Differential expression of a phenylalanine ammonia-lyase gene, *OsPAL4*, in bacterial blight-susceptible and *Xa21*-mediated resistant indica rice cultivars

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Abstract

Most agronomically important traits, including resistance against pathogens, are controlled by quantitative trait loci (QTL). Identification of genes involved in QTL-based disease resistance is essential for breeding for cultivars exhibiting stably high resistance to invading pathogens. Several defense-related genes have been successfully used as potential indicators and contributors to QTL-based resistance against these devastating rice diseases. In this study, the researchers examined the expression of *OsPAL4*, a phenylalanine ammonia-lyase (PAL) gene, in BB-susceptible Thai indica rice cultivar RD47 and its improved BB-resistant progenies BC_3F_3 (*Xa21/Xa21*) inoculated with *Xanthomonas oryzae pv. oryzae* (*Xoo*). Based on the results, *OsPAL4* was expressed both in the Thai indica cultivar RD47 and BC_3F_3 lines. Nucleotide sequence analysis also confirmed that the partial cDNA sequence of *OsPAL4* from these rice samples showed 100% nucleotide sequence similarity to the *OsPAL4* nucleotide sequence in IR64. Meanwhile, expression analysis of *OsPAL4* under BC_3F_3 inoculation showed that the gene was induced as early as one-hour post inoculation in BC_3F_3 lines. Also, its expression increased at two-hour post inoculation. No sign of gene upregulation was observed in BC_3F_3 lines after the two-hour post inoculation. In RD47, the expression of *OsPAL4* was induced after two-hour post inoculation and is disposed to increase again after 6 to 24-hour post inoculation. The findings suggest that *OsPAL4* is induced by *Xoo* inoculation thereby indicating its potential activity in the earlier responses of rice against invading pathogens.

Keywords: bacterial blight, gene expression, *OsPAL4*, rice, *Xanthomonas*

Introduction

Most of the world's population depends on rice (*Oryza sativa* L.) as its primary food source. However, limiting factors such as insect pests and diseases caused by various types of pathogens tend to reduce the total production volume of rice because of the damages they inflict on the crop. Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the costliest among the most destructive diseases of rice as it incurs up to 70% reduction in yield if not properly controlled (IRRI, 2018).

In plants, the ability to recognize both the general elicitors and specific pathogens through gene-mediated resistance, is essential for their defense mechanisms (Bittel and Robatzek, 2007). Thus, it is necessary to fully understand the molecular mechanisms underpinning the plant's defense against invading pathogens. Most agronomically important traits of plants, including resistance against pathogens, are governed by quantitative trait loci (QTL). Identification of genes involved in QTL-based disease resistance is essential for breeding for cultivars exhibiting stably high resistance to invading pathogens. Several defense-related genes have been successfully used as potential indicators and contributors to QTL-based resistance against these devastating rice diseases (Boyd et al., 2013). The study on the expression of these various genes in plants has laid the foundation in understanding their unique activities and functions in different processes and mechanisms in the whole plant system.

Recently, *OsPAL4*, a phenyalanine ammonia-lyase (PAL) gene, was found to be associated with broad spectrum disease resistance against sheath blight and bacterial blight in rice (Tonnessen et al., 2015). To date, no resources are yet available on its relative expression under various conditions. In the present study, the researchers evaluated whether the PAL gene *OsPAL4* could be identified in the elite Thai rice cultivar RD47 and in its near isogenic lines carrying the *Xa21* gene. They also analyzed the expression of this gene under *Xoo* infection in different times post inoculation.

Methodology

A. Plant samples and growing conditions

The rice (*Oryza sativa* L. ssp. indica) cultivar RD47 was 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. The rice plants were grown under greenhouse condition.

B. BC_3F_3 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 bacterial isolates were 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 hours. A *Xoo* inoculum (OD600 of 0.2) was prepared and

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Table 1. List of genes evaluated in this study

Gene name	Primer sequence (5'-3')	Amplicon length	Reference
OsPAL4	CTTCACAACAGCTAATCGAG	104 bp	Tonnessen et al. (2015)
LOC_Os02g41680	CGCACTCCATTTCAGTACCA		
Triosephosphate isomerase (*TI*)	CGACATCATCAACTCCGCCAC	83 bp	Wang et al. (2016)
LOC_Os01g05490	CCTCTTCAGACATCTTCCCACG		
Endothelial differentiation factor (*Edf*)*	TCCGAACCAGCAGATCATCG	158 bp	Wang et al. (2016)
LOC_Os08g27850	GCATGGTATCAAAAGACCCAGC		
*Ubiquitin-5**	CCAGTACCTCAGCCATGGA	69 bp	Hu et al. (2015)
LOC_Os03g13170	GGACACAATGATTAGGGATC		

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

used to inoculate 60-day-old plants using the clipping method of inoculation (Kauffman, 1973). Mock (water) inoculation was used as the control. Plant samples corresponding to 5 cm of the leaves directly below the inoculation sites were collected at 0-, 1-, 2-, 6-, and 24-hour post inoculation (hpi). Leaf samples collected were frozen in liquid nitrogen immediately.

C. 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.

D. 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 *OsPAL4* (Tonnessen et al., 2015) was used to amplify the *OsPAL4* gene. The PCR products were cloned using RBC TA cloning vector (RBC Bioscience; Taiwan) following the instructions in the manual. Plasmids carrying the *OsPAL4* gene were extracted and subjected to sequence analysis.

E. 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 ran on an Eco™ PCR Max (Biosystem; Argentina) for 35 cycles (95°C for 10 s, 60°C for 10 s, 72°C for 20 s) followed by melting curve analysis.

Results and Discussion

Expression of *OsPAL4* in RD47 cultivar and its near isogenic lines carrying Xa21 gene (BC_3F_3)

In the study, the researchers evaluated whether the gene *OsPAL4* can be identified and isolated from the RD47 cultivar and its near isogenic lines carrying *Xa21* gene (BC_3F_3) through polymerase chain reaction (PCR) amplification using the primer *OsPAL4* (Tonnessen et al., 2015). Based on the results, a 104 bp single DNA band was predicted in all tested samples (Fig 1a). The amplified DNA was then isolated, purified, and cloned into an *E. coli* species to carry out the specificity of the desired gene of interest. Positive colonies carrying the *OsPAL4* gene were selected and were assessed through PCR assays and gel electrophoresis (Fig. 1b). Predicted positive DNA bands were extracted, purified, and subjected to sequence analysis to further check the specificity of the DNAs that were amplified. Results of the analysis showed that the amplified partial cDNA sequence of *OsPAL4* from all the samples displayed 99% similarity to the *OsPAL4* sequence from the IR64 cultivar. Both of the samples tested yielded 100% nucleotide sequence similarity to he *OsPAL4* gene (Fig. 1c). These findings indicate that *OsPAL4* is expressed in all test samples, and implies that *OsPAL4* is conserved both in the IR64 and RD47 cultivars.

Relative expression of *OsPAL4* in RD47 cultivar and BC_3F_3 lines under *Xoo* inoculation

After confirming the nucleotide sequence of *OsPAL4* in the test samples, the researchers analyzed its expression under *Xoo* inoculation. Results of the analyses showed that the expression *OsPAL4* gene was induced in the early hours after *Xoo* inoculation but in different time posts for the two tested rice samples. As shown, the expression of *OsPAL4* was induced after 1 hpi in BC_3F_3 lines (Fig 2a) and after 2 hpi in RD47 (Fig 2b). The expression of the gene was also maintained after 2 hpi in

Figure 1a. Migration of *OsPAL4* gene in agarose gel (1.5%). Template DNA was taken from leaf samples of 60-day-old plants in each of the cultivar assessed. Legend: M- marker (100 bp DNA Ladder, Invitrogen™, USA); (-) – no template control

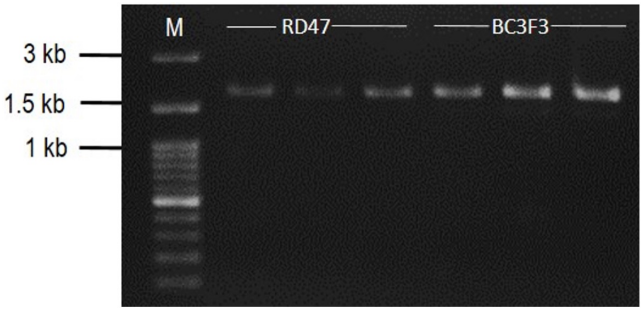

Figure 1b. Migration of *OsPAL4* gene from *E. coli* plasmids in agarose gel (1.5%). Template DNA was taken from three (3) positive white colony samples in each cultivar. The *OsPAL4* primers (Tonnessen et al., 2015) were used in the PCR amplification process. Legend: M- marker

BC_3F_3 lines but not in the RD47 cultivar. *OsPAL4* expression diminished after 6 hpi in both of the RD47 cultivar and its improved BC_3F_3 lines. Surprisingly, an increase in the expression of *OsPAL4* was observed after 24 hpi in RD47 but not in BC_3F_3 lines.

Previous studies have reported that genes in the phenylpropanoid pathway are activated in the early hour responses to pathogen attacks (Kostyn et al., 2012; Naoumkina et al., 2010). PAL genes are also found to be activated during Pattern Triggered Immunity (PTI) and Effector Triggered Immunity (ETI) and are induced by various types of pathogens (Giberti et al., 2012; Gupta et al., 2012; Li et al., 1999; Sana et al., 2010). In this study, it was found that the expression of a PAL gene *OsPAL4* was induced in the early hours after BC_3F_3 infection. When compared with mock inoculation, the expression of *OsPAL4* was induced in the early hours of *Xoo* infection and was maintained until 2hpi. Interestingly, it was also found that the expression of the gene was induced earlier in the BB-resistant BC_3F_3 lines than in the BB-susceptible cultivar RD47, thus implicating that *OsPAL4* gene is activated earlier in BB-resistant BC_3F_3 lines than in the BB-susceptible cultivar RD47. These findings of the study suggest that *OsPAL4* is associated with broad spectrum disease resistance against *Xoo* as the expression of the gene is activated in the early hours post inoculation by the pathogen. These findings supplement the findings of Tonnessen et al. (2015), thus *OsPAL4* may be involved in a broad-spectrum resistance in rice and could be a target for breeding interventions in the future.

Conflicts of Interest

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

Acknowledgements

This study was supported by Research Grants numbers R2559B020, R2560B122 and R2560C030 from Naresuan University, Thailand.

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RD47      CTTCACAACAGCTAATCGAGTAGCTAGAACCATTATATACTCTTCTCTCGACGCTTTTTG
BC3F3     CTTCACAACAGCTAATCGAGTAGCTAGAACCATTATATACTCTTCTCTCGACGCTTTTTG
IR64      CTTCACAACAGCTAATCGAGTAGCTAGAACCATTATATACTCTTCTCTCGACGCTTTCTG
          ************************************************************ **

RD47      TGCTAGGTTAACCGATCCATCTTCTGGTACTGAAATGGAGTGCG
BC3F3      TGCTAGGTTAACCGATCCATCTTCTGGTACTGAAATGGAGTGCG
IR64      TGCTAGGTTAACCGATCCATCTTCTGGTACTGAAATGGAGTGCG
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Figure 1c. Multiple sequence alignment of *OsPAL4* partial cDNA sequences in RD47 and BC_3F_3 as compared with the IR64 cultivar (CLUSTAL O 1.2.4). Partial cDNA sequence of *OsPAL4* in IR64 was taken from the MSU Rice Genome Annotation Project (available at http://rice.plantbiology.msu.edu/)

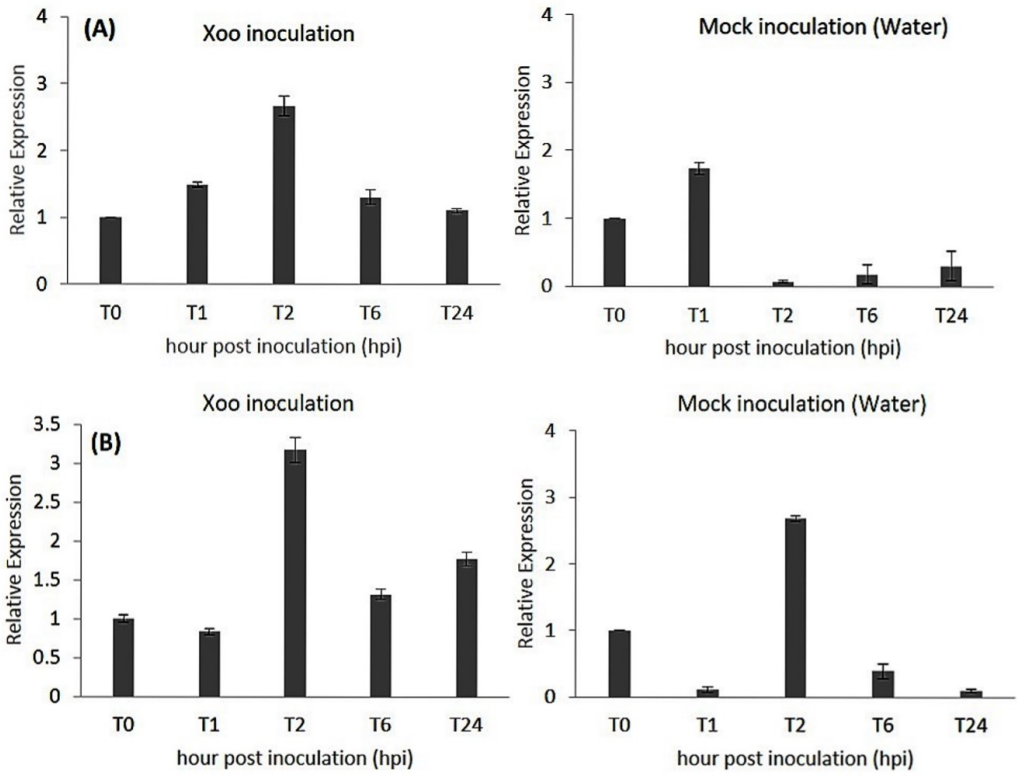

Figure 2. Relative Expression of *OsPAL4* in A) BC_3F_3 lines and B) RD47 after *Xoo* inoculation. Three independent biological experiments were repeated with similar results. Error bars indicate standard deviation of the data analyzed. Data were normalized with *Edf* and *Tl*.

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