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Original Article

Searching for and analysis of bacterial blight resistance genes from Thailand rice germplasm

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ABSTRACT

In Thailand, rice (*Oryza sativa* L.) is the main food and agricultural product for export. However, the production of rice has faced many problems and bacterial blight disease is one of the problems in rice growing. Bacterial blight resistance *Xa4*, *Xa7*, *xa5* and *xa13* genes were discovered in 155 Thai rice cultivars using polymerase chain reaction-based gene-linked and gene-specific markers and the specific allele was compared with susceptible rice cultivars and with rice carrying each resistance gene. The results showed the same alleles as in the *Xa4* resistance gene (89.68%), followed by the *Xa7* resistance gene (11.61%), while the same alleles as *xa5* and *xa13* resistance genes were not found in the Thai rice germplasm studied. A new allele of *Xa7* was found and part of the DNA sequence was analyzed and compared to alleles obtained from resistant and susceptible rice lines. Sixteen cultivars carried the same alleles as the *Xa4* and *Xa7* resistant alleles. Phenotyping of 12 rice cultivars was performed using the leaf-clipping method and inoculation with three isolates of *Xoo* (TB0002, *Xoo*5 and *Xoo*6). Three rice cultivars (Kan Phu Daeng, Phuyai Li and RD23) had resistance to all three *Xoo* isolates. These cultivars will be useful as genetic sources in rice breeding programs in the future.

Introduction

Rice (*Oryza sativa* L.) is an important food grain for more than half of the world's population and global rice production is estimated at 480 million tonnes per year (Sumithra et al., 2014). More than 90% of rice is grown in Asian countries, such as China, Japan, India, Pakistan, Vietnam and Thailand (Sumithra et al., 2012). In Thailand, rice is the most important agricultural product and has been a primary export product on the world market for a long time; in 2015, Thailand exported 9.8 million tonnes and was the world's second largest exporter of rice (Arunmas, 2016). However, the yield and quality of paddy rice is dependent on biotic and abiotic stresses and one of the problems is bacterial blight disease (Ullah et al., 2012).

Bacterial blight (BB) or bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae*, *Xoo*, (a Gram-negative bacteriam) is one of the most destructive diseases of rice and was first found in 1884 in Fukuoka province, Japan (Ou, 1985), while in Thailand it was first reported in Pathum Thani province in 1963 (Eamchit and Mew, 1982). BB disease has spread to many rice growing regions, especially in irrigated and rainfed lowland ecosystems (Mew, 1987). In general, BB damages about 20–30% of the rice product (Ou, 1985), but in severe cases it can cause yield losses up to 80% (Akhtar et al., 2004; Srinivasan and Gnanamanickam, 2005; Perumalsamy et al., 2010). However, the damage caused by BB disease depends on many factors including location, the stage of the crop, the environment (season and weather) and cultivar (Reddy and Reddy, 1989; Ullah et al., 2012). BB disease is controlled using chemicals, nitrogen management and host plant resistance. However, a resistant cultivar would be an effective, economical and environmentally safe mean of controlling this disease (Sidhu et al., 1978; Khush et al., 1989). Therefore, most researchers are interested in identifying a resistant cultivar and searching for available resistance genes against BB disease (Korinsak et al., 2009).

Currently, 40 genes which confer resistance to various *Xoo* strains have been designated in a series from *Xa1* to *Xa40* (Kim et al., 2015). The greatest number of resistance genes against BB disease have been identified in *O. sativa* L. ssp. *indica* rice and wild

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rice (*O. longistaminata*, *O. rufipogon*, *O. minuta* and *O. officinalis*), but some resistance genes have been identified from *O. sativa* L. ssp. *japonica* rice (Lee et al., 2003; Korinsak et al., 2009). Nine genes have been cloned—*Xa1*, *Xa3*/*Xa26*, *xa5*, *Xa10*, *xa13*, *Xa21*, *Xa23*, *xa25* and *Xa27* (Akhtar et al., 2004; Liu et al., 2011; Tian et al., 2014; Wang et al., 2015; Dilla-Ermita et al., 2017). A rice cultivar carrying many resistance genes can show a broader spectrum and higher level of resistance to pathogens than a cultivar with a single resistance gene as the latter may lead to the cultivar being susceptible to pathogen mutation (Huang et al., 1997; Singh et al., 2001; Jeung et al., 2006; Rajpurohit et al., 2010). Rice cultivars of Indonesia, India, China and the Philippines carrying a single resistance gene for defense against BB have become susceptible to the pathogen later (Huang et al., 1997). Consequently, pyramiding cultivars which have many resistance genes have been developed against BB disease (Singh et al., 2001; Rajpurohit et al., 2010).

Nowadays, a molecular marker tool is used to determine germplasm containing resistance genes and assists to develop resistant rice cultivars with single and multiple resistance genes (Blair and McCouch, 1997; Perumalsamy et al., 2010; Rajpurohit et al., 2010). Many researchers have reported the use of polymerase chain reaction (PCR) markers for searching for resistance genes in germplasm rice cultivars, such as the study of *Xa4* and *Xa7* resistance genes in Pakistani rice germplasm (Arif et al., 2008; Muhammad et al., 2015) and of *Xa4*, *xa5*, *Xa7* and *xa13* resistance genes in basmati rice (Ullah et al., 2012). The current searched for *Xa*-resistance genes such as *Xa4*, *xa5*, *Xa7* and *xa13* using PCR-based gene-linked and gene-specific markers in Thai local rice germplasm which was considered to be carrying high genetic value and could diversify the beneficial genes for a rice breeding program. The outcome of this study will be useful for research on rice and rice improvement programs.

Materials and methods

Plant materials and DNA extraction

The study used 155 rice samples, including Thai local rice or native landraces and improved rice cultivars from Rice Department, Thailand (Table 1), together with the comparison lines: near-isogenic lines carrying resistance genes—IRBB4 (*Xa4*), IRBB5 (*xa5*), IRBB7 (*Xa7*) and IRBB13 (*xa13*) and the susceptible line IR24. DNA samples were extracted from young leaves using the cetyltrimethylammonium bromide (CTAB) method modified from the protocol of Doyle and Doyle (1990). The quality of genomic DNA was analyzed in 1% agarose gel and the concentration was measured using spectrophotometry. Total genomic DNA samples were diluted to 100 ng/μL using sterilized distilled water and stored at 4 °C for PCR amplification.

Polymerase chain reaction amplification and analysis

Amplification of DNA fragments was carried out using specific primers of *Xa4* (Ma et al., 1999), *xa5* (Iyer and McCouch, 2007), *Xa7* (Porter et al., 2003) and *xa13* (forward primer; Chu et al., 2006 and reverse primer developed based on a sequence in GenBank DQ421394.1). The primer sequences are shown in Table 2. Each PCR amplification reaction was in a total volume of 25 μL containing 100 ng of genomic DNA, 1X PCR buffer, 0.2 mM of each of dATP, dCTP, dGTP and dTTP (Thermo Scientific; Waltham, MA, USA), 5 pmol of each primer, 2 mM $MgCl_2$ and 1 U of *Taq* DNA polymerase (Invitrogen; Waltham, MA, USA). The thermal cycle consisted of an initial denaturation of 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 46–60.5 °C for 45 s and 72 °C for 1 min and then a final elongation of 72 °C for 1 min. The PCR products were stored at 4 °C for analysis.

The PCR products of *Xa4* were analyzed in 6% denaturing polyacrylamide gel in 1X TBE buffer at 300 V 90 min, while the products of *Xa7* gene were analyzed in 1.5% agarose gel in 1X TBE buffer. The PCR products of *xa5* gene were analyzed in both 1.5% agarose gel and 6% non-denaturing polyacrylamide gel according to the single-strand conformational polymorphism (SSCP) technique (Orita et al., 1989) in 1X TBE buffer at 300 V 5 h. Products of *xa5* gene were also cut with *Smo*I (5′-C↓TYRA↑G-3′) (Thermo Scientific; Waltham, MA, USA) before analysis in 1.5% agarose gel. The PCR products of *xa13* were analyzed in 1.0% agarose gel and 6% non-denaturing polyacrylamide. The agarose gel was stained with ethidium bromide, while polyacrylamide gel was stained with silver nitrate (Caetano-Anolles, 1997). The amplified fragments/genotypes of all samples were compared with the near-isogenic line (IRBB) and a susceptible line (IR24). The amplified products were purified using a Favorprep GEL/PCR Purification Mini Kit (Favorgen; Ping-Tung, Taiwan) and sent to 1st BASE, (Seri Kembangan, Selangor, Malaysia) for sequencing. The obtained sequences were analyzed using BLAST (The Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/cgi-bin/blast), Clustal Omega (http://www.ebi.ac.uk) and ExPaSy (http://web.expasy.org/translate/).

Phenotyping

Xanthomonas oryzae pv. *oryzae* (*Xoo*) isolates TB0002, *Xoo*5 and *Xoo*6, provided by the Department of Plant Pathology, Faculty of Agriculture, (Kasetsart University, Bangkok, Thailand) were used for testing rice against BB. Each isolate was grown in yeast extract-dextrose-$CaCO_3$ medium (Schaad, 1988) for 72 h at 25–30 °C. The bacterial cells were suspended in sterile water and adjusted to 1×10^8 colony forming units (CFU)/mL (optical density = 0.2 at 600 nm). Plants were inoculated using the leaf-clipping method (Jennings et al., 1979) at 30–45 d after planting and were scored at 14 d after inoculation. The reaction of resistance was expressed in lesion length (resistant: less than 3 cm, moderately resistant: 3–5 cm, susceptible: more than 5 cm), following the protocol of Suh et al. (2009).

Results and discussion

Thailand has a long history of rice production, leading to occurrence of many native landraces with high genetic variation (Ariyatanakatawong et al., 2014). The variation could diversify the gene pool for rice breeding programs. Conventional breeding is limited because it requires time and depends on environmental changes, while marker-assisted breeding is a highly efficient and precise strategy for targeted improvement of elite varieties (Raman et al., 2014). The current study provided molecular characterization in the Thai local rice germplasm for four resistance genes—*Xa4*, *xa5*, *Xa7* and *xa13* (Table 1).

Genotyping

One hundred fifty-five Thai rice samples were evaluated to resistant and susceptible status of BB resistance genes; including *Xa4*, *xa5*, *Xa7* and *xa13* using PCR-based gene-linked and gene-specific markers. The near-isogenic line carrying BB resistance genes—IRBB4 (*Xa4*), IRBB5 (*xa5*), IRBB7 (*Xa7*) and IRBB13 (*xa13*), as well as IR24 (no resistance gene)—were used to compare the difference between resistant and susceptible status.

The *Xa4* gene-linked SSR marker was used to analyze for resistant or susceptible alleles in the 155 rice samples using *Xa4*-primers from Ma et al. (1999). The *Xa4* gene-linked marker was located on chromosome 11 and was mapped between RZ536 and G2132b (Li et al., 1999). After electrophoresis in 6% denaturing polyacrylamide gel, 2

Table 1
Rice samples, origin and allele of resistance genes.

Number	Name	Type	Source	*Xa4*	*Xa7*	*xa5*	*xa13*
1.	Khao Malet Yao	Native landrace	Central, Thailand	R	N	S	S
2.	Khao Setthi	Native landrace	Central, Thailand	R	N	S	S
3.	Khao Hom	Native landrace	Central, Thailand	R	S	S	S
4.	Kot Fom	Native landrace	Central, Thailand	R	S	S	S
5.	Bai Bua	Native landrace	Central, Thailand	R	N	S	S
6.	Wang Pao	Native landrace	Central, Thailand	R	N	S	S
7.	Chet Sip Sam Won	Native landrace	Central, Thailand	R	N	S	S
8.	Khao Daeng	Native landrace	Central, Thailand	S	N	S	S
9.	Khao Kan Tang	Native landrace	Central, Thailand	R	N	S	S
10.	Sa Kae Kang	Native landrace	Central, Thailand	R	R	S	S
11.	Bang Na	Native landrace	Central, Thailand	R	N	S	S
12.	Leuang Roi-et	Native landrace	Central, Thailand	R	N	S	S
13.	Phuang Ma Lai	Native landrace	Central, Thailand	R	R	S	S
14.	Niaw Kham Noi	Native landrace	Central, Thailand	R	N	S	S
15.	Leuang Khai La	Native landrace	Central, Thailand	R	N	S	S
16.	Leuang I Duan	Native landrace	Central, Thailand	R	N	S	S
17.	Kaen Taeng	Native landrace	Central, Thailand	R	R	S	S
18.	Lot Chong	Native landrace	Central, Thailand	R	N	S	S
19.	Leuang Ra Ngae	Native landrace	Central, Thailand	R	N	S	S
20.	Leuang La	Native landrace	Central, Thailand	R	N	S	S
21.	Khao Luang Nak	Native landrace	Central, Thailand	R	N	S	S
22.	Kan Phu Daeng	Native landrace	Central, Thailand	R	N	S	S
23.	Leuang Bang Khem	Native landrace	Central, Thailand	R	N	S	S
24.	Leuang Kum	Native landrace	Central, Thailand	R	N	S	S
25.	Ka Duk Chang	Native landrace	Central, Thailand	R	N	S	S
26.	Leuang Pa Kim	Native landrace	Central, Thailand	R	R	S	S
27.	Khao Mali	Native landrace	Central, Thailand	R	N	S	S
28.	Khao Thong Suk	Native landrace	Central, Thailand	R	N	S	S
29.	Ko Diao Bao	Native landrace	Central, Thailand	R	N	S	S
30.	Nim Nuan	Native landrace	Central, Thailand	R	N	S	S
31.	Ta Suan	Native landrace	Central, Thailand	R	N	S	S
32.	Lueang Phra	Native landrace	Central, Thailand	R	N	S	S
33.	Khao Akat	Native landrace	Central, Thailand	R	N	S	S
34.	Khao Phak Chi	Native landrace	Central, Thailand	R	N	S	S
35.	Lueang Kon Chut	Native landrace	Central, Thailand	R	S	S	S
36.	Niaw Thurian	Native landrace	Central, Thailand	R	N	S	S
37.	Khao Rachini	Native landrace	Central, Thailand	S	N	S	S
38.	Hom Chiang Mai	Native landrace	Central, Thailand	R	S	S	S
39.	Phuang Hang Mu	Native landrace	Central, Thailand	R	N	S	S
40.	Lueang Kwat Thung	Native landrace	Central, Thailand	R	N	S	S
41.	Lueang I Thong	Native landrace	Central, Thailand	R	R	S	S
42.	Fong Khai	Native landrace	Central, Thailand	R	N	S	S
43.	Lon Khrok	Native landrace	Northeast, Thailand	R	N	S	S
44.	Ma Kheng	Native landrace	Northeast, Thailand	R	N	S	S
45.	Ton Mey Dam	Native landrace	Northeast, Thailand	R	R	S	S
46.	Pla Khaeng	Native landrace	Northeast, Thailand	R	N	S	S
47.	Pet Nam	Native landrace	Northeast, Thailand	R	S	S	S
48.	Hom Thung	Native landrace	Northeast, Thailand	R	S	S	S
49.	Kam Liao	Native landrace	Northeast, Thailand	R	S	S	S
50.	Pla Lot	Native landrace	Northeast, Thailand	R	N	S	S
51.	Nang Kham Phai	Native landrace	Northeast, Thailand	R	N	S	S
52.	Kathi	Native landrace	Northeast, Thailand	R	S	S	S
53.	Dok Jan	Native landrace	Northeast, Thailand	R	N	S	S
54.	Kam Phai	Native landrace	Northeast, Thailand	R	N	S	S
55.	Chek Choei Bao	Native landrace	Northeast, Thailand	R	N	S	S
56.	Ma Bang	Native landrace	Northeast, Thailand	R	N	S	S
57.	Niang Kha Mao Muang Khem	Native landrace	Northeast, Thailand	R	R	S	S
58.	Som Daeng	Native landrace	Northeast, Thailand	R	N	S	S
59.	Chek Kradot	Native landrace	Northeast, Thailand	R	N	S	S
60.	Mali	Native landrace	Northeast, Thailand	R	S	S	S
61.	Khao Ruam	Native landrace	Northeast, Thailand	R	R	S	S
62.	Khao Phama	Native landrace	Northeast, Thailand	R	N	S	S
63.	Ma Ue	Native landrace	Northeast, Thailand	S	S	S	S
64.	Dok Kha	Native landrace	Northeast, Thailand	R	S	S	S
65.	Khiao Hang Nak	Native landrace	Northeast, Thailand	R	N	S	S
66.	I Soi	Native landrace	Northeast, Thailand	R	R	S	S
67.	Phuyai Li	Native landrace	Northeast, Thailand	R	N	S	S
68.	Pong Aeo	Native landrace	Northeast, Thailand	R	S	S	S
69.	Khao Ku	Native landrace	Northeast, Thailand	R	R	S	S
70.	Makok	Native landrace	Northeast, Thailand	R	N	S	S
71.	I Khao Do	Native landrace	Northeast, Thailand	S	R	S	S
72.	Pla Lookkhrok	Native landrace	Northeast, Thailand	R	N	S	S
73.	Khao Ban Phot	Native landrace	Northeast, Thailand	R	N	S	S

(*continued on next page*)

Table 1 (*continued*)

Number	Name	Type	Source	*Xa4*	*Xa7*	*xa5*	*xa13*
74.	Lueang Super	Native landrace	Northeast, Thailand	R	S	S	S
75.	Lueang On	Native landrace	Northeast, Thailand	R	N	S	S
76.	Bak Nai	Native landrace	Northeast, Thailand	R	S	S	S
77.	Chao Sawoei	Native landrace	Northeast, Thailand	R	R	S	S
78.	Mak Yom	Native landrace	Northeast, Thailand	R	S	S	S
79.	Luea Ubon	Native landrace	Northeast, Thailand	R	N	S	S
80.	Do Hang Yi	Native landrace	Northeast, Thailand	R	S	S	S
81.	Khao Tut Ngon	Native landrace	Northeast, Thailand	R	N	S	S
82.	Hang Yi	Native landrace	Northeast, Thailand	R	N	S	S
83.	Khao Yai Kon Dam	Native landrace	Northeast, Thailand	R	N	S	S
84.	Leuat Pla Lai	Native landrace	Northeast, Thailand	R	S	S	S
85.	Phua Mia	Native landrace	Northeast, Thailand	R	R	S	S
86.	Mak Lueam	Native landrace	Northeast, Thailand	R	N	S	S
87.	Mi Don	Native landrace	Northern, Thailand	R	R	S	S
88.	Do Pralat	Native landrace	Northern, Thailand	R	N	S	S
89.	Chao Mum	Native landrace	Northern, Thailand	R	N	S	S
90.	Bi I Ko	Native landrace	Northern, Thailand	R	N	S	S
91.	Nong Hoi 1	Native landrace	Northern, Thailand	R	N	S	S
92.	Khiao Nok Kraling	Native landrace	Northern, Thailand	R	N	S	S
93.	Lon Yung	Native landrace	Northern, Thailand	R	N	S	S
94.	Khao Hao	Native landrace	Northern, Thailand	R	N	S	S
95.	Do Lueang Nam Phueng	Native landrace	Northern, Thailand	R	N	S	S
96.	Lueang	Native landrace	Northern, Thailand	R	R	S	S
97.	Do Lai	Native landrace	Northern, Thailand	R	S	S	S
98.	Pha Phueng	Native landrace	Northern, Thailand	R	S	S	S
99.	Lueang Phichit	Native landrace	Northern, Thailand	R	N	S	S
100.	Pi Rok	Native landrace	Northern, Thailand	R	S	S	S
101.	Tak Haeng	Native landrace	Northern, Thailand	R	N	S	S
102.	Do Kak Phet	Native landrace	Northern, Thailand	R	S	S	S
103.	Do O	Native landrace	Northern, Thailand	R	S	S	S
104.	Do Pong Aeo	Native landrace	Northern, Thailand	R	R	S	S
105.	Do Sadao	Native landrace	Northern, Thailand	R	S	S	S
106.	Chet Ruang	Native landrace	Northern, Thailand	R	N	S	S
107.	Pha Kong Luang	Native landrace	Northern, Thailand	R	N	S	S
108.	Buea Nam	Native landrace	Northern, Thailand	R	S	S	S
109.	Khao Kam	Native landrace	Northern, Thailand	R	N	S	S
110.	San Pa Tong Do	Native landrace	Northern, Thailand	R	S	S	S
111.	Pha Bong	Native landrace	Northern, Thailand	S	S	S	S
112.	Huang Node	Native landrace	Southern, Thailand	S	N	S	S
113.	Thai Dam	Native landrace	Southern, Thailand	R	S	S	S
114.	Hoi Sang	Native landrace	Southern, Thailand	S	N	S	S
115.	Look Non	Native landrace	Southern, Thailand	S	R	S	S
116.	Sai Buk Ya	Native landrace	Southern, Thailand	R	N	S	S
117.	Nuai Khuea	Native landrace	Southern, Thailand	S	N	S	S
118.	Yot Muang	Native landrace	Southern, Thailand	S	N	S	S
119.	Nang-Ek	Native landrace	Southern, Thailand	S	N	S	S
120.	Khao Tah Haeng 17	Improved cultivar	Rice Department	R	N	S	S
121.	Khao Pank Maw 148	Improved cultivar	Rice Department	R	S	S	S
122.	Chiang Phatthalung	Improved cultivar	Rice Department	R	N	S	S
123.	Chum Phae 60	Improved cultivar	Rice Department	R	N	S	S
124.	Nahng Mon S4	Improved cultivar	Rice Department	R	S	S	S
125.	Puang Rai 2	Improved cultivar	Rice Department	R	S	S	S
126.	Phatthalung 60	Improved cultivar	Rice Department	S	S	S	S
127.	Phitsanulok 60-1	Improved cultivar	Rice Department	R	S	S	S
128.	Look Daeng Pattani	Improved cultivar	Rice Department	R	N	S	S
129.	Hahng Yi 71	Improved cultivar	Rice Department	R	N	S	S
130.	Muey Nawny 60 M	Improved cultivar	Rice Department	R	R	S	S
131.	Niaw Ubon 1	Improved cultivar	Rice Department	R	S	S	S
132.	Jow Hawm Khlong Luang 1	Improved cultivar	Rice Department	R	S	S	S
133.	Chai Nai 1	Improved cultivar	Rice Department	R	N	S	S
134.	Pathum Thani 1	Improved cultivar	Rice Department	R	S	S	S
135.	Phatthalung	Improved cultivar	Rice Department	R	S	S	S
136.	Phitsanulok 60-2	Improved cultivar	Rice Department	R	S	S	S
137.	Suphan Buri 60	Improved cultivar	Rice Department	R	S	S	S
138.	Chek Choei	Improved cultivar	Rice Department	R	N	S	S
139.	Lueang Yai 148	Improved cultivar	Rice Department	R	S	S	S
140.	Leuang Pratew 123	Improved cultivar	Rice Department	R	N	S	S
141.	Surin 1	Improved cultivar	Rice Department	R	S	S	S
142.	Hahng Yi 17	Improved cultivar	Rice Department	R	N	S	S
143.	RD1	Improved cultivar	Rice Department	S	S	S	S
144.	RD3	Improved cultivar	Rice Department	S	S	S	S
145.	RD6	Improved cultivar	Rice Department	R	S	S	S
146.	RD9	Improved cultivar	Rice Department	R	S	S	S
147.	RD11	Improved cultivar	Rice Department	S	S	S	S
148.	RD13	Improved cultivar	Rice Department	S	N	S	S

Table 1 (*continued*)

Number	Name	Type	Source	*Xa4*	*Xa7*	*xa5*	*xa13*
149.	RD15	Improved cultivar	Rice Department	R	S	S	S
150.	RD17	Improved cultivar	Rice Department	R	N	S	S
151.	RD19	Improved cultivar	Rice Department	R	N	S	S
152.	RD21	Improved cultivar	Rice Department	R	S	S	S
153.	RD23	Improved cultivar	Rice Department	R	N	S	S
154.	RD25	Improved cultivar	Rice Department	R	S	S	S
155.	RD27	Improved cultivar	Rice Department	R	S	S	S

R = allele same as found in resistant line, S = allele same as found in susceptible line, N = new allele.

different DNA fragments (150 bp and 120 bp) were observed (Fig. 1A). One hundred thirty-nine rice samples (89.68%) and IRBB4 (resistant line with *Xa4*) showed a homologous 150 bp fragment (the size of the allele was the same as found in the *Xa4* resistant line), while 16 rice samples (10.32%) and IR24 (susceptible line) showed a homologous 120 bp fragment (the size of the allele was the same as found in the susceptible allele). The *Xa4* resistance gene has been widely used in many Asian rice breeding programs and found to have durable resistance in many commercial rice cultivars (Mew et al., 1992; Sun et al., 2003). Arif et al. (2008) found 49% *Xa4* resistance gene in Pakistan rice germplasm. Moreover, it was also found in cultivars from different countries, such as Manipur Black Rice1 from India, Rexoro from the USA and Sathra from Thailand (Muhammad et al., 2016).

The *Xa7* gene-linked marker was used to analyze for resistant or susceptible alleles in 155 rice samples using *Xa7*-primers from Porter et al. (2003). This gene was first studied in cultivar DV85 and subsequently developed to cultivar IRBB7 (Sidhu et al., 1978). The *Xa7* gene-linked sequence was located on chromosome 6 between GDSSR02 and RM20593 (Chen et al., 2008). The PCR products showed homology of three different sizes of approximately 294 bp, 1170 bp and 1400 bp in 1.5% agarose gel (Fig. 1B). Eighteen rice samples (11.61%) and IRBB7 (resistant line with *Xa7*) showed a 294 bp fragment size (the size of the allele was the same as found in the *Xa7* resistant line), while 49 rice samples (31.61%) and IR24 (susceptible line) showed a 1170 bp fragment size (the size of the allele was the same as found in the *Xa7* susceptible allele). Eighty-eight rice samples (56.78%) had a fragment size of approximately 1400 bp, which has not been reported before. A partial sequence of this new allele compared to resistant and susceptible alleles obtained from Porter et al. (2003) is shown in Fig. 2. Insertion of the DNA sequence in the middle of this allele produced a new allele with a longer size. Porter et al. (2003) reported two alleles of 294 bp and 1170 bp, consisting of similar sequences at both ends of DNA fragments with insertion/deletion in the middle of sequences. Similar to this finding, the *Xa7* resistance gene was found in some cultivars in Pakistan rice germplasm (Muhammad et al., 2015).

The *xa5* gene-specific marker was used to analyze for resistant or susceptible alleles in all 155 rice samples using the *xa5*-primers from Iyer and McCouch (2007). The PCR products showed homology of approximately 625 bp in all rice samples, IRBB5 (resistant line with *xa5*) and IR24 (susceptible line) in 1.0% agarose gel (Fig. 1C) and produced two DNA patterns in 6% non-denaturing polyacrylamide gel electrophoresis (SSCP technique). Pattern I consisted of all rice samples and IR24 and pattern II consisted of only IRBB5. The same result was observed when the PCR products were digested with restriction enzyme *Smo*I and digested products were analyzed in 1.5% agarose gel (Fig. 1C).

The *xa5* gene-specific PCR products of five rice cultivars—Kan Phu Daeng, RD6, Pi Rok, Hom Chiang Mai and Phuyai Li—were sent for sequencing and analyzed using the BLAST and Clustal Omega programs. A partial sequence of these PCR products covered part of the *TFIIA*γ gene (*xa5* disease resistance gene for bacterial blight, GenBank #AY643716), consisting of a partial sequence of 5′UTR (22 bp) – coding sequence of exon 1 (129 bp) – intron 1 (94 bp) – exon 2 (51 bp) – partial sequence of intron 2 (184 bp). The DNA sequences in this region of the five rice samples were very similar. There was only one base difference in the coding sequence of exon 1, causing one amino acid difference between Phuyai Li and the other four rice samples. Sixty amino acids translated from exon 1 and exon 2 of the five rice cultivars were compared with amino acids of *xa5* disease resistance gene. The amino acids at position 15 and position 39 in exon 1 were different among the five rice samples and the *xa5* gene sequence (Fig. 3). The amino acid at position 39 could be used to distinguish resistant (glutamic acid, E) and susceptible (valine, V) alleles, as was reported by Iyer and McCouch (2004).

The *xa13* gene-specific marker was used to analyze for resistant or susceptible alleles in 155 rice samples using the *xa13*-primer from Chu et al. (2006) and primer developed based on the *xa*13 gene sequence (GenBank #DQ421394) (Table 2). The PCR products showed homology of approximately 700 bp fragment size in IRBB13 (resistant line with *xa13*), while all rice samples and IR24 (susceptible line) showed homology of approximately 500 bp fragment size in 1.0% agarose gel (Fig. 1D). A similar result was observed in 6% non-denaturing polyacrylamide gel electrophoresis (SSCP technique). The partial DNA sequences at 5′UTR and the coding sequence of exon 1 of Kan Phu Daeng, Phuyai Li and RD23 were compared with sequences of IRBB13 (GenBank #DQ421394.1),

Table 2
Oligonucleotide primers and annealing temperature used in this study.

Gene	Primer sequence (5′ → 3′)	Total (bp)	Annealing temperature	Reference
Xa4 (gene linked)	F: ATCGATCGATCTTCACGAGG	20	55 °C	Ma et al. (1999)
	R: TCGTATAAAAGGCATTCGGG	20		
xa5 (gene specific)	F: CTGGAAGAAGCTCTTAATTT	20	46 °C	Iyer and McCouch (2007)
	R: GATTCCTTTAGCAAGGTGTG	20		
Xa7 (gene linked)	F: CGATCTTACTGGCTCTGCA ACTCTGT	26	55 °C	Porter et al. (2003)
	R: GCATGTCTGTGTCGATTCG TCCGTACGA	28		
xa13 (gene specific)	F: AGCTCCAGCTCTCCAAATG	19	60.5 °C	Chu et al. (2006) and developed based on GenBank DQ421394.1
	R: CATTGCTACTGGTGATGAAGG	21		

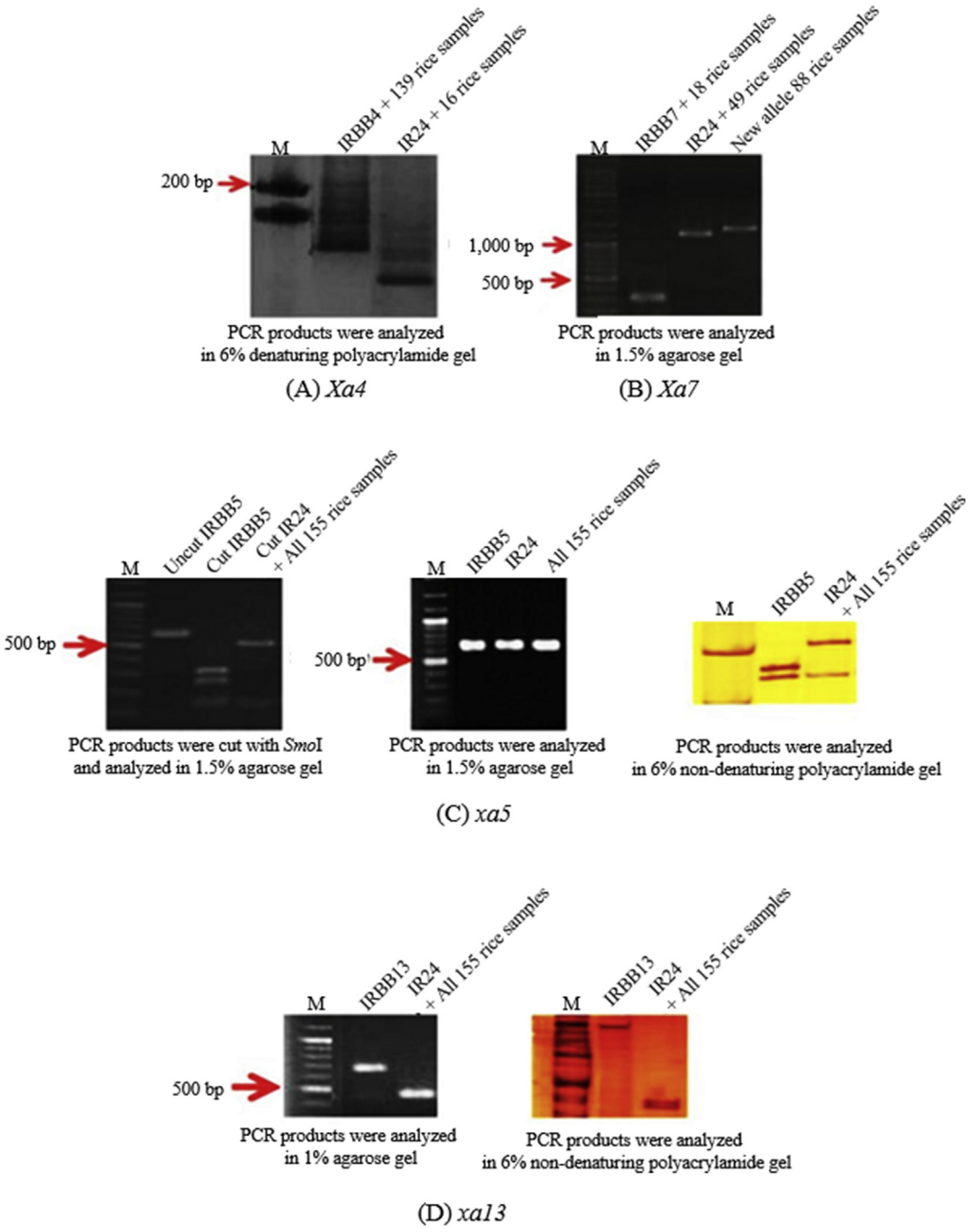

Fig. 1. Status of bacterial blight alleles *Xa4* (A), *Xa7* (B), *xa5* (C) and *xa13* (D) in 155 rice samples compared to resistant (IRBB) and susceptible (IR24) lines, where M = DNA ladder marker.

IR24 (GenBank #DQ421396.1) and IR64 (GenBank #DQ421395.1) as shown in Fig. 4. Both IR24 and IR64 carried the susceptible allele of *xa13* and had 98.89% sequence similarity to the alleles of Kan Phu Daeng, Phuyai Li and RD23. Only one base difference was found between Kan Phu Daeng and Phuyai Li/RD23 and gap of about 215 bp in the promoter region of this gene was found between the resistant and the susceptible alleles (Fig. 4). Based on these results, only the susceptible allele of *xa5* and *xa13* was found in rice samples. This result was supported by Cobelli et al. (2014) in that both resistance genes were not found in Thai local rice cultivars.

When considering the resistance alleles in the 155 rice samples, 125 samples had at least one allele the same as found in resistant cultivars; 123 samples carried only the *Xa4* allele, and two samples carried only the *Xa7* allele (Table 1). Sixteen samples carried a combination of the *Xa4* and *Xa7* resistance alleles, whereas 14 samples did not have any *Xa4*, *xa5*, *Xa7* or *xa13* resistance alleles. However, the rice samples carrying the new allele of the *Xa7* gene have not been proved to have resistance for BB disease at this point. Most Thai local rice samples in this study have one allele the same as found in resistant cultivars, especially the *Xa4* allele. The pyramided lines with *Xa4* and other BB resistance genes showed a wider spectrum and a higher level of resistance than the lines with a single resistance gene (Huang et al., 1997; Zhang et al., 1998). For example, the combination of *Xa4* + *xa5* + *Xa21* genes provided a

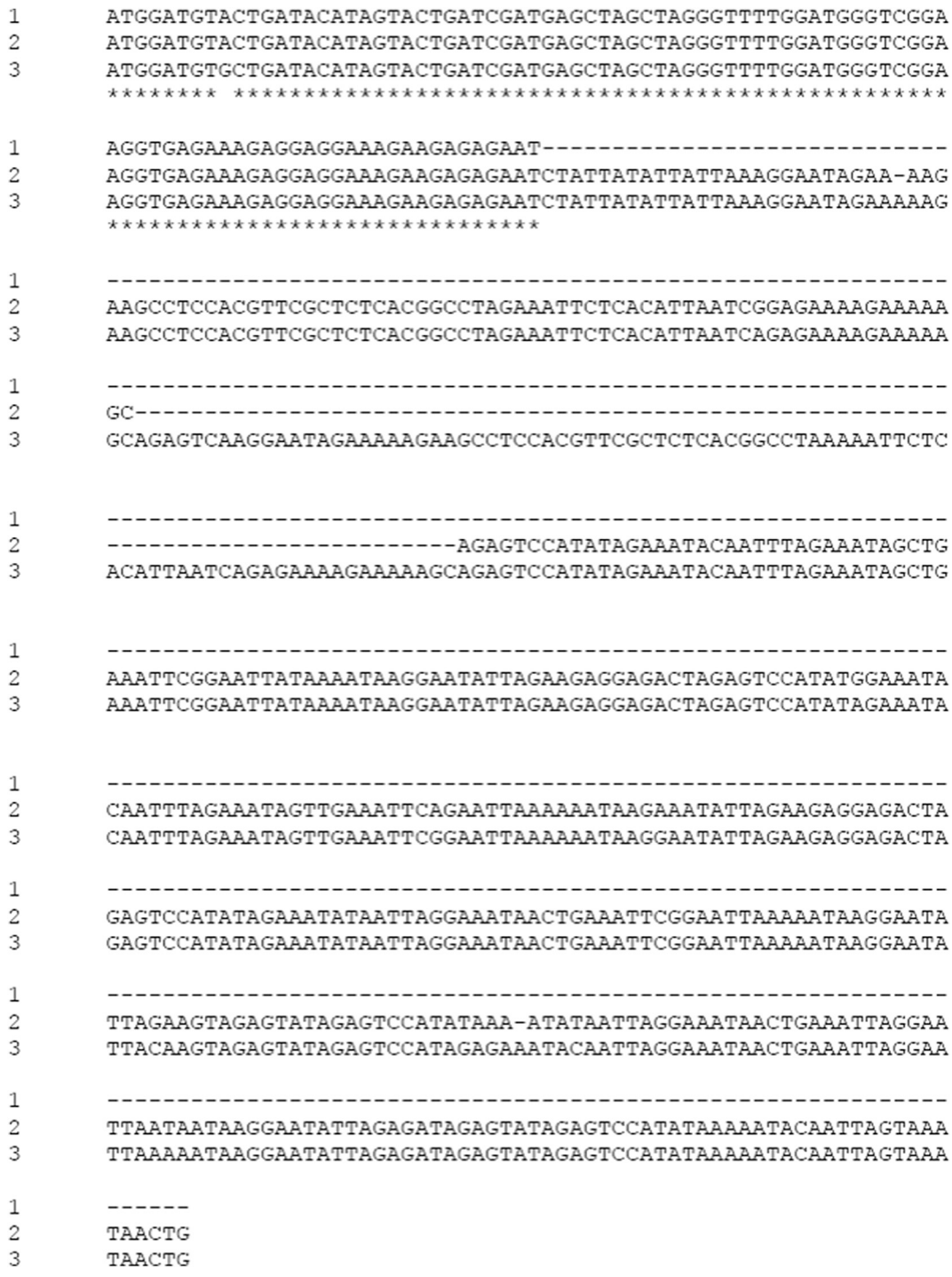

Fig. 2. Partial DNA sequence comparison of allele 1 (resistant allele, Porter et al., 2003), 2 (susceptible allele, Porter et al., 2003) and 3 (new allele) of *Xa7* gene linked marker, where the similar nucleotide of three sequences is shown as *.

higher resistance to *Xoo* than the individual resistance gene (Suh et al., 2013). However, in the current study, there were 16 Thai rice samples carrying two alleles the same as found in the resistant cultivars, *Xa4* and *Xa7*, in the germplasm used in this study. These rice cultivars would be a beneficial genetic resource for rice breeding program. According to Raman et al. (2014), the combination of the *Xa4* and *Xa7* resistance genes also conferred resistance to *Xoo* at high temperature.

Phenotyping

Twelve Thai rice samples, together with four near-isogenic resistant lines and one susceptible line, were inoculated with three isolates of *Xoo*, TB0002, *Xoo*5 and *Xoo*6, kindly provided by the Department of Plant Pathology, Faculty of Agriculture (Kasetsart University, Bangkok, Thailand) and scored for resistant status (Table 3, Fig. 5). Three rice samples—Kan Phu Daeng, Phuyai Li and

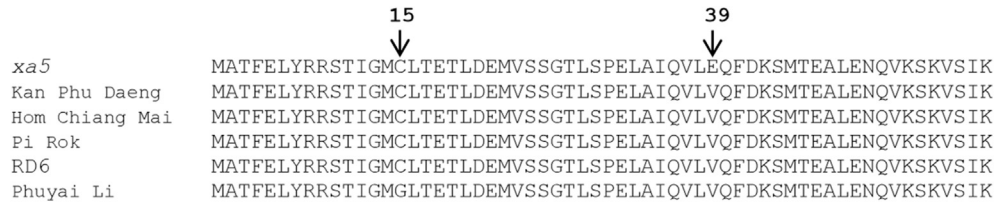

Fig. 3. Partial protein sequence comparison of *xa5* gene specific marker of Kan Phu Daeng, Hom Chiang Mai, Pi Rok, RD6 and Phuyai Li compared with partial protein sequence of TFIIAγ (*xa5* disease resistance gene for bacterial blight, AY643716), where the 15 and 39 text labels indicate positions of different amino acids among resistant and susceptible rice samples, with allelic difference shown at amino acid position 39.

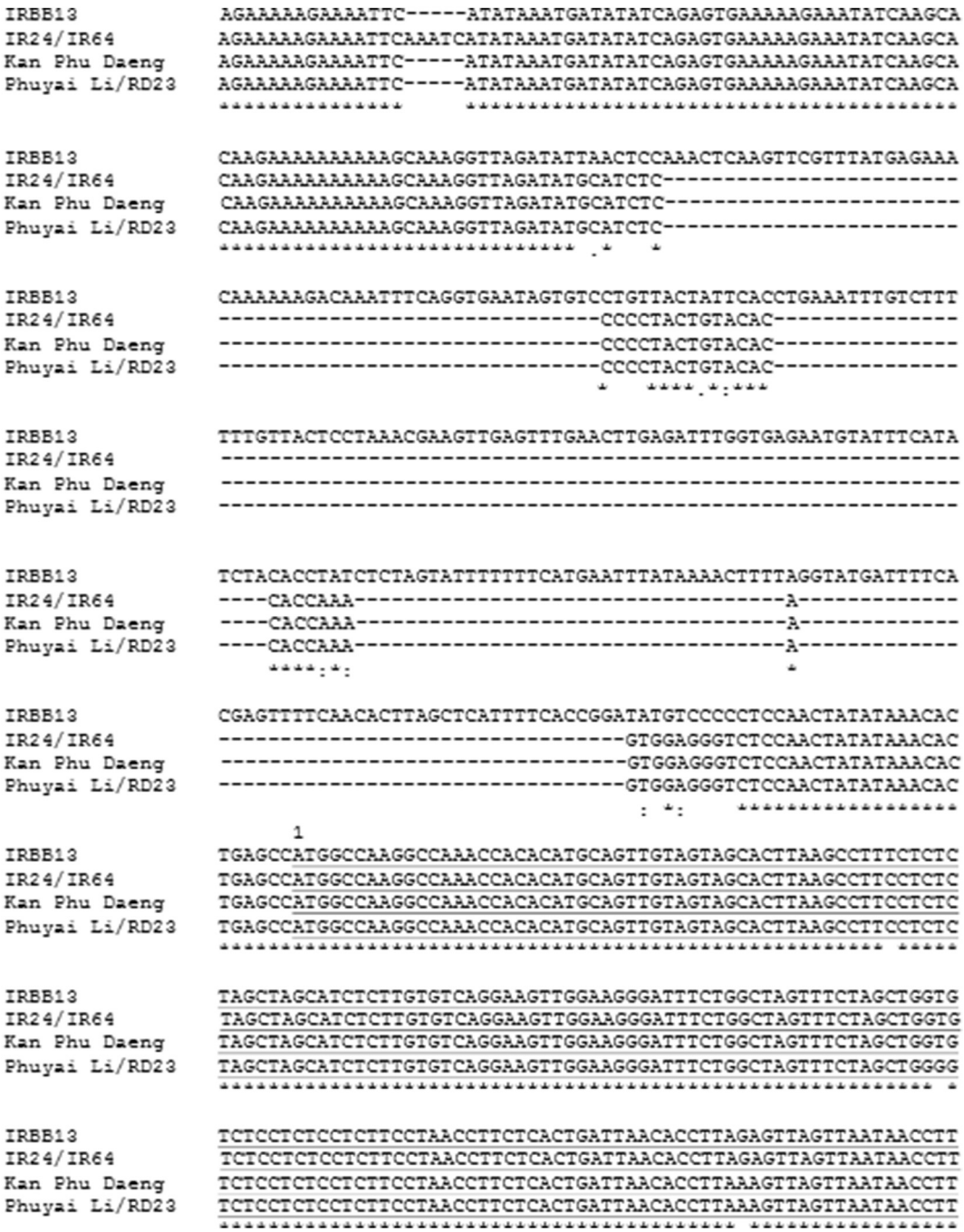

Fig. 4. Partial DNA sequences at 5′UTR and coding sequence of exon 1 of Kan Phu Daeng, Phuyai Li and RD23 compared with sequences of IRBB13 (GenBank #DQ421394.1), IR24 (GenBank #DQ421396.1) and IR64 (GenBank #DQ421395.1), where underlining indicates coding sequence, 1 indicates start codon and * indicates similar nucleotides among all samples.

Table 3
Reaction of 12 rice cultivars to three isolates of *Xanthomonas oryzae* pv. *oryzae* where bracketed numbers indicate average lesion length from three replicates.

Number	Name	Genotype[a]				Phenotype[b]		
		Xa4	*Xa7*	*xa5*	*xa13*	TB0002	*Xoo*5	*Xoo*6
22.	Kan Phu Daeng	R	N	S	S	(2.3) R	(2.5) R	(2.1) R
26.	Leuang Pa Kim	R	R	S	S	(4.5) MR	(2.5) R	(2.3) R
38.	Hom Chiang Mai	R	S	S	S	(6.1) S	(3.2) MR	(4.6) MR
67.	Phuyai Li	R	N	S	S	(2.9) R	(2.7) R	(2.6) R
80.	Do Hang Yi	R	S	S	S	(4.1) MR	(2.2) R	(1.3) R
98.	Pha Phueng	R	S	S	S	(5.3) S	(4.4) MR	(6.0) S
115.	Look non	S	R	S	S	(3.6) MR	(3.0) MR	(3.6) MR
145.	RD6	R	S	S	S	(7.5) S	(5.4) S	(4.0) MR
147.	RD11	S	S	S	S	(3.7) MR	(3.3) MR	(3.3) MR
148.	RD13	S	N	S	S	(5.5) S	(6.0) S	(3.0) MR
149.	RD15	R	S	S	S	(7.7) S	(4.0) MR	(4.2) MR
153.	RD23	R	N	S	S	(0.6) R	(2.0) R	(2.5) R
_	IRBB4	R	–	–	–	(1.6) R	(2.1) R	(1.3) R
_	IRBB5	–	–	R	–	(4.3) MR	(0.5) R	(2.8) R
_	IRBB7	–	R	–	–	(0.4) R	(0.5) R	(0.4)R
_	IRBB13	–	–	–	R	S	(4.0) MR	S
_	IR24	–	–	–	–	S	S	S

[a] Genotypes: R = allele same as found in resistant line; S = allele same as found in susceptible line; N = new allele.
[b] Phenotypes: R = resistant, < 3 cm; MR = moderately resistant, 3–5 cm; S = susceptible, >5 cm (Suh et al., 2009).

RD23—were resistant to all three isolates. These three rice cultivars carried an allele the same as was found in the resistant line of *Xa4* and the new allele of *Xa7*. Leuang Pa Kim carrying a combination allele the same as was found in the resistant line of *Xa4* and *Xa7* exhibited moderate resistance to TB0002 and resistance to *Xoo*5 and *Xoo*6. Five cultivars carrying only the allele the same as was found in the *Xa4* resistant line, showed moderate resistance to at least one isolate, but Do Hang Yi exhibited moderate resistance to TB0002 and resistance to *Xoo*5 and *Xoo*6. Look Non carrying only the allele that was the same as found in the *Xa7* resistant line showed moderate resistance to all *Xoo* isolates. RD13 carrying the new allele of the *Xa7* gene was susceptible to TB0002 and *Xoo*5 and moderately resistant to *Xoo*6. RD11 had no resistant allele of the four resistance genes, but showed moderate resistance to all *Xoo* isolates. Near-isogenic lines which have a single resistance gene showed resistance or moderate resistance to all stains, except IRBB13 which was susceptible to TB0002 and *Xoo*6, and showed moderate resistance to *Xoo*5.

In the current study, 12 rice samples showed resistance or moderate resistance to at least one *Xoo* isolate, although the samples didn't present alleles of the resistant line. The relationship between the resistance level and the genotype of the resistant allele could not be concluded explicitly because the same resistance allele or combination of resistance alleles showed different levels of

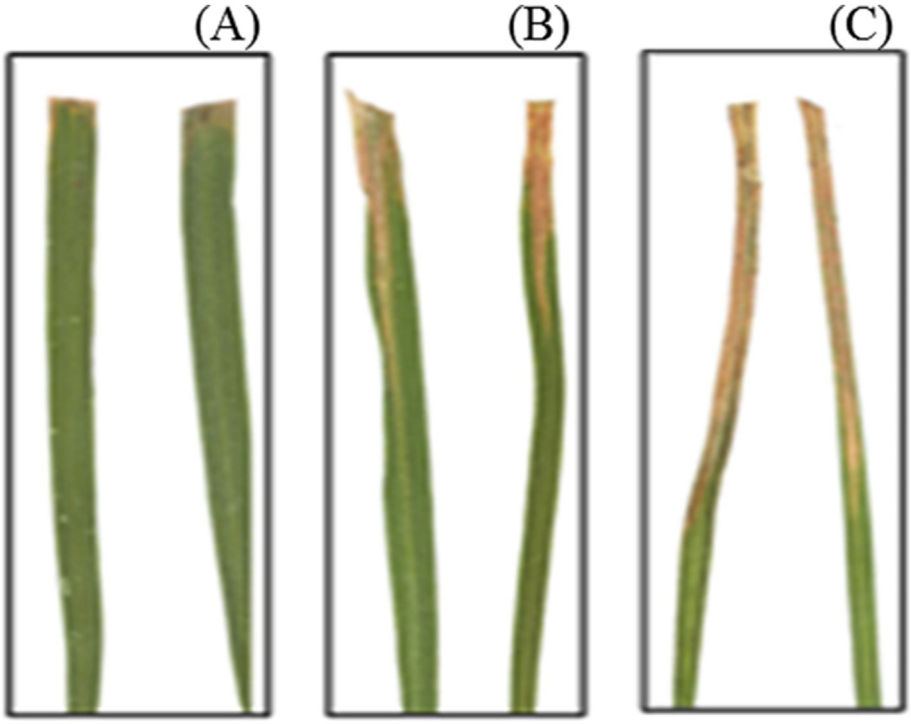

Fig. 5. Level of lesion on rice leaves indicating resistance status: (A) resistant, < 3 cm; (B) moderate resistance, 3–5 cm; (C) susceptible, > 5 cm (Suh et al., 2009).

resistance. Different *Xoo* isolates responded to specific resistance genes differently. However, rice carrying one resistance allele can exhibit resistance to *Xoo*, while a combination of resistance genes provides more effective resistance against the disease. In Vietnam, rice with a high resistance level has the *Xa4* + *Xa7* + *Xa21* combination of resistant genes (Du and Loan, 2007). An allele that was the same as found in the *Xa4* resistant line is found mostly in Thai local rice germplasm, while the allele that was the same as was found in the Xa7 resistant line was found in some rice samples. These two genes are the major resistance genes that have been incorporated into rice cultivars and have been used to develop new resistant varieties against BB disease (Perumalsamy et al., 2010). Suh et al. (2013) did transfer *Xa4, xa5* and *Xa21* resistance genes from Indica rice into an elite japonica rice cultivar using SSR marker-assisted breeding in backcross progenies. The pyramiding of the resistance genes *Xa4, xa5* and *Xa21* provided a higher resistance to *Xoo* than the introduction of the individual resistance genes. Zhang et al. (2001) improved a rice restorer line "Minghui63" through introgression of two resistance genes (*Xa7* + *Xa21*), resulting in pyramided double resistance lines and their hybrids with high level of resistance to BB. In the current study, IRBB5 showed resistance to all three isolates at the resistant and moderate resistance levels. According to Win et al. (2013), the improved aromatic Manawthukha rice lines (Myanmar rice) carrying triple and double resistance genes (*xa5Xa21xa33* or *xa5Xa21* or *xa5xa33* or *Xa21xa33*) had a higher resistance level and a wider resistance spectrum against Thai and Myanmar *Xoo* strains. These improved lines carrying *xa5* had higher resistance against Thai *Xoo* strains. Therefore, studying the combination of bacterial blight resistance genes is very important in order to provide information for the development of improved rice cultivar in the future.

In this study, alleles the same as four resistance alleles (*Xa4*, *xa5*, *Xa7* and *xa13*) in resistant lines were screened in 155 samples of Thai rice germplasm from different cultivars. The *Xa4* resistance allele was found in more rice samples than the other resistance alleles, while no *xa5* and *xa13* resistance alleles were found in any of the cultivars in this study. The combination of *Xa4* and *Xa7* alleles was found in 16 rice cultivars. Based on the phenotyping of 12 rice cultivars using the leaf-clipping method and inoculation with three isolates of *Xoo* (TB0002, *Xoo*5 and *Xoo*6), three rice cultivars (Kan Phu Daeng, Phuyai Li and RD23) were found to have resistance to all three *Xoo* isolates.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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