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DEVELOPMENT OF CRISPR/CAS9 SYSTEMS TO INDUCE TARGETED MUTATIONS IN THE PROMOTER REGION OF THE *OsSRFP1* GENE IN RICE

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SUMMARY

Rice is one of the most important staple food crops worldwide, especially in Asia. However, climate change has been negatively affecting the arable land area, which leads to a severe reduction in the rice yield. Salt stress is considered one of the most critical abiotic stresses. The creation of rice varieties with salt tolerance is an urgent research direction. Besides traditional gene transfer and plant breeding methods, gene editing technology has emerged in recent years with outstanding advantages. It assists in producing plants with the desired mutation without the presence of foreign genes. In this study, we successfully created two CRISPR/Cas9 vectors to introduce targeted mutations of two different positions in the promoter region of the *OsSRFP1*, a gene involves in the ubiquitination pathway and regulates important responses to multiple stresses. Cali from mature seeds of the Khang Dan 18 rice cultivar was used for *Agrobacterium*-mediated transformation. A total 6 T0 rice lines showed CRISPR/Cas9 induced mutations in the targeted sites identified through PAGE results and sequencing results. Two of them carrying mutations in different positions were further utilized for validating inheritance and segregation of induced mutations at the T1 generation. The results shown that mutations from the T0 generation were passed on to the next generation. Importantly, three homozygous mutant lines lacking the transgene were successfully identified at the T1 generation. These mutant lines are valuable materials for us to continue to assess gene expression, plant growth and development as well as salt tolerance in the further research work.

Keywords: *CRISPR/Cas9, promoter, rice, OsSRFP1 gene, Agrobacterium-mediated method*

INTRODUCTION

Rice is one of the most important crops, providing staple food for half of the global population. Currently, rice is cultivated in more than 100 countries in the world. In which, Asia is the main rice-producing region, accounting for 90% of both output

and area (Muthayya *et al.*, 2012). India, Thailand, and Vietnam are the top 3 rice exporting countries in the world. In which, Vietnam was the third largest rice exporter, with about 6.8 million metric tons for a period of 2022/2023. However, rice production has been faced a lot of challenges including salinization due to the climate change, recently.

Under saline stress conditions, physiological and biochemical processes in plants are negatively affected at all stages of development. At the salinization, plants show underdeveloped root systems, wilt leaves, reduced growth biomass and yield (Hussain *et al.*, 2017; Yadav *et al.*, 2020). In recent years, many studies have shown that protein regulation by the ubiquitin/proteasome system to be one of the plant's survival and adaptation strategies in various environmental stresses (Cui *et al.*, 2012; Tian *et al.*, 2015; Xu, Xue, 2019; Wang *et al.*, 2020). The most prominent function of ubiquitin is to mark proteins for destruction in the proteolytic system. In ubiquitination process, E3 ligase plays a role in determining which protein substrates bind to ubiquitin (Metzger *et al.*, 2014), deciding which proteins are degraded. The enhanced expression of several genes encoding E3 ligases (*OsRHA2a, OsDSG1, OsSIRP1/2, OsSRFP1, OsRINGC2-1*) showed increase in salt tolerance in plants (Dametto *et al.*, 2015; Xu, Xue, 2019). In which, the *OsSRFP1* (Stress-related RING finger protein 1) is a potential candidate gene located on rice chromosome 3 and involves in resistance to abiotic stresses. This gene consists of 12 exons encoding a RING E3 ligase. In previous reports, the *OsSRFP1*-down-regulation by the RNAi technology showed increases in salt and cold tolerances, whereas, the overexpression of the *OsSRFP1* gene reduced the tolerance of rice plants to those stresses (Fang *et al.*, 2015). This result indicated that the *OsSRFP1* gene may have a negative regulatory role in responding to multiple abiotic stresses, including salt stress in rice. Reducing or inactivating the *OsSRFP1* gene expression could enhance salt and cold tolerance in rice.

The promoters are binding regions of the RNA polymerase, which is responsible for the generation of RNAs (Peng *et al.*, 2016). The promoter region contains special DNA sequences (*cis*-acting elements), capable of binding to transcription factors, which enhances or inhibits the initiation of gene transcription (Peng *et al.*, 2016; Jin *et al.*, 2017). Previous studies have shown that mutations in the promoter region could lead to a change in gene expression levels (Yin *et al.*, 2011; Li *et al.*, 2019). In this study, we isolated and analysed the promoter sequence of the *OsSRFP1* gene. Then, different CRISPR/Cas9 vectors were designed and constructed to induce targeted mutations in the *OsSRFP1* promoter region to reduce the expression levels of this candidate gene. Different CRISPR/Cas9 induced mutations in the the *OsSRFP1* promoter region were detected and validated at the T0 generation. In addition, the inheritance and segregation of these indels were successfully confirmed at the progeny.

MATERIALS AND METHODS

Materials

Mature seeds of the Khang Dan 18 (KD18) rice variety were provided by the ThaiBinh Seed company. The pHUE411 vector (Plasmid #62203, Addgene, USA), *Escherichia coli* (*E. coli*) strain G10 and *Agrobacterium tumefaciens* (*A. tumefaciens*)

strain AGL1 were from the Plant Cell Biotechnology, Institute of Biotechnology. The primer list used in the study was shown in Table 1.

Table 1: Primers used in this research.

Primers	Nucleotide sequence (5' - 3')	Note
prSRFP1-F1	GAGGAGATTATCTGCGGCTCT	For PCR, sequencing
prSRFP1-R1	GCCTTAAATGCCAGCACTGT	
prSRFP1-F2	CGAATTTCCCAAGCAGAAGA	
prSRFP1-R2	ATCCTGCACCTCCTCCTGTA	
prSRFP1-F7	GGCGTAAGATCGGTTAGTTTGTCA	
prSRFP1-F11	ACTATAGCAAAAGTACCGTAG	
prSRFP1-R11	CGAATCCGTGGCGAGAC	
hptII-F	TTTATCGGCACTTTGCATCG	
hptII-R	GAGCATATACGCCCGGAGTC	
pUC18-F	CAGGGTTTTCCCAGTCACGA	
prSRFP1-g7-F	NNCGCCTCCTCAATCCAACCTTGC	For annealing
prSRFP1-g7-R	ANNCGCAAGGTTGGATTGAGGAGG	
prSRFP1-g11-F	NNCGGATCACTGTAGCGATAAATC	
prSRFP1-g11-R	ANNCGATTTATCGCTACAGTGATC	

Methods

Amplification and sequence analysis of OsSRFP1 promoter

Two primer pairs prSRFP1-F1/R1 and prSRFP1-F2/R2 (Table 1) were designed to amplify the promoter region of the *OsSRFP1* gene in the KD18 rice variety. The PCR thermal cyclers consisted of a 3 min initial denaturation at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 45 s at 72 °C, and a 5 min final extension at 72 °C. PCR products were electrophoresed on a 1% agarose gel, purified and sequenced on the ABI 3100 machine (Applied Biosystems, USA). The sequences were analyzed using MUSCLE 3.8.31 program.

CRISPR/Cas9 vector construction

Base on the stress-related cis-acting element sequences in the amplified promoter region of the *OsSRFP1* gene (Fang *et al.*, 2016) and the results of CCtop analysis (CRISPR/Cas9 target online predictor), 2 potential gRNAs were selected for the CRISPR/Cas9 vector construction. For each gRNA, forward and reverse primers (Table 1) were annealed and ligated into the pHUE411 vector (Plasmid #62203, Addgene, USA), which was digested with BsaI. The designed vector was transformed into *E. coli* cells. The PCR was performed and plasmids from the positive colonies were cultured, extracted, purified and sequenced to confirm

the constructed vector. Finally, the constructed vectors were mobilized into the *A. tumefaciens* strain AGL1 for further rice transformation.

Rice transformation and genotyping

The *Agrobacterium*-mediated method as described by our previous reports (Thao *et al.*, 2022) was used for rice transformation. Transgenic rice lines were selected and transferred into the greenhouse condition (16:8 h, light/dark photoperiod; temperature and humidity depend on environmental conditions). Total genomic DNA was extracted from leaf tissues of tested plants using the CTAB method (Doyle *et al.*, 1990). Specific primers (Table 1) were used to amplify the expanding targeted regions of the *OsSRFP1* promoter. CRISPR/Cas9 induced mutations on the targeted gene were detected by DNA banding shifts between mutant lines and wild-type on the heteroduplex analysis using PAGE (Zhu *et al.*, 2014). The PCR products were also purified by the GeneJET™ Gel Extraction Kit (Thermo Scientific, USA), followed the manufacture's protocol and used for sequencing by Sanger's method. The sequences were analyzed by the MUSCLE 3.8.31 program. In addition, the presence of *hptII* gene will be also confirmed in transgenic lines by PCR with specific primers.

RESULTS AND DISCUSSION

Characterization of the *OsSRFP1* promoter in KD18 variety

From the *OsSRFP1* reference gene sequence in opened sources (Stress-related RING finger protein 1, MSU ID LOC_Os03g22680), we designed prSRFP1-F1/R1 and prSRFP1-F2/R2 primers (Table 1) to amplify two DNA fragments in the *OsSRFP1* promoter region in the KD18 variety. PCR and gel electrophoresis showed the two expected DNA bands (> 1000 bp) (Figure 1). These DNA fragments were then purified and used for sequencing.

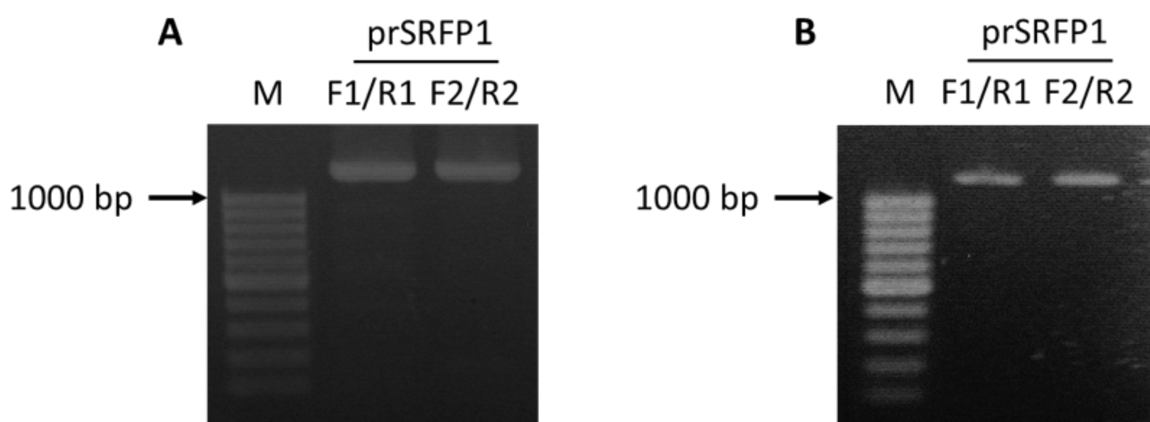

Figure 1. Electrophoresis of PCR product with prSRFP1-F1/R1 and prSRFP1-F2/R2 primers before (A) and after (B) purification (M: GeneRuler 100 bp DNA Ladder).

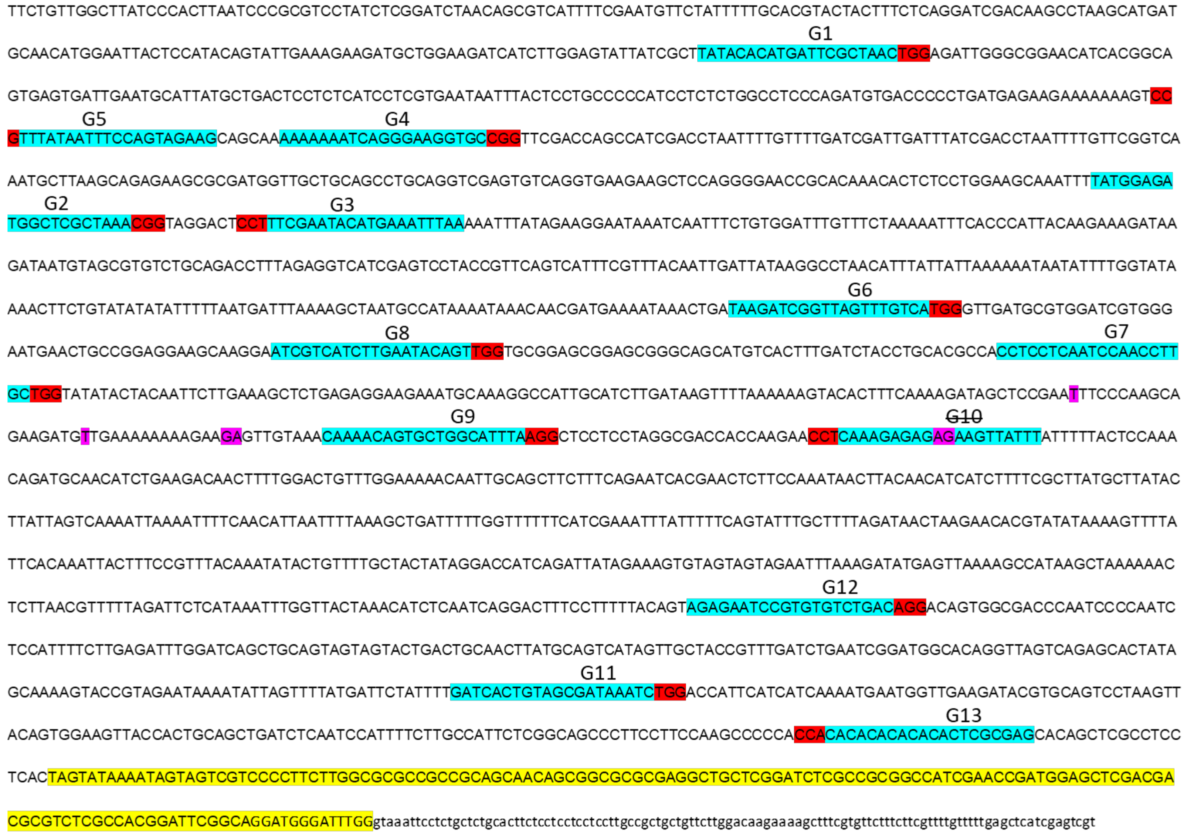

Figure 2. The locations of gRNAs on the *OsSRFP1* promoter region of KD18. Blue highlighted sequences: selected gRNA sequences, purple highlighted nucleotides: mismatched nucleotides, yellow highlighted sequence: coding sequence, lowercase sequence: intron region, red highlighted sequences: PAM sequences, strikethrough gRNA: rejected gRNA.

The sequencing results showed high similarity in the *OsSRFP1* promoter sequences between KD18 and the reference rice variety from the Oryzabase database with few mismatches (0.3%) (Figure 2). This sequence was also used to analyze stress-related *cis*-acting elements in the promoter region of *OsSRFP1* as reported by Fang and colleagues (Fang *et al.,* 2016). We found all stress-related cis-acting elements distributed in the *OsSRFP1* promoter of KD18 cultivar. Therefore, the promoter region (approximately 2 kb) of the *OsSRFP1* gene was successfully isolated and analyzed from the local rice cultivar (KD18). This sequence was used for analyzing and selecting potential gRNAs to construct CRISPR/Cas9 vectors.

Selection of gRNAs

Using the CCTop to analyse the *OsSFRP1* promoter sequence of KD18, we identified 13 potential gRNA sequences (Figure 2, Table 2). In which, G10 was not considered due to the mismatched nucleotides occurred in the *OsSFRP1* promoter of KD18 as compared to the reference sequence. Other gRNA sequences showed no mismatch, located right before the PAM position "NGG",

harbored GC ratio > = 30% and had no possibility to create hairpin structures. In addition, most off-target sites contained at least 4 different nucleotides with the on-targets and located mostly in the non-coding (I-intron) or the intergenic region (i) which should have no effect on gene function and expression.

Table 2. Selection of gRNA sequences (I: intron, i: intergenic region, purple highlighted nucleotides: undefined nucleotides, strikethrough gRNA: rejected gRNA).

Name	Strand	Sequence	Efficacy score	Off-target	Cis-acting elements (Fang et al., 2016)
G1	-	TATACACATGATTCGCTAACTGG	0.57	3I + 1i	MYCL + MYBL
G2	-	TATGGAGATGGCTCGCTAAACGG	0.73	3I + 4i	
G3	+	TTAAATTTCATGTATTCGAAAGG	0.59	8I + 3i	HEAT (2)
G4	-	AAAAAAATCAGGGAAGGTGCCGG	0.58	13I + 7i	MYBL
G5	+	CTTCTACTGGAAATTATAAACGG	0.73	12I + 8i	CNAC
G6	-	TAAGATCGGTTAGTTTGTCATGG	0.73	4I + 4i	MYBL (3)
G7	-	CCTCCTCAATCCAACCTTGCTGG	0.69	10I + 5i	NACF (2) + MYBS (2)
G8	-	ATCGTCATCTTGAATACAGTTGG	0.69	5I + 10i	EREF + GBOX + MYBL + MYCL + MIIG
G9	-	CAAAACAGTGCTGGCATTTAAGG	0.58	7I + 13i	
G10	+	AAATAACTTCTCTCTCTTTGAGG	0.69	8I + 12i	
G11	-	GATCACTGTAGCGATAAATCTGG	0.66	2I + 2i	HEAT
G12	-	AGAGAATCCGTGTGTCTGACAGG	0.61	8I + 8i	
G13	+	CTCGCGAGTGTGTGTGTGTGTGG	0.67	6I + 14i	SALT

Finally, 2 gRNAs (G7 and G11) were selected for the CRISPR/Cas9 vector construction. In which, targeted mutations in the G7 position could affect two NACF (NAC transcriptional factors) and two MYBS (MYB proteins with single DNA binding repeat) *cis*-element sequences. NACF *cis*-element is a binding site of the NAC transcription factors and plays important roles in the regulation of the transcriptional reprogramming associated with plant stress responses (Nuruzzaman *et al.,* 2013; Tweneboah, Oh, 2017). MYB is a large transcription factor family involved in

controlling various processes in plants such as responses to biotic and abiotic stresses, plant development, cell differentiation (Ambawat *et al.,* 2013). On the other hand, targeted mutations in the G11 site could damage HEAT *cis*-element sequences and affect transcription levels of the *OsSRFP1* gene.

CRISPR/Cas9 vector construction

Two gRNAs for targeting G7 and G11 sites in the OsSRFP1 promoter region of the KD18 cultivar were constructed in CRISPR/Cas9 systems. Particularly, two pairs of primers (prSRFP1-g7-F/R and prSRFP1-g11-F/R) (Table 1) were designed to generate 2 cassettes of gRNA scaffolds of G7 and G11 targets. After digestion by the restriction enzymes (BsaI), vector pHUE411 and the two cassettes were ligated to generate the recombinant vector (pHUE411/SRFP1-gRNAs). The recombinant vectors were transferred to *E. coli* cells and confirmed by PCR reactions. Two colonies (G7-4 and G11-6) showed expected PCR products (500 bp) on the gel electrophoresis. Plasmids were then isolated from these colonies and used for sequencing. The sequencing results confirmed the accuracy of the recombinant vectors (Figure 3). Two gene editing vectors pHUE411/SRFP1-G7 (S7) and pHUE411/SRFP1-G11 (S11) were created and mobilized into *A. tumefaciens* strain AGL1 for further rice transformation (Figure 4).

Figure 3. Plasmid sequencing analysis (yellow highlighted sequence: gRNA sequence, red highlighted sequences: PAM sequence).

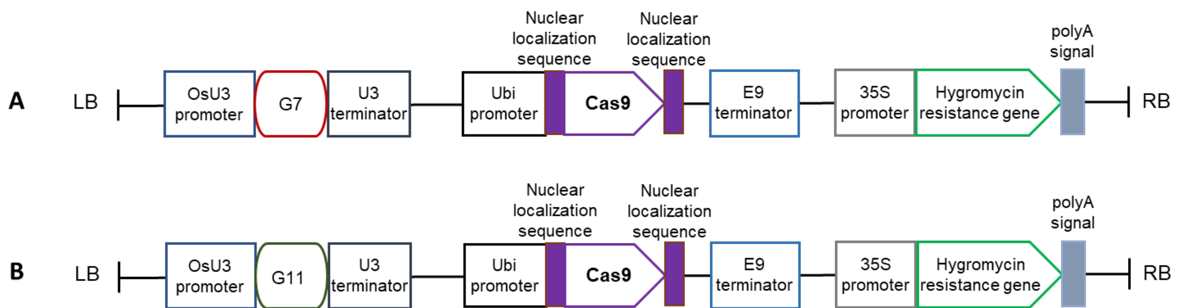

Figure 4. Diagram of CRISPR/Cas9 vectors. A: pHUE411/SRFP1-G7. B: pHUE411/SRFP1-G11.

Generation of T0 mutant rice from the pHUE411/SRFP1-G7 construct

A. tumefaciens strain AGL1 containing the CRISPR/Cas9 construct (pHUE411/SRFP1-G7) was used for rice transformation. On selection medium, 7 T0 lines were regenerated, total DNA was extracted from these plants and used for further analysis.

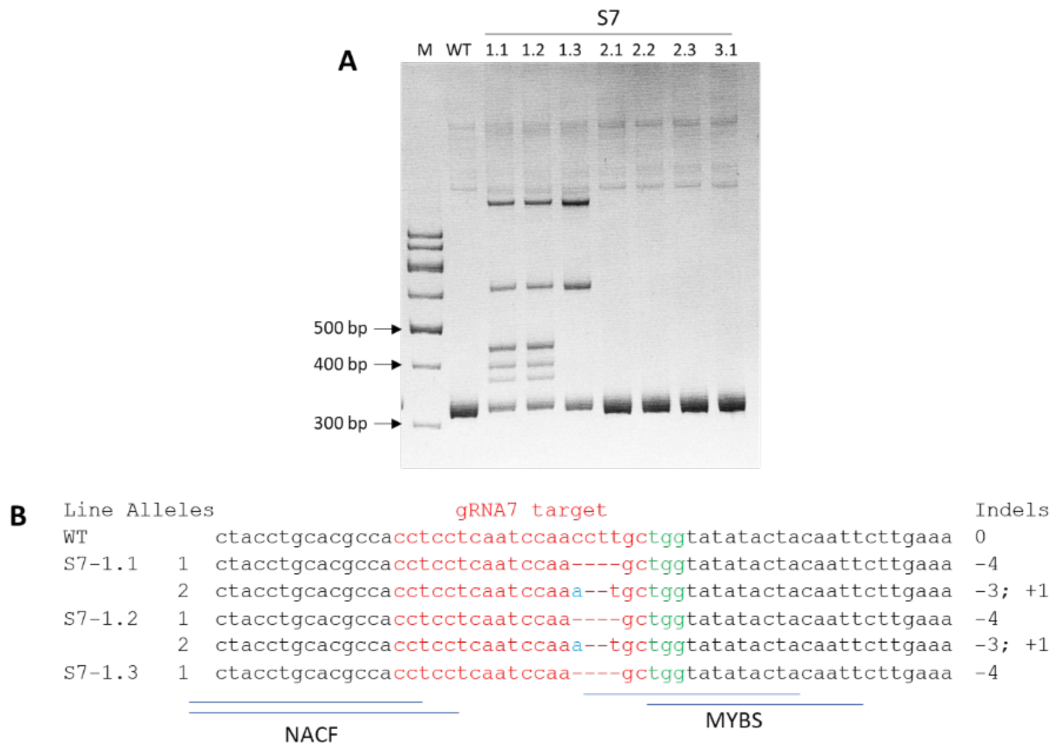

Figure 5. Identification and characterization of CRISPR/Cas9 induced mutations in T0 lines from the S7 construct. A: PAGE of T0 transgenic rice lines. M: GeneRuler 100 bp DNA Ladder, 1.1-3.1: T0 rice lines. B: Sequencing of targeted regions in the promoter of *OsSRFP1* gene. Targeted sequences (gRNAs) are indicated by red color, while the PAM sequences are indicated by green. The inserted sequence was shown in blue. Indels indicates targeted sequence changes: 0 for no change, − for deletion and + for insertion.

PAGE results showed that 3/7 regenerated plants carried mutations in the *OsSRFP1* promoter region indicating by DNA shifted bands as compared to wild-type (Figure 5A). In which, S7-1.1 and S7-1.2 lines exhibited the same DNA patent demonstrating the expectedly similar mutations (Figure 5A). The sequencing results were highly consistent to the PAGE analysis and showed that S7-1.1 and S7-1.2 lines carried biallelic mutations (-4; -3, +1 bp) (Figure 5B). Meanwhile, only one mutant allele (- 4 bp) was found from the S7-1.3 line. All mutations occurred at the target sites in the *OsSRFP1* promoter indicating the precise of the constructed CRISPR/Cas9 system in this study.

Generation of T0 mutant rice from the pHUE411/SRFP1-G11 construct

Using the same above *Agrobacterium*-mediated method, 26 T0 plants were

regenerated from the selection medium and transferred to the rooting medium. The PAGE analysis showed DNA shifted bands in 4/26 T0 plants (S11-3.7, S11-3.9, S11-3.11 and S11-3.13) as compared to the wild-type non-transgenic rice (Figure 6A). Sequencing results indicated that the 3 T0 lines carried heterozygous mutant forms (one mutant allele and one wild-type allele) (Figure 6B). The indel size was small and varied from -1 to -3 bp. In which, S11-3.11 and S11-3.13 lines had the same -1 bp mutant allele, while S11-3.9 harbored a -3 bp mutant allele. Similar to what observed from mutant lines of the pHUE411/SRFP1-G7 construct, all indels were found in the target sites (G11) of the *OsSRFP1* promoter. Furthermore, the mutations obtained at the target sites (G7 or G11) were all small deletion mutations, which had been reported in previous research in rice using CRISPR/Cas9 vectors with a single guide RNA (Shen *et al.,* 2017; Zhang *et al.*, 2019)

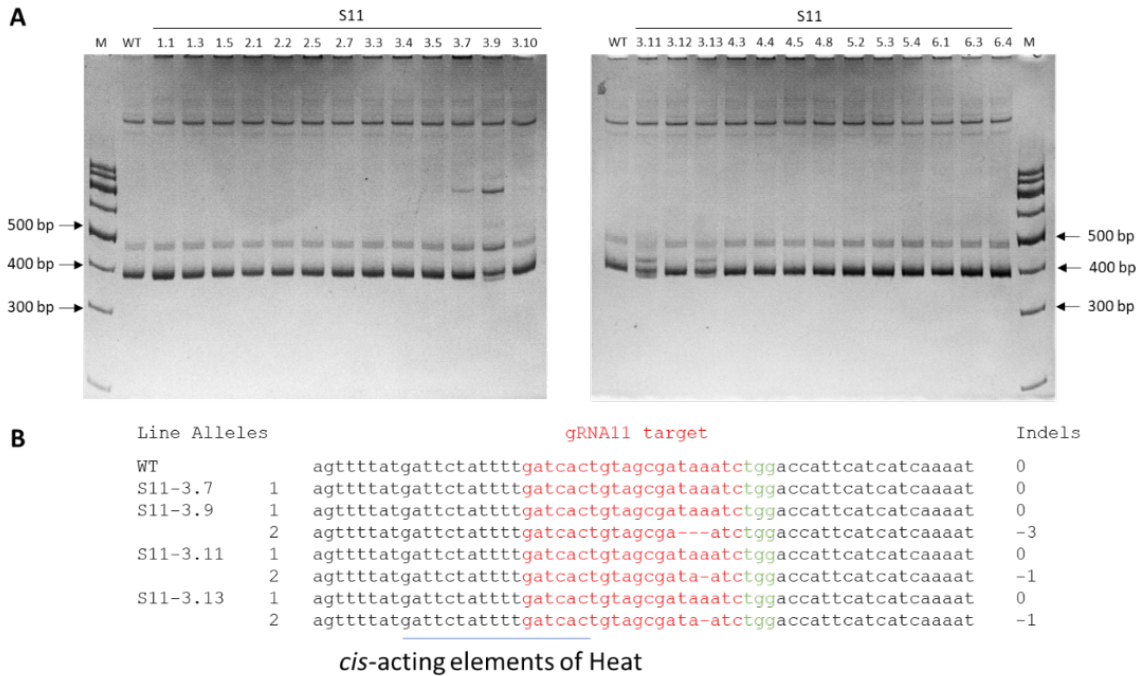

Figure 6. Identification and characterization of CRISPR/Cas9 induced mutations in T0 lines from the S11 construct. A: PAGE of T0 transgenic rice lines. M: GeneRuler 100 bp DNA Ladder, 1.1-6.4: T0 rice lines. B: Sequencing of targeted regions in the promoter of *OsSRFP1* gene. Targeted sequences (gRNAs) are indicated by red color, while the PAM sequences are indicated by green. Indels indicates targeted sequence changes: 0 for no change, − for deletion.

CRISPR/Cas9 induced mutant inheritance

To analyze the inheritance and segregation of CRISPR/Cas9 induced mutations and transgenes, different T1 seeds of two selected mutant lines (S7-1.1 and S11-3.13) were grown under the greenhouse condition. Total DNA was extract from these T1 plant and used for PCR with specific primers of transgenes (*hptII*) and for sequencing of the targeted regions.

In the S7-1.1 line

For the biallelic mutant line (S7-1.1), 4/12 lines were PCR positive for hptII-F/R primers indicating the inheritance of the transgene (*hptII*) in these plants (Figure 7A). PAGE results showed that 5 T1 plants (S7-1.1.1, S7-1.1.4, S7-1.1.6, S7-1.1.12 and S7-1.1.17) had the same DNA banding pattern as the S7-1.1 line, indicating they inherited all mutant alleles from the T0 generation (Figure 7B). Three T1 plants (S7-1.1.5, S7-1.1.10 and S7-1.1.15) showed an identical DNA banding pattern, while the other T1 plants (S7-1.1.7, S7-1.1.8, S7-1.1.9 and S7-1.1.16) carried another DNA banding pattern. These results indicated the inheritance of only one of the detected alleles from the T0 line (Figure 7B). In addition, the mutant alleles were observed at a ratio of 3:5:4 from the T1 progenies indicating the expected Mendelian segregation. This segregation result is consistent with a previous research by Zhang and colleagues in rice utilizing the CRISPR/Cas9 system (Zhang *et al.*, 2019).

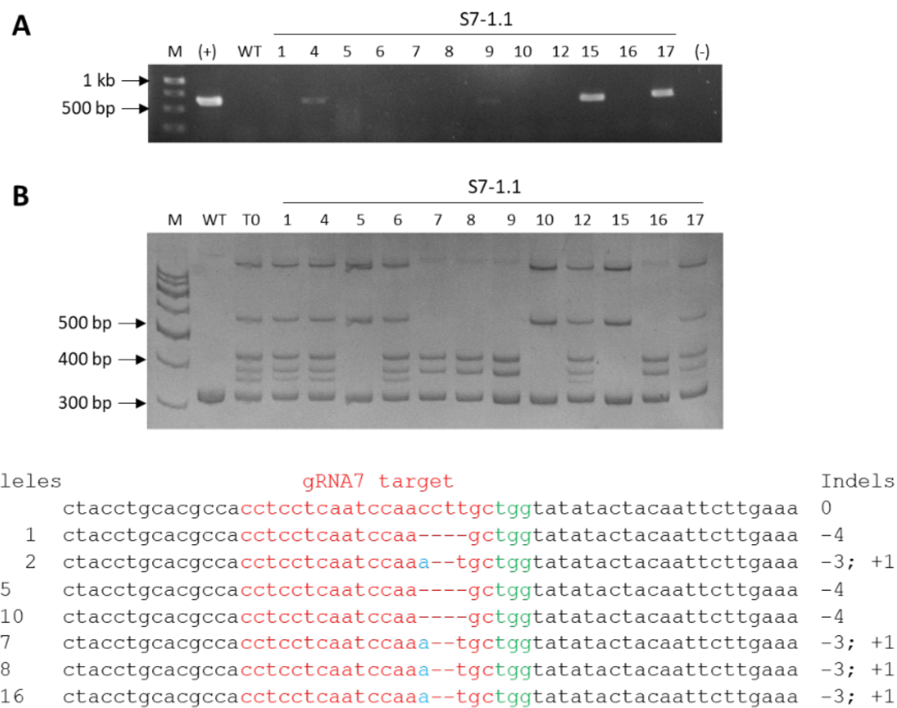

Figure 7. Genotyping analysis of T1 plants of S7-1.1 T0 line. A: Electrophoresis results on 1% agarose gel of PCR products with primer hptII-F/R. M: GeneRuler 1 kb DNA Ladder; (+): vector S7; (-): Negative control; 1-17: Different plants of T1 generation. B: PAGE of T1 transgenic rice lines. M: GeneRuler 100 bp DNA Ladder, 1-17: T1 rice lines. C: Sequencing of targeted regions in promoter of *OsSRFP1* gene. Targeted sequences (gRNAs) are indicated by red color, while the PAM sequences are indicated by green. The inserted sequence was shown in blue. Indels indicates targeted sequence changes: 0 for no change, − for deletion and + for insertion.

Sequencing data of selected T1 lines were completely consistent to the PAGE analysis results. In which, both S7-1.1.5 and S7-1.1.10 lines harbored -4 bp mutant allele

while three T1 lines (S7-1.1.7, S7-1.1.8 and S7-1.1.16) all carried the -3, +1 bp mutant allele (Figure 7C). This result indicated the CRISPR/Cas9 induced mutations on the G7 sites of the *OsSRFP1* promoter were stably passed to the progeny. In addition, we observed homozygous mutant lines as early as at the T1 generation.

In the S11-3.13 line

Analogous investigations were conducted utilizing hetero mutant line S11-3.13. In which, half of the tested T1 plants were free transgene (Figure 8A). PAGE results showed that most of T1 plants (except plant S11-3.13.2) had the same DNA banding pattern as the S11-3.13 line, indicating they inherited mutant allele from the T0 generation (Figure 8B). Sequencing data of selected T1 lines were entirely compatible with the outcomes of the PAGE analysis. In which, 3 T1 plants (S11-3.13.7, S11-3.13.11, S11-3.13.15) carrying the -1bp homozygous mutation were identified and they were all free transgene (Figure 8C).

In total, we generated 3 homozygous mutant lines (two mutant lines at position G7 and one mutant line at position G11) and they were all free transgene at the T1 generation. This result was also similar to previous publications with CRISPR/Cas9 genome editing on rice, indicating that free-transgene mutant lines could be generated as early as at the T1 generation (Wang *et al.*, 2016; Kim *et al.*, 2019; Zhang *et al.*, 2019).

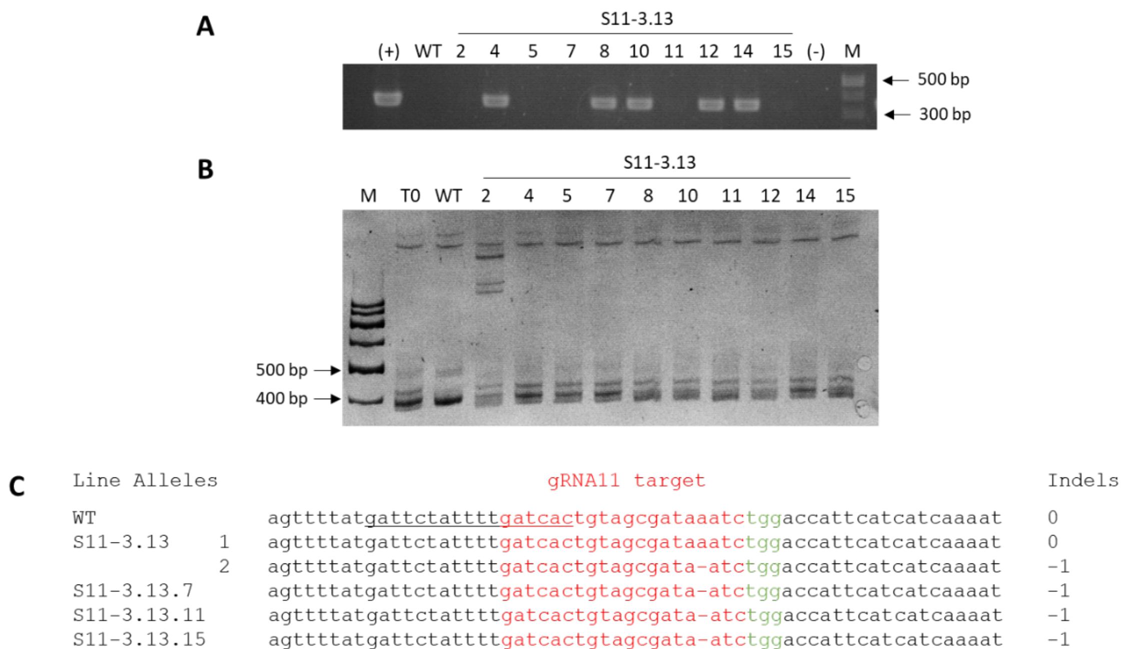

Figure 8. Genotyping analysis of T1 plants of S11-3.13 T0 line. A: Electrophoresis results on 1% agarose gel of PCR products with primer hptII-F/R. M: GeneRuler 1 kb DNA Ladder; (+): vector S11; (-): Negative control; 1-14: Different plants of T1 generation. B: PAGE of T1 transgenic rice lines. M: GeneRuler 100 bp DNA Ladder, 1-14: T1 rice lines. C: Sequencing of targeted regions in promoter of *OsSRFP1* gene. Targeted sequences (gRNAs) are indicated by red color, while the PAM sequences are indicated by green. Indels indicates targeted sequence changes: 0 for no change, − for deletion.

CONCLUSION

We successfully designed and constructed CRISPR/Cas9 vectors to induce targeted mutation of the *OsSRFP1* promoter in a local rice cultivar KD18. These vectors were transferred into the rice genome via *Agrobacterium*-mediated method and created induced mutations in target sites of the *OsSRFP1* promoter. The inheritance and segregation of CRISPR/Cas9 induced mutations were validated in the next generation. The homozygous mutant forms of the *OsSRFP1* promoter were created and selected at the T1 generation. Further research should be carried out to evaluate *OsSRFP1* expression, growth, development and salt tolerance of mutant plants.

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