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Research Article

Expression Analysis of Defense Related Genes in Rice Response to *Bipolaris oryzae*, the Causal Agent of Rice Brown Spot

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Abstract
The rice defense mechanism was studied against *Bipolaris oryzae*, the rice brown spot fungus, in two Thai rice varieties, Khao Dawk Mali 105 (KDML 105) and Jao Hom Nin (JHN) (showing highest and lowest susceptibility to *B. oryzae*, respectively). The expression was evaluated of eight genes through real-time quantitative reverse transcription polymerase chain reaction. The gene involved in the salicylic acid (SA) signaling pathway (*OsPAL*) and the pathogenesis related genes (*OsPR1b* and *OsPBZ1*) were upregulated in both varieties with no significant differences. Despite higher expression of the genes involved in the jasmonic acid (JA) signaling pathway (*OsLOX* and *OsAOS2*) in JHN, the expression of *JiOsPR10* was not significantly different in both varieties. The genes involved in the ethylene (ET) signaling pathway (*OsACS1* and *OsEIN2*) were expressed more highly and far more rapidly in KDML 105 than JHN. Overall, our results demonstrated that the investigated genes related to SA, JA and ET defense pathways may not play a major role in rice resistance against *B. oryzae*. Furthermore, the high level of transcript accumulation of genes related to the ET signaling pathway may interfere with the ability of rice to resist *B. oryzae*. The study provided information for a better understanding of rice defense mechanisms against *B. oryzae*.

Keywords: *Bipolaris oryzae*, Brown spot, Defense mechanism, Gene expression, Rice

1 Introduction

Rice (*Oryza sativa*) is one of the main staple foods and is considered as a key food source for half of the world's population [1]. Unfortunately, rice production confronts many adversities including plant diseases which influence crop production annually. Rice brown spot is caused by *Bipolaris oryzae* (Breda de Haan) Shoemaker (telemorph = *Cochliobolus miyabeanus*), an ascomycetous necrotrophic fungus. This disease occurs worldwide in rice growing areas [2] and is well known for being one of the major causal factors for the "Great Bengal Famine" during 1942–1943 [3]. Loss of up to 67% in the quantity and quality of rice grain in several fields and different degrees of disease severity from slight to 75% have been documented [4], [5]. Typical symptoms are cylindrical or oval shaped and brown spots with a grey or whitish center, sometimes with yellow halo, on rice leaves [2], with grain discoloration of the rice also being noted [6]. Far less is known about the disease mechanism of *B. oryzae*; nonetheless, ophiobolin, a non host selective toxin, produced in fluids during *B. oryzae* conidial germination, has been reported to be associated with

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susceptibility inducing activity and phytotoxicity [7]. Furthermore, ophiobolin A reduced the phenolic content which have an impact on rice resistance against *B. oryzae* [8]. In addition, *B. oryzae* infection was shown to affect rice leaf physiology by interfering with the cell membrane of plant tissues [9].

Implementation of management strategies available for brown spot disease includes the use of cultivars expressing disease resistance, an appropriate supply of plant nutrients, fungicide application and biological control methods [2], [10], [11]. Although employing resistant cultivars is effective economically as well as being environmental-friendly for controlling disease, so far there have been no reports of major resistant genes against the rice brown spot pathogen [12]. Disease resistance against the pathogen is polygenically controlled with recent successful attempts in the identification of some quantitative trait loci (QTLs) associated with rice brown spot resistance [12]–[15].

Plants combat to invading pathogens using two branches of innate immune system including a pattern triggered immunity (PTI) and an effector triggered immunity (ETI) [16]. PTI corresponds to the recognition of pattern recognition receptors (PRRs) to common molecules of various classes of microbes, known as pathogen associated molecular pattern (PAMPs) or microbe associated molecular patterns (MAMPs). The latter immunity, ETI, responds to pathogens via plant resistance protein (R proteins) via the recognition of pathogen effectors or the virulent factors. Perception of corresponding stimuli by PRRs or R proteins leads to the induction of defense response to repel pathogen attacks where PTI yields slow and low nature of host defense and ETI produces robust and rapid mode of defense in host [16]–[19]. Once pathogens initiate their local infection, plants establish the induced immunity such as systemic acquired resistance (SAR) and induced systemic resistance (ISR) for increasing the protection of uninfected plant tissues against the pathogen [20]. In this regard, the phytohormones are key factors mediated systemic defense signaling [21].

Generally, plant defense responses against pathogens are regulated through a complex network of signaling pathways in which the plant hormones play key roles in this regard. Among others, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are known as key players that act sequentially, antagonistically or synergistically to fine tune the plant defense response to specific invaders [18], [22], [23]. Indeed, our understanding of the mechanisms that plants use for protecting themselves against pathogens has been based on research on the dicotyledonous model plant, Arabidopsis. Although, there are exceptions, the SA pathway is typically known to play a role in plant defense against biotrophic and hemibiotrophic pathogens, while the JA/ET pathways are commonly involved in defense against necrotrophs and chewing insects [24], [25]. Although both positive and negative regulatory interplay of SA and JA pathways are documented, interaction between the SA and JA signaling pathways is more antagonistic [26]. However, in rice there are no discrete actions of the SA and JA pathways in the response to a pathogen. Both these pathways have been reported to be effective against either biotrophs/hemibiotrophs or necrotrophs. Additionally, the JA signaling pathway can play a role in positive SA pathway regulation in the rice defense response [23].

Until now, the understanding of rice-*B. oryzae* interaction, especially on a molecular basis, has been limited. The information on rice response through defense relating gene expression upon *B. oryzae* attack is still poorly understood. In this study, we used real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR) to analyze the differential expression of some defense related genes at various time points in two distinct rice varieties that display different levels of brown spot disease severity following *B. oryzae* infection.

2 Materials and Methods

2.1 *Brown spot fungal isolate*

The *B. oryzae* isolate KUSBR9 was used throughout the experiments due to its ability to produce large number of spores and its pathogenicity (data not published). The isolate of *B. oryzae*, KUSBR9 was obtained from a rice leaf showing a brown spot symptom collected from a paddy field in Thailand. Its morphological characteristics and nucleotide sequence of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) gene were confirmed as *B. oryzae*. The DNA sequence was deposited in the GenBank with the accession no. MH059554.

2.2 *Plant varieties and brown spot severity evaluation*

Brown spot disease expression was investigated on several rice varieties: Khao Dawk Mali 105 (KDML 105), a Thai jasmine rice susceptible to many diseases and insects; Jao Hom Nin (JHN), a Thai glutinous rice conferring broadspectrum resistance to rice blast disease; and two certified rice varieties from the Rice Department of Thailand consisting of RD31, a variety with moderate brown spot disease resistance and Pathum Thani 1 (PTT1), a variety usually recommended for brown spot disease management in central Thailand.

Preparation of plants for *B. oryzae* inoculation involved soaking seeds of each variety in water overnight before placing them on moist tissue paper until they germinated. Subsequently, 7-day-old germinated seeds were grown in five 20 cm pots containing a commercial soil mixture with each pot divided into quarters where each quarter contained five plants of one of the four tested varieties. The pots were maintained in a greenhouse with a temperature range of 28 to 33°C for 21 days. In addition, granular urea (46-0-0) fertilizer was applied at 7 days before inoculation.

A spore suspension of *B. oryzae* isolate KUSBR9 was prepared for plant inoculation using mycelia from the stock culture which were placed on potato dextrose agar (PDA) and incubated at 28°C for 7 days. Subsequently, the culture medium containing the mycelia was induced to sporulate by cutting the mycelia into small square pieces (1 cm × 1 cm) and placing them on moist, sterilized filter paper for 7 days at 28°C. Spores were harvested and prepared in sterilized distilled water with 0.05% Tween 20 added. The concentration of spores was adjusted to 5×10^4 spores/mL.

All pots were inoculated with the spore suspension of *B. oryzae* by spraying all leaves until they were covered with fine droplets. Inoculated plants were kept overnight in sealed plastic bags containing water with approximately 100% relative humidity and then transferred to a greenhouse. Brown spot disease expression of each plant variety was observed and photographed at 72 hour post inoculation (hpi). The percentage of leaf area affected on individual plants was calculated. The experiment was replicated twice. Mean comparisons of each rice variety infected with *B. oryzae* were tested for significance using Tukey's test at the 0.05 probability level.

2.3 *B. oryzae-infected plant tissue preparation*

The plant varieties showing the highest and lowest brown disease expression from the above experiment were selected to prepare *B. oryzae* infected leaf material for defense related gene expression analysis. The seeds of two rice varieties were prepared using the same procedure as above. The 7-day-old germinated seeds of each variety were grown in twenty-four 10 cm pots containing a commercial soil mixture with each pot divided into two parts where each part contained three plants of the tested variety. The pots were maintained in a growth chamber at 26°C and approximately 80% relative humidity with a 12 h light/dark photoperiod for 21 days. In addition, granular urea (46-0-0) fertilizer was applied at 7 days before inoculation.

A spore suspension of *B. oryzae* isolate KUSBR9 for plant inoculation was prepared following the method described earlier. All pots were separated into two treatments where each treatment contained twelve pots. For the first treatment, the plants were inoculated with sterilized distilled water plus 0.05% Tween 20, while for the second treatment, the pots were inoculated with the spore suspension of *B. oryzae* by spraying all leaves until they were covered with fine droplets. Inoculated plants were kept overnight in sealed plastic bags containing water with approximately 100% relative humidity and then transferred to a growth chamber at 26°C under 80% relative humidity with a 12 hour light/dark photoperiod. Tissue samples of each treatment from three inoculated pots were randomly collected and pooled together at 0, 12, 24 and 48 hpi. Collected plant tissues were immediately transferred in liquid nitrogen after sample harvesting and subsequently kept at –80°C prior to RNA extraction and cDNA preparation. The experiment was duplicated in independent experiments.

2.4 *RNA extraction and cDNA preparation*

Tissue samples were ground in liquid nitrogen and then 100 mg of ground tissue samples were subjected to total RNA extraction using an RNeasy® Plant Mini Kit (Qiagen; Hilden, Germany). The RNA samples were subsequently treated with RNase-free DNase I (Thermo Scientific; Waltham, MA, USA) before reverse transcription (RT). First-strand cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific; Waltham, MA, USA).

2.5 *Gene expression analysis by real-time qRT-PCR*

Real-time qRT-PCR was performed in 0.2 mL 8-tube strips using a CFX96™ Deep Well real-time PCR detection system (Bio-Rad Corporation; Hercules, CA, USA) with a HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX) (Solis BioDyne; Tartu, Estonia). Each reaction contained 2 µL of 5x HOT FIREPol® EvaGreen® qPCR Mix Plus, 10 ng of cDNA and 0.25 µL of each 10 µmol gene specific primer set in a final volume of 10 µL. Template denaturation was conducted for 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 58–60°C for 1 min and extension at 72°C for 30 s. All primers of the eight defense related genes and also one reference gene, *OsActin* used in this study had been previously used in other studies [27]–[30] (Table 1).

Table 1: Sequences of defense related gene and reference gene specific primers used for the real-time quantitative reverse transcription polymerase chain reaction

Rice gene (Gene description) and Primer sequence (5'–3')	Ref.
OsPAL (Phenylalanine ammonia lyase) F: CTACAACAACGGGCTGACCT R: TCTGGACATGGTTGGTGATG	[27]
OsPR1b (Pathogenesis related gene 1) F: GGCAACTTCGTCGGACAGA R: CCGTGGACCTGTTTACATTTTC	[28]
OsPBZ1 (Probenazole induced gene 1) F: CCCTGCCGAATACGCCTAA R: CTCAAACGCCACGAGAATTTG	[27]
OsLOX (Lipoxygenase) F: GCATCCCCAACAGCACATC R: AATAAAGATTTGGGAGTGACATA	[28]
OsAOS2 (Allene oxide synthase 2) F: CTCGTCGGAAGGCTGTTGCT R: ACGATTGACGGCGGAGGTT	[28]
JiOsPR10 (Jasmonate Inducible Pathogenesis-related gene 10) F: CGGACGCTTACAACTAAATCG R: AAACAAAACCATTCTCCGACAG	[29]
OsACS1 (1-Aminocyclopropane-1-carboxylic acid synthase) F: AAGGCCGAGAGATAATCGCCAAGA R: ACGAAAGGAATCTGCTACTGCTGC	[30]
OsEIN2 (Ethylene insensitive protein 2) F: CAAGGAACCAGTGACAACCA R: GCAGTCGTCTCCGCAGTTAG	[28]
OsActin (Actin) F: CAGCACATTCCAGCAGAT R: GGCTTAGCATTCTTGGGT	[28]

Relative expression of genes was calculated by the $2^{-\Delta\Delta C_t}$ method [31] using the equation, as indicated in the Equation (1) below,

$$\text{Relative expression} = 2^{-\Delta\Delta C_t} \quad (1)$$

Where C_t is the threshold cycle and $\Delta\Delta C_t$ is indicated in the Equation (2) below,

$$\Delta\Delta C_t = \Delta C_t \text{ (target)} - \Delta C_t \text{ (reference)} \quad (2)$$

Indeed, ΔC_t (target) and ΔC_t (reference) are indicated in the Equations (3) and (4) below,

$$\Delta C_t \text{ (target)} = [C_t, \text{ target gene of treated sample} - C_t, \text{ target gene of control sample}] \quad (3)$$

$$\Delta C_t \text{ (reference)} = [C_t, \text{ reference gene of treated sample} - C_t, \text{ reference gene of control sample}] \quad (4)$$

Three samples were analyzed for each treatment. The values were expressed as the mean ± standard deviation (SD). Statistical significance was tested using Tukey's test at the 0.05 probability level.

3 Results

3.1 *Comparison of the variety response to B. oryzae*

Four Thai rice varieties: KDML 105, RD31, PTT1 and JHN were evaluated against brown spot disease. Among all tested rice varieties, no complete disease resistance was observed after inoculation with *B. oryzae* isolate KUSBR9 on 21-day-old seedlings for 72 hour. Indeed KDML 105 was the most susceptible variety to the disease, while JHN showed the highest degree of resistance compared with the others [Figure 1(a) and (b)]. The affected leaf area caused by *B. oryzae* isolate KUSBR9 in KDML 105 was about 50% or greater than 50% of those of JHN at 24–72 h after *B. oryzae* inoculation (hpi) at the 21- to 23-day-old seedling stage (Figure 2). In particular, significant differences according to Tukey's test, $p < 0.05$ were observed between the two varieties tested. Phenotypic analysis of *B. oryzae* infected KDML 105 and JHN showed the individual pinpoint gray spots as early as 24 hpi in both varieties. At 48 hpi, each individual spot enlarged and became a cylindrical shaped spot with a grey or whitish center.

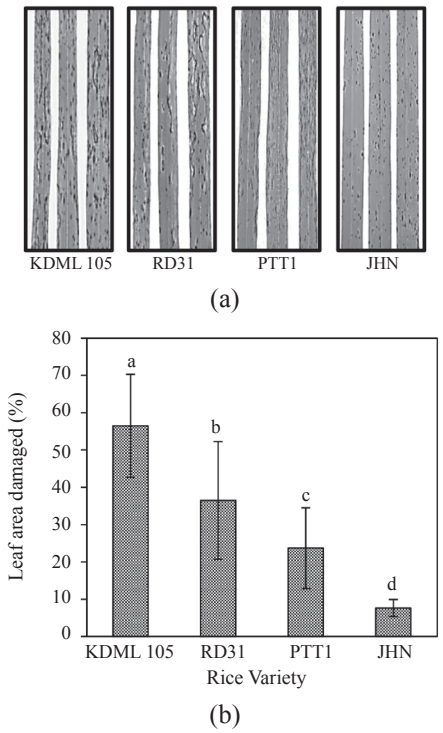


Figure 1: Rice brown spot expression on the leaves of different rice varieties after *B. oryzae* infection. (a) Disease formation on KDML 105, RD31, PTT1 and JHN after 72 h of *B. oryzae* infection on 21-day-old seedling plants. (b) Mean ± SD percentage leaf damage of the four rice varieties after 72 hours of *B. oryzae* infection on 21-day-old seedling plants. Leaf damage was based on the direct measurement of the chlorotic leaf tissue. Percentage of leaf damage was based on the chlorotic and necrotic leaf area/total leaf area. The bars represent results obtained in each of three independent experiments (N = 40). The error bars represent the standard deviation of the means. Means followed by different letters are significantly different as indicated by Tukey's test at $p < 0.05$.

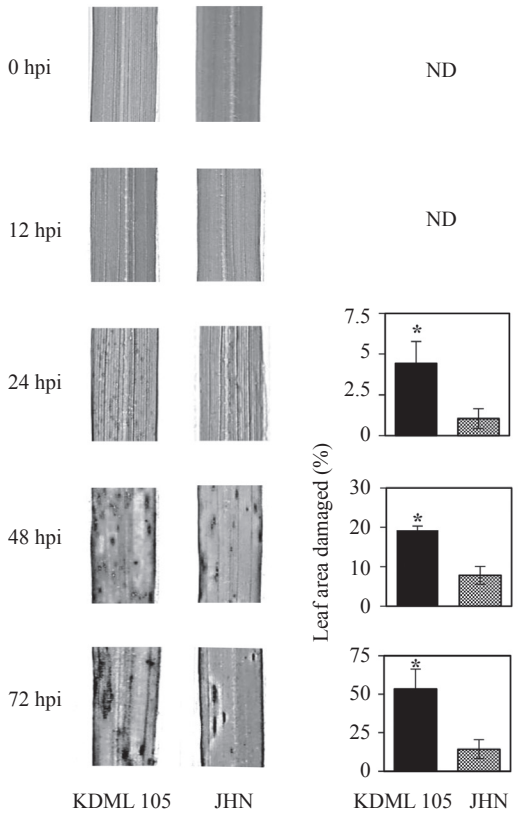


Figure 2: Comparative brown spot disease progression in rice KDML 105 and JHN varieties challenged with *B. oryzae*. Temporal disease progression of rice variety KDML 105 and JHN after 0, 12, 24, 48 and 72 hpi with *B. oryzae*. The bars represent mean ± SD percentage leaf damage of an individual rice variety (N = 5). Asterisk represents significant difference as indicated by Tukey's test at $p < 0.05$. ND indicates not determined. Note that the leaf tissues represented in this figure were taken from the same batch of rice *B. oryzae* infected tissue samples used for gene expression analysis.

Indeed, leaf tissue of KDML 105 turned yellow as the spot enlarged but not with JHN. At 72 hpi, oval shaped spots with a yellow halo could be observed in JHN, whereas individual spots had merged together and caused the leaf to become necrotic in KDML 105 (Figure 2). Therefore, with the differences in degree of brown spot severity and in symptom progress between these two varieties, KDML105 and JHN were selected for further studies.

3.2 *SA dependent responses of rice after B. oryzae infection*

A time course analysis was performed to determine the expression of genes involving the SA pathway in KDML 105 and JHN under treatment of *B. oryzae* isolate KUSBR9. The time course expression analysis of these genes at 0, 12, 24 and 48 hpi was investigated but not a 72 hpi due to the fact that after this period

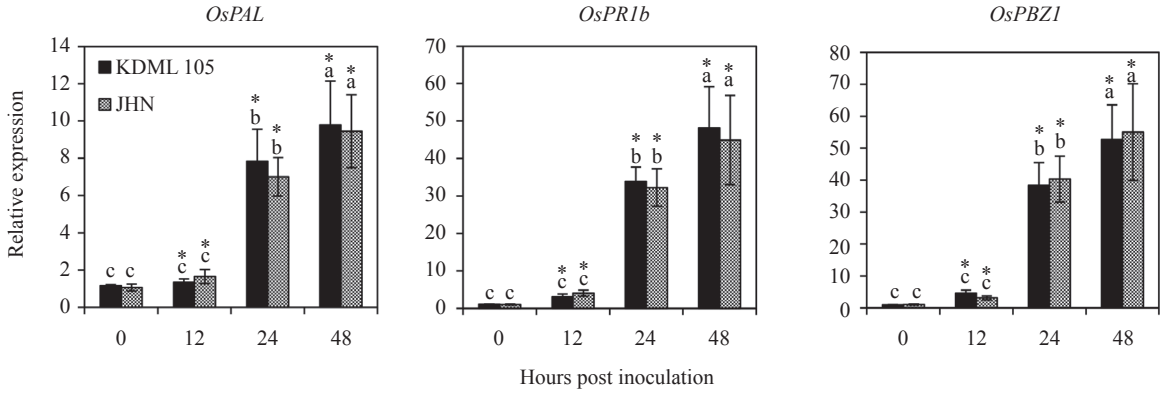
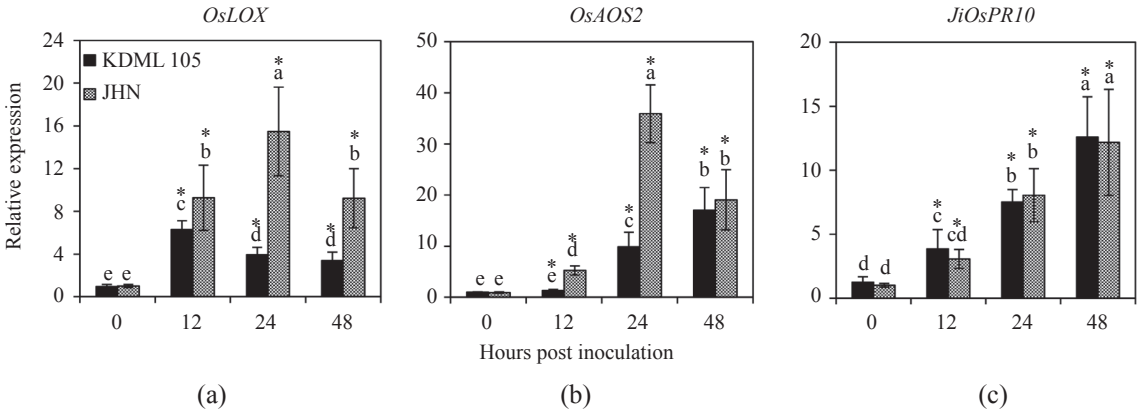


Figure 3: Temporal expression analysis of the relative expression levels of genes involved in the salicylic acid (SA) related pathway in rice KDML 105 and JHN varieties challenged with *B. oryzae* at 0, 12, 24 and 48 hpi using real-time quantitative reverse transcription polymerase chain reaction. The relative expression levels of the genes, *OsPAL* (a), *OsPR1b* (b) and *OsPBZ1* (c), were normalized to those of the rice *Actin* gene (*OsActin*). The bars represent mean ± SD from two independent biological experiments ($N = 16$). Asterisk indicates significant difference at $p < 0.05$ between control tissue samples and infected tissue samples as determined by Tukey's test. Means followed by different letters are significantly different among individual tissue samples with the treatment of *B. oryzae* at different hpi periods as indicated by Tukey's test at $p < 0.05$. The experiments were independently duplicated with similar results.

more than half of the KDML 105 leaf tissue had become necrotic. The upregulation of *OsPAL*, a key gene in the SA biosynthesis, was investigated in both rice varieties after the treatment with *B. oryzae*; however, there was no significant deference in the relative expression of *OsPAL* between these two varieties at all periods investigated. The *OsPAL* transcript accumulation in KDML 105 and JHN increased substantially from 12 hpi (1.34 fold in KDML 105 and 1.65 fold in JHN) to the greatest level (9.79 fold in KDML 105 and 9.46 fold in JHN) at 48 hpi [Figure 3(a)]. We further investigated the expression of SA responsive genes (*OsPR1b* and *OsPBZ1*). Similar results were observed in the expression of *OsPR1b* and *OsPBZ1* where there were no significant differences in the transcript accumulation between the two varieties [Figure 3(b) and (c)]. The relative expression of *OsPR1b* increased from 12 hpi (3.12 fold in KDML 105 and 4.06 fold in JHN) and reached the highest values at 48 hpi (48.18 fold in KDML 105 and 44.93 fold in JHN), whereas the relative expression of *OsPBZ1* increased from 12 hpi (4.61 fold in KDML 105 and 3.15 fold in JHN) to a peak at 48 hpi (52.69 fold in KDML 105 and 55.08 fold in JHN).

3.3 *JA dependent responses of rice after B. oryzae infection*

The *OsLOX* and *OsAOS2* genes (involved in JA biosynthesis) and the *JiOsPR10* (a JA responsive gene) were further investigated in the two rice varieties, KDML 105 and JHN, after *B. oryzae* infection. All three genes showed upregulated expression in both rice varieties during *B. oryzae* infection at 12–48 hpi. The transcript accumulation of *OsLOX* increased more in JHN than KDML 105 for every period of inoculation with the highest value of 15.48 fold at 24 hpi [Figure 4(a)]. In terms of *OsAOS2*, the transcript accumulation increased more in JHN than KDML 105 at 12 and 24 hpi with the greatest value being 35.91 fold, but not at 48 hpi [Figure 4(b)]. Although *OsLOX* and *OsAOS2* were likely expressed more highly in JHN than KDML 105, the JA responsive gene, *JiOsPR10*, showed no significant difference in relative expression level between JHN and KDML 105 for every period of inoculation. The relative expression of *JiOsPR10* reached a peak at 48 hpi (12.59 fold in KDML 105 and 12.18 fold in JHN) [Figure 4(c)].



(a) (b) (c)

Figure 4: Temporal expression analysis of the relative expression levels of genes involved in jasmonic acid (JA) related pathway in rice KDML 105 and JHN varieties challenged with *B. oryzae* at 0, 12, 24 and 48 hpi using real-time quantitative reverse transcription polymerase chain reaction. The relative expression levels of the genes, *OsLOX* (a), *OsAOS2* (b) and *JiOsPR10* (c), were normalized to those of the rice *Actin* gene (*OsActin*). The bars represent mean ± SD from two independent biological experiments ($N = 16$). Asterisk indicates significant difference at $p < 0.05$ between control tissue samples and infected tissue samples as determined by Tukey's test. Means followed by different letters are significantly different among individual tissue samples with the treatment of *B. oryzae* at different hpi periods as indicated by Tukey's test at $p < 0.05$. The experiments were independently duplicated with similar results.

3.4 *ET dependent responses of rice after B. oryzae infection*

OsACS1, the gene involved in ET biosynthesis and *OsEIN2*, the gene involved in ET signaling were used as marker genes for this investigation. *OsACS1* was upregulated at 12 and 24 hpi in both rice varieties; however, in KDML 105 the expression of this gene reached a peak (3.79 fold) at 12 hpi which was earlier and greater than JHN (2.89 fold, 24 hpi) [Figure 5(a)]. The transcript accumulation of *OsEIN2* increased more in KDML 105 (3.93 fold) than JHN (1.93 fold) at 48 hpi but not after the other periods [Figure 5(b)].

4 Discussion

Plant defense responses against invading pathogens rely on massive changes in gene expression mediated by complex signaling webs integrated by small signaling molecules and hormone pathways [18], [22], [23], [32]. Among these, SA, JA, and ET are known to function as primary signal molecules and the balancing of these hormone networks leads to optimization of the defense outputs against different pathogen lifestyles [33]. Rice brown spot caused by *B. oryzae* is one of the important rice diseases affecting grain yield and quality [4], [5]. Despite the detrimental potential of *B. oryzae*, information is limited on the molecular mechanism of rice defense responses to *B. oryzae*, a necrotroph fungus. In this study, we evaluated rice brown spot expression in four Thai rice varieties at the seedling stage. This growth stage of rice was reported to be highly susceptible to *B. oryzae* besides the heading stage of growth [34]. Our results showed that no complete resistance to *B. oryzae* observed among the tested varieties. Alternatively, a range in disease resistance to *B. oryzae* was observed. This result is in agreement with the previous studies where varietal differences in the rice brown spot resistances among the cultivars have been reported in various countries [34]. Subsequently, some rice cultivars expressing high resistance against *B. oryzae* were further used as the resistant sources for QTL mapping [12], [14], [15]. In this study, JHN had the highest quantitative (incomplete) resistance compared to the other varieties tested whereas KDML105 was the most susceptible variety to *B. oryzae*. The response of both varieties against virulent *B. oryzae* were used to investigate the transcript levels of defense-related genes to gain an understanding of the rice defense mechanism toward this necrotrophic fungus.

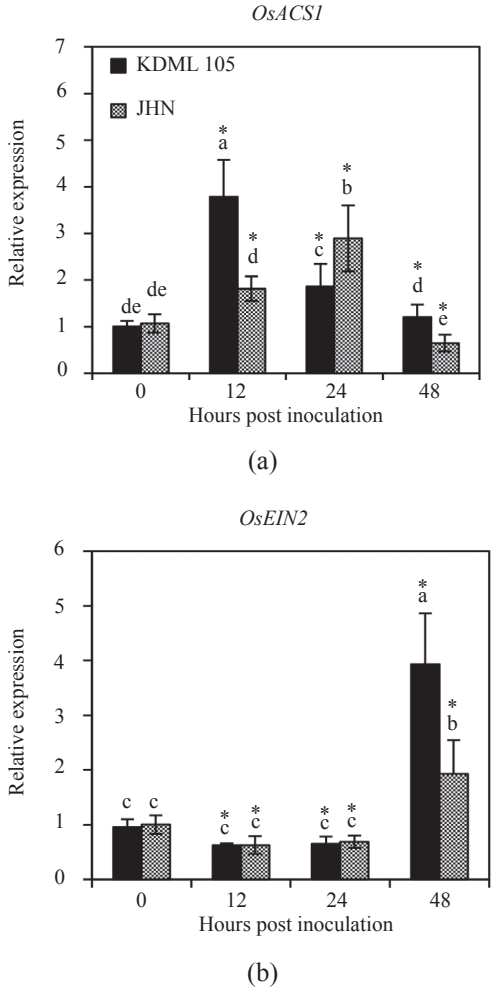


Figure 5: Temporal expression analysis of the relative expression levels of genes involved in ethylene (ET) related pathway in rice KDML 105 and JHN varieties challenged with *B. oryzae* at 0, 12, 24 and 48 hpi using real-time quantitative reverse transcription polymerase chain reaction. The relative expression levels of the genes, *OsACS1* (a) and *OsEIN2* (b), were normalized to those of the rice *Actin* gene (*OsActin*). The bars represent mean ± SD from two independent biological experiments (N = 16). Asterisk indicates significant difference at $p < 0.05$ between control tissue samples and infected tissue samples as determined by Tukey's test. Means followed by different letters are significantly different among individual tissue samples with the treatment of *B. oryzae* at different hpi periods as indicated by Tukey's test at $p < 0.05$. The experiments were independently duplicated with similar results.

Typically the plant hormones SA, JA and ET are principally involved in plant defense against invading pathogens by exerting influence through complex networks of diverse signaling pathways with either synergistic or antagonistic interaction [22]. Here we report that the genes investigated in this study which are the common known genes related to SA, JA and ET defense pathway may not play a major role in rice resistance to *B. oryzae*.

Phenylalanine ammonia lyase is an important plant enzyme in the phenylpropanoid metabolism involving in the biosynthesis of phenolic phytoalexins and SA hormone which is an important signal for the systemic resistance of a plant [35], [36]. Despite the evidence of increased accumulation of *OsPAL*, there was no significant difference in the relative expression of this gene in both rice varieties challenged with *B. oryzae*. This result suggests that *OsPAL* may play a role in the defense response during rice-*B. oryzae* interaction, but it does not contribute to the resistance to the rice brown spot fungus. The expression of *PAL* in response to various stimuli such as pathogen infection, wounding and stress has been documented in several studies [37]–[39]. Disruption of *PAL* genes in some plant species was shown to increase disease susceptibility to the virulent and avirulent pathogens [37]–[39], indicating the important role of *PAL* in the plant defense response to pathogens during PTI and ETI. Similar results were observed in the expression of *OsPR1b* and *OsPBZ1*, as defense marker genes associated with the SA signaling [40]–[42]. These inducible defense response through the induction of *OsPR1b* and *OsPBZ1* may be the consequence of the induced changes in plant cell metabolism, initially in the enzyme activity including PAL. Overall, the results are consistent with the earlier report of Ahn *et al*. [43] showing that the application of benzothiadiazole (BTH), a chemical analog of SA, produced no effect in protecting rice against *B. oryzae*. This finding suggested that SA is not a key factor contributing to defense activation for resistance against *B. oryzae*.

JA plays a role as a signal transducer in several physiological processes during plant development and in responses against biotic and abiotic stress [44]. The JA hormone and its components are recognized as one of the key players in plant immunity to necrotrophs [45], [46]; however, our results showed that rice resistance against *B. oryzae* may not rely on JA signal

molecule as supported by the expression analysis of the JA-related genes in this study. Although *OsLOX* and *OsAOS2*, as JA biosynthesis genes [47], [48], were more highly expressed in JHN at some particular periods after inoculation, there was no significant difference in the accumulation of the JA responsive gene, *JIOsPR10* [49] in both plant genotypes. These results are consistent with previous reports [29], [43] which documented the failure to protect rice against *B. oryzae* after exogenous JA application despite the transcript induction of some *PR* genes, indicating the minor role of the JA dependent pathway on the activation of defense against *B. oryzae*. In this study, only *JIOsPR10* was used as a *PR* gene marker for studying the JA dependent response of rice against *B. oryzae*; however, it cannot be ruled out that this JA responsive protein may not function as the key protein for rice to mediate defense response against *B. oryzae*. Therefore, the expression of other rice JA responsive *PR* genes such as *PR3*, *PR4* and *PR12* [50] should be further investigated.

ET acts as a small hydrocarbon gaseous hormone of plant and is involved in many plant aspects such as development and defense responses [51], [52]. Generally, ET is accepted as acting in cooperation with JA to elicit resistance against necrotrophic pathogens [18], [29]; however, the negative effect of ET on plant resistance to necrotroph has also been documented as shown for *B. oryzae*, the rice brown spot fungus [29]. Our results on a far more rapid and greater level of the transcript accumulation of *OsACS1*, the gene involved in ET biosynthesis [53] and *OsEIN2*, the gene involved in ET signaling [54] in susceptible KDML105 are in accordance with the previous reports wherein the robust activation of ET signaling was observed in the susceptible plant but not in the resistant one [29]. In addition, rice treated with ethephon, an ET-releasing chemical, conferred high susceptibility to *B. oryzae*, and the disruption of the ET signaling related gene in rice increased the resistant to *B. oryzae* [29]. From all the previous findings, it has been proposed that ET involves susceptibility promotion of *B. oryzae* [23], [29], [32].

5 Conclusions

We investigated the defense response of rice plants to *B. oryzae* through the backbone of defense hormones (SA, JA, and ET). We have shown that rice resistance to *B. oryzae* may not rely chiefly on SA, JA, and ET related immune signaling. Furthermore, the higher level of gene transcript accumulation related to the ET signaling pathway may be involved in the interference of rice resistance to *B. oryzae*. More recently, there is increasing evidence suggesting that other plant hormones such as abscisic acid, cytokinins, auxin, brassinosteroids, and gibberellins are involved in defense regulation either in negative or positive ways, by entering into the SA-JA-ET hormone pathway. Interplay between these individual hormone pathways is important in fine tuning the plant's defense to distinct types of pathogen [24], [32], [55]. Previously investigation were undertaken into the interaction of ABA and the individual core hormone pathway (SA, JA, and ET) regarding resistance against *B. oryzae* [29]. The pathological outcomes in rice interplay between other ranges of hormone during rice-*B. oryzae* interaction should be carried out to complete understanding on how hormone crosstalk does influence rice immune responses.

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