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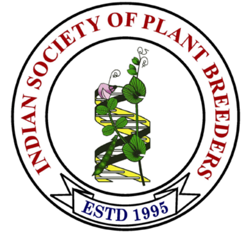

Research Article

Targeted editing of *OsSWEET11* promoter for imparting bacterial leaf blight resistance in rice

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Abstract
Rice is one of the most cultivated cereal crops worldwide and its productivity is affected by several biotic and abiotic factors. The gram-negative bacterium, *Xanthomonas oryzae pv. oryzae (Xoo)* severely impacts rice productivity by causing bacterial leaf blight disease. During infection, *Xoo* secretes a TALe protein which binds to effector binding element (EBE) present in the promoter sequence of susceptible genes, such as *SWEET* genes in order to make the plants vulnerable to infection. Mutations in EBE were shown to prevent binding of TALe and in turn result in enhanced resistance to the pathogen. In an attempt to engineer resistance in ASD16 to *Xoo* strains that secrete PthXo1, the EBE of promoter of *SWEET11* gene was edited through CRISPR/Cas9 tool. Genome editing of ASD16, through *Agrobacterium*-mediated transformation resulted in seventeen transgenic events. Thirty one plants belonging to thirteen independent transgenic events turned out to be mutants possessing biallelic or homozygous mutations. Bioassay studies on twelve T_0 mutant plants against *Xoo* revealed that eleven mutant plants were found to be resistant/ moderately resistant to the *Xoo* strain, indicating the potential of CRISPR technology in creating allelic variations which could be exploited in disease resistance breeding programmes.

Keywords: Rice, Bacterial Leaf Blight, *Xanthomonas oryzae pv. oryzae*, Effector Binding Element,CRISPR/Cas9

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most consumed staple foods in the world. It is cultivated in an area of 150 million hectares globally (Fahad *et al.,* 2019) and more than 3.5 billion human population depends on rice for 20% of their daily calorie requirement (Chukwu *et al.,* 2019; Mishra *et al.,* 2021). It is anticipated that world population may reach 9.3 billion by the year of 2050 which is 34% higher than current population level, so there is an immediate need for doubling rice production to meet up with the global food demands (Nayak *et al.,* 2021). However, a number of biotic and abiotic factors negatively impact rice production. *Xanthomonas oryzae pv. oryzae (Xoo)*, a gram-negative proteo-bacterium, causes vascular disease called bacterial leaf blight (BLB),

predominantly in rice growing areas of South-east Asia and sub-Saharan Africa (Mishra *et al.,* 2021; Borkar and Yumlembam, 2016). *Xoo* invades plant through wounds or damaged regions and results in yield losses upto 15% at early stages and 20 to 40% at maximum tillering stage (Blanvillain-Baufume *et al.,* 2017). During infection, *Xoo* invades the host machinery and releases transcriptional activator like effector molecules (TALe) which binds to effector binding element (EBE) present in promoter region of *SWEET* (Sugar will eventually be exported transporters) genes and activates them (Zafar *et al.,* 2020). Activation of *SWEET* genes enhances sugar production and its release into apoplast serves as nutritional source for *Xoo*, making host plant vulnerable to pathogen (Chen *et al.,* 2012). There are 20 *SWEET* genes whose products are responsible for sugar production and mediate sugar transport which are important for normal plant growth and development (Gupta 2020). *Xoo* targets clade III genes, such as *SWEET11*, *SWEET13* and *SWEET14* which are regarded as susceptible genes (Antony *et al.,* 2010; Streubel *et al.,* 2013). These three susceptible genes are targeted by six different TALes in nature. *Xoo* strains which encode PthXo1 and PthXo2 TALes target *SWEET11* and *SWEET13* genes, respectively and interfere with their expression. *SWEET14* gene is targeted by strains encoding any one of the four TALes, AvrXa7, PthXo3, TalC and TalF. Variations in the *SWEET* gene promoters, particularly in EBE sequences, generated through natural mutations have resulted in resistance to *Xoo* as the sequence variations in EBE prevents the binding of TALe to the promoter, leading to non-activation of *SWEET* genes during pathogen infection. These mutants are valuable resources for breeding rice cultivars with resistance to BLB (Chen *et al.,* 2010). Three recessive *R* genes, *xa13*, *xa25* and *xa41* which are mutants of *OsSWEET11*, *OsSWEET13* and *OsSWEET14,* respectively (Xu *et al.,* 2019). Novel mutations in EBEs of *SWEET* genes can be generated through sequence specific gene editing tools such as CRISPR/Cas9 with a view to developing novel recessive resistant alleles and in turn imparting resistance to *Xoo* in rice cultivars of choice (Zafar *et al.,* 2020; Arulganesh *et al.,* 2021).

Clustered regularly interspaced short palindromic repeats (CRISPR) and their associated proteins (Cas) are part of an adaptive immune system in prokaryotes which employs a small guide RNA for generating sequence-specific double stranded break of invading nucleic acids in order to resist their invasion. Repurposing the CRISPR/Cas9 system for genome editing exploits the cells' DNA repair mechanism. The double-strand DNA break created by CRISPR/Cas9 system is repaired by the cell through non-homologous end joining (NHEJ) repair mechanism. This repair pathway is error-prone leading to point mutations, deletions or / and insertions. As a result, there is a frame-shift of the reading frame leading to truncation of gene product and abolition of its function. The CRISPR/Cas system has been successfully employed to impart novel traits, such as disease resistance or product quality.

The previous studies on editing of *SWEET* genes has shown that the mutations could be introduced in the EBE region of *SWEET* gene's promoters using CRISPR/Cas9 technology (Oliva *et al.,* 2019; Xu *et al.,* 2019) and that such mutants were resistant to the *Xoo* strains. In this study, an attempt was made to edit EBE in the promoter region of *OsSWEET11* gene, to prevent the binding of PthXo1 TALe and impart BLB resistance in an elite cultivar, ASD16.

MATERIALS AND METHODS

Development of pRGEB32-*OsSWEET11* construct: The promoter sequence of *OsSWEET11* gene with locus ID BGI0SGA026582 was retrieved from the online database EnsemblPlants (https://plants.ensembl.org) (**Fig. 1**). Based on the sequence information available in the database, a 20 bp target sequence (top strand : 5'-ACTTTTGGTGGTGTACAGTA-3' and bottom strand: 5'-TACTGTACACCACCAAAAGT-3') upstream of PAM in EBE region of *OsSWEET11* promoter was chosen (CRISPR-P 2.0, http://crispr.hzau.edu.cn). The target sequence was incorporated with *Bsa*I adapters such as 5'-GGCA-3' on top strand and 5'-AAAC-3' on bottom strand, respectively to facilitate cloning of target sequence in *Bsa*I site, immediately downstream to snoRNA U3 promoter of pRGEB32 plasmid (A gift from Dr. Yinong Yang's lab; Addgene plasmid #63142). The binary vector possesses snoRNA U3 promoter of rice for the expression of sgRNA, polyubiquitin promoter for the expression of *Cas9* gene and *CaMV35S* promoter for the expression of selectable marker, *hpt* gene (**Fig. 2**). The cloning was performed in DH5α strain of *E. coli* competent cells. The recombinant pRGEB32, harbouring *OsSWEET11* sgRNA was mobilized into LBA4404 strain of *Agrobacterium tumefaciens* with the help of helper strain pRK2013 *via* triparental mating. The transconjugant *Agrobacterium* was used for transformation of an elite rice cultivar, ASD16 which is susceptible to BLB.

Generation of T_0 transgenic lines: *Agrobacterium*-mediated transformation of ASD16 cultivar: Immature seeds were collected about 12-15 days post anthesis and dehusked. The dehusked seeds were subjected to surface sterilization with 70% ethanol for 2 minutes, 2% sodium hypochlorite for 3 minutes and finally 3 washes with double autoclaved distilled water, each for 5 minutes. Followed by surface sterilization, immature embryos were isolated aseptically and used as explants for *Agrobacterium*-mediated transformation according to the protocol recommended by Hiei and Komari (2008). The isolated embryos were kept on NB-As medium (Hiei and Komari, 2008) supplemented with acetosyringone. The embryos were then infected with 3 µl of freshly prepared infection medium. The infected explants were incubated in dark at 25 ˚ C for 20 minutes and transferred to fresh NB-As medium and subjected to incubation for 7 days under dark at 25 ˚ C. After 7 days, the germinated explants (**Fig. 3a**) were sub-cultured onto resting medium (CCMC; Hiei and Komari, 2008) by removing elongated

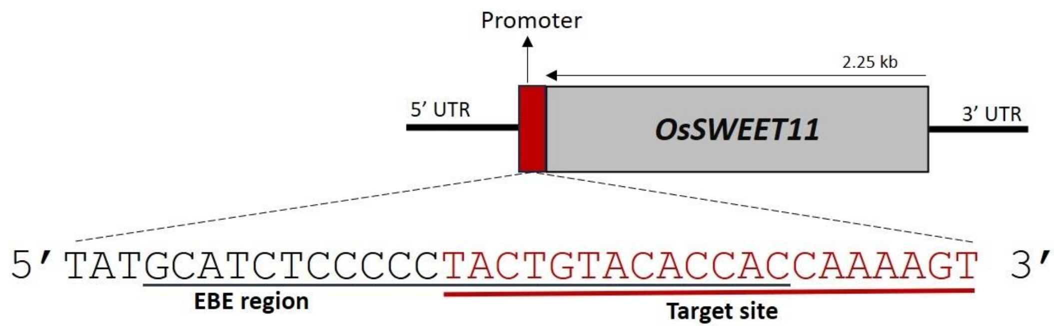

Fig. 1. Illustration of target EBE region in the promoter of *OsSWEET11* gene

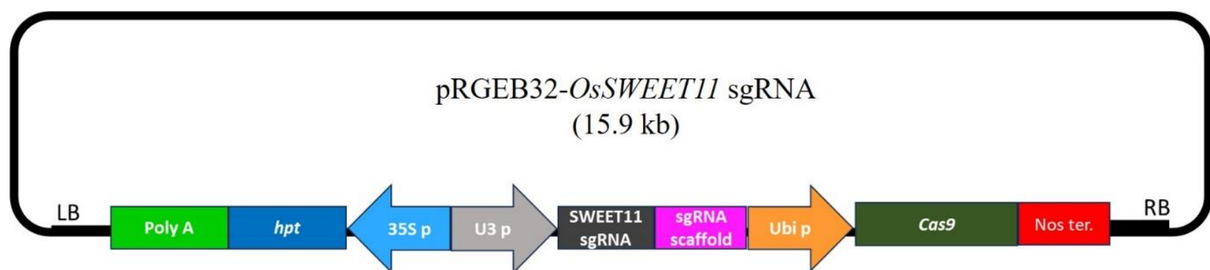

Fig. 2. Physical map of the pRGEB32 plasmid harbouring *OsSWEET11* sgRNA expression cassette

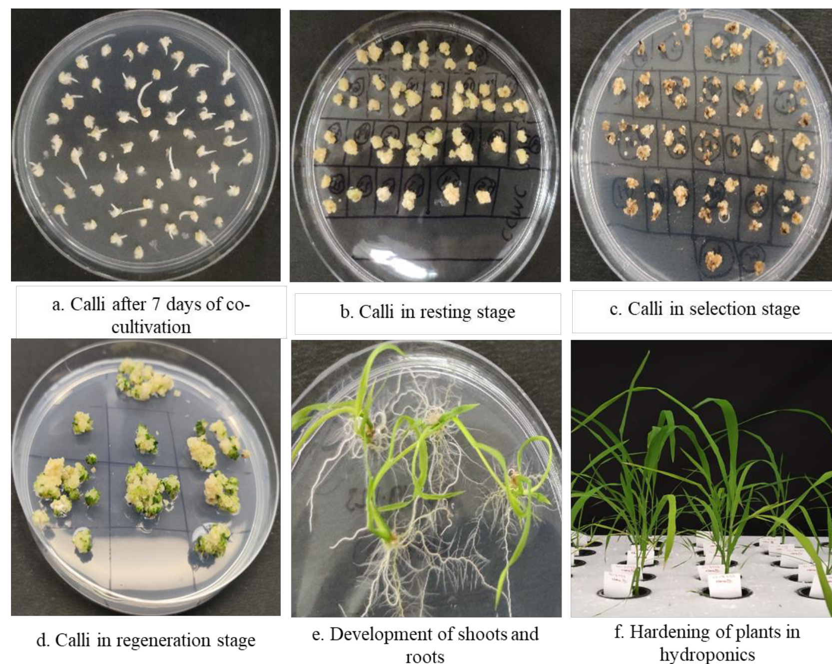

Fig. 3. *Agrobacterium*-mediated transformation of rice

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shoots and incubated with continuous illumination at 30 ˚ C for 5 days (**Fig. 3b**). After 5 days, calli were sub-cultured onto a fresh CCMC medium for 10 days followed by two stringent selections for 17 days (10+7) on CCMC medium supplemented with hygromycin (50 mg/l) (**Fig. 3c)**. After 17 days of selection, healthy calli were sub-cultured onto pre-regeneration medium (NBPRCH40; Hiei and Komari, 2008) and then to regeneration medium (RNMH30; Hiei and Komari, 2008; **Fig. 3d**). Green shoots from regenerated calli were transferred to rooting medium (**Fig. 3e**) supplemented with hygromycin (30 mg/l) and well-developed plantlets were transferred to bottles containing rooting medium (Murashige and Skoog, 1962). Plantlets with well-established roots were transferred to hydroponics and maintained under greenhouse conditions (**Fig. 3f**). The transformation efficiency (%) was computed as follows: (Number of co-cultivated embryos that produced plants / Total number of embryos co-cultivated) × 100.

Molecular characterization of T_0 edited plants: Genomic DNA of T_0 plants was isolated following CTAB method (Porebski *et al.,* 1997) and analyzed through PCR for the presence of *Cas9* and *hpt* gene sequences (**Table 1**). Later, the target region was amplified with *OsSWEET11* gene specific primers (**Table 1**) and the amplified PCR product was sequenced (Biokart India Pvt. Ltd., Bangalore). The Sanger sequencing results were analysed for mutations in the target region through online tools such as DSDecodeM (http://skl.scau.edu.cn/dsdecode/) and CRISP-ID (http://crispid.gbiomed.kuleuven.be/). The mutation efficiency (%) was calculated as follows: (Number of events with mutations/ Total number of events generated) × 100.

Evaluation of T_0 mutants against DX 170 PT-5 strain: The *Xoo* strain, DX 170 PT-5 obtained from Central University of Kerala, Kasaragod was used in bioassay studies. The culture was maintained at 28 ˚ C by continuous sub-culturing to sustain its virulence. Serial dilution was performed with 3 days old culture in 10 mM $MgCl_2.6H_2O$ under aseptic conditions and optical density was adjusted to 0.5 at 600 nm in spectrophotometer. Healthy plants in each event having homozygous/biallelic mutations were selected and inoculated with bacterial suspension using leaf clipping method (Kauffman *et al.,* 1973). In each plant, second, third and fourth leaves of primary tiller were inoculated with bacterial suspension and leaves of other tiller were inoculated with $MgCl_2.6H_2O$ (without *Xoo* culture). Inoculated wild type plants were also maintained as control. The plants were maintained at controlled environment and lesion length was recorded after 14 days post inoculation.

Based on the BLB disease severity scale (IRRI, 2013), the plants were categorised as follows:

Lesion Length (cm)	Host response
0	Highly Resistant (HR)
> 0 – 5	Resistant (R)
> 5 – 10	Moderately Resistant (MR)
> 10 – 15	Moderately Susceptible (MS)
> 15	Susceptible (S)

RESULTS AND DISCUSSION

Resistance breeding programmes and cultivation of inherently resistant cultivars are sustainable approaches to mitigate the loss of agricultural production due to diseases. Traditional plant breeding programmes, though successfully employed in developing disease resistant cultivars, besides being tedious and time consuming, are limited by the availability of genes for resistance. However, new plant-breeding technique, such as gene editing has become a powerful tool that allows breeders to improve the agronomic traits by enabling targeted modification of relevant genes. Gene editing tools are recognized as new methods for crop improvement, particularly in resistance breeding programme. Besides, the genome edited plants which are free from transgene sequences are considered

Table 1. List of primers used in this study

Name of the gene	Primer sequence (5' to 3')	Amplicon size (bp)	PCR conditions
hpt	Forward primer : GCTGTTATGCGGCCATTGGTC Reverse primer : GCCTCCAGAAGAAGATGTTG	686 bp	95 °C for 5 minutes – 1 cycle 94 °C for 45 seconds 57.8 °C for 45 seconds 30 cycles 72 °C for 45 seconds 72 °C for 10 minutes – 1 cycle
Cas9	Forward primer : CTTCTGGCGGTTCTCTTTAG Reverse primer : TGCTGTTTGATCCGTTGTTG	478 bp	95 °C for 5 minutes – 1 cycle 95 °C for 45 seconds 52 °C for 45 seconds 30 cycles 72 °C for 45 seconds 72 °C for 10 minutes – 1 cycle
OsSWEET11	Forward primer : ACGTGTCATATTGCCCCTCA Reverse primer : TCAGTTGCATTTGTCCATGGA	574 bp	95 °C for 5 minutes – 1 cycle 95 °C for 45 seconds 61.8 °C for 45 seconds 30 cycles 72 °C for 45 seconds 72 °C for 10 minutes – 1 cycle

non-transgenic and require lighter regulation before environmental release in several countries including India. Among different genome editing tools, CRISPR has established itself as a most popular platform for genetic manipulation with high efficiency and specificity (Bao *et al.,* 2019).

Development of pRGEB32-*OsSWEET11* **construct:** Earlier studies demonstrated that the introduction of minor changes in EBE of *SWEET* gene promoter conferred resistance to *Xoo* (Oliva *et al.,* 2019; Arulganesh *et al.,* 2021; Diana *et al.,* 2022). In order to introduce minor changes in EBE of promoter region of *OsSWEET11* gene to prevent binding of TALe (PthXo1), a target sequence (20 bp) was cloned in *Bsa*I site of binary vector, pRGEB32. The presence of target sequence was confirmed through Sanger sequencing. The above binary vector was used to transform an elite *indica* rice genotype, ASD16. Genetic transformation of ASD16: Rice cv. ASD16 which is susceptible to BLB was used as the genetic background for CRISPR/Cas9 based gene editing in this study. The recombinant binary vector harbouring *OsSWEET11* sgRNA was mobilized into LBA4404 strain of *Agrobacterium tumefaciens via* triparental mating. *Agrobacterium* suspension harbouring *OsSWEET11* construct was used for co-cultivation of immature embryos of ASD16. After two rounds of stringent selection under hygromycin selection system, 17 transgenic events were generated with a transformation efficiency of 1.07 per cent (**Table 2; Fig. 3**).

Molecular characterization of edited (T_0) plants: Analysis of T_0 plants for presence of *Cas9* and *hpt* genes, revealed expected amplicon size of 478 bp and 686 bp, respectively in all regenerated plants (**Fig. 4** & **Fig. 5**) indicating the stringency and efficiency of hygromycin selection system in rice transformation experiments. Target specific amplification of *OsSWEET11* gene showed an amplicon size of 574 bp which was further analyzed through Sanger sequencing for identifying mutations in the target region. Mutations were identified in 31 plants belonging to thirteen different independent events (**Table 3**) with a mutation efficiency of 76.5 per cent (**Table 2**). Among them, nine plants had biallelic mutations and sixteen plants had homozygous mutations and six plants had chimeric mutations. Three types of mutations *viz.,* deletions, insertions and substitutions were observed in the mutant plants with deletion mutants being the predominant ones. Deletion mutation was observed with varying number of nucleotides being deleted which ranged between 1 and 39 in the present study. Such variation in deletions with large numbers of base pairs being deleted have been reported earlier (Hu *et al.,* 2019; Tang *et al.,* 2017; Zhou *et al.,* 2014; Kumam *et al.,* 2022).

Table 2. ***Agrobacterium*-mediated genome editing of ASD16**

Number of co-cultivated embryos	Number of calli survived after hygromycin selection	Number of events regenerated	Number of events with mutation	Transformation efficiency (%)	Mutation efficiency (%)
1587	241	17	13	1.07	76.5

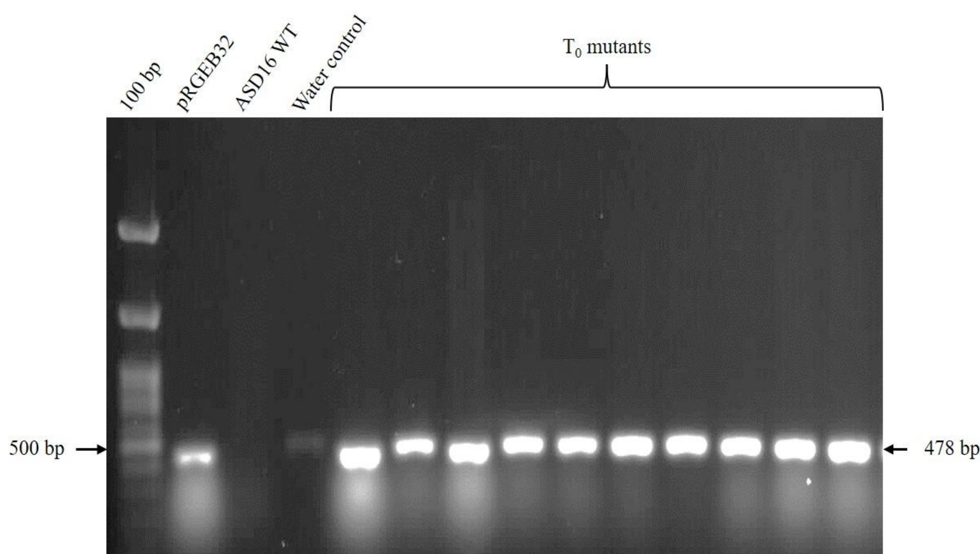

Fig. 4. PCR analysis for the presence of *Cas9* gene in T_0 mutants

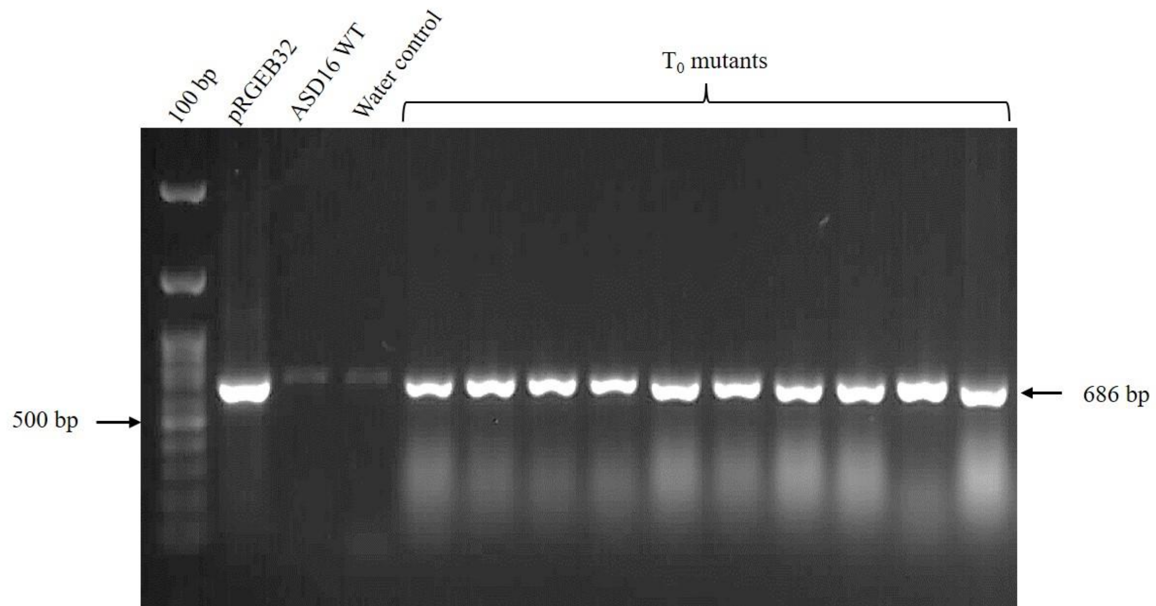

Fig. 5. PCR analysis for the presence of *hpt* gene in T_0 mutants

Table 3. Details of nucleic acid sequence in the target region of ASD16 T_0 mutants

Event I.D	Number of plants	Plant I.D.	Nucleotide sequence	Zygosity
Wild type	-	ASD16	WT: TATGCATCTCCCCCTACTGTACACCACCAAAAGTGGAGGG	-
BSN-ASD16-1	2	BSN-ASD16-1/1	A1: TATGCATCTCCCCCTATTTGACCCCA---AAAATGAAGGG A2: TATGCATCTCCCCCTATTTGACCCCA---AAAATGAAGGG	Homozygous (3d, 6s)
		BSN-ASD16-1/2	A1: TATGCATCTCCCCCTATTTG----CCCCCAAAAATGAAGGG A2: TATGCATCTCCCCCTATTTG----CCCCCAAAAATGAAGGG	Homozygous (4d,1i,6s)
BSN-ASD16-2	2	BSN-ASD16-2/1	A1: TATGCATCTCCCCCTAT--------------ATTGGAGGG A2: TATGCATCTCCCCCTA-TGT-------------TGGAGGG	Biallelic (14d,2s/14d)
		BSN-ASD16-2/1	A1: TATGCATCTCCCCCTA-TGT-------------TGAAGGG A2: TATGCATCTCCCCCTA-TGTA-------------GAAGGG	Biallelic (14d,1s/14d,1s)
BSN-ASD16-3	3	BSN-ASD16-3/1	A1: TATGCATCTCCCCCTACTTGAAAACGA----------AGGG A2: TATGCATCTCCCCCTACTTGAAAACGA----------AGGG	Homozygous (10d,1i,3s)
		BSN-ASD16-3/2	A1: TATGCATCTCCCCCTACCTGAAAACGA---AGGGTCTCGAAGGG A2: TATGCATCTCCCCCTACCTGAAAACGA---AGGGTCTCGAAGGG	Homozygous (3d,4i,6s)
		BSN-ASD16-3/3	A1: TATGCATCTCCCCCTACTTGAAA--ACGAAGGGTCTCGAAGGG A2: TATGCATCTCCCCCTACTTGAAA--ACGAAGGGTCTCGAAGGG	Homozygous (2d,3i,7s)
BSN-ASD16-5	3	BSN-ASD16-5/1	A1: TATGCATCTCCCC-TACCCCACCCAACCAGAAGGGGAGGC A2: TATGCATCTCCCC-TACCCCACCCAACCAGAAGGGGAGGC	Homozygous (1d,8s)
		BSN-ASD16-5/2	A1: TATGCATCTCCCCC-ACCCCACCCAACCAGAAGGGGAGGG A2: TATGCATCTCCCCC-ACCCCACCCAAACAGAAGGGGAGGC	Biallelic (1d,7s/1d,8s)
		BSN-ASD16-5/3	A1: TATGCATCTCCCCCTACT--ACCCAAACTGAAGGGGAGGC A2: TATGCATCTCCCCCTACT--ACCAAACCAGAAGGGGAGGC	Biallelic (2d,7s/2d,6s)

Table 3. Continued..

Event I.D	Number of plants	Plant I.D.	Nucleotide sequence	Zygosity
BSN-ASD16-7	2	BSN-ASD16-7/1	A1: TATGCATCTCCCCCTACT--ACACCACCAAAAGTGGAGGG A2: TATGCATCTCCCCCTACT--ACACCACCAAAAGTGGAGGG	Homozygous (2d)
		BSN-ASD16-7/2	A1: TATGCATCTCCCCCTACT--ACACCACCAAAAGTGGAGGG A2: TATGCATCTCCCCCTACT--ACACCACCAAAAGTGGAGGG	Homozygous (2d)
BSN-ASD16-10	3	BSN-ASD16-10/1	A1: TATGC--------------------------------------- A2: TATGC---------------------------------------	Homozygous (39d)
		BSN-ASD16-10/2	A1: TATGC--------------------------------------- A2: TATGC---------------------------------------	Homozygous (39d)
		BSN-ASD16-10/3	A1: TATGC--------------------------------------- A2: TATGC---------------------------------------	Homozygous (39d)
BSN-ASD16-12	3	BSN-ASD16-12/1	A1: TATGCATCTCCCCCTAC-----ACCACCAAAAAAGGAGGG A2: TATGCATCTCCCCCTAC-----ACCACCAAAAAAGGAGGG	Homozygous (5d)
		BSN-ASD16-12/2	A1: TATGCATCTCCCCCTACT-TACACCCCCAAAAATGGGGGG A2: TATGCATCTCCCCCTACT-TACACCCCCCAAAATGGGGGG	Biallelic (1d,3s/1d,4s)
		BSN-ASD16-12/3	A1: TATGCATCTCCCCCTACT-TACACCCCCCAAAATGGGGGG A2: TATGCATCTCCCCCTACT-TACACCCCCAAAAATGGGGGG	Biallelic (1d,2s/1d,1s)
BSN-ASD16-13	1	BSN-ASD16-13/1	A1: TATGCATCTCCCCCTACT-TGCACCCAAAAAAGAGGGGGG A2: TATGCATCTCCCCCTACT-TGCCCCCAAAAAAGGGGGGGG	Biallelic (1d,6s/1d,7s)
BSN-ASD16-14	1	BSN-ASD16-14/1	A1: TATGCATCTCCCCCTACTGTACACCACCAAAAGGGGAGGG A2: TATGCATCTCCCCCTACTGTACACCACCAAAAGGGGAGGG	Homozygous (1s)
BSN-ASD16-15	2	BSN-ASD16-15/1	A1: TATGCATCTCCCCCTACT---CACCCCCCAAAAAGGGGGGGG A2: TATGCATCTCCCCCTACT---CACCCCCCAAAAAGGGGGGGG	Homozygous (3d,2i,4s)
		BSN-ASD16-15/2	A1: TATGCATCTCCCCCTACT---CACCCCCCAAAATGGGGGGG A2: TATGCATCTCCCCCTACT---CACCCCAAAAAATGGGGGGG	Biallelic (3d,1i,5s/3d,1i,5s)
BSN-ASD16-16	2	BSN-ASD16-16/1	A1: TATGCATCTCCCCCTACT--ACACCACCAAAAGTGGAGGG A2: TATGCATCTCCCCCTACT--ACACCACCAAAAGTGGAGGG	Homozygous (2d)
		BSN-ASD16-16/2	A1: TATGCATCTCCCCCTACT--ACACCACCAAAAGTGGAGGG A2: TATGCATCTCCCCCTACT--ACACCACCAAAAGTGGAGGG	Homozygous (2d)
BSN-ASD16-17	1	BSN-ASD16-17/1	A1: TATGCATCTCCCCCTAC----CACAACCAAAAGGGGAGGG A2: TATGCATCTCCCCCTAC----CACCACCAAAAGGGGAGGG	Biallelic (4d,1s/4d)

'd' denotes deletion; 'i' denotes insertion; 's' denotes substitution; the underlined sequence represents EBE.

Bioassay of T_0 mutants for resistance to bacterial leaf blight : Twelve T_0 plants (belonging to eight transgenic events) including five biallelic, six homozygous and one chimeric mutant plants were screened for BLB resistance by inoculating a *Xoo* strain, DX 170 PT-5 which targets the EBE of *OsSWEET11* gene. After 14 days of inoculation, water-soaked lesions were developed in all inoculated leaves with differences in lesion length. The wild type ASD16 (non-transformed) has developed typical blight symptoms as expected with lesion length of 18.6 cm showing susceptible reaction. Out of twelve plants, eight showed resistance reaction, three exhibited moderate resistance and one showed susceptible reaction (**Table 4, Fig. 6**). A homozygous mutant plant, #BSN-ASD16-10/3 exhibited a resistance reaction with a lowest lesion length of 2.13 cm. The mutant #BSN-ASD16-14/1 with a single nucleotide substitution behaved similar to wild type ASD16, showing susceptible reaction with a lesion length of 16.6 cm (**Table 4, Fig. 6**). Though three of the mutants showed moderate resistance as per IRRI disease severity scale, the lesion lengths of these plants were significantly smaller compared to wild type plant and marginally larger

Table 4. Mean lesion length of infected ASD16 T_0 mutants

S. No.	Mutants	Zygosity/ Mutation	Mean lesion length (cm)	Host response
0	Wild type ASD16	Nil	18.60 ± 1.84^{a}	Susceptible
1	BSN-ASD16-5/3	Biallelic (2d,7s/2d,6s)	3.6 ± 0.80^{b}	Resistant
2	BSN-ASD16-7/1	Homozygous (2d)	2.6 ± 0.50^{b}	Resistant
3	BSN-ASD16-10/1	Homozygous (39d)	5.8 ± 1.10^{b}	Moderately Resistant
4	BSN-ASD16-10/3	Homozygous (39d)	2.13± 1.44^{b}	Resistant
5	BSN-ASD16-12/2	Biallelic (1d,3s/1d,4s)	4.1 ± 1.49^{b}	Resistant
6	BSN-ASD16-12/3	Biallelic (1d,2s/1d,1s)	2.54 ± 0.77^{b}	Resistant
7	BSN-ASD16-13/1	Biallelic (1d,6s/1d,7s)	2.2 ± 0.55^{b}	Resistant
8	BSN-ASD16-13/2	Chimera	2.48 ± 0.67^{b}	Resistant
9	BSN-ASD16-14/1	Homozygous (1s)	16.6 ± 5.90^{a}	Susceptible
10	BSN-ASD16-15/1	Homozygous (3d,2i,4s)	5.5 ± 1.38^{b}	Moderately Resistant
11	BSN-ASD16-15/2	Biallelic (3d,1i,5s/3d,1i,5s)	5.0 ± 0.76^{b}	Resistant
12	BSN-ASD16-16/2	Homozygous (2d)	5.9 ± 1.33^{b}	Moderately Resistant

Table represents the mean lesion lengths of T_0 mutants after 14 days of inoculation of DX 170 PT-5.
Mean values were compared at C.D value 6.763.
Means denoted with same superscript do not differ significantly at P<0.05.

Fig. 6. Phenotypes of disease reaction in gene edited mutants of ASD16

compared to those plants exhibiting resistance reaction. In the present study, eight plants showed resistance reaction and three plants were moderately resistant based on the phenotype of the infected leaves. In all these plants, a significant level of sequence variation in EBE was observed. However, the mutant, BSN-ASD16-14/1 which had a substitution just outside the EBE, showed susceptible reaction similar to the wild type. In the earlier studies, it was reported that some of the mutations observed in T_0 generation were not inherited to T_1 generation (Kumam *et al.,* 2022). Moreover, some of the mutants were in biallelic condition and they would probably segregate in subsequent generations. It is important that the resistance against *Xoo* needs to be confirmed in subsequent generations also. None of the resistant or moderately resistant mutants had a single nucleotide deletion, insertion or substitution. Oliva *et al.* (2019) has proposed that the polymorphisms created in EBE through gene editing need to be larger than a single nucleotide change as TALe proteins with minor variations may bind to the modified EBE. They also suggested that the significant modifications (modifying the whole EBE) in EBE of the promoter would prevent TALe adaptation as the adaptation to new binding sites is inversely related to the number of novel nucleotides in the target sequence. Mutagenesis of coding region of *S* genes, such as *SWEET* genes will result in abnormal plants, as *OsSWEET* genes are required for sugar efflux and phloem loading, which provides roots and other tissues with energy (Chen *et al.,* 2010). In contrast, plants with edited EBE were shown to be resistant to *Xoo* infection (Xu *et al.,* 2019; Arulganesh *et al.,* 2021) but did not have any side-effects. However, Xu *et al*. (2019) observed reduced plant height in edited plants. In the present study, the plants were maintained under hydroponic conditions and no significant differences in agronomic traits were observed. Further studies on agronomic performance of edited plants needs to be undertaken in T_1 and T_2 generations, after eliminating heterologous sequences.

In conclusion, the eleven *Xoo* resistant / moderately resistant plants with mutations in the EBE region and reduced lesion length are promising candidates for further breeding processes. The finding indicates that editing of *OsSWEET11* gene, an *S* gene *via* CRISPR/Cas9 tool is an effective approach to develop BLB resistant cultivars in rice.

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