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 genes promoter regions, of which four different TALEs viz., PthXo3, AvrXa7, TaIc, and TaIF (previously known as TaI5), 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<sub>0</sub> generation itself. At the same time, mutants without exogenous T-DNA can be segregated out in the T<sub>1</sub> 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’ ATATAAACCCCTCAACC 3’ and bottom strand: 5’ GGTTGGAGGGGGTTTATATAT 3’. During the synthesis of these DNA oligomers, BsaI adaptors (5’GGCA 3’ for the top strand and 5’ AAAC 3’ for the bottom strand) were added to facilitate cloning in the BsaI 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/addedgene: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.](image-url)
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 callus that survived two rounds of stringent selection in 50 mg l⁻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,

\[
\text{Transformation efficiency} = \left( \frac{\text{Number of cocultivated embryos that produced plants}}{\text{Total number of embryos cocultivated}} \right) \times 100
\]

To determine the mutations at the target site, molecular characterization of putative T₀ mutants was done. Genomic DNA was isolated from wild type and T₀ 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 T₀ 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 T₀ 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 MgCl₂. 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 T₀ adult plants as well as wild type plants. Additionally, sterilized scissors dipped in MgCl₂ 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

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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: GCCCTCCAGAAAGAGATGTTG | 686 | 94 °C for 5 minutes; 94 °C for 45 seconds; 57.8 °C for 45 seconds; 72 °C for 10 minutes ×30 cycles |
| cas9*        | Forward primer: CTTCTGGCGGTTCTCTTTAG; Reverse primer: TGCTGGTGTATCCGTGTTGG | 478 | 95 °C for 5 minutes; 95 °C for 45 seconds; 52 °C for 45 seconds; 72 °C for 10 minutes ×30 cycles |
| SWEET14      | Forward primer: CATGGCTGTGATTGATCAGG; Reverse primer: GTTTGGTGGGAGGAGATCAA | 344 | 94 °C for 5 minutes; 94 °C for 45 seconds; 54 °C for 45 seconds; 72 °C for 10 minutes 4 °C for infinite hold ×30 cycles |

* 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 T₀ mutants

Fig. 4. PCR analysis for the presence of hpt gene in putative T₀ mutants
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 < 8.0 cm) 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.

Table 3. Details of nucleotide sequence of the target region in CO51 mutants (T<sub>0</sub>)

| Event I.D       | Plant I.D. number | Nucleotide sequence | zygosity         |
|-----------------|-------------------|---------------------|------------------|
| CO51            | WT:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | Wild type         |
| TAG-CO51-1      | TAG-CO51-1-1      | A1:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | Biallelic        |
|                 |                   | A2:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | (1/2s1i)         |
|                 | TAG-CO51-1-2      | A1:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | Homozygous       |
|                 |                   | A2:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | (1/1i)           |
|                 | TAG-CO51-2-1      | A1:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | Wild type        |
|                 |                   | A2:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC |                  |
|                 | TAG-CO51-2-2      | A1:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | Wild type        |
|                 |                   | A2:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC |                  |
|                 | TAG-CO51-3-1      | A1:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | Biallelic        |
|                 |                   | A2:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | (1d/1i)          |
|                 | TAG-CO51-4-1      | A1:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | Biallelic        |
|                 |                   | A2:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | (1d/1i)          |
|                 | TAG-CO51-4-2      | A1:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | Monoallelic      |
|                 |                   | A2:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | (WT/2d)          |
|                 | TAG-CO51-5-1      | A1:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | Biallelic        |
|                 |                   | A2:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | (1/1i)           |
|                 | TAG-CO51-5-2      | A1:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | Biallelic        |
|                 |                   | A2:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | (1/1i)           |
|                 | TAG-CO51-6-1      | A1:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | Homozygous       |
|                 |                   | A2:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | (1/1i)           |
|                 | TAG-CO51-6-2      | A1:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | Homozygous       |
|                 |                   | A2:TCCTTCTAGCAGCTATAAAACCCCTCCAACCAGGTGCTAACTGCTC | (1/1i)           |

'-' 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 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 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<sub>0</sub> 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-
Table 4. Lesion length in T<sub>0</sub> mutants of CO51 and wild type CO51

| Treatment (Mutations in the T<sub>0</sub> events) | Mutation / zygosity | Mean lesion length (cm) * (Mean ± S.E) | Resistance reaction |
|------------------------------------------------|---------------------|----------------------------------------|---------------------|
| Non-transformed Wild Type CO51 | Wild type | 15.50±1.803<sup>a</sup> | S |
| TAG-CO51-2-1 | Wild type | 15.16±1.691<sup>a</sup> | S |
| TAG-CO51-4-1 | Biallelic (1d/1i) | 11.00±0.764<sup>ab</sup> | MR |
| TAG-CO51-4-2 | Monoallelic (WT/2d) | 19.33±2.048<sup>a</sup> | S |
| TAG-CO51-5-1 | Biallelic (1i/1i) | 6.50±2.566<sup>b</sup> | R |
| TAG-CO51-6-1 | Homozygous (1i/1i) | 15.33±1.424<sup>a</sup> | 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).

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.
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