Received: 07 Oct 2021 | Revised: 09 Nov 2021 | Accepted: 13 Nov 2021

Electronic Journal of Plant Breeding

Research Article

Genome editing of elite rice cultivar CO51 for bacterial leaf blight resistance

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Abstract
Bacterial leaf blight (BLB) of rice caused by *Xanthomonas oryzae* pv *oryzae* (*Xoo*) is a major constraint in rice production. Natural resistance to BLB in some of the wild type cultivars of rice is due to mutations in effector binding element (EBE), present in the promoter region of *SWEET* genes. *SWEET14* is one of the most commonly targeted genes by majority of the TALEs of *Xoo* pathogen. Thus, the present study was aimed at creating novel mutations in the EBE of *OsSWEET14* gene in *indica* rice cultivar, CO51 through CRISPR/Cas9 mediated genome editing technology to impart resistance against BLB. *Agrobacterium*-mediated transformation using immature embryos followed by regeneration resulted in eleven transgenic plants from six independent transformation events, of which nine plants (belonging to five events) had mutations in the target sequence. Bioassay studies on four mutant plants (belonging to three events) resulted in the identification of two plants (belonging to two events) with resistance/moderately resistance against BLB.

Key words: Rice, *SWEET* genes, CRISPR/Cas9, Bacterial Leaf Blight, Effector Binding Element

INTRODUCTION

Rice (*Oryza sativa*) is a cereal crop that is consumed by half of the global population (Fukagawa and Ziska, 2019). High population growth, limited arable land, shortage of other natural resources and outbreak of crop diseases limit rice cultivation and production (FAO, 2017). Among these constraints, yield loss due to diseases is a serious concern, contributing up to 16 per cent globally (Ficke *et al.,* 2018). Bacterial leaf blight (BLB) is one of the major foliage diseases that attack the rice crop seasonally. Yield losses due to BLB have been reported to vary from 2 to 74 per cent depending on location, season, crop growth stage and cultivar (Rao and Kauffman, 1977; Reddy *et al.,* 1978). Major symptoms of BLB include water soaked yellowish stripes on leaf blades, curling of leaves and early morning appearance of bacterial ooze on young lesions on the leaves (Nino-Liu *et al*., 2006). The pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo)* targets *SWEET (Sugar will eventually be exported transporters)* genes in rice during BLB infection. Even though the rice genome has more than 20 *SWEET* genes, only three (*OsSWEET11, OsSWEET13* and *OsSWEET14*) are targeted by the *Xoo* pathogen, and these genes are called 'susceptible (S) genes' (Oliva *et al.,* 2019). During infection with BLB, the *Xoo* pathogen secretes Transcription Activator like Effector (TALE) molecules which bind to the effector binding element (EBE) in the promoter region of some of the rice *SWEET* genes. The binding of TALEs to the promoter region results in the over-expression of *SWEET* genes. Over-expression of *SWEET* genes results in the production of sugars,

rendering the plant susceptible to the pathogen (Oliva *et al.,* 2019). There are six known TALE proteins that target *OsSWEET* gene promoter regions, of which four different TALEs *viz.,* PthXo3, AvrXa7, TalC, and TalF (previously known as Tal5), target EBE present in *OsSWEET14* gene (Blanvillain-Baufume *et al.,* 2017). The remaining two TALEs, PthXo1 and PthXo2 target EBE present in *OsSWEET11* and *OsSWEET13* genes respectively (Oliva *et al.,* 2019). Naturally occurring recessive alleles of these *SWEET* genes confer resistance against BLB (Zafar *et al.,* 2020). Recessive alleles, *xa13* and *xa25* for the genes *OsSWEET11* and *OsSWEET13* were found to confer resistance against BLB caused by *Xoo* race 6 (Chu *et al*., 2006) and *Xoo* strain PXO339 (Chen *et al.,* 2002), respectively. With respect to *the OsSWEET14* gene, *xa41* recessive allele identified in a few of the African wild type cultivars, *Oryza barthii* and *O. glaberrima* conferred resistance to BLB (Hutin *et al.,* 2015). Most of the recessive alleles contain nucleotide polymorphisms in the EBE of *OsSWEET* genes (Oliva *et al.,* 2019).

Genome editing is a novel biotechnological approach that promises to create nucleotide modifications at the target region within a gene. Previous studies have used genome editing technologies such as CRISPR-Cas9 to generate resistance against BLB by creating targeted mutations in the EBE of *OsSWEET14* genes (Oliva *et al.,* 2019; Blanvillain-Baufume *et al.,* 2017; Zafar *et al.,* 2020). Though there are several types of CRISPR systems available, the Cas9 nuclease from *Streptococcus pyogenes* (a type II CRISPR/Cas9 system) is most commonly used (Li *et al.,* 2020b). Cas9 creates a double stranded break at the target region based on PAM (proto-spacer adjacent motif) sequence 'NGG' which is later repaired either by non-homologous end joining (NHEJ) or homology directed repair (HDR) mechanism (Jiang and Doudna, 2017). Since the CRISPR/cas9 induced mutation occurs in the T-DNA transformed rice calli before the regeneration stage, chances are there to identify homozygous mutants with high mutation frequencies in the T_0 generation itself. At the same time, mutants without exogenous T-DNA can be segregated out in the T_1 generation. These mutants are also known as "transgene-free" mutants. (Ricroch *et al.,* 2017). Considering these available opportunities, the present study was aimed towards the development of BLB resistant lines in the genetic background of a short duration *indica* rice cultivar CO51, by using CRISPR/Cas9 technology.

MATERIALS AND METHODS

Plants of the high yielding, popular short duration rice genotype, CO51 were maintained at Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore for regular supply of immature seeds. The immature seeds were collected from the field and used as the source of explants.

The nucleotide sequence of promoter region encompassing the EBE of *OsSWEET14* gene (Os11g0508600) was retrieved from the online database tool, EnsemblPlants (https://plants.ensembl.org/index.html). This sequence information was used to design single guide RNA (sgRNA) using the web-based tool, CRISPR P 2.0 (http://crispr.hzau.edu.cn/) (**Fig. 1**). The design of sgRNA was restricted to the 29 bp EBE sequence of the promoter region. This identified a promising sgRNA sequence with top strand: 5' ATATAAACCCCCTCCAACC 3' and bottom strand 5' GGTTGGAGGGGGTTTATAT 3'. During the synthesis of these DNA oligomers, *Bsa*I adaptors (5'GGCA 3' for the top strand and 5' AAAC 3' for the bottom strand) were added to facilitate cloning in the *Bsa*I restriction site of the binary vector, pRGEB32. The plasmid, pRGEB32, was a gift from Dr Yinong Yang's lab (Addgene plasmid # 63142; http://n2t.net/addgene:63142; RRID: Addgene_63142) (Xie *et al*., 2015). The T-DNA of the binary vector harbouring the sgRNA sequence is represented in **Fig. 2.**

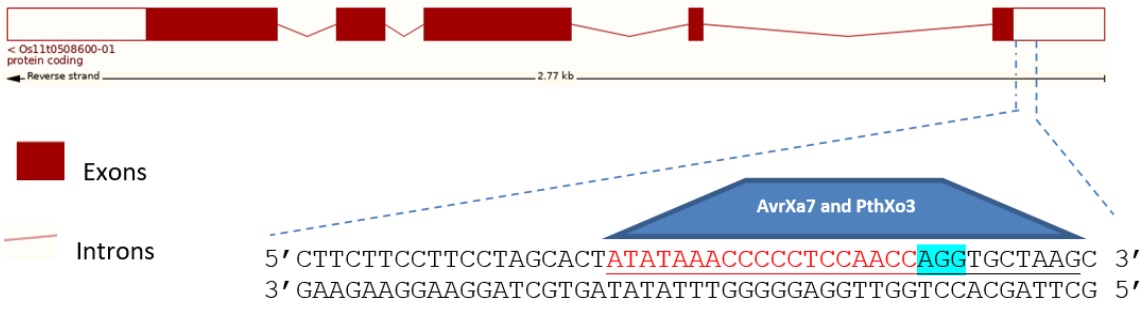

Fig. 1. Genomic structure of *OsSWEET14* gene containing effector binding elements (EBEs) for the Transcription-Activator-Like Effectors (TALE) proteins AvrXa7 and PthXo3. The 29 bp EBE is underlined. The sgRNA sequence is in red and the protospacer adjacent motif PAM sequence (AGG) is highlighted in blue.

Fig. 2. T-DNA of binary vector pRGEB32 harbouring the sgRNA of *OsSWEET14* gene

The recombinant binary vector, pRGEB32 harbouring the Cas9/*OsSWEET14sgRNA* was mobilized into *Agrobacterium* strain, LBA4404 by triparental mating. Immature embryos of CO51 were collected from maturing panicles (12-14 days after anthesis). *Agrobacterium*-mediated transformation was done using these immature embryos, following the protocol given by Hiei and Komari (2008) with minor modifications (Nithya *et al*., 2020; Shweta *et al*., 2020). Friable, well proliferated yellow calli that survived two rounds of stringent selection in 50 mgl^{-1} hygromycin were successfully regenerated into complete plants on regeneration medium (RNMH30 medium; Hie and Komari, 2008) and rooted on half strength MS medium (Murashige and Skoog, 1962). Finally, the plants were hardened in pro-trays and maintained in the transgenic greenhouse. The transformation efficiency (%) was calculated by using the following formula,
Transformation efficiency=

$$\frac{\text{Number of cocultivated embryos that produced plants}}{\text{Total number of embryos cocultivated}} \times 100$$

To determine the mutations at the target site, molecular characterization of putative T_0 mutants was done. Genomic DNA was isolated from wild type and T_0 mutant plants using the CTAB method (Porebski *et al*., 1997). PCR analyses for the presence of *cas9* and *hpt* genes in these mutants were performed using sequence specific primers with respective temperature profiles (**Table 1**). Further, the target region of about 344 bp encompassing the sgRNA sequence was also amplified using sequence specific primers (**Table 1**). The amplified products were purified and sequenced by the Sanger method (Eurofins, Bengaluru). The sequence results were analyzed using the online software, DS DecodeM (http://skl.scau.edu.cn/dsdecode/) (Xie *et al.,* 2017; Liu *et al.,* 2015) and CRISP I.D. (http://crispid.gbiomed.kuleuven.be/) (Dehairs *et al.,* 2016) to detect the mutants by comparing the sequence of transgenic plants with those of wild type plant. The mutation efficiency was calculated by using the following formula,

$$\text{Mutation efficiency} = \frac{\text{Number of events with mutations}}{\text{Total number of events generated}} \times 100$$

Bioassay on T_0 rice mutant plants for resistance against BLB was performed in the transgenic greenhouse. The leaf clipping method proposed by Kauffman *et al.* (1973) was followed for bioassay of T_0 mutant plants against *Xoo* strain, 1X020 which was obtained from ICAR-Indian Institute of Rice Research, Hyderabad. For the leaf clipping method, a three-day-old culture of *Xoo* maintained on nutrient agar medium was used. Fresh *Xoo* suspension was prepared by suspending a loopful of culture in 10 Mm sterile $MgCl_2$. The optical density of the bacterial suspension was adjusted to 0.5 at 600 nm using a spectrophotometer (Yinggen *et. al.*, 2017). Forty five day-old plants belonging to four mutant plants (from three events), a transformed (wild type) plant and a non-transformed CO51 (wild type) plant were subjected to bioassay. For this, sterilized scissors (in 70 % ethanol) were dipped in *Xoo* suspension and used to cut about 4-5 cm of first three fully extended leaves of T_0 adult plants as well as wild type plants. Additionally, sterilized scissors dipped in $MgCl_2$ without *Xoo* suspension were also used to cut the leaves and it served as the negative control. Observations on symptom development in the plants were recorded 14 days post inoculation (dpi). Lesion length on the inoculated leaves was measured and recorded. The data obtained were subjected to Tukey's HSD test using the online statistic tool OPSTAT (Sheoran *et al.,* 1998) to identify any significant difference between the mutant and wild type plants.

RESULTS AND DISCUSSION

Within a decade of the development, CRISPR/Cas9 based genome editing technology has emerged as a revolutionary genetic engineering technology accelerating crop improvement programs. The edited plants developed are a result of small indels or substitutions of nucleotides within the endogenous target gene, resulting in novel alleles. Using CRISPR tools, several novel alleles for grain number, grain size, panicle structure (Li *et al*., 2016) disease resistance (Li *et al*., 2020a; Oliva *et al*., 2019), herbicide tolerance (Sun *et al.,* 2016), aroma (Ashokkumar *et al.,* 2020), grain quality (Huang *et al*., 2020), nutritional quality (Zafar *et al*., 2020) and herbicide tolerance (Sun *et al*., 2016) have been generated in rice.

Various approaches have been used in the past to develop disease resistant rice cultivars, which include the introgression of resistance genes (R genes), host-derived resistance, and loss of susceptibility through mutation

Table 1. List of primers used in this study

Name of gene	Primer sequence (5' to 3')	Amplicon size (bp)	PCR conditions
*hpt**	Forward primer: GCTGTTATGCGGCCATTGGTC Reverse primer: GCCTCCAGAAGAAGATGTTG	686	94˚C for 5 minutes 94 ˚C for 45 seconds 57.8 ˚C for 45 seconds ×30 cycles 72 ˚C for 45 seconds 72 ˚C for 10 minutes
*cas9**	Forward primer: CTTCTGGCGGTTCTCTTTAG Reverse primer: TGCTGTTTGATCCGTTGTTG	478	95 ˚C for 5 minutes 95 ˚C for 45 seconds 52 ˚C for 45 seconds ×30 cycles 72 ˚C for 45 seconds 72 ˚C for 10 minutes
SWEET14	Forward primer: CATGGCTGTGATTGATCAGG Reverse primer: GTTTGGTGGGAGGAGATCAA	344	94 ˚C for 5 minutes 94 ˚C for 45 seconds 54 ˚C for 45 seconds ×30 cycles 72 ˚C for 45 seconds 72 ˚C for 10 minutes 4 ˚C for infinite hold

* Nithya *et al.* (2020)

in recessive R genes. Even though the introgression of R genes is very effective against *Xoo* pathogens, the large scale deployment of the R gene may result in the emergence of new virulent *Xoo* strains (Ji *et al*., 2016). So there is a necessity for alternative approaches which include biotechnological tools such as genome editing for imparting resistance against bacterial blight disease in rice.

One of the BLB susceptible genes in rice is *SWEET14* and mutations in its promoter region, particularly in EBE, resulted in BLB resistance in rice (Oliva *et al.*, 2019; Duy *et al*., 2021). A sgRNA was designed based on the EBE of *SWEET14* and cloned in a binary vector, pRGEB32, containing sgRNA expression cassette, *cas9* gene and *hpt* gene. The recombinant pRGEB32 was mobilized into *Agrobacterium* strain, LBA4404 and the resultant *Agrobacterium* was used in transformation experiments.

Immature embryos (232 embryos) collected from rice variety CO51 were co-cultivated with the *Agrobacterium* strain harbouring recombinant pRGEB32 possessing *cas9* and *SWEET14* sgRNA expression cassettes. Calli derived from 70 co-cultivated embryos survived two rounds of hygromycin selection and they were subcultured onto regeneration medium for shoot development. Eleven plants belonging to six independent events were generated with a transformation efficiency of 2.6% (**Table 2**). Each of the transformation events had two plants except the event TAG-CO51-3 which had only one plant (**Table 3**).

Molecular analyses by PCR for the presence of *cas9* and *hpt* genes confirmed their presence in all the putative mutants generated (**Fig. 3 and Fig. 4**). Analysis of Sanger sequencing after amplification of the 344 bp target region identified mutants in five events, thereby resulting in high mutation efficiency of 83.3 % (**Table 2**). These five events had 9 plants, of which five had a mutation in biallelic, one in monoallelic (heterozygous) and three in biallelic-homozygous forms (**Table 3**). Five out of the nine mutant plants had mutations at 3 bp upstream of the PAM sequence (**Table 3**). This indicated the specificity of the CRISPR/Cas9 editing system in the rice genome.

Four mutant plants, one transformed plant (but turned as wild type due to absence of mutation) and CO51 wild type (non-transformed) were subjected to bioassay (**Table 4**). Other mutant plants could not be included in this study as they were relatively older than mutants tested. Symptom development of BLB infection was observed at 14 days post inoculation. The infection among the mutants and

Table 2. Transformation and mutation efficiency of *Agrobacterium*-mediated transformation of rice cultivar CO51

No. of immature embryos co-cultivated	No. of calli survived two rounds of selection	No. of events generated	Events with mutation	Transformation efficiency (%)	Mutation efficiency (%)
232	70*	6	5	2.5	83.3

* Calli derived from 70 co-cultivated embryos survived two rounds of selection.

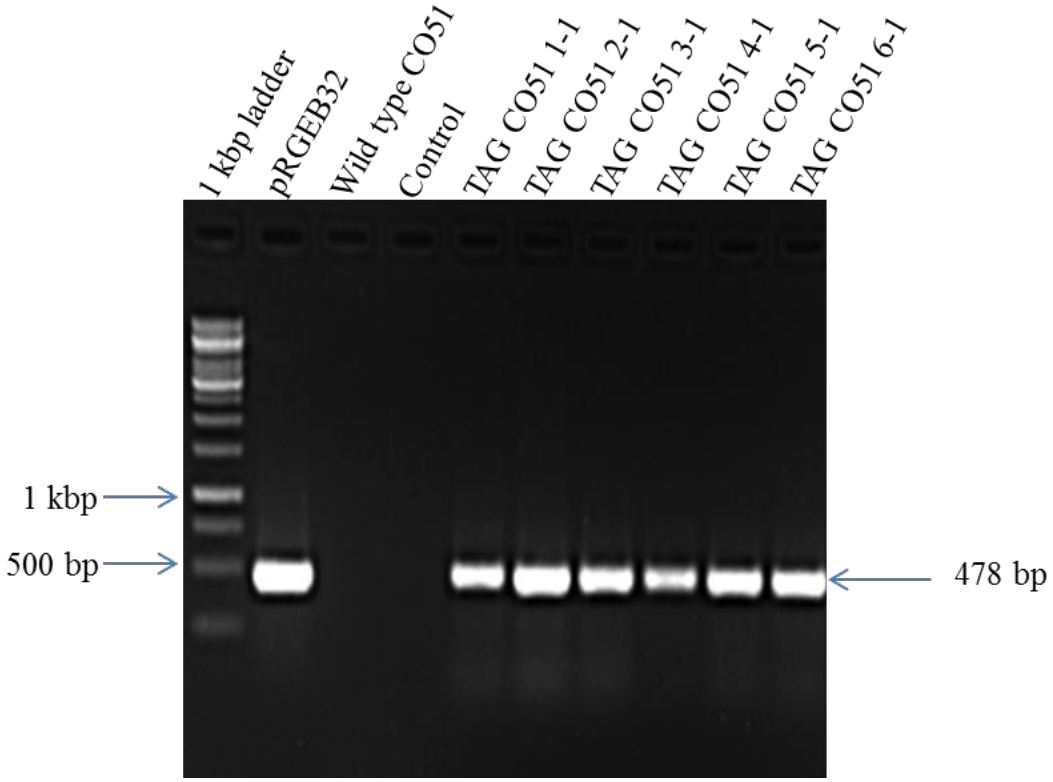

Fig. 3. PCR analysis for the presence of *cas9* gene in putative T_0 mutants

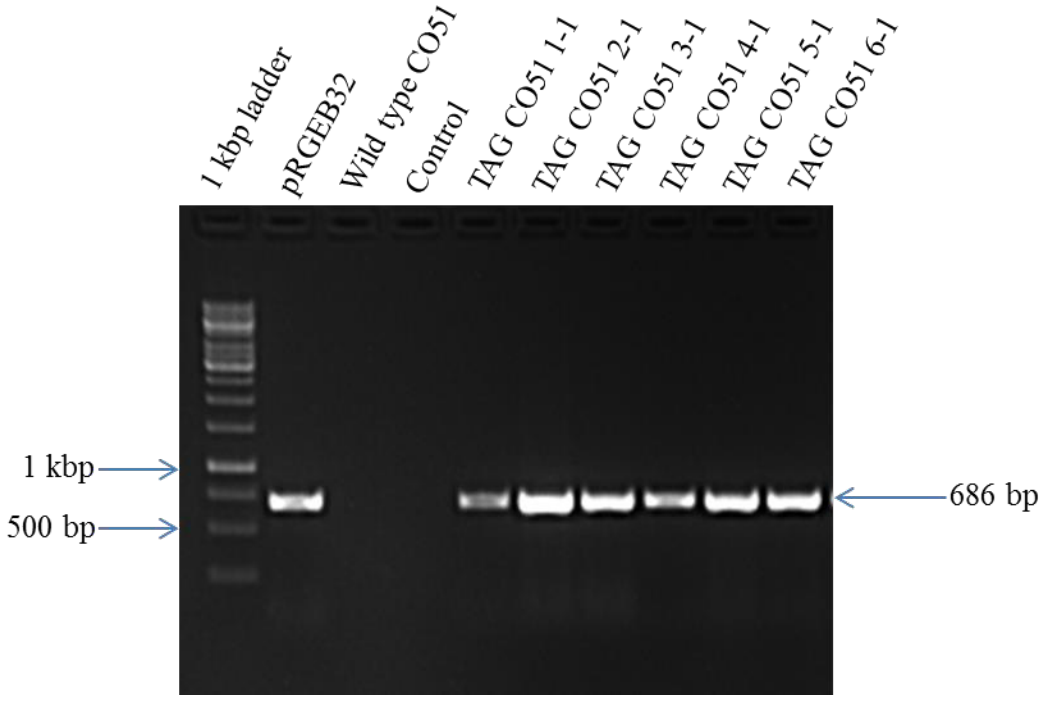

Fig. 4. PCR analysis for the presence of *hpt* gene in putative T_0 mutants

Table 3. Details of nucleotide sequence of the target region in CO51 mutants (T_0)

Event I.D	Plant I.D. number	Nucleotide sequence	zygosity
	CO51	WT:TCCTTCCTAGCACTATATAAACCCCCTCCAACCAGGTGCTAAGCTC	Wild type
TAG-CO51-1	TAG-CO51-1-1	A1:TCCTTCCTAGCACTATATAAACCCCCTCCATACCAGGTGCTAAGCTC A2:TCCTTCCTAGCACTATATCCACCCCCTCCATACCAGGTGCTAAGCTC	Biallelic (1i/2s1i)
	TAG-CO51-1-2	A1:TCCTTCCTAGCACTATATAAACCCCCTCCAAACCAGGTGCTAAGCTC A2:TCCTTCCTAGCACTATATAAACCCCCTCCAAACCAGGTGCTAAGCTC	Homozygous (1i/1i)
TAG-CO51-2	TAG-CO51-2-1	A1:TCCTTCCTAGCACTATATAAACCCCCTCCAACCAGGTGCTAAGCTC A2:TCCTTCCTAGCACTATATAAACCCCCTCCAACCAGGTGCTAAGCTC	Wild type
	TAG-CO51-2-2	A1:TCCTTCCTAGCACTATATAAACCCCCTCCAACCAGGTGCTAAGCTC A2:TCCTTCCTAGCACTATATAAACCCCCTCCAACCAGGTGCTAAGCTC	Wild type
TAG-CO51-3	TAG-CO51-3-1	A1:TCCTTCCTAGCACTATATAAACCCCCTCCA-CCAGGTGCTAAGCTC A2:TCCTTCCTAGCACTATATAAACCCCCTCCAAACCAGGTGCTAAGCTC	Biallelic (1d/1i)
TAG-CO51-4	TAG-CO51-4-1	A1:TCCTTCCTAGCACTATATAAACCCCCTCCA—CCAGGCTAAGCTC A2:TCCTTCCTAGCACTATATAAACCCCCTCCAGACCAGGCTAAGCTC	Biallelic (1d/1i)
	TAG-CO51-4-2	A1:TCCTTCCTAGCACTATATAAACCCCCTCCAACCAGGTGCTAAGCTC A2:TCCTTCCTAGCACTATATAAACCCCCTCCA—-CAGGTGCTAAGCTC	Monoallelic (heterozygous) (WT/2d)
TAG-CO51-5	TAG-CO51-5-1	A1:TCCTTCCTAGCACTATATAAACCCCCTCCAAACCAGGTGCTAAGCTC A2:TCCTTCCTAGCACTATATAAACCCCCTCCATACCAGGTGCTAAGCTC	Biallelic (1i/1i)
	TAG-CO51-5-2	A1:TCCTTCCTAGCACTATATAAACCCCCTCCAAACCAGGTGCTAAGCTC A2:TCCTTCCTAGCACTATATAAACCCCCTCCATACCAGGTGCTAAGCTC	Biallelic (1i/1i)
TAG-CO51-6	TAG-CO51-6-1	A1:TCCTTCCTAGCACTATATAAACCCCCTCCAAACCAGGTGCTAAGCTC A2:TCCTTCCTAGCACTATATAAACCCCCTCCAAACCAGGTGCTAAGCTC	Homozygous (1i/1i)
	TAG-CO51-6-2	A1:TCCTTCCTAGCACTATATAAACCCCCTCCAAACCAGGTGCTAAGCTC A2:TCCTTCCTAGCACTATATAAACCCCCTCCAAACCAGGTGCTAAGCTC	Homozygous (1i/1i)

'-' denotes deletion. Insertions are in red. PAM (AGG) sequence is highlighted in blue and EBE sequence in the wild type is underlined.

wild type plants was compared in terms of lesion length covered by the pathogen in the inoculated leaf. All the inoculated leaves of both wild type and mutant plants expressed typical water soaked lesions of BLB. Among them, event TAG-CO51-5-1 showed the lowest lesion length of 6.5 cm (**Fig. 5**). Mean comparisons of the lesion lengths using Tukey's HSD test further indicated that it was significantly different from the wild type CO51 which had a lesion length of 15.5 cm. In contrast, no significant difference was observed in the remaining events (**Table 4**). No lesion was observed in the leaves of uninoculated (cut with scissors without *Xoo*) wild type plants. The disease resistance scorecard proposed by Duy *et al.* (2021) was used in this study. The events TAG-CO51-5-1 and TAG-CO51-4-1 with lesion lengths of 6.5 cm and 11 cm were classified as resistant (lesion length < 8.0cm) and moderately resistant (lesion length 8-12 cm) against BLB respectively. Both these mutants were biallelic. The only mono-allelic (heterozygous) mutant TAG-CO51-4-2 with a lesion length of 19.33 cm was highly susceptible to BLB, as it has an intact EBE in one of its alleles. This functional allele would allow *Xoo* to bind its TALE protein and cause the disease. Identification of only two resistant lines in the present study may be attributed to earlier reports suggesting that larger nucleotide polymorphisms need to be created in the EBE region (Oliva *et al*., 2019). All the mutants generated in the study had deletions or insertions restricting to only 1 bp. This highlights the need for not only developing larger nucleotide polymorphisms but also screening a larger T_0 mutant population so that more plants with desired mutations could be identified.

Three of the events had mutations in homozygous condition, indicating that CRISPR/Cas9 system induces mutation in the embryogenic cell even before it undergoes the first cell division. Besides, all the three homozygous T_0 plants had 1 bp insertion which is in agreement with the earlier report of Zhang *et al.* (2014). Such precise, desirable, targeted mutation in a short time frame demonstrates the high mutation efficiency of the CRISPR/Cas9 system in rice. Besides, this tool greatly saves the time taken to develop a product compared to traditional mutational breeding. In our study, the mutation efficiency of CRISPR/Cas9 for targeting the *OsSWEET14* promoter was 83.3 per cent, which was higher than the mutation efficiency of TALENS, which was 51 per cent for the same target sequence (Blainvillain-

Fig. 5. Bacterial leaf blight lesion lengths at 14 days post inoculation in T_0 mutants

Table 4. Lesion length in T_0 mutants of CO51 and wild type CO51

Treatment (Mutations in the T_0 events)	Mutation / zygosity	Mean lesion length (cm) * (Mean ± S.E)	Resistance reaction
Non-transformed Wild Type CO51	Wild type	15.50±1.803[a]	S
TAG-CO51-2-1	Wild type	15.16±1.691[a]	S
TAG-CO51-4-1	Biallelic (1d/1i)	11.00±0.764[ab]	MR
TAG-CO51-4-2	Monoallelic (WT/2d)	19.33±2.048[a]	S
TAG-CO51-5-1	Biallelic (1i/1i)	6.50±2.566[b]	R
TAG-CO51-6-1	Homozygous (1i/1i)	15.33±1.424[a]	S

*Significant difference was calculated at C.D value 5.5346.
Data represent mean ± S.E of lesion length of three independent replications at 14 dpi *Xoo* strain, IX020.
Means followed by the same superscript do not differ significantly at P<0.05 (one-way ANOVA with Tukey's HSD test)

baufume *et al.,* 2017). This shows the superiority of CRISPR/Cas9 over other genome editing tools. Moreover, previous reports suggested that RNA mediated silencing of *OsSWEET14* in BLB resistant Kitaake lines caused a negative impact on seed filling and seed production (Antony *et al.,* 2010). In contrast, CRISPR/Cas9 generated mutants of the *OsSWEET14* gene did not show significant differences in major agronomic traits compared to the respective wild type (Duy *et al.,* 2021). So, it is conceivable that minor changes in promoter regions using CRISPR/Cas9 have no effect on the expression of *SWEET* genes as well as the growth and development of plants.

In conclusion, the two BLB resistant / moderately resistant mutants, TAG-CO51-5-1 and TAG-CO51-4-1 identified from the present investigation, with mutations in the EBE region and reduced lesion length can be promising candidates. . Since there is a chance of chimeric mutation and both of the resistant lines are biallelic, their resistance against BLB, however, needs to be proven by bioassay on homozygous progeny population. Besides, their agronomic performances also need to be characterized, although the majority of previous studies suggested that CRISPR/Cas9 induced mutations in the *OsSWEET14* promoter did not negatively impact the main agronomic traits (Duy *et al*., 2021). Taken together, our findings indicate that targeted modifications of susceptibility (S) *OsSWEET14* gene *via* CRISPR/Cas9 is effective in imparting resistance against BLB in rice.

ACKNOWLEDGEMENT

The authors thank Dr G.S. Laha, Principal Scientist, Department of Plant Pathology, ICAR-IIRR, Hyderabad for providing the *Xanthomonas* strains for conducting bioassay. The first author expresses his sincere gratitude to DBT for providing the fellowship.

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