Plant Pathol. J. 40(5) : 451-462 (2024)
https://doi.org/10.5423/PPJ.OA.04.2024.0070
eISSN 2093-9280

The Plant Pathology Journal

Research Article Open Access

Development and Validation of an SNP Marker for Identifying *Xanthomonas oryzae* pv. *oryzae* Thai Isolates That Break *xa5*-Mediated Bacterial Blight Resistance in Rice

Tebogo Balone[1], Ananda Nuryadi Pratama[2], Werapat Chansongkram[2], Thanita Boonsrangsom[1,2], Kawee Sujipuli[1,2], and Kumrop Ratanasut[1,2]*

[1]*Department of Agricultural Science, Faculty of Agriculture Natural Resources and Environment, Naresuan University, Phitsanulok 65000, Thailand*
[2]*Center of Excellence in Research for Agricultural Biotechnology, Naresuan University, Phitsanulok 65000, Thailand*

(Received on April 30, 2024; Revised on July 12, 2024; Accepted on August 12, 2024)

***Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a pathogenic bacterium responsible for bacterial blight (BB) disease in rice, primarily mediated by the interaction between the plant and pathogen. The virulence mechanism involves the activation of the *Sugars Will Eventually be Exported Transporter* (*SWEET*) gene family in rice by transcription activator-like effectors derived from *Xoo*. The BB resistance gene *xa5* has been identified as one of the most effective genes against Thai *Xoo* isolates, but *xa5*-mediated resistance-breaking *Xoo* strains have emerged. This study aimed to develop a single nucleotide polymorphism (SNP) marker for precise identification of *xa5*-mediated resistance-breaking *Xoo*. Comparative genomics of Thai *Xoo* isolates Xoo16PK001 and Xoo16PK002, which were incompatible and compatible with rice variety IRBB5 carrying *xa5*, respectively, identified eight SNP positions for the development of an SNP marker. The SNP marker XooE6 yields a specific 1,143 bp PCR product unique to Xoo16PK002. Screening 61 Thai isolates using XooE6 identified two positives: Xoo20PL010 and Xoo20UT002. Inoculation tests on rice varieties IRBB5 and IRBB13 demonstrated compatibility with IRBB5 and incompatibility with IRBB13, which bears *Xa5* and *xa13*. Xoo16PK001 (XooE6-negative) showed different virulence. Inoculation on IRBB21 harboring *Xa5*, *Xa13*, and *Xa21* resulted in partial resistance to both XooE6-positive and -negative strains. XooE6-positive strains up-regulated *SWEET11* and suppressed *SWEET14* in IRBB5, while Xoo16PK001 slightly induced *SWEET11* but activated *SWEET14* in IRBB13. This highlights the potential of XooE6 to identify *xa5*-mediated resistance-breaking *Xoo* strains and elucidate their pathogenic mechanisms through the upregulation of *SWEET11*.**

Keywords : IRBB5, *SWEET11*, *SWEET14*, whole-genome sequencing, *Xoo*

The virulence of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) depends on the mechanism of plant-pathogen interaction, primarily revolving around the recognition between specific effector binding elements (EBEs) in the promoters of the *Sugars Will Eventually be Exported Transporter* (*SWEET*) gene family in rice and transcription activator-like effectors (TALEs) from *Xoo*. TALEs, integral components of the type III secretion systems, have been identified and characterized as either direct or indirect activators of *SWEETs*, which act as susceptibility genes in rice. TALEs possess the capability to bind specifically to the EBE sites on the promoters of *SWEET*s (Oliva et al., 2019). Moreover, certain TALEs also bind to transcription factor (TF) subunits, such as TFIIAγ1 or TFIIAγ5, to form a transcription complex. This complex subsequently interacts with the EBE of *SWEET* (White et al., 2009), ultimately precipitating bacte-

*Corresponding author.
Phone) +66-850645084, FAX) +66-55962709
E-mail) kumropr@nu.ac.th

Handling Editor : Young-Su Seo

rial blight (BB) disease in rice.

Comparative analysis of the central repeat region in various TALE proteins, consisting of 33-35 amino acids, has revealed that two consecutive variable diresidues at the 12th and 13th positions specifically correspond to binding with a 28-bp DNA sequence in the EBEs located on the *SWEET* promoter (Erkes et al., 2017). Upregulation of *SWEET* genes, which encode sucrose or fructose transporters, has the potential to increase the abundance of apoplastic sugar, thereby facilitating the proliferation of pathogenic *Xoo* and contributing to the development of symptomatic BB (Garcia-Ruiz et al., 2021). Recent studies have reported that only six known TALEs of *Xoo* target three major rice *SWEET* genes (*SWEET11*, *SWEET13*, and *SWEET14*) out of more than 20 *SWEET* genes in nature, leading to a strong virulence effect in causing BB symptoms (Oliva et al., 2019).

The *SWEET* genes in rice are targeted by a variety of TALEs from different *Xoo* strains. The initial discovery of an interaction between an EBE and a TALE revealed that the EBE on the *SWEET11* promoter binds to PthXo1, a TALE found in several *Xoo* strains such as PXO99A and PXO71 (Chen et al., 2010). This interaction between the EBE and PthXo1 leads to the induction of *SWEET11* expression, characteristic of the susceptible dominance *Xa13* allele. However, mutation of the EBE in the *SWEET11* promoter eliminates the specific sequence required for PthXo1 binding, converting the susceptible dominance *Xa13* allele to the resistant recessive *xa13* allele (Zhou et al., 2015). Consequently, the reduced expression of *xa13* confers BB resistance against PthXo1-carrying *Xoo* strains. Moreover, the expression of *SWEET13* is induced by *Xoo* strains carrying PthXo2, whereas the activation of *SWEET14* is mediated by AvrXa7, PthXo3, TalC, and TalF (Doucouré et al., 2018). These findings underscore the role of TALEs as virulence factors in the context of BB disease in rice. Each TALE performs a specific function by binding to its target EBE on the *SWEET* promoter, thereby triggering gene expression in rice. Additionally, TALE proteins are hypothesized to interact with the products of resistance (*R*) genes such as *xa5* (Huang et al., 2016). The *xa5* resistant recessive allele in rice encodes a TF subunit TFIIAγ5, which disrupts the interaction with TALEs, consequently diminishing their ability to activate *SWEET* alleles (Huang et al., 2016).

Phylogenomic analysis has revealed high polymorphisms among TALE genes across various *Xoo* strains isolated from different geographical regions. This *Xoo* evolution impacts the virulence dynamics of BB disease against rice cultivars, depending on the specific interaction between the *Xoo* strain and the rice variety (Song et al., 2023). In Thailand, the *Xoo* strain SK2-3 (referred to as a *xa5*-mediated BB resistance breaking strain) was initially isolated from Sukhothai province and comprises 15 TALEs. While the Tal7 (ortholog of PthXo7) in the *Xoo* strain SK2-3 reduces TF accumulation in rice variety IRBB5 (*xa5/xa5*) by interacting with TFs (TFIIAγ1 and TFIIAγ5), another TALE, such as the Tal1c effector in the *Xoo* strain SK2-3, strongly activates *SWEET11* despite the dampening effect of IRBB5 (Carpenter et al., 2020). These findings suggest that *Xoo* strain SK2-3 may overcome the *xa5* gene through the Tal1c effector, which strongly induces the expression of *SWEET11*, leading to virulent BB symptoms in IRBB5.

Our preliminary study on virulence of various pathogenic *Xoo* isolates, collected from the Lower Northern Thailand, revealed two groups of *Xoo* pathogenicities: IRBB5- and IRBB13-compatible *Xoo*. All *Xoo* isolates were selectively either IRBB13- or IRBB5-compatible; thus, no collected *Xoo* isolates caused BB virulence on both IRBB13 and IRBB5, which carry BB resistance genes *xa13* and *xa5*, respectively. A few of the collected *Xoo* isolates are IRBB5-compatible *Xoo*, whereas the rest of the isolates are IRBB13-compatible *Xoo*. The *xa5* gene has been reported to be the most effective BB resistance gene against most Thai *Xoo* strains and has been used for improving BB resistance in Thai rice (Wongkhamchan et al., 2018). However, the appearance of IRBB5-compatible *Xoo* strains indicates that these *Xoo* strains can overcome *xa5*-mediated resistance and may pose a risk for outbreaks of these *Xoo* strains in the future, particularly considering the effects of global warming. Identifying *Xoo* strains in each rice-growing region contributes to managing the proper strategy to control the outbreak of specific *Xoo* strains.

In this study, we aimed to develop and validate a single nucleotide polymorphism (SNP) molecular marker for identifying IRBB5-compatible *Xoo* strains. Additionally, we determined the responses of *SWEET11* and *SWEET14* to IRBB5- and IRBB13-compatible *Xoo* in rice varieties IRBB5, IRBB13, and IRBB21, which carry BB resistance genes *xa5*, *xa13*, and *Xa21*, respectively. These findings could provide a novel SNP marker for categorizing *Xoo* strains that break *xa5*-mediated resistance in rice for the first time. Such implications are important for developing effective strategies for managing BB disease in rice production systems and could aid in further improving BB resistance in rice cultivars.

Materials and Methods

Xoo isolates and DNA extraction. Sixty-three *Xoo* isolates, collected and isolated from rice paddy fields in the Lower Northern Thailand (Table 1) along with a reference *Xoo* strain SK2-3 provided by Dr. Sujin Patarapuwadol's Lab at Kasetsart University, Kamphaeng Saen campus, Nakorn Pathom, Thailand, were utilized in this study. Single colonies of each isolate were cultured on nutrient agar (NA) medium (0.5% peptone, 0.3% yeast extract/beef extract, 0.5% NaCl, and 0.5% agar) at 37°C for 3-4 days. Subsequently, they were streaked on fresh NA plates and cultured under the mentioned condition for 1-2 days. Genomic DNA extraction from each *Xoo* culture was carried out using the genomic DNA extraction kit (PureDirex, Taipei, Taiwan) following the manufacturer's protocol. The extracted DNA was quantified and assessed for quality using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The integrity of the DNA was verified through a 1.0% agarose gel electrophoresis assay.

Comparative genome analysis and SNP primer design. Each of IRBB5- and IRBB13-compatible *Xoo* isolates,

Table 1. List of *Xanthomonas oryzae* pv. *oryzae* isolates collected from Lower Northern Thailand, and the preliminary test of *Xoo* compatibilities on rice varieties IRBB5 and IRBB13

No.	Bacterial isolates	Source	IRBB5/ IRBB13 compatibility	No.	Bacterial isolates	Source	IRBB5/ IRBB13 compatibility
1	Xoo16PK001	PL	IRBB13	33	Xoo19SK001	SK	IRBB13
2	Xoo16PK002	PL	IRBB5	34	Xoo19SK002	SK	IRBB13
3	Xoo16PK003	PL	IRBB13	35	Xoo19SK003	SK	IRBB13
4	Xoo19PL001	PL	IRBB13	36	Xoo19KP001	KP	IRBB13
5	Xoo19PL002	PL	IRBB13	37	Xoo19KP002	KP	IRBB13
6	Xoo19PL003	PL	IRBB13	38	Xoo19KP003	KP	IRBB13
7	Xoo19PL004	PL	IRBB13	39	Xoo19KP004	KP	IRBB13
8	Xoo19PL005	PL	IRBB13	40	Xoo20KP005	KP	IRBB13
9	Xoo19PL006	PL	IRBB13	41	Xoo20KP006	KP	IRBB13
10	Xoo19PL007	PL	IRBB13	42	Xoo19NW001	NW	IRBB13
11	Xoo19PL008	PL	IRBB13	43	Xoo20NW002	NW	IRBB13
12	Xoo19PL009	PL	IRBB13	44	Xoo20NW003	NW	N/A
13	Xoo19PL010	PL	IRBB5	45	Xoo20NW004	NW	IRBB13
14	Xoo19PL011	PL	IRBB13	46	Xoo20NW005	NW	N/A
15	Xoo19PL012	PL	N/A	47	Xoo20NW006	NW	N/A
16	Xoo19PL013	PL	IRBB13	48	Xoo20UN001	UN	IRBB13
17	Xoo16PT001.1	PC	N/A	49	Xoo20UN002	UN	N/A
18	Xoo16PT001.2	PC	N/A	50	Xoo20UN003	UN	N/A
19	Xoo16PT002	PC	IRBB13	51	Xoo20UN004	UN	IRBB13
20	Xoo16PT003.1	PC	N/A	52	Xoo19UT001	UT	IRBB13
21	Xoo16PT003.2	PC	N/A	53	Xoo20UT002	UT	IRBB5
22	Xoo16PT004	PC	IRBB13	54	Xoo20UT003	UT	N/A
23	Xoo16PT005	PC	IRBB13	55	Xoo20UT004	UT	IRBB13
24	Xoo19PC001	PC	IRBB13	56	Xoo20UT005	UT	IRBB13
25	Xoo19PC002	PC	IRBB13	57	Xoo20UT006	UT	IRBB13
26	Xoo19PC003	PC	IRBB13	58	Xoo20UT007	UT	N/A
27	Xoo19PC004	PC	IRBB13	59	Xoo20UT008	UT	N/A
28	Xoo19PC005	PC	IRBB13	60	Xoo20UT009	UT	N/A
29	Xoo19PC006	PC	IRBB13	61	Xoo20UT010	UT	N/A
30	Xoo19PC007	PC	N/A	62	Xoo20UT011	UT	N/A
31	Xoo19PC008	PC	N/A	63	Xoo19PB001	PB	N/A
32	Strain SK2-3	SK	IRBB5	64	Xoo22PRRC	PL	N/A

Abbreviations of sample collecting sources (provinces in Thailand) were designated as follows: PL, Phitsanulok; PC, Phichit; SK, Sukhothai; KP, Kamphaeng Phet; NW, Nakhon Sawan; UN, Uthai Thani; UT, Uttaradit; PB, Phetchabun.
Xoo, *Xanthomonas oryzae* pv. *oryzae*; N/A, not assessed.

named Xoo16PK002 and Xoo16PK001, respectively, was selected for whole-genome sequencing using the Illumina HiSeq-PE150 platform (Novogene Bioinformatics Technology Co., Beijing, China). Primer designs to classify both *Xoo* isolates were carried out using bioinformatic software as shown in Fig. 1. Briefly, the DNA sequence reads from both *Xoo* isolates were mapped and aligned to the reference genome of *Xoo* strain SK2-3 (Carpenter et al., 2020). Unique SNPs among *Xoo* isolates were generated and identified using the Snippy program in Galaxy software.

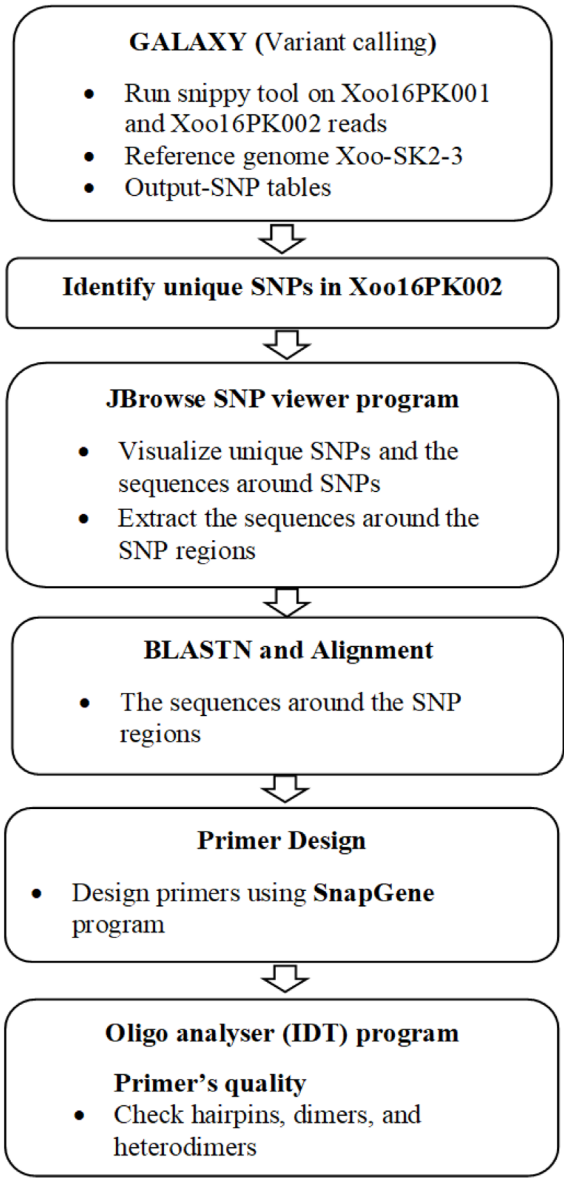

Fig. 1. Computational pipeline used for single nucleotide polymorphism (SNP) analysis and Xoo16PK002 SNP marker design from whole-genome sequencing data. Xoo, *Xanthomonas oryzae* pv. *oryzae*.

The unique SNPs, along with the sequences around each SNP, were detected and visualized using the JBrowse Snip Viewer software (https://jbrowse.org/jb2/), and then aligned using BLASTN and alignment software in NCBI. Primers were designed from the unique Xoo16PK002 SNPs using SnapGene 7.0 software (https://www.snapgene.com/). At each SNP site, SNP-forward and SNP-reverse primers were designed using the criterion that an SNP marker contained the 1st original-nucleotide mismatch at the 3′ end of its primer. Lastly, the designed forward and reverse primer sequences were validated for potential primer-dimer and intramolecular hairpin formation using Oligo Analyzer primer design tool provided by Integrated DNA Technologies (IDT) (https://www.idtdna.com/pages/tools/oligoanalyzer). The oligonucleotide primers were synthesized by Macrogen Laboratories, Inc. (Seoul, Korea).

PCR optimization and validation of the SNP primers. Two designed primer set were validated for specific detection of the Xoo16PK002 isolate, compared to Xoo16PK001, and the *SK2-3* strain using PCR assay (Bio-Helix, New Taipei, Taiwan). The PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at various temperatures (either 55°C, 57°C, 59°C, 61°C, 63°C or 65°C) for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

The PCR products corresponding to expected sizes of 1,143 and 386 bp were purified using the PCR Clean-Up and gel Extraction Kit (Bio-Helix) following the manufacturer's protocol. Subsequently, they were subjected to sequencing to compare the sample sequences with the progenitor genome Xoo16PK002.

Artificial Xoo inoculation in rice. The resistance rice varieties IRBB5, IRBB13, and IRBB21, carrying *xa5*, *xa13*, and *Xa21*, respectively, were individually grown in pots with three seedlings per pot. They were cultured for 40 days under natural light and temperature conditions in the greenhouse at Naresuan University, Phitsanulok, Thailand. The *Xoo* inoculum was prepared by suspending bacterial colonies in sterilized distilled water and adjusting the dilution to an OD_{600} of approximately 0.2 (cell density of 2.5×10^8 cfu/ml). The inoculum was then applied to leaves of 40-day-old experimental plants using the leaf-clipping method. Each *Xoo* isolate was artificially inoculated onto six leaves of each plant of all the cultivars planted. Three biological replicates of each cultivar were carried out. The negative control for rice leaf was clipped with sterilized distilled water. At 7, 14, and 21 days after *Xoo* inoculation

(DAI), the symptomatic response to BB disease was evaluated and photographed by measuring the lesion lengths of 9 leaves per rice cultivar. The lesions were then scored according to the International Rice Research Institute Standard Evaluation System for greenhouse (http://www.knowledgebank.irri.org/images/docs/rice-standard-evaluation-system.pdf). The scoring criteria were as follows: resistance (R, lesion length 0-5 cm), moderate resistance (MR, lesion length > 5-10 cm), moderate susceptibility (MS, lesion length > 10-15 cm), susceptibility (S, lesion length > 15-20 cm), and high susceptibility (HS, lesion length > 20).

Expression analysis of *SWEET* genes. The inoculated leaf region approximately 5 cm in size was harvested at specific time points of 0 (immediately collected after clipping) and 7 DAI. Total RNA was extracted using the FavorPrep Plant Total RNA Mini kit (Favorgen, Ping Tung, Taiwan) following the manufacturer's recommendations. Genomic DNA contamination in the total RNA samples was further eliminated with RNase-Free DNaseI treatment (Promega, Madison, WI, USA). For real-time reverse transcriptase (RT)-qPCR, the first-strand cDNA was synthesized from 50 ng of total RNA using the first-strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), following the manufacturer's instructions. The RT-qPCR conditions were as follows: initial denaturation at 95°C for 12 min, followed by 40 cycles (95°C for 15 s, 60°C for 30 s, and 72°C for 30 s) in the Eco48 Real-Time PCR system (Eco 48, PCRmax Limited, Cheshire, UK). Primers used for detecting *SWEET11*, *SWEET14*, and a reference gene, *endothelial differentiation factor* (*Edf*), are listed in Table 2. The Ct value was extracted for both the reference gene and target gene and used to calculate the fold change of gene expression using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen,

Table 2. A list of primers utilized for the quantitative expression analysis of *SWEET11* and *SWEET14* in response to the tested *Xoo* isolates

Gene	Primer name	Primer sequence (5′→3′)	Amplicon size (bp)	Reference
SWEET11	SWEET11-F	GTCAAGTTCCTCGGCAGCG	156	Verdier et al. (2012)
	SWEET11-R	GCAGAACCACGCGACGGC		
SWEET14	SWEET14-F	GGCGACCGCCGCATCGTGGTT	216	Verdier et al. (2012)
	SWEET14-R	GCCCAGCACGTTGGGAAGAGCG		
Edf	Edf-F	TCCGAACCAGCAGATCATCG	226	Sagun et al. (2020)
	Edf-R	GCATGGTATCAAAAGACCCAGC		

Xoo, *Xanthomonas oryzae* pv. *oryzae*.

Table 3. SNP-designed primers and their target loci in the Xoo16PK002 genome

SNP marker name	Primer set	Primer Sequence (5′-3′)	SNP loci	SNP location
XooE1	XooE-F1	GTGACTCTTTGCGGGCTT**T**	T/G	392,999
	XooE-R1	CAGATCAGGTCATCGGT**G**	C/T	394,201
XooE2	XooE-F2	GACGGCTGAATGACCC**C**	C/T	1,831,983
	XooE-R2	GTACTTGCTTGAAGGCTG**G**	C/T	1,832,226
XooE3	XooE-F3	AAGGGTATAAGGATCTGTA**C** GCAAT-	C/T	1,832,206
	XooE-R3	GCGGCTCCCTC**C**	G/T	1,832,552
XooE4	XooE-F4	ACTCAGGATGGTGTTGGC	-	-
	XooE-R1	CAGATCAGGTCATCGGT**G**	C/T	394,201
XooE5	XooE-F1	GTGACTCTTTGCGGGCTT**T**	T/G	392,999
	XooE-R4	GTCGCCATCGCCACCTT	-	-
XooE6	XooE-F5	ACCGCCTACAACGACTA**C**	C/T	1,928,910
	XooE-R5	TCAGTTCCAGGTTCACTTC**A**	T/G	1,930,016
XooE7	XooE-F5	ACCGCCTACAACGACTA**C**	C/T	1,928,910
	XooE-R6	GAAATACCATTCCAGCCCCA	-	-
XooE8	XooE-F6	ACCTCACCGTGTTCTACGAG	-	-
	XooE-R5	TCAGTTCCAGGTTCACTTC**A**	T/G	1,930,016

SNPs are denoted by bold letters at the 3′ ends of primers.
SNP, single nucleotide polymorphism; *Xoo*, *Xanthomonas oryzae* pv. *oryzae*.

```
CLUSTAL O (1.2.4) multiple sequence alignment

        SNP marker: (XooE1 and XooE5)-F1    Nucleotide:392,999
SK2-3       GTGACTCTTTGCGGGCTTTACCGCGTCGCTGGCGGGACATCTGGTGCCGGACGCTGC
Xoo16PK002  GTGACTCTTTGCGGGCTTTACCGCGTCGCTGGCGGGACATCTGGTGCCGGACGCTGC
Xoo16PK001  GTGACTCTTTGCGGGCTTCACCGCGTCGCTGGCGGGACATCCGGTGCCGGACGCTGC
            ****************** ********************** **************

        SNP marker: (XooE1 and XooE4)-R1    Nucleotide:394,201
 SK2-3      TGATGCGCGAAAACACGCTGACCACCGATGACCTGATCTGGCCGGTGTTCGTGCACGCCT
Xoo16PK002  TGATGCGCGAAAACACGCTGACCACCGATGACCTGATCTGGCCGGTGTTCGTGCACGCCT
Xoo16PK001  TGATGCGCGAAAACACGCTGACTACCGATGACCTGATCTGGCCGGTGTTCGTGCACGCCT
            ********************** ************************************

        SNP marker: XooE2-F2    Nucleotide: 1,831,983
 SK2-3      TTGCGGTGGAGGGACGGCTGAATGACCCCGCACACAAGCGGGTGCATGTGGACATCGACC
Xoo16PK002  TTGCGGTGGAGGGACGGCTGAATGACCCCGCACACAAGCGGGTGCATGTGGACATCGACC
Xoo16PK001  TTGCGGTGGAGGGACGGCTGAATGACCCTGCACACAAGCGGGTGCATGTGGACATCGACC
            **************************** ******************************

        SNP marker: XooE2-R2    Nucleotide: 1,832,226
SK2-3       CACCTAATGTTCAAGGGTATAAGGATCTGTACCAGCCTTCAAGCAAGTACGGGTAAGCAC
Xoo16PK002  CACCTAATGTTCAAGGGTATAAGGATCTGTACCAGCCTTCAAGCAAGTACGGGTAAGCAC
Xoo16PK001  CACCTAATGTTCAAGGGTATAAGGATCTGTAACAGCCTTCAAGCAAGTACGGGTAAGCAC
            ****************************** ****************************

        SNP marker: XooE3-F3    Nucleotide: 1,832,206
SK2-3       CCTAATGTTCAAGGGTATAAGGATCTGTACCAGCCTTCAAGCAAGTACGGGTAAGCACTC
Xoo16PK002  CCTAATGTTCAAGGGTATAAGGATCTGTACCAGCCTTCAAGCAAGTACGGGTAAGCACTC
Xoo16PK001  CCTAATGTTCAAGGGTATAAGGATCTGTAACAGCCTTCAAGCAAGTACGGGTAAGCACTC
            ****************************** ******************************

        SNP marker: XooE3-R3    Nucleotide: 1,832,552
SK2-3       AGGGAGGGAGCCGCATTGCTGGCGTGGCCAACGTCTCTCAGGTCAGGCCTCCAATGCAAG
Xoo16PK002  AGGGAGGGAGCCGCATTGCTGGCGTGGCCAACGTCTCTCAGGTCAGGCCTCCAATGCAAG
Xoo16PK001  AGTGAGGGAGCCGCATTGCTGGCGTGGCCAACGTCTCTCAGGTCAGGCCTCCAATGCAAG
            ** ************************************************************

        SNP marker: (XooE6 and XooE7)-F5    Nucleotide: 1,928,910
SK2-3       TTCCACCGACAAGGTGACCGCCTACAACGACTACTTCTTCGACGGCGCCGACGGCAATCC
Xoo16PK002  TTCCACCGACAAGGTGACCGCCTACAACGACTACTTCTTCGACGGCGCCGACGGCAATCC
Xoo16PK001  TTCCACCGACAAGGTGACCGCCTACAACGACTATTTCTTCGACGGCGCCGACGGCAATCC
            ********************************** *************************

        SNP marker: (XooE6 and XooE8)-R5    Nucleotide: 1,930,016
SK2-3       TCGGTGACGGCCGGGCTGTAGTTGAAGTGAACCTGGAACTGAGGT
Xoo16PK002  TCGGTGACGGCCGGGCTGTAGTTGAAGTGAACCTGGAACTGAGGT
Xoo16PK001  TTGGCGGCGGCTGGGTTGTAGTGGAAGTGAACCTGGAACTGAGGT
            * ** *.**** *** ****** **********************
```

Fig. 2. Alignment of selected single nucleotide polymorphism (SNP) regions between Xoo16PK001 and Xoo16PK002 compared to the Xoo strain SK2-3 as a reference (CLUSTAL O (1.2.4)). Bold nucleotides indicate SNPs in each region. The sequence of the SNP primer is underlined. Xoo, *Xanthomonas oryzae* pv. *oryzae.*

2001). All reactions were technically repeated three times with two to three biological replicates.

Statistical analysis. The results were expressed as the mean value ± standard error. Data analysis was carried out using IBM SPSS statistics version 19 (IBM Corp., Armonk, NY, USA). Statistically significant differences in mean values among the treatments were determined by Duncan's new multiple range test at a significance level of $P \leq 0.05$.

Results

The SNP marker designed specifically for the *Xoo* isolate Xoo16PK002. The nucleotide sequence comparison between Xoo16PK001 (4,871,124 bp) and Xoo16PK002 (4,923,613 bp), generated by an Illumina HiSeq-PE150 platform, revealed that most nucleotide sequences, along with both *Xoo* isolates, shared high sequence identity (99.75%). Galaxy variant calling detected 8,338 SNPs when comparing Xoo16PK001 to Xoo16PK002, and 8,352

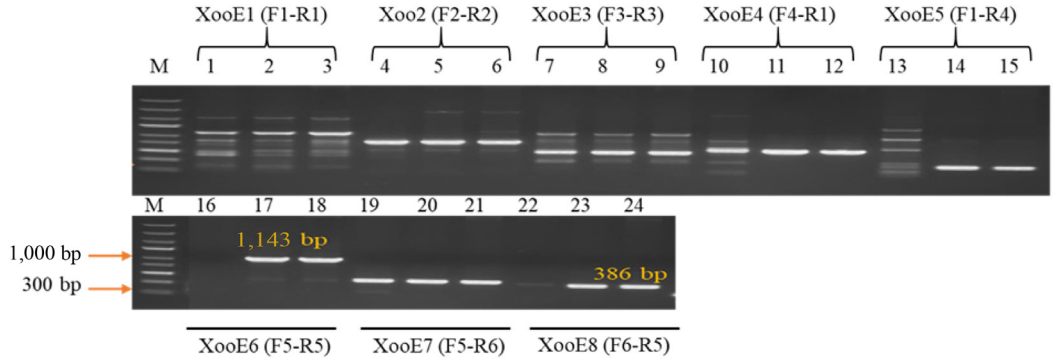

Fig. 3. Evaluation of the single nucleotide polymorphism markers XooE1-XooE8 specifically for identifying Xoo16PK002. Lanes 1, 4, 7, 10, 13, 16, 19, and 22 correspond to *Xoo*16PK001; lanes 2, 5, 8, 11, 14, 17, 20, and 23 correspond to *Xoo*16PK002, and lanes 3, 6, 9, 12, 15, 18, 21, and 24 correspond to *Xoo*-SK2-3. Lane M represents 100 bp DNA ladder (Bio-Helix, Taipei, Taiwan). Xoo, *Xanthomonas oryzae* pv. *oryzae*.

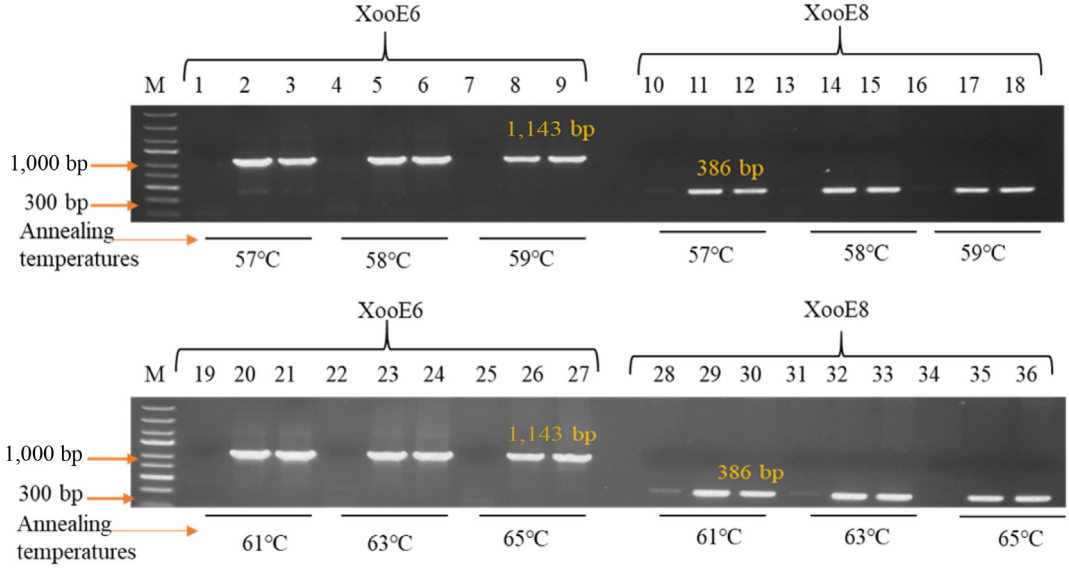

Fig. 4. Optimization of the annealing temperatures for the single nucleotide polymorphism markers XooE6 and XooE8 to identify Xoo16PK002. Lanes 1, 4, 7, 10, 13,16, 19, 22, 25, 28, 31, and 34 represent Xoo16PK001. Lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, and 35 represent Xoo16PK002. Lanes 3, 6, 9, 12, 15,18, 21, 24, 27, 30, 33, and 36 represent *Xoo* strain SK2-3. Lane M represents 100 bp DNA ladder (Bio-Helix, Taipei, Taiwan). Xoo, *Xanthomonas oryzae* pv. *oryzae*.

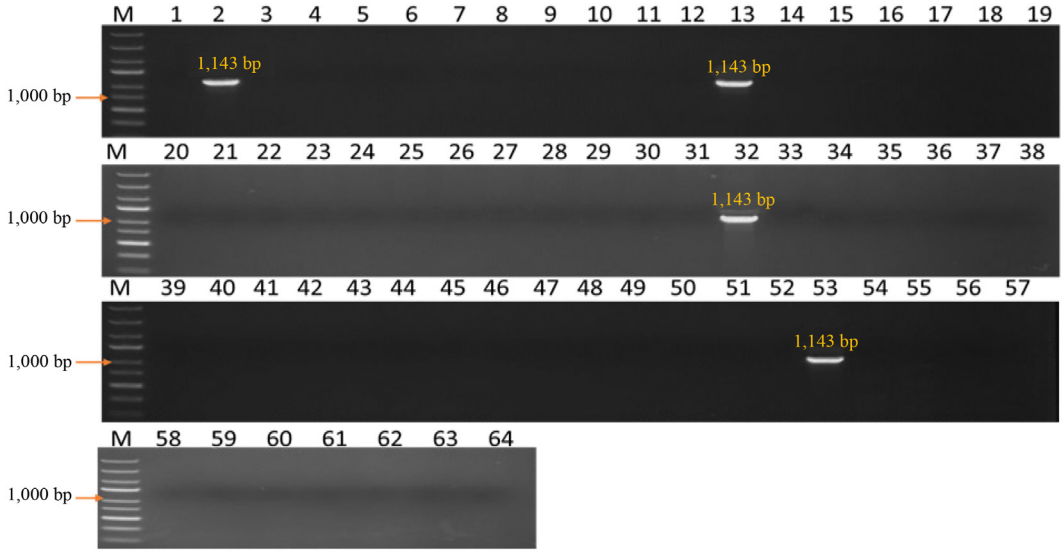

Fig. 5. Identification of 64 *Xoo* isolates using the single nucleotide polymorphism marker XooE6. Lanes 1-64 correspond to *Xoo* isolates listed in Table 3. Lanes 2, 13, 32, and 53 represent Xoo16PK002, Xoo20PL010, *Xoo* strain SK2-3, and Xoo20UT002, respectively. Lane M represents the 100-bp DNA ladder (Bio-helix, Taipei, Taiwan). Xoo, *Xanthomonas oryzae* pv. *oryzae.*

SNPs between Xoo16PK001 and XooSK2-3. Various regions of the genome were randomly chosen for marker development, with the PCR product sizes ranging between approximately 300 and 1,200 bp. A higher abundance of distant SNPs in the Xoo16PK002 genome resulted in a decreased selection of SNPs, leading to the selection of eight SNPs for primer design (Table 3, Fig. 2). The SNPs found in both Xoo16PK002 and XooSK2-3 were identical and differed from those in Xoo16PK001. Based on PCR screening, only two SNP markers, XooE6 and XooE8, generated satisfactory molecular markers, providing expected PCR products (Fig. 3). The two SNP loci (C/T and T/G) were identified along both alignment sequences, corresponding to nucleotide positions at 1,928,910, and 1,930,016, respectively (Table 3, Fig. 2). These SNP regions were predicted as hypothetical proteins (accession no. CP019226.1) within the Xoo16PK002 genome using the Blast program. The primer sequences based on these SNP regions were confirmed to be specific to the target region by sequence alignment against the entire genome or target database. The primer set of the SNP markers XooE6 and XooE8 were specifically designed to target DNA regions unique to the Xoo16PK002 isolate, with the expected amplicon sizes of 1,143 bp and 386 bp, respectively (Fig. 3). These SNP markers provided specific amplification in Xoo16PK002 and *Xoo* strain SK2-3 but not in Xoo16PK001, with the optimal annealing temperature at 65°C (Fig. 4). BLASTN analysis of the amplified PCR products using the XooE6 (1,143 bp) and XooE8 (386 bp) markers from Xoo16PK002 revealed that the DNA sequences exhibited homology to *Xoo* strain SK2-3 (GenBank no. CP019515.1), corresponding to 99.63% and 99.15% identity, respectively. However, the XooE6 marker had a slightly higher percentage identity compared to the XooE8 marker. Based on the optimization of primer conditions, XooE6 is primarily chosen for validation across isolates collected from Lower Northern Thailand owing to its consistent specificity across a range of annealing temperatures. In contrast, XooE8 demonstrated specificity only at 65°C and was therefore not used in the validation process to detect variability, resulting in its disqualification as a suitable marker.

Identification of *Xoo* isolates using the SNP marker XooE6. The SNP marker XooE6 conferred four positive *Xoo* isolates out of 64, including isolates Xoo16PK002, Xoo20PL010, Xoo20UT002, and *Xoo* strain SK2-3. Meanwhile, the rest of the *Xoo* isolates tested negative for the XooE6 marker (Fig. 5). To confirm the correspondence of the XooE6 marker and *xa5*-mediated resistance breaking *Xoo* isolates, four of the XooE6-positive *Xoo* isolates were inoculated on three BB resistance rice varieties: IRBB5, IRBB13, and IRBB21. At 21 DAI, these four *Xoo* isolates significantly increased BB severity only in IRBB5, characterized by BB lesion lengths longer than 20 cm. In contrast, a significant enhancement of BB resistance in IRBB13 and IRBB21 was evidenced by a reduction in BB lesion lengths on leaves to less than 5 and 10 cm, respectively,

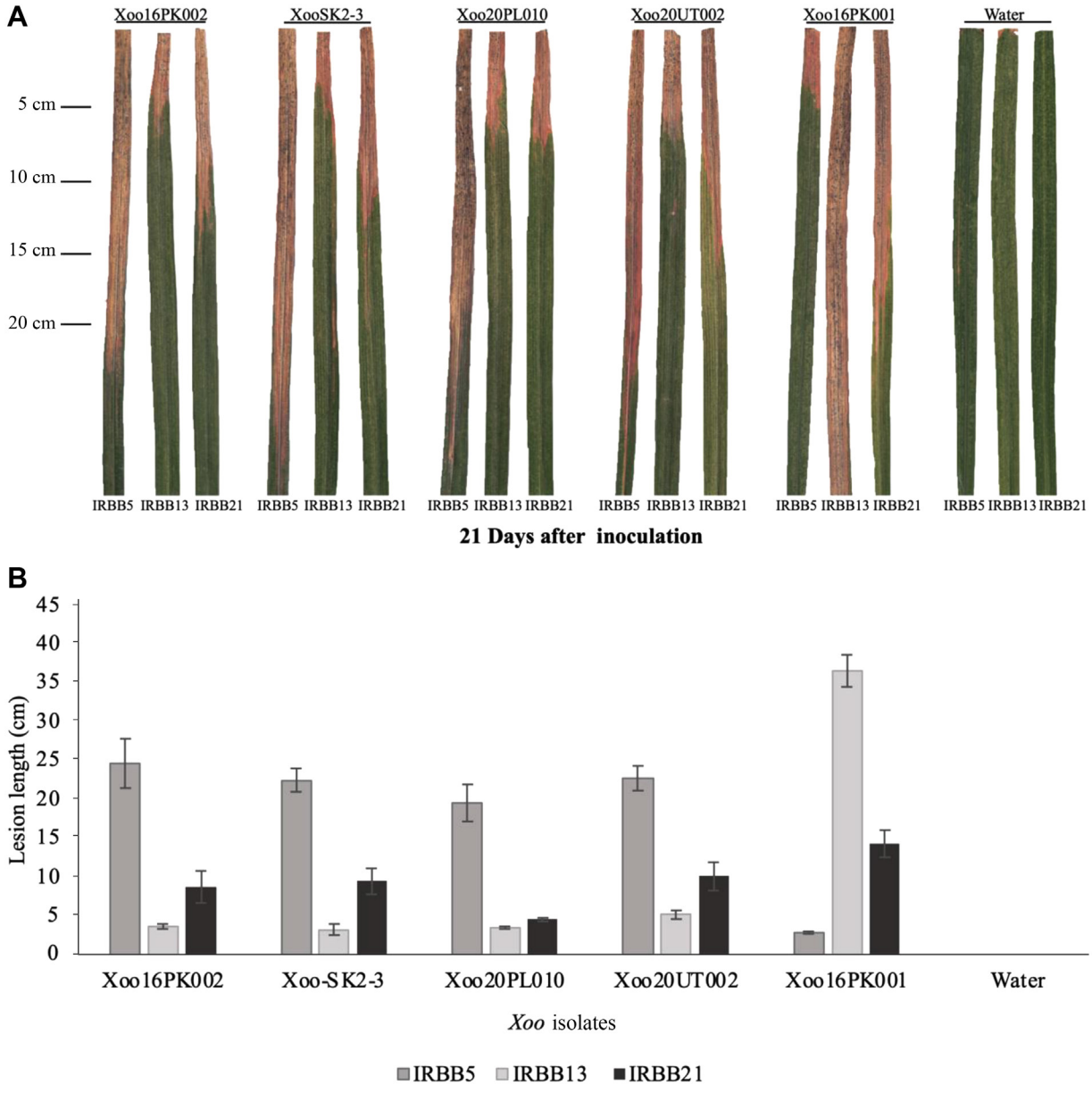

Fig. 6. Artificial inoculation test of four XooE6-positive *Xoo* isolates (Xoo16PK002, Xoo20PL010, Xoo20UT002, and *Xoo* strain SK2-3) in rice varieties IRBB5, IRBB13, and IRBB21. (A) The representatives of the bacterial blight (BB) lesions on the leaves of three varieties inoculated with each *Xoo* isolate. (B) The average BB lesion lengths of each variety inoculated with each *Xoo* isolate ($n = 9$). The Xoo16PK001 isolate represents the XooE6-negative *Xoo* isolate. The BB lesions were examined at 21 DAI. The control inoculation consisted of H_2O application. Xoo, *Xanthomonas oryzae* pv. *oryzae.* The resistance score is as follows: R = high resistance (0-5 cm); MR = moderate resistance (>5-10 cm); MS = moderate susceptibility (>10-15 cm); S = susceptibility (>15-20 cm); HS = high susceptibility (>20 cm).

indicating high and moderate resistance. In contrast, only Xoo16PK001 isolate exhibited significant BB resistance in IRBB5, with the lesion length on leaves less than 5 cm (2.80 ± 0.1 cm), but it caused significant BB severity in IRBB13 and IRBB21, by enhancing the BB lesion length on leaves (Fig. 6).

Expression analysis of *SWEET11* and *SWEET14* in IRBB5, IRBB13, and IRBB21 inoculated with XooE6-positive and -negative *Xoo* isolates. In rice variety IRBB5, the expression level of *SWEET11* dramatically increased nearly 150 folds at 7 DAI with four XooE6-positive *Xoo* isolates, whereas it increased approximately 2.5 folds when inoculated with the XooE6-negative isolate Xoo16PK001

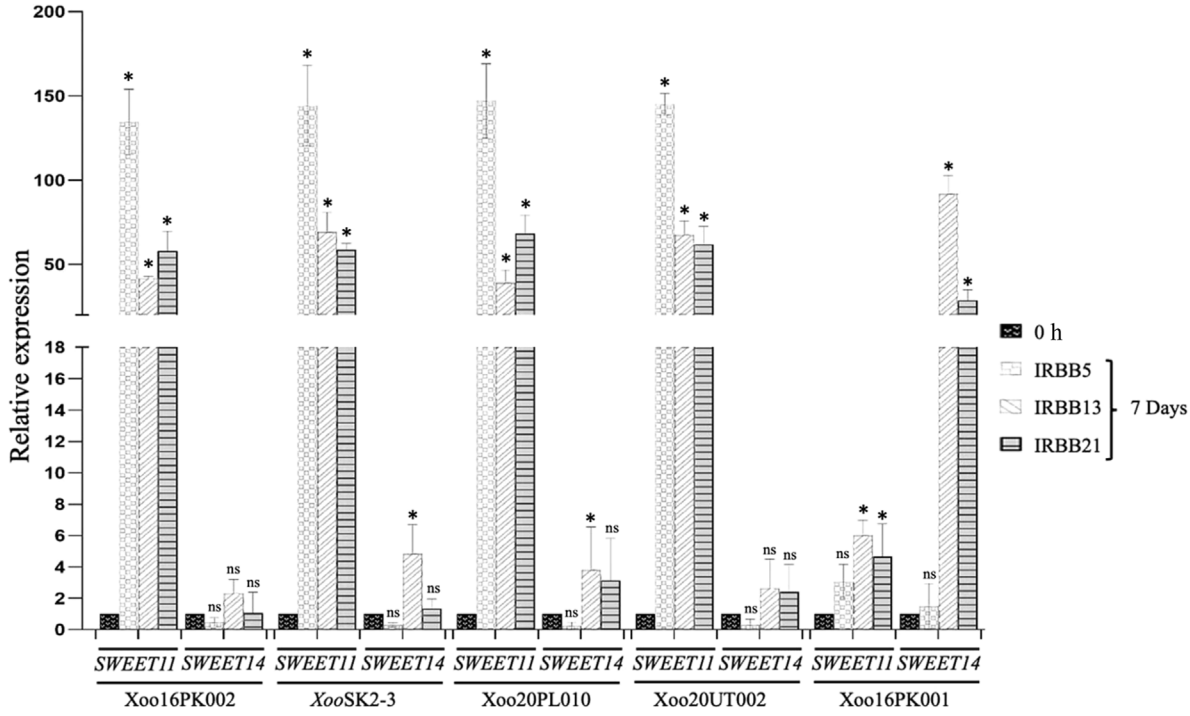

Fig. 7. Expression of *SWEET11* and *SWEET14* in rice varieties IRBB5, IRBB13, and IRBB21 challenged with the XooE6-positive (Xoo16PK002, Xoo20PL010, Xoo20UT002, and *Xoo* strain SK2-3) and -negative (Xoo16PK001) *Xoo* isolates. The relative expression values of the *SWEET* genes were measured at 7 days after *Xoo* inoculation and normalized to the uninfected control. Each expression level was compared to the baseline expression at 0 h (set to 1). The asterisk (*) within each *Xoo* isolate denotes a significant difference ($P \leq 0.05$) in the expression of individual *SWEET* genes in each rice variety (either IRBB5, IRBB13, or IRBB21) compared to the control (at 0 h after *Xoo* inoculation). Each bar graph represents the mean of three independent biological replicates, and the error bar indicates the standard error. Xoo, *Xanthomonas oryzae* pv. *oryzae*; ns, not significant.

(Fig. 7). The expression of *SWEET11* was also induced in IRBB13 and IRBB21 inoculated with four XooE6-positive *Xoo* isolates ranging between 39-69 folds and 58-68 folds, whereas the XooE6-negative isolate Xoo16PK001 induced only 6 and 5 folds, respectively (Fig. 7). In contrast, the expression of *SWEET14* was suppressed by all four XooE6-positive *Xoo* isolates, but slightly induced by the XooE6-negative isolate Xoo16PK001 (less than 4 folds), which significantly activated the expression of *SWEET14* in IRBB13 and IRBB21, approximately 92 and 46 folds, respectively (Fig. 7). Unlike IRBB5, the expression of *SWEET14* was slightly induced, but not significantly, by all four XooE6-positive *Xoo* isolates in both IRBB13 and IRBB21. Noticeably, the expression of *SWEET11* was slightly induced when inoculated with water in either IRBB5, IRBB13, or IRBB21.

Discussion

The SNP marker XooE6 specific to IRBB5-compatible *Xoo*. The comparative nucleotide sequences between the IRBB5-compatible *Xoo* isolate Xoo16PK002 (4,923,613 bp) and the IRBB13-compatible *Xoo* isolate Xoo16PK001 (4,871,124 bp), exhibiting a high sequence identity (99.75%), suggested that they were lowly diverged in nucleotide sequences. However, there were over 8,000 SNPs throughout these two genomes, but due to the prevalence of distant SNPs, only eight SNP regions were selected for developing the PCR-based markers to distinguish these two Xoo isolates, as they provided PCR product sizes ranging between approximately 300 and 1,200 bp. One out of the eight designed SNP markers, designated as XooE6, could provide a specific PCR product in Xoo16PK002 but not Xoo16PK001. *Xoo* strain SK2-3, previously reported as an *xa5*-mediated resistance breaking *Xoo* strain (Carpenter et al., 2020), was also positive for the XooE6 marker. Rice variety IRBB5 also carries the BB resistance gene *xa5*; therefore, Xoo16PK002 could be the same strain as SK2-3. Other two *Xoo* isolates, Xoo20PT010 and Xoo20UT002, were also positive for the SNP marker XooE6. The pathogenicity test for these four XooE6-positive *Xoo* isolates exhibited similar virulence patterns on rice varieties IRBB5

and IRBB13, which were compatible and incompatible, respectively. In contrast, Xoo16PK001, representative of the XooE6-negative isolates, showed opposite virulence patterns: incompatible with IRBB5 but compatible with IRBB13. These results confirm that the SNP marker XooE6 is specific to the IRBB5-compatible *Xoo* strains; therefore, XooE6 can be applied to identify *Xoo* strains that break *xa5*-mediated resistance in rice. Our results also demonstrate that IRBB21, carrying *Xa21*, exhibited higher resistance than IRBB5 when plants were inoculated with four XooE6-positive *Xoo* isolates, and showed higher resistance than IRBB13 when plants were inoculated with the XooE6-negative Xoo16PK001. This confirms the broad-spectrum resistance of *Xa21* to Thai *Xoo* isolates as well as other strains previously reported (Win et al., 2012).

Responses of *SWEET11* and *SWEET14* to XooE6-positive and -negative *Xoo* isolates. Previous publications have reported that *Xoo* strains are well known to target BB susceptibility genes in rice, particularly *SWEET11* and *SWEET14*, causing a strong virulence effect of BB symptoms (Oliva et al., 2019). These *SWEET* genes are classified as a class of passive sugar transporters, playing a crucial role in the transport of glucose or sucrose into or out of a cell along their concentration gradients (Eom et al., 2015).

Here, *SWEET11* in rice variety IRBB5 showed the highest expression at 7 DAI with four XooE6-positive *Xoo* isolates, compared to its expression levels in IRBB13 and IRBB21, and this difference is statistically significant. This finding suggests that upregulation of *SWEET11* in IRBB5 was specifically activated by XooE6-positive *Xoo* isolates. One possible explanation is that XooE6-positive *Xoo* produced the TALE protein, which specifically and directly bound to the EBE target located in the promoters of *SWEET11* in IRBB5, leading to the activation of gene transcription. In agreement with previous findings, the Tal1c effector in the *Xoo* strain SK2-3 identified recently was predicted to target the EBE of *SWEET11*, subsequently inducing a strong expression of *SWEET11* in rice variety IRBB5 higher than in IR24, carrying no BB resistance gene (Carpenter et al., 2020). Similarly, *SWEET11* expression in rice 'Kitaake' was up-regulated after inoculation with *Xoo* strain PXO99A harboring PthXo1 (Kim et al., 2019; Yang and White, 2004). The upregulation of *SWEET11* caused a significant increase in sucrose concentration in the rice cells, enabling the proliferation of pathogenic *Xoo* cells, which are responsible for the susceptibility of rice to BB disease.

In contrast, the expression of *SWEET11* in IRBB13 was lower than in IRBB5 after infection of *xa5*-mediated resistance breaking *Xoo*. These might be explained by the mutated EBE in the promoter region of *SWEET11* (known as *xa13*) in IRBB13, which prevented its binding with TALE-PthXo1 from *Xoo* strains PXO99A (Chu et al., 2006) and SK2-3 (Carpenter et al., 2020).

Furthermore, the XooE6-negative Xoo16PK001 isolate significantly influenced a more considerable increase in *SWEET14* expression in rice varieties IRBB13 and IRBB21 compared to IRBB5. The reason for this could be that IRBB13 and IRBB21, which carry the dominant *Xa5* gene, also harbor the basal TF gamma subunit TFIIAγ1 protein. This protein exhibits a stronger binding affinity with various TALE proteins (such as AvrXa7, PthXo3, TalC or Tal5, produced in African and most Asian *Xoo* strains) than the recessive *xa5* gene (present in IRBB5, carrying TFIIAγ5). This interaction complex of the TALE and TFIIAγ1 subsequently results in a greater increase in *SWEET14* expression compared to the interaction of TALE and TFIIAγ5 (Carpenter et al., 2020; Ma et al., 2018). In contrast, the expression of *SWEET14* showed a trend towards a decrease in all studied rice varieties after infection with the XooE6-positive *Xoo* isolates, whereas its expression slightly increased when challenged with the XooE6-negative Xoo16PK001 isolate. However, these expression levels were not statistically significant.

In this study, the SNP marker XooE6 displayed high specificity in detecting IRBB5-compatible (*xa5*-mediated resistance breaking) *Xoo* isolates, which are widespread in several provinces of Lower Northern Thailand. Consequently, this marker should be further utilized for ongoing development into a practical testing kit for the specific detection of *xa5*-mediated resistance breaking *Xoo* isolates in fields, facilitating *Xoo* surveillance and long-term BB disease management. Additionally, this knowledge could aid in the selection or improvement of suitable rice cultivars for planting in areas affected by outbreaks of *xa5*-mediated resistance breaking *Xoo* isolates.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

This work was supported by the Program Management Unit for Human Resources & Institutional Development, Research and Innovation (PMU-B), Thailand [grant number B05F640157]; Naresuan University [grant num-

ber R2566B027]; Naresuan University [grant number R2565B003]; and the Thailand International Cooperation Agency (TICA) scholarship.

References

Carpenter, S. C. D., Mishra, P., Ghoshal, C., Dash, P. K., Wang, L., Midha, S., Laha, G. S., Lore, J. S., Kositratana, W., Singh, N. K., Singh, K., Patil, P. B., Oliva, R., Patarapuwadol, S., Bogdanove, A. J. and Rai, R. 2020. An *xa5* resistance gene-breaking Indian strain of the rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* is nearly identical to a Thai strain. *Front Microbiol.* 11:579504.

Chen, L.-Q., Hou, B.-H., Lalonde, S., Takanaga, H., Hartung, M. L., Qu, X.-Q., Guo, W.-J., Kim, J.-G., Underwood, W., Chaudhuri, B., Chermak, D., Antony, G., White, F. F., Somerville, S. C., Mudgett, M. B. and Frommer, W. B. 2010. Sugar transporters for intercellular exchange and nutrition of pathogens. *Nature* 468:527-532.

Chu, Z., Yuan, M., Yao, J., Ge, X., Yuan, B., Xu, C., Li, X., Fu, B., Li, Z., Bennetzen, J. L., Zhang, Q. and Wang, S. 2006. Promoter mutations of an essential gene for pollen development result in disease resistance in rice. *Genes Dev.* 20:1250-1255.

Doucouré, H., Pérez-Quintero, A. L., Reshetnyak, G., Tekete, C., Auguy, F., Thomas, E., Koebnik, R., Szurek, B., Koita, O., Verdier, V. and Cunnac, S. 2018. Functional and genome sequence-driven characterization of *tal* effector gene repertoires reveals novel variants with altered specificities in closely related Malian *Xanthomonas oryzae* pv. *oryzae* strains. *Front. Microbiol.* 9:1657.

Eom, J.-S., Chen, L.-Q., Sosso, D., Julius, B. T., Lin, I. W., Qu, X.-Q., Braun, D. M. and Frommer, W. B. 2015. *SWEETs*, transporters for intracellular and intercellular sugar translocation. *Curr. Opin. Plant Biol.* 25:53-62.

Erkes, A., Reschke, M., Boch, J. and Grau, J. 2017. Evolution of transcription activator-like effectors in *Xanthomonas oryzae. Genome Biol. Evol.* 9:1599-1615.

Garcia-Ruiz, H., Szurek, B. and Van den Ackerveken, G. 2021. Stop helping pathogens: engineering plant susceptibility genes for durable resistance. *Curr. Opin. Biotechnol.* 70:187-195.

Huang, S., Antony, G., Li, T., Liu, B., Obasa, K., Yang, B. and White, F. F. 2016. The broadly effective recessive resistance gene *xa5* of rice is a virulence effector-dependent quantitative trait for bacterial blight. *Plant J.* 86:186-194.

Kim, Y.-A., Moon, H. and Park, C.-J. 2019. CRISPR/Cas9-targeted mutagenesis of *Os8N3* in rice to confer resistance to *Xanthomonas oryzae* pv. *oryzae*. *Rice* 12:67.

Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25:402-408.

Ma, W., Zou, L., Zhiyuan, J. I., Xiameng, X., Zhengyin, X., Yang, Y., Alfano, J. R. and Chen, G. 2018. *Xanthomonas oryzae* pv. *oryzae* TALE proteins recruit OsTFIIAγ1 to compensate for the absence of OsTFIIAγ5 in bacterial blight in rice. *Mol. Plant Pathol.* 19:2248-2262.

Oliva, R., Ji, C., Atienza-Grande, G., Huguet-Tapia, J. C., Perez-Quintero, A., Li, T., Eom, J.-S., Li, C., Nguyen, H., Liu, B., Auguy, F., Sciallano, C., Luu, V. T., Dossa, G. S., Cunnac, S., Schmidt, S. M., Slamet-Loedin, I. H., Vera Cruz, C., Szurek, B., Frommer, W. B., White, F. F. and Yang, B. 2019. Broad-spectrum resistance to bacterial blight in rice using genome editing. *Nat. Biotechnol.* 37:1344-1350.

Sagun, C. M., Grandmottet F., Suachaowna N., Sujipuli K. and Ratanasut K. 2020. Validation of suitable reference genes for normalization of quantitative reverse transcriptase- polymerase chain reaction in rice infected by *Xanthomonas oryzae* pv. *oryzae*. *Plant Gene* 21:100217.

Song, Z., Zheng, J., Zhao, Y., Yin, J., Zheng, D., Hu, H., Liu, H., Sun, M., Ruan, L. and Liu, F. 2023. Population genomics and pathotypic evaluation of the bacterial leaf blight pathogen of rice reveals rapid evolutionary dynamics of a plant pathogen. *Front. Cell Infect. Microbiol.* 13:1183416.

Verdier, V., Triplett, L. R., Hummel, A. W., Corral, R., Cernadas, R. A., Schmidt, C. L., Bogdanove, A. J. and Leach, J. E. 2012. Transcription activator-like (TAL) effectors targeting *OsSWEET* genes enhance virulence on diverse rice (*Oryza sativa*) varieties when expressed individually in a TAL effector-deficient strain of *Xanthomonas oryzae*. *New Phytol.* 196: 1197-1207.

White, F. F., Potnis, N., Jones, J. B. and Koebnik, R. 2009. The type III effectors of *Xanthomonas. Mol. Plant Pathol.* 10:749-766.

Win, K. M., Korinsak, S., Jantaboon, J., Siangliw, M., Lanceras-Siangliw, J., Sirithunya, P., Vanavichit, A., Pantuwan, G., Jongdee, B., Sidhiwong, N. and Toojinda, T. 2012. Breeding the Thai jasmine rice variety KDML105 for non-age-related broad-spectrum resistance to bacterial blight disease based on combined marker-assisted and phenotypic selection. *Field Crops Res.* 137:186-194.

Wongkhamchan, A., Chankaew, S., Monkham, T., Saksirirat, W. and Sanitchon, J. 2018. Broad resistance of RD6 introgression lines with *xa5* gene from IR62266 rice variety to bacterial leaf blight disease for rice production in Northeastern Thailand. *Agric. Nat. Resour*. 52:241-245.

Yang, B. and White, F. F. 2004. Diverse members of the AvrBs3/PthA family of type III effectors are major virulence determinants in bacterial blight disease of rice. *Mol. Plant-Microbe Interact.* 17:1192-1200.

Zhou, J., Peng, Z., Long, J., Sosso, D., Liu, B., Eom, J.-S., Huang, S., Liu, S., Vera Cruz, C., Frommer, W. B., White, F. F. and Yang, B. 2015. Gene targeting by the TAL effector PthXo2 reveals cryptic resistance gene for bacterial blight of rice. *Plant J.* 82:632-643.