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OsNPR1 GENE EXPRESSION IN RESPONSE TO SALYSILIC ACID IN RICE

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Abstract:

NPR1 gene plays an important role in the activation of systemic acquired resistance (SAR). This research was conducted to examine the expression profiles of *OsNPR1* gene in four rice varieties: Pongsu Seribu, MR219, MRQ74 and CO39 in response to treatment with salicylic acid (SA). All four varieties vary in their level of resistance to fungal sheath blight disease while Pongsu Seribu showed the highest tolerance. SA treatment did not result in an increase in the level of expression in all varieties except for CO39 which is highly susceptible to blast disease and *OsNPR1* expressed in untreated CO39 was lower than the other three varieties. The overall results of this study show that SA did not result in increased expression of *OsNPR1* gene in all varieties. We therefore may conclude that exogenous SA does not induce *NPR1* gene in rice due to the higher endogenous levels of SA in rice. In addition SAR in rice may be controlled by non SA mediated pathways or combinatorial pathways.

Keywords

OsNPR1 gene; Rice; *Rhizoctonia solan;*, Systemic Acquired Resistance (SAR); Sheath blight disease.

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INTRODUCTION

Plants are sessile organisms that have a unique immune system that protects them from pathogen infections. This natural immunity is divided into two types of existing defense mechanisms, which are the structural and chemical defense system (Moerschbacher & Mendgen, 2001). In compatible interactions however the pathogens are able to overcome these defences and enter plant cells where they produce proteins that are identified as effectors during infection (Jones & Dangl, 2006). Effectors will be detected by the resistance proteins (R proteins) in plants which in turn would trigger plant's immune system (Zipfel, 2008). Hypersensitive response (HR) is the most frequent response found in the defense systems which results in programmed cell death at the infection site (Jones and Dangl 2006). At the same time, HR leads to systemic acquired resistance (SAR) confers secondary resistance to a broad spectrum of infection (Dong, 2004). SAR is an induced immune system rendering broad spectrum defense response in plants (Fu & Dong, 2013).

SAR is mediated by salicylic acid (SA) accumulation which in turn coordinates the expression of pathogenesis-related (PR) genes and disease resistance in plants (Gaffrey et al., 1993; Ryals et al., 1996; Paherowska-Mukhtar et al., 2013). SA is a well known important signal component in the activation of the SAR system in a wide variety of plants (Rivas-San Vicenta & Plasencia, 2011). Transgenic *Arabidopsis* plants expressing the *nahG* gene, that codes salicylate hydroxylase that prevents the accumulation of SA, diminishes SAR in plants (Lawton et al., 1995). The relationship between SA and SAR has been studied extensively and high level of SA induced SAR is reported in Arabidopsis, tobacco and tomato during pathogen infections (Shern et al., 2001).

NPR1 (*nonexpressor of pathogenesis-related gene1*) gene is a key signal transducer in SA-mediated SAR and it was cloned in 1997 (Cao et al., 1997). NPR1 is constitutively expressed and is increased two fold following exogenous application of SA in a wide variety of plants (Ryals et al., 1996). SA treatment induces the transition of NPR1 oligomer to monomer that in turn promotes nuclear localization which is essential for the activation of *PR* genes (Mou et al., 2003; Dong et al., 2004). The induction of *PR* gene expression which are marker genes of SAR confers enhanced defense on secondary infection adjacent to the local infection site in *Arabidopsis* (Ryals et al., 1996). The over-expression of *npr1* gene in Arabidposis and *A. thaliana NPR1* gene in transgenic rice resulted in elevated *PR* gene expression and increased resistance to bacterial blight disease (Shern et al., 2001). This therefore strengthens the regulatory role of NPR1 in SAR. In addition, NPR1 mutants that were unable to recognize the SAR inducing agent failed to trigger SAR and showed susceptibility to avirulent pathogens. Thus, NPR1 protein plays an essential role in SA-mediated SAR. The *NPR1-like* gene, *OsNPR1,* had been isolated in rice and the over-expression of *OsNPR1* showed an increase resistance in rice plants to bacterial blight disease (Chern et al., 2005; Yuan et al., 2007).

Basal SA levels in rice is very much higher compared to tobacco, Arabidopsis and the level has little or no change post pathogen induction (Silverman et al., 1995). This shows that the SA-mediated SAR in rice maybe different from others plants. High endogenous SA levels in rice appear to be insensitive to exogenous SA treatment (Yang et al., 2004; Fu & Dong, 2013). Thus, it raises a question as to whether high levels of SA in rice will confer greater NPR1 nuclear localization and further activate *PR* gene expression in a non-induced condition. To further examine the action of SA, exogenous SA treatment was applied in four rice varieties to determine the NPR1 transcripts levels and defense response in rice. Here we observed the effect of exogenous SA on *Rizoctonia solani* infected rice varieties on the level of *OsNPR1* gene expression in rice with and without SA treatment.

Materials and Method

Plant materials

Four rice varieties, Pongsu Seribu, MR219, MRQ74 (Maswangi) and CO39 (Amaravathi) were used in this study. Pongsu Seribu, MR219 and MRQ74 seeds were obtained from the Malaysian Agricultural Research and Development Institute while CO39 was obtained from the rice seed collection at University of Minnesota, USA. These four rice varieties were selected based on their varied response to *Rhizoctonia solani* infections which are either susceptibility, moderately susceptible and moderately resistant to rice sheath blight disease.

Pathogens Inoculation and disease evaluation

Five weeks old rice plants (7-8 leaves stage) were inoculated with *Rhizoctonia solani* strain 1802/KB as described by Park et al., (2008). The inoculums were prepared on the potato dextrose agar (PDA). Agar plugs were cut from the outer edge of three day old cultures and placed on the base of sheath. The inoculated leaf sheath with agar plug was covered instantly with aluminium foil. The aluminium foil was removed three days post inoculation. Infected rice plants were left in a humidity chamber for disease development where the humidity was maintained between 80 to 100%. Seven days post infection, the degree of disease severity of sheath blight symptoms was recorded as described previously by Park et al., (2008). The degree of disease severity was assigned as in table 1.

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Table 1. Disease severity of sheath blight described by Park et al., (2008)

Scale	Description
0	No lesion
1	Appearance of water-soaked lesions
2	Arise of necrotic lesions
3	< 50% of the leaf sheath cover by necrotic lesions
4	> 50% of the leaf sheath cover by necrotic lesions
5	Entire leaf sheath cover with necrotic lesions

Treatment with Salicylic Acid (SA)

2.0 mM SA was dissolved in 0.1% (v/v) ethanol and sprayed onto the leaves of rice plants till they were drenched. The rice plants were treated one hour before inoculation with the fungal plugs that were prepared as above. Control plants were treated with 0.1% (v/v) ethanol solution only (Liu et al., 2007).

Isolation of Total RNA

Total RNA was isolated via CTAB method from leaves collected at 0, 6, 12 , 24 and 48 hpi (hour post inoculation) (Zhang et al., 2012; Untergasser, 2008). About 100 mg of leaves were ground into fine powder in liquid nitrogen. The ground tissue was used in the CTAB extraction method (Untergasser, 2008).

PCR and RT-qPCR analysis

RNA samples were treated with DNases I according to the manufacturer's protocol (Fermentas). High-Capacity cDNA Reverse Transcription Kits, (Applied Biosystems®) was used to synthesize the first strand cDNA, through reverse transcription as per manufacturer's recommendations. PCR was carried out by pre-denaturation for 5 minutes at 94 °C, followed by 30 cycles of denaturation for 30 seconds at 94 °C, 30 seconds of annealing at 56 °C and 30 seconds of elongation at 72 °C. Each 20 µL of reaction mixture contains 200 ng of cDNA template, 2.0 mM of $MgCl_2$, 1X of PCR buffer (Invitrogen), 0.80 mM of dNTP mixtures, 2 U of *Taq* DNA polymerase, 1.0 pmol of forward primer (5'GCAAGGATTATGTTTCCGATG-3') and reverse primer (5'GTCTTTCAGGAGGTGGATTTG-3'). RT-qPCR reaction was performed with Power SYBR® Green PCR Master Mix and AmpliTaq Gold® DNA polymerase. The primers used for RT-qPCR amplification was the same primer set used in the PCR analysis. Amplification of actin gene was performed as reference with the primers as described in Narsai et al., (2010).

RESULTS

Disease evaluation in different rice varieties

The levels of resistance in a plant may correspond to the SA levels within the plant. Four rice varieties with different capabilities in resistance to sheath blight disease were used. Preliminary observation showed that variety Pongsu Seribu is moderately resistant with the lowest disease severity scale shown among the four varieties studied (Table 2). However, Pongsu Seribu did not show an increase in resistance post SA treatment (Figure 1A and 1B). Meanwhile MR219 and MRQ74 were moderately susceptible to *R. solani* with a severity index of 4 (Figure 1C and 1E) where more than 50% of the total sheath length was covered with lesions. SA treatments at 2.0 mM did not lead to an increase in disease resistance in MR219 and MRQ74 (Figure 1D and 1F). However, the susceptible variety CO39 showed a reduction in susceptibility level to *R. solani* infection post SA treatment. The disease symptom shown in the leaf sheaths pre-treated with SA were less than 50% of the total sheaths length (Figure 1G) while white lesions spread throughout the leaf sheaths without SA pre-treatment (Figure 1H). Table 2 show that the level of disease index in a SA treated CO39 was comparable to the moderately resistant rice cultivar Pongsu Seribu. In order to determine if this morphologically detected difference in disease severity level was accompanied by changes internally, we monitored the changes in *OsNPR1* gene expression levels.

RT-PCR *OsNPR1* gene and Sequence Analysis

The product amplified by the primers designed for this study resulted in an approximately 100bp product. The product was sequenced and the resulting 115bp product is presented in Figure 2. The BLAST analysis performed showed that the amplified product of the *OsNPR1* gene in this studied was identical to part of the *NPR1-like* genes in *Oryza sativa indica* (GenBank accession No. AY923983.1) and shared 94% of homology with the *NPR1-like* genes. The sequence of the PCR products corresponds to the sequence of *NPR1-like* genes from nucleotides 1397 to 1505 in the *Oryza sativa indica* (Figure 2).

There are six nucleotides in the amplified products that differ with the sequence of *NPR1-like* genes from nucleotides 1397 to 1505 (Figure 2). In addition, each end of the sequence of PCR product has three extra nucleotides which differ from the *NPR1-like* genes sequence. The differences shown between the sequences of the *OsNPR1* gene and *NPR1-like* gene may be due to different rice varieties used. The genome sequences of different varieties are almost similar, yet there are differences that exist at the genome sequence level between each variety. Therefore, the differences at the sequence level in the amplified product of the *OsNPR1* gene in this study may be due the existence of natural variant among *NPR1*

gene in different indica rice cultivar. The rice cultivar used in this study is a Malaysian indica rice variety, MR219 whilst the *NPR1-like* cDNA used in comparison was from cultivar IRBB21.

Analysis of OsNPR1 Expression Profiles via RT-PCR

As *NPR1* gene is a key regulator in SA-mediated defense response in plants, disease resistance level is thought to be correlated with the increase of NPR1 transcript which activates *PR* gene expression. In order to evaluate the effect of SA treatment on *NPR1* gene expression in *R. solani* infected rice, the *OsNPR1* expression levels were monitored in four rice varieties via RT-PCR.

There was an increase in OsNPR1 transcript levels in the susceptible variety CO39 in response to SA treatment pre inoculation with *R. solani*. Higher level of OsNPR1 transcript observed corresponds to the enhanced level of resistance observed in CO39 rice plants (Figure 1 and Figure 3). Non SA treated plants only showed detectable levels of OsNPR1 expression at point of inoculation (0 hrs) and later again at 48 hpi. In MR219, the levels of expression in non SA treated inoculated plants were similar to that observed in control plants with only a slightly higher level of expression detected in the SA and inoculated plant samples. Although the highest level of OsNPR1 expression was observed in MRQ74, their profiles were similar to MR219 where the transcript levels were at the same levels for control and SA treated plants i.e. almost constant at all time points. Though MRQ74 showed higher level of transcript levels in treated and non treated plants compared to the other varieties used in this study, the level of disease resistance exhibited by this variety is similar to that observed in MR219 (Figure 1; Table 2). Similarly the results obtained from Pongsu Seribu also showed that the level of transcripts present in treated and non treated lines were comparable to those observed in MR219 (Figure 3) and does not correspond to the level of disease severity observed in the variety with or without SA treatment (Figure 1; Table 2). No direct correlation could be made from comparing the levels of OsNPR1 transcript detected in varieties and the level of disease resistance afforded.

Expression Analysis by RT-qPCR

Further, we performed a quantitative real time PCR (qPCR) on elite rice variety MR219 to detect the relative NPR1 transcript level at several time points, (hpi) to examine the difference of *OsNPR1* gene expression level between SA-treated and untreated MR219 with reference actin gene (Figure 4). The single peak detected in melt curve analysis indicated that these two pairs of primer designed in this study can efficiently amplify *OsNPR1* and actin genes (Figure 5). Expression of actin gene was stably expressed in every sample at all studied time points. However, the expression of actin gene was not constant throughout the 48 hpi. Though reference genes such as actin are supposed to be constitutively expressed at a stable level, there have been reports that certain housekeeping genes may be influenced by internal and external factors which may result in fluctuation of expression levels in experimental hosts (Volkov et al., 2003). In quantitative PCR the selection of reference gene is crucial. Several genes have been tested as reference genes in different organisms and different tissues. Actin has been used widely as a reference gene in rice research however there are others genes that have been included as reference gene for rice such as β-tubulin (Nicot et al., 2005; Chandna et al., 2012).

However though the actin gene showed some fluctuation in the level expressed at each time point, these increase or decrease corresponded to the levels expressed in the control plants. In observing the expression levels of OsNPR1, we noticed a rhythmic profile. The *OsNPR1* gene has been reported to be a gene that is regulated by circadian rhythm and is light regulated (Zhang et al., 2012). This may explain the rhythmic expression of OsNPR1 in control, treated and untreated samples. The induced relative expressions of *OsNPR1* gene observed in SA-treated MR219 at 6 hpi and 24 hpi were 0.74 folds and 1.00 folds respectively (Figure 4). The results from the RT-qPCR analysis corresponds to the results obtained from the RT-PCR analysis where the highest expression levels were detected at 6 hpi and 24 hpi (Figure 3 and Figure 4).

Further scrutiny of the RT-qPCR results obtained with MR219 shows that the expression levels of OsNPR1 in SA treated and inoculated samples did not differ significantly to the data obtained with the non SA treated *R.solani* inoculated plants. This therefore indicates that the induction of expression observed in the plants compared to control plants was most likely due to pathogen invasion. Though the application of exogenous SA did increase the relative expression levels of MR219, but the difference was not substantial (Figure 4). The increment of gene expression at 6 hpi was probably induced by the effectors secreted by pathogen. Once the effectors are recognized by resistance protein (R protein) of plants, R protein will trigger the effector-triggered immunity (ETI) and activate the host immune system. As the effectors were suppressed, the *OsNPR1* revealed reduced expression at 12 hpi. However, the *OsNPR1* gene expression increased again at 24 hpi. The recognition of newly acquired effectors triggers the ETI again, causing the increased expression of *OsNPR1*(Jones & Dangl, 2006).

DISCUSSIONS

We have evaluated four rice varieties for *R. solani* infection in SA treated and non treated rice plants. Disease evaluation shows rice variety Pongsu seribu was the most resistant (moderately resistant) followed by MR219 and MRQ74 where CO39 was the most susceptible line in this study. Three out of four rice varieties used in this research showed no enhanced disease resistance when treated with exogenous application of SA. This is in agreement with a previous study conducted by Silverman et al., (1995) who reported that exogenously applied SA did not enhance disease resistance in rice. However, the susceptible rice variety CO39 showed increase in resistance to *R. solani* when treated with SA. Therefore in susceptible lines and more specifically in lines that have lower basal levels of endogenous SA, the application

of exogenous SA may help increase the internal SA levels and thus increase the levels of defense gene expression (Fu & Dong, 2013).

Herbers et al., (1996) hypothesized that an SA-independent, sugar-mediated route may play a significant role in the early stages of defense against plant–pathogen interactions, before the onset of SA-mediated SAR (Mohammad Reza & Wim Van den Ende, 2012). NPR1 plays a crucial role in SA-mediated SAR in dicots (Cao et al., 1994) and NPR1 homologs such as OsNPR1 which activates *PR* genes via SA signal transduction (Hiroshi et al., 2010). In addition previous reports have shown that OsNPR1 activation may be mediated through the antagonistic cross-talk between the SA- and JA-dependent pathways in rice (Spoel et al., 2003; Yuan et al., 2007). However, little is known about the defense network or the true role of SA induced disease resistance in rice (Silverman et al., 1995; Yang et al., 2004).

High basal levels of SA in rice suggest that SA mediated defense pathway in monocots might be different from that of dicots. The possibility cannot be excluded that fine-tune regulation or components of the *NPR1*-mediated defense pathway may be somewhat different in rice compared with *Arabidopsis* (Chern et al., 2001), given the fact that *PR* genes are differently expressed in *Arabidopsis* and rice. Alternatively, the rice SA receptor (if any) might perceive the SA signal in a manner different from that in *Arabidopsis*. Thus, most probably, SAR-like disease resistance activation against sheath blight through SA signaling, may be less dependent on the elevation of SA levels in rice cells. In addition we can not preclude the possibility that there may be another regulatory switch for SAR activation in rice which may not involve *OsNPR1* gene.

CONCLUSIONS

Although SA is an important component in the activation of SAR, the SA mediated defense system in rice may be different from others plant as rice plants have a high level of endogenous SA and the application of exogenous SA did not significantly contribute towards the activation of the defense mechanism in rice.

Acknowledgements

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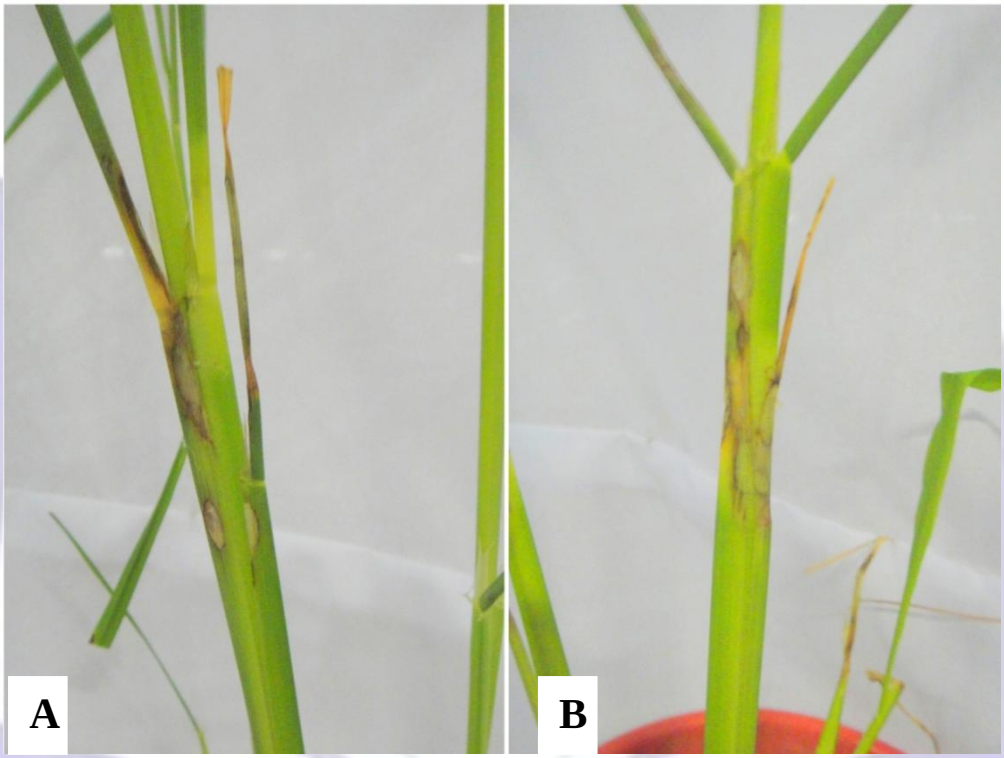
TABLES AND FIGURES

Table 2. Disease severity scale recorded seven days post inoculation

Rice Variety	Disease Severity Scale	
	With SA Treatment	Without SA Treatment
Pongsu Seribu	3	3
MR219	4	4
MRQ74	4	4
CO39	3	5



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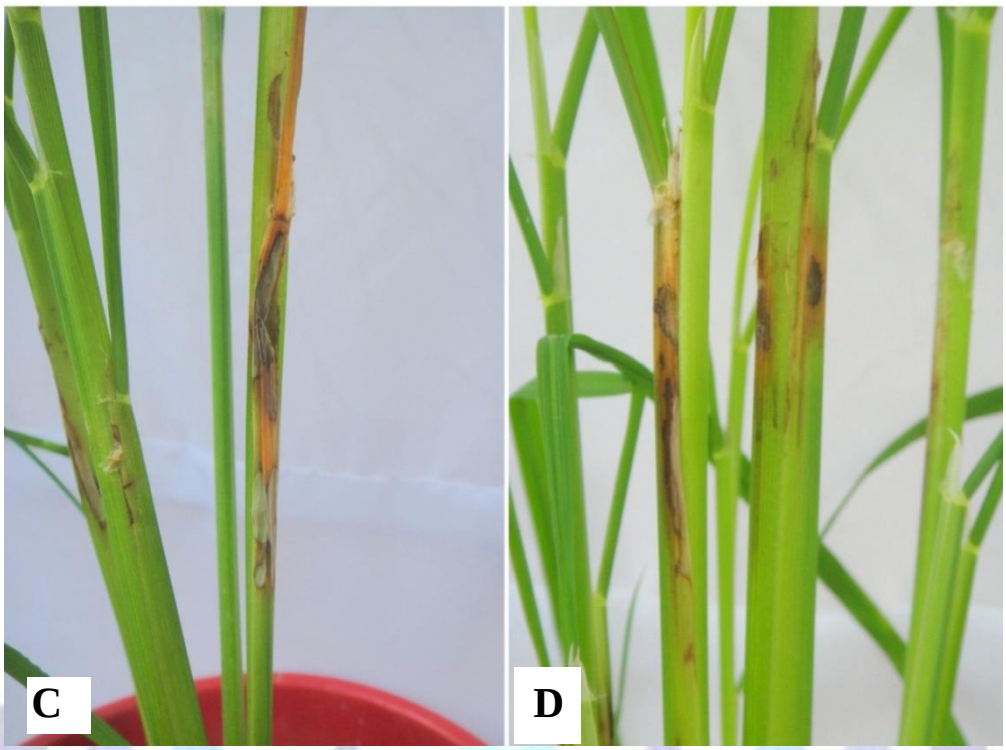

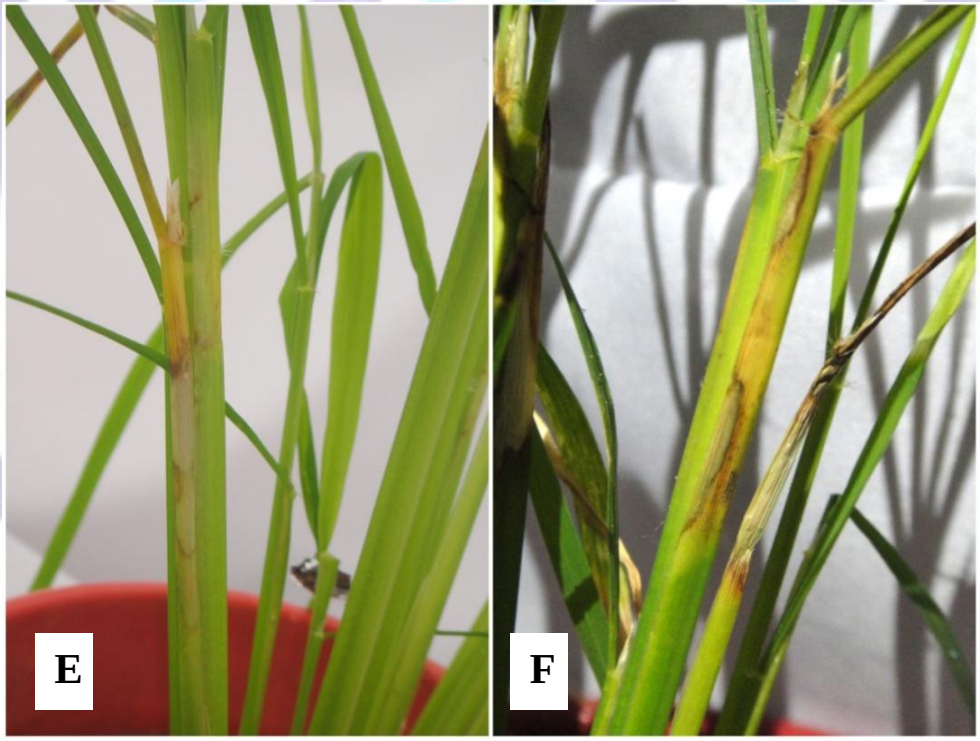

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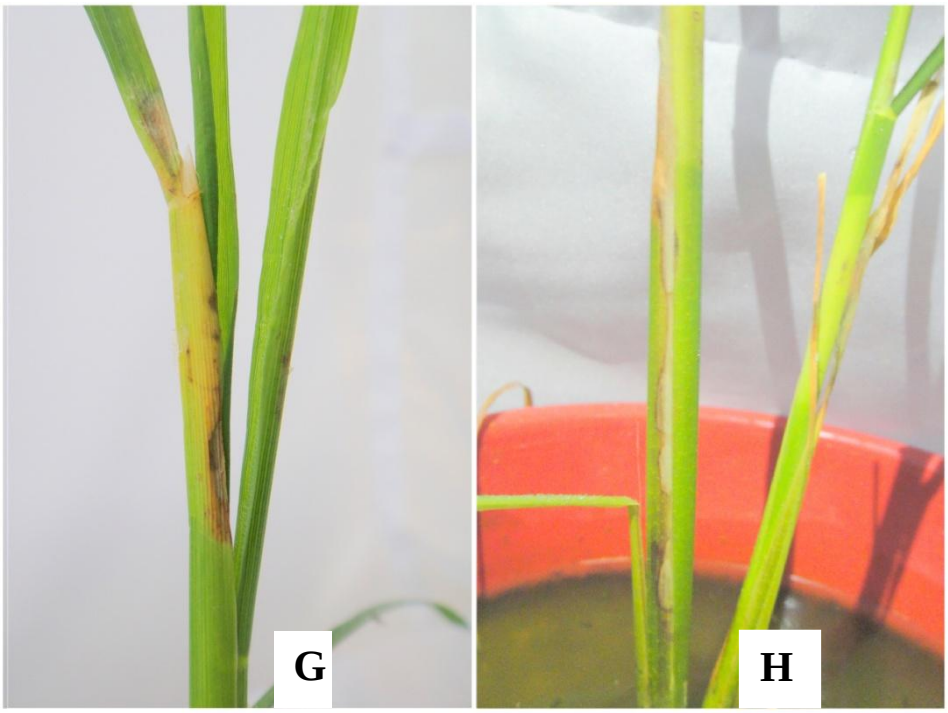

Fig. 1: Lesions formed of leaf sheath seven days after inoculation for SA-treated and untreated rice plants: A, SA-treated variety Pongsu Seribu; B, Untreated variety Pongsu Seribu; C, SA-treated variety MR219; D, Untreated variety MR219; E, SA-treated variety MRQ74; F, Untreated variety MRQ74; G, SA-treated variety CO39; H, Untreated variety CO39

Oryza sativa (indica cultivar-group) NPR1-like 1 mRNA, complete cds

Sequence ID: gb|AY923983.1| **Length:** 2119 **Number of Matches:** 1

Range 1: 1397 to 1505 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
169 bits(91)	6e-39	108/115(94%)	6/115(5%)	Plus/Plus

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Sbjct  1454  GGAACTTTGGAATTTAACCTG--GGTTCTGGTGCAAATC-CACCTCCTGAAAGAC  1505
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Fig. 2: BLASTn analysis of the amplification product, *OsNPR1* gene with *O. sativa* Cv Indica NPR1-like 1 mRNA (Gene bank id: AY923983.1)

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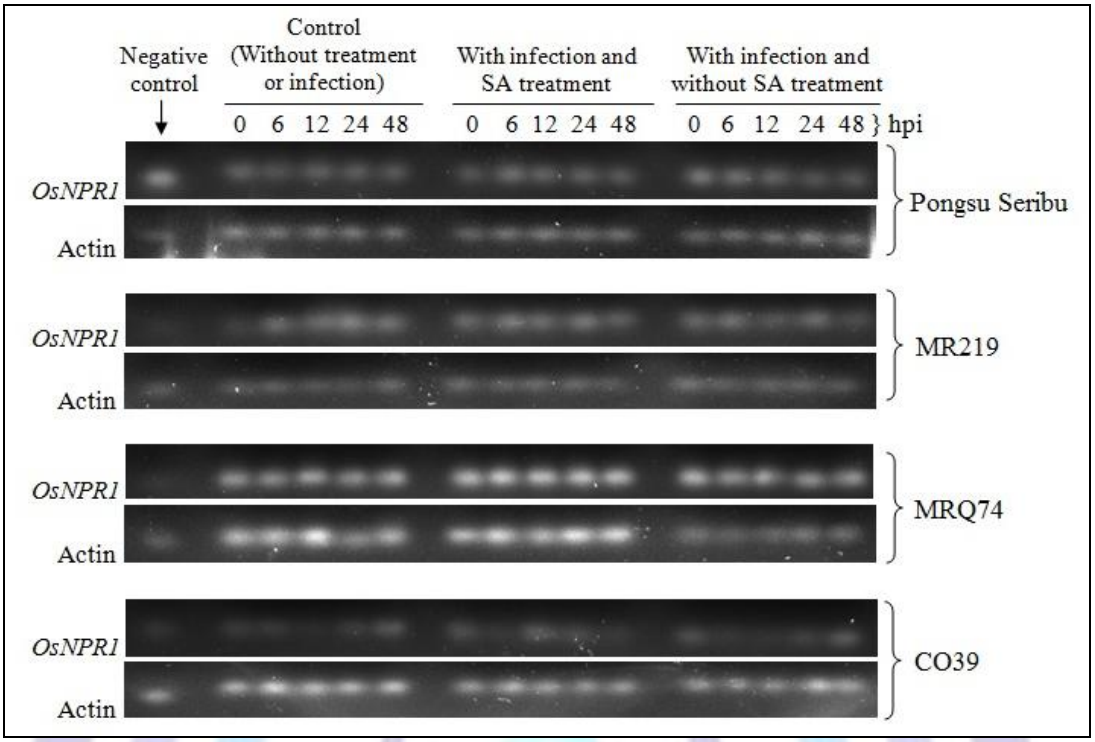

Fig. 3: Amplification products of *OsNPR1* and actin gene for the varieties examined at different hours post inoculation

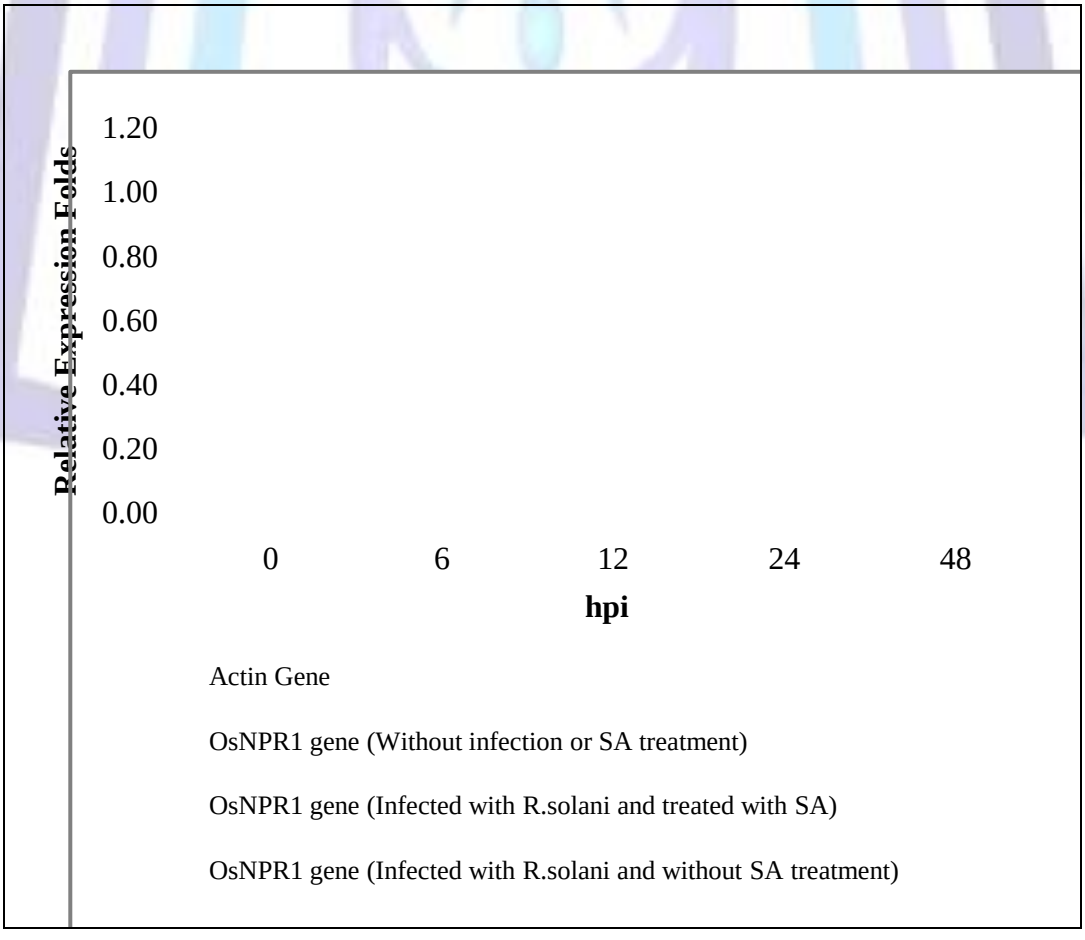

Fig. 4: Expression analysis of *OsNPR1* gene in MR219 in SA treated and non-treated rice plants

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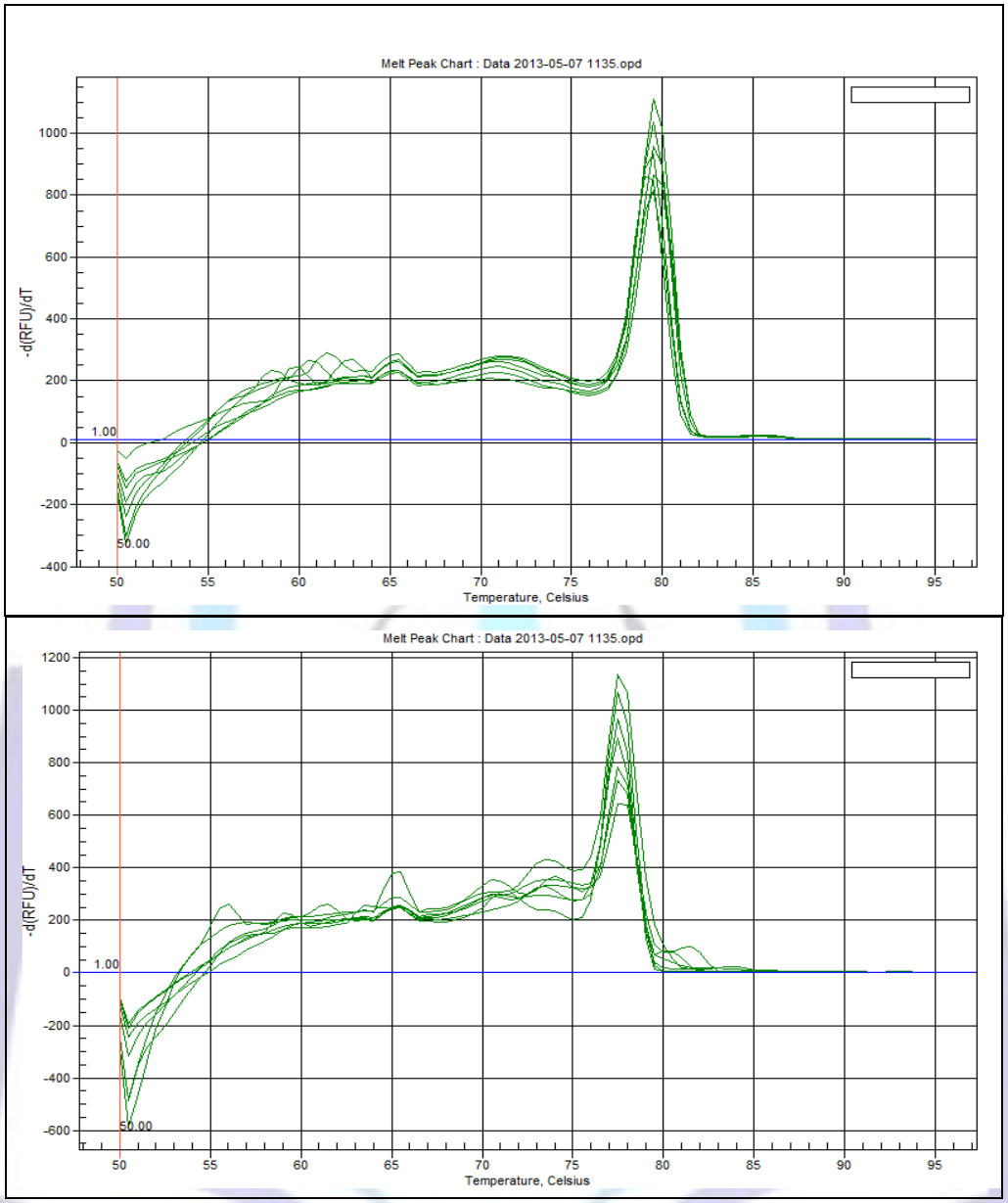

Fig. 5: Melt curve analysis of *Actin* and *OsNPR1* gene via RT-qPCR

Author' biography with Photo

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Kalaivani Nadarajah was born and raised in Malaysia. She graduated from Universiti Kebangsaan Malaysia, with a bachelor's degree in Microbiology. She than obtained a Masters in Microbiology specializing in Fungal Genetics. She earned a doctorate in Plant Molecular Biology specifically in the area of plant microbe interactions from JIC United Kingdom. She is an Associate Professor and lectures at the School of Environmental Sciences and Natural Resources, Universiti Kebangsaan Malaysia. Her past and current researches concentrates on plant diseases, plant microbe interactions, modulation of stress responses in plants and plant omics. She has authored 45 papers in indexed journals.