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Research Article

Targeted mutation in *eIF4G* gene in rice

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Abstract
Rice tungro disease (RTD), caused by the interaction between rice tungro spherical virus (RTSV) and rice tungro bacilliform virus (RTBV) is a serious constraint in rice production. Natural RTSV resistance is due to a mutation in the translation initiation factor 4 gamma (*eIF4G)* gene. To develop novel alleles for resistance to RTD through CRISPR/Cas9 mediated genome editing, a guide RNA sequence, having higher GC content and fewer off -targets, targeting exon 7 of *eIF4G* was designed and cloned into a binary vector, pRGEB32. This construct was used for generating mutations in rice cultivar ASD16 through *Agrobacterium*-mediated transformation. A target region in the thirteen putative mutants generated in this experiment was sequenced, of which six were found to possess indels. The analysis of the predicted amino acid sequence identified deletion mutants that possess near full -length eIF4G gene which can impart resistance to RTSV.

Keywords
CRISPR/Cas9, Rice tungro spherical virus, *eIF4G*

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most widely consumed staple food crops in the world. As the population increases exponentially, the demand for food derived from cereals continues to increase and necessitates us to produce more. However, the attempts to enhance rice production are often constrained by outbreaks of diseases including viral diseases. Among the viral diseases, rice tungro disease (RTD) causes severe yield loss and affect more than 350,000 ha throughout the main rice-producing countries (Azzam and Chancellor, 2002; Chancellor *et al*., 2006; Muralidharan *et al*., 2003). The plants affected by RTD show symptoms similar to most of the physiological disorders like stunting or yellow discoloration of leaves at the early stages, and reduced tillering and sterile panicles at later stages (Hull, 1996). RTD is caused by the interaction between two viruses namely rice tungro spherical virus (RTSV), possessing a single-stranded RNA genome, and rice tungro bacilliform virus (RTBV), a double-stranded DNA genome (Hull, 1996). RTSV and RTBV are transmitted by the green leafhopper (GLH) such as *Nephotettix virescens* and N. *nigropictus* (Hibino, 1983). The virus which is responsible for the development of disease is RTBV, while RTSV acts as a helper virus in the transmission of RTBV by GLH and enhancing the symptoms (Hull, 1996). Rice tungro spherical virus resistance is a recessive trait, controlled by translation initiation factor 4 gamma (*eIF4G*) gene (Lee *et al.* 2010). Lee *et al.* (2010) reported that the resistance phenotype is due to deletion and/or single nucleotide polymorphism that affects valine residue that is present at 1060th position in eIF4G protein. Such deletion and substitution in the eIF4G gene are observed in the naturally occurring RTSV resistant genotypes such as Utri Rajappan and TKM6. Eukaryotic translation initiation factor 4 G (eIF4G) is a protein involved in eukaryotic translation initiation and is a component of the eIF4F cap-binding complex. The eIF4G protein expression in the host cell is essential for the translation of host proteins as well as proteins of the RNA viruses which utilize the host cellular translational machinery.

Since 2013, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system has been widely used for targeted genome modifications in plants. Though there are various types of CRISPR systems that can be used for targeted modifications, the most commonly used system is the Cas9 nuclease from the type II CRISPR/Cas9 system of *Streptococcus pyogenes*. The Type II CRISPR system requires an NGG (N, any nucleotide; G, guanine) PAM sequence for DNA targeting.

In rice, CRISPR/Cas9-induced editing events take place mainly in the T-DNA transformed callus before regeneration. Therefore, homozygous mutants with high mutation frequencies can be obtained in the T_0 generation and after segregation of the T_1 generation, mutants without exogenous T-DNA can be isolated. These mutants are also labelled as "transgene-free" (Ricroch *et al.,* 2017). Several novel alleles for grain number (Shen *et al*., 2017), disease resistance (Li *et al*., 2020a), herbicide tolerance (Sun *et al.,* 2016), aroma (Usman *et al.,* 2020), high amylase content, grain quality (Li *et al.,* 2020b), have been generated in rice using this method.

In the present experiment, an attempt was made to generate novel mutants in a high yielding RTD susceptible rice genotype, ASD16 using CRISPR/Cas9 system. The mutants generated were analysed for the presence of mutations.

MATERIALS AND METHOD

Guide RNA (gRNA) sequence was identified using CRISPR-Plant and CRISPR P 2.0 tools to target exon 7 of the *eIF4G* gene (LOC_Os07g36940). The DNA oligo duplex corresponding to gRNA sequences (Forward oligo – 5'- AAGACTTTCCGGCCAAATTA-3'; Reverse oligo – 5' - TAATTTGGCCGGAAAGTCTT -3') were cloned into *Bsa*I restriction site of the binary vector, pRGEB32 vector (**Fig.1**), a gift from Yinong Yang (Addgene plasmid # 63142; http://n2t.net/addgene:63142; RRID; add gene 63142). The plasmid harbours*Cas9* gene under the control of maize ubiquitin promoter, hygromycin phosphotransferase gene driven by *CaMV35S* promoter, gRNA scaffold and cloning site for the introduction of sgRNA sequence under the control of *OsU3* promoter. The construct was subsequently mobilized into *Agrobacterium* strain LBA4404 through triparental mating. The *Agrobacterium* transconjugant with the construct was used in transformation experiments.

Immature embryos were isolated under sterile conductions from field collected healthy immature seeds (7-10 days after flowering) and immersed in 1 ml sterile water in a sterile microfuge tube. The embryos were subjected to heat treatment at 42°C for 30 minutes and cold treatment in ice for 1 minute. The tube was centrifuged for 10 minutes at 1100 rpm. After centrifugation, the embryos were dispensed on a sterile petri dish and later, carefully placed onto the NBAS medium (Hiei and Komari, 2008; Rajadurai *et al.,* 2018; Shweta *et al.,* 2020)

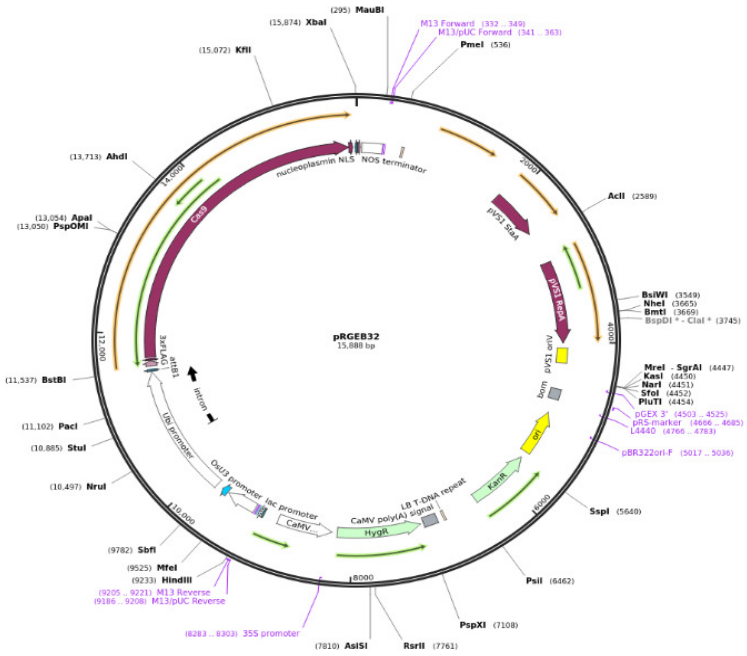

Fig 1. Physical map of pRGEB32 vector

with the scutellum face up. These embryos were infected with the freshly prepared infection medium. The infection medium was prepared by adding a half loopful of grown culture in AA medium (Hiei and Komari, 2008). The infected embryos were kept in the dark at 25°C for 20-25 minutes and then transferred onto fresh NBAS medium in the same fashion as mentioned earlier. The co-cultivated plate was kept for incubation in the dark at 25°C. On the 7th day, emerging from the embryos was removed and the calli were transferred onto CCMC medium (Hiei and Komari, 2008) and incubated for 15 days. The well proliferated calli was transferred onto the selection medium (CCMCH containing hygromycin 50 mg/l) and incubated at 30°C for two rounds of selection of 10 and 7 days respectively. Yellow friable calli that survived the second round of selection were cultured onto a pre-regeneration medium (NBPRCH40) for 7 days, followed by regeneration medium (RNMH30) for 15 days. The regenerated shoots were transferred on to the rooting medium containing hygromycin 30 mg/l. Well-developed plants were transferred to potting medium and maintained in the transgenic greenhouse.

Genomic DNA was isolated from the genome edited rice plants and control plants using the CTAB method (Sambrook, 1989). Molecular analysis of the plants was carried out by PCR to check the presence of *hpt* and *cas9* genes using gene specific primers (**Table 1**). PCR reactions were performed in an Eppendorf Thermal Cycler with 0.5 µl (50-100 ng/µl) of the genomic DNA, 2.0 µl of 10X PCR buffer, 0.5 µl of 100 mMdNTPs, 1.0 µl each of 10 mM respective forward and reverse primers, 0.3 µl of 3 U *Taq* DNA polymerase and 15.7 µl of sterile distilled water. Further, the target region of the *eIF4G* gene was amplified using gene specific primers **(Table 1**) and the PCR protocol described above. The amplified product was purified and sequenced using the Sanger sequencing method (Eurofins, Bengaluru). The sequences were analyzed using DSDecoedM (http://skl.scau.edu.cn/dsdecode/) and CRISPID (http://crispid.gbiomed.kuleuven.be/) softwares.

Table 1. PCR primers used in the study

Name of gene	Primer sequence	Amplicon size	PCR conditions
hpt	Forward primer 5'GCTGTTATGCGGCCATTGGTC-3' Reverse primer 5'-GCCTCCAGAAGAAGATGTTG-3'	686 bp	One cycle of 94°C for 5 min 30 cycles of 94°C for 1 min; 58°C for 30 s; 72°C for 30 s One cycle of 72°C for 2 min
Cas9	Forward primer 5'-CTTCTGGCGGTTCTCTTTAG-3' Reverse primer 5'-TGCTGTTTGATCCGTTGTTG-3'	478 bp	One cycle of 95°C for 5 min, 30 cycles of 95°C for 45 s; 52°C for 45 s; 72°C for 45 s 72°C for 10 min:1 cycle
eIF4G	Forward primer 5'- AAGACTTTCCGGCCAAATTA-3' Reverse primer 5'-TAATTTGGCCGGAAAGTCTT-3'	280 bp	One cycle of 94°C for 5 min 30 cycles of 94°C for 1 min; 58°C for 30 s; 72°C for 30 s One cycle of 72°C for 2 min

Table 2. Details of putative mutants of ASD16

Sl No.	Event ID	Mutation	Zygosity
1.	NS-ASD16-P4-1	wt	-
2.	NS-ASD16-P4-2	wt	-
3.	NS-ASD16-P4-3	5d/wt	Monoallelic
4.	NS-ASD16-P4-4	6d/4d	Biallelic
5.	NS-ASD16-P4-5	wt	-
6.	NS-ASD16-P4-6	wt	-
7.	NS-ASD16-P4-7	1d/wt	Monoallelic
8.	NS-ASD16-P4-8	wt	-
9.	NS-ASD16-P4-9	3d/4d	Biallelic
10.	NS-ASD16-P4-10	wt	-
11.	NS-ASD16-P4-11	1i/wt	Monoallelic
12.	NS-ASD16-P4-12	wt	-
13.	NS-ASD16-P4-13	1d/wt	Monoallelic

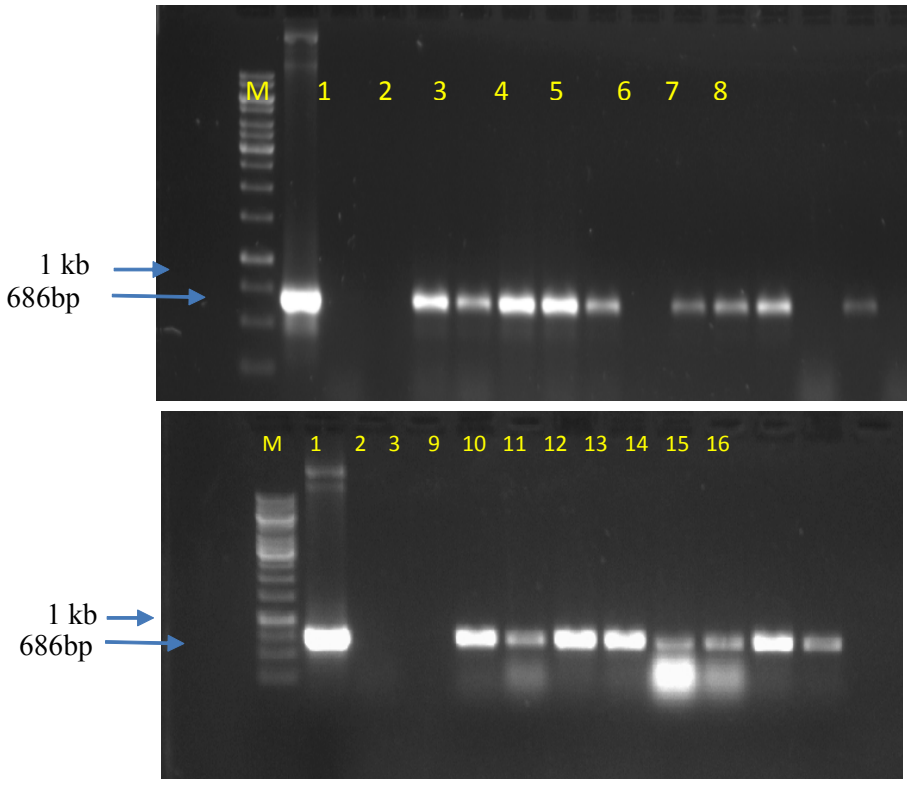

Fig 2a: PCR analyses of T0 transgenic plants for presence of hpt gene
M - 1kb ladder; 1- Positive control (pREB32 plasmid); 2- Negative control (ASD16); 3- Negative control (Water); 4-16: NS-P4-4-1 to NS-ASD16-P4-4-13

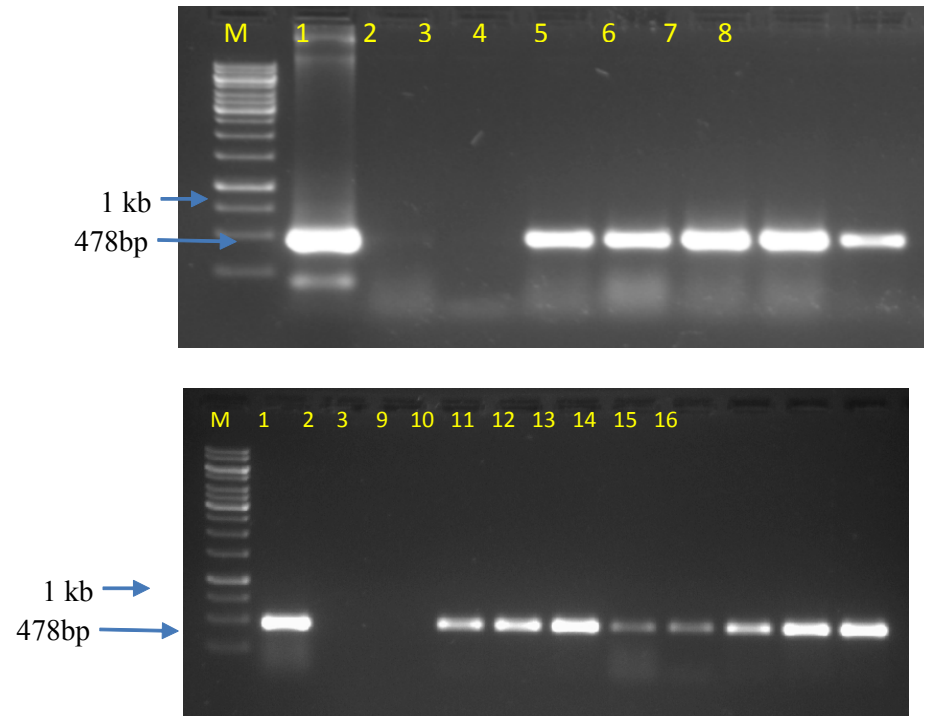

Fig 2b: PCR analyses of T_0 transgenic plants for presence of *cas9* gene
M - 1kb ladder; 1- Positive control (pREB32 plasmid); 2- Negative control (ASD16); 3- Negative control (Water); 4-16: NS-P4-4-1 to NS-ASD16-P4-4-13

RESULTS AND DISCUSSION

Twenty four batches comprising 1680 immature embryos were co-cultivated with *Agrobacterium* strain, LBA4404 harbouring pRGEB32-eIF4G construct. The co-cultivated embryos were cultured on hygromycin selection. Hygromycin resistant calli lines, on transferring to regeneration medium, developed into complete plants. Thirteen plants were generated with a transformation efficiency of 0.8 %. All the thirteen lines were positive to PCR amplification for *hpt* and *cas9* genes (**Fig 2a and 2b**). Sequencing analysis of PCR amplicon encompassing the target region identified six genome edited mutants (**Table 2**). The mutations observed in these six lines are represented in **Table 3**. In these mutants, a combination of both monoallelic and biallelic mutations was observed. No indels was detected in the rest of the seven events.

Analysis of translated protein sequence identified truncation of the protein, terminating prematurely at VLCC* residue in one of the alleles in all the six mutants (**Table 4**). Of the six mutant lines, in two events, NS-ASD16-P4-4 and NS-ASD16-P4-9, the mutation was found to be in the frame.

Table 3. Details of nucleotide sequence of target region in putative mutants

Sl No	EVENT ID	Nucleotide sequence
CONTROL ASD16		GTTCTGTTCCCTAATTTGGCCGGAAAGTCTTATGTTGTTGATCACCCA
1	NS-ASD16-P4-3	A1 GTTCTGTTCCCTA-----GCCGGAAAGTCTTATGTTGTTGATCACCCA A2 GTTCTGTTCCCTAATTTGGCCGGAAAGTCTTATGTTGTTGATCACCCA
2	NS-ASD16-P4-4	A1GTTCTGTTCCCT------GCCGGAAAGTCTTATGTTGTTGATCACCCA A2 GTTCTGTTCCCTAA----GCCGGAAAGTCTTATGTTGTTGATCACCCA
3	NS-ASD16-P4-7	A1 GTTCTGTTCCCTAATT-GGCCGGAAAGTCTTATGTTGTTGATCACCCA A2 GTTCTGTTCCCTAATTTGGCCGGAAAGTCTTATGTTGTTGATCACCCA
4	NS-ASD16-P4-9	A1 GTTCTGTTCCCTAA---GGCCGGAAAGTCTTATGTTGTTGATCACCCA A2 GTTCTGTTCCCTAA----GCCGGAAAGTCTTATGTTGTTGATCACCCA
5	NS-ASD16-P4-11	A1 GTTCTGTTCCCTAATTTTGGCCGGAAAGTCTTATGTTGTTGATCACCCA A2 GTTCTGTTCCCTAATTTGGCCGGAAAGTCTTATGTTGTTGATCACCC
6	NS-ASD16-P4-13	A1 GTTCTGTTCCCTAATT-GGCCGGAAAGTCTTATGTTGTTGATCACCCA A2 GTTCTGTTCCCTAATTTGGCCGGAAAGTCTTATGTTGTTGATCACCCA

'-' denotes deletions; red letter denotes insertion in the nucleotide sequence

Table 4. Predicted protein sequence of genome edited mutants

Sl No	EVENT ID	Predicted Amino acid Sequence
CONTROL ASD16		VLFPNLAGKSYVVDHP
1	NS-ASD16-P4-3	A1 VLFPS--RKVLCC* A2 VLFPNLAGKSYVVDHP
2	NS-ASD16-P4-4	A1 VLFP—AGKSYVVDHP A2 VLFPN--RKVLCC*
3	NS-ASD16-P4-7	A1 FCSLIGRKVLCC* A2 VLFPNLAGKSYVVDHP
4	NS-ASD16-P4-9	A1 VLFPK-AGKSYVVDHP A2 FCSLSRKVLCC*
5	NS-ASD16-P4-11	A1 VLFPNFGRKVLCC* A2 VLFPNLAGKSYVVDHP
6	NS-ASD16-P4-13	A1 FCSLIGRKVLCC* A2 VLFPNLAGKSYVVDHP

'-' denotes deletions; red letter denotes change in amino acid residue; '*' denotes truncation

The target gene *eIF4G* is essential for translation in plants as well as for RNA viruses that exploit the host translational system. Mutation in a critical region of eIF4G protein will affect viral translation without affecting the translation of plant proteins. This demands the development of mutants with a nearly full length of the gene to sustain it's function in the plant while guarding against the viral attack simultaneously. An earlier report on the need for selecting in-frame mutants in *eIF4G* edited lines compliments this theory (Macovei *et al*., 2018). This explains our identification of the six T_0 mutants with a trimmed VLCC* residue in one of the alleles. No mutant homozygous for truncated protein was observed in any of the T_0 lines as their survival itself would have been terminated during callus stage.

Besides the valine residue at 1060th position of eIF4G protein, mutation in the SVLFPNLAGKS (especially NL, 1053 and 1054 positions of amino acid) residue can also account for resistance against tungro disease (Macovei *et al*., 2018). The two lines identified in this study have a mutation in the NL residue and thus are promising candidates. Their resistance against Rice tungro spherical virus, however, needs to be proven by bioassay in homozygous lines.

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