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Research article

Differential expression of *Xoo-induced kinase 1 (XIK1)*, a *Xanthomonas oryzae* pv. *oryzae* responsive gene, in bacterial blight-susceptible and *Xa21*-mediated resistant indica rice cultivars

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Abstract

Bacterial blight (BB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a costly disease in rice that threatens global rice production. The *Xa21* gene is a broad spectrum BB-resistance gene that is extensively used for improving BB resistance in rice. The *Xoo* responsive gene, *Xoo-induced kinase 1* (*XIK1*), recently characterized in japonica rice, was also found to be involved in Xa21-mediated resistance. The current study investigated the differential expression of *XIK1* in the BB-susceptible indica rice cultivar RD47 and its improved BB-resistant progenies BC_3F_3 (*Xa21*/*Xa21*) in various growth stages and during *Xoo* inoculation. The results showed that the expression of *XIK1* was development-dependent and induced earlier in BB-resistant progenies BC_3F_3 than in the susceptible cultivar RD47 after *Xoo* inoculation. However, the expression levels of *XIK1* substantially dropped after a peak of expression. Interestingly, the *XIK1* expression tended to increase again in the succeeding hours post-inoculation in BC_3F_3 but not in RD47. Similar development and induction patterns were also observed when the expression of *XIK1* and *Xa21* was analyzed in the same runs in the BB-resistant BC_3F_3 under different growth stages and during *Xoo* inoculation. The findings suggested that XIK1 may also be involved in the Xa21-mediated resistance pathway of indica rice.

Introduction

Most of the world's population depends on rice (*Oryza sativa*) as the primary food source and this requires large-scale-production volumes to meet the growing demand (IRRI, 2006). However, limiting factors such as insect pests and diseases tend to reduce the yield by 30–80% (Reissig et al., 1985; IRRI, 2018a). Among the major diseases of rice, bacterial blight (BB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the costliest as it reduces production by up to 70% (IRRI, 2018b). Its symptoms include a vascular wilt at the seedling stage, a leaf blight, and unfilled panicles in mature plants which result from the invasion of the vascular system by *Xoo* bacteria (Mew, 1987). At the molecular level, *Xoo* secretes transcription activator-like (TAL) effectors which invade and highjack the host cells by activating the transcription of genes that enhance plant susceptibility and support bacterial virulence (Boch and Bonas, 2010; Bogdanove et al., 2010; Römer et al., 2010).

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In plants, the ability to recognize both general elicitors and specific pathogens through gene-mediated resistance is essential to their defense mechanisms (Andersen et al., 2018). Hence, studying the variations in the expression of various genes provides a perspective of the mechanisms of plant responses to BB. To date, more than 30 BB resistance genes have been identified in *Oryza sativa* and its closely related species; among these, *Xa21* has probably been the most commonly used for rice variety improvement as it provides for high level and broad spectrum BB resistance (Song et al., 1995; Win et al., 2012). *Xa21* codes for a plasma membrane which recognizes the tyrosine-sulfated protein RaxX and triggers the Xa21-mediated immunity (Pruitt et al., 2015). It is known that this response involves Xa21-binding proteins (Wang et al., 2006; Chen et al., 2010; Park et al., 2017) as well as the direct interaction of a cleaved XA21 subunit with the WRKY62 transcription factor (Peng et al., 2008; Park et al., 2012; He et al., 2017). Nonetheless, the precise mechanisms of *Xa21* resistance have not yet been completely elucidated.

The current study centered on the recently discovered *Xoo-induced kinase 1* (*XIK1*, Loc_Os02g4790) which encodes a LRR-RLK protein and is hypothesized to act as a co-receptor of *Xa21* since it positively regulates Xa21-mediated BB resistance (Hu et al., 2015). Since *XIK1* was previously identified and conserved in the japonica rice cultivar Kitaake, the current study characterized the expression levels of this gene in the BB-susceptible cultivar RD47 and its improved BB resistant progenies BC_3F_3 (*Xa21*/*Xa21*) in various growth and developmental stages and during *Xoo* inoculation.

Materials and Methods

Plant samples and growing conditions

Rice (*Oryza sativa* L. ssp. indica) cultivars RD47 and IRBB21 were provided by the Bureau of Rice Research and Development, Phitsanulok, Thailand. The *Xa21* gene from IRBB21 was introgressed in RD47 through backcross breeding and marker-assisted selection (data not shown) until homozygous-*Xa21* BC_3F_3 lines were obtained. Leaf samples were collected from the 2-leaf, 4-leaf, 6-leaf and reproductive stages, respectively.

Xoo isolate and inoculation test

BB-infected leaves were collected from paddy fields in Phitsanulok province and *Xoo* was isolated on nutrient agar (peptone-bovine-agar). The isolated bacteria was identified as *Xoo* through polymerase chain reaction (PCR) assays using *Xoo* specific primers TXT (Sakthivel et al., 2001) and Xoo80 (Lu et al., 2014). Before infection, the *Xoo* isolate labeled as "xoo16PK002" was re-streaked and incubated at 28°C for 48 hr. A *Xoo* inoculum (the optical density at 600 nm (OD600) of 0.2) was prepared and used to inoculate 60-day-old plants using the clipping method of inoculation (Kauffman, 1973). Mock (water) inoculation was used as a control. Samples corresponding to 5 cm of the leaves directly below the inoculation sites were collected at 0 hr, 1 hr, 2 hr, 6 hr and 24 hr post inoculation (hpi); leaf samples were frozen in liquid nitrogen immediately.

RNA extraction and cDNA synthesis

Total RNA was extracted from each 100 mg leaf sample using an RNAprep Pure Kit (Tiangen Biotech Ltd.; China) following the manufacturer's instructions. Each RNA sample was treated with Rnase-Free Dnase I (RBC Bioscience; Taiwan) to remove possible gDNA contaminants. Total RNA samples were quantified using a Synergy H1 microplate reader (Biotek; USA) and their integrity was assessed using agarose gel electrophoresis. The qScript™ XLT cDNA synthesis kit (QuantaBio; USA) was used to reverse transcribe 1 µg of total RNA templates in order to synthesize first strand cDNAs according to the manufacturer's protocol.

Polymerase chain reaction and cloning of XIK1 partial cDNA sequence

PCR was performed using a BioRad T100™ Thermal Cycler for 35 cycles (95°C for 10 s, 60°C for 10 s, 72°C for 20 s). The primer XIK1Ri (Hu et al., 2015) was used to amplify the *XIK1* gene. The PCR products were cloned using RBC TA cloning vector (RBC Bioscience; Taiwan) following the instructions in the manual. Plasmids carrying the *XIK1* gene were extracted and subjected to sequence analysis.

Quantitative real-time polymerase chain reaction

For the quantitative real-time polymerase chain reaction (qPCR) analyses, fast SYBR Green Master Mix (Quantabio; USA) was used to prepare 20 µl qRT-PCR reactions containing 1 µl of the cDNA templates and 0.5 µM of each primer. The specific primers used to amplify the genes of interest (GOIs) evaluated in this study are shown in Table 1. Non-RT PCR was performed to confirm no gDNA contamination. The specificity of the PCR and qPCR products was carefully assessed using gel electrophoresis and melting curve analysis, respectively. Technical triplicates and no template controls (NTCs) were run on an Eco 48 Real Time PCR System (PCR Max; United Kingdom) for 35 cycles (95°C for 10 s, 60°C for 10 s, 72°C for 20 s) followed by melting curve analysis.

Results

Expression of XIK1 and Xa21 in different growth stages

The amplified *XIK1* product using the primers based on japonica rice Kitaake showed 100% nucleotide identicality to the annotated *XIK1* sequence (Fig. 1). The relative expression of *XIK1* in different growth stages (2-leaf, 2S; 4-leaf, 4S; 6-leaf, 6S; reproductive stage, RS) showed that *XIK1* gene expression levels progressively increased from the 2S to 6S stages and eventually decreased in the RS stage in both the BB-susceptible cultivar (RD47) and its improved BB-resistant progenies (BC_3F_3) as shown in Fig. 2A and 2B, respectively. The expression of *Xa21* in the improved BB-resistant (BC_3F_3) progenies showed a similar trend to the *XIK1* gene (Fig. 2C). As expected, no amplification of *Xa21* was detected in the BB-susceptible cultivar RD47 (data not shown).

Table 1 List of genes evaluated in this study

Gene name	Primer sequence (5'-3')	Amplicon length	Reference
Xoo-induced kinase 1 (XIK1)	GACCAGGCGAAATCAACTTT ATGTAAGGCAGTGAGTTTAGTCAA	187 bp	Hu et al., 2015
Xa21	CAGAGTATGGCGTTGGGCT CGGGTCTGAATGTACTGTCA	114 bp	Promma et al., 2016
Triosephosphate isomerase (TI)	CGACATCATCAACTCCGCCAC CCTCTTCAGACATCTTCCCACG	83 bp	Wang et al., 2016
Endothelial differentiation factor (Edf)	TCCGAACCAGCAGATCATCG GCATGGTATCAAAAGACCCAGC	158 bp	Wang et al., 2016
Ubiquitin-5	CCAGTACCTCAGCCATGGA GGACACAATGATTAGGGATC	69 bp	Hu et al., 2015

Gene names and all their details are presented using the style in the reference cited.

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RD47     TTGGGCCATTGCCAACAAGCTTGCTAAACTGCAAAACCCTGGTTAGAGTTCGTCTTGAGC
BC3F3    TTGGGCCATTGCCAACAAGCTTGCTAAACTGCAAAACCCTGGTTAGAGTTCGTCTTGAGC
Kitaake  TTGGGCCATTGCCAACAAGCTTGCTAAACTGCAAAACCCTGGTTAGAGTTCGTCTTGAGC
         ************************************************************

RD47     GAAATCAACTTGAAGGAGATATCTCCGAGATGGGCCTTCATCCAAACCTTGTCTATATTG
BC3F3    GAAATCAACTTGAAGGAGATATCTCCGAGATGGGCCTTCATCCAAACCTTGTCTATATTG
Kitaake  GAAATCAACTTGAAGGAGATATCTCCGAGATGGGCCTTCATCCAAACCTTGTCTATATTG
         ************************************************************

RD47     ACATGAGCTCAAATAAACTATATGGACAATTATCTCATCGCTGGGGTGAGTGCGCCAAAC
BC3F3    ACATGAGCTCAAATAAACTATATGGACAATTATCTCATCGCTGGGGTGAGTGCGCCAAAC
Kitaake  ACATGAGCTCAAATAAACTATATGGACAATTATCTCATCGCTGGGGTGAGTGCGCCAAAC
         ************************************************************

RD47     TTACCAC
BC3F3    TTACCAC
Kitaake  TTACCAC
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Fig. 1 Multiple sequence alignment of *XIK1* sequences in RD47 and improved BB-resistant BC_3F_3 progenies compared with the japonica cultivar Kitaake (CLUSTAL O 1.2.4).

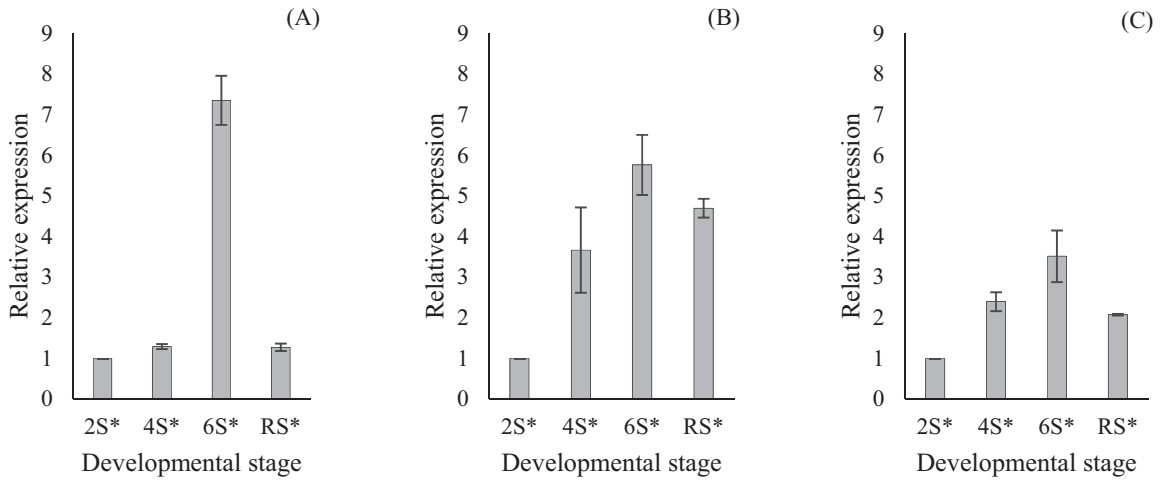

Fig. 2 Relative expression of *XIK1* in: (A) RD47; (B) BC_3F_3 progenies; (C) relative expression of *Xa21* in BC_3F_3 progenies in various growth stages, where 2S, 4S, 6S and RS represent the 2-leaf, 4-leaf, 6-leaf, and reproductive stages, respectively and asterisks indicate significant differences among datasets based on analysis of variance at $p < 0.05$ and error bars indicate SD of the analyzed data that have been normalized relative to *Ubiquitin 5*.

Response of XIK1 and Xa21 to Xoo infection

The 60-day-old RD47 and BC_3F_3 plants corresponding to the 6S stage were subjected to *Xoo* infection and samples were collected at different times post inoculation for qPCR analyses. The results showed that the expression of *XIK1* was induced by *Xoo* inoculation for both the RD47 and BC_3F_3 lines but in different times post inoculation. The expression of *XIK1* in the RD47 cultivar was suppressed in the first hour after *Xoo* inoculation and was later induced after 2 hpi. Notably, the expression level substantially dropped after the time of induction and there was no clear indication of up-regulation in the succeeding time post inoculation (Fig. 3A). On the other hand, the expression of *XIK1* in the BC_3F_3 lines was quickly induced after 1 hpi but the expression level suddenly decreased after 2 hpi. Interestingly, the expression of the gene was up-regulated again after 6 hpi until 24 hpi, thus indicating that the expression of *XIK1* tended to increase again (Fig. 3B). The expression of *Xa21* in BC_3F_3 was also significantly induced after 1 hpi and eventually decreased in the succeeding time post inoculation (Fig. 3C). No signal of *Xa21* up-regulation was observed, even after 24 hpi.

Discussion

The genetics of resistance to bacterial blight has been studied in depth and was first carried out using the resistance (R) gene *Xa21*. As there is diversity in the *Xoo* strains of different rice-producing countries, the mechanism of *Xa21*-mediated resistance has not yet been completely elucidated. Since *Xa21* functions as a pattern recognition receptor and a tyrosine-sulfated protein *RaxX* is required for the activation of Xa21-mediated immunity, the activity of *Xa21* in the plant's plasma membrane may accelerate subsequent responses to the conserved bacterial molecule of *Xoo* (Gómez-Gómez and Boller, 1999). Previous reports have reported that several Xa21-binding proteins are directly involved and play important roles in the early events of the *Xa21* signaling pathways (Wang et al., 2006; Lee et al., 2009; Chen et al., 2010; Park et al., 2010; Park et al., 2012). However, no changes of expression after *Xoo* inoculation were found in the genes encoding these proteins. Hu et al. (2015) found a BB-resistant gene (*XIK1*) that shared a structural motif with *Xa21* and positively regulated Xa21-mediated immunity. However, that study did not indicate whether the expression of the *XIK1*gene was stable in the time post inoculation after *Xoo* infection and whether the gene expression in the various leaf stages was the same for both BB-resistant and BB-susceptible cultivars. The current results showed that the expression levels of *XIK1* were induced earlier post inoculation by *Xoo* in both the BB-susceptible cultivar (RD47) and the BB-resistant BC_3F_3 progenies. However, the induction on the expression was earlier in BC_3F_3 than in RD47. Though the expression of *XIK1* significantly decreased after the induction, its expression tended to accumulate in the succeeding time post inoculation in the resistant BC_3F_3 but not in the susceptible RD47. These results suggested that *XIK1* is activated earlier and is progressively expressed in the succeeding hours after *Xoo* infection in BB-resistant BC_3F_3 but not in the BB-susceptible cultivar RD47. In addition, *XIK1* was either induced by *Xoo* or wounding (data not shown). Considering the reports that *Xa21* expression is development-dependent and its expression is either induced by *Xoo* or wounding (Mazzola et al., 1994; Century et al., 1999), the same findings for *XIK1* expression for both BB-susceptible and BB-resistant cultivars were also presented in this study.

The gradual increase in the expression of *XIK1* during the plant's growth and development shares similarity with the expression of *Xa21* and the responses of both genes to *Xoo* inoculation in the BB-resistant BC_3F_3 progenies carrying the *Xa21* gene are in commonality after 1 hpi and in the succeeding time post inoculation, Thus, *XIK*1 might act as a co-receptor of *Xa21* in regulating the early events of XA21-mediated signaling and thereby conforming

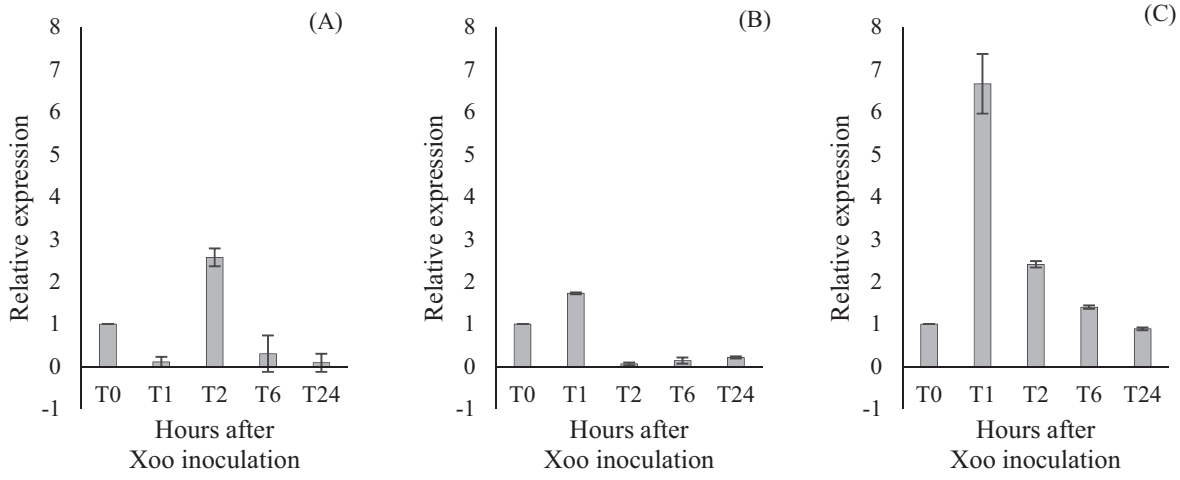

Fig. 3 Relative expression of *XIK1* in: (A) RD47; (B) BC_3F_3 lines; (C) relative expression of *Xa21* in BC_3F_3 lines after *Xoo* inoculation, where 0 hr, 1 hr, 2 hr, 6 hr and 24 hr post inoculation are presented as T0, T1, T2, T6, and T24, respectively and error bars indicate SD of the data analyzed that have been normalized with the reference genes *Edf* and *TI*.

to the findings of Hu et al. (2015). Data on the disease development by *Xoo* in both tested cultivars also confirmed that BC_3F_3 progenies expressing the genes *Xa21* and *XIK1* established resistance against the *Xoo* pathogen (Fig. 4). In this case, the current study supports the mechanism of defense for *Xa21*-mediated rice plants in both indica and japonica subspecies as characterized by *XIK1* and *Xa21* genes expression as being alike.

Fig. 4 Disease development of *Xoo* on indica rice cultivars RD47 and BC_3F_3 progenies 21 d post inoculation, where RD47 shows complete susceptibility while BC_3F_3 shows moderate resistance

The results revealed that the expression of *XIK1* was development-dependent and was induced by *Xoo* in the tested indica rice cultivar RD47 and its improved BB-resistant progenies BC_3F_3 (*Xa21*/*Xa21*). Furthermore, it was shown that the *XIK1* gene was induced earlier in the BB-resistant BC_3F_3 progenies than in the susceptible cultivar RD47 after *Xoo* inoculation, thus indicating that *XIK1* was activated earlier in resistant plants than in susceptible ones. Moreover, similar expression patterns of *Xa21* and *XIK1* were identified in the various growth stages and after *Xoo* inoculation.

Conflict of Interest

The authors declare that there were no conflicts of interest and all ideas reflected herein have the agreement of all authors.

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