Efficient CRISPR/Cas9-mediated Targeted Mutagenesis in Populus in the First Generation

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Recently, RNA-guided genome editing using the type II clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (Cas) system has been applied to edit the plant genome in several herbaceous plant species. However, it remains unknown whether this system can be used for genome editing in woody plants. In this study, we describe the genome editing and targeted gene mutation in a woody species, Populus tomentosa Carr. via the CRISPR/Cas9 system. Four guide RNAs (gRNAs) were designed to target with distinct poplar genomic sites of the phytoene desaturase gene 8 (PtoPDS) which are followed by the protospacer-adjacent motif (PAM). After Agrobacterium-mediated transformation, obvious albino phenotype was observed in transgenic poplar plants. By analyzing the RNA-guided genome-editing events, 30 out of 59 PCR clones were homozygous mutants, 2 out of 59 were heterozygous mutants and the mutation efficiency at these target sites was estimated to be 51.7%. Our data demonstrate that the Cas9/sgRNA system can be exploited to precisely edit genomic sequence and effectively create knockout mutations in woody plants.

The CRISPR/Cas9 system is highly efficient at generating targeted mutations in stable transgenic poplar plants, and homozygous mutations at the desired sites can be created in the first generation. In recent years, genome editing technologies using engineered nucleases have been developed as effective genetic engineering methods to target and digest DNA at specific locations in the genome. To date, there are three main types of engineered nucleases for genome editing: zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and CRISPR (clustered regularly interspaced short palindromic repeats)/Cas¹. Among these genome editing approaches, due to its simplicity, design flexibility, and high efficiency, the CRISPR/Cas9 system has now been utilized widely for editing the genome of various organisms, including bacteria, yeast and animals². Most recently, it has also been reported to successfully and specifically edit the genome in plants³–⁴.

Since the first reports of CRISPR/Cas9 directed genome editing in two model plant species, Arabidopsis thaliana⁵–⁶ and Nicotiana benthamiana⁶–⁷, this system has been shown to effectively work in at least five crop species, including monocots: rice⁵–⁸, wheat⁹–¹⁰, sorghum¹¹, maize¹² and dicots: tomato¹³. The transient expression of Cas9 and sgRNA successfully directs genome modifications in leaf cells or protoplast of Arabidopsis⁵ and tobacco¹⁰–¹¹, and in protoplast of wheat¹⁰. The stable transformation process is also applied to create genetically 1 edited lines carrying a mutation in the gene of interest in all these plant species using CRISPR/Cas9¹. Moreover, in the stable transgenic lines of Arabidopsis, rice and tomato, the sequence changes in first generation of transformants were persist in the next generation¹⁴–¹⁵. Although CRISPR/Cas9 is rapidly becoming a primary choice for gene editing in plants, there is no report on the
efficacy of CRISPR/Cas9 in woody species yet. Therefore, more extensive investigation is still needed to
determine whether the efficacy of this system will be universal.

As a most widely planted fast-growing tree, poplars have tremendous economic and ecological value.
Since the whole-genome sequence of *Populus trichocarpa* was released in 2006\(^{16}\), extensive genomic
resources are now available for functional genomics studies in this species, which has been used as a
model in forest genetics and woody plant studies. Thus, understanding of the molecular mechanisms of
gene function and transcriptional regulation in *Populus* is crucial for genetic engineering in trees and
sustainable forest management. Compared to *Arabidopsis*, rice and other annual model plants, how-
ever, functional genomics researches in woody plants are more difficult due to long vegetative periods,
low efficiency of genetic transformation and a limited number of mutants\(^{17}\). Despite some promising
approaches to create knockout mutants in poplar\(^{18}\), large-scale gene mutant resources are still lacking
so far.

Here we report an improved approach to delivery Cas9 coding gene and multiple sgRNAs into plant
cells by a single plasmid. Based on this system, an poplar endogenous phytoene desaturase gene (*PtoPDS*)
was disrupted site-specifically in the first generation of transgenic plants. The mutagenesis mediated
by Cas9/sgRNA in poplar was highly efficient and both homoallelic and heteroallelic *pds* mutant were
detected by DNA sequencing. Taken together, our data suggest that the CRISPR/Cas9 system is a highly
efficient and powerful tool for genome modifications in woody plants.

### Materials and Methods

#### Growth and transformation of *P. tomentosa* Carr. plants.
*Populus tomentosa* Carr. (clone 741) is grown in the greenhouse at 25 °C under a 14-h light/dark cycle with supplemental light (4500 lx). Leaf discs from *P. tomentosa* Carr. were transformed as described previously by Jia *et al.*\(^{19}\). Leaves of Chinese white poplar (*P. tomentosa* Carr.) were excised from *in vitro* plantlets, cut into disks and dipped in the diluted *Agrobacterium* culture for 8–10 min. After excess liquid on the surface was absorbed by sterilized paper, the leaf disks were transferred to woody plant medium (WPM) (2 mg/L zeatin, 1 mg/L 1-naphthalene acetic acid [NAA]). The infected disks were co-cultivated in dark for 2 days and then transferred to callus-inducing medium containing 2 mg/L zeatin, 1 mg/L NAA, 400 mg/L cefotaxime, 9 mg/L hygromycin and 0.8% (w/v) agar. After 2–3 weeks of culture without light, these leaf disks with

#### Cloning of *PtoPDS* gene.
The genomic DNA fragment of *PtoPDS* was amplified with gene-specific primers (*PtoPDS-F*: 5′-GTTGAATTTGGTTTTGGAGAAATG-3′; *PtoPDS-R*: 5′-CATTTAATGGTGCAGGGAGAAC-3′) designed based on its homologous gene (Potri.014G148700): 5′-sequence in the *P. trichocarpa* genome. The PCR reaction was carried out with pfu DNA polymerase (Takara, China) in a total volume of 50 μL. The PCR product was cloned and sequenced. The sequence result was supplemented in online materials.

#### CRISPR/Cas9 target sites selecting.
The sequence of *PtoPDS* was input in the online tool ZIFIT Targeter Version 4.2 (http://zifit.partners.org/ZIFIT/Introduction.aspx)\(^{20}\), which could find the CRISPR/
Cas9 target sites within an input sequence. Four of output target sites were selected for designing the
gRNA sequences based on their location in gene and their GC content.

#### Assemble Cas9/sgRNA construct.
The binary pYLCRIPSR/Cas9 multiplex genome targeting vector system carrying Cas9 coding gene and four plasmids with sgRNA cassettes driven by *AtU3b*, *AtU3d*, *AtU6-1* and *AtU6-29*, respectively, and the multiple sgRNA assembly instruction based on Golden Gate Cloning\(^{21-22}\), were provided by Yao-Guang Liu of South China Agricultural University\(^{23}\). A CRIPSR/Cas9 construct carrying four sgRNA cassettes was generated.

#### Genomic DNA extraction.
For analysis the mutation of PDS gene in transgenic T0 poplars, the genomic DNA was extracted from stable transgenic and wild-type plants following a typical CTAB
method. About 0.1 g tissues of poplars were ground in liquid nitrogen and 400 μL of pre-heated CTAB
buffer was added for each sample. After incubated at 65 °C for 30 min, 200 μL of chloroform was added and the resulting mixtures stay in room temperature for 10 min. After centrifugation at 16,000 g for 5 min, the supernatant was transferred to a new tube, mixed with 300 μL of isopropanol and incubation in 4 °C for 30 min. Then, genomic DNA was precipitated by 16,000 g centrifuge for 10 min and the DNA pellet was washed with 0.5 mL of 70% ethanol. Pellet of genomic DNA was dissolved in 100 μL of H₂O and concentration was determined using spectrophotometer.

#### Detection of mutation.
The genomic DNA extracted from poplars was then used as template to amplify the endogenous PDS fragment by PCR. PCR was performed using specific primers for each targets: the pair of *PtoPDS-F1*: 5′-GTTGAATTTGGTTTTGGAGAAATG-3′ and *PtoPDS-R1*: 5′-GCAGAGGAGAAC-3′. The PCR product was cloned and sequenced. The sequence result was supplemented in online materials.
5’- GCGGAGAAGAACGAAAGG -3’ cover the region of target site 1, 2 and 3; the pair of PtoPDS-F2: 5´- TAGAGGCAGTGAATCAATGGG -3´ and PtoPDS-R2:5´- CCTAAAACATCTCTTGCTTCAAGC -3´ cover the region of target site 2, 3 and 4. The PCR product was separated on an ethidium bromide-stained agarose gel (1.5%) and bands were recovered and cloned into the pMD19-T Simple vector (Takara, China). The mutations were identified through Sanger sequencing of individual clones. All sequence results were compared with the reference sequence of PtoPDS gene by alignment in DNAMAN (version 7.0). The mutation rate in transgenic plants was calculated according to the ratio of mutated clonal amplicons versus total sequenced clonal amplicons.

Results

Strategy for detection the CRISPR/Cas9-mediated mutagenesis of poplar endogenous gene. In order to test whether the CRISPR/Cas9 system could effectively direct gene-specific editing in Populus, we selected the poplar phytoene desaturase gene (PtoPDS), which is required for chlorophyll biosynthesis and its mutant shows an albino phenotype in other plant species9,24, as the target of Cas9 endonuclease. Four 20-bp sequences with tandem guanosine nucleotides (PAM) on their 3´-regions in PtoPDS locus were elected as sgRNA complementary sites, including one in 5´ of first exon and three in the second exon (Supplementary Figure S1 and Fig. 1A). By a two-step assemble strategy, the four targeting sequences aiming to PtoPDS were first inserted in the sgRNA expression cassettes (Fig. 1B), and then their cassettes were combined with the Cas9 endonuclease coding sequence in a single plant binary vector, pYLCRIPSR/Cas9P35S-H (Fig. 1C). Using this system and Agrobacterium-mediated transformation, Cas9 and four sgRNAs could simultaneously express in transgenic poplar.

CRISPR/Cas9-mediated mutagenesis of PtoPDS in transgenic poplar. We found that, most (89%) of leaf discs transformed with the pYLCRIPSR/Cas9+sgRNA generated at least one albino shoots, which lost green color in the whole plants (Fig. 2).
chlorophyll biosynthesis, albino phenotype in these transgenic plants indicates the loss of PtoPDS function. The ratio of albino phenotype occurred in T0 transgenic poplar is more than 50% (30 out of 59), much higher than that previous reported in other species such as Arabidopsis, rice, wheat, sorghum and tomato, while no albino phenotype was observed in the control transformed with an empty vector (Table 1).

The poplar PDS gene was mutated at desired targets. To further verify whether loss of green in the transgenic poplar was caused by generation of mutations in the PtoPDS gene by the CRISPR/Cas9 system, more than 100 clones from 8 independent transgenic T0 plants were randomly selected for sequencing (Table S1). The results confirmed that all of these transgenic plants with albino phenotype contained mutant alleles in the PDS gene. Most of the PDS mutant alleles were small insertions or deletions (indels, less than 15 bp) at the desired target sites, as the consequence of repairing through non-homologous end joining (NHEJ) following sgRNA-directed Cas9 cleavages (Fig. 3A). However, some alleles with a sequence inversion (Fig. 3B) between two sgRNAs targeting sites or a big fragment deletion (Fig. 3C–E) were also detected in our experiments. As a result of sequence rearrangement in the exons, the translation of the endogenous PDS gene was frame-shifted or prematurely terminated in the Cas9+PtoPDS sgRNAs transgenic T0 poplars. Interestingly, we also found that the contributions of the four selected sgRNAs to directing Cas9 and mutating target gene were not equal, and the highest efficiency (89.3%) of mutagenesis appeared in the sites T2&T3 of the target gene, middle in the site T1.
(56.9%) and no mutation was detected in the site T4 (Fig. 3A and Table S1), indicating that the appropriate selection of sgRNA pairs is important to effectively generate indels.

In addition, at least two copies of the PDS gene in Chinese white poplar genome were amplified by the gene-specific primers we used, and they could be distinguished based on the nucleotide polymorphism in their sequences flanking the target regions (Figure S2). The Cas9 could direct mutation on both of the two PtoPDS copies in a single transgenic plant (Figure S2). This suggests the ability of this multiple sgRNA-Cas9 system to knock-out two or multiple loci in the poplar genome at once.

Figure 3. CRISPR/Cas9-mediated gene editing in transgenic poplar plants. (A) A range of insertion and deletions (indels) were found in the other T0 plants. Red number in right side means the no. of detected clones with such mutant allele and in which plant such mutant allele is found. (B) A big fragment inversion mutant of PtoPDS. The sequence between the target site1 and target site 2 (296 bp) of PtoPDS were cut down by Cas9 and ligated back to the genome reversely, with 1 and 3 bp of nucleotides around the up- and downstream cut sites were lost. (C) DNA samples from independent transgenic poplar were analyzed for mutations by PCR assays. In the top row, F1 and R1 were used; in the bottom row, F2 and R2 were used. T0-1 represents a heterozygous mutant of PDS, in which two amplification bands with different length were obtained in this assay: “a” indicates the full-length fragment of the PDS gene, “b” indicates a shorter fragment of the PDS gene with mutation. (D) The PCR products from T0-1 plant were separated by their size and cloned and sequenced to validate the deletion directed by CRISPR/Cas9 in the PDS gene. (E) A big fragment deletion mutant of PtoPDS. The 116 bp sequence upstream of target site 2 was lost in the DNA repairing after Cas9 mediated double strain break. In B and E, Red and blue quadrangles indicate the target site 1 and 2 in PtoPDS locus respectively; the red number in the right side means the no. of detected clones with such mutant allele.
The Cas9/sgRNA system generated both biallelic homozygous and heterozygous mutants in transgenic poplar. Sequencing analysis of randomly selected clones from individual transgenic T0 plants revealed that, among the 65 clones that could cover target site 1, 2 and 3, most (61) of them were mutated in at least one of these three target sites (Fig. 3A). Actually, 7 (Plant ID 2–8) out of 8 transgenic T0 plants analyzed, contained only mutated alleles of the PtoPDS gene, suggesting that these transgenic lines should be biallelic homozygous pds mutants (Table S1). In contrast, for another transgenic T0 plant (Plant ID 1) which exhibited the half-white phenotype in their 1 organs (Fig. 2B,C), two bands were observed (Fig. 3C) when amplified with the gene-specific primers for the poplar PDS gene. Sequencing analysis confirmed the presence of the innate allele and the mutated allele with 116 bp deletion (Fig. 3D,E), indicating that this transgenic line could be a heterozygous pds mutant. Using our approach, most (28 out of 30, 93.3%) of the pds mutant plants were homozygotes, only 2 transgenic lines (6.7%) were considered as heterozygotes (Table 1). These results implies that the generation of DNA double stranded breaks (DSB) by Cas9 may occurred in an early stage in the regeneration T0 poplars from transgenic calli.

Discussion
In a most recent report, the CRISPR/Cas9 system was delivered into sweet orange (Citrus sinensis) by transient infiltration of A. rhizogenes25, but stable transformation remains a preferred method for delivering nuclease constructs into woody species. To our knowledge, we first successfully applied a CRISPR/Cas9 approach for obtaining stable transgenic woody plants, and demonstrated that this system can efficiently and site-specifically mutate endogenous genes. Our data suggest that, by means of the CRISPR/Cas9 technology, locus-specific gene editing has become a routine practice not only in model plants and crops, but also in trees.

The multiple sites editing by the simultaneous expression of two or more sgRNAs has been reported in Arabidopsis6–26, rice23 and tomato13. But the pervious ways to delivery sgRNAs into plants are based on co-transformation of more than one plasmid, and relatively low efficiency, which restrict the application of multiple genes editing by CRISPR/Cas9 in plant genome. The pYLCRISPR/Cas9 multiplex genome targeting vector system is designed to introduce multiple sgRNA expression cassettes by single vector transformation13. In this study, we used this vector system to successfully target multiple sites of the PtoPDS gene in poplar. Thus, one can foresee an application of the CRISPR/Cas9 system for knocking-out of whole gene family, as shown in rice23.

Besides, it is possible that the insertion or deletion mediated by single sgRNA guided Cas9 did not create a frameshift of target gene, consequently no defect in phenotype. Actually, the indels such as −3bp or +1/−1 bp, which theoretically not lead to frameshift, were detected in the target 2 region (Fig. 3A). Because there are 4 sgRNAs for one locus being used in our experiment, the possibility that the changes in 3–4 target regions consequently escaped from frameshift is extremely low. Based on our data, we did not find that a transgenic plant with indels in the PtoPDS gene was still green. Therefore, the rate of modifications on multiple target sites affecting the reading frame was much lower in our results.

Our approach can also generate homozygous knockout mutation in predicted loci in T0 generation of transgenic poplar (Fig. 3), which is very important for genetic modification of trees with a long life span. With the advantages of both high efficiency in editing target gene sequence and synchronous mutation in two homologous chromosomes, poplar knockout mutants of interested genes could be generated in a very short time (approximately two months) and with a high ratio using the CRISPR/Cas9 method. Thus, our study proves that, using the CRISPR/Cas9 system, it is not only possible to investigate the function and mechanism of genes in woody trees, but also raises the possibility to construct a mutant library for poplar using the CRISPR/Cas9 system, instead of the T-DNA method used in Arabidopsis.

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Author Contributions
D.F. and K.M.L. designed the research. D.F., T.T.L., B.J. and C.F.L. performed gene cloning, vector construction, transgenic plants generation, and drafted the manuscript. D.F. and S.L. performed phenotype analysis and qRT-PCR analysis. D.F. and K.M.L. modified the manuscript. All authors reviewed the manuscript.

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