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Expression of Rice Resistance Gene *OsNPR1* Against Bacterial Leaf Blight on Black Rice Cultivar 'Cempo Ireng' after Salicylic Acid Treatment

Ikhsan Maulana[1], Triyaningsih[1], Tri Rini Nuringtyas[1,2], Yekti Asih Purwestri[1,2*]

[1]*Biochemistry Laboratory, Department of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada, Indonesia*
[2]*Research Center for Biotechnology, Universitas Gadjah Mada*
**Corresponding author: yekti@ugm.ac.id*

***Abstract*—** Black rice is an alternative staple food better than white rice. It has lower carbohydrates, but higher anthocyanin compared to white rice. Nowadays, black rice consumption has increased, production needs to be increased to accommodate the demand. However, to our knowledge, there is a lack of information about black rice resistance against biotic stresses, especially Cempo Ireng as one of its cultivars. This information needed for optimal Cempo Ireng cultivation. In this research we applied salicylic acid (SA) treatment to improve black rice resistance to bacterial leaf blight (BLB) caused by *Xanthomonas oryzae (Xoo)*. We determined chlorophyll level as it exhibits the ability of plants in producing yield. We also analyzed rice resistance gene *OsNPR1* expression level from three cultivars: Java14 (resistant control), Cempo Ireng, and IR64 (susceptible control), against *Xoo*. This gene is supposed to suppress chlorophyll production and the photosynthesis process. We sprayed all the plants with SA before inoculated with *Xoo*. IR64 24h had the lowest chlorophyll level (0.576 ± 0.066 mg/g), meanwhile Java14 72h had the highest level (2.358 ± 1.301 mg/g). *OsNPR1* expression did not show any significant change in Java14 and Cempo Ireng after being inoculated. However, IR64 showed increasing *OsNPR1* in 72h and did not change in 96h after inoculation. It showed that *OsNPR1* played an important role in IR64 resistance against *Xoo*, but not in Cempo Ireng and Java14.

***Keywords*— *Xanthomonas oryzae*, *OsNPR1*, Cempo Ireng, chlorophyll**

INTRODUCTION

Black rice is consumed more nowadays due to its health benefit to humans. This rice contains high anthocyanin in the pericarp which gives purple-blackish color to its grain. It also has lower carbohydrate content compared to white rice (Apridamayanti et al., 2017). Anthocyanin has many health benefits including eyesight improvement for glaucoma patients and cancer cell prevention (Hock et al., 2017). To meet the demand, the production of black rice is needed to be increased. However, both biotic and abiotic stresses in the environment inhibit the optimal production of rice. One of the most devastating biotic stress is bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* (*Xoo).*

BLB causes major damage and compromises plant physiological processes resulting in mass decrease productivity (yield grain), up to 60%. In some cases, it decreased yield grain up to 100%. *Xoo* attacks rice in several regions in Asia including Malaysia, China, and Indonesia (Shanti et al., 2010; Solekha, 2016). Rice with this disease produces greyish lesions along the side part of the leaf at tillering stage. The symptoms increase with plant growth until the entire leaf turns yellow and wilt. Besides, *Xoo* infection reduces chlorophyll, which in turn decreasing plant ability to perform photosynthesis (Gnanamanickam, 2009; Shi et al., 2015).

Plants have several defense mechanisms against the pathogen. One of them is systemic acquired resistance (SAR). This defense is

generating resistance against secondary infection in systemic tissue from a vast type of pathogens, which, in turn reduces the severity of next infection from the same pathogen. This defense induces reactive oxygen species (ROS) production, hypersensitive responses (HRs), cell wall fortification, resistance enzyme, and others pathogen growth-inhibiting substances (Thanh et al., 2017). SAR is effective, long-lasting, and may be passed to the next generation via DNA methylation changes in several genes (Luna et al., 2012).

SAR is induced upon increasing salicylic acid (SA) content in the plant body via pathogen attack or exogenous application of SA and its analog. Interestingly, exogenous induction does not show a negative impact on plant growth when applied at appropriate doses. Furthermore, SA induces resistance without directly affect the pathogen. Therefore, it is less likely to cause pathogens resistance (Takatsuji, 2014). To achieve SAR, SA affects gene expression via two separated pathways, *OsNPR1*-dependent and *WRKY45*-dependent pathways. Both pathways can generate SAR against many pathogens, even toward the same pathogen. However, *WRKY45* may have a different role in different rice cultivar against *Xoo*. Tao et al. (2009) found *WRKY45* overexpression in *japonica* rice resulting in increasing susceptibility, while Shimono et al. (2012) found contradicted results. However, to our knowledge, there were no similar results in *OsNPR1* overexpression. It conferred resistance against *Xoo* in both *japonica* and *indica* rice. In this study, we performed chlorophyll determination and *OsNPR1* expression analysis on three rice cultivars, IR64, Java14, and especially black rice cultivar Cempo Ireng.

MATERIALS AND METHODS

A. *Plant Material*

Three cultivars, Cempo Ireng, Java14 (resistant), and IR64 (susceptible), used in this study were obtained from rice seed collection Research Center for Biotechnology Universitas Gadjah Mada.

B. *Salicylic Acid Treatment*

Fourty five days old rice plants were sprayed with SA solution (500 μM) and kept in plastic shade for 24 h prior to inoculation of *Xoo* and mock treatment.

C. *Xoo Inoculation*

Xoo pathotype IV isolate used in this study was a collection of Research Center for Biotechnology Universitas Gadjah Mada. Peptone Sucrose Agar (PSA) was prepared with 5 g of sucrose, 1.25 g peptone, 0.125 g K_2PO_4, 0.0625 g $MgSO_4.7H_2O$, 5 g agar, and 250 ml of dH_2O. The mixture was boiled until all the ingredients dissolved, then poured into the Petri dish. *Xoo* was cultured on PSA medium and incubated for 72 h at room temperature. The inoculum was harvested from the dish and was suspended in an Erlenmeyer flask containing deionized water. The suspension was adjusted to 10^8 colony-forming units (CFU)/mL with absorbance measurement (absorbance=0.3 at 600 nm).

Inoculation was performed between 3.00 and 5.00 p.m. in a greenhouse to avoid heat and evaporation. Each cultivar was divided into two groups including *Xoo* treatment and mock. Inoculation was done using the leaf clipping method. Sterile scissors were dipped into *Xoo* suspension then used to cut leaf tip. In mock treatment, scissors were dipped into sterile water before being used. All the plants were kept in the greenhouse.

D. *Chlorophyll and Carotenoid Content Determination*

Samples were taken at 0, 24, 72, and 96 h after *Xoo* inoculation by cutting 4-5 cm below the inoculated tip. The samples were stored in zip lock bag and put in a -20°C freezer. The leaf sample was grounded using a mortar and liquid nitrogen. Chlorophyll and carotenoid content were determined using UV-Vis Spectrophotometer at 470, 645, and 664 nm wavelength. The absorbance value was used to determine the content in mg/g fresh weight (FW) using protocol from Harborne (1984) with modification.

E. *OsNPR1 Gene Expression Analysis*

Total RNA of the sample was extracted using FavorPrep™ Plant Total RNA Mini Kit (Favorgen). The total RNA quantity of each sample was determined using NanoDrop™ Spectrophotometer (Thermo Scientific™). One

microgram of RNA was subjected to synthesize complementary DNA (cDNA) using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo). Then, the quality of cDNA was determined by PCR amplification of a housekeeping gene, Ubiquitin (*Ubq*) followed by electrophoresis and UV visualization of amplicon.

Semiquantitative analysis of resistance gene was performed using *OsNPR1* as the gene target and *Ubq* as an internal control. The *OsNPR1* primer was created using Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/) from *O. sativa* Japonica Group sequence data. Primer *Ubq* was obtained from Sutrisno et al. (2018). *OsNPR1* primer sequence was forward 5'-GAACACCATTGCTCCCAAAT-3' and reverse 5'- ACGGGGCAATAGCTGTAAGA-3' (amplicon size: 152 bp), and *Ubq* primer sequence was forward 5'-CTCAGGCTCCG- TGGTGGTATG-3' and reverse 5' GTGATAGTTTT-CCCAGTCAACGTC-3' (amplicon size: 200 bp). *OsNPR1* was expressed and amplified by quantitative real-time PCR (qRT-PCR) with *Ubq* as an internal control. In this study, we used cDNA of the sample as a template and kit ExcelTaq™ 2X Fast Q-PCR Master Mix (Smobio). The relative expression levels were analyzed using the method from Livak and Schmittgen (2001).

F. *Statistical Analysis*

We performed descriptive and quantitative data analysis for chlorophyll and carotenoid content and relative gene expression level using ANOVA, followed by Duncan's multiple range test (DMRT), and Pearson's correlation coefficient (PCC).

RESULTS AND DISCUSSIONS

Chlorophyll and Carotenoid Content Determination

The chlorophyll content is the biggest factor affecting the rate of photosynthesis, because of its function in absorbing energy from light to drive photosynthetic reactions. Even so, not all light energy that can be absorbed by chlorophyll is useful in photosynthesis. Conversely, this energy can also cause chlorophyll breakdown. In addition, pathogenic infections can cause chloroplast damage and inhibit chlorophyll production. Plants have additional pigments in the form of carotenoids which function to protect chlorophyll from damage caused by light energy (Urry et al., 2021). Carotenoids have antioxidative abilities that can increase resistance to pathogens, which is why chlorophyll and carotenoid content are important parameters to determine plant ability in pathogens resistance (Boba et al., 2011; Shi et al., 2015).

Based on **Figure 1.,** it is known that the lowest total chlorophyll content was in susceptible cultivar *Xoo* IR64 at 24 h (0.576 ± 0.066 mg / g), while the highest was in resistant cultivar Mock Java14 at 0 h (2.456±0.0156 mg / g). However, all three cultivars, both Mock and *Xoo* treatment, showed no decreasing trend in chlorophyll levels. Shi et al. (2015) stated that chlorophyll levels remained relatively constant until day 3 (72 h), a decrease was seen from day 5 (120 h) after *Xoo* inoculation. Decrease in leaf chlorophyll content can be caused

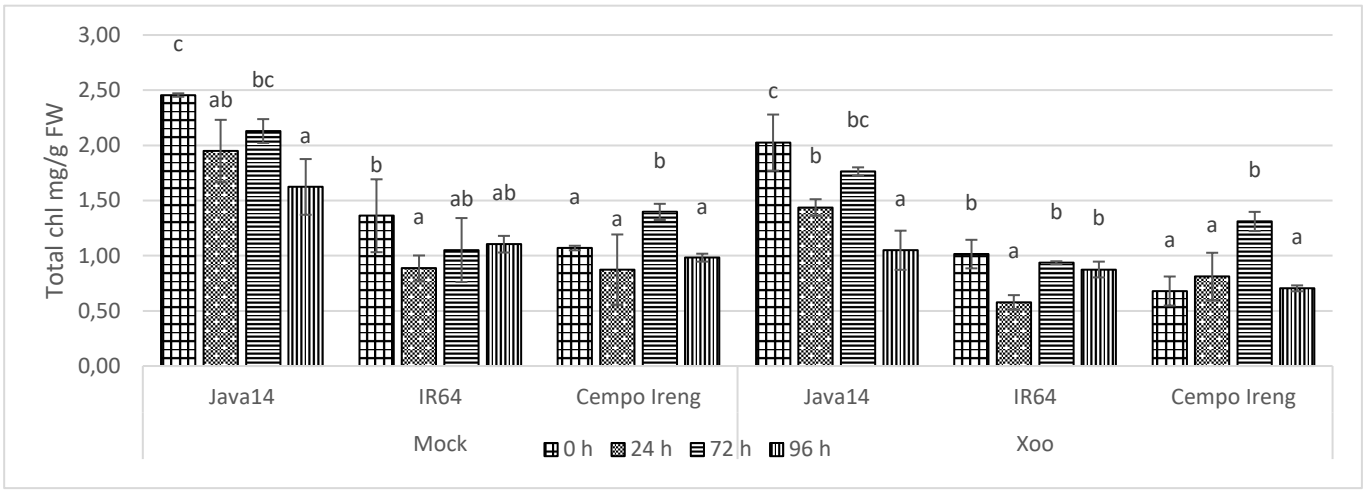

Figure 1. Total chlorophyll in three rice cultivars. Different letters in the same group and cultivars were significantly different (P<0.05)

by the production of free radicals around the infection site which will damage leaf cells and bacterial cells. In addition, it can also be produced by inhibition of chlorophyll synthesis as a compensation for the defense response against pathogens (Xu et al., 2018).

The lowest carotenoid content was in susceptible cultivar (IR64) infected by Xoo at 24 h (0.188±0.008 mg / g), while the highest was in resistant cultivar (Java14) under mock treatment at 0 h (0.525±0.0738 mg / g). Carotenoid content in cultivars IR64 and Cempo Ireng fluctuated, but there was no time-related trend, while Java14 showed a time-related downward trend (**Figure 2**). According to Kumar et al. (2013), carotenoid content decreased drastically after 15 days after *Xoo* inoculation. Changes in carotenoid content can be caused by pathogenic infection, or the formation of other antioxidative compounds in the isoprenoid pathway such as tocopherol (Boba et al., 2011).

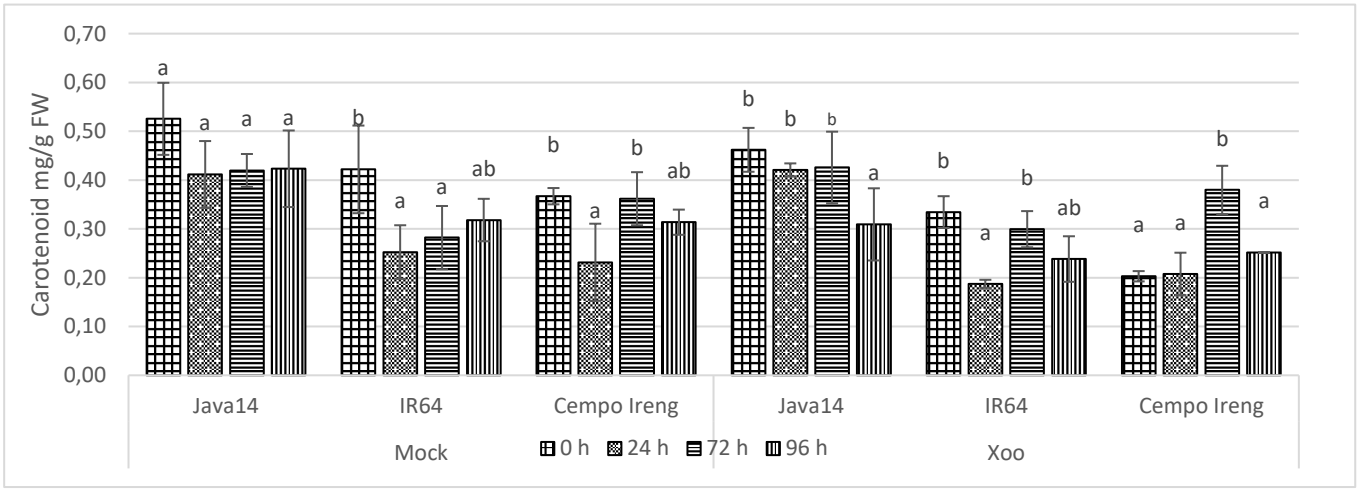

Figure 2. Carotenoid content in three rice cultivars. Different letters in the same group and cultivars were significantly different (P<0.05)

Resistance Gene Expression

Rice resistance against *Xoo* is determined by its ability to recognize and establish response to the infection. The faster a plant recognize the infection, the better it generates resistance. Plants have several mechanisms and genes involved in pathogen recognition and defense induction. Rice establishes SAR via *OsNPR1* pathway under SA induction to encounter *Xoo* infection. We used three cultivars of rice: IR64, Java14, and Cempo Ireng, to analyze *OsNPR1* expression. We sprayed all the plants using SA a day before *Xoo* inoculation to establish SAR.

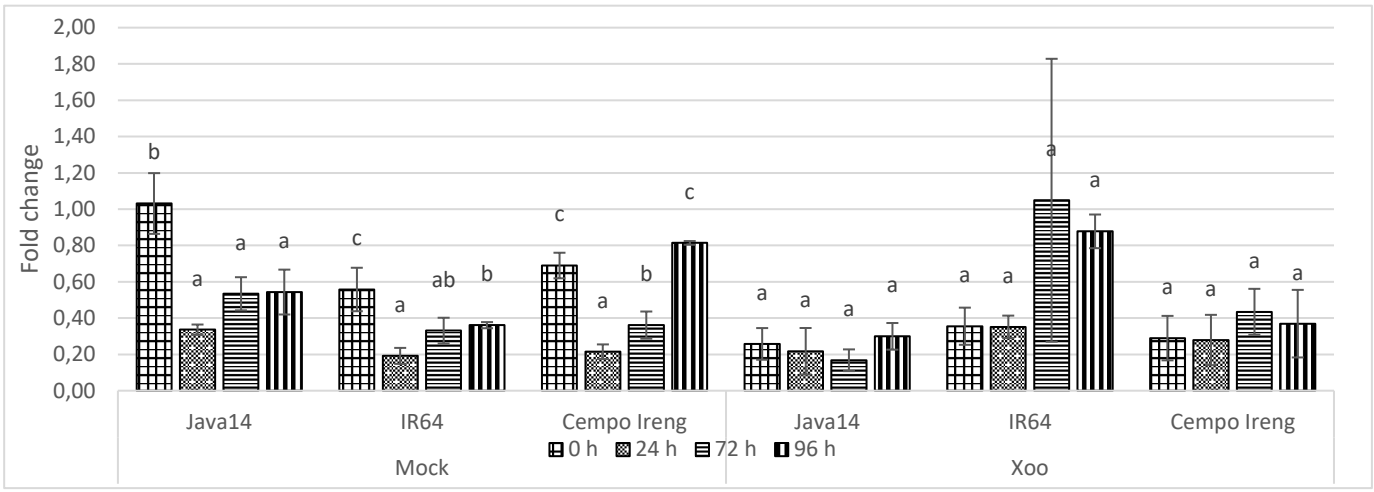

Figure 3. The expression level of *OsNPR1* in three rice cultivars. We used Mock Java14 0 h as a reference point. Different letters in the same group and cultivars were significantly different (P<0.05)

Based on **Figure 3**., it is known that the expression levels of *OsNPR1* remained relatively the same in Cempo Ireng and Java14 at 0, 24, 72, and 96 h. These results indicate that *OsNPR1* expression may not be the main pathway in *Xoo* resistance in Cempo Ireng and Java14. Another possible explanation is that the expression level increased until 4 hours after inoculation, and decreased after that (Jiang et al., 2020). Conversely, the expression level of *OsNPR1* in IR64 showed a major increase at 72 h. It contradicted the result found by Jiang et al. (2020). However, this result was coherence with Xu et al. (2013), in which the expression level of *OsNPR1* remained increasing after 48 h after inoculation. Despite this contradictory result, *OsNPR1* may be an important pathway in *Xoo* resistance due to its major increase.

To verify the previous result, we also compared the expression level of *OsNPR1* with mock treatment. **Figure 3**. showed that *OsNPR1* was downregulated in all mock treatments. It indicated that resistance was induced by wounding may repressed expression of *OsNPR1* in all the cultivars. It is known that wounding is strongly correlated with the defense mechanism induced by jasmonic acid (JA). Lee et al. (2004) stated that wounding decreased SA content in plant tissues. Therefore, it supported the previous assumption that wound inhibits *OsNPR1* expression.

Correlation between Chlorophyll Content and OsNPR1 Expression

When plants establish resistance against the pathogen, they reallocated resources from growth to defense mechanisms. This generates negative effects such as growth retardation and decreasing yield. It is called fitness cost, which represents the price of resistance to protect the plants. Sugano et al. (2010) found that SA induction repressed photosynthetic processes through the expression of resistance genes. One of them is the inhibition of magnesium-chelatase, an important enzyme in chlorophyll biosynthesis. Therefore, we performed Pearson's coefficient correlation (PCC) test. Based on **Table 1.,** it is known that total chlorophyll and carotenoid content were positively correlated to each other, while they negatively correlated with *OsNPR1* expression. It indicated that *OsNPR1* expression might inhibit chlorophyll and carotenoid synthesis.

Table 1. Correlation between *OsNPR1* level of expression, total chlorophyll, and carotenoid content.

	OsNPR1	Total chl	Carotenoid
OsNPR1	1		
Total chl	-0.278	1	
Carotenoid	-0.092	0.361*	1

*Correlation is significant at the 0.05 level.

CONCLUSIONS

Total chlorophyll and carotenoid content in Cempo Ireng did not show a significant effect caused by *Xoo* inoculation, compared with the

susceptible control IR64. The level expression of *OsNPR1* in Cempo Ireng was lower than IR64, but slightly higher than Java14 at each time. *OsNPR1* expression negatively correlated with total chlorophyll and carotenoid content.

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