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 \( Nephrotettix virescens \) 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 1060\(^{th} \) 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.
Targeted mutation in eIF4G gene

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 T₀ generation and after segregation of the T₁ 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’- AAAGACTTTCGCGACAAATT-3’; Reverse oligo – 5’ - TAATTTGCCGGAAATGCTT -3’) were cloned into BsaI restriction site of the binary vector, pRGEB32 vector (Fig.1), a gift from Yinong Yang (Addgene plasmid # 63142; http://n2t.net/addgene:63142; RRID; addgene 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](image-url)
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 mM dNTPs, 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’GCTGTATGCGGCCATTGGTC-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 |
|              | Reverse primer 5’-GCCTCCAGAAGAGATGTTG-3’ |  | One cycle of 72°C for 2 min |
| Cas9         | Forward primer 5’-CTTCTGGCGGTTCTCTTAG-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 |
|              | Reverse primer 5’-TGCTGTTGTGATCCGTTGGT-3’ | | 72°C for 10 min:1 cycle |
| eIF4G        | Forward primer 5’-AAGACTTTCCGCGCAAAATT-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 |
|              | Reverse primer 5’-TAATTTGGCCGGAAAGTCTT-3’ | | 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 T0 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.

| Sl No | EVENT ID   | Nucleotide sequence                        |
|------|------------|--------------------------------------------|
| 1    | NS-ASD16-P4-3 | A1 GTTCTGTTCCCTA------GCCGGAAAGTCTTATGGTTGTGATCACCCA A2 GTTCTGTTCCCTAATTTGGCCGGAAAGTCTTATGGTTGTGATCACCCA |
| 2    | NS-ASD16-P4-4 | A1 GTTCTGTTCCCTA---GCCGGAAAGTCTTATGGTTGTGATCACCCA A2 GTTCTGTTCCCTA---GCCGGAAAGTCTTATGGTTGTGATCACCCA |
| 3    | NS-ASD16-P4-7 | A1 GTTCTGTTCCCTAATTTGGCCGGAAAGTCTTATGGTTGTGATCACCCA A2 GTTCTGTTCCCTAATTTGGCCGGAAAGTCTTATGGTTGTGATCACCCA |
| 4    | NS-ASD16-P4-9 | A1 GTTCTGTTCCCTAATTTGGCCGGAAAGTCTTATGGTTGTGATCACCCA A2 GTTCTGTTCCCTAATTTGGCCGGAAAGTCTTATGGTTGTGATCACCCA |
| 5    | NS-ASD16-P4-11| A1 GTTCTGTTCCCTAATTTGGCCGGAAAGTCTTATGGTTGTGATCACCCA A2 GTTCTGTTCCCTAATTTGGCCGGAAAGTCTTATGGTTGTGATCACCCA |
| 6    | NS-ASD16-P4-13| A1 GTTCTGTTCCCTAATTTGGCCGGAAAGTCTTATGGTTGTGATCACCCA A2 GTTCTGTTCCCTAATTTGGCCGGAAAGTCTTATGGTTGTGATCACCCA |

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

| Sl No | EVENT ID   | Predicted Amino acid Sequence                        |
|------|------------|------------------------------------------------------|
| 1    | NS-ASD16-P4-3 | A1 VLFP---RKVLCC* A2 VLFPNLAGKSYVVDHP |
| 2    | NS-ASD16-P4-4 | A1 VLFP---AGKSYVVDHP A2 VLFPNLAGKSYVVDHP |
| 3    | NS-ASD16-P4-7 | A1 FCSLIGRKLCC* A2 VLFPNLAGKSYVVDHP |
| 4    | NS-ASD16-P4-9 | A1 VLFPK-AGKSYVVDHP A2 FCSLIRKVLCC* |
| 5    | NS-ASD16-P4-11| A1 VLFPNFGRKLCC* A2 VLFPNLAGKSYVVDHP |
| 6    | NS-ASD16-P4-13| A1 FCSLIRKVLCC* 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 T0 mutants with a trimmed VLCC* residue in one of the alleles. No mutant homozygous for truncated protein was observed in any of the T0 lines as their survival itself would have been terminated during callus stage.
Besides the valine residue at 1060th position of elf4G 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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