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CRISPR/Cas9-mediated efficient targeted mutagenesis of *RAS* in *Salvia miltiorrhiza*

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ABSTRACT

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 (CRISPR-associated) system is a powerful genome editing tool that has been used in many species. In this study, we focused on the phenolic acid metabolic pathway in the traditional Chinese medicinal herb *Salvia miltiorrhiza*, using the CRISPR/Cas9 system to edit the rosmarinic acid synthase gene (Sm*RAS*) in the water-soluble phenolic acid biosynthetic pathway. The single guide RNA (sgRNA) was designed to precisely edit the most important Sm*RAS* gene, which was selected from 11 family members through a bioinformatics analysis. The sequencing results showed that the genomes of 50% of the transgenic regenerated hairy roots had been successfully edited. Five biallelic mutants, two heterozygous mutants and one homozygous mutant were obtained from 16 independent transgenic hairy root lines when the sgRNA was driven by the *Arabidopsis* U6 promoter, while no mutants were obtained from 13 independent transgenic hairy root lines when the sgRNA was driven by the rice U3 promoter. Subsequently, expression and metabolomics analysis showed that the contents of phenolic acids, including rosmarinic acid (RA) and lithospermic acid B, and the *RAS* expression level were decreased in the successfully edited hairy root lines, particularly in the homozygous mutants. In addition, the level of the RA precursor 3,4-dihydroxyphenyllactic acid clearly increased. These results indicated that the CRISPR/Cas9 system can be utilized to identify important genes in a gene family with the assistance of bioinformatics analysis and that this new technology is an efficient and specific tool for genome editing in *S. miltiorrhiza*. This new system presents a promising potential method to regulate plant metabolic networks and improve the quality of traditional Chinese medicinal herbs.

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1. Introduction

Genomic mutation is one of the most important methods used in gene functional studies in plants. With frameshift mutations generated by genome engineering technologies, the expression of the target sequence will be modified, and a new phenotype might be observed. In recent years, the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 (CRISPR-associated) system has been developed rapidly as a new form of genome engineering (Jinek et al., 2012). Compared with transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs), the CRISPR/Cas9 system has many advantages, such as ease of use, high efficiency and adaptation to diverse organisms (Bortesi and Fischer, 2015; Chen and Gao, 2014). All these genome engineering techniques generate double strand breaks (DSBs) in the target genomic DNA, which cause DNA sequence modification in two ways (Cong et al., 2013). One is non-homologous end joining (NHEJ), which results in nucleotide insertions, deletions and substitutions. The other is homologous recombination (HR), which can be performed if homologous donor templates are present when DSBs occur.

The CRISPR/Cas9 system contains two functional parts, CRISPR-associated protein 9 (Cas9) and a short single guide RNA (sgRNA), that can execute specific genome editing and other functions. In combination with the interaction between CRISPR RNAs (crRNA) and trans-activating RNAs (tracrRNA), the endogenous CRISPR/Cas9 system can induce DSBs in a target sequence (Deltcheva et al., 2011; Gasiunas et al., 2012). Scientists have designed a single chimeric

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RNA composed of these two RNA moieties to recruit Cas9 in a site-specific manner, making the applications of CRISPR/Cas9 more versatile (Jinek et al., 2012). The sgRNA guides the Cas9 endonuclease to recognize a specific sequence, and the protospacer-associated motif (PAM), an NGG sequence downstream of the target side, assists the CRISPR/Cas9 system to select the target site more precisely (Gasiunas et al., 2012).

Plant-based Chinese medicines have been used for thousands of years, and bioactive natural compounds from medicinal plants play an important role in protecting health and well-being. With the accumulation of studies on medicinal plants, scientists are concentrating more on mining critical genes in plant metabolic pathways and finding synthetic ways to increase their production of active compounds (Kiss and Piwowarski, 2016). Although the CRISPR/Cas9 system has been used in many model plants and crops, such as *Arabidopsis thaliana* (Li et al., 2013), *Oryza sativa* (Feng et al., 2013), *Nicotiana benthamiana* (Nekrasov et al., 2013), and *Triticum aestivum* (Shan et al., 2013a), few reports exist of this technology in medicinal plants, because of their complex genetic background. It is essential and meaningful to confirm whether this new system can be applied to gene functional studies of medicinal plants.

As a highly prized Chinese herbal medicine, *Salvia miltiorrhiza* contains two types of medicinal components: lipid-soluble compounds known as tanshinones and water-soluble phenolic acids, such as rosmarinic acid (RA) and lithospermic acid (Ma et al., 2006). In a previous report, the diterpene synthase gene (*SmCPS1*) from the tanshinone biosynthetic pathway was successfully targeted for knockout with the CRISPR/Cas9 system (Li et al., 2017). Through sequencing and metabolomic analysis, the results showed that a mutation rate of approximately 42.3% occurred in 26 transgenic lines. Tanshinone content was absent in homozygous mutants and reduced in heterozygous as well as biallelic mutants.

Owing to their remarkable biological activities and the convenient decoction method for use of the herb, water-soluble phenolic acids, including lithospermic acid B (LAB) and its precursor RA, have drawn considerable interest to their biosynthetic pathway and key enzymes. Rosmarinic acid synthase (*RAS*), which links 3′,4′-dihydroxyphenyllactic acid (DHPL) and 4-coumaroyl-CoA, has been suggested as an important enzyme catalysing RA biosynthesis and leading to the accumulation of LAB (Sander and Petersen, 2011; Di et al., 2013). In the future, *RAS* will be a candidate for metabolic projects to improve the quality of *S. miltiorrhiza*. To evaluate the effects of a CRISPR/Cas9 system applied to the biosynthetic pathway of water-soluble phenolic acids and deeply explore the role of Sm*RAS*, we selected one *RAS* gene to knock out with this new technology and assessed its effect on the metabolic pathway in hairy root culture.

Here, we report phenolic acid metabolic pathway gene editing in *S. miltiorrhiza* via the CRISPR/Cas9 system by the introduction of a binary vector with a specific sgRNA. The target gene was selected from a family of eleven *RAS* genes based on bioinformatics analysis, and a CRISPR/Cas9 system was constructed to specifically edit the target. The results showed that target loci can successfully be edited with this system. RA and LAB content decreased, whereas DHPL accumulation increased, in transgenic hairy roots. Mutagenesis in transgenic regenerated hairy roots was detected by PCR amplification and Sanger sequencing. The contents of RA, LAB and DHPL in hairy roots were also measured by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Gene expression levels in the mutant samples, as measured by semi-quantitative PCR and quantitative real-time PCR (qRT-PCR), were reduced. In conclusion, these results demonstrated that a specific mutation was successfully induced by the CRISPR/Cas9 system, and variations in phenolic acid levels were observed, indicating the feasibility of this new technology as a tool for genome modification in medicinal plants and *RAS* as a crucial enzyme regulating the biosynthesis of phenolic acids.

2. Results

2.1. Identification and characterization of RAS genes in S. miltiorrhiza

The eleven RAS genes were obtained from the genome database of *S. miltiorrhiza* via homologous blast. The genes were then verified through a BLAST on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and were confirmed via comparison with the Conserved Domain Database of NCBI (Supplementary Table 1, Xu et al., 2016). To obtain clues about the evolutionary relationships and the topological structures of these *RAS* genes, multiple alignments of the amino acid sequences encoded these genes were used to build a neighbour-joining (NJ) tree with 1000 bootstrap replicates and a pairwise deletion gaps/missing data treatment. An *RAS* gene in *Melissa officinalis*, *MoRAS*, catalysing RA biosynthesis was selected as control (Weitzel and Petersen, 2011). The result indicated the SMil_00025190 has high homology with the *MoRAS*. We analysed the different expression levels of these genes in *S. miltiorrhiza* organs, including the root, stem, leaf and flower, through a heat map using a new transcriptomic database which integrally analysed and formed by three published transcription profiles (SRP049543 SRP051564 and SRP028388) (Fig. 1C). Among the eleven SMil_*RAS* genes, the expression of SMil_00025190 was high in multiple organs, suggesting that this *RAS* gene may play an important role in RA biosynthesis. These results provided useful information for further target selection with the CRISPR/Cas9 system in *S. miltiorrhiza*.

2.2. sgRNA design and CRISPR/Cas9 expression vector construction

SMil_00025190 was selected as the target locus for editing, and the sgRNA was designed by an online web tool (http://crispr.mit.edu/). Because no *S. miltiorrhiza* genome sequence data was available in this web tool, the genome data of another dicotyledonous plant, *A. thaliana*, was used. All potential 20-bp sequences followed by 5′-NGG (PAM) from open reading frame (ORF) of *RAS* were scored and analysed based on several factors, including mismatches and number of off-target sites. Sequences with high scores were screened and cloned into a CRISPR/Cas9 vector (Supplementary Table 2).

The CRISPR/Cas9 system was kindly provided by Jian-Kang Zhu's laboratory at the Shanghai Center for Plant Stress Biology, Chinese Center for Plant Stress Biology. In this vector, sgRNA and hSpCas9 were combined to support co-delivery. The optimized coding sequence of hSpCas9 was driven by a cauliflower mosaic virus (CaMV) 35S promoter, and that of the sgRNA was driven by native plant promoters: either the U6-26 promoter of *A. thaliana* or the U3 promoter of *O. sativa* (Zhang et al., 2016).

To express this vector in medicinal plants, the main expression cassette was subcloned into a plant expression vector, pCAMBIA1300, for transformation (Fig. 2). After two-step construction, a 20-bp fragment from the *RAS* gene was inserted into the CRISPR/Cas9 vector driven by different promoters. The empty binary vector without an sgRNA sequence was called *EV-RAS*, whereas the vectors with sgRNA sequences driven by the AtU6 and OsU3 promoters were named *At-RAS* and *Os-RAS*, respectively.

2.3. Screening and identification of transgenic regenerated hairy roots

Two weeks after *Agrobacterium rhizogenes*-mediated

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