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Research Article

Targeted editing of *OsSWEET13,* a bacterial leaf blight susceptible gene in rice using CRISPR tool

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Abstract
Bacterial leaf blight (BLB) of rice is considered to be a disease of economic importance as the disease causes severe yield losses in all rice growing regions. Transcription activator like effector (TALE) molecules are produced by the pathogen, *Xanthomonas oryzae pv. oryzae* (*Xoo*) bind to the effector binding element (EBE) of the promoter of *SWEET* gene and activates transcription of *SWEET* genes, making the plant susceptible to the disease. Innate resistance to *Xoo* in certain rice genotypes is due to mutations in EBE present in the upstream regulatory region of *SWEET* genes. CRISPR–mediated targeted modification of susceptibility gene/promoter is an effective approach to develop BLB resistance in rice. The present study was an attempt to repress TALE triggered signalling *via* introducing *indels* in the EBE of *OsSWEET13* gene in a local popular rice genotype, CO51 employing CRISPR/Cas9 mediated genome editing tool with a view to imparting BLB resistance. *Agrobacterium*-mediated transformation using immature embryos followed by regeneration resulted in four independent transformation events. Five plants representing three events were found to have one nucleotide deletion in the target sequence. These deletion mutations in EBE could potentially interfere with the binding of the corresponding TALE to confer resistance against certain strains of BLB.

Keywords: Rice, Bacterial Leaf Blight, *SWEET13* gene, Effector Binding Element, CRISPR/Cas9.

INTRODUCTION

Rice (*Oryza sativa*) is a major staple food for almost half of the world's population due to its adaptability to different environmental conditions (Long-ping, 2014). Because of its economic and social importance, there is a constant requirement for developing new varieties with improved agronomic characteristics, such as tolerance to different biotic and abiotic stresses. Bacterial leaf blight (BLB), one of the most devastating diseases of rice, occurs predominantly in India, South-East Asia, Africa and Japan, causing yield losses ranging from 20 to 50 per cent, and is considered a potential threat to rice cultivation, especially during the South-West monsoon season (Lal *et al*., 2017). Bacterial leaf blight disease in rice is caused by the *Xanthomonas oryzae pv. oryzae* (*Xoo*). *Xoo* belongs to the family of gram-negative proteobacteria (Jonit *et al*., 2016). The pathogen enters the host plant system *via* natural openings and colonizes the xylem vessels. The symptoms begin to appear at the tillering stage and reach peak at the flowering stage. Droplets of bacterial ooze can be observed on younger lesions. In an advanced stage, the infection spreads and the leaves wilt completely, leading to 'kresek' symptoms (Raju *et al*., 2020).

During the infection process, *Xoo* pathogen secretes TALE molecules that bind to the EBE present in the upstream regulatory region of the *SWEET* genes of rice. These genes are negative players of BLB resistance and major BLB susceptibility genes in rice are *OsSWEET11, OsSWEET13* and *OsSWEET14.* Due to mutations in the EBEs of the promoter of *SWEET* genes, some rice cultivars show natural resistance genes against BLB. Given the above, mutations in EBE of *OsSWEET* genes through genome editing are expected to impart resistance in susceptible rice varieties (Zafar *et al*., 2020).

Oliva *et al*. (2019) have demonstrated that the upregulation of *SWEET* genes had resulted in over-accumulation of sugars, rendering the plants susceptible to the BLB pathogen. Six TALE proteins are known to bind to the promoters of *OsSWEET* genes. TALEs such as, PthXo3, AvrXa7, TalC, and TalF are shown to bind to EBE present in the promoter of *OsSWEET14* gene (Blanvillain-Baufume *et al.,* 2017). The TALE, PthXo1 bind to EBE of *OsSWEET11 while* PthXo2 bind to EBEs of *OsSWEET13* gene (Oliva *et al.,* 2019). Mutant alleles of *SWEET* 11 (*xa13*), *SWEET13* (*xa25*) and *SWEET14* (*xa41*) were shown to be resistant to certain strains of *Xoo* (Chu *et al*., 2006; Hutin *et al*., 2015). These mutant alleles were reported to be due to indels in the upstream regulatory region of the rice *SWEET* genes (Oliva *et al.,* 2019).

Plant genome editing *via* the CRISPR/*Cas9* (Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein) tool is a popular approach in crop improvement programmes in recent times due to its ease, efficiency and simplicity. (Manghwar *et al*., 2019). Oliva *et al*. (2019) generated rice lines with broad spectrum resistant against *Xoo* strains of Asian origin that produce TALEs that bind to EBE of all the susceptible *SWEET* genes. Xu *et al*. (2019) used genome editing to impart durable broad spectrum resistance against BLB in rice by mutating the EBEs of all three *OsSWEET* genes. Zafar *et al*. (2020) engineered BLB resistance in Super basmati rice by targeting four EBEs found in the promoter region of *OsSWEET14* gene. Duy *et al*. (2021) used a CRISPR/Cas9-mediated editing method to improve BLB resistance of the rice variety TBR225 by affecting the binding of TALEs, AvrXa7, PthXo3 and TalF on the *OsSWEET14* promoter. Yu *et al*. (2021) used the CRISPR/Cas12 a genome editing tool to mutate EBE in the *Xa13* promoter to impart BLB resistance in rice. In another study, Arulganesh *et al*. (2021) produced indels in the EBE of *OsSWEET14* gene in an elite variety, CO51 *via* CRISPR/Cas9 tool. Bioassay studies on four mutant plants resulted in the identification of two plants with resistance/moderately resistance against BLB. These studies demonstrated that the CRISPR/Cas9 genome editing tool is efficient in conferring BLB resistance in rice. In the present study, an attempt was made to introduce BLB resistance in rice cultivar CO51 against *Xoo* strains *via* interfering with the binding of TALE onto the EBE of *SWEET13* gene.

MATERIALS AND METHODS

A popular short duration rice variety, CO51 was used in our study. Immature panicles were collected from the Department of Rice, Tamil Nadu Agricultural University and used as explants. The sequence and structure information of rice *OsSWEET13* gene (Locus ID. BGIOSGA037364) retrieved from the Ensembl Plants (https://plants.ensembl.org/index.html) database were used to design single guide RNA (sgRNA) for the study. A single guide RNA (sgRNA) targeting EBE (TATAAAGCACCACAACTCCCTTC; Oliva *et al*., 2019) of promoter region of *OsSWEET13* gene were designed (CRISPR-P 2.0; http://cbi.hzau.edu.cn/crispr/) tool (**Fig. 1**). The sgRNA sequence with upper strand: 5'-GCTCAACACCACGAAAATATA-3' and lower strand: 5'-GTATATTTTCGTGGTGTTGAG-3' which is located exactly in the EBE region were designed with appropriate adaptors to facilitate cloning in pRGEB32 binary vector (a gift from Dr Yinong Yang's lab, Addgene plasmid # 63142; http://n2t.net/addgene:63142;RRID: Addgene_63142) (Xie *et al*., 2015). A schematic diagram of pRGEB32 harbouring the *OsSWEET13* sgRNA sequence is illustrated in **Fig. 2**. The recombinant pRGEB32 plasmid, harbouring the expression cassette of *Cas9*/*OsSWEET13* sgRNA was mobilized into *Agrobacterium* strain, LBA4404 by triparental mating.

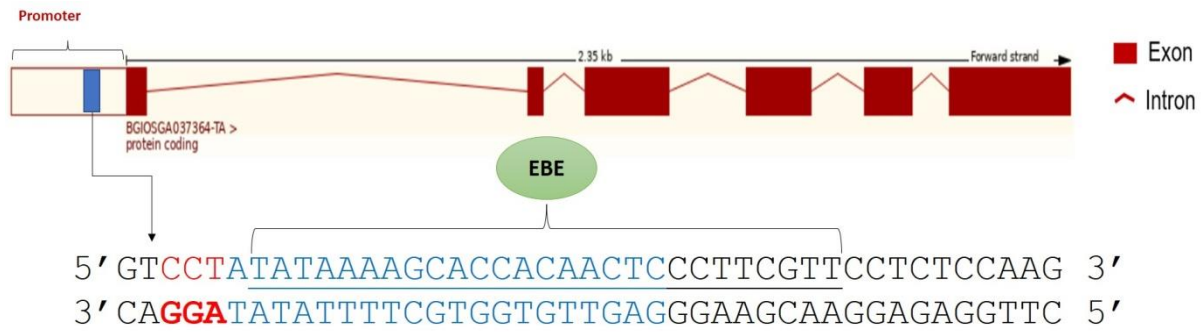

Fig. 1. Structure of *OsSWEET13* gene showing upstream regulatory sequences including EBEs for the TALE protein, PthXo2. The EBE is highlighted; sgRNA sequence is in blue and the PAM sequence is AGG

Fig. 2. T-DNA of pRGEB32 with sgRNA for targeting EBE of *OsSWEET13* gene

Agrobacterium-mediated rice genetic transformation was carried out using immature embryos collected from a high yielding popular *indica* cultivar, CO51. The genetic transformation method suggested by Hiei and Komari (2008) was followed with a few modifications (Nithya *et al*., 2020; Shweta *et al*., 2020; **Fig. 3**). Embryogenic calli survived after two rounds of hygromycin selection regenerated into shoots. The shoots were rooted on half-strength MS medium (Murashige and Skoog, 1962). The regenerated plants were acclimatized and grown to maturity in a transgenic greenhouse.

The plant DNA was extracted from non-transformed and putative edited mutants (T_0 generation) of CO51 using the CTAB method (Porebski *et al*., 1997). T_0 lines were screened for the presence of *Cas9* and *hpt* genes using specific primers as mentioned in **Table 1**. In PCR positive plants, the upstream regulatory region of about 408 bp which includes the EBE sequence was amplified using sequence specific primers (**Table 1**). The amplified products were Sanger sequenced. The sequences were analyzed using the online tool, DSDecodeM (http://skl.scau.edu.cn/dsdecode) (Xie *et al*., 2017; Liu *et al*., 2015) to detect the indels.

RESULTS AND DISCUSSION

In during recent years, genome editing has become a powerful tool for introducing precise alterations in any gene of interest to create novel alleles which could confer resistance to pest and pathogen resistance, abiotic stress tolerance and enhance nutritional value. Intensive research in this area led to the development of CRISPR/*Cas9* system and found to be efficient and easier than other systems such as ZFNs and TALENs. CRISPR/*Cas9* induced mutagenesis is equivalent to mutants generated through conventional mutation breeding as well as natural mutants but has a much higher specificity and efficiency. Besides, the exogenous genetic elements such as *Cas9* and marker genes can be segregated out in subsequent generations, generating plants that are free from foreign DNA (Zhou *et al*., 2014). Due to the above characteristics of the edited plants, several countries do not consider these plants, particularly edited plants belonging to 'site directed nuclease' (SDN) 1 and 2 types as transgenic and do not regulate. In India, SDN 1 and 2 type edited plants are lesser regulated (Anonymous, 2022). Consequently, crop improvement researchers across the globe consider CRISPR technology as a potential tool for crop improvement.

Rice bacterial leaf blight disease caused by *Xoo* results in severe yield losses in rice growing regions (Yu *et al*., 2021). TALEs from *Xoo* are injected into plant cells by the pathogen using a type III secretion system and once inside the host nucleus the TALEs recognize and bind to effector-binding elements (EBEs) present in susceptible *SWEET* genes' promoters, resulting in upregulation of *SWEET* genes leading to accumulation of sugars that provide a favourable condition for the pathogen (Oliva *et al*., 2019). Since, the TALEs produced by the pathogen bind to specific nucleotide sequences, these BLB susceptible genes serve as good targets in genome editing experiments for conferring resistance against BLB in rice (Hutin *et al*., 2015). Earlier reports have successfully demonstrated that the mutations in EBE will effectively prevent the binding of respective

Table 1. Oligomers used in PCR assays

Particular	Nucleotide sequence (5' - 3')	Amplicon size (bp)	PCR conditions	
hpt	hpt F (GCTGTTATGCGGCCATTGGTC) hpt R (GCCTCCAGAAGAAGATGTTG)	686	94 °C for 5min: 94 °C for 1 min 58 °C for 30 sec 72 °C for 30 sec 72 °C for 10 min:	1 cycle 30 cycles 1 cycle
Cas9-pRGEB32	Cas9 F (CTTCTGGCGGTTCTCTTCAG) Cas9 R (TGCTGTTTGATCCGTTGTTG)	478	95 °C for 5 min: 95 °C for 45 sec 52 °C for 45 sec 72 °C for 45 sec 72 °C for 10 min:	1 cycle 30 cycles 1 cycle
OsSWEET13	*SWEET 13* F (TATGGCTAGTGAGAGGTGCG) *SWEET 13* R (AGGAATTGAGCTTGTTGTTGC)	408	95˚C for 5 min : 95 ˚C for 45 sec 59 ˚C for 45 sec 72 ˚C for 45 sec 72 ˚C for 10 min :	1 cycle 30 cycles 1 cycle

Fig. 3. Stages of rice genetic transformation

TALEs and in turn impart resistance to BLB (Oliva *et al*, 2019; Xu *et al*., 2019; Zafar *et al*., 2020; Duy *et al*., 2021; Yu *et al*., 2021; Arulganesh *et al*., 2021).

Taking lead from these earlier studies, an attempt was made to create targeted *indels* in EBE of *SWEET13* gene in CO51. An appropriate sgRNA sequence (which targets the EBE) was designed, cloned into a popular binary vector, pRGEB32 possessing *Cas9* gene, subsequently mobilized into *Agrobacterium* strain, LBA4404 and used in transformation experiments. Immature embryos (250 embryos) of CO51 were infected with the *Agrobacterium* strain harbouring recombinant pRGEB32 possessing *SWEET13* sgRNA expression cassette. Co-cultivated calli were subjected to hygromycin selection for selective growth of transformed cells and recovery of edited plants. Thirteen plants (T_0) belonging to four independent transgenic events were generated with a transformation efficiency of 1.6 per cent.

Molecular characterisation of mutants by PCR assays confirmed the presence of *Cas9* and *hpt* genes with an expected amplicon size of about 478 bp and 686 bp. respectively in the putative mutants (**Fig. 4 and Fig. 5**). PCR amplification of mutant plants to amplify the target region in the promoter of *SWEET13* gave an expected amplicon of 408 bp which included EBE and sgRNA region. Sanger sequencing results revealed the occurrence of mutation in three events, with a mutation efficiency of 75% (**Table 2**). These three events had 5 plants and all of them were found to possess a single base pair deletion in homozygous condition (**Table 3**), while rest of the regenerated plants were found to be wild type (Data not shown).

All the mutant plants had indels more than 3 bp away from the PAM sequence on the upstream side (**Table 3**). All five homozygous T_0 plants had one base pair deletion as observed by earlier workers (Zhang *et al.,* 2014). These

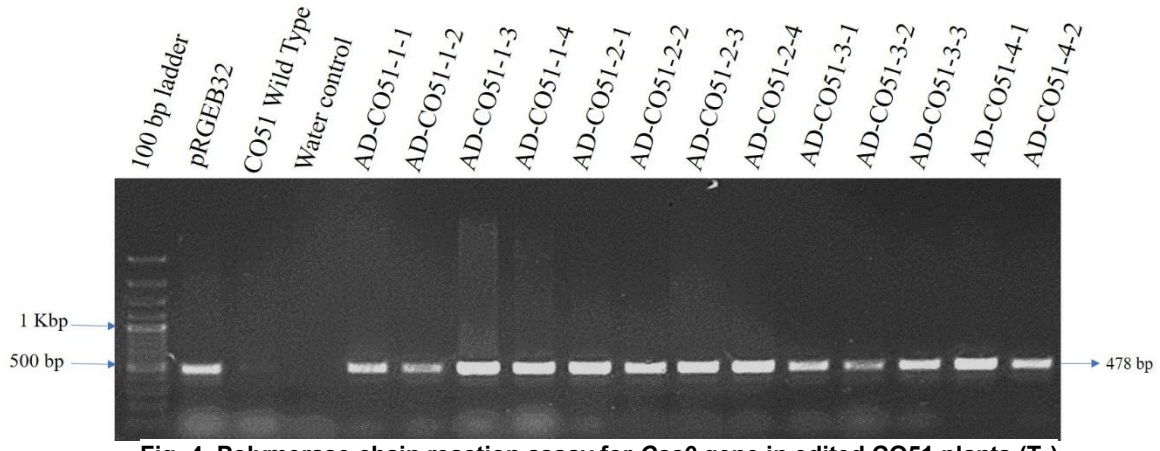

Fig. 4. Polymerase chain reaction assay for *Cas9* gene in edited CO51 plants (T_0)

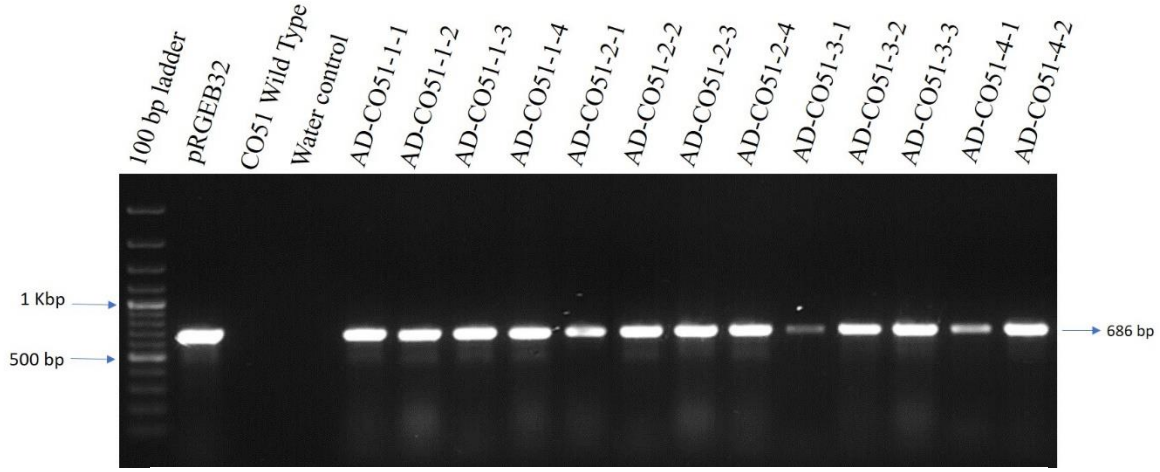

Fig. 5. Polymerase chain reaction assay for *hpt* gene in edited CO51 plants (T_0)

Table 2. Genetic transformation of rice cultivar CO51

Number of explants co-cultivated	Number of calli survived hygromycin selection	Number of events recovered	Events with *indels*	Transformation efficiency (%)*	Mutation efficiency (%)#
250	26	4	3	1.6	75.0

*Transformation efficiency: Percentage of embryos that produced PCR positive transgenic events
#Mutation efficiency: Percentage of transgenic events that turned out to be mutants

Table 3. Nucleic acid sequence of target region in the edited plants (T_0)

Event I.D	Plant I.D number	Nucleotide sequence	Zygosity
	Wild type CO51	WT: CCTATATAAAAGCACCACAACTCCCTTC	Wild type
AD-CO51-1	AD-CO51-1-2	A1:CCTATATAAA-GCACCACAACTCCCTTC A2:CCTATATAAA-GCACCACAACTCCCTTC	1 bp deletion (Homozygous)
	AD-CO51-1-3	A1:CCTATATAAA-GCACCACAACTCCCTTC A2:CCTATATAAA-GCACCACAACTCCCTTC	1 bp deletion (Homozygous)
AD-CO51-3	AD-CO51-3-1	A1:CCTATATAAA-GCACCACAACTCCCTTC A2:CCTATATAAA-GCACCACAACTCCCTTC	1 bp deletion (Homozygous)
	AD-CO51-3-2	A1:CCTATATAAA-GCACCACAACTCCCTTC A2:CCTATATAAA-GCACCACAACTCCCTTC	1 bp deletion (Homozygous)
AD-CO51-4	AD-CO51-4-1	A1:CCTATATAAA-GCACCACAACTCCCTTC A2:CCTATATAAA-GCACCACAACTCCCTTC	1 bp deletion (Homozygous)

'-' denotes deletion. PAM sequence is highlighted

above observations indicate the precision and reliability of the CRISPR tool. Besides, this tool significantly reduces the time taken to develop a variety as all plants generated are homozygous for the mutation, reducing the time required for product development. Oliva *et al.* (2019) reported that the mutants of *SWEET13* had indels, ranging from two-nucleotide insertions (+2) to seven-nucleotide deletion (−7), and these mutations conferred resistance. In yet another study, nine *SWEET14* mutants generated in a Vietnamese cultivar, TBR225 had deletions ranging from 2 to 5 nucleotides or insertion of one nucleotide (Duy *et al*., 2021). One of the T_2 lines, harbouring the homozygous 6-bp deletion, showed reduced *OsSWEET14* expression and total resistance to a Vietnamese strain. These authors also reported that the shorter modifications on the 3'end of this EBE could not affect *OsSWEET14* expression after infection and do not impart resistance against the corresponding strains. However, cultivars (Guihong 1 and Zhonghua 11) that are naturally resistant to *Xoo* secreting TALE, PthXo2 have one-base deletion in the EBE upstream of *OsSWEET13* and this deletion blocks the binding of the corresponding TALE, PthXo2 to the EBE and the induction of *OsSWEET13* expression (Ni *et al.,* 2021).

Earlier reports suggested that RNA mediated silencing or mutation of *SWEET* genes may negatively impact seed development (Antony *et al.,* 2010; Van Schie and Takken, 2012) 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, genome-edited rice plants with modified EBEs in the promoters of *OsSWEET* were resistant to *BLB*, but developed normally (Xu *et al*., 2019; Duy *et al.,* 2021; Yu *et al*., 2021). So, it is conceivable that a single nucleotide deletion in promoter region of the plants generated in this study will have no effect on the *SWEET* gene expression and growth and development of plants.

In conclusion, the putative mutants generated from the present investigation, with a deletion in the EBE could be promising candidates for BLB disease management. These mutants need to be evaluated for their resistance to BLB strains that express PthXo, for the absence of any unintended mutations through off-target analysis as well as agronomic performance. Based on published studies on imparting resistance to BLB using genome editing tools, our findings demonstrated that targeted modifications of *OsSWEET13* gene are an effective approach to develop BLB resistance in rice.

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