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Research review paper

The CRISPR/Cas9 system for plant genome editing and beyond

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ABSTRACT

Targeted genome editing using artificial nucleases has the potential to accelerate basic research as well as plant breeding by providing the means to modify genomes rapidly in a precise and predictable manner. Here we describe the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system, a recently developed tool for the introduction of site-specific double-stranded DNA breaks. We highlight the strengths and weaknesses of this technology compared with two well-established genome editing platforms: zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). We summarize recent results obtained in plants using CRISPR/Cas9 technology, discuss possible applications in plant breeding and consider potential future developments.

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Introduction

Genome editing with site-specific nucleases allows reverse genetics, genome engineering and targeted transgene integration experiments to be carried out in an efficient and precise manner. It involves the introduction of targeted DNA double-strand breaks (DSBs) using an engineered nuclease, stimulating cellular DNA repair mechanisms. Different genome modifications can be achieved depending on the repair pathway and the availability of a repair template (Fig. 1). Two different DSB repair pathways have been defined: non-homologous end joining (NHEJ) and homologous recombination (HR). In most cases, NHEJ causes random insertions or deletions (indels), which can result in frameshift mutations if they occur in the coding region of a gene, effectively creating a gene knockout. Alternatively, when the DSB generates overhangs, NHEJ can mediate the targeted introduction of a double-stranded DNA template with compatible overhangs (Cristea et al., 2013; Maresca et al., 2013). When a template with regions of homology to the sequence surrounding the DSB is available, the DNA damage can be repaired by HR, and this mechanism can be exploited to achieve precise gene modifications or

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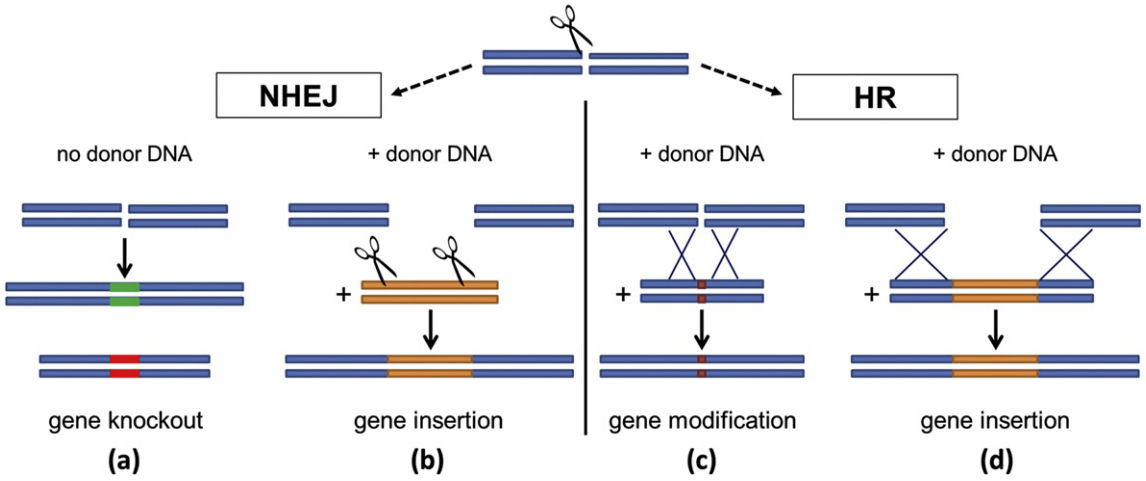

Fig. 1. Genome editing with site-specific nucleases. Double-strand breaks induced by a nuclease at a specific site can be repaired either by non-homologous end joining (NHEJ) or homologous recombination (HR). (a) Repair by NHEJ usually results in the insertion (green) or deletion (red) of random base pairs, causing gene knockout by disruption. (b) If a donor DNA is available, which is simultaneously cut by the same nuclease leaving compatible overhangs, gene insertion by NHEJ can also be achieved. (c) HR with a donor DNA template can be exploited to modify a gene by introducing precise nucleotide substitutions or (d) to achieve gene insertion.

gene insertions. Even though the generation of breaks in both DNA strands induces recombination at specific genomic loci, NHEJ is by far the most common DSB repair mechanism in most organisms, including higher plants, and the frequency of targeted integration by HR remains much lower than random integration (Puchta, 2005). Strategies such as the overexpression of proteins involved in HR or the use of negative selection markers outside the homology regions of the insertion cassette to prevent the survival of random integration events can achieve moderate improvements in gene targeting efficiency (reviewed in Puchta and Fauser, 2013).

The CRISPR/Cas9 system

Until 2013, the dominant genome editing tools were zinc finger nucleases (ZFNs; Kim et al., 1996) and transcription activator-like effector nucleases (TALENs, Christian et al., 2010). Both are artificial fusion proteins comprising an engineered DNA-binding domain fused to the nonspecific nuclease domain of the restriction enzyme FokI, and they have been used successfully in many organisms including plants (reviewed in Jankele and Svoboda, 2014; Palpant and Dudzinski, 2013). The latest ground-breaking technology for genome editing is based on RNA-guided engineered nucleases, which already hold great promise due to their simplicity, efficiency and versatility. The most widely used system is the type II clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 (CRISPR-associated) system from *Streptococcus pyogenes* (Jinek et al., 2012). CRISPR/Cas systems are part of the adaptive immune system of bacteria and archaea, protecting them against invading nucleic acids such as viruses by cleaving the foreign DNA in a sequence-dependent manner. The immunity is acquired by the integration of short fragments of the invading DNA known as spacers between two adjacent repeats at the proximal end of a CRISPR locus. The CRISPR arrays, including the spacers, are transcribed during subsequent encounters with invasive DNA and are processed into small interfering CRISPR RNAs (crRNAs) approximately 40 nt in length, which combine with the transactivating CRISPR RNA (tracrRNA) to activate and guide the Cas9 nuclease (Barrangou et al., 2007). This cleaves homologous double-stranded DNA sequences known as protospacers in the invading DNA (Barrangou et al., 2007). A prerequisite for cleavage is the presence of a conserved protospacer-adjacent motif (PAM) downstream of the target DNA, which usually has the sequence 5′-NGG-3′ (Gasiunas et al., 2012; Jinek et al., 2012) but less frequently NAG (Hsu et al., 2013). Specificity is provided by the so-called 'seed sequence' approximately 12 bases upstream of the PAM, which must match between the RNA and target DNA (Fig. 2).

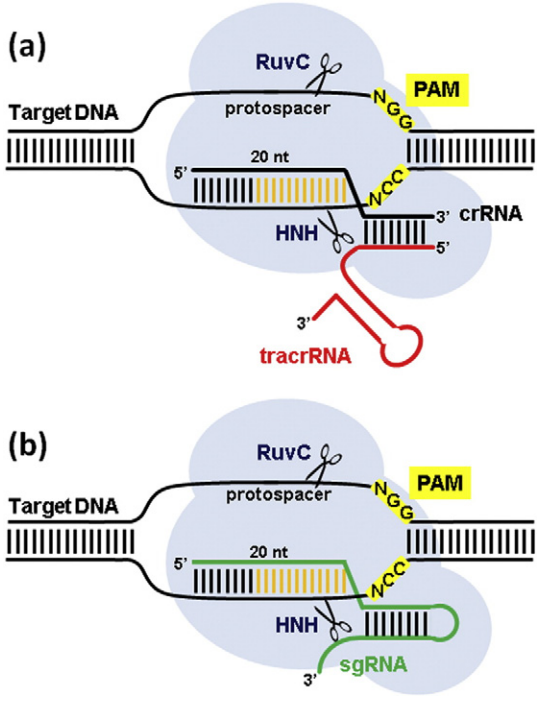

Fig. 2. RNA-guided DNA cleavage by Cas9. (a) In the native system, the Cas9 protein (light blue) is guided by a structure formed by a CRISPR RNA (crRNA, in black), which contains a 20-nt segment determining target specificity, and a trans-activating CRISPR RNA (tracrRNA, in red), which stabilizes the structure and activates Cas9 to cleave the target DNA (protospacer). The presence of a protospacer-adjacent motif (PAM, in yellow), i.e., an NGG (or less frequently NAG) sequence directly downstream from the target DNA, is a prerequisite for DNA cleavage by Cas9. Among the 20 RNA nucleotides determining target specificity, the so-called seed sequence of approximately 12 nt (in orange) upstream of the PAM is thought to be particularly important for the pairing between RNA and target DNA. (b) Cas9 can be reprogrammed to cleave DNA by a single guide RNA molecule (gRNA, in green), a chimera generated by fusing the 3′ end of the crRNA to the 5′ end of the tracrRNA.