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Identification of a Susceptibility Gene for *Xanthomonas oryzae* pv. *oryzae* in Vietnamese Elite Rice (*Oryza sativa* L.) Cultivar Bacthom 7

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ABSTRACT

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is the causal agent of bacterial leaf blight (BLB) disease in rice, exerting a detrimental impact on the yield of various rice cultivars in Vietnam, notably the Bacthom 7 (BT7) variety. *Xoo* possesses transcription activator-like effectors (TALEs) which modulate the expression of host genes by specifically binding to the effector-binding element (EBE) sequence in target promoter regions, thereby facilitating bacterial proliferation and pathogenesis on rice plants. Notably, *OsSWEET13*, a member of the *Sugar Will Eventually be Exported Transporter* (*SWEET*) gene family encoding sugar transporter proteins, has been identified as a prominent susceptibility (*S*) gene for BLB. This investigation focuses on elucidating the role of *OsSWEET13* in the susceptibility of BT7 to BLB disease in Vietnam. Vietnamese *Xoo* (*VXO*) strains were observed to elicit characteristic BLB symptoms on BT7 and trigger the expression of *OsSWEET13* during the course of infection. To further probe this, a clustered regularly interspaced palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) construct targeting the EBE *PthXo2A* site on the *OsSWEET13* promoter was designed and introduced into BT7 rice plants through *Agrobacterium tumefaciens*, resulting in the creation of rice lines with *OsSWEET13* promoter modifications. Upon conducting detailed genotype and phenotype analyses of the gene-edited rice plants, it was ascertained that the mutation exhibited no adverse effects on the principal agronomic traits under scrutiny. Notably, a transgene-free homozygous 3-nucleotide deletion modified rice line displayed diminished *OsSWEET13* gene expression and a significant reduction in BLB development upon inoculation with *VXO* strains. This study unequivocally establishes *OsSWEET13* as an *S* gene for *VXO*, underscoring its significance in breeding programs aimed at enhancing BLB resistance in the BT7 variety through the utilization of CRISPR/Cas9 technology.

Nguyen Duy Phuong and Dong Huy Gioi equally contributed to this article.

1 | Introduction

Bacterial leaf blight (BLB) disease, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), poses a significant threat to global rice cultivation, documented extensively in rice-producing areas, including Vietnam (Lee et al. 2011). *Xoo* infection entails the secretion of transcription activator-like effectors (TALEs) into host cells through the Type III secretion system. Notably, these TALEs act as key virulence factors, influencing transcriptional activation by selectively binding to effector-binding elements (EBEs) on susceptibility genes (*S* genes) within the host plant genome (Streubel et al. 2013; Yang et al. 2006; Zhou et al. 2015).

In rice, the *Sugar Will Eventually be Exported Transporter* (*SWEET*) family genes, particularly *OsSWEET11*, *OsSWEET13* and *OsSWEET14*, encoding sugar transporter proteins, are repeatedly used as *S* genes by field *Xoo* bacteria (Oliva et al. 2019; Wang et al. 2017). Pathogenic TALEs from *Xoo* play a crucial role in activating the expression of these *SWEET* genes, purportedly leading to the release of sugars into the apoplast as a nutritional source for the bacteria (Blanvillain-Baufumé et al. 2017). *OsSWEET13*, also known as *xa25/Xa25 (*Zhou et al. 2015*)*, is a primary target for Asian *Xoo* strains carrying the *pthXo2* gene, encoding the TALE PthXo2 (Zhou et al. 2015; Oliva et al. 2019). Specifically, several *Xoo* strains employ PthXo2 to directly activate *OsSWEET13* by recognizing the *PthXo2* EBE on the *OsSWEET13* promoter (Zhou et al. 2015; Liu et al. 2011). In contrast to the highly conserved EBEs found on the *OsSWEET11* and *OsSWEET14* promoters (Oliva et al. 2019), the *PthXo2* EBE exhibits considerable polymorphism. No less than six *PthXo2* EBE alleles have been identified in various rice cultivars (Oliva et al. 2019; Zaka et al. 2018). These alleles are recognized by at least three distinct PthXo2 TALEs, which differ from each other in their repeat variable residues (RVDs) (Oliva et al. 2019). These RVDs enable the TALEs to detect specific DNA base pairs. Notably, PthXo2A, formerly known as PthXo2 (Zhou et al. 2015), can target two disctinct EBE alleles that differ from each other at the fourth nucleotide position (A/T). Conversely, PthXo2B and PthXo2C, which differ from each other at the 8th EBE-binding position, target the same EBE variant (Figure S6).

BT7, the major rice variety in Northern Vietnam, is highly susceptible to BLB disease (Sam et al. 2019). Previous research indicates that three *VXO* strains, VXO_11, VXO_60 and VXO_96, target the *S* gene *OsSWEET14* in BT7 (Quyen et al. 2022). Recently, a *PthXo2A* EBE was identified on the BT7 *OsSWEET13* promoter (Huong et al. 2020). Here, we utilize the CRISPR/Cas9 gene editing tool to investigate the interaction between *VXO* TALEs and BT7 *OsSWEET13*, elucidating the pivotal role of the *PthXo2A* EBE in regulating the expression of BT7 *S* gene *OsSWEET13* under *VXO* infection. Our research results provide a foundation for initiating breeding programs focused on enhancing broad-spectrum resistance against BLB disease in the major rice variety BT7, utilizing gene editing technology in the future.

2 | Materials and Methods

2.1 | Plant and Pathogen Materials

Wild-type (WT) rice cultivars (*Oryza sativa* L. ssp. *indica*) BT7 and IR24 were collected from the Plant Resources Center in Vietnam. The rice plants were cultivated in a net-house under the following conditions: 30°C for 14 h (light) and 25°C for 10 h (dark) with 80% humidity.

The *Xoo* strains (Table S1) were isolated from diseased leaves collected in Vietnam between 2013 and 2018 (Quyen et al. 2022; Dai et al. 2022; Duy et al. 2021; Ngoc et al. 2022). *Xoo* cells were cultured following the procedure outlined by Zhou et al. (2015).

2.2 | Gene Expression Analysis

Rice leaves were subjected to infiltration (Duy et al. 2021) with *VXO* strains; total RNA extraction was performed 48 h postinoculation. One microgram of RNA was utilized with an oligo (dT) primer, followed by PCR using *OsSWEET13*-specific primers (Blanvillain-Baufumé et al. 2017). The PCR cycles (31 cycles) were carried out using an *Eppendorf Mastercycler ep Gradient S*. The expression of targeted genes was normalized to the *OsEF1α* gene (Blanvillain-Baufumé et al. 2017). All PCR products were separated through agarose gel electrophoresis, and the resulting gel images were subjected to analysis using ImageJ v1.1 software to study the signal of PCR gel electrophoresis. The digital value of the *OsSWEET13* band was normalized against the corresponding digital value of the *OsEF1α* band from the same cDNA sample. Subsequently, the relative ratios were divided by the relative ratio of the non-inoculated sample.

2.3 | Vector Construction and Rice Transformation

Two gRNA sequences (Figure 2A) targeting the *PthXo2A* EBE region on BT7 *OsSWEET13* promoter (Huong et al. 2020) were used for vector construction. In detail, gRNAs were designed using the CRISPR-P v2.0 tool and prioritized based on the following: (1) ability to form stable secondary structures, (2) high on-target score and (3) predicted DSB generation site on or near the target sequence. Off-target sequences were anticipated with the CCTop tool (Stemmer et al. 2015) against the *OsSWEET13* promoter sgRNAs and the rice Nipponbare genome with default parameters (Table S2).

Complementary oligonucleotides with appropriate 4-bp overhangs were synthesized by Phusa Biochem (Vietnam). Following heat denaturation, two pairs of complementary oligonucleotides (5′-gtgtGGAGAGGAATGAAGGGAGTTG-3′/5′-aaacCAACTCCCTTCATTCCTCTCC-3′ and 5′-gtgtGAAAACTCTTGGAGAGGAATGA-3′/5′-aaacTCATTCCTCTCCAAGAGTTTTC-3′) were initially annealed, phosphorylated and subsequently cloned into the *Btg*ZI and *Bsa*I sites downstream of *OsU6* promoter, respectively, on the sgRNA expression vector pENTR4-sgRNA (Zhou et al. 2014). The integrity of the inserted fragment

was confirmed through sequencing. Following this, both sgRNA expression cassettes were integrated into plant transformation vector pCas9 (Herbert et al. 2020) using the Gateway LR clonase (Life Technologies). The final construct pCas9/sgRNA-OsSWEET13 (Figure 2A) was validated by Sanger sequencing of the insertion junctions.

The resulting construct was introduced into *Agrobacterium tumefaciens* EHA105 via electroporation, and the recombinant strain was used for rice transformation using the previous method (Sam et al. 2021). The presence of the transgene in the genome of regenerated T_0 plants was assessed through PCR with *Cas9*-specific primers (5′-ATGGCCCCAAAGAAGAAG-3′ and 5′- GCCTCGGCTGTCTCGCCA-3′). T_1 individuals underwent PCR analysis using *Cas9* and *HPT* (5'-AAACTGTGATGGACGACACCGT-3′ and 5′- GTGGCGATCCTGCAAGCTCC -3′) specific primer pairs to identify transgene-free lines.

2.4 | *OsSWEET13* Promoter Mutation Analysis

Genomic DNAs (50 ng) from all transgenic T_0 or transgene-free T_1 plants were used for amplification with *OsSWEET13*-specific primers (5′-CCGTATCAGGATTCAGGAATA-3′ and 5′- CCAGCCATTTTTGTGTGCTA-3′) (Huong et al. 2020). Subsequently, the PCR products were subjected to direct sequencing using the Sanger method. Decoding of the sequencing chromatograms was performed using the degenerate sequence decoding method (Ma et al. 2015) to identify the mutations. TALE binding score of mutant alleles was analysed by Talvez v3.2 tool (Pérez-Quintero et al. 2013), where a score of 0 indicates that the TAL effector fails to bind to the target DNA sequence.

2.5 | Pathogen Inoculation

Disease assays (Blanvillain-Baufumé et al. 2017) were conducted using *VXO* strains (Table S1) and 45-day-old BT7 plants at the tillering stage. Bacteria were cultured at 28°C for 2 days (Bai et al. 2000) and grown at an optical density (OD_{600}) of 0.5 (for leaf-infiltrations) or 0.4 (for leaf-clipping) in water. Lesion length measurements were taken 14 days postinoculation (dpi) on at least three inoculated leaves per plant, with three plants per line. The scoring criteria (International Rice Research Institute (IRRI) 2013) were defined as follows: high resistance (lesion length < 5 cm), moderate resistance (lesion length 5–10 cm), moderately susceptibility (lesion length > 10–15 cm) and susceptibility (lesion length > 15 cm). For gene expression analyses, 4-cm leaf sections infiltrated with bacterial suspensions were collected at 48 h post inoculation (hpi) to extract RNA for RT-PCR analysis. Each experiment comprised samples from three pooled biological replicate leaves. Negative controls consisted of plants inoculated with distilled water only.

2.6 | Assessment of Major Agronomic Traits of T_2 Edited Plants

The wild type (WT) and chosen mutant BT7 plants were grown in a controlled environment under net-house conditions, following a randomized pot design experiment. When the plants reached maturity, five plants from each line were examined for various agronomic traits, including growth duration, plant height, tiller count per plant, panicle grain count, filled grain count per panicle and yield (seed mass) per plant. This experiment was repeated three times, resulting in a total evaluation of 15 plants for each line.

3 | Results

3.1 | Virulence of *VXO* Strains and Expression of *OsSWEET13* in Pathogen-Inoculated BT7 Rice Cultivar

The cultivation of the BT7 rice cultivar is significantly impacted by rice leaf blight disease; however, there have only been a few limited studies on the susceptibility of BT7 to the *VXO* population (Sam et al. 2019; Le et al. 2014). Our disease assays (Figure 1A) demonstrated that all *VXO* strains induced typical BLB symptoms with lesion lengths > 20 cm. Significantly, the disease severity in BT7 was comparable to that of the standard susceptible cultivar IR24, implying a high vulnerability of BT7 to *VXO* and emphasizing the imperative for disease management strategies.

The RT-PCR analysis indicates a significant difference in the expression levels of the *OsSWEET13* gene between the samples inoculated with *VXO* strains and the control sample (Figures 1B and S1). The *OsSWEET13* mRNA levels were notably higher in samples inoculated with VXO_15, VXO_59, VXO_60, VXO_80, VXO_96 and VXO_100 strains. Conversely, the inoculation with VXO_11 strains resulted in minimal changes in the *OsSWEET13* expression level in the sample. These findings suggest a strong correlation between the virulence of *VXO* strains, particularly six out of the seven strains tested (VXO_15—VXO_100) and the expression of *OsSWEET13* during the bacterial infection process.

3.2 | Construction of Vector pCas9/sgRNA-OsSWEET13 and Generation of BT7 Transgenic Plants

Based on two designed gRNA sequences, a rice transformation vector was constructed to express the CRISPR/Cas9 complex, targeting the EBE *PthXo2A* on the BT7 *OsSWEET13* promoter (Figure 2A). PCR and sequencing analysis confirmed the presence and integrity of the *Cas9* and *OsSWEET13*-targeting sgRNA expression constructs under the control of the *ZmUbiquitin* and *OsU6* promoters, respectively, on our vector (Figures S2 and S3). This *OsSWEET13*-editing vector was transformed into the target rice variety BT7 using the *Agrobacterium*-mediated method.

During the transformation process, pCas9/gRNA-SW13 was introduced into calli derived from BT7 mature embryos (Table S3). The results demonstrated a remarkable survival rate of the calli, surpassing 50% after each selection step of the gene transformation. Notably, the shoot regeneration rate of calli that underwent three stages of selection reached an

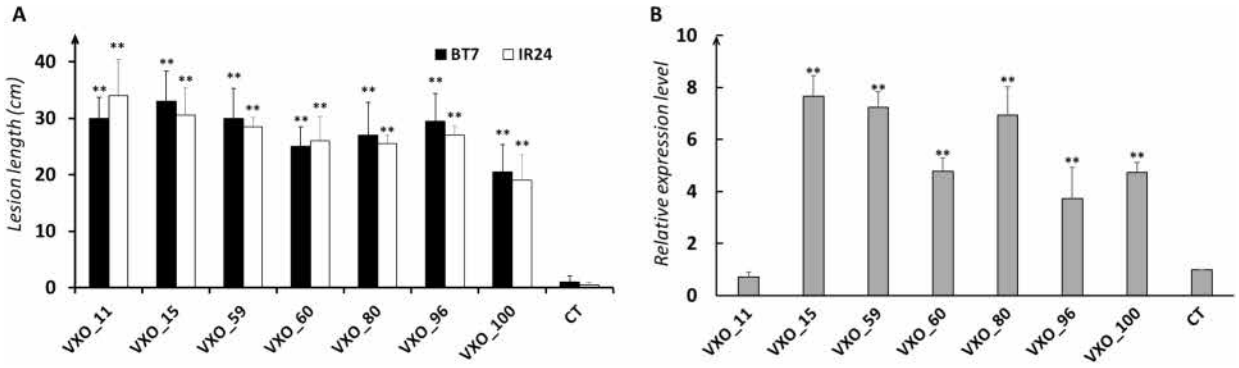

FIGURE 1 | OsSWEET13 is likely an S gene for VXO strains in rice variety BT7. (A) BT7 susceptibility to VXO strains. Lesion lengths at 14 dpi; bars show mean ± SD ($n = 3$). (B) OsSWEET13 upregulation post-VXO inoculation. Control expression set to 1.0; bars represent mean ± SD from three independent experiments. CT: negative control (Xoo-free buffer). Asterisks: significant differences vs. control (Tukey's HSD test; $p < 0.05$).

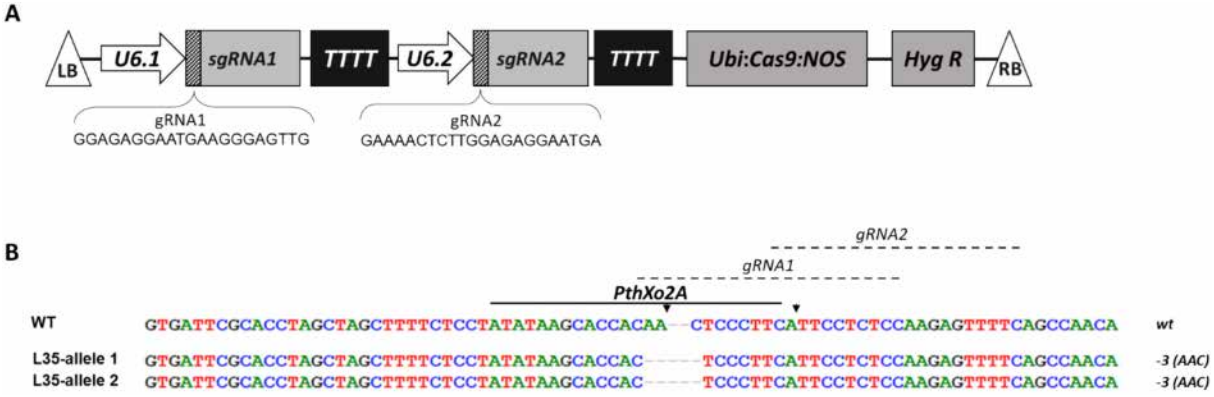

FIGURE 2 | CRISPR/Cas9-induced OsSWEET13 promoter mutation in BT7 rice. (A) T-DNA region with OsSWEET13 gRNAs and key components: ZmUbiquitin promoter (Ubi); OsU6 promoters (U6.1, U6.2); hygromycin resistance cassette (HygR); terminators (TTTT); left and right borders (LB, RB). (B) Alignment of edited OsSWEET13 promoter in T_0 transgenic BT7 line L35, showing CRISPR/Cas9 cutting site (arrow), PthXo2A binding site (solid line), gRNA sites (dashed lines) and mutations (right labels).

impressive 44.02%. As a result, 43 regenerated plants were successfully obtained, and they exhibited normal growth under net-house conditions. To confirm the presence of transgenes in the genome, all these plants were subjected to PCR analysis. There were 13 out of 43 generated plants exhibiting positive PCR results with specific primers for *Cas9* expression construct, corresponding to a 30.23% true positive rate.

3.3 | Characterization of *OsSWEET13* Mutation in Transgenic BT7 Plants

To evaluate the mutation efficiency of the CRISPR/Cas9 system, the *OsSWEET13* promoter fragment was extracted from 13 transgenic rice lines and subjected to sequencing analysis. Analysis of the obtained sequences revealed mutations in the targeted *OsSWEET13* promoter region in 7 out of the 13 transgenic rice lines (Table 1). Thus, the rate of transgenic plants harbouring mutations reached 53.9%, indicating a gene editing efficiency of the entire process at 1.4%. The observed mutations include base insertions and deletions, with no substitution mutations detected (Table 2). These mutations had indels, ranging from two-nucleotide insertions (+2) to three nucleotide deletion (−3) and specifically occurred around the site located 3 bp upstream from the PAM sequence of gRNA1 but not gRNA2 on the *OsSWEET13* promoter (Figures 2B and S4), highlighting the significant impact of gRNA design on CRISPR/Cas9 editing efficiency. Additionaly, three different mutant genotypes were identified for the *OsSWEET13* promoter, including homozygous (14.3%), heterozygous (71.4%) and biallelic (14.3%) mutations (Table 2). The allele mutation rate was relatively high, with 34.6% of alleles (9 out of 26 alleles) showing mutations. These results demonstrated the effectiveness of the CRISPR/Cas9 system in inducing specific mutations in the BT7 *OsSWEET13* promoter. However, the analysis conducted using the Talvez tool

TABLE 1 | OsSWEET13 genotype of BT7 transgenic rice lines.

BT7 *OsSWEET13* genotype		Number of plants
Mutant BT7 *OsSWEET13*	Heterozygous	5
	Homozygous	1
	Bialellic	1
Wild type		6

(Tables 3 and S4) predicted that the variants of PthXo2, including PthXo2A, retained the capability to interact with 7 out of 9 obtained mutant *OsSWEET13* alleles, with the exception of two 3-nucleotide deletion mutations observed in the L35 line. Consequently, the L35 line was selected for subsequent analysis.

3.4 | Selection of Transgene-Free Mutant BT7 Rice Lines

To generate a desired *OsSWEET13*-edited BT7 line without T-DNA construct in the genome, T_0 transgenic rice BT7 line L35 carrying a homozygous promoter mutation (−3/−3) was allowed to self-pollinate. PCR analysis (Table S5) of all T_1 plants with the primers specific for both *Cas9* and *HPT* genes revealed that around one-fourth of the tested plants did not contain the transgene. This ratio is consistent with the Mendelian ratio ($\chi^2 < \chi^2_{0.05,\,1} = 3.84$), suggesting that their T_0 parental plant carried a single copy of transgene which was transmitted as expected to the next generation. Interestingly, the *OsSWEET13* sequencing results indicated that all these T_1 plants possessed the same allele as their parent (data not shown), demonstrating the stable inheritance of Cas9-induced *OsSWEET13* mutation to the next generation.

TABLE 2 | Frequency of OsSWEET13 mutation types in T_0 generation transgenic BT7 rice lines.

	Mutation type ratios[a] (%)	
Type of mutations	**Plant[b]**	**Allele[a]**
Deletion	57,1 (4/7)	35,7 (5/14)
Insertion	42,9 (3/7)	28,6 (4/14)
Substitution	0 (0/7)	0 (0/14)

[a](Numbet of allele with specific mutation type/total number of mutant allele) x 100%.
[b](Number of plants with specific mutation type/total number of mutant plants) × 100%.

3.5 | Expression of *OsSWEET13* in T_2 BT7 Edited Plant Inoculated With *VXO* Strains

To assess the impact of the mutation in *PthXo2A* EBE on the expression of *OsSWEET13*, L35.24 plants (propagated from the selected transgene-free T_1 individuals) were subjected to artificial inoculation with seven representative VXO strains. The results of the RT-PCR analysis, quantified using ImageJ (Figures 3A and S5), revealed no significant increase in the expression level of the target gene in the leaves of all edited BT7 samples after 48 hpi of *VXO* inoculation compared to the control experiment conducted without using *Xoo* bacteria. This stands in contrast to the WT BT7 plants, which, upon inoculation with all *VXO* strains but VXO_11, exhibited substantial upregulation of *OsSWEET13* expression. Moreover, a comprehensive assessment of the predicted off-target sites of our designed CRISPR/Cas 9 system (Table S2) revealed that they did not localize on or near any functional gene. This supports the hypothesis that the 3-bp deletion in the *OsSWEET13* promoter is responsible for the observed discrepancy in gene expression between *OsSWEET13*-edited BT7 line and WT BT7 line. These results collectively suggested that the mutation in the novel EBE *PthXo2A* disrupts the transcriptional activation of the target gene induced by the *VXO* strains.

3.6 | BLB Disease Resistance and Agronomic Traits of T_2 BT7 Edited Plants

In order to characterize the BLB resistance phenotype of the generated rice mutants, 8-week-old edited T_2 and WT BT7 plants were inoculated by leaf-clipping with *VXO* strains for BLB susceptibility assays. The inoculated leaves of all *Xoo*-inoculated plants exhibited typical symptom of BLB disease, with lesion lengths

TABLE 3 | Binding specificity of PthXo2 TAL effector variants to mutant OsSWEET13 alleles in Bacthom 7 rice cultivar.

	Talvez core[b]			
Name of allelle[a]	**PthXo2A**	**PthXo2B**	**PthXo2C**	**PthXo2.1**
WT	14.219	0	0	0
L2-allele 1	10.439	0	6.642	10.439
L18-allele 1	13.792	12.115	13.725; 6.264[c]	13.792
L19-allele 1	6.371	0	0	6.371
L35-allele1/2	0	0	0	0
L31-allele 1	7.064	0	0	7.064
L31-allele 2	13.792	12.115	13.725; 6.264[c]	13.792
L38-allele 1	6.364	0	0	6.364
L42-allele 1	9.389	0	0	9.389

[a]OsSWEET13 alleles of gene-edited and wild-type (WT) BT7 rice lines.
[b]Values calculated by Talvez tool (Pérez-Quintero et al. 2013) represent the binding ability of known TAL effectors (Oliva et al. 2019; Zaka et al. 2018) to OsSWEET13 alleles; higher values indicate stronger specific binding ability.
[c]Binding affinity of TAL effector to two distinct sequences on the mutant allele.

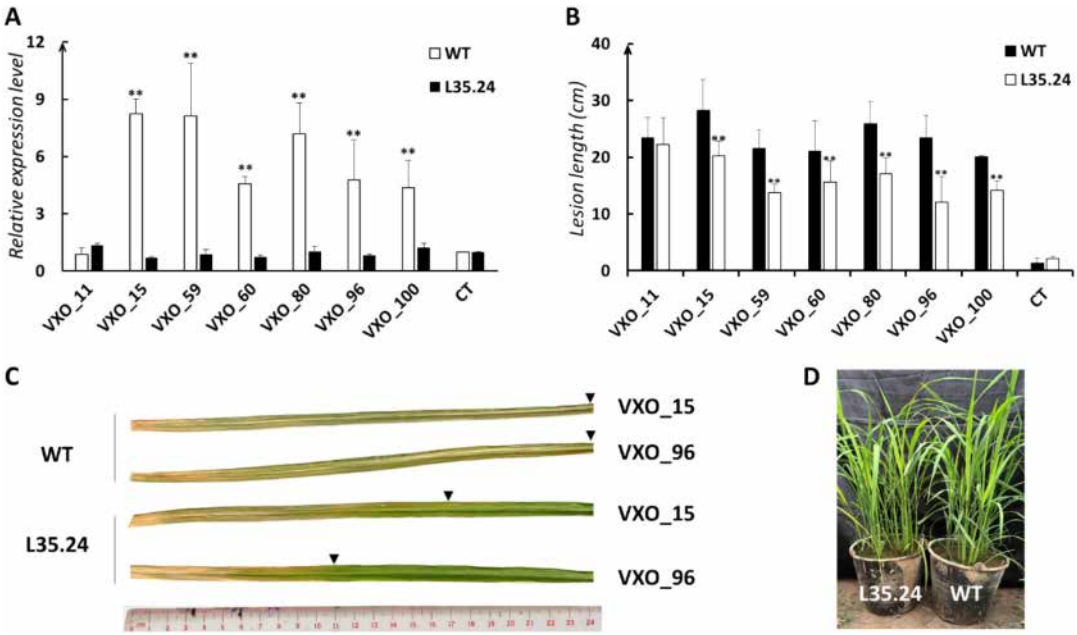

FIGURE 3 | Gene expression and phenotyping analysis of free-transgene OsSWEET13 homozygous mutant line L35.24. (A) OsSWEET13 expression pattern (RT-PCR) 2 days postinfiltration with VXO strains. Bars: mean ± SD from three independent experiments; asterisks: significant differences vs control plants (Tukey's HSD test; $p < 0.05$). (B) BLB resistance assays. Bars: mean lesion lengths ± SD, measured 14 days post-leaf clipping inoculation (VXO_15 and VXO_96). Data from at least three leaves from each of three plants, repeated thrice. Asterisks denote significant differences between edited and WT lines (Tukey's HSD test; $p < 0.05$). (C) BLB resistance phenotype. Leaves photographed 14 days post-inoculation; arrowheads indicate lesion end. (D) Growth phenotype of BT7 edited line in net-house condition.

TABLE 4 | Agronomic traits evaluation of homozygous T_2 mutant line L35.24.

Lines	Growth duration (day)	Plant height (cm)	No. of filled grains per panicle	1000 grains weight (g)
WT	103.9 ± 1.2	105.2 ± 3.2	86.9 ± 10.5	17.6 ± 0.4
L35.24	103.4 ± 0.9	104.4 ± 4.9	88.3 ± 9.4	17.6 ± 0.5

Note: Five plants per line were measured. Data represent the mean values from three independent experiments, with SD shown. Means followed by the same letter do not differ significantly ($p < 0.05$).

ranging from 12.1 to 28.3 cm (Figure 3B,C). Tukey's HSD analysis further indicated that upon inoculation with the VXO_11 strain, the mean lesion lengths measured on the edited line or WT line were not significantly different. Remarkably, the edited BT7 displayed decreased susceptibility to the remaining *VXO* strains, as evidenced by average lesion lengths ranging from 12.1 to 22.2 cm, in comparison to WT plants with average lesion lengths of 21.1–28.3 cm. The findings indicated that the decreased expression of *OsSWEET13*, resulting from CRISPR/Cas9-induced mutation, has detectable effects on the virulence of VXO on BT7 rice.

Loss-of-function mutations in certain *SWEET* family genes, such as *OsSWEET14*, have the potential to diminish both plant growth and reproductive processes (Antony et al. 2010). However, the assessment of various phenotypic characteristics, including growth duration, plant height, number of filled grains per panicle and yield per plant (Table 4), revealed no discernible differences between edited BT7 line and WT line when cultivated under net-house conditions (Figure 3D). These results substantiated that the 3-bp deletion mutation within the *OsSWEET13* promoter region does not exert adverse effects on the principal agronomic traits of BT7 rice.

4 | Discussion

4.1 | The Susceptibility of BT7 Rice to Leaf Blight Disease Is Contingent Upon Gene–Gene Interactions Between the Host Plant and the Pathogen

The susceptibility of a rice variety to leaf blight disease is contingent upon the specific pathogen to which it is exposed, or in other words, on the bacterial strain present in the field (Streubel et al. 2013; Antony et al. 2010; Triplett et al. 2011). BT7, being the major rice variety in Northern Vietnam and possessing exceptional rice quality, exhibits high sensitivity to leaf blight disease, possibly due to the lack of effective resistance genes against *VXO* bacterial strains (Le et al. 2014; Furuya et al. 2012). Our disease assays data (Figure 1A) indicated that despite potential fluctuations in the *VXO* population structure over time, as evidenced by variations in effective BLB resistance genes (Tho et al. 2022), *VXO* consistently exhibits strong virulence against the rice varieties persisting in cultivation across extensive areas for prolonged durations.

The interaction between TALE of *Xoo* bacteria and host *S* genes significantly contributes to the susceptibility of rice to BLB disease (Jiang et al. 2020; Xu et al. 2022). Our identification of increased expression of *OsSWEET13* in *VXO*-inoculated BT7

rice plants serves to further substantiate the previous observation that *OsSWEET13* plays a crucial role as a *S* gene in the infection and pathogenesis of Asian *Xoo* bacterial strains (Oliva et al. 2019; Zaka et al. 2018). This study underscored the significance of *OsSWEET13* in the interaction between rice and *Xoo* bacteria, particularly in the context of susceptibility to BLB disease. Additionally, our previous investigation noted an upregulation of *OsSWEET14* transcription upon inoculation of BT7 with all representative *VXO* bacterial strains (Sam et al. 2019). These findings collectively suggested that the broad-spectrum susceptibility of BT7 rice to *VXO* bacteria may be attributed to both *OsSWEET13* and *OsSWEET14* genes. Moreover, the observation that VXO_11 failed to induce *OsSWEET13* expression in BT7 rice (Figure 1B) has further strengthened the previous hypothesis, suggesting that *OsSWEET14* acts as the primary susceptibility gene dictating the virulence of the VXO_11 strain on BT7 rice (Quyen et al. 2022). The results here contributed to the ongoing understanding of the interplay between specific *S* genes, including *OsSWEET13* and *OsSWEET14*, and the virulence of *Xoo* bacterial strains on rice plants, shedding light on the mechanisms of molecular interaction between host rice plant and *Xoo* pathogen.

4.2 | Change in Gene Expression via EBE Site Mutations in the Promoter Region

It is imperative to recognize that gene editing researches often instigate gene knock-out mutations through the modification of the gene's coding sequence (Li et al. 2022). Notably, *Xoo* bacteria frequently target multifunctional genes, and the inactivation of these genes may have a substantial impact on the normal productivity of the host plant (Fei et al. 2021). Consequently, disrupting the molecular interaction between host plant susceptibility genes and *Xoo* bacterial effectors stands as a primary strategy in the endeavour to develop resistance for the commercial rice varieties, as it offers the potential to confer resistance to bacterial blight while minimizing adverse effects on plant growth and productivity.

TALEs of *Xoo* induce the expression of *S* genes in host plants by specifically binding to EBE sequences in the promoter region of these genes (Xu et al. 2022). This interaction mechanism was further exemplified by the identification of a novel *PthXo2*-like EBE sequence, previously named as *PthXo2A (*Oliva et al. 2019*)*, on the *OsSWEET13* promoter in the BT7 genome (Huong et al. 2020). Furthermore, a CRISPR/Cas9-induced 3-bp deletion mutation at this EBE site resulted in the basal expression level of *OsSWEET13* in T_2 BT7 plants (Figure 3A). This finding aligned with several previous researches, supporting the notion that small mutations at the EBE site can prevent the activation of *S* gene expression by *Xoo* pathogens (Zhou et al. 2015; Oliva et al. 2019; Blanvillain-Baufumé et al. 2017; Zaka et al. 2018; Ancy Diana et al. 2022).

4.3 | Flexibility and Specificity of TALE-DNA Interactions: Focus on PthXo2A

Xoo bacteria rely on specific TALE interactions with EBE sequences to activate *S* genes and induce disease symptoms in host plants. In nature, a prevalent evolutionary strategy in rice involves modifying EBE-containing sequences within *S* gene promoter regions, enabling resistance to *Xoo* bacteria, contingent upon corresponding TALEs (Zhou et al. 2015; Zaka et al. 2018). Selective pressure from *Xoo* may be the primary cause of polymorphism in EBE sites within rice genomes in production, especially in the case of EBE *PthXo2* on the OsSWEET13 promoter (Zaka et al. 2018; Xu et al. 2022; Xu et al. 2019). Due to the specificity of TALE-DNA interactions, even small changes in EBE sequences, including PthXo2A, can disrupt the interaction with the corresponding TALE and confer resistance to Xoo (Oliva et al. 2019; Zaka et al. 2018; Xu et al. 2019). For instance, a single nucleotide difference (A deletion) between the EBE-containing region on the *OsSWEET13* promoter of the *indica* rice variety IR24 and the *japonica* rice varieties Kitaake or Nipponbare (Figure S6) prevented PthXo2A (which recognizes the EBE *PthXo2A* of IR24) from interacting with the corresponding EBE of Kitaake or Nipponbare. Consequently, bacterial strains carrying only PthXo2A were unable to cause disease in these varieties (Xu et al. 2019). In this study, our gene-edited rice line L35 did not express *OsSWEET13* when infected with *Xoo* carrying the PthXo2A gene, suggesting that the 3-nucleotide deletion mutation on the EBE *PthXo2A* of BT7 may have disrupted its interaction with the corresponding TALE. These findings demonstrate that using gene editing tools such as CRISPR/Cas9 to disrupt interactions between TALEs and target EBEs is a potential and feasible strategy for breeding rice resistant to BLB (Oliva et al. 2019; Xu et al. 2019).

Xoo bacteria can adapt to variations in *S* genes under selective pressure from resistant rice varieties by modifying their TALEs (Oliva et al. 2019; Zaka et al. 2018; Xu et al. 2022). They employ the degeneracy of specific TALE-EBE interactions, particularly with PthXo2A, to enhance their pathogenicity (Xu et al. 2022). The specificity of some RVDs is known to be low, such as NI (Asn-Ile, recognizes A/C), NN (Asn-Asn, recognizes G/A/C/T), NG (Asn-Gly, recognizes T/A/C), HG (His-Gly, recognizes T/A/C), HD (His-Asp, recognizes C/A/T) and NS (Asn-Ser, recognizes A/C/G) (Bogdanove and Voytas 2011). Thus, a given TALE can interact with several different DNA sequences, as is the case with PthXo2A, or several different TALEs could interact with the same EBE sequence, as with PthXo2B and PthXo2C (Oliva et al. 2019). This flexibility explains why the majority of mutant *OsSWEET13* alleles obtained in this study were predicted to still interact with PthXo2A (Tables 3 and S3). This aligns with our previous research on *OsSWEET14* promoter editing, where only one of three CRISPR/Cas9-edited lines with EBE *AvrXa7* mutations failed to express *OsSWEET14* when infected with AvrXa7-encoding *Xoo* strains (Dai et al. 2022; Duy et al. 2021). However, the mechanism of flexibility in TALE-DNA specific interactions remains unclear, especially with nucleotide insertion/deletion changes, as they can induce frameshifts. A notable example is the EBE on the *OsSWEET13* promoter of rice varieties Zhenshan 97 (Oliva et al. 2019; Xu et al. 2019), Ejaili, and Khama1183 (Zaka et al. 2018) (similar to BT7), which is considered a 2-nucleotide (AA) deletion mutation compared to EBE *PthXo2A* of rice variety IR24 (Oliva et al. 2019; Zaka et al. 2018; Xu et al. 2019). However, this deletion mutation causes a rearrangement and creates a new *PthXo2A*-like sequence that differs by only 1 nucleotide (A to T) at the fourth position compared to the original IR24 EBE *PthXo2A* (Figure S6). Furthermore, the interaction of PthXo2A with these EBEs varied among studies. Xu et al. (2019) and Zaka et al. (2018) reported compatible

interactions between PthXo2A-containing *Xoo* strains and IR24 *OsSWEET13* but incompatible interactions with Zhengshan 97, Ejaili, and Khama1183 *OsSWEET13 (*Zaka et al. 2018; Xu et al. 2019*)* (Figure S6). Contrastingly, Oliva et al. (2019) demonstrated that TALE PthXo2A can recognize both different EBE sequences on Zhengshan 97 and IR24 *OsSWEET13* promoters to induce disease symptoms, with lower target gene expression in Zhengshan 97 than IR24 (Oliva et al. 2019), possibly due to the reduced interaction frequency of the 4th RVD (NN) with Thymine (T) in Zhengshan 97 versus Adenine (A) in IR24 (Figure S6) (Bogdanove and Voytas 2011).

In this study, BT7 *OsSWEET13*, with a predicted EBE sequence similar to Zhengshan 97 (Figure S6), was induced by VXO strains, suggesting the presence of PthXo2A in these strains. However, Talvez analysis (Tables 3 and S3) indicated that PthXo2A maintained the ability to interact with seven out of eight obtained mutants. Notably, we demonstrated that a 3-nucleotide deletion mutation could completely disrupt the interaction between studied Xoo Vietnam strains and the *PthXo2A* EBE (Figure 3). These results support the notion that the location and type of mutation variably influence *S* gene interaction with *Xoo*.

These findings highlight the complexity of TALE-EBE interactions, particularly regarding PthXo2A, which underscores the need for further research to understand the mechanisms underlying these interactions and their implications for developing durable rice resistance strategies. The results emphasize the importance of assessing promoter mutations' impact on native *OsSWEET* expression and carefully evaluating base pair indel quantity and placement to ensure successful disruption of TALE-target gene interactions.

4.4 | Modulating the Expression of Susceptibility Genes Alters the Virulence of *Xoo* Bacteria Towards the Host Rice Plant

In efforts to impart pathogen resistance to host plants, a commonly employed strategy involves the inactivation of crucial susceptibility genes. Some researchers have achieved resistance to BLB disease in certain rice varieties by identifying and editing the coding region of *S* genes such as *OsSWEET14 (*Zeng et al. 2020*)* or *OsSWEET11*/*Os8N3 (*Kim et al. 2019*)*. However, this approach often results in frameshift mutations and the potential generation of defective mRNA and/or protein products. Especially, for *SWEET* family genes responsible for sugar transport in plants, including *OsSWEET13 (*Zhou et al. 2015*)*, complete gene knockout may lead to undesirable effects on the normal processes of plant growth and reproduction (Yang et al. 2018). Consequently, altering the expression pattern of *S* genes through editing the noncoding/promoter region may offer a more suitable approach to bestow effective resistance without compromising other agronomic properties of the crops.

The editing of the EBE site on the promoter has been shown to induce changes in the gene expression pattern, resulting in resistance to the corresponding TALE-dependent *Xoo* strains. For instance, in the study by Li et al. (2022), two rice varieties, Kongyu131 and Huanghuazhan, carrying a small deletion mutation in the EBE *PthXo1*, did not express *OsSWEET11* and displayed resistance to infection by strain PXO99 (Li et al. 2022). Furthermore, Zafar et al. (2020) and Duy et al. (2021) successfully induced mutations at the EBE position *AvrXa7/PthXo3/TalF* on the *OsSWEET14* promoter of commercial rice varieties Basmati and TBR225, respectively, resulting in complete resistance to the given *Xoo* strains by preventing the activation of the *OsSWEET14* gene (Duy et al. 2021; Zafar et al. 2020).

Our study indicated that although the BT7 edited line did not exhibit an increase in *OsSWEET13* expression after inoculation with representative *VXO* strains (Figure 3A), it still displayed typical BLB disease symptoms (Figure 3B). This observation can be attributed to the fact that *OsSWEET13* is not the sole major *S* gene of BT7 for *VXO*. This aligns with previous research results, which established that the VXO_11 strain exclusively targets *OsSWEET14*, while other strains such as VXO_60, VXO_80, VXO_96, and VXO_100 attack at least one other *S* gene besides *OsSWEET14* in BT7 (Quyen et al. 2022; Dai et al. 2022). Furthermore, the significant reduction in lesion length of the *OsSWEET13*-edited line L35.24, compared to the WT control plants, suggested that mutating *OsSWEET13* alone is necessary but insufficient to confer complete resistance to the BT7 rice against *VXO* strains. Previous research demonstrated the necessity of simultaneously mutating five EBEs on three genes, *OsSWEET11*, *OsSWEET13* and *OsSWEET14*, to establish a broad-spectrum resistance for the IR64 and Ciherang-Sub1 rice varieties (Oliva et al. 2019). Similarly, to achieve comprehensive BLB resistance for the major BT7 rice cultivar, it may be effective to utilize the CRISPR/Cas9 tool to concurrently mutate all EBE positions on the major *S* genes targeted by *VXO* strains, based on a comprehensive analysis of the talome of the *VXO* population.

Author Contributions

Nguyen Duy Phuong: data curation, investigation, formal analysis, methodology, visualization, validation, writing – original draft preparation. **Dong Huy Gioi:** data curation, funding acquisition, investigation, project administration, validation, visualization. **Cao Le Quyen:** investigation. **Nguyen Xuan Hung:** investigation. **Ha Hai Yen:** investigation. **Bui Thi Thu Huong:** investigation. **Sebastien Cunnac:** conceptualization, methodology, resources, writing – review and editing. **Tran Dang Xuan:** validation, writing – review and editing. **Pham Xuan Hoi:** conceptualization, project administration, resources, supervision, validation, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

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Supporting Information

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