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FUNCTIONAL CHARACTERIZATION OF THE *OsSWEET13* PROMOTER INVOLVED IN THE INFECTION OF *Xanthomonas oryzae* pv. *oryzae (Xoo)* IN RICE CULTIVAR TBR225

Pham Phuong Ngoc[1], Tran Lan Dai[2], Do Thi Hanh[3], Nguyen Quang Huy[1], Nguyen Duy Phuong[4,*]

[1]Hanoi University of Sciences, VNU, Vietnam
[2]Quy Nhon University, Vietnam
[3]Hanoi University of Industry, Vietnam
[4]Insitute of Agricultural Genetics, Vietnam

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ABSTRACT

Xanthomonas oryzae pv. *oryzae* (*Xoo*) causes severe bacterial leaf blight (BLB) disease to many Vietnamese major rice cultivars, including the TBR225. *OsSWEET13* belongs to group III of the *OsSWEET* gene family encoding sugar transport proteins, which is considered one of the "susceptibility" genes (*S* genes) necessary for BLB disease. In this study, the rice cultivar TBR225 was determined to be susceptible to 19/20 Vietnamese *Xoo* isolates collected from the Northern provinces. Two of the three tested isolates (VXO_60 and VXO_96 isolates) were shown to up-regulate *OsSWEET13* upon the *Xoo* infection of the TBR225 cultivar. The TBR225 *OsSWEET1*3 promoter was isolated for sequencing analysis. The isolated DNA fragment was 615 bp in size, contained an effector binding element (EBE) *PthXo2* that was recognized by the type III-secretory transcription activator-like (TAL) proteins of the *Xoo*. This promoter showed a similarity of more than 99% to the published *OsSWEET13* promoter sequences (AP014967.1 and CP018167.1). Our findings are basic for the generation of highyielding rice varieties with resistance to BLB disease by genetic engineering in Vietnam.

Keywords: Bacterial leaf blight, *OsSWEET13*, TAL effector, TBR225, *Xanthomonas oryzae*.

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**Corresponding author email*: phuongnd.bio@gmail.com

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INTRODUCTION

Bacterial leaf blight disease is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), occurs quite commonly in rice-growing areas of Vietnam, especially in the Red River Delta. The BLB disease causes more damage in summer-autumn rice crops than that in winter-spring crops due to wet, foggy weather and high air humidity. It appears and causes damage from tillering stage to the ripening stage. BLB disease may cause 25–50% grain yield depending on the severity of infection (Singh et al., 2020). For some northern provinces of our country, TBR225 is one of the major rice varieties with a great significant proportion of rice production (https://www.gso.gov.vn/en/agriculture-forestry-and-fishery/). It was developed by selective breeding from hybrids of two China domestic breeds K2 and TBR27, which was confirmed as Vietnam's national seed in 2015 by scopes of attributes. However, the biggest limit of TBR225 cultivars is the susceptibility to BLB disease, leading to severely affected yields when *Xoo* pathovars occur on the fields (Manh Bao et al., 2016). Until now, there was not any effective way to control the bacterial leaf blight disease on rice, including agronomic, chemical, and biological solutions. Therefore, research on the breeding of TBR225 rice varieties resistant to blight is very urgent, helping to ensure the sustainability and efficiency of the rice industry.

Xoo causes rice disease through the type III TAL (transcription activator-like) secretory protein system. TAL protein binds to a specific target sequence (effector binding element - EBE) on the promoter region of the *S* genes in the host genome and induces the targeted genes for the growth of *Xoo* (Boch et al., 2010). Member of the targeted gene family *OsSWEET* encode the corresponding sucrose protein transporter, which leads the sucrose from phloem parenchyma cells to the apoplast near the sieve-element-companion cell complex (Chen et al., 2012). Thereotically, the TAL effector of *Xoo* strains binds to the EBE, causing the upregulation of the assisted sucrose protein transporter encoded by the *OsSWEET* gene family, which makes loading sucrose become the pathogen supplying source. For this reason, TAL protein is a contributed factor that decides the plant susceptibility to *Xoo* strains.

Several genes belonging to clade III of the *SWEET* gene family, including *OsSWEET11*, *OsSWEET13* and *OsSWEET14*, have been identified to be *S* gene for *Xoo* (Blanvillain et al., 2017; Streubel et al., 2013). The promoter regions of *OsSWEET13* and *OsSWEET14* genes contain the well-known EBEs recognized by the TAL effectors (TALEs) PthXo1 and PthXo2 secreted by the majority of the Asian *Xoo* strains (Streubel et al., 2013). The EBE mutations on the promoter region of these *S* genes can generate the resistance for susceptible rice plants against the corresponding *Xoo* strains (Antony et al., 2010; Chen et al., 2012; Chu et al., 2006; Hutin et al., 2015; Zhou et al., 2015). Zhou et al. (2015) showed that *OsSWEET13* is a potential target for leaf blight-resistant rice varieties breeding research. Recently, the *OsSWEET14* promoters of TBR225 and Bacthom 7 rice cultivars were identified that both contain 4 EBEs, including *AvrXa7*, *PthXo3*, *Tal5/TalF* and *TalC*, which may be involved in the virulence of *Xoo* (Huong et al., 2018; Sam et al., 2019).

In this study, we identified the role of *OsSWEET13* in the infection of some representative Vietnamese *Xoo* isolates into Rice cultivar TBR225. The obtained results are the premise for generating BLB-resistant rice cultivar TBR225 by gene editing technology in the future.

MATERIALS AND METHODS

Material

Rice cultivar TBR225 and IR24 were provided by Thai Binh Seed Corporation and The International Rice Research Institute (Philippines), respectively.

Xoo isolates used in this study were annually collected and identified by the Department of Molecular Pathology (Institute of Agricultural Genetics) (Table 1) (Sam et al., 2019).

Table 1. List of *Xoo* isolates used in the study

No.	Name	Place of collection	Year	No.	Name	Place of collection	Year
1	VXO_11	Gia Lam, Ha Noi	2013	11	VXO_82	Thuong Tin, Ha Noi	2016
2	VXO_16	An Lao, Hai Phong	2013	12	VXO_42	Hai Hau, Nam Dinh	2017
3	VXO_41	Cam Xuyen, Ha Tinh	2016	13	VXO_86	Nho Quan, Ninh Binh	2017
4	VXO_40	Chuong My, Ha Noi	2016	14	VXO_84	Hiep Hoa, Bac Giang	2017
5	VXO_91	Thanh Oai, Ha Noi	2017	15	VXO_85	Dong Hung, Thai Binh	2017
6	VXO_93	Chuong My, Ha Noi	2017	16	VXO_44	Hai Hau, Nam Dinh	2017
7	VXO_90	Tu Son, Bac Ninh	2016	17	VXO_95	Thanh Liem, Ha Nam	2017
8	VXO_100	Nam Dinh, Nam Dinh	2016	18	VXO_89	Xuan Truong, Nam Dinh	2017
9	VXO_49	Yen Lac, Vinh Phuc	2016	19	VXO_43	Thach That, Ha Noi	2017
10	VXO_120	Hai Hau, Nam Dinh	2016	20	VXO_80	Dien Chau, Nghe An	2017

Method

Bacterial growth

Bacteria were cultured in PSA media (10 g/L peptone, 10 g/L sucrose, 1 g/L glutamic acid, 15 g/L Bacto Agar) at 28 ^{o}C for two days. *Xoo* bacteria solution is prepared by suspending the bacterial cells in sterile distilled water at an optical density (OD_{600}) of 0.5 (infiltrations) or 0.4 (leaf clipping) prior to inoculation.

Bacterial blight inoculation

For lesion length measurements, 2-week-old TBR225 rice plants were grown in 20 cm diameter pots (3 plants/pot) under net house condition (average temperature about 28 ^{o}C, average humidity about 70%) for 2 months. Inoculation was done by the leaf-clipping method (Ke et al., 2017). Each *Xoo* bacterial isolate was infected on 10 to 15 leaves. At 14 days after inoculation, lesion length from the cut leaf tip were measured in centimeter. BLB disease reaction was scored as follows: high resistance (lesion length < 8 cm), moderate resistance (lesion length 8-12 cm) and susceptibility (lesion length > 12 cm) (Ton et al., 2013). The experiment was repeated three times.

For gene expression analyses, 4 cm leaf sections of 2-week-old TBR225 rice plants were infiltrated with *Xoo* bacterial suspensions as described in the previous study (Blanvillain et al., 2017). Each *Xoo* bacterial isolate was infected on 3 leaves of 3 plants. The plants inoculated with distilled water only were used as negative controls. The leaf sections infiltrated with bacterial suspensions were collected at 48 hours after inoculation and stored in liquid nitrogen for RNA extraction.

Evaluation of gene expression by qPCR

Total RNA was extracted from *Xoo*-inoculated rice leaves using the *RNA-spinTM Total RNA Extraction* kit according to the instructions of Intron (Korea). One µg of purified RNA sample was used for cDNA synthesis using the *Maxima H Minus First Strand cDNA Synthesis* kit according to the instructions of Thermo Fisher Scientific (USA) with an oligo dT primer. cDNA was used as a template for PCR with *OsSWEET*-specific primers (SW14-qPCR-F: 5'-ACTTGCAAGCAAGAACAGTAGT-3'/SW14-qPCR-R: 5'-ATGTTGCCTAGGAGACCAAAGG-3'; SW13-qPCR-F: 5'-GGCCTGTCCCTGCAGCATC-3'/SW13-qPCR-R: 5'-CCCGAACACCCCCACGTTC-3' and SW11-qPCR-F: 5'-AGTCGACGGGAGGGTACAG-3'/SW11-qPCR-R: 5'-CCCGAACACCCCCACGTTC-3'). The PCR was performed with 40 cycles of 94 C, 30 s; 56 ^{o}C, 30 s and 72 ^{o}C, 30 s. The *OsEF1α* gene was used as the constitutive control using specific primers (forward 5'-GAAGTCTCATCCTACCTGAAGAAG-3' and reverse 5'-GTCAAGAGCCTCAAGCAAGG-3') (Blanvillain-Baufumé et al., 2017).

Isolation and sequencing analysis of the OsSWEET13 promoter

Total DNA was extracted from leaf tissue according to the method of Doyle & Doyle (1990), using 2% CTAB solution. Promoter *OsSWEET13* of TBR225 rice (referred to as *OsSWEET13-TBR*) was isolated from TBR225 genomic DNA by PCR (Sambrook et al., 2001), using specific primer pair (SW13-F: 5'-CCGTATCAGGATTCAGGAATA-3'/SW13-R: 5'-CCAGCCATTTTTGTGTGC TA-3'). The PCR was performed with 35 cycles of 94 ^{o}C, 30 s; 55 ^{o}C, 30 s and 72 ^{o}C, 45 s. The 642 bp PCR product was purified using the GenJET-TM Gel Extraction kit (Thermo Fisher Scientific, USA) and directly sequenced using the Sanger method (Smith et al., 1986). The sequencing chromatograms were analyzed by BioEdit 2.0 software.

Statistical analysis

All experiments were repeated three times with the indicated factors. Statistical analyses were carried out using Microsoft Excel version 2013 software.

RESULTS AND DISCUSSION

Rice cultivar TBR225 is susceptible to most Vietnamese *Xoo* isolates

Most Vietnamese major rice cultivars are susceptible to BLB disease. To confirm the susceptibility of rice cultivar TBR225 to the BLB disease, 20 representative Vietnamese *Xoo* isolates collected from 2013 to 2017 (Table 1) were used for leaf-clipping experiments. After 2 weeks, it was observed that all *Xoo* isolates caused typical BLB symptoms on the surface of infected rice leaves, however, disease severity was different. The measurement of lesion length showed that most of the isolates (19/20 isolates) were virulent to the Rice cultivar TBR225, with an average lesion length > 20 cm. In particular, VXO_89 (isolated in Xuan Truong, Nam Dinh province) exhibited to be the most virulent pathogen. The mean lesion length reached 36,5 cm and 34,9 cm in the experiments using TBR225 and IR24 cultivars, respectively. In contrast, both TBR225 and IR24 cultivars were moderately resistant to the VXO_84 isolate; the lesions on 14-day-inoculated leaves had an average length of 15 cm and 13.5 cm, respectively. These experimental results show that the TBR225 is very susceptible to *Xoo* bacteria collected in the North of Vietnam.

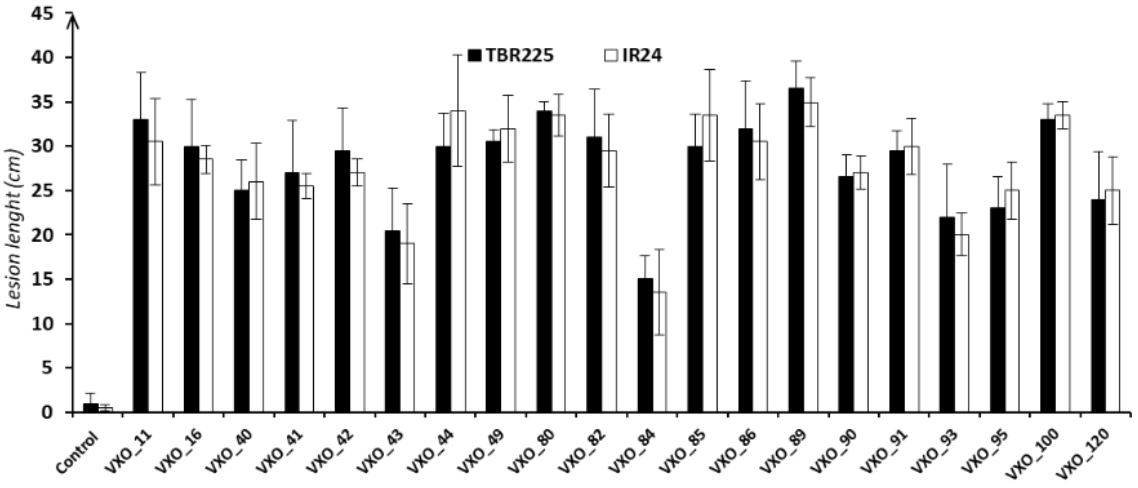

Figure 1. Pathogenicity of *Xoo* bacteria on rice cultivar TBR225

Note: TBR225 rice plants were inoculated with 20 Vietnamese Xoo isolates. (Control) TBR225 rice plants were inoculated with Xoo-free solution. The graph shows the mean lesions on the leaf after 14 days of inoculation. Three replicates were performed for each experiment

The leaf-clipping method is a popular tool in the study to evaluate the virulence of *Xoo* bacteria (Lien et al., 2012; Ton et al., 2013; Blanvillain-Baufumé et al., 2017; Oliva et al., 2019). Ton et al. (2013) used this method to assess the resistance of Vietnamese sticky rice varieties carrying resistant genes *Xa4, Xa5* and *Xa7* against *Xoo* strains. Lien et al. (2012) also determined the virulence of 5/70 *Xoo* isolates collected from Southern provinces on Jasmine 85 rice cultivar by using similar procedures. In North Vietnam, the major rice cultivar TBR225 exhibits are susceptible to BLB disease, but there is no study to comprehensively evaluate the susceptibility of TBR225 to Vietnamese *Xoo*. The results obtained in this study showed that TBR225 had apparent disease symptoms when inoculated with 19/20 *Xoo* isolates from the updated collection of *Xoo* pathovars in Vietnam, demonstrating the susceptibility of this cultivar to the disease.

Vietnamese *Xoo* isolates exhibit *OsSWEET*-induced activity

Several members of the rice sugar transporter encoding gene family *SWEET*, including *OsSWEET11*, *OsSWEET13* and *OsSWEET14*, have been identified as susceptibility genes of many *Xoo* strains (Streubel et al., 2013). In order to determine the role of *OsSWEET* genes in the infection of *Xoo* into the TBR225 rice plant, 3 representative *Xoo* isolates (VXO_11, VXO_80 and VXO_100) from 3 rice-growing provinces were randomly selected for the gene expression analysis.

The resulting qPCR data showed that the expression levels of the target genes were different between rice samples inoculated with each *Xoo* isolate (Fig. 2). All three examined *Xoo* isolates did not induce the expression of *OsSWEET11* at 2-day post inoculation in all experiments. In contrast, it showed a significant increase in the mRNA level of *OsSWEET13* and *OsSWEET14* in TBR225 plants inoculated with bacteria. Notably, two isolates VXO_80 and VXO_100 simultaneously induced the expression of *OsSWEET13* and *OsSWEET14*; while the VXO_11 isolate activated the expression level of *OsSWEET14*. This result proved that both *OsSWEET13* and *OsSWEET14* genes are involved in the infection of Vietnamese *Xoo* in TBR225, but the susceptibility gene of each isolate may be different from the others.

Recently, the study by Oliva et al. (2019) has demonstrated the virulence of many Asian *Xoo* strains, including Chinese, Japanese, Philippine, Taiwanese, Thai, Indian, Korean, Nepalese, South Korean strains, are simultaneously related to *OsSWEET13* and *OsSWEET14*. Meanwhile, *OsSWEET11* is mainly targeted by the African *Xoo* bacteria. Huang et al. (2016) analyzed 31 Asian *Xoo* strains and identified 9, 14 and 22 strains, respectively, that induced the expression of *OsSWEET11*, *OsSWEET13* and *OsSWEET14*. However, all *OsSWEET11*-induced strains are located somewhere far from Vietnam; while all *Xoo* strains from China induced only *OsSWEET13* or *OsSWEET14* or both of these genes. In this study, gene expression analysis by semi-quantitative real-time RT-PCR showed that TBR225 *OsSWEET13* and *OsSWEET14* but not *OsSWEET11* expressions were increased upon infections of representative *Xoo* isolates. These results are comparable to previous studies in which *OsSWEET13* and/or *OsSWEET14* were identified as the major susceptibility genes of Asian *Xoo* strains (Huang et al., 2016; Oliva et al., 2019). Our findings supported the previous hypothesis of that three EBEs *Tal5*, *PthXo3* and *AvrXa7* located on the TBR225 *OsSWEET14* promoter are targeted by virulent TALE of Vietnamese *Xoo* bacteria (Huong et al., 2018). To further reinforce the prediction of susceptibility gene *OsSWEET13* in the TBR225 rice cultivar, the DNA fragment of the TBR225 *OsSWEET13* promoter was isolated for sequencing analysis.

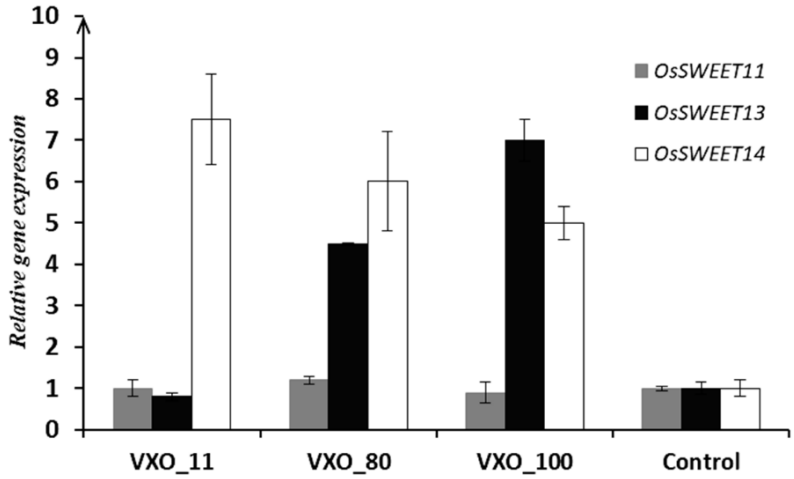

Figure 2. Profiling Vietnamese isolates *OsSWEET*-induction activities on TBR225 rice cultivar
Note: Relative expression levels of OsSWEET11, OsSWEET13 and OsSWEET14 were examined in rice samples inoculated with Vietnamese *Xoo* isolates (VXO_11, VXO_80, VXO_100) by qPCR. The barplot reports least-square means from a mixed linear model of the $\log_2$-transformed OsSWEET induction ratio relative to water treatment of the corresponding rice plant. OsEF1α was used as the internal standard gene. (Control) TBR225 rice plants were inoculated with *Xoo*-free solution. The values shown in the graph are the average results of three independent experiments

TBR225 *OsSWEET13* promoter contains an alternative target sequence of TALE PthXo2

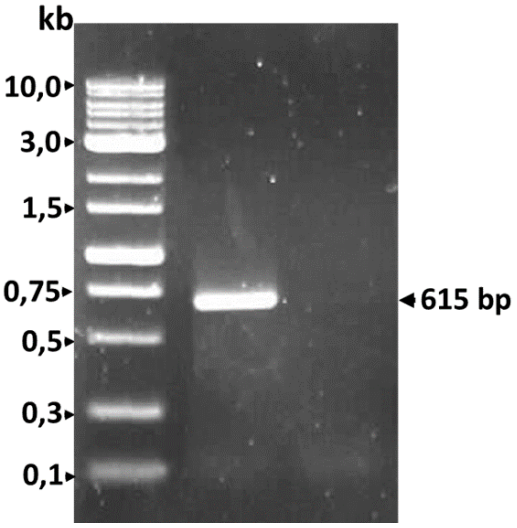

Figure 3. PCR isolation of TBR225 *OsSWEET13* promoter from genomic DNA
Note: Lane M: DNA ladder 1 kb; lane 1: the genomic DNA extract from TBR225 plants was used as a template; lane 2: negative control (no template DNA)

The promoter fragment upstream of the *OsSWEET13* gene was isolated from the total DNA of TBR225 rice plants by PCR with a specific primer pair based on the *OsSWEET13* reference published in Genbank. The results of electrophoresis showed a single DNA band with a size of about 0.6 kb corresponding to the expected size of the *OsSWEET13* promoter (Fig. 3). To confirm that the isolated DNA fragment is the *OsSWEET13* promoter, the PCR product was purified from gel and sequenced (Fig. 4). The sequencing analysis indicated that the isolated *OsSWEET13* promoter fragment was 615 bp in length. The *OsSWEET13* promoter of TBR225 was 99% and 100% similar to the published *OsSWEET13* promoter sequences of the Niponbare variety (Japonica rice; AP014968.1) and Shuhui498 variety (Indica rice; CP018167.1), respectively. Furthermore, the TBR225 *OsSWEET13* promoter also contains the characteristic TATA Box sequence at -259 position. In particular, there was an identified *cis*-element located in the *OsSWEET13* promoter of TBR225 that perfectly match with the sequence of the EBE

recognized by the major virulence effector PthXo2 from Asian *Xoo* strains (Fig. 4) (Oliva et al., 2019). These results, combined with the above profiles of *Xoo* pathogenicity and *OsSWEET13* gene expression, further strengthen the hypothesis that *OsSWEET13* functions as a major susceptibility gene in the infection of *Xoo* in the TBR225 rice cultivar.

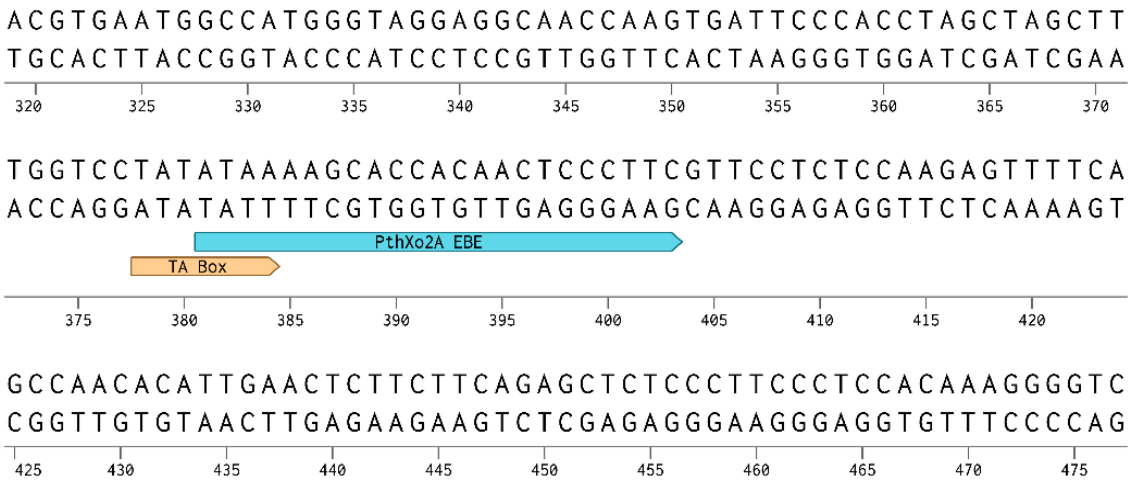

Figure 4. Nucleotide sequence of TBR225 *OsSWEET13* promoter
Note: The TATA box and EBE position were marked with the arrows.

SWEET is a family of genes coding for sugar transport proteins, which are considered to be involved in the infection mechanism of the *Xoo* through the release of sugar molecules into the apoplast for bacterial growth. For rice, several genes such as *OsSWEET11*, *OsWEET13*, and *OsSWEET14* have been identified as *S* genes for *Xoo* which are targeted by major virulent TAL effectors (Blanvillain et al., 2017). In the previous study on the role of *OsSWEET13* in *Xoo* infection mechanism in IRBB13 rice variety, 341 bp DNA fragment located on *OsSWEET13* promoter was also isolated for functional characterization (Hutin et al., 2015). Later studies have demonstrated that the virulence of many *Xoo* strains is mainly or partly dependent on the activation of *OsSWEET13* through the interaction of the TAL protein PthXo2 with the specific EBE sequence on the promoter region of the target gene (Oliva et al., 2019). In this study, the EBE *PthXo2* was also detected on the 615 bp sequence of the TBR225 *OsSWEET13* promoter. Thus, it is possible that Vietnamese *Xoo* isolates secrete the virulent TAL effectors in the infection of TBR225 rice plants that bind specifically to the EBEs on the promoter region of target host genes, including *OsSWEET13*, and induce gene expressions for bacterial growth. Based on the archived complete nucleotide sequence of the *S* genes, the BLB resistance of the TBR225 cultivar can be improved via editing the EBE sites by using precise mutagenesis techniques such as CRISPR/Cas9.

CONCLUSION

In summary, the rice cultivar TBR225 was susceptible to 19/20 Vietnamese *Xoo* isolates. TBR225 *OsSWEET13* and *OsSWEET14* expressions were induced by the 2-day inoculation with representative Vietnamese *Xoo* isolates (VXO_11, VXO_80 and VXO_100). The isolated 615 bp *OsSWEET13* promoter fragment of TBR225 had 99% and 100% nucleotide similarity to the published sequence of Japonica (AP014968.1) and Indica (CP018167.1) rice varieties, respectively, and contained the *PthXo2* EBE recognized by the virulent TAL effectors of *Xoo*. Our findings showed the prospect of

improving BLB resistance of elite rice variety TBR225 through editing specific *PthXo2* EBE via the CRISPR/Cas9 tool.

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