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Genome Editing of Rice *PFT1* Gene to Study its Role in Rice Sheath Blight Disease Resistance

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A B S T R A C T

The CRISPR/Cas9 system has been used predominantly for precise editing of the plant genomes. In the present study, we have made an application of CRISPR/Cas9 system for targeted mutagenesis in rice(*Oryza sativa* L.) targeting the *Phytochrome and Flowering Time 1* (*PFT1*) gene known to be involved in disease susceptibility in *Arabidopsis* for root-infecting hemibiotrophic fungal pathogen *Fusarium oxysporum.* Hence, to confirm the role of *OsPFT1* gene in rice sheath blight disease, CRISPR-Cas9 gene editing tool is being employed for generating *OsPFT1* gene knock out in rice. Two guide RNAs (gRNAs) were designed to pair with distinct *OsPFT1* gene region followed by protospacer-adjacent motif (PAM). Rice genome editing constructs were cloned and mobilized into the *Agrobacterium* strain LBA4404. The transgene (Cas9-gRNA) introduction into *indica* rice variety ASD16was done via *Agrobacterium*-mediated rice transformation by using immature embryos as explants. The gRNA will direct the Cas9 nuclease to generate double-strand breaks (DSB) at the specific sites of *OsPFT*1 gene, thereby introducing mutations at the DSB by error-prone non-homologues end joining repair mechanism. Through PCR analysis, the presence of the *hpt* transgene was identified. Homozygous gene-edited transgenic rice plants will be identified and further will be subjected to sheath blight disease screening.

Introduction

Genome targeted mutagenesis plays a crucial role in crop improvement. CRISPR/Cas9 has been an emerging molecular tool in the field of genome editing (Sun *et al.,* 2016). It is a powerful RNA-guided DNA targeting platform for editing the genome of different organisms (Jiang and Doudna 2017). This technology makes use of the specific ~20bp guide RNA for targeting the specific genomic loci (Ran *et al.,* 2013). Rice (*Oryza sativa* L.) is susceptible to many diseases including sheath blight. Rice sheath blight (ShB) is caused by *Rhizoctonia solani* Kuhn, [Teleomorph stage, *Thanatephorus cucumeris* (Frank) Donk]. It is regarded as a second internationally important disease after rice blast (Dasgupta, 1992). Yield loss of 8% to 50% was reported due to ShB (Savary *et al.,*

2000). The study made by Backstrom *et al.,* (2007) has suggested that *PFT1* protein (MEDIATOR25) is a part of the conserved multiprotein mediator complex that plays an essential role in initiating transcription by acting as a "universal adaptor" between RNA polymerase II and DNA-bound transcription factors. Arabidopsis *PFT1* gene is involved in disease susceptibility to root-infecting hemibiotrophic fungal pathogen *Fusarium oxysporum* (Thatcher *et al.,* 2009). A homolog of wheat of *PFT1*/MED25 complemented the defensive and developmental phenotypes of the *pft*1 mutant, which suggests about the conserved function of *PFT1* in wheat and possibly in rice as well (Kidd *et al.,* 2009). Hence, an attempt was made to characterize the functional role played by *OsPFT1* gene in rice ShB disease resistance and knocking out of *OsPFT1* by CRISPR-Cas9 to study its role in resistance.

Materials and Methods

Designing candidate Cas9/gRNA target site in rice genome

Targeted sites of *OsPFT1* were chosen (Fig. 1) in exon 1 and exon 3 and 20-nt unique guide sequences (5'A N20 GG 3') were identified by using the web based tool such as 'CRISPR PLANT' database (Xie *et al.,* 2014) (http://www.genome.arizona.edu/crispr/) and CRISPR-P2.0 (Liu *et al.,* 2017). The selected 20-nt sgRNA-binding sequence precedes immediately the NGG PAMto make the target sequence unique. The CRISPR-Cas9 based pRGEB32 vector was used for expressing the selected guide RNA. The pRGEB32 express Cas9 gene under rice ubiquitin promoter and possess hygromycin resistance gene (*hpt)* driven by *CaMV35S* promoter as plant selectable marker (Fig. 2). The PS-I and PS-II construct harbouring gRNA were prepared as follows: Firstly, the empty pRGEB32 vector was digested with *Bsa*I and gel eluted. Then, oligo-duplex was prepared by using *OsPFT1* gRNAs F and R sequence of IgRNA and IIgRNA respectively (Table 2). For ligation of the oligo-duplex with the appropriate adapters into the purified *Bsa*I digested vector, the oligo duplex was diluted 1:200 and separate ligation reaction was incubated at 4 °C overnight. The confirmation of ligated product was done by amplifying the ligated product using pRGEB32 specific forward and *OsPFT1*gRNA specific reverse primers or vice-versa (Table 2). The two PS-I and PS-II CRISPR-Cas9-gRNA constructs based on pRGEB32 harboring oligo duplex (gRNA) for knocking out *OsPFT1* gene. The ligated products were then used to transform competent *E. coli* (DH5α) cells. The constructs were further confirmed by restriction digestion by *Bsa*I and *Xba*I of the plasmid DNA isolated from the transformed colonies followed by sequencing of the isolated plasmid DNA for PS-I and PS-II construct respectively.

Transformation of Cas9 and gRNA constructs

Agrobacterium-mediated transformation of *indica* rice cultivar ASD16using immature embryos derived calli was performed as described by Hiei and Komari (2008). Hence the PS-I and PS-II CRISPR/Cas9–gRNA constructs were mobilized into the *Agrobacterium* strain (LBA4404) by using tri-parental mating. Randomly isolated mobilized transconjugants colonies were selected for PCR screening for the presence of *vir* and *hpt*II specific primer sets with appropriate PCR conditions. Then the isolated immature embryos were infected by *Agrobacterium* carrying the PS-I and PS-II constructs respectively. After 7 days of co-cultivation, the elongated shoots from immature embryos were removed and calli were transferred to resting medium, CCMC containing cefotaxim 250mg/l to control *Agrobacterium*

overgrowth. Further for the selection of transformed calli, the calli were placed on the selection medium, CCMCH50 supplemented with 50g/l hygromycin. Hygromycin resistant calli were selected over 17 days. Proliferating calli were then transferred to the regeneration media supplemented with 30g/l glutamine and 40g/l hygromycin. The regenerated shoots after 14 days were transferred to rooting medium followed by hardening in green house.

Results and Discussion

Target selection and construction of the CRISPR/Cas9 system

In rice (*Oryza sativa)*, the *PFT1* gene is located on chromosome 9 and the locus id is LOC_Os09g13610. Two sgRNAs (IgRNA and IIgRNA) targeting the first exon and third exon of *PFT1* respectively were designed (Fig. 1) by the aid of web based tool 'CRISPR PLANT' database (http://www.genome. arizona.edu/crispr/) and CRISPR-P2.0. The designed sgRNA along with the various parameters are present in the Table 1.

The PS-I (pRGEB32 + IgRNA) and PS-II (pRGEB32 + IIgRNA) construct were prepared by ligating oligo duplex for IgRNA and IIgRNA with the appropriate adapters into the *Bsa*I digested vector and were confirmed by the PCR analysis. The pRGEB32 vector specific forward and *OsPFT1* gRNA specific reverse primer showed an expected amplification of 280bp while the other set of primer gRNA specific forward primer and pRGEB32 vector specific reverse primer (Table 2) shows specific amplification of 208 bp. The *Bsa*I digested plasmid was used as negative control which does not show any amplification. The confirmed ligated products were transformed into the *E. coli* DH5α competent cells, the recombinant colonies were screened through colony PCR by the pRGEB32 vector specific forward and *OsPFT1*gRNA specific reverse primer (Table 2). The positive colonies were selected and subsequently the plasmid DNA was isolated from the recombinant colonies. Further confirmation was made by restriction digestion of the plasmid DNA by *Bsa*I and *Xba*I. The transformed colonies will not be digested by *Bsa*I as due to the insertion of gRNA, the recognition site of *Bsa*I was disturbed so there will be only single digestion by *Xba*I. Finally, PS-I and PS-II constructs were confirmed by sequencing.

CRISPR/Cas9- mediated genome editing of the *PFT1* gene of *indica* rice for developing ShB resistance

Agrobacterium-mediated transformation of *indica* rice was done to knock out the *PFT1*gene using the CRISPR/Cas9 construct PS-I (pRGEB32 + IgRNA) and PS-II (pRGEB32 + IIgRNA), harboring *OsPFT1* gene specific sgRNA and a hygromycin as plant selectable marker respectively. The gene constructs were mobilized into Agrobacterium strain LBA4404separately. The presence of the Cas9 plasmid, PS-I (pRGEB32 + IgRNA) and PS-II (pRGEB32 + IIgRNA) in the *Agrobacterium* strains were confirmed by colony PCR for the presence of 440bp *virG*, 686 bp *hptII* and integrity of construct was further confirmed by back transformation. Negative controls did not show any amplification in *hptII/virG* specific primers sets (Fig. 5). Total *Agrobacterium* genomic DNA was isolated from three PS-I (pRGEB32 + IgRNA) and PS-II (pRGEB32 + IIgRNA) transconjugant respectively followed by transformation in *E. coli*. Plasmid DNA was isolated from *E. coli* transformants and was used for restriction enzyme digestion. Restriction digestion of plasmid isolated from back-transformed *E. coli* for three PS-I (pRGEB32 + IgRNA) and PS-II (pRGEB32 + IIgRNA) respectively was done with *Bsa*I and

*Xba*I to confirm the presence of the *OsPFT1* gene specific gRNA into the PS-I and PS-II constructs transconjugants. Restriction enzyme digestion resulted in only single digestion by *XbaI* as expected (Fig. 1–5).

Fig.1 Schematic map of the gRNA target site selection in the target *OsPFT1* gene. Two sgRNAs (I gRNA and II gRNA) of the *PFT1* gene were selected, corresponding to the sites in the first and the third exon, respectively

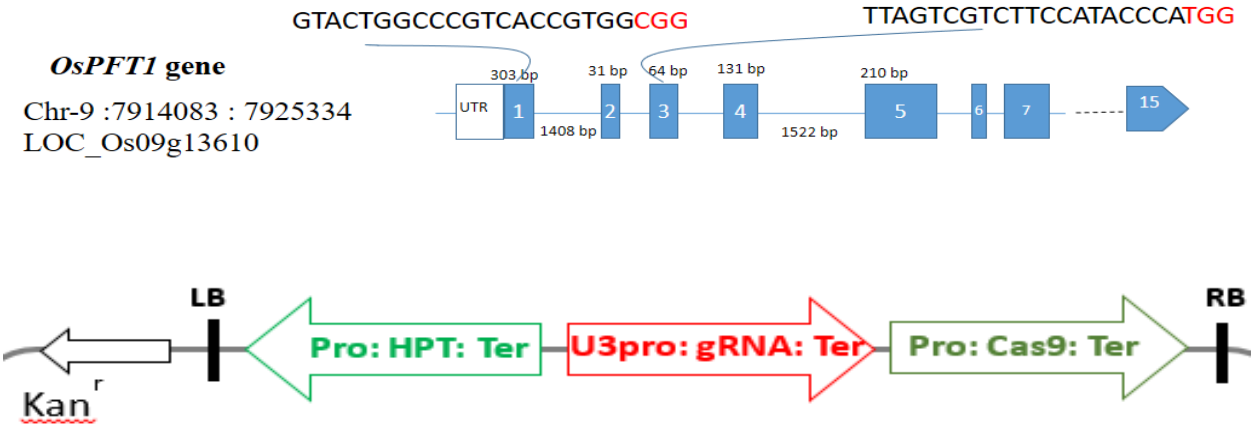


Fig. 2 CRISPR-Cas9-gRNA construct

Fig.3 PCR analysis of ligated products (a) I gRNA products with pRGEB32 F-primer and OsPFT1 R-primer (280 bp) and OsPFT1 F-primer and pRGEB32 R-primer (208 bp) (b) II gRNA products with pRGEB32 F-primer and OsPFT1 R-primer (280 bp) and OsPFT1 F-primer and pRGEB32 R-primer(208 bp) (Table 2) and (c) Restriction analysis of clones from the ligation of two dsOligo (I gRNA and II gRNA) fragments into *Bsa*I digested pRGEB32 individually. The individual recombinant clones of PS-I construct (pRGEB 32 + I gRNA) and PS-II (pRGEB 32 + II gRNA) with *Bsa*I and *Xba*I. The *Bsa*I undigested clones are the positive ones that are ready for mobilization into *Agrobacterium*.

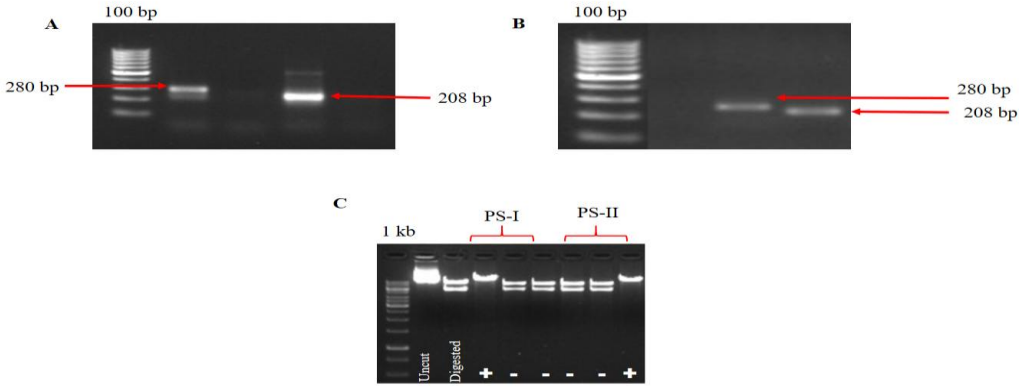


Fig.4 Sequence of the PS-I and PS-II cloned construct. Yellow label bars indicate the cloned I and II sgRNA sequence in the pRGEB32 vector

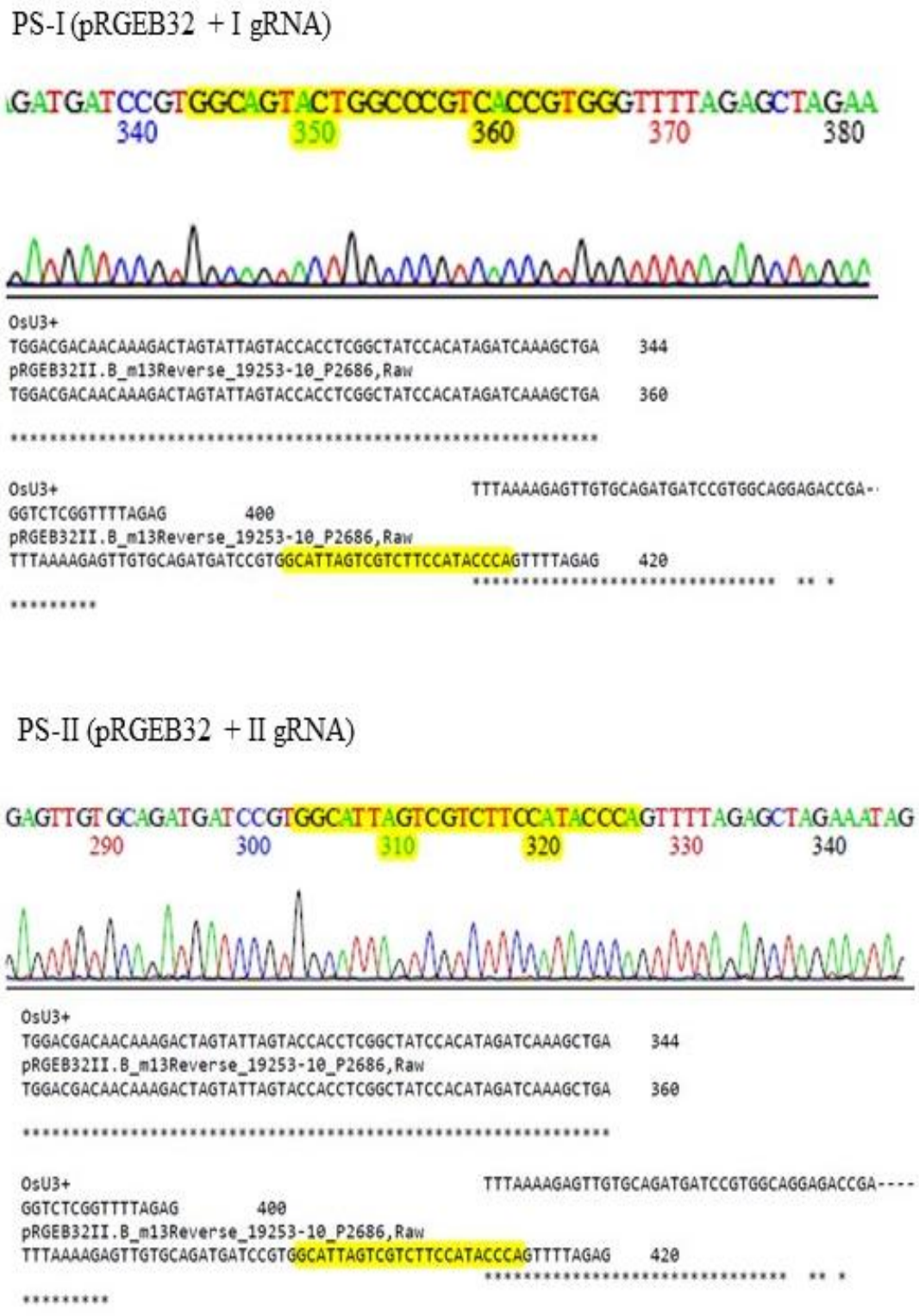


Fig.5 The clones of PS-I and PS-II construct were mobilized into *Agrobacterium* and PCR analysis of the TC (Transconjugants Colonies) by a and b *virG*and c and d*hpt*II for PS-I and PS-II construct respectively

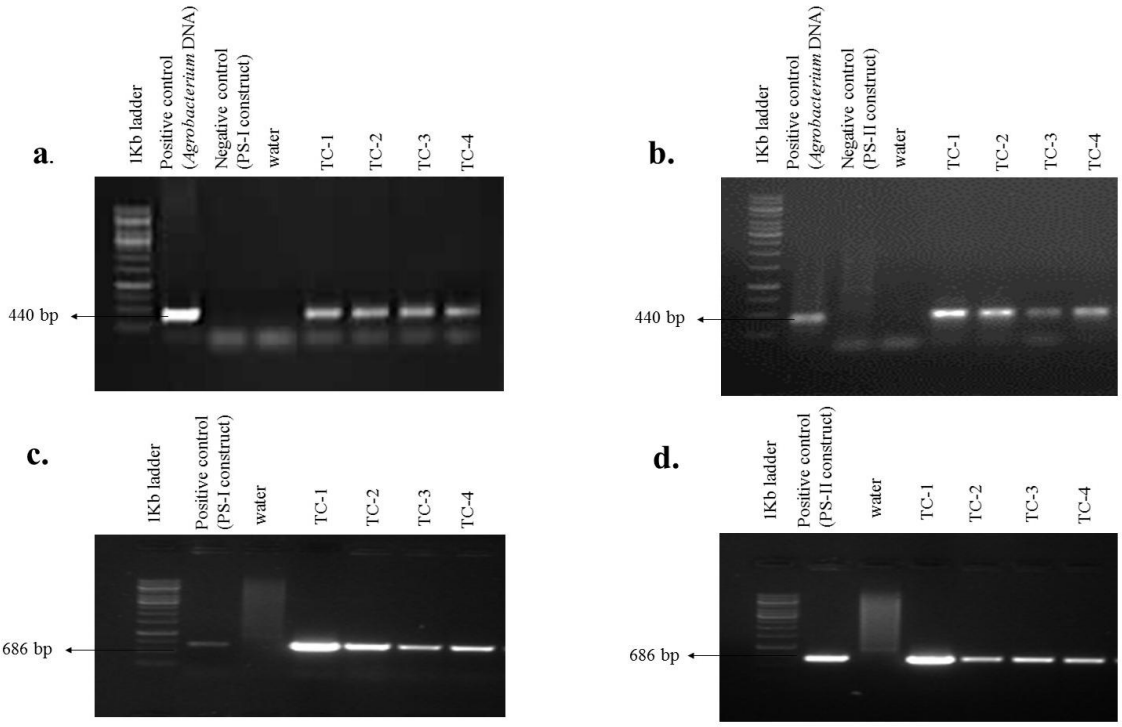
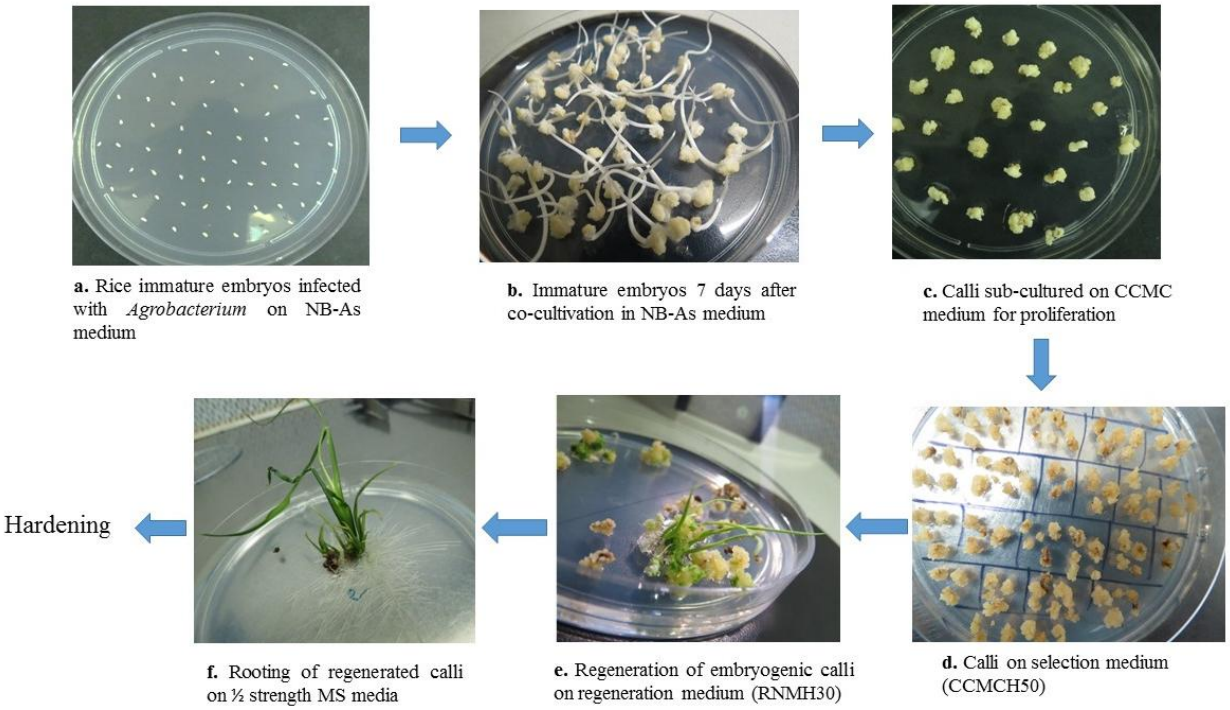


Fig.6 *Agrobacterium* mediated transformation of immature embryos of rice cultivar ASD16



a. Rice immature embryos infected with *Agrobacterium* on NB-As medium

b. Immature embryos 7 days after co-cultivation in NB-As medium

c. Calli sub-cultured on CCMC medium for proliferation

d. Calli on selection medium (CCMCH50)

e. Regeneration of embryogenic calli on regeneration medium (RNMH30)

f. Rooting of regenerated calli on ½ strength MS media

Table.1 Selected gRNAs on the basis of the various parameters

Sequence ID	Spacer sequence(5'- 3')	PAM	Strand	Location	Exon No.	On Target	Off Target	Restriction sites
Chr9:7914340-7914360	GTACTGGCCCGTCACCGTGG	CGGACTACGT	+	Exon	**1**	**0.61**	**22**	HpyCH4IV cut ACGT EciI cut GGCGGA
Chr9:7915955-7915975	TTAGTCGTCTTCCATACCCA	TGGTCCTTAT	+	Exon	**3**	**0.75**	**19**	NlaIII cut CATG CviAII cut CATG FatI cut CATG NcoI cut CCATGG

Table.2 Primer and sequences used in the protocol

S. No.	Sequences (5'- 3')	Purpose
pRGEB32-F	AGGCGTCTTCTACTGGTGCT	PCR for ligated product and clones verification
pRGEB32-R	CCTCCGTATTTGCTGACGTG	PCR for ligated product and clones verification
OsPFT1 g 1 F	GGCAGTACTGGCCCGTCACCGTGG	Cloning sgRNA into pRGEB32
OsPFT1 g 1 R	CATGACCGGGCAGTGGCACCCAAA	PCR for ligated product and clone verification Cloning sgRNA into pRGEB32
OsPFT1 g 2 F	GGCATTAGTCGTCTTCCATACCCA	PCR for ligated product and clone verification Cloning sgRNA into pRGEB32
OsPFT1 g 2 R	AATCAGCAGAAGGTATGGGTCAAA	PCR for ligated product and clone verification Cloning sgRNA into pRGEB32
		PCR for ligated product and clone verification

The protocol reported by Hiei and Komari (2008), was used for rice transformation. Immature embryos of ASD16 rice plants were used as explants in *Agrobacterium*-mediated transformation experiments with PS-I and PS-II gene construct. After co-cultivation, the developed embryogenic calli were sub-cultured twice on hygromycin containing selection medium. The hygromycin resistant calli survived selection process and grew well

in two rounds of selection, but the untransformed calli turned brown and dried. The calli lines of ASD16 were recovered after two rounds of hygromycin selection. The selected embryogenic calli obtained after two rounds of selection were transferred to pre-regeneration and then to regeneration medium for shoot induction.

On regeneration medium, calli exhibited greening and small shoots started emerging within 8-10 days (Fig. 6). The transgenic plants obtained will then be subjected to the target mutation analysis by PCR/RE assay(Shan *et al.,* 2014, Char *et al.,* 2017). In PCR/RE assay, target locus includes restriction enzyme site that is destroyed by CRISPR/Cas-induced mutation. The CRISPR/Cas-PFT1 mutant will remain undigested while the non-mutant shows the digested bands. Further characterization of uncleaved bands can be made by sequencing. So as to validate these transgenic plants further molecular analysis has to be done by the PCR/RE assay as well as by sequencing to observe the expected mutation in the regenerated plant. In addition, the performance against the sheath blight disease will be assessed in the transgenic plants mutated for *OsPFT1*.

CRISPR/Cas9 has proven to be widely emerging and applicable genome editing tool for the production of new improved varieties, which exhibits enhanced disease resistance and other improved traits such as stress tolerance, nutritional improvement, and yield increment (Zong *et al.,* 2017). The production of rice is hampered by various biotic and abiotic constraints among these the fungal, bacterial and viral constraints are the major ones. One such fungal constraint is rice sheath blight (ShB) and the causal agent for it is *Rhizoctonia solani.* For control of ShB in rice via biotechnological approaches, an attempt was made for knocking out of the susceptibility gene *PFT1* (Kidd *et al.,* 2009). The ~20bp guide RNAs were designed for targeting the initial exon that is the exon 1 and 3 so that initially there is no expression of *PFT1*gene.A ShB susceptible *indica* rice cultivar, ASD16 was selected for the transformation with PS-I and PS-II CRISPR/Cas9 construct mobilized into *Agrobacterium*. In this study, immature embryos were used as target tissue for transformation since the immature embryo has high regeneration and transformation efficiency, although both immature embryos and mature seeds have been reported to be an excellent starting material for rice transformation *via Agrobacterium* method (Hiei and Komari 2008). After several rounds of *Agrobacterium*-mediated transformation, putative transgenic plants were recovered. Transgenic plants generated will be tested for its performance against the sheath blight disease.

In conclusions, rice (*Oryza sativa* L.) is one of the most widely cultivated, at the same time affected by various biotic and abiotic factors. Rice trait improvement appears really promising for the future and will surely be influenced by the developments in CRISPR/Cas9 technologies. This induced mutation will help to generate disease resistant rice varieties. In our paper, we efficiently applied targeted mutagenesis in immature embryos rice cultivar ASD16 using the CRISPR/Cas9 system and transgenic rice plants were generated. The CRISPR/Cas9 system can be used for targeted mutations and facilitating rice genetic improvement.

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