Using *Staphylococcus aureus* Cas9 to Expand the Scope of Potential Gene Targets for Genome Editing in Soybean

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Abstract: The CRISPR (clustered regularly interspaced short palindromic repeat)/Cas (CRISPR-associated) is a revolutionary genome editing technology that has been used to achieve site-specific gene knock-out, large fragment deletion, or base editing in many plant species including soybean (*Glycine max*). The *Streptococcus pyogenes* Cas9 (SpCas9) is widely used in plants at present, although there are some reports describing the application of CRISPR/Cpf1 in soybean. Therefore, the selection range of PAM (protospacer adjacent motif) sequences for soybean is currently limited to 5′-NGG-3′ (SpCas9) or 5′-TTTN-3′ (Cpf1), which in turn limits the number of genes that can be mutated. Another Cas9 enzyme from *Staphylococcus aureus* (SaCas9) recognizes the PAM sequence 5′-NNGRRT-3′ (where R represents A or G), which can provide a wider range of potential target sequences. In this study, we developed a CRISPR/SaCas9 system and used this tool to specifically induce targeted mutations at five target sites in the *GmFT2a* (*Glyma.16G150700*) and *GmFT5a* (*Glyma.16G044100*) genes in soybean hairy roots. We demonstrated that this tool can recognize the PAM sequences 5′-AAGGGT-3′, 5′-GGGGAT-3′, 5′-TTGAAT-3′, and 5′-TAGGGT-3′ in soybean, and it achieved mutation rates ranging from 34.5% to 73.3%. Our results show that we have established a highly efficient CRISPR/SaCas9 tool that is as suitable as SpCas9 for genome editing in soybean, and it will be useful for expanding the range of target sequences for genome editing.

Keywords: soybean; gene editing; CRISPR/Cas9; *Staphylococcus aureus* Cas9; PAM

1. Introduction

The broad application of CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9) system for genome editing in diverse organisms is a far-reaching and fundamental technological revolution in the life sciences. The diverse characteristics and continued development of CRISPR/Cas9 provide new methods to achieve site-specific gene knock-out, large fragment deletion, base editing, site-specific knock-in, target fragment replacement, and transcriptional activation and inhibition in plants [1–4].

Many previous studies have attempted to clarify how CRISPR/Cas9 system mediates RNA-guided DNA recognition and cleavage. The Cas9 protein contains two endonuclease domains, HNH and RuvC-like, which cleave the DNA strands that are complementary and non-complementary to the guide RNA, respectively [5]. Cas9-catalyzed DNA cleavage requires the recognition of a protospacer adjacent motif (PAM) located immediately downstream of the target sequence [5–7]. The widely used SpCas9 protein from *Streptococcus pyogenes* primarily recognizes a 5′-NGG-3′ PAM sequence [5,8]. The PAM recognition characteristics of the SpCas9 protein limit the range of possible target sequences [9]. Therefore, one feasible solution to overcome the target range limitations is to engineer Cas9 variants that recognize novel PAM sequences [9]. For example, SpCas9-VQR recognizes...
NGA or NGNG PAMs; SpCas9-EQR recognizes NGAG PAMs; SpCas9-VRER recognizes NGCG PAMs; SpCas9-NG recognizes NG PAMs; and xCas9 recognizes NG, GAA, and GAT PAMs [9–12]. In addition, three modified SpCas9 variants (SpCas9-NRRH, SpCas9-NRTH, and SpCas9-NRCH) have robust editing activity on non-G PAM sequences (NRNH, where R represents A or G, H represents A, C, or T) in human cells and plants [13,14]. Another SpCas9 variant named SpRY was engineered for nearly PAM less genome editing and base editing [14–16]. The PAM sequences recognized by the orthologous CRISPR-Cas systems are diverse. Previous studies have also reported that several CRISPR-Cas systems targeting different PAMs have been isolated from diverse microbes and engineered as effective genome editing tools in various organisms [17]. For example, the SaCas9 protein from Staphylococcus aureus (1053 amino acids) is smaller than SpCas9 (1368 amino acids) and is thus is easier to deliver into cells for gene editing [18]. In addition, SaCas9 recognizes a 5′-NNGRRT-3′ (where R represents A or G) PAM sequence, which is distinct from that of SpCas9 and provides additional potential target sequences [19,20]. The Scas9 protein was isolated from Streptococcus canis based on its sequence similarity (89.2%) to SpCas9, and this ortholog recognizes a more relaxed PAM sequence (5′-NNG-3′) than that recognized by SpCas9 [21]. CRISPR-Cpf1 (CRISPR from Prevotella and Francisella 1) is a novel class 2 CRISPR system that recognizes a T-rich (5′-TTTN-3′) PAM sequence and cleaves DNA strands with robust genome editing activity [22–24].

Soybean (Glycine max (L.) Merr.) is an important legume crop used for food production and animal feed worldwide due to its abundant protein and oil contents. However, at present, the progress of agronomic improvement using only traditional breeding is not enough to meet the demands of a rapidly increasing human population. Thus, improving soybean breeding through innovative breeding technology is urgently needed. Although CRISPR/Cas9 systems have been widely used for soybean genome editing, SpCas9 is the main nuclease used at present [25,26]. However, there are also a few reports describing the successful application of the CRISPR/Cpf1 system in soybean [27,28]. Therefore, the range of potential PAM sequences for soybean genome editing is currently limited to 5′-NGG-3′ or 5′-TTTN-3′, which is not enough to meet the future development demand.

In this study, we developed a CRISPR/SaCas9 system and demonstrated that this tool can recognize the PAM sequences 5′-AAGGGT-3′, 5′-GGGGAT-3′, 5′-TTGAAT-3′, and 5′-TAGGGT-3′ in soybean. Our results suggest that SaCas9 can direct highly specific genome editing activity, and it will be useful for expanding the range of targets for soybean genome editing.

2. Results

2.1. Testing the Efficiency of the CRISPR/SaCas9 System for Genome Editing in Soybean

In order to determine the capacity and efficiency of the CRISPR/SaCas9 system for genome editing in soybean, five sgRNAs were designed to edit different regions of two soybean genes, GmFT2α (Glyma.16G150700) and GmFT5α (Glyma.16G044100), in soybean hairy roots. Because the PAM sequence recognized by SaCas9 is 5′-NNGRRT-3′, we designed three sgRNAs (named GmFT2α-SaCas9-SP1, GmFT2α-SaCas9-SP2, and GmFT2α-SaCas9-SP3) that target sites located in the first exon of GmFT2α. The PAM sequences of these three sgRNAs are 5′-AAGGGT-3′, 5′-GGGGAT-3′, and 5′-TTGAAT-3′, respectively (Figure 1A). We also selected two sgRNAs (GmFT5α-SaCas9-SP1 and GmFT5α-SaCas9-SP2) that target sites located in the first exon of GmFT5α. The PAM sequences of these two sgRNAs are 5′-TAGGGT-3′ and 5′-GGGGAT-3′, respectively (Figure 2A). The five corresponding vectors targeting the aforementioned five sites were constructed and then transformed into the soybean hairy roots to induce site-specific mutations. As shown in Table 1, mutations in the GmFT2a-SaCas9-SP1 site were identified in 73.3% (22/30) of independently transformed transgenic hairy roots, mutations in the GmFT2a-SaCas9-SP2 site were identified in 34.8% (8/23) of independent transgenic hairy roots, and mutations at GmFT2a-SaCas9-SP3 were identified in 34.5% (10/29) of independent transgenic hairy roots. At the same time, mutations in the GmFT5a-SaCas9-SP1 site were identified in 42.9%
(12/28) of independent transgenic hairy roots, and mutations at \textit{GmFT5a-SaCas9-SP2} were identified in 57.1% (16/28) of independent transgenic hairy roots.

Table 1. Summary of CRISPR/SaCas9 genome editing assays in the present study.

| Target Gene | Target Site        | No. of Hairy Roots Examined | No. of Hairy Roots with Mutations | Mutation Rates |
|-------------|--------------------|-----------------------------|----------------------------------|----------------|
| \textit{GmFT2a} | \textit{GmFT2a-SaCas9-SP1} | 30                          | 22                               | 73.3%          |
| \textit{GmFT2a} | \textit{GmFT2a-SaCas9-SP2} | 23                          | 8                                | 34.8%          |
| \textit{GmFT2a} | \textit{GmFT2a-SaCas9-SP3} | 29                          | 10                               | 34.5%          |
| \textit{GmFT5a} | \textit{GmFT5a-SaCas9-SP1} | 28                          | 12                               | 42.9%          |
| \textit{GmFT5a} | \textit{GmFT5a-SaCas9-SP2} | 28                          | 16                               | 57.1%          |
2.2. Targeted Mutagenesis in GmFT2a Induced by the CRISPR/SaCas9 System

In this study, we used subcloning and DNA sequencing to detect typical types of mutations induced by CRISPR/SaCas9 in soybean hairy roots. There were five types of mutations identified at target site GmFT2a-SaCas9-SP1 that included a 4-bp deletion, a 5-bp deletion, a 1-bp deletion, a 1-bp A insertion, and a 1-bp G insertion (Figure 1B). Three types of mutations were identified at target site GmFT2a-SaCas9-SP2; a 2-bp deletion, a 1-bp deletion, and a 1-bp A insertion (Figure 1C). We also identified five types of mutations at target site GmFT2a-SaCas9-SP3, including a 12-bp deletion, a 9-bp deletion, an 8-bp deletion, and a 1-bp deletion (Figure 1D). These results clearly demonstrated that the CRISPR/SaCas9 system can recognize the 5′-AAGGGT-3′, 5′-GGGGAT-3′, and 5′-TTGAAT-3′ PAM sequences to induce gene site-specific knockouts in soybean.

2.3. Targeted Mutagenesis in GmFT5a Induced by the CRISPR/SaCas9 System

Subsequently, four types of mutations were identified at the target site GmFT5a-SaCas9-SP1. These were a 14-bp deletion, a 15-bp deletion, a 1-bp deletion, and a 2-bp T insertion (Figure 2B). Moreover, four types of mutations were identified at the target site GmFT5a-SaCas9-SP2, including a 2-bp deletion, a 1-bp T insertion, a 1-bp A insertion, and a 1-bp transversion (A changed to T) (Figure 2C). These results demonstrated that the CRISPR/SaCas9 system can recognize the 5′-TAGGGT-3′ and 5′-GGGGAT-3′ PAM sequences to induce gene site-specific knockouts in soybean.

Figure 2. Site-specific mutations induced by the CRISPR/SaCas9 in target gene GmFT5a. (A) Gene structures of GmFT5a with two target sites of CRISPR/SaCas9. Green stripe, exon; black solid line, intron; gray stripe, UTR (untranslated region). The red letters are PAM (protospacer adjacent motif) sequences. (B,C) Sequences of WT (wild type) and representative mutation types induced at target sites GmFT5a-SaCas9-SP1 and GmFT5a-SaCas9-SP2 are presented, respectively. Underline, insertions. Dashes, deletions. Base substitution is indicated with the capital letter on a green background. The red arrowheads indicate the location of mutations.
3. Discussion
3.1. SaCas9 Has Comparable Genome Editing Efficiency to SpCas9 in Soybean

The successful application of a new genome editing tool requires that it be efficient at inducing targeted mutations. In 2015, CRISPR/SpCas9 was first used to knock out target genes in soybean hairy roots. Targeted DNA mutations were detected in 95% of 88 hairy-root transgenic events analyzed [29]. To select a more suitable promoter for the CRISPR/SpCas9 system in soybean, the soybean U6-10 and Arabidopsis U6-26 promoters were compared. Mutation efficiencies ranged from 3.2 to 9.7% using the Arabidopsis U6-26 promoter and 14.7–20.2% with the soybean U6-10 promoter [30]. Similarly, mutation efficiencies of 43.4–48.1% were achieved with CRISPR/SpCas9 using the soybean U6-16g-1 promoter in two editing targets, GmPDS11 and GmPDS18 [31]. In a previous study, we also demonstrated that CRISPR/SpCas9 can be successively applied to generate mutations in the desired target genes (bar transgene, GmFEI1/2, and GmSHR) in soybean hairy roots with mutagenesis efficiencies of 10.0–93.3% [32]. In this study, we developed a CRISPR/SaCas9 system and employed this tool to specifically induce targeted mutations in five target sites of two soybean genes, GmFT2a and GmFT5a, and achieved mutation frequencies ranging from 34.5% to 73.3%. These results indicated that the efficiency of the SaCas9 enzyme is comparable to that of SpCas9 in soybean; that is to say, SaCas9 is equally suitable for genome editing in soybean.

3.2. CRISPR/SaCas9 Can Be Used to Expand the Targeting Scope for Soybean Genome Editing

In recent years, the use of various CRISPR technologies in soybean has been widely reported. CRISPR/SpCas9 has been successfully used to achieve the site-specific knockout of target genes in both soybean hairy roots and regenerated plants for improving important agronomic characteristics [29,32,33]. For example, the CRISPR/SpCas9 tool was used to broaden the latitudinal adaptability of soybean by regulating flowering time. Plants carrying the ft2a, ft2b, and ft5a mutations generated by CRISPR/SpCas9 exhibit late flowering phenotypes [33–35]. Early flowering soybean mutants have also been generated with CRISPR/Cas9 technology by knocking out E1 [36]. The soybean quadruple lcls mutants generated by CRISPR/SpCas9 exhibit an extreme short-period circadian rhythm and a late flowering phenotype [37]. The gnprrr37 mutants generated by CRISPR/SpCas9 show an early flowering phenotype under long-day conditions [38]. CRISPR/SpCas9 has also been used to optimize soybean plant architecture (plant height, internode length, and the number of nodes and branches) by knocking out four SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9 (SPL9) homologues and four soybean LATE ELONGATED HYOCOTYL (LHY) genes [39,40]. CRISPR/SpCas9 could also be used to improve the quality of soybean seeds, such as by regulating the oleic acid content and the taste of soybean oil and protein products [41,42]. However, SpCas9 can only recognize the 5′-NGG-3′ PAM sequences, which means that in some cases, it cannot meet the needs of target gene selection.

In addition to single-gene knock-out, the CRISPR/SpCas9-mediated deletion of large genomic fragments and target base editing have also been successfully applied to soybean to improve agronomic traits [43,44]. The deletion of large DNA fragments can be used to study the function of specific sequence elements in genes. In addition, base editing is routinely used to regulate gene function by targeting specific single nucleotides. Therefore, compared to gene knock-out, an expanded range of PAM sites is required for the deletion of specific DNA sequence elements or for base editing. In this study, we developed a CRISPR/SaCas9 system and demonstrated that this tool can recognize the PAM sequences 5′-AAGGGT-3′, 5′-GGGGAT-3′, 5′-TTGAAT-3′, and 5′-TAGGGT-3′ in soybean genomic DNA. Moreover, the CRISPR/SaCas9 system will be more accurate in creating mutations, because SaCas9 recognizes a longer PAM motif than SpCas9. Our results suggest that SaCas9 can direct highly specific genome editing activity, and it will therefore be useful for expanding the scope of potential target genes for genome editing in soybean.
4. Materials and Methods

4.1. SgRNA Design and Construction of the CRISPR/SaCas9 Vectors

The genome sequences and detailed information of the soybean genes \( \text{GmFT2a} \) and \( \text{GmFT5a} \) were downloaded from the Phytozome website (www.phytozome.net, accessed on 11 March 2021). Because the PAM sequence recognized by SaCas9 is 5′-NNGRRT-3′ (where R represents A or G), we designed three sgRNAs (named \( \text{GmFT2a-SaCas9-SP1} \), \( \text{GmFT2a-SaCas9-SP2} \), and \( \text{GmFT2a-SaCas9-SP3} \)) to target three sites located in the first exon of \( \text{GmFT2a} \). The PAM sequences of these three sgRNAs are 5′-AAGGGT-3′, 5′-GGGGAT-3′ and 5′-TTGAAT-3′, respectively. We also designed two sgRNAs (\( \text{GmFT5a-SaCas9-SP1} \) and \( \text{GmFT5a-SaCas9-SP2} \)) that target sites located in the first exon of \( \text{GmFT5a} \). The PAM sequences of these two sgRNAs are 5′-TAGGGT-3′ and 5′-GGGGAT-3′, respectively. The specificity of these five target sequences were identified by sequence comparison with the soybean genome database in the NCBI website (www.ncbi.nlm.nih.gov, accessed on 11 March 2021). For each sgRNA, a pair of DNA oligos were synthesized by Tsingke Biotechnology (Beijing, China) and annealed to generate dimers using PCR amplification. These dimers were subsequently ligated upstream of the sgRNA scaffolds in the plasmid vector for the simultaneous expression of SaCas9 and sgRNA. The sgRNA expression cassette was driven by the \( \text{Arabidopsis U6} \) promoter (Supplementary Data S1). The SaCas9 gene sequence with two NLS (nuclear localization signal) sequences was codon-optimized for dicotyledons (Supplementary Data S2) and placed downstream of the CaMV 2 × 35S promoter. The \( \text{GFP} \) gene driven by the CaMV 35S promoter was used for the rapid visual screening of transgenic hairy roots.

4.2. Hairy Root Transformation Using Agrobacterium rhizogenes K599

The corresponding CRISPR/SaCas9 vectors were introduced into \( \text{Agrobacterium rhizogenes} \) K599 via electroporation. The soybean cultivar ‘Jack’ was used for hairy root transformation in the present study according to the protocol previously described [32,45]. After cultivation for 15 days, each individual hairy root that had grown to a length of 5 to 6 cm was numbered and examined using a stereoscopic fluorescence microscope (Nikon SMZ1500, Nikon, Tokyo, Japan). The hairy roots harboring the desired CRISPR/SaCas9 vectors gave GFP fluorescence signals (Supplementary Figure S1) and were used for further analysis.

4.3. Determination of the Site-Specific Mutations Induced by the CRISPR/SaCas9 in Target Genes

Each transgenic hairy root is an independent transformation event. The genomic DNA of each transgenic hairy root was extracted using the CTAB method and subsequently used as templates for PCR amplification with specific primers followed by DNA sequencing of the amplicons. To detect mutations in the \( \text{GmFT2a} \) gene, the primers \( \text{GmFT2a-SaCas9-F} \) (5′-AAGCAAAACGAGTATATAAGAAAGCA-3′) and \( \text{GmFT2a-SaCas9-R} \) (5′-TGGATGGTCAAAAACAATAAACGTC-3′) were designed to amplify a 585 bp amplicon containing the three target sequences \( \text{GmFT2a-SaCas9-SP1} \), \( \text{GmFT2a-SaCas9-SP2} \), and \( \text{GmFT2a-SaCas9-SP3} \). To detect mutations in the \( \text{GmFT5a} \) gene, the primers \( \text{GmFT5a-SaCas9-F} \) (5′-GCAGATGCTAAGGTGGAAAAATA-3′) and \( \text{GmFT5a-SaCas9-R} \) (5′-TGCATCCACCAT AACCTGAGAT-3′) were designed to amplify a 462 bp amplicon containing the two target sequences \( \text{GmFT5a-SaCas9-SP1} \) and \( \text{GmFT5a-SaCas9-SP2} \). The heterozygous mutations showed overlapping peaks from the target sites to the end, while the wild-type and homozygous mutations had no overlapping peaks at the targeted sites [26]. The mutation rates were calculated using the following formula: mutation rate = number of hairy roots with mutations/number of hairy roots examined × 100%. In order to identify the specific types of mutations, the PCR products were further characterized by subcloning, DNA sequencing and alignment with the reference sequence.
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