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Efficient targeted mutagenesis in potato by the CRISPR/Cas9 system

Shaohui Wang[1] · Shuaibin Zhang[2] · Wanxing Wang[2] · Xingyao Xiong[2] · Fanrong Meng[1] · Xia Cui[2]

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Technologies to achieve the specific and precise knockout of genes are critical for understanding gene functions and fundamental biological processes. Targeted genome editing as a new and efficient method to mutate genes has been rapidly used in many organisms. Compared with the earlier systems, such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), the bacterial clustered regularly interspaced short palindromic repeats (CRISPR)/associated protein 9 nuclease (Cas9) is an easier and more efficient system, and it has been widely used in recent years (Gaj et al. 2013). The Cas9 endonuclease is dictated by a 20-base pair (bp) sequence at the 5′ end of the single-guide RNA (sgRNA), which then acts as a guide to the specific site of the genome, where Cas9 is able to cleave double-stranded DNA, leading to deletion, insertion, or substitution at the target sites (Sander and Joung 2014).

Since 2013, CRISPR/Cas9 has been successfully applied by transient expression and/or stable transgenic lines in several plant species, such as Arabidopsis, *Nicotiana benthamiana*, rice, wheat, maize, and tomato (Brooks et al. 2014; Jiang et al. 2013; Li et al. 2013; Miao et al. 2013; Nekrasov et al. 2013; Shan et al. 2013). Moreover, the mutations generated in the primary transgenic plants by the CRISPR/Cas9 system can be stably transmitted to the next generation (Brooks et al. 2014; Feng et al. 2014). Thus, the CRISPR/Cas9 system is becoming a powerful tool for genome editing in plants, whereas the reports of the usage and efficiency of the CRISPR/Cas9 system-mediated plant genome engineering are still limited.

Potato is a very important crop for world food security. Environmental changes and extended cultivation regions are challenges for potato cultivars. Understanding the functions of genes will help us to improve agronomic traits using molecular engineering technologies. Since cultivated potatoes are tetraploid, the functional approach of genes by molecular genetics is very difficult. Similar to other plant species in which the CRISPR/Cas9 has been successfully used, gene transformation in potato is efficient, and the genome sequence of double-haploid DM and diploid RH is available, which made potato an ideal candidate for this genome editing system (The Potato Genome Sequencing Consortium 2011). Here, we used the CRISPR/Cas9 system to produce knockouts of genes in potato successfully, which will provide an excellent foundation for future gene function studies.

In order to construct a CRISPR/Cas9 plasmid which would function efficiently in potato, we first cloned a native promoter for potato *U6* RNAs *(StU6P)* from DM

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✉ Fanrong Meng
mengfanrong73@henau.edu.cn

✉ Xia Cui
cuixia@caas.cn

[1] College of Life Science, Henan Agricultural University, Zhengzhou 450002, China

[2] Key Laboratory of Biology and Genetic Improvement of Horticultural Crops of the Ministry of Agriculture, Sino-Dutch Joint Laboratory of Horticultural Genomics, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 100081, China

(Supplementary Fig. S1), which drove the sgRNA. The optimized coding sequence of *SpCas9* in rice (*OsCas9*) (Shan et al. 2013) driven by the CaMV *35S* promoter was cloned into the same binary plasmid with *StU6P*::sgRNA (Fig. 1a). To test whether this native *U6* promoter is able to promote the expression of sgRNA and then direct Cas9 targeting, we performed an *Agrobacterium tumefaciens*-mediated transient expression assay in *N. benthamiana* as mentioned by Nekrasov et al. (2013). The *sgRNA* with the guide sequence matching a 20-bp region within the *PHYTOENE DESATURASE* (*PDS*) gene in *N. benthamiana* was placed under the potato *U6* promoter (Supplementary Fig. S2). Two days later, the injected leaves were collected and the DNA was extracted. The genomic DNA was

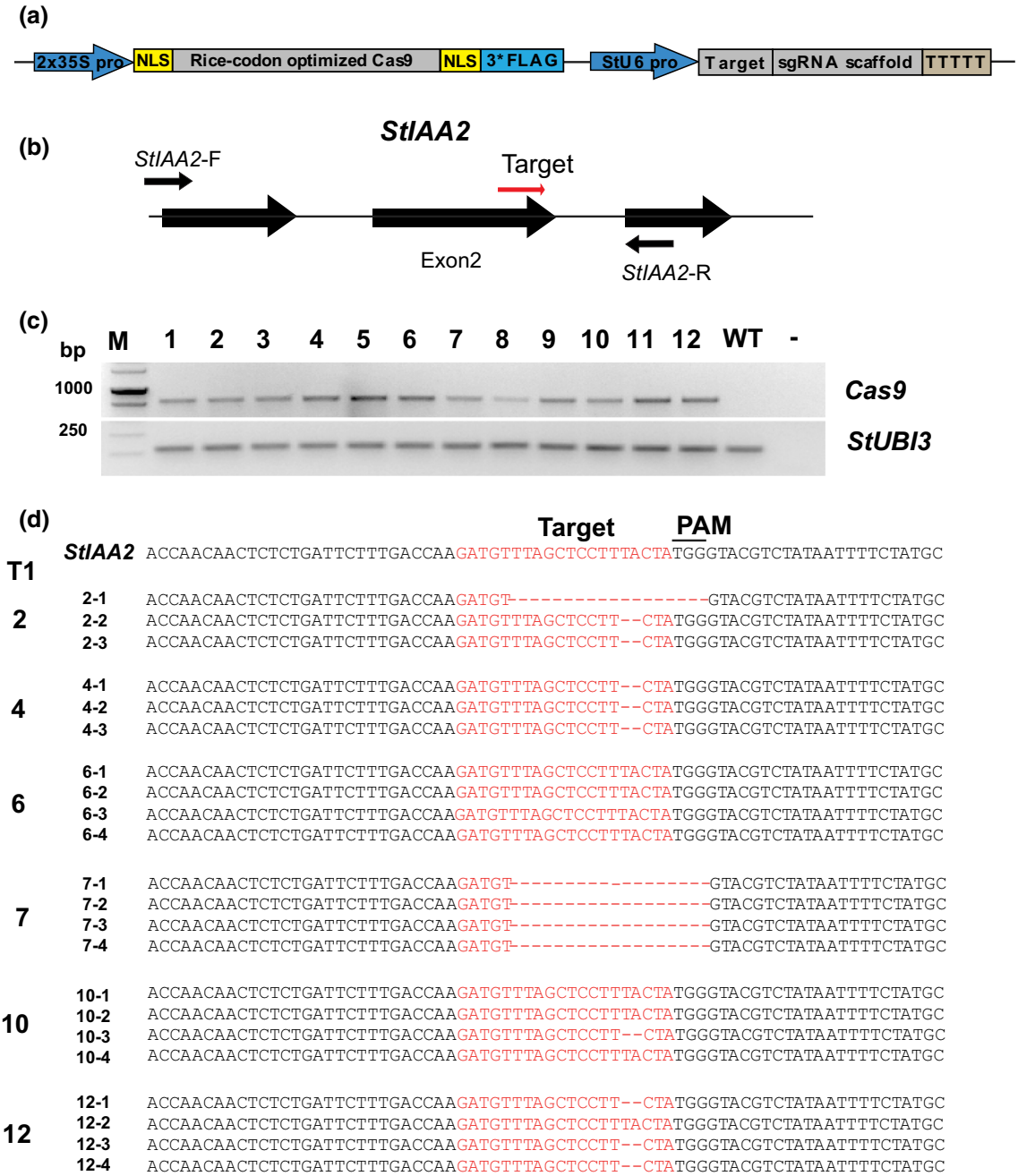

Fig. 1 CRISPR/Cas9-mediated gene editing in stable transgenic potato plants. **a** Schematic illustrating the engineered CRISPR/Cas9 vector. **b** Schematic illustrating the guide RNA (sgRNA, *red arrows*) targeting the *StIAA2* coding sequence. *Black arrows* indicate the PCR primers used to evaluate the mutation types. **c** RT-PCR for the transcripts of *Cas9* in wild-type and transgenic potatoes. **d** PCR genotyping of six representative *StIAA2* mutation plants showing the types of DNA lesions generated

digested with the endonuclease *Mly*I, which recognizes and digests the target sequence near the protospacer adjacent motif (PAM) within the *PDS* gene. Polymerase chain reaction (PCR) was then performed with primers flanking the target site (Supplementary Fig. S2a). After doing so, the samples with the mutated DNA that disrupted the *Mly*I site were enriched. We detected the *Mly*I-resistant amplicons in five different injected leaves (lanes 1–5 show PCR products, and lane 6 shows the negative control; Supplementary Fig. S2b). The nondigested genomic DNA of *N. benthamiana* as a positive control is shown in lane 7 (Supplementary Fig. S2b). The PCR products from lanes 1 and 7 were cloned, and the individual clones were sequenced. The 11 clones derived from the PCR products in lane 1 revealed the presence of 6 different mutant alleles, from 1 bp substitutions up to 2 bp insertions or 9 bp deletions (Supplementary Fig. S2c). Sequences of the 4 clones derived from the control PCR products (lane 7) were all wild-type. We further evaluated the off-target effects of the sgRNA:Cas9 construct targeting the *NbPDS* gene. Three potential targets described by Nekrasov et al. (2013) were tested and no off-target effect happened in injected leaves (Supplementary Fig. S2d). These results indicate that the native *U6* promoter of potato is efficient for targeted mutagenesis in *N. benthamiana*.

To test whether this CRISPR/Cas9 construct can introduce gene knockouts in potato, we transformed the CRISPR/Cas9 plasmid into DM targeting the *StIAA2* gene. *StIAA2* encoding an Aux/IAA protein in potato has been previously cloned and analyzed (Kloosterman et al. 2006). The CRISPR/Cas9 construct which we designed to target the second exon of *StIAA2* contained a 20 bp seed sequence beginning with a G nucleotide and followed by PAM, NGG (Fig. 1b). Twelve T1 transgenic plants were generated. We tested the transcripts of *Cas9* in the independent transgenic plants by RT-PCR, which indicated that the *Cas9* is expressed in all of these plants (Fig. 1c).

To test whether the *StIAA2* mutations were generated in transgenic potato plants by the CRISPR/Cas9 system, we performed PCR on 6 representative T1 plants using primers flanking the target sequence (Fig. 1b). The PCR products were cloned and sequenced. Among them, two plants were monoallelic homozygous mutants for a 2 or 18 bp deletion (Fig. 1d, e.g., line #4 and 7), and one additional plant was a biallelic homozygous mutant, carrying an allele with the 18 bp deletion and an additional allele with a smaller deletion of 2 bp (Fig. 1c, e.g., line #2). Additionally, one of the plants was wild-type (Fig. 1c, e.g., line #6), and two mutants were heterozygous (Fig. 1c, e.g., line #10 and 12). Our results demonstrate that the CRISPR/Cas9 system is highly efficient for targeted mutations of the first generation of transgenic potato plants. We further evaluated the off-target effects of the sgRNA:Cas9 construct targeting the *StIAA2* gene. One sequence which is identical to the target site of *StIAA2* was identified in the DM genome (Supplementary Fig. S3a), whereas the PAM motif, which followed the seed sequence, was not present in it. Using the PCR/sequencing assay, no CRISPR/Cas9-induced mutations were found, indicating that no off-target effects of this construct occurred in potato (Supplementary Fig. S3b).

Taken together, our findings demonstrate that the CRISPR/Cas9 system could induce mutations in stable transgenic potato. By this method, we can obtain monoallelic and biallelic homozygous mutants in the T1 generation. Thus, the CRISPR/Cas9 system is an effective tool to promote the functional studies of uncharacterized genes in potato.

Materials and methods

Vector construction

We first synthesized *StU6p*::sgRNA by GenScript Company, and then cloned it into pCAMBIA2300 after NOS terminator by *Hin*dIII and *Pme*I to generate pCAMBIA2300-*StU6p*::sgRNA. The *OsCas9* was amplified from the vector provided by Shan et al. (2013), and constructed into pCAMBIA2300-*StU6p*::sgRNA after *35S* promoter by *Spe*I and *Bam*HI to generate the final vector pCAMBIA2300-*35S*::Cas9-*StU6p*::sgRNA. The guided RNA obtained by annealing two primers with the *Aar*I complemented sequence at their 5′ end was ligated into the final vector by *Aar*I (Supplementary Fig. S1). Primers used are listed in Supplementary Table 1.

Agrobacterium-mediated transformation

The *A. tumefaciens*-mediated transient expression in *N. benthamiana* was performed according to our previous paper (Lu et al. 2011). *A. tumefaciens*-mediated transformations of potato double-haploid DM were performed as following. In brief, stem segments from 8 to 10-day-old seedlings were pre-cultured for 2 days at 28 °C followed by inoculation with *A. tumefaciens* strain EHA105 containing the CRISPR/Cas9 constructs of interest. After 2 days of co-cultivation, the stem segments were transferred to a selective regeneration medium that contained 50 mg/l kanamycin. When shoots were approximately 1.5 cm tall, they were transferred to a selective rooting medium that also contained 50 mg/l kanamycin.

DNA extraction and sequencing

Leaflets were collected from each T1 and DM plant, and genomic DNA was extracted using a standard cetrimonium

bromide (CTAB) protocol. Each plant with root growth on medium containing 50 mg/l kanamycin was genotyped with primers designed to amplify a region spanning the 3′ end of the Cas9 and 5′ end of NOS terminator. The six positive transgenic lines were further genotyped for insertion/deletion (indel) polymorphisms using a pair of primers deposited on the two sides of the seed sequence. All PCR products were resolved on 1 % agarose gels at 140 V for 30 min. Selected PCR products were excised and purified for cloning into the pEASY-Blunt vector (Transgen). A minimum of three clones per PCR product was sequenced using M13F and M13R primers. Primers for genotyping are listed in Supplementary Table 1.

RNA extraction and RT-PCR

Total RNA was extracted from the Leaflets of each T1 and DM plant using Trizol reagent (Invitrogen, 15596018). RNAs (1 μg) were digested by DNaseI (Ambion, Turbo DNaseI kit, 2238G) and then RT reactions were performed using TransScript II First-Strand cDNA Synthesis Super MIX (TransGen AH301-02). *StUBI3* (*PGSC0003DMG400005199*) was used as the internal control of RT-PCR. Primers for RT-PCR are listed in Supplementary Table 1.

Plant materials and growth conditions

Potato wild-type DM and the T1 mutated plants were grown on 1/2 MS medium under LD (16-h light/8-h dark) conditions with an intensity of 80–120 $\mu E/m^{-2}\ s^{-1}$ of white light at 22 °C.

Author contribution statement F. R. Meng and X. Cui conceived and designed the study. S. H. Wang and S. B. Zhang performed most of the experiments with the help from W. X. Wang, X. Y. Xiong, F. R. Meng; and X. Cui wrote the paper.

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

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