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RESEARCH ARTICLE

Genome Engineering for Thermo-sensitive Genic Male Sterilty (TGMS) in rice using CRISPR/Cas9 editing system

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ABSTRACT

Thermosensitive genic male sterile (TGMS) rice genotypes, which are male-sterile at the restrictive temperature (>26°C) but becomes male-fertile at the permissive temperature (< 22°C) are widely used in hybrid rice breeding. Mutation in *tms5* gene located on chromosome 2 is responsible for the male-sterility trait in rice. Through gene editing, it is possible to create a specific mutation in the *tms5* locus of male fertile rice lines and convert them into male sterile lines. The CRISPR/Cas9 editing system was employed to induce specific mutation in *tms5* gene of rice variety ASD16. CRISPR/Cas9 vector with guide RNA targeting the second exon of *tms5* gene was constructed and used for rice transformation. Four putative transgenic ASD16 events were generated through rice immature embryo transformation and analysed for the presence of *hpt* and mutation in *tms5* locus in T_0 generation.

Keywords: *Thermosensitive genic male sterility (TGMS), tms5 gene, two-line hybrid rice, CRISPR/Cas9.*

INTRODUCTION

Rice is the staple food crop that feeds over half of the world's population (Khush 2005). The population will reach 10 billion in 2050 to feed the world the production has to be increased by 60–100% (FAOSTAT, 2016). Hybrid rice has an advantage over inbreeding varieties to increase yield by 10-20 percent (Li *et al.,* 2007). Conventionally, the production of hybrid rice is based on two-line and three-line breeding systems. Three line breeding systems utilize the cytoplasmic male sterile line (A line), maintainer line (B line) and restore line (R line) for hybrid rice production. It is difficult to maintain those line due to limited genetic resources of restorer and male sterile line. The two-line breeding system uses either photo-sensitive genic male sterility or thermo-sensitive genic male sterility (Chen and Liu 2014). In thermo-sensitive genic male sterile line, fertility is restored under permissive temperature and become sterile under the restrictive temperature.

Indica rice variety AnnongS-1 (AnS-1) is the first TGMS line found to be controlled by a recessive gene *tms5* (Wang *et al.,* 2003). *TMS5* gene encodes ribonuclease Z (RNase Z), has a major role in thermo-sensitive genic male sterility (Yang *et al.,* 2007). RNase Z^{S1} controls the TGMS trait by degrading the temperature sensitive ubiquitin fusion ribosomal protein L40 (UB_{L40}) mRNA. The RNase Z^{S1} expression is not affected by an increase or decrease in temperature. RNase Z^{S1} has its function in the cytoplasm but not in the nucleus. During permissive temperature, it will produce normal pollen grains. During restrictive temperature in the *tms5* mutant, no RNase Z^{S1} is produced and it will lead to unprocessed the Ub_{L40} mRNA and finally leads to pollen sterility. More $UbL40_1$ and $UbL40_4$ mRNA will be produced in the transgenic plant by over-expression of $UbL40_1$ and $UbL40_4$, leading to partial pollen abortion. Whereas $UbL40_1$ and $UbL40_4$ knockdown plants produce reduced $UbL40_1$ and $UbL40_4$ mRNA and partially restored the pollen fertility (Zhou *et al.,* 2014).

A few sequence-specific nucleases including zinc finger nucleases (ZFNs) and TAL effector nucleases (TALENs) have been used in plants for their genetic variation effectively over the past decade (Voytas and Gao 2014). TAL effector nucleases (TALENs) and zinc finger nucleases (ZFNs) comprise the DNA binding domain as well as the *FokI* nuclease domain as their role in recognizing and cleaving the targets present in the DNA sequence (Kim *et al.,* 1996). There are high chances of failure in recognizing and cutting the targeted site by using those techniques (Voytas 2013). The newly emerging CRISPR/Cas9 technique is therefore simple and is based on the RNA guided genome editing (RGE) and used in different organisms (Xie and Yang, 2013).

CRISPR/Cas (clustered regularly interspaced short palindromic repeat/CRISPR-associated proteins) system initially performs as an adaptive immunity in opposition to invasive phage and

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plasmids in a wide range of archaea and bacteria (Barrangou 2013). Modern CRISPR/Cas approach is based on the *Streptococcus pyogenes,* which is of type II classification and it has an important role in genome engineering. Cas9 protein obtained from *S. pyogenes* will involve in genome engineering by utilizing the nuclease activity (Tsai and Joung 2016). The Type II CRISPR system, together with two non-coding RNAs, the crRNA and the trans-activating crRNA (tracrRNA), plays an important role in identity and cleaving the target DNA via the RNA-guided endonuclease activity Cas9 (Deltcheva *et al.,* 2011). The crRNA hybridizes along with the tracrRNA will produce a crRNA: tracrRNA duplex. This duplex is manipulated into Cas9 to target similar DNA sequences along with suitable Protospacer-adjacent motifs (PAM). PAM is the preserved sequence which is of 2- 6 nucleotides length present after the target site. In general, the PAM sequence plays an important role in the recognitionof the targets (Mojica *et al.,* 2009). By using these two nuclease domains NHN and RuvC, the cas9 will cut the complementary and non-complementary strand on the gRNA insertion region (Nishimasu *et al.,* 2014). The Cas9 protein which is guided by the gRNA will chop the targeted sequence 3 nucleotides upstream of the protospacer-adjacent motif (PAM) and produces the site-specific DNA double-strand breaks (DSBs) (Tsai and Joung 2016). These double strand breaks are repaired by the cell endogenous repair mechanisms, such as NHEJ and HDR. In NHEJ the break ends are ligated directly without the use of any homologous template DNA. In NHEJ, the ends were joined by DNA IV Ligase enzyme. In the case of the NHEJ pathway, there is a possibility of both insertion or deletion in the target site and it causes mutation which leads to gene disruption. In the HDR pathway, it uses the donor template to repair the DSB by replacing the single nucleotide at the target site. Various modification of the gene carried out due to insertion it may produce the novel genes or knockout the present gene.

MATERIAL AND METHODS

Designing of sgRNA

The gene model for *tms5* (LOC_Os02g12290) *was predicted from* the rice genome annotation project database. The *tms5* gene has 6 exons and 5 intron regions present in chromosome 2 of the rice from 6397342 to 6399236 nucleotide position and the size of the gene is approximately 1.89 kb (Fig.1a).

Guide RNA was designed using web based tool CRISPR-PLANT (http://www.genome.arizona.edu/crispr) and CRISPR-$P_{2.0}$ (http://cbi.hzau.edu.cn/crispr/). In this experiment, the second exon of *tms5* gene was targeted. The gRNA was selected based on high target score with low off-target sites. For cloning gRNA into pRGEB32 vector appropriate adaptors specific to the forward primer (5'-GGCA-3 ') and reverse primer (5'-AAAC-3') were included.

Construct preparation

Target oligonucleotide was cloned into binary vector, pRGEB32, possessing cas9 gene, selectable marker gene *hpt* and cloning site for sgRNA downstream of OsU3 promoter between the right border and left border was used as a vector (Fig. 1b). pRGEB32 by restriction digestion with *BsaI* enzyme followed by ligation of oligos.(Xie *et al.,* 2014).The ligated product was transformed into *E.coli* and recombinant clones identified by restriction digestion analysis followed by DNA sequencing. The confirmed colonies were mobilized into *Agrobacterium* strain LBA4404 through triparental mating. The construct was confirmed for the presence of *hpt* gene, *vir* gene and gRNA in the vector.

Agrobacterium-mediated transformation in immature embryo

Agrobacterium-mediated transformation of rice immature embryos of a local elite rice cultivar ASD16 was carried out following protocol suggested by Hiei and Komari 2008. The immature seeds of ASD16 were collected from the field 12 -15 days after pollination. The immature seeds were de-husked and surface sterilized with 70 % ethanol for 1 min, 1.5 % of sodium hypochlorite for 5 min, followed by three washes with sterile distilled water. Embryos were dissected out aseptically under the microscope and placed on an agar plate (semi-solid medium) containing 0.8 % (w/v) Agar. The immature embryos were immersed in 1.0 ml of sterile distilled water on a sterile microcentrifuge tube for pretreatment. The embryos were subjected to heat-shock treatment at 42 °C for 30 min, and immediately cool down on the ice for 1 min and centrifuged at 1100 rpm for 10 min. The pretreated immature embryos were placed on the NB-AS medium (Hiei and Komari 2008) and infected with 5 µl of freshly prepared AA infection medium. The infected embryos were kept in dark at 25 °C for 20-25 min and the infected embryos were then placed on the fresh NB-AS medium with their scutellum facing up. The co-cultivated plate has been incubated for 7 days in the dark at 25 °C. The elongated shoots were trimmed off and the embryogenic calli were transferred to the first resting in CCMC Medium (Hiei and Komari 2008) and kept for incubation at 30 °C in light for 5 days. The culture was transferred to second resting in a fresh CCMC medium and kept for incubation in light at 30 °C for 10 days. The proliferated calli were transferred on to the selection medium (CCMC containing hygromycin 50mg/l) and incubated at 31 °C. While selecting the calli, browning and watery calli were discarded

and only friable yellowish ones were advanced. Well proliferated calli which survived first selection were transferred to a fresh selection medium and incubated for 7 days. Calli that survived the second round of selection was transferred to the NBPRCH40 pre-regeneration medium (Hiei and Komari 2008) and incubated at 30 °C for 7 days under continuous light. Healthy yellowish calli were transferred to the regeneration medium RNMH30 (Hiei and Komari 2008) and incubated under continuous illumination at 30 °C for 14 days. The regenerated shoots were transferred to a rooting medium 1/2 strength MS containing 0.3 percent agar and hygromycin. The plants were incubated under growth chamber for 14 days in light and then transferred to the transgenic greenhouse and planted in protray followed by larger pots (Fig: 2).

PCR analysis of transgenic plants

Genomic DNA was isolated from the transgenic plant by the CTAB method (Sambrook and Russell 2006). Presence of the *tms5* and *hptII* genes in transgenic plants were analyzed with gene-specific primers (Table 1). These primers amplify, 650 bp and 686 bp internal fragment of *tms5* and *hptII* gene, respectively. Then the amplified PCR product was subjected to electrophoresis with 0.8 percent agarose gel and visualized under UV light and documented in the gel documentation system. (Fig. 3a and 3b)

RESULTS AND DISCUSSION

CRISPR/Cas9 has become a powerful tool for genome editing. By inducing mutation in the *tms5* gene, RNase Z^{S1} expression will be reduced and the UB_{L40} mRNA will not be processed, leading to the production of defective pollen and cause male sterility. According to a recent report, when the plants were grown under a high-temperature condition, several *tms5* mutants generated in a *japonica* type background using the CRISPR/Cas9 system exhibited a high level (85.3 %) of pollen sterility (Zhou *et al.,* 2016). We have selected the *tms5* gene because previous studies have reported that mutation in the *tms5* gene will lead to the production of the male sterile line (Barman *et al.,* 2019). To develop a TGMS line by conventional method takes several years. In this study, sgRNA was designed by CRISPR-P and CRISPR-$P_{2.0}$ which is targeting the second exon of the *tms5* gene present in chromosome 2 between 6397965- 6397985 nucleotide position. In this experiment, we used pRGEB32 plasmid as a CRISPR/Cas9 vector. gRNA was cloned into pRGEB32 by restriction digestion with *BsaI* enzyme. The pRGEB32 vector plasmid along with gRNA was transformed into *E.coli* DH5α strain. Plasmid DNA was isolated from the *E.coli* colonies and the presence of *tms5* was confirmed by colony PCR. The confirmed colonies were sent for sequencing and sequenced with the help of M13 reverse primer. The sequence was analyzed by pairwise sequence alignment to find the similarity between pRGEB32 plasmid and the pRGEB32 plasmid harboring gRNA. After confirmation, the construct was mobilized into *Agrobacterium* strain LBA4404 by triparental mating. Genome edited rice plant has been generated and analyzed for *hpt* gene and *tms5* gene.

Table 1. List of primers used for PCR analysis.

S.No	Gene	Forward primer (5'- 3')	Reverse primer(5'- 3')	Amplification size
1	*tms5*	GCCAGGTGAAGGGTTCATCA	CAGGCTGAATAGCAACAATGACA	650 bp
2	*hptII*	GACGTCTGTCGAGAAGTT	CCTCCAGAAGAAGATG	686 bp

Figure 1a . Structure of *tms5* gene

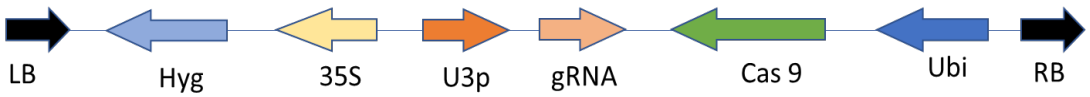

Figure 1b. Vector structure of CRISPR/Cas9 (pRGEB32)

Molecular confirmation of putative transgenic rice

The total genomic DNA isolated from the putative transgenic plants was subjected to PCR analysis for *tms5* and *hptII* genes with their respective primers (Table.1). PCR analysis was performed to check the presence of *hpt* and *tms5* gene, all the putative events were found to be positive for *tms5* and *hptII* genes with the amplification of 650 bp and 686 bp internal sequences, respectively.

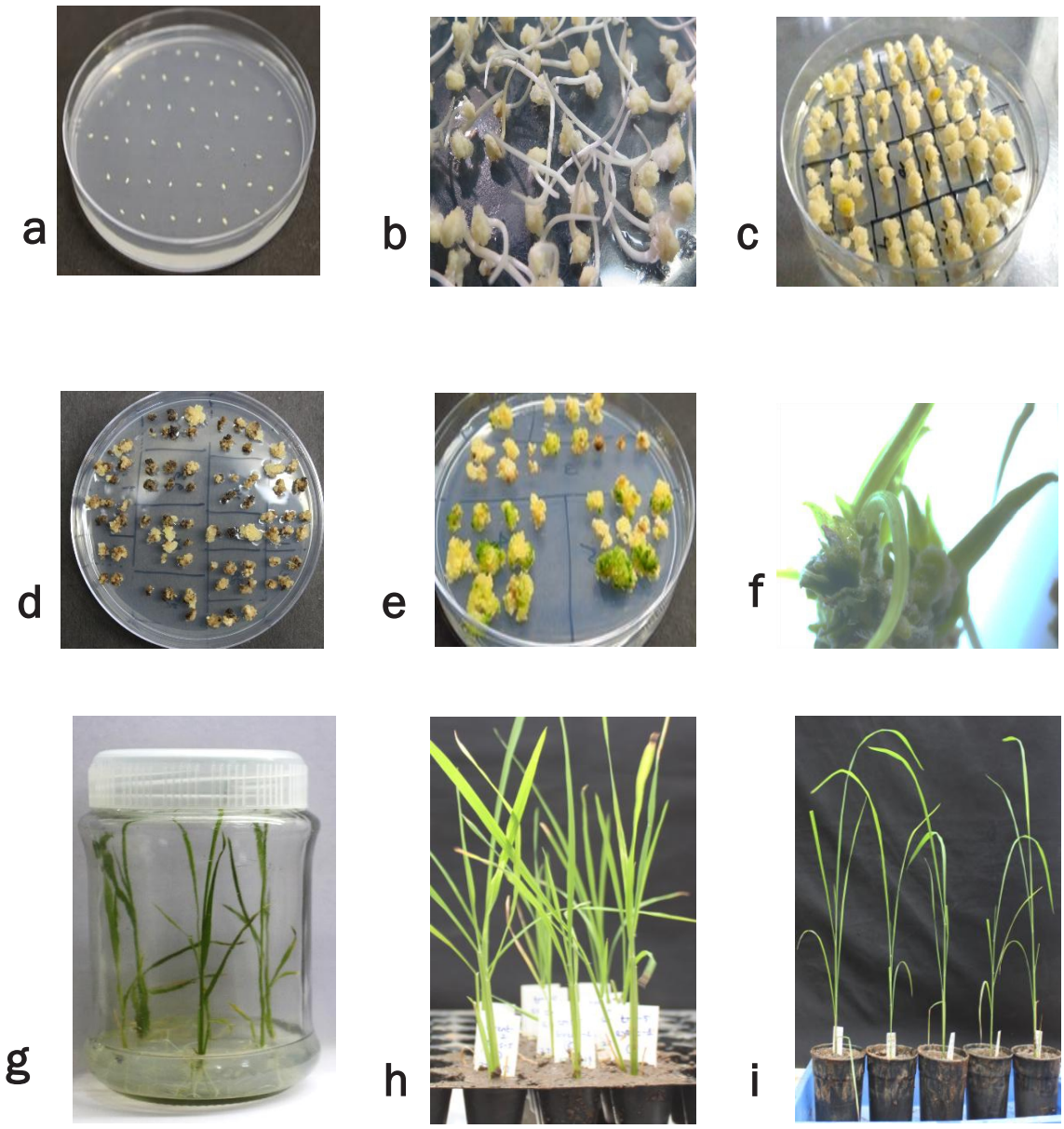

Figure 2. Stages through Agrobacterium mediated transformation of rice cv. ASD 16.

a. Pre-cultured rice immature embryos infected with Agrobacterium on NB-As medium

b. Immature embryos after co-cultivation on NB-As medium

c. Subcultured calli on resting medium (CCMC)

d. Subcultured calli on selection medium (CCMCH50) after second round of selection

e. Embryogenic calli on pre-regeneration medium (NBPRH40)

f. Shoot development on regeneration medium (RNMH30)

g. Root Development on rooting medium (half strength MS medium)

h. Hardening of putative transgenic plants in transgenic greenhouse

i. Transgenic plants in pots at transgenic greenhouse

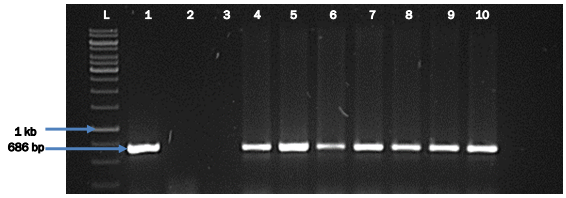

Figure 3a. A 686 bp internal sequence of *hptII* gene was amplified by PCR from the DNA isolated from putative transgenic plants.

L - 1kb ladder; 1- Positive (pRGEB32 plasmid); 2- Negative control (ASD16); 3 – Negative control (Water) and 4- 10– putative transgenic events.

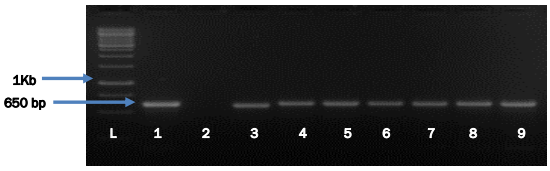

Figure 3b. A 650 bp internal sequence of *tms5* gene was amplified by PCR from the DNA isolated from putative transgenic plants.

L - 1kb ladder control (ASD16); 2- Negative control (water) and 3- 9– putative transgenic events. ; 1- Positive

CONCLUSION

By using the conventional method it will take several years to generate the new TGMS line. Whereas by using CRISPR/Cas9 it is possible to induce mutation in the *tms5* gene which will lead to the production of the new TGMS line. The present study is undertaken to develop a new TGMS line in ASD16 rice variety which will be useful in the production of hybrid rice.

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