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Analysis of secondary metabolites and genes related to the pathogenicity of the rice blast fungus *Pyricularia oryzae*

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Abstract *Pyricularia oryzae* causes rice blast disease and is one of the most significant plant pathogens worldwide. The rice blast fungus can produce a variety of phytotoxic secondary metabolites and has been found to be essential for host invasion. Two isolates, virulent isolate RBR55003 and non-virulent isolate CCO56003 were selected which based on their abilities to cause blast symptoms on rice leaves. Pyriculol and picolinic acid were detected in culture filtrate of both isolates during the exponential growth phase. Expression of genes involved in fungal pathogenicity and plant defense were analyzed. The expression of *MoPks19* gene in isolate RBR55003 was higher than avirulent isolate CCO56003, indicating that *MoPks19* may play a role in the pathogenesis of RBR55003. The expression of the *OsWRKY30* gene that plays an important role in the regulation of a defense-related gene in rice was higher in CCO56003 inoculated than in RBR55003 inoculated rice sample, indicating the increased resistance of rice plants occurring in rice after non-virulent isolate infection, while the expression of the *OsCPS4* gene involved in phytoalexin synthesis in rice was not different after isolate infections. Gene expression analysis revealed different responses according to plant-pathogen relationships.

Keywords: Phytotoxin, Secondary metabolite, Rice blast, *Pyricularia oryzae*

Introduction

Rice blast disease is caused by *Pyricularia oryzae*, a fungus that can infect rice plants at all growth stages. Symptoms appear on the leaves, collar, node, neck, and panicle (Boddy, 2016). Rice blast disease causes 70 – 80 % loss of yield (Simkhada and Thapa, 2022). Leaf blast also increases the respiration rate and reduces the leaf photosynthetic rate of rice plants (Puri *et al.*, 2009). Neck blast disease increased grain sterility percentages, and reduced grain size, as well as the yield and quality traits of seeds (Khan *et al.*, 2014). This fungus has a relatively high genetic variation. The pathogen can adapt to tackle newly released blast-resistant cultivars in a short time (Sirithunya *et al.*,

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2008). The population of *P. oryzae* in Thailand is more variable than in other rice-growing regions. The pathogenic properties of fungi vary according to rice-planting sites, growing seasons, and rice-growing stages (Mekwatanakarn *et al.,* 2000). Thailand suffered from an outbreak of rice blast disease in 2019 covering an area of 432,118 rai in the Northeast, the North, the East, and the South, including Surin, Sisaket, Maha Sarakham, Sakaeo, Lampang, Loei, Phrae, Lamphun, Kalasin, Songkhla, Chaiyaphum, Mukdahan, and Chanthaburi. The most infested areas comprised plots with susceptible rice varieties such as Khao Dawk Mali 105, RD15, and RD6, as well as plots with planting density and high nitrogen fertilizer application (Department of Agricultural Extension, 2019). In 2020, a rice blast outbreak occurred in 22 provinces with an epidemic area of 39,475 rai, which reduced rice yields. Jasmine rice tends to be most affected by blast disease outbreaks (Ministry of Agriculture and Cooperatives, 2020). *P. oryzae* is a hemibiotrophic fungal pathogen that derives nutrients from living host tissues and dead or dying cells (Glazebrook, 2005). In the necrotrophic phase, the fungus synthesizes various secondary metabolites that affect the development of the rice plant and play a role in causing rice blast disease or promoting more severe disease symptoms (Teraoka, 2015). Many secondary metabolites are poisonous substances, or phytotoxins, that are toxic to the growth of plants (Collemare *et al*., 2008). Secondary metabolites such as pyriculol, pyriculariol, and tenuazonic acid are substances that induce blast-like lesions on rice leaves. In addition, pyriculone and pyrichalasin can induce necrotic lesions and inhibit the growth of rice seedlings (Teraoka, 2015).

The objectives were to assess the synthesis of secondary metabolites in the rice blast fungus that is related to pathogenicity and to examine the expression of genes involved in the synthesis of fungal secondary metabolite and defense-related genes in rice.

Materials and methods

Strains, culture, and growth conditions

Two isolates of *P. oryzae* were used in this study, including RBR55003 (virulent) and CCO56003 (non-virulent). Fungal isolates were re-growth from the stock cultures on Potato Dextrose Agar (PDA) medium (for 1 L of PDA; 200 g potato, 20 g dextrose, 20 g agar) and incubated at 28 ℃ for 5 days. The mycelium plugs 0.5 mm in diameter were transferred as inoculum to the Potato Dextrose Broth (PDB) medium and then incubated at 28 ℃ under shaking conditions (120 rpm) for 3 days. The inoculum from the PDB (10%) was

transferred to the Starch Broth (SB) medium (for 1 L of SB; 20 g starch, 2 g yeast extract). These cultures were grown at 28 ℃ with shaking at 120 rpm for 3 days.

Extraction of secondary metabolites from P. oryzae culture

The cultures were filtered through filter paper (Whatman No.1, UK). The culture filtrate was extracted with ethyl acetate (1:2, v/v) and shaken overnight (12 – 15 h). The ethyl acetate phase was collected and evaporated to dryness at 40 ℃ in a rotary evaporator. The crude extracts were dissolved in methanol and further used for LC-MS analysis.

LC-MS analysis of secondary metabolites

The crude extracts of *P. oryzae* were analyzed by LC-QTOF-MS (Agilent 1290 Infinity II LC-6545 Quadrupole-TOF) equipped with ZORBAX Eclipse Plus C18 columns (150 mm ×1.2 mm; 0.25 μm, Agilent) under the following conditions: The temperature of the column was set to 40 ℃, and 2 μl of a sample was injected. The elution was performed with a gradient of water and acetonitrile, and a flow rate of 0.2 ml/min, as described by Jacob *et al.* (2017).

Plant material and inoculation

Blast-susceptible rice varieties Khao Dawk Mali 105 (KDML 105) were planted in seedling trays and placed on plastic trays containing water. Seedlings were grown for 21 days in a greenhouse. Rice seedlings were inoculated with a spore suspension at 1×10^5 spore/ml in 0.5% gelatin solution using sprayers. Leaf samples were collected at 12, 24, and 48 hours after inoculation, and leaves collected from non-inoculated plants served as a control. Leaf samples were kept in liquid nitrogen and stored at -80 ℃.

RNA extraction and cDNA Preparation

The rice plant tissues were ground into a fine powder with liquid nitrogen. RNA was extracted from inoculated and non-inoculated rice plant tissue using Trizol Reagent (Ambion®) following the manufacturer's instructions. The RNA pellet was then dissolved in DEPC-treated water and stored at -80 ℃. One microgram of RNA was reverse transcribed with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Quantitative real-time PCR (qPCR) analysis

Real-time PCR was performed using a BioRad CFX96 Real-time PCR system (BioRad, USA) with a reaction mix (20 µl) containing 13 µl DEPC water, 4 µl of 5x EvaGreen® qPCR Mix Plus (Solis BioDyne, Estonia), 0.5 µl each of 10 µM forward and reverse primers, and 2 µl of cDNA. The primers used in this study are shown in Table 1. The real-time PCR cycling conditions consisted of an initial denaturation step (95 °C for 12 min), followed by 40 cycles of 95 ℃ for 15 s, 60 ℃ for 20 s, and 72 ℃ for 20 s. The quantification cycles (Cq) were analyzed for each gene, and gene expression levels were presented as relative expression compared with the reference gene including *ACT1* for rice and *Pot2* for *P. oryzae* gene expression.

Table 1. Primer sequences for qPCR experiments

Gene	Sequence (5' - 3')	Annealing temperature (℃)	Product size (bp)	Reference
MoPKS19	F: GCGTACCTGTCCCAATTCCA R: TGCTTCGCTTCCACAGAGAG	60	244	This research
Pot2	F: ACGACCCGTCTTTACTTATTTGG R: AAGTACGCTTGGTTTTGTTGGAT	60	99	Park *et al.* (2012)
OsCPS4	F: CTGCAGCGCTATTAACAGAC R: AGTGTAGATGAGTCGGGGTAA	59	161	Toyomasu *et al.* (2008)
OsWRKY30	F: CTACCAATGGTCTTCTTCACC R: GTTCACCTTCATCTCACCTCT	60	248	Ryu *et al.* (2006)
ACT1	F: CTTCATAGGAATGGAAGCTGC R: CGACCACCTTGATCTTCATGC	60	197	This research

Results

Detection of secondary metabolites by LC-MS analysis

The crude extract was analyzed by the LC-MS technique, which showed the retention time, molecular formula, and molecular weights of several compounds. The results of analysis for a crude extract of isolate RBR55003 (virulent) revealed compounds related to blast disease and toxicity to rice plants. The secondary metabolites detected in the filtrate culture were picolinic acid, *m*-coumaric acid, *trans*-cinnamic acid, *cis*-*p*-coumaric acid, ferulic acid, oryzalin, and pyriculol (Table 2). For analysis of the crude extract from isolate CCO56003 (non-virulent), four compounds were found including picolinic acid, *m*-coumaric acid, *trans*-cinnamic acid, and *cis*-*p*-coumaric acid (Table 3). The compounds found in both isolates were picolinic acid, a toxin that causes blast lesions on rice leaves, while the other compounds were *m*-coumaric acid, *trans*-cinnamic acid, *cis*-*p*-coumaric acid, and ferulic acid, which is a substance that can inhibit seedling growth and seed germination.

Table 2. Secondary metabolites detected in the culture filtrate of isolate RBR55003 by LC-MS analysis

No.	RT	Name of compound	Molecular formula	MW
1	1.96	picolinic acid	$C_6H_5NO_2$	123.03
2	2.51	*m*-coumaric acid	$C_9H_8O_3$	164.05
3	4.72	*trans*-cinnamic acid	$C_9H_8O_2$	148.05
4	8.77	*cis-p*-coumaric acid	$C_9H_8O_3$	148.05
5	9.83	ferulic acid	$C_{10}H_{10}O_4$	194.06
6	16.91	oryzalin	$C_{12}H_{18}N_4O_6S$	346.10
7	20.65	pyriculol	$C_{14}H_{16}O_4$	248.11

Table 3. Secondary metabolites detected in the culture filtrate of isolate CCO56003 by LC-MS analysis

No.	RT	Name of compound	Molecular formula	MW
1	1.86	picolinic acid	$C_6H_5NO_2$	123.03
2	2.51	*m*-coumaric acid	$C_9H_8O_3$	164.05
3	4.73	*trans*-cinnamic acid	$C_9H_8O_2$	148.05
4	8.77	*cis-p*-coumaric acid	$C_9H_8O_3$	164.05

Expression of the secondary metabolite-related gene in P. oryzae

This study compared the expression of genes involved in secondary metabolite synthesis between the RBR55003 (virulent) and CCO56003 (non-virulent) isolates by quantitative real-time RCR (qPCR). The *MoPks19* gene is a gene involved in the biosynthesis of compounds causing blast symptoms in rice. The results showed that both isolates were expressed at 12 h and continued until 48 h after inoculation. At 12 h, the expression levels of the virulent RBR55003 were significantly higher than the non-virulent CCO56003 (Turkey HSD, $p < 0.05$). The isolate CCO56003 showed the highest expression levels at 24 h, while the isolate RBR55003 showed the highest expression levels at 48 h, and the expression level was significantly higher than the isolate CCO56003 (Turkey HSD, $p < 0.05$) (Figure 1).

Expression of defense-related genes in rice

In plants, the *OsWRKY30* gene plays a role in regulating the genes involved in plant defense. The results showed that rice plants inoculated with non-virulent CCO56003 were expressed at 12 h and the highest expression levels were at 24 h after inoculation. The gene expression levels in rice after inoculation with both isolates at 24 and 48 h were significantly different (Turkey HSD, $p < 0.05$) (Figure 2). Verification of the expression of *OsCPS4* gene, a gene involved in rice phytoalexin biosynthesis. The results showed that rice plants inoculated with both isolates were expressed at 12 h and continued

until 48 h after inoculation, with the highest expression levels at 48 h. However, there was no significant difference between the rice inoculated with the isolate CCO56003 and RBR55003 in expression levels (Turkey HSD, $p < 0.05$) (Figure 2).

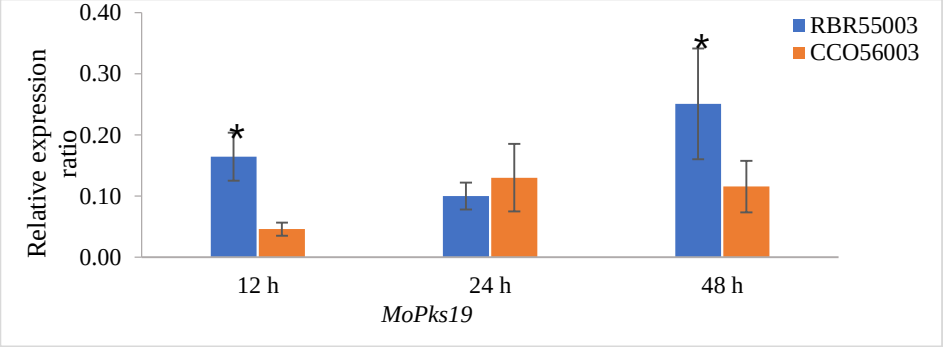

Figure 1. Expression analysis of the *MoPks19* gene in *P. oryzae* by quantitative real-time RCR (qPCR). Samples were taken at 12, 24, and 48 h after inoculation on rice plants. * indicates a significant difference between the two fungal isolates (Turkey HSD, $p < 0.05$)

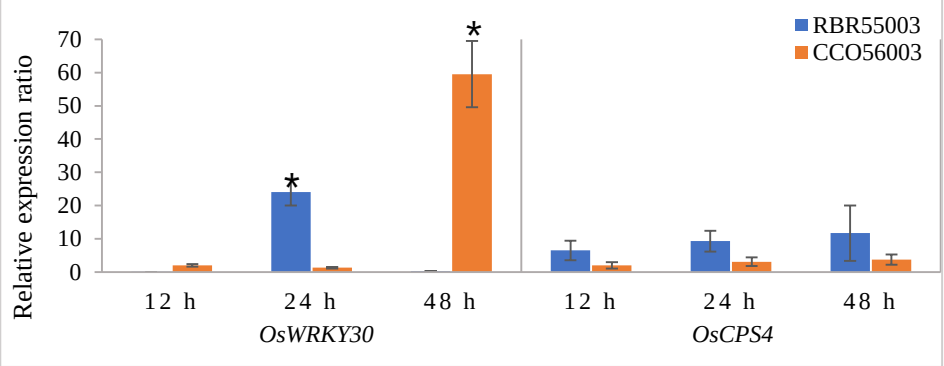

Figure 2. Expression analysis of *OsWRKY30* and *OsCPS4* genes in rice at 12, 24, and 48 h after inoculation with *P. oryzae* by quantitative real-time RCR (qPCR). * indicates a significant difference between the two fungal isolates (Turkey HSD, $p < 0.05$)

Discussion

Analysis of the secondary metabolite extracted from the culture filtrate of *P. oryzae* by LC-MS technique revealed picolinic acid and pyriculol, which are important compounds causing rice blast disease (Iwahashi *et al.*, 1999; Zhang *et al.*, 2004; Tsurushima *et al.*, 2009; Jacob *et al.*, 2017). In this study, the

secondary metabolite was compared between the RBR55003 (virulent) and CCO56003 (non-virulent) isolates. The results showed that both isolates of fungi were able to produce picolinic acid, a substance involved in causing blast symptoms on rice leaves, though the pathogenesis of the two fungi was different. Therefore, the pathogenicity of fungus in causing blast disease may depend on other factors. In addition, toxic substances that inhibit seedling growth and seed germination were also found, such as *m*-coumaric acid, *trans*-cinnamic acid, *cis*-*p*-coumaric acid, oryzalin, and ferulic acid (Li *et al.*, 1993; Hugdahl and Morejohn, 1993), as in the experiment.

The expression of the *MoPks19* gene in *P. oryzae* showed that both fungal isolates were expressed at 12 h and the highest at 48 h after inoculation. This is consistent with the results of the experiment by Jacob *et al*. (2017), which found high expression at 48 – 72 h after inoculation, during invading rice plants of the fungus. The virulent isolate RBR55003 showed higher expression than the non-virulent isolate CCO56003. Therefore, the expression of the *MoPks19* gene may be a factor involved in the pathogenesis of isolate RBR55003.

The expression of the *OsWRKY30* gene in rice showed that rice plants inoculated with CCO56003 had faster and higher expression than rice plants inoculated with RBR55003, indicating that the non-virulent isolate was able to induce *OsWRKY30* gene expression in rice better than the virulent isolate. Peng *et al.* (2012) found that *OsWRKY30* gene expression increased resistance to fungi in rice, and the expression of genes involved in the synthesis of jasmonic acid (JA) and *pathogenesis-related* (*PR*) genes were induced. The expression of the *OsCPS4* gene, a gene involved in phytoalexin biosynthesis in rice, showed that rice plants inoculated with both fungal isolates were continuously expressed at 12 – 48 h, indicating that phytoalexin was produced continuously during infection. Thus, CCO56003 infection can be inhibited, while isolate RBR55003 can cause rice blast disease, which may depend on other pathogenic factors such as effector protein synthesis.

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