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A detailed procedure for CRISPR/Cas9-mediated gene editing in *Arabidopsis thaliana*

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Abstract The newly developed CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) system has emerged as an efficient tool for genome-editing, providing an alternative to classical mutagenesis and transgenic methods to study gene function and improve crop traits. CRISPR/Cas facilitates targeted gene editing through RNA-guided DNA cleavage followed by cellular DNA repair mechanisms that introduce sequence changes at the site of cleavage. Here we describe a detailed procedure for our previously developed and highly efficient CRISPR/Cas9 method that allows the generation of heritable-targeted gene mutations and corrections in *Arabidopsis*. This protocol describes the strategies and steps for the selection of targets, design of single-guide RNA (sgRNA), vector construction and analysis of transgenic lines. We also offer a method to target two loci simultaneously using vectors containing two different sgRNAs. The principles described in this protocol can be applied to other plant species to generate stably inherited DNA modifications.

Keywords CRISPR/Cas9 · Targeted gene editing · Genome engineering · *Arabidopsis thaliana*

1 Introduction

Targeted gene editing encompasses a number of technologies to precisely manipulate the genomes in living cells. These technologies are appealing to plant biologists as they can be used for many purposes, from the study of gene functions to the improvement in crop traits. The primary tools to perform precise gene editing are site-specific endonucleases. To date, four endonuclease-based genome-engineering tools have been developed: meganucleases [1], zinc finger nucleases (ZFNs) [2–5], transcription activator-like effector nucleases (TALENs) [6–11] and CRISPR/Cas9 [12–14]. These site-specific endonucleases can generate double-stranded breaks (DSBs) at selected gene loci. The repair of DSBs can be harnessed to introduce a piece of exogenous DNA via homologous recombination (HR) or can cause the introduction of insertion or deletion mutations (indels) via the error-prone, nonhomologous end-joining (NHEJ) repair pathway [15–17]. These two cellular DSB repair pathways are highly conserved in eukaryotes, and thus, it can be exploited to perform precise gene editing in a wide range of species.

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The recently developed RNA-guided CRISPR/Cas9 system is derived from the adaptive immune system used by *Streptococcus pyrogenes* to fight invasive viruses and plasmids [18]. An optimized type II CRISPR/Cas9 system is composed of a Cas9 endonuclease for double-stranded DNA (dsDNA) cleavage and a chimeric single-guide RNA (sgRNA) for target recognition [13, 14]. The assembled Cas9/sgRNA complex binds to a 20-bp DNA target (protospacer) via Watson–Crick base pairing, and the only premise of its cleavage activity is the presence of a protospacer-adjacent motif (PAM) at the 3′ end of targeted sequences [19]. The PAM sequence for Cas9 is 5′-NGG-3′, in which "N" means any nucleotide [12]. Theoretically, Cas9 can be directed to any DNA sequence located 20-bp upstream of an NGG motif by simply introducing the corresponding 5′ sequence in the sgRNA. Thus, the simplicity, flexibility and high efficiency of this versatile CRISPR/Cas9 system have boosted its application for targeted gene mutagenesis, correction or rearrangement in many species [20, 21]. Since 2013, application of the type II CRISPR/Cas9 system has been extensively reported in model species and commercial crops using transient expression and stable transformation with satisfactory gene-targeting efficiency [22–35].

We previously reported the development of two CRISPR/Cas9 systems designed to perform multiplex gene editing in both *Arabidopsis* and rice [25]. These systems allow construction of two individual sgRNAs in a single vector and thus can be used to perform multiplex gene mutagenesis, gene correction, long fragment gene deletion and gene inversion. Transgenic plants generated by our multiplex CRISPR/Cas9 system were analyzed by whole-genome sequencing, confirming the specificity of the system by the lack of detectable off-target mutations. Meanwhile, although all of the examined *T*1 seedlings were chimera, homozygotes for targeted gene modifications were segregated at an average frequency of 22 % [36]. It is fair to expect that this CRISPR/Cas9-based gene-editing technology will be widely adopted for routine use in the near future.

To facilitate the application of this system, we provide an easy-to-follow procedure as well as practical advices for the diverse application of our CRISPR/Cas9 system for targeted gene modifications in *Arabidopsis*. We also offer a range of solutions for the different steps in the process, from the design of sgRNAs and vector construction to the detection of specific gene modifications and the selection of gene-edited plants. In general, the principles described here to customize the versatile CRISPR/Cas9 system can be applied to other plant species for gene editing.

2 Materials and methods

2.1 Selection of sgRNA targets

There are a number of web-based sites for the selection of sgRNA targets. We provide here the one used by our own group [37] and three additional online CRISPR-design tools recommended for generating a list of candidate sgRNAs by inputting the sequence of interest.

(1.) http://www.plantsignal.cn/CRISPR/crispr_primer_designer.html
(2.) http://www.genome-engineering.org/crispr/?page_id=41
(3.) https://www.dna20.com/eCommerce/cas9/input
(4.) http://www.genome.arizona.edu/crispr/index.html

Alternatively, sgRNAs can be designed manually. In principle, the specificity of the Cas9 nuclease is determined by the 20-nt guide sequence within the sgRNA. A potential target sequence must immediately precede a PAM (5′-NGG-3′) to be recognized by the first 20-nt of sgRNA via Watson–Crick base pairing. The recognition between sgRNA and its target will lead to Cas9 cleavage ~3 bp upstream from the PAM. Note that the PAM sequence (5′-NGG-3′) is not a part of the sgRNA. For different research purposes, the requirements regarding the specificity and relative location of a designed target might differ.

2.2 Design of the guide oligonucleotides

Once a 20-nt target site is selected, a pair of DNA oligos can be synthesized as follows,

Forward oligo: 5′-gattGNNNNNNNNNNNNNNNNNNN-3′
Reverse oligo: 5′-aaacNNNNNNNNNNNNNNNNNNNC-3′

Here the successive "N"s in the forward oligo correspond to the 5′ 20-nt preceding the PAM (5′-NGG-3′) and those in the reverse oligo are just the reverse complementary sequence. Note that the PAM sequence is not included in the oligos. For seamless ligation of the synthesized DNA oligos to the sgRNA backbone vector, psgR-Cas9-At (Supporting Information), two adapters were added to the 5′ of both oligos to generate a 20-bp oligo duplex with 4-nt overhangs at both ends after annealing. Since the resulting overhangs are compatible with the two BbsI sites in psgR-Cas9-At, the annealed oligos can be ligated into the sgRNA-Cas9 cassette seamlessly.

Notes: a. For sgRNA driven by different promoters, the adapter sequences might be different. b. The guide oligos cannot contain any *Eco*RI and *Hin*dIII sites.