Original Research Article

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 ASD16 was 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 OsPFT1 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.

Keywords
Gene editing, CRISPR/Cas9, Rice transformation, Phytochrome and flowering time 1, Sheath blight resistance

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., 1992).
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 pft1 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 PAM to 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 Bsal 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 Bsal 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 OsPFT1gRNA 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 Bsal and XbaI 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 ASD16 using 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 triparental mating. Randomly isolated mobilized transconjugants colonies were selected for PCR screening for the presence of vir and hptII 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 BsaI 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 BsaI 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 OsPFT1gRNA 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 BsaI and XbaI. The transformed colonies will not be digested by BsaI as due to the insertion of gRNA, the recognition site of BsaI was disturbed so there will be only single digestion by XbaI. 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 LBA4404 separately. 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 BsaI and
XbaI 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.

**OsPFT1 gene**

Chr-9 :7914083 : 7925334
LOC_Os09g13610

![Schematic map of the gRNA target site selection in the target OsPFT1 gene.](image)

**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 BsaI digested pRGEB32 individually. The individual recombinant clones of PS-I construct (pRGEB 32 + I gRNA) and PS-II (pRGEB 32 + II gRNA) with BsaI and XbaI. The BsaI 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 *dhptII* for PS-I and PS-II construct respectively.

**Fig. 6** *Agrobacterium* mediated transformation of immature embryos of rice cultivar ASD16.
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                  |
|-----------------|-------------------------|--------------|--------|------------|----------|-----------|------------|------------------------------------|
| Chr9:7914-340-7914360 | GTACTGGCCCGTCACC GTGG | CGGACTACGT   | +      | Exon       | 1        | 0.61      | 22         | HpyCH4IV cut ACGT EciI cut GGCGGA |
| Chr9:7915-955-7915975 | TTAGTCGTCTTCCATACC CCA | 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 | AGGCCTTCTTACTGGTGCT | PCR for ligated product and clones verification |
| pRGEB32-R | CCTCCGTATTTGCTGACGTG | PCR for ligated product and clones verification |
| OsPFT1 g 1 F | GGCA GTACTGGCCCGTCACC GGG | Cloning sgRNA into pRGEB32 |
| OsPFT1 g 1 R | CATGACCGGCAGTGACCGACC AAA | PCR for ligated product and clone verification Cloning sgRNA into pRGEB32 |
| OsPFT1 g 2 F | GCCATTAGTCGTCTTCCATACC CA | PCR for ligated product and clone verification Cloning sgRNA into pRGEB32 |
| OsPFT1 g 2 R | AATCAGGAGGTTATGGCCGT AAA | PCR for ligated product and clone verification Cloning sgRNA into pRGEB32 |

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 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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