al.,2009; Vogt, 2010). PAL is also a key enzyme for biosynthesis of SA, a plant hormone required to initiate SAR in plants (Fig.1) (Lee et al., 1995; Mauch-Mani and Slusarenko, 1996; Coquoz et al., 1998; Achnine et al., 2004; Beckers and Spoel, 2006; Gruner et al., 2013; Roycewicz and Malamy et al., 2014)

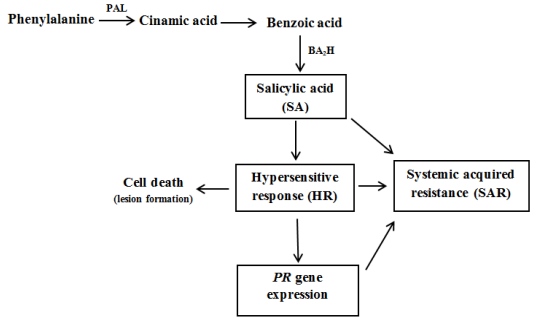

Figure 1 Schematic diagram of systemic acquired resistance (SAR) via a salicylic acid (SA)-dependent pathway (modified from Borowiak, 2007).

Objective of the study

In this study, we aimed to characterise expression of *Xa21* and defence-related genes in rice RD47 and IRBB21 used as female and male parental lines, respectively,in backcross breeding program for production of BB-resistance RD47, and their F_1 hybrid and F_2 progenies carrying heterozygous *Xa21* and null *Xa21* genotypes under wounding and *Xoo* infection conditions.

Materials and Methods

Plant materials and growth conditions

Rice cultivar RD47 and IRBB21 obtained from Phitsanulok Rice Research Center, were selected for their susceptibility and high resistance to *Xoo*, respectively. Rice plants were grown and maintained in the greenhouse at Naresuan University, Phitsanulok province, Thailand.

Breeding procedure and PCR marker

RD47 as a recurrent parent (recipient) was crossed with IRBB21 as a resistance donor (*Xa21* donor) to obtain F_1, and then F_1 was self-pollinated to obtain F_2.The F_1 and F_2 progenies were selected for plants carrying *Xa21* gene using PCR-based marker pTA248 closely linked to *Xa21*gene (Magar et al., 2014).

PCR was carried out according to the Phire plant direct PCR master mix (Thermo scientific, EU) using forward primer, 5'-AGACGCGGAAGGGTGG TTCCCGGA-3', and reverse primer, 5'-AGACGCG GTGTAATCGAAAGATGAAA-3'.

Xoo inoculation

Xoo used for inoculation was kindly provided by Phitsanulok rice research center, Phitsanulok, Thailand. Inoculum was prepared by streaking *Xoo* onto nutrient agar and incubating at 28°C for 24 h. A single colony was transferred into 1 ml of sterile distilled water and spread onto a nutrient agar plate. After 48 h, *Xoo* culture was harvested from a plate and mixed with 150 ml of sterile distilled water. This *Xoo* prep was used for inoculation by the clipping method (Kauffman et al., 1973). At the 6-leaf stage, rice leaves were cut at the tip by scissors with and without *Xoo* inoculation. The wounded and inoculated leaves were collected at different time points after treatments for RNA analysis.

Expression analysis of *Xa21* and defence-related genes by reverse transcriptase (RT)-PCR

Total RNA was extracted using total RNA extraction kit (RBC Bioscience, Taiwan), and then

treated with RQ1 RNase-Free DNase (Promega, USA) before reverse transcription (RT). The concentration of total RNA was measured using Qubit® fluorometer (Invitrogen, USA). First-strand cDNA was synthesized using ImProm-II™ Reverse Transcription System (Promega, USA). PCR was performed using MyTaq™ HS Mix (Bioline, USA). The specific primers were used to amplify four genes of interest; *Xa21* (114 bp) 5'-CAGAGTATGGCGTTGGGCT-3' and 5'-CGGGTCTGAATGTACTGTCA-3' (based on GenBank no. U37133), *OsPR1b* (90 bp), 5'-AGCTGGCCATTGCTTTGG-3' and 5'-CGTTGTGGAGCCTCACGTAGT-3', *OsPR10a* (86 bp) 5'-C GCCGCAAGTCATGTCCTA-3' and 5'-GCTTCGTCTCCGTCGAGTGT-3', *PAL* (141 bp) 5'-GCACA TCTTGGAGGGAAGCT-3' and 5'-GCGCGGATAA CCTCAATTTG-3', and *18s rRNA* internal standard (Jain et al., 2006).

Amplification reactions were carried out in a 20 µl volume mixture containing 12.5 µl of My Taq HS Mix, 0.2 µM of each primer, 1 µl of cDNA template, and 4.5 µl of RNase-Free ddH_2O. Template denaturation was conducted for 5 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s.

Results

Identification of *Xa21* genotypes using the pTA248 marker

RD47, IRBB21, F_1 and F_2 plants were inspected by the pTA248 co-dominant marker. The result shows three different patterns of the PCR products. A single 730-bp product exhibited in RD47 and F_2-1 plants indicating no *Xa21* gene (homozygous susceptible) whereas a single 925-bp product exhibited in IRBB21 indicating homozygous *Xa21* genotype (homozygous resistance). The PCR product patterns in F_1 and F_2-2 exhibited both 730-bp and 925-bp fragments indicating heterozygous *Xa21* genotype (heterozygous resistance) (Fig. 2).

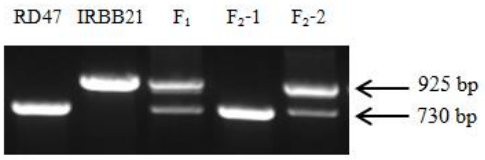

Figure 2 The *Xa21* genotype of RD47, IRBB21, F_1 and F_2 identified by the pTA248 marker. F_2-1 and F_2-2 carried null *Xa21* and heterozygous *Xa21* genotypes, respectively.

Effect of wounding and*Xoo*infection on expression of *Xa21*

Determination of *Xa21* expression in RD47 and IRBB21 plants given wounded and *Xoo* inoculated conditions revealed that the *Xa21* transcripts were detected only in IRBB21 plants even at 0 h post wounding and *Xoo* inoculation. However, the *Xa21* transcript levels in IRBB21 plants increased to the maximum level at 24 h post wounding and *Xoo* inoculation before decreasing at 48 h post wounding and *Xoo* inoculation (Fig. 3). The *Xa21* expression in wounded F_2 (derived from self-pollinated F_1RD47 x IRBB21) carrying null *Xa21* and heterozygous *Xa21* genotypes showed similar expression patterns to RD47 and IRBB21 parental plants, respectively (Fig.3). No *Xa21* transcript was detected in *Xoo* inoculated F_2 carrying null *Xa21*. In *Xoo* inoculated F_2 carrying heterozygous *Xa21* genotype, the

maximum level of the *Xa21* transcripts was detected at 48 h post inoculation (Fig. 3).

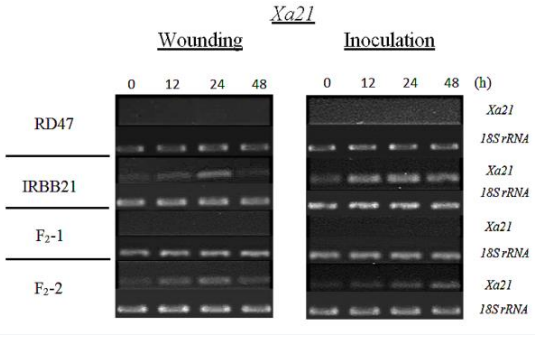

Figure 3 RT-PCR analysis of *Xa21* expression in rice RD47, IRBB21 and RD47 x IRBB21 F_2at 0, 12, 24 and 48 h post wounding and *Xoo* inoculation. F_2-1 and F_2-2 carried null *Xa21* and heterozygous *Xa21* genotypes, respectively. The 18S rRNA was used as the internal control.

Effect of wounding and *Xoo* infection on expression of *PAL*

PAL is the major gene involved in the SA-synthesis pathway. Examination of *PAL* expression in RD47, IRBB21 and F_2 carrying null *Xa21* and heterozygous *Xa21* genotypes which were wounded and *Xoo* inoculated revealed that no *PAL* transcripts were detected in RD47 and F_2 carrying null *Xa21* plants. The *PAL* transcript levels in IRBB21 and F_2 carrying heterozygous *Xa21* genotype rapidly increased to the maximum level at 12 h post wounding before decreasing after 24 h post wounding (Fig. 4). In IRBB21, *PAL* was highly expressed at 12 and 24 h post inoculation with *Xoo* before decreasing at 48 h whereas the *PAL* expression level in F_2 carrying heterozygous *Xa21* genotype was slightly high at 48 h post inoculation (Fig. 4).

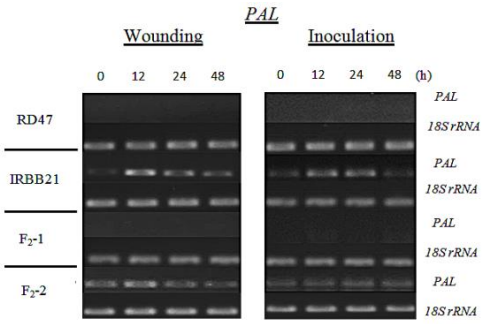

Figure 4 RT-PCR analysis of *PAL* expression in rice RD47, IRBB21, RD47 x IRBB21 F_2at 0, 12, 24 and 48 h post wounding and *Xoo* inoculation. F_2-1 and F_2-2 carried null *Xa21* and heterozygous *Xa21* genotypes, respectively. The 18S rRNA was used as the internal control.

Expression of defence-related genes in F_1 hybrid infected with *Xoo*

To study the interaction of defence genes and the *Xa21* gene in response to *Xoo*developmental disease resistance in the F_1 hybrid (RD47 x IRBB21), expression of *Xa21*, *PR1b*, *PR10a* and *PAL* was determined at 21 days post inoculation when the symptom of the BB disease was obviously noticed in RD47 but not in the F_1 plants. The result demonstrates that the transcript levels of *PR1b* and *PR10a* were increased in both *Xoo* infected RD47 and F_1 plants whereas *PAL* transcripts were increased only in *Xoo* infected F_1 plants. Meanwhile, the *Xa21* transcript levels were similar between wounded and *Xoo* infected F_1 plants (Fig. 5).

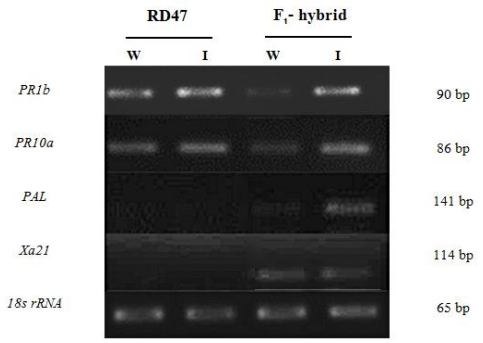

Figure 5 RT-PCR analysis of *Xa21, PR1b, PR10a and PAL* expression in rice F_1 hybrid RD47 x IRBB21 at 21 days post *Xoo* inoculation. The 18S rRNA was used as the internal control. W, wounding, I, inoculation.

Discussion

Identification of the *Xa21* genotypes in RD47, IRBB21 and F_1 plants by the pTA248 marker indicated that the parental lines, RD47 and IRBB21, were homozygous *Xa21* whereas F_1 was heterozygous. This marker was also used to identify the *Xa21* genotype of F_2 individuals. Expression of *Xa21* was correlated with the *Xa21* genotypes. The *Xa21* expression in IRBB21 and heterozygous F_2 plants was detected at 0 h post wounding and *Xoo* inoculation indicated that the *Xa21* gene was constitutively expressed and induced by wounding and *Xoo* inoculation. However, expression analysis of *Xa21* in wounded and *Xoo* infected F_1 plants suggested that the expression of *Xa21* transcripts was not correlated with expression of *Xa21*-mediated disease resistance. Century et al. (1999) proposed that the developmental regulation of *Xa21*-mediated disease resistance in rice is either controlled post-transcriptionally or by other factors.

The transcripts of *PR1b* and *PR10a* was expressed in both RD47 (no *Xa21*) and F_1 hybrid (with *Xa21*) inoculated with *Xoo*. Ponciano et al. (2007) suggested that the *PR1b* expression is important for mounting the resistance response to *Xoo* while the *PR10a* expression is not important. On the other hand, the *PAL* expression was triggered either by wounding or *Xoo* inoculation only in rice carrying the *Xa21* gene. Wound-induced *PAL* expression in rice was also demonstrated in the previous report that the expression level of *PAL* was significantly higher in resistant Kasalath rice than in the susceptible Wuyujing3 rice in response to small brown-planthopper feeding (Duan et al., 2014). Our results indicate that *Xa21*-mediated resistance in F_1 hybrid and their F_2 progenies carrying the *Xa21* gene would be *PAL*- and SA- dependent pathway.

Conclusions

We have shown that *Xa21* and *PAL*was constitutively expressed in rice carrying the *Xa21* gene. The *Xa21*-mediated resistance against *Xoo* isolated from Phisanulok, Thailand in F_1 hybrid (RD47 x IRBB21) would be *PAL*-, but not *PR1b* and *PR10a*, dependent pathway.

Acknowledgements

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