lettuce protoplasts for genome editing, instead of directly importing the DNA plasmids encoding Cas9 structural protein and gRNA. The regenerated plants originating from a single engineered protoplast inherit the mutation in a Mendelian fashion. In addition, in contrast to the plasmid-based system, foreign DNA is not inserted. When Cas9 RGEN is administered as DNA, a fragmented DNA is inserted into the genome. Figure 1 illustrates the procedure of protein-based genome editing in plants.

The emergence of DNA-free genome editing in plants raises the question of whether plants genetically edited with this technology should be classified as GMOs. The United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) designates a crop as GM if it is produced using a plant pathogen and contains foreign genes. Once plants have been genetically modified using *Agrobacterium tumefaciens* and the transgenic plants harbour a foreign gene, such as an antibiotic selection marker, the plants are legally designated as GMOs and are subjected to strict regulatory procedures before commercialization. Despite advantages of GM technologies, such as the rapid introduction of novel traits, small- and mid-sized seed companies tend to produce non-GMO seeds, to avoid the cost involved in obtaining ‘de-regulation’ status of their GMOs. Therefore, there

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**Figure 2** Lettuce plants at the T1 generation. Mutations generated by DNA-free genome engineering with pre-assembled CRISPR-Cas9 in vitro are stably inherited in the T1 generation.
is an interest in developing novel technologies to produce genetically edited crops in such a manner that they are not categorized as GMOs. The newly reported techniques of DNA editing that are based on conventional chemical transfection methods rather than Agrobacterium and do not involve foreign DNA offer an alternative approach to producing genetically edited crops that would not legally be designated as GMOs. Once granted a non-GMO designation, the technology could expedite the development of genetically edited seeds that give rise to plants with desirable traits, such as enhanced nutritional value, disease resistance, tolerance to abiotic stress, energy efficient architecture and increased yield.

**Patent issues**

In addition to these regulations, a patent war is delaying the widespread use of CRISPR-Cas9 technology (see ‘Who owns gene editing? Patents in the time of CRISPR’ p26). At least three offices representing the Broad Institute, University of California at Berkeley and ToolGen, Inc. filed patent applications for CRISPR-Cas9 technology in eukaryotic systems with the United States Patent and Trademark Office (USPTO). As of February 2016, the USPTO has issued patents with claims to CRISPR-Cas9 to the Broad Institute, MIT and affiliated groups to cover the use of the technology in mammalian cells. CRISPR-Cas9 technology is a game-changing method that could greatly help scientists in both academia and industry resolve humanitarian issues, such as food shortage, environmental protection and pharmacological treatment. Therefore, the rapid resolution of license negotiations and establishment of licensing fee structures would be a tremendous boost for start-ups aiming to use this powerful technology to create next-generation bioproducts.

A recent report that a gene-edited CRISPR mushroom was not subject to US regulation signals that future CRISPR seeds may be exempt from regulation. Once patent issues have been resolved, a variety of CRISPR produce with desirable qualities, such as improved nutritional value and pesticide free, are likely to appear in grocery stores.

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