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FOCUS ARTICLE

Targeted mutagenesis by CRISPR/Cas9 system in the model legume *Medicago truncatula*

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In recent years, several genome-editing technologies based on engineered nucleases have been developed and successfully reported to mutate specific loci in various organisms. Compared to the early developed genome-editing methods, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), which need substantial protein engineering to each DNA target, the clustered regularly interspersed short palindromic repeats (CRISPR)/Cas9 system only requires a change in a 20-nt targeting sequence within the single-guide RNA (sgRNA). The advantage of the simplicity of the cloning strategy and less limitations on potential target sites make the CRISPR/Cas9 system the standout choice for targeted genome editing in understanding gene function and developing valuable traits in model plants and food crops (Li et al. 2013; Shan et al. 2013).

Alfalfa (*Medicago sativa*) is a perennial legume cultivated as one of the most important forage crops in the world. Alfalfa is well recognized as "The queen of forage crops" because of its high feed value and high yield productivity, yet the complex autotetraploid genome and limited genomics tools hamper the genetic modifications and improvements in alfalfa. *Medicago truncatula* is a close relative of alfalfa with a diploid genetics, small genomes, short lifecycle, and highly natural diversity. With the completion of its genome sequencing, *M. truncatula* has been adopted as a model for the study of genetics and genomics for forage legume. Recently, the CRISPR/Cas9 system has been reported to mutate targeted genes in somatic cells of *M. truncatula* and soybean by root hair transformation suggesting the application potential of CRISPR/Cas9 in legumes (Michno et al. 2015). However, CRISPR/Cas9-based stable transformation in forage legumes has not been reported. In this study, we developed an efficient CRISPR/Cas9 system through agrobacterium-mediated transformation for targeted gene mutations in the model legume *M. truncatula*, which will facilitate investigation in Medicago gene function and also expand the forage legume's genomics tool box.

To promote the expression efficiency of guide RNA in *M. truncatula*, we first cloned a native Medicago *U6* promoter to drive the expression of specific sgRNA (Supplementary Figs. S1, S2). To accommodate the Medicago CRISPR/Cas9 system to *Agrobacterium*-mediated transformation, we constructed a binary vector pFGC5941-Cas9 based on the backbone of pFGC5941 for co-expression of the guide RNA and Cas9. The designed binary vector contains a codon-optimized *streptococcus pyogenes Cas9* gene driven by the 2 × CaMV *35S* promoters (Shan et al.

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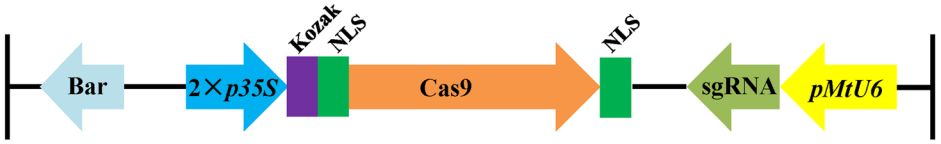
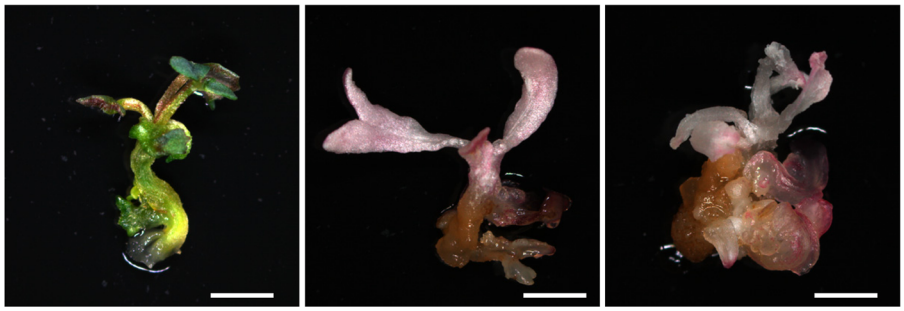
(a)

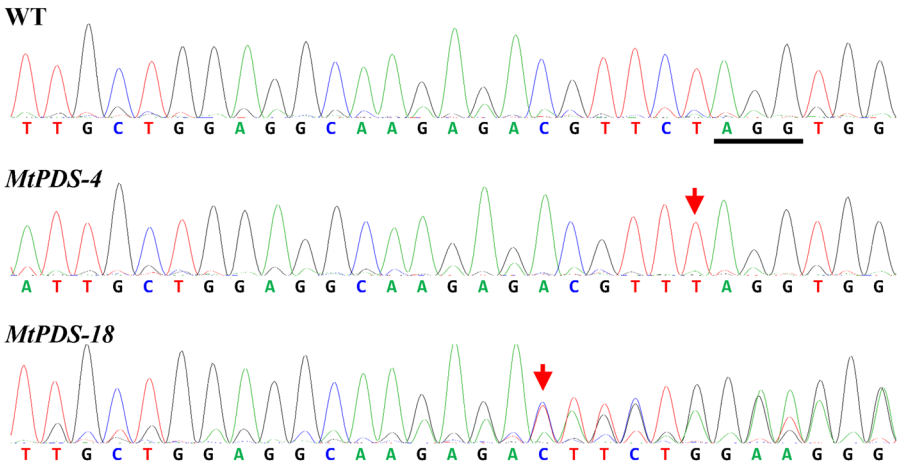


(b)



WT *MtPDS-4* *MtPDS-10*

(c)



(d)

MtPDS

Homozygous monoallelic mutants

WT: TATATTGCTGGAGGCAAGAGACGTTCTAGGTGGAAAGGTTTTCTGACTAATTTAATCTCATTTG

MtPDS-4: TATATTGCTGGAGGCAAGAGACGTT-TAGGTGGAAAGGTTTTCTGACTAATTTAATCTCATTTG -1

MtPDS-21: TATATTGCTGGAGGCAAGAGACGT-CTAGGTGGAAAGGTTTTCTGACTAATTTAATCTCATTTG -1

Homozygous biallelic mutants

WT: TATATTGCTGGAGGCAAGAGACGTTCTAGGTGGAAAGGTTTTCTGACTAATTTAATCTCATTTG

MtPDS-3: TATATTGCTGGAGGCAAGAGACG-TCTAGGTGGAAAGGTTTTCTGACTAATTTAATCTCATTTG -1
MtPDS-3: TATATTGCTGGAGGCAAGAGAC--TCTAGGTGGAAAGGTTTTCTGACTAATTTAATCTCATTTG -2

MtPDS-10: TATATTGCTGGAGGCAAGAGAC--TCTAGGTGGAAAGGTTTTCTGACTAATTTAATCTCATTTG -2
MtPDS-10: TATATTGCT----------TCTatTTG -48/+2

MtPDS-18: TATATTGCTGGAGGCAAGAGAC-TTCTAGGTGGAAAGGTTTTCTGACTAATTTAATCTCATTTG -1
MtPDS-18: TATATTGCTGGAGGCAAGAGA-----TAGGTGGAAAGGTTTTCTGACTAATTTAATCTCATTTG -5

◀**Fig. 1** Targeted mutagenesis in *Medicago truncatula* using the CRISPR/Cas9 system. **a** Schematic illustration of CRISPR/Cas9 construct used in this study. **b** Phenotypes of the *MtPDS* mutants. *Bars* 1 cm. **c** Sequencing chromatography of wild type, the homozygous monoallelic mutant *MtPDS-4*, and the homozygous biallelic mutant *MtPDS-18*. *Red arrows* indicate mutation sites. **d** SgRNA:Cas9-induced *MtPDS* mutations in albino transgenic plants. *Red bases* mean the targeting sequence; *Green bases* mean the PAM (color figure online)

2013). To reconstitute Cas9 ribonucleoprotein complex in the nucleus, the *Cas9* gene was attached with a nuclear localization signal (NLS) after the Kozak sequence. In addition, the destination vector pFGC5941-Cas9 contains a single *Xba*I restriction site located downstream of the 35S::Cas9 cassette, which is designed for integrating the gene-specific gRNA (Fig. 1a).

To test whether the CRISPR/Cas9 system can be used to specifically disrupt an endogenous gene in *M. truncatula*, we designed a specific sgRNA, which targeting the second exon of the medicago *phytoene desaturase* (*MtPDS*) gene (Supplementary Fig. S3), using a web single-guide RNA design tool, CRISPR-P (http://cbi.hzau.edu.cn/cgi-bin/CRISPR) (Lei et al. 2014). The destination construct containing the *Cas9* and the specific *pMtU6::gRNA* expression cassettes was introduced into *M. truncatula* ecotype R108 by agrobacterium-mediated transformation. We generated a total of 309 T0 transgenic R108 plants and 32 of them exhibited the albino phenotype with some of them showing the phenotype even at the regeneration stage (Fig. 1b; Supplementary Fig. S4), suggesting that the *MtPDS* gene might be disrupted in vivo. To determine whether the targeted gene was mutated, we randomly selected 16 transgenic plants with albino phenotype and conducted genotyping analysis on these lines using specific primers flanking the target sequence. Sequencing analysis showed that, compared with the wild type, all 16 albino plants carry mutations at the targeted site of the *MtPDS* gene. Among them, two plants were monoallelic homozygous mutants carrying respective one-nucleotide deletion and 14 plants were biallelic homozygous mutants with diverse deletion or base substitution (Fig. 1c, d). In addition, we performed genotyping analysis on 12 out of 277 transgenic plants without albino phenotype. Sequencing analysis showed that three plants carried heterozygous mutations, while the other nine plants were wild type at the *MtPDS* gene. We further examined the off-target effect of the CRISPR/Cas9 construct targeting the *MtPDS* gene. Two potential off-target sites were predicted using the web tool CRISPR-P but neither of them was detected with mutations by sequencing analysis (Supplementary Table 1), suggesting that the sgRNA is specific for its recognition site and no off-target effects of this construct occurred in *M. truncatula*. Taken together, these results indicate that the Medicago *MtPDS* gene, which involved in the carotenoid biosynthesis, was successfully disrupted in the T0 generation using the CRISPR/Cas9 technology.

In conclusion, we developed an optimized agrobacterium-delivered CRISPR/Cas9 system, which could successfully induce targeted genome modifications in the model legume *M. truncatula*. Using this system, we obtained monoallelic and biallelic homozygous mutants (10.35%) for *MtPDS* in the T0 generation which suggested that our strategy for vector construction was amenable and this CRISPR/Cas9 system could serve as an effective tool to facilitate studies of gene function in *M. truncatula*. Further study will extend the range of applications of CRISPR/Cas9 to cultivated legume forages with more complicated and bigger genomes, such as alfalfa and clover, which will benefit and speed up the forage legume improvement.

Materials and methods

Plant materials and growth conditions

The *M. truncatula* ecotype R108 were grown in the green house with 25 °C/16-h (day) and 23 °C/8-h (night) photoperiods at 60–70% humidity and 150–200 μmol m^2 s light intensity.

CRISPR/Cas9 vector constructs

CRISPR/Cas9 vector constructs was performed, as described in Supplementary Materials and Methods.

Agrobacterium-mediated plant transformation

The agrobacterium-mediated transformation in *M. truncatula* was performed as previously described (Niu et al. 2015).

Genome DNA extraction and sequencing

Genome DNA extraction and sequencing were performed, as described in Supplementary Materials and Methods.

Author contribution statement YYM, BL, LFN, and HL designed the experiments. YYM, YLH, HW, and RHJ performed the experiments. LFN, JW, and HL wrote the paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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