Madras Agric. J., 2021; https://doi.org/10.29321/MAJ.10.000548 (online first)

RESEARCH ARTICLE

Adenine Base Editor Creates Novel Substitution Mutations in *eIF4G* Gene of Rice

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ABSTRACT

Two single nucleotide polymorphic mutations and deletion affecting $Y^{1059}V^{1060}V^{1061}$ amino acid residues in a host translation initiation factor four gamma (eIF4G) gene in rice are reported to confer resistance to rice tungrospherical virus in resistant genotypes. A CRISPR-based adenine base editing vector was used to target these residues in a susceptible indica cultivar, ASD16.Agrobacterium-mediated transformation of ASD16 generated 16 missense mutants and two deletion mutants. Substitution mutations occurred at $A_5 > G_5$ and $A_4 > G_4$, where 5.5 % and 3.37 % of adenosines got converted to guanosines, respectively. The mutantsgenerated had missense mutations affecting the YVV residues and the residues immediately adjacent to YVV.Thus,these novel mutationsare promising candidates in imparting resistance against rice tungro disease.

Keywords: *Rice; Adenine base editors; eIF4G*

INTRODUCTION

Rice tungro disease (RTD) causes severe yield losses in rice-growing endemic regions of South and Southeast Asia (Herdt, 1988; Azzam and Chancellor, 2002; Muralidharan *et al*., 2003). Rice tungro spherical virus (RTSV), along with its counterpart RTBV (Rice tungro bacilliform virus, a dsDNA virus), is responsible for RTD (Bunawan *et al*., 2014). Management of tungro disease *via* the development of broad-spectrum resistance through suppression of RTSV has been the preferred choice since rice plants infected with RTBV exclusively are incapable of spreading the disease. Thus, RTSV resistant cultivars rather than RTBV could successfully reduce the incidence of tungro disease in the field (Hibino, 1996; Anjaneyulu *et al.,* 1995; Lee *et al.,* 2010). Host-pathogen interaction studies of disease-causing plant viruses reveal that most RNA viruses exploit the host machinery to perform their life cycle (Dreher and Miller, 2006; Pyott *et al*., 2016; Li, 2019). RTSV is one such RNA virus that leverages a host translation initiation factor four gamma (*eIF4G*) gene of rice to replicate and establish within rice plant (Lee *et al*., 2010).

Lee *et al*. (2010) identified that naturally available cultivars resistant to RTSV had nucleotide polymorphisms and deletions affecting $Y^{1059}V^{1060}V^{1061}$ amino acid residues in *eIF4G* gene in *japonica* genotypes. The mutations resulted from substitutions at nucleotide positions 4387 (A > G) and 4390 (T > C). This suggested that mimicking such naturally occurring mutations in susceptible genotypes would successfully impart tungro disease resistance in target cultivars.

The latest genome editing tool *via* base editors has unravelled the possibilities of creating highly specific targeted substitution mutations in the host genome (Komor *et al*., 2016). Adenine base editors (ABEs) convert an A•T base pair to a G•C base pair, while Cytosine base editors (CBEs) convert a C•G base pair into a T•A base pair (Gaudelli *et al*., 2017; Komor *et al*., 2016). Unlike the Cas9 of CRISPR/Cas9 system, which has two nuclease domains, the Cas9 in base editors have only one active cleavage domain and hence, the Cas9 in base editors is referred to as nCas9/Cas9n (nickase Cas9) or dCas9 (dead Cas9). This nCas9 or dCas9 is fused to cytosine deaminase (in case of CBEs) or adenosine deaminase (in case of ABEs) that characterize a base editor pair (Gaudelli *et al*., 2017; Komor *et al*., 2016). Of the two base editors, ABE has been widely accepted for base editing in rice crop as CBEs were found to induce unintended off-target mutations and have low editing efficiency (Jin *et al*., 2019; Hao *et al*., 2019). A series of adenine base editing vectors have been developed within a short span of four years, to yield maximum A > G substitution

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with negligible non-canonical substitutions (Gaudelli *et al*., 2017; Li *et al*., 2018; Kim *et al*., 2019). An adenine base editor, ABE7.10 has been reported to create high substitution mutations, up to 59.1 % in a *japonica* rice variety, Zhonghua 11 (Lee *et al*., 2018). Considering the efficacy and specificity of ABE, the plasmid harboring ABE7.10, pH-PABE-7-esgRNA (Lee *et al*., 2018) was used in the present study to create substitution mutations in the YVV residues of *eIF4G* gene of *indica* cultivar, ASD16, to impart resistance against rice tungro disease.

MATERIAL AND METHODS

Design of sgRNA and construct development

The nucleotide sequence of *eIF4G* gene in *indica* genotype, bearing gene ID: BGIOSGA025931 was retrieved from EnsemblPlants. Single guide RNA (sgRNA) was designed using this sequence information with the web-based tool, Chop Chop (Labun *et al.,* 2016). A sgRNA sequence encoding for GKSYVVD amino acid residues was designed with top strand: 5' ATCAACAACATAAGACTTTC 3' and bottom strand: 5' GAAAGTCTTATGTTGTTGAT 3' (Fig 1). Additional A was added to the top strand at 5' end for which corresponding T was added to the bottom strand at 3' end to compliment ligation. *Bsa*I adaptors, 5' GGCG 3' and 5' AAAC 3' were added to the oligos during synthesis to complement their ligation into *Bsa*I restricted plant adenine base editing vector, pH-PABE7-esgRNA. The pH-PABE-7-esgRNA was a gift from Dr. Caixia Gao (Addgene plasmid # 115620; http://n2t.net/addgene:115620; RRID: Addgene_115620) (Li *et al*., 2018). The recombinant vector (Fig. 2) harboring the sgRNA was mobilized into *Agrobacterium* strain, LBA4404, by triparental mating.

Agrobacterium-mediated rice transformation

Agrobacterium culture harboring the recombinant plasmid was used to transform an RTD susceptible *indica* cultivar ASD16, a cross derivative of ADT 39 and CO 51. Immature seeds (14-16 days after flowering) of ASD16 were collected from Paddy Breeding Station, Tamil Nadu Agricultural UNIVERSITY. Embryos were isolated from these seeds and used as explants for *Agrobacterium*-mediated transformation following the protocol of Hiei and Komari (2008). Well proliferated and friable yellow calli were subjected to two rounds of stringent selection in 50 mgL^{-1} of hygromycin B. The calli that survived on hygromycin selection were subcultured onto pre-regeneration, regeneration and rooting media. Regenerated plants with well-developed roots were hardened and maintained in transgenic greenhouse.

On-target mutation analysis of putative T_0 mutants by Sanger sequencing

Plant genomic DNA was isolated from T_0 putative mutants and ASD16 wild type following CTAB method (Porebski *et al.,* 1997). Molecular analyses by PCR for *hpt* (hygromycin phosphotransferase) and *cas9* genes were performed using sequence-specific primers to confirm that the putative mutants developed had T-DNA with genes required for editing (Table 1). The target region encompassing the sgRNA sequence was amplified with *eIF4G* primers (*eIF4G* F and *eIF4G* R; Table 1) in PCR positive mutants. PCR amplicons were purified (NucleoSpin Gel and PCR Clean-up Kit, Machery Nagel) and Sanger sequenced (Eurofins, Bengaluru).

Table 1. List of primer sequences along with temperature profiles used in the study

Name of gene/Region	Forward (F) and reverse (R) primers (5' to 3')	Amplicon size (in bp)	PCR conditions	
hpt	hpt F: GCTGTTATGCGGCCATTGGTC hptR: GCCTCCAGAAGAAGATGTTG	686	94 °C for 5 min 94 °C for 1 min 58 °C for 30 s 72 °C for 30 s 72 °C for 2 min	35 cycles
cas9	cas9 F: ACTAACTCTGTTGGCTGGGC cas9 R: GCGCAATGAGATTCCCGAAC	694	95 °C for 5 min 95 °C for 45 s 58 °C for 45 s 72 °C for 45 s 72 °C for 10 min	30 cycles
eIF4G	eIF4G F: AAGACTTTCCGGCCAAATTA eIF4G R: TAATTTGGCCGGAAAGTCTT	577	95 °C for 5 min 95 °C for 45 s 53 °C for 1 min 72 °C for 45 s 72 °C for 2 min	30 cycles

Analysis of results obtained from Sanger sequencing was performed using web-based tools, DSDecodeM (http://skl.scau.edu.cn/dsdecode/) (Xie *et al*., 2017; Liu *et al*., 2015) and CRISPR-ID (http://crispid.gbiomed.kuleuven.be/) (Dehairs *et al*., 2016) to identify the position of substitution mutations in the sgRNA sequence. The percentage of substitution contributed by a base at a specific position in the sgRNA sequence was predicted using web-based tool, EditR (http://baseeditr.com) (Kluesner *et al*., 2018). Base substituted mutants with missense mutations were identified based on these results.

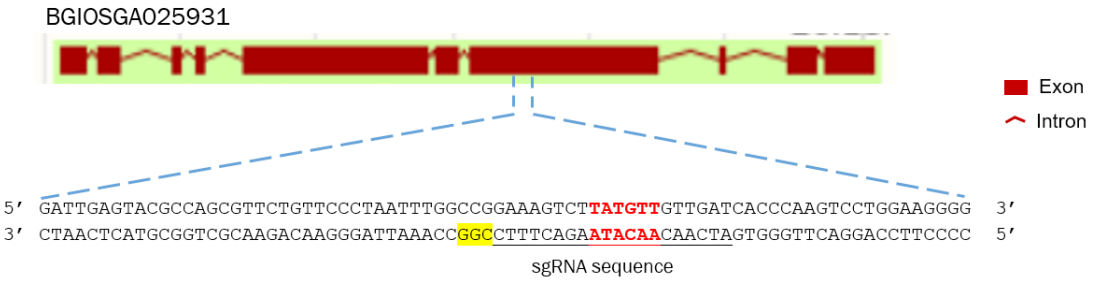


Figure 1. Genomic structure of *eIF4G* gene (Gene ID: BGIOSGA025931; Plant Ensemble). (The sequence region encompassing the sgRNA sequence is given in double stranded form. The sgRNA sequence is underlined and PAM region is highlighted in yellow. The nucleotide sequences encoding for the YV residues are in red.)

RESULTS AND DISCUSSION

Agrobacterium-mediated transformation of ASD16

Agrobacterium-mediated transformation of elite rice cultivar ASD16, with the strain LBA4404, harboring the recombinant plasmid pH-PABE7-esgRNA+sgRNA was successful in generating 139 independent events from 22 batches of co-cultivation, comprising of 2220 immature embryos. This gave an average transformation efficiency of 6.26 % (Table 2).

Table 2. Agrobacterium-mediated transformation of ASD16 and mutants generated

No. of batches co-cultivated	No. of immature embryos co-cultivated	No. of events generated	Transformation efficiency (%)*	Events with missense mutations	Events with deletion mutations	Mutation Frequency (%)#
20	2220	139	6.26	16	2	12.95

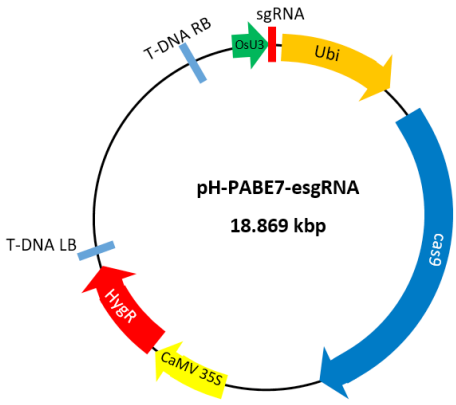
* Transformation efficiency = (Number of events generated)/(Total number of embryos co-cultivated) × 100

Mutation efficiency = (Events with mutation)/(Total number of events generated) × 100

Identification of on-target mutations harboring missense mutations

Molecular characterization of T_0 putative mutants by PCR for the presence of *hpt* and *cas9* genes confirmed successful integration of T-DNA in all the 139 independent events generated (Fig. 3a and Fig. 3b). Amplification of the target region in these T-DNA positive events gave expected amplification of 577 bp (Fig. 3c). The sgRNA sequence has 4 adenine (A) residues in the editing window at positions A_4, A_5, A_7 and A_8. Substitution mutations at A_5 and A_8 lead to missense mutations as $A_5 > G_5$ and $A_8 > G_8$ would result in V > A missense mutations at both the positions, while $A_4 > G_4$ and $A_7 > G_7$ would result in silent mutations. Sanger sequencing analysis of the T-DNA positive mutants identified 16 events harboring missense mutations and two harboring deletion mutations (Table 2). All the substitution mutations observed were in monoallelic form. Out of the 16 missense mutants, 14 had one missense mutation, while two had two missense mutations (YK-ASD16-141 and YK-ASD16-150) (Table 3).

Figure 2. Physical map of pH-PABE7-esgRNA harbouring sgRNA



The majority of the mutants (13 mutants) had substitution mutations at A_4 and A_5. No substitution mutations were detected at A_7 and A_8 positions. At A_5, 5.5 % of the adenosines were converted to guanosine, while at A_4, 3.37 % of adenosines were converted to guanosine (Fig. 4). No silent mutation, arising from A_4 > G_4 alone, was observed and all the 5 mutants which had A_4 > G_4 substitution were observed along with A_5 > G_5. Four mutants (YK-ASD16-141, YK-ASD16-150, YK-ASD16-147 and YK-ASD16-151B) had substitutions immediately upstream of the YVV residues, resulting in S > F. In contrast, one mutant (YK-ASD16-234) had substitution downstream, resulting in D > H. These novel mutations are in close proximity to the YVV residues, which were reported earlier by Lee *et al.* (2010) in naturally available resistant genotypes. Macovei *et al*. (2018) reported that CRISPR/Cas9-mediated genome-edited rice mutants targeting a stretch of 14 amino acid residues 'SVLFPNLAGKSYVV', could successfully confer resistance against rice tungro disease. Thus, the 16 missense mutants with substitutions affecting YVV residues and residues immediately adjacent to YVV residues could serve as promising candidates for imparting resistance against rice tungro disease.

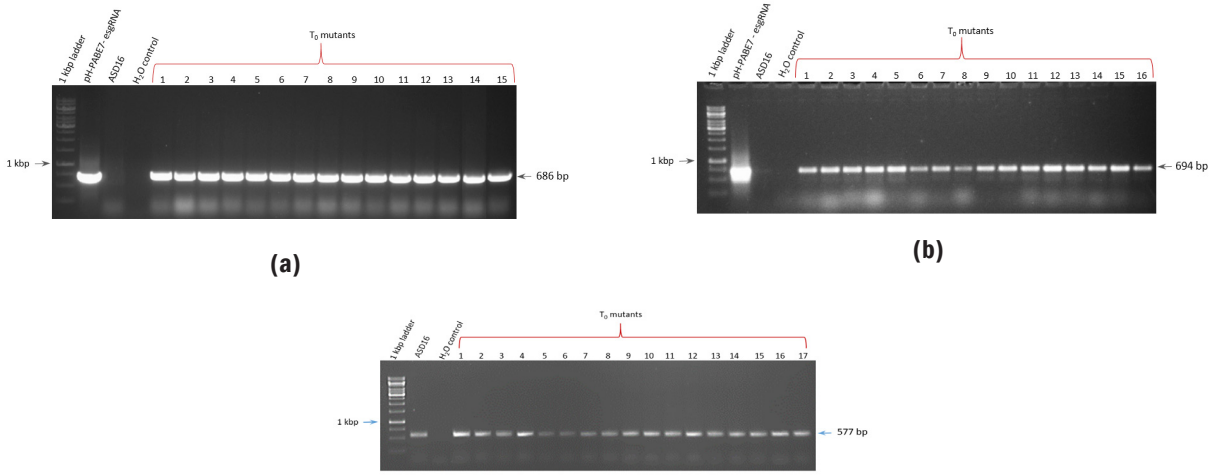


Figure 3. PCR analysis of putative mutants (T0). (a) hpt, (b) cas9, (c) target region of eIF4G gene

The two deletion mutants (YK-ASD16-246 and YK-ASD16-354) had similar homozygous deletion mutations of three nucleotides 'GTT', encoding valine in the target GKSYVVD residues (Table 3). Observations of such deletion mutants with adenine base editors have also been reported earlier with low frequency (Li *et al.,* 2018; Li *et al.,* 2021). This mutation observed in the two deletion mutants was similar to that of the naturally available resistant genotype, TKM 6, which had deletion of the V residue (Lee *et al*., 2010). They serves as promising candidates in imparting resistance against tungro disease. Thus, 18 mutants were identified, giving a mutation efficiency of 12.95 % (Table 2).

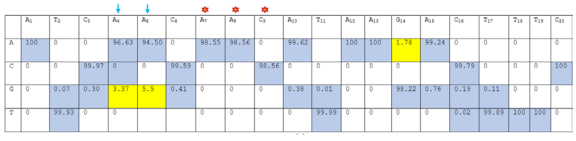


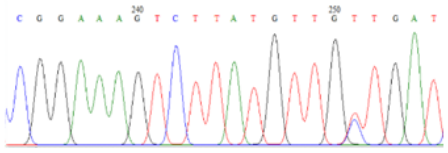
Figure 4. Nucleotide frequencies at each position in the target sequence of eIF4G gene obtained using EditR web-based tool.

(Arrows indicate expected base substitution of A > G. Frequency of expected nucleotides are highlighted in blue and those of substituted nucleotides are highlighted in yellow.

✸ As deletion mutation was observed at these nucleotide positions, total percentage value is less than 100.)

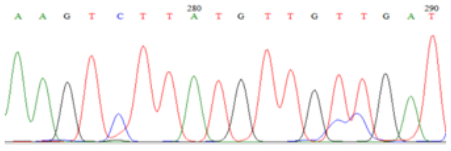
Besides the canonical A > G substitution, we have also observed non-canonical substitution of G > A at 14th position of the sgRNA sequence. At G_{14}, 1.78 % of the guanosines got converted to adenosine (Fig. 4). Similar observations on non-canonical editing using adenine base editors and, more precisely, ABE7.10 have also been reported earlier (Lee *et al*., 2018; Kim *et al*., 2019, Jeong *et al*., 2020). A possible explanation for this observation is the role of the adenosine deaminase enzyme. Unlike cytosine base editing, adenine base editing does not occur spontaneously *in vivo* as no enzymes are known to deaminate adenine in DNA (Gaudelli *et al*., 2017). Thus, the deaminase enzyme used in the construction of ABE 7.10 is sourced from *E.coli* (ecTadA, *E.coli* tRNA specific adenosine deaminase). The ecTadA enzyme harbors a common catalytic site for deamination of cytosine and adenine residues (Jeong *et al*., 2020). This explains the non-canonical substitutions of cytosine to adenine/thymine/guanine while using an ABE7.10 in human and mouse cells (Lee *et al*., 2018; Kim *et al*., 2019, Jeong *et al*., 2020). Such cytosine substitutions were favored when C is present in a TC*N fashion and the editing window was limited between 5-7 bp (Lee *et al*., 2018; Kim *et al*., 2019).

YK-ASD16-115



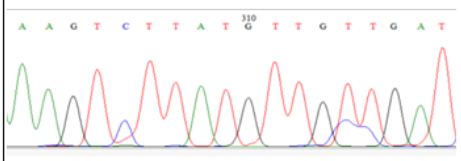
A1: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)
A2: CCGGAAAGTCTTATGTTGCTGAT (GKSYVAD)
WT: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)

YK-ASD16-116



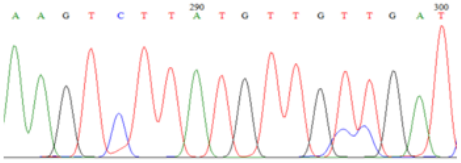
A1: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)
A2: CCGGAAAGTCTTATGTTGCCGAT (GKSYVAD)
WT: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)

YK-ASD16-118



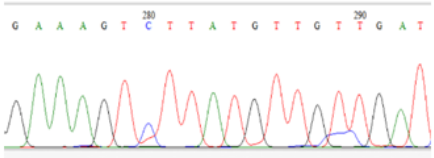
A1: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)
A2: CCGGAAAGTCTTATGTTGCCGAT (GKSYVAD)
WT: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)

YK-ASD16-124



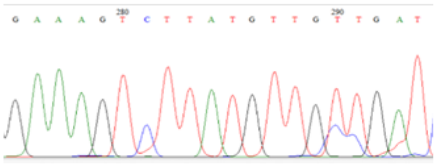
A1: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)
A2: CCGGAAAGTCTTATGTTGCCGAT (GKSYVAD)
WT: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)

YK-ASD16-132



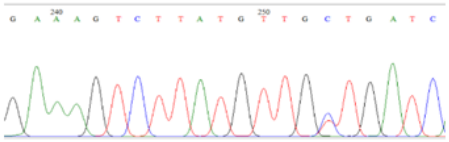
A1: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)
A2: CCGGAAAGTCTTATGTTGCCGAT (GKSYVAD)
WT: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)

YK-ASD16-135



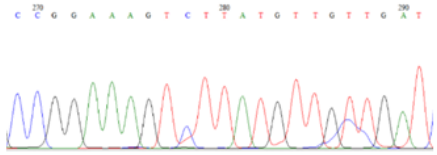
A1: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)
A2: CCGGAAAGTCTTATGTTGCCGAT (GKSYVAD)
WT: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)

YK-ASD16-117



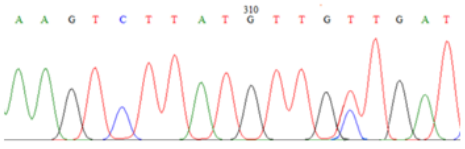
A1: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)
A2: CCGGAAAGTCTTATGTTGCTGAT (GKSYVAD)
WT: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)

YK-ASD16-123



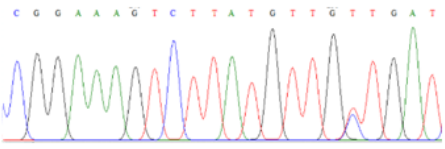
A1: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)
A2: CCGGAAAGTCTTATGTTGCTGAT (GKSYVAD)
WT: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)

YK-ASD16-136



A1: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)
A2: CCGGAAAGTCTTATGTTGCTGAT (GKSYVAD)
WT: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)

YK-ASD16-151A



A1: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)
A2: CCGGAAAGTCTTATGTTGCTGAT (GKSYVAD)
WT: CCGGAAAGTCTTATGTTGTTGAT (GKSYVVD)

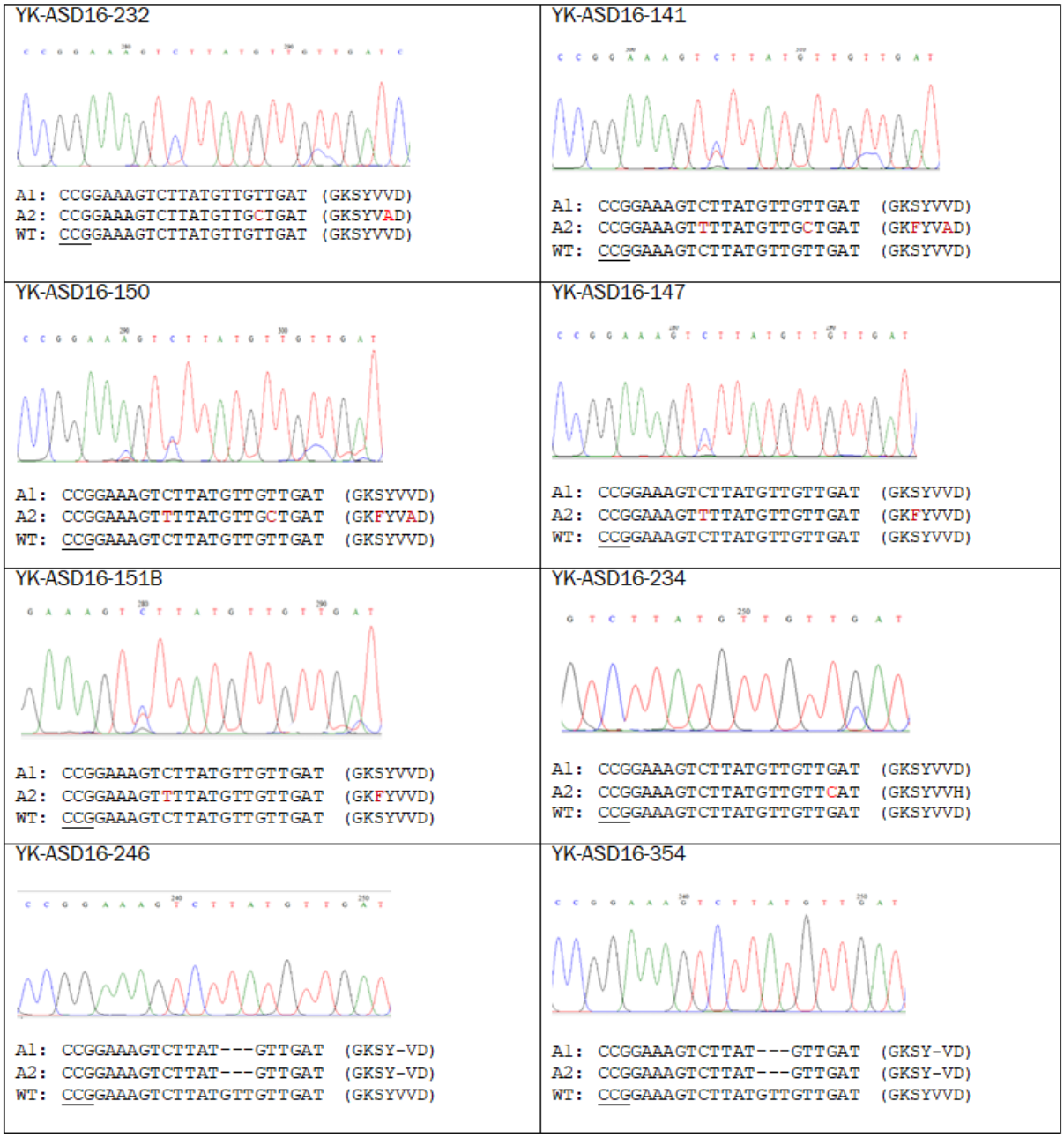


Table 3. Nucleotide traces and predicted protein sequences in T0 mutants.
The PAM sequence is underlined in the wild type allele. Substitutions in the nucleotide sequences and predicted protein sequences are denoted in red.A1: Allele 1, A2: Allele 2, WT: Wild type.

CONCLUSION

The present study is a report on the application of adenine base editing vector systems in creating targeted base substitution in *indica* rice. The successful generation of *eIF4G* mutants in the local elite cultivar ASD16 harboring mutations similar to that of naturally available tungro resistant genotypes can impart tungro disease resistance. The inheritance of mutation needs to be studied in subsequent T_1 and T_2 generations. Their performance needs to be assessed in homozygous T_2 progeny by conducting bioassay against rice tungro virus. Besides, their agronomic performance also needs to be compared with the ASD16 wild type in the T_2 population. These promising mutants in ASD16 background can be used directly for cultivation or as a parent to introgress the trait to other elite genotypes, once characterized for RTD resistance.

FUNDING AND ACKNOWLEDGMENT

The authors would like to thank ICAR-NASF (ICAR/CRISPR-Cas-7003/2017-18) for the funding and Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore for the facilities. YK also thank ICAR-NASF for the fellowship.

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