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Classification and diversity assessment of *Pyricularia oryzae* based on leaf and neck blast pathogenesis on Khao Dawk Mali 105

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Abstract Forty isolates of *P. oryzae* were obtained from disease outbreak areas in various regions of Thailand. The fungi were divided into two groups; group 1 included 2 isolates which cause only neck blast with disease score of 7 – 9 and group 2 included 23 isolates which cause leaf and neck blast symptoms with disease scores of 5 – 6 and 7 – 9, respectively. The two groups of fungi were tested on 31 NILs. The avirulence genes including *Avr-Pik* (*Avr-Pik, Avr-Pikm, Avr-Pikp*), *Avr-Pi1, Avr-Pi3, Avr-Pi5(t), Avr-Pi7(t), Avr-Pi9, Avr-Pi20, Avr-Pii* and *Avr-Pita* were found in both groups whereas *Avr-Pi11, Avr-Pi12, Avr-Pi19, Avr-Pia, Avr-Pib, Avr-Pikh, Avr-Piks, Avr-Pish, Avr-Pita, Avr-Piz, Avr-Piz-5* and *Avr-Piz-t* were detected only in the group 2. The results explained the relationship between fungi and the avirulence genes functioned at different stages of rice.

Keywords: leaf blast, neck blast, *Pyricularia oryzae*, avirulence (*Avr*) genes

Introduction

Rice blast disease caused by *Pyricularia oryzae* (teleomorph *Magnaporthe oryzae*), is considered as major disease of rice cultivation in Thailand. The fungus can infect rice plants at any growth stage and produce lesions on several organs of the rice plant: leaves, stem, nodes, necks and panicles (Castilla *et al.*, 2010). Leaf blast disease stunts the plant, reduces the number of bearing panicles and weight of individual grains (Puri *et al.*, 2009). Neck blast affects more significant on yield and quality than leaf blast (Hai-Yan *et al.*, 2006). Losses of up to 80% have been recorded in fields attacked by neck blast. An outbreak of neck blast in Sakon Nakhon province of Thailand was reported in 2013 which damaged 2.9 thousand acres of rice growing areas and 80 – 100% yield reduction (Kantajun *et al.*, 2014). In the study of the indica rice in Zhejiang Province, avirulent isolates to leaf blast at the seedling stage are

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virulent to neck blast, while some virulent isolates to leaf blast are avirulent to neck blast. The genetic mechanisms of the resistance to blast at different development stages might vary in rice (Hai-Yan *et al.*, 2006) and the disease development depends on inoculum pressure, crop growth stage at infection, environmental conditions, varietal resistance and cultural management (Groth, 2006). It is well known that highly pathogenic variability in *P. oryzae* often leads to rapid breakdown of resistant cultivars (Ma *et al.*, 2015). Specificity of blast fungi to infect at different stages of plant growth may result from the presence of avirulence gene factors. To date, several *Avr* genes has been recognized in plant pathogens (Lopez *et al.*, 2019). The fungus may use different *Avr* genes to infected rice plants at the seedling and the heading stages. The dynamic interaction between host and fungus in the rice blast pathosystem can be explained by the gene-for-gene theory, in which the product of a host disease resistance (*R*) gene detects the presence of a corresponding avirulence (*Avr*) gene in the pathogen (Flor, 1971). A successful interaction results in the activation of the host's defense response, thereby suppressing the infection (Zhang *et al.*, 2015).

The objectives of this study were to classify the fungi according to their ability in causing leaf blast and neck blast symptoms on Khao Dawk Mali 105 and examine the *Avr* gene in the pathogen by testing on near-isogenic lines (NILs), with single blast resistance genes for assessing pathotype and *Avr* gene diversity of the fungus.

Materials and methods

Fungal isolate

Rice plants expressed blast symptoms were collected from rice fields in Thailand. The fungus was purified by single spore isolation technique and cultured on Potato Dextrose Agar medium (for 1 L of PDA; 200 g potato, 20 g dextrose, 20 g agar) and incubated at 27 ℃ for 5 days. The mycelium plugs were transferred to the Rice Flour Agar medium (for 1 L of RFA; 20 g rice flour, 2 g yeast extract, 20 g agar) with filter paper on top of the medium. The fungal colony was allowed to grow for 5 days and fungal stock was prepared as described by Sirithunya *et al.* (2007). The stock of blast fungus isolates was stored at -20 ℃.

Production of inoculum

Fungal isolates were re-grown from the stock cultures on RFA medium and incubated at 28 ℃ for 8 – 10 days. The sporulation was induced by scraping

the mycelium in each plate. After that, the open-lid plates were placed at room temperature for 2 days. The spores were harvested and the concentration was adjusted to 1×10^5 spores/ml in 0.5% gelatin solution to be used in inoculation methods.

Seedling preparation for leaf inoculation

The blast susceptible rice varieties Khao Dawk Mali 105 (KDML105) and resistance varieties including Jao Hom Nin (JHN) and IR64 were used as control varieties in this study. Germinated seeds were planted in seedling trays (42 × 28 cm) and placed on plastic trays containing water. Fertilizer was supplied at 7 days after planting by adding ammonium sulphate at 1 g/tray. The seedlings were grown for two weeks in a greenhouse.

The leaf blast inoculums were sprayed onto two-week-old rice plants. The inoculated plants were placed in a high humidity chamber for 18 – 24 h at 25 ℃ and then transferred to a greenhouse until disease scoring. Disease evaluations were recorded at 7 days after inoculation using standard evaluation system of rice in 0 – 6 scale (Roumen *et al*., 1997). Classification of fungal isolates was based on disease symptoms into three groups was done as follows: 1) isolates induced scoring 0 – 2 were considered avirulent. 2) isolates induced scoring 3 – 4 were considered moderately virulent and 3) isolates induced scoring 5 – 6 were considered virulent. The fungus group 1 and group 3 were selected to test for neck blast.

Preparation of rice plants for neck inoculation

Seeds of KDML105 were planted in seedling trays as described above. Two-week old seedlings were transplanted in a pot (30 cm diameter × 20 cm deep), three plants were maintained per pot and placed inside a glass house. Fertilizer (ammonium sulphate) was supplied at 30 days and 60 days after seedling. The experiments were conducted at the booting stage of the rice plant.

The inoculum concentration was adjusted to 1×10^5 spores/ml and then injected into the leaf sheath at booting stage using a 1 ml syringe. One neck was inoculated with 1 ml of spore suspension. The inoculated plants were kept in 90 – 100% humidity for 18 – 24 h at 25 ℃ and then transferred to a greenhouse until disease scoring (Pradapphai and Parinthawong, 2017). Neck blast scoring was recorded at 21 days after inoculation. Severity of symptoms was rated on infected neck using a 0 – 9 scale (IRRI, 2002), where: 0 = no lesions or lesions only on pedicels, 1 = lesions on several pedicels or secondary branches, 3 = lesions on a few primary branches or the middle part of panicle axis, 5 = lesions partially around the base or the uppermost internode or the lower part of panicle

axis, 7 = lesion completely around panicle base or uppermost internode or panicle axis near base with more than 30% of filled grains, 9 = lesions completely around panicle base or uppermost internode or the panicle axis near the base with less than 30% of filled grains.

Detection of the avirulence (Avr) gene

Examination of the *Avr* gene of each fungal group was done at seedling stage by testing on 31 near-isogenic lines (NILs), carrying 23 rice blast resistance gene/allele (Table 1). Seeds of 31 NILs and susceptible variety LTH as well as KDML105 were planted in seedling trays (42 × 28 cm) as described above. The concentration of spore was adjusted to 1×10^5 spores/ml in 0.5% gelatin solution. Inoculation of each fungal isolate was performed by spraying 100 ml/trays of inoculum onto two-week-old rice plants. The inoculation and scoring were following the protocol described above.

Table 1. List of near-isogenic lines (NILs) and resistance (*R*) genes

No.	Cultivar	*R* gene	No.	Cultivar	*R* gene
1	IRBLa-A	*Pia*	17	IRBLsh-B	*Pish*
2	IRBLa-C	*Pia*	18	IRBL1-CL	*Pi1*
3	IRBL1-F5	*Pii*	19	IRBL3-CP4	*Pi3*
4	IRBLks-F5	*Piks*	20	IRBL5-M	*Pi5(t)*
5	IRBLks-S	*Piks*	21	IRBL7-M	*Pi7(t)*
6	IRBLk-Ka	*Pik*	22	IRBL9-W	*Pi9*
7	IRBLkp-K60	*Pikp*	23	IRBL12-M	*Pi12*
8	IRBLkh-K3	*Pikh*	24	IRBL19-A	*Pi19*
9	IRBLz-Fu	*Piz*	25	IRBLkm-Ts	*Pikm*
10	IRBLz5-CA	*Piz-5*	26	IRBL20-IR24	*Pi20*
11	IRBLzt-T	*Piz-t*	27	IRBLta2-Pi	*Pita-2*
12	IRBLta-K1	*Pita*	28	IRBLta2-Re	*Pita-2*
13	IRBLta-CT 2	*Pita*	29	IRBLta-CP1	*Pita*
14	IRBLb-B	*Pib*	30	IRBL11-Zh	*Pi 11(t)*
15	IRBLt-K59	*Pita*	31	IRBLz5-CA	*Piz-5*
16	IRBLsh-S	*Pish*			

Result

Isolation and pathogenicity of P. oryzae causing leaf and neck blast

Forty *P. oryzae* isolates were obtained from disease outbreak areas in various regions of Thailand. Nine blast isolates were from the North, 15 isolates

from the North East, 10 isolates from the Central, 3 isolates from the East, 2 isolates from the West and 1 isolate from the South (Table 2). Assessment of leaf blast symptom were tested on KDML105 at the seedling stage. The fungi were divided into 3 groups according to the severity of the disease as follows: avirulent group included 11 isolates with disease scoring 0 – 2, moderately virulent group included 6 isolates with disease scoring 3 – 4 and virulent group included 23 isolates with disease scoring 5 – 6, as shown in Table 3. The avirulent group and virulent group, a total of 34 isolates were selected to test for neck blast.

Table 2. List of names and sources of *Pyricularia oryzae* isolates collected and used in experiments

Region	Province	Isolates
North	Chiang Rai	Chiangrai34.1, CRI59004.1, CRI59004.2 and CRI59005
	Mae Hong Son	MSN60013, MSN60014 and MSN60018
	Phrae	PRE59006.1 and PRE59006.2
North East	Khon Kaen	KKN2008 7357 and KKN2009 61067
	Buriram	BRM60012.2
	Sakon Nakhon	SKN2008 60867
	Surin	SRN54005
	Nong Khai	NKI2010 47181
	Nong Bua Lamphu	NBP60001, NBP60003.1 and NBP60002
	Udon Thani	UDN60001.1
	Ubon Ratchathani	UBN2009 11308, UBN2010 11351, UBN2010 195167, UBN2010 195171 and UBN2010 7384
Central	Bangkok	BKK55001 and BKK55003
	Phitsanulok	Phitsanulok40.4
	Phetchabun	PNB59001.1, PNB59001.2, PNB59001.3, PNB59001.4, PNB59003.3 and PNB60001
	Lopburi	LBR59003.2
East	Chachoengsao	CCO55002, CCO56003 and CCO56004
West	Ratchaburi	RBR55001 and RBR55003
South	Phatthalung	PL2

Pathogenicity of 34 *P. oryzae* isolates causing neck blast was tested on KDML105 at the booting stage. No symptom was detected when isolates in avirulent group were used as inoculum, except the isolate CCO56003 and UBN2010 7384. All 23 isolates of the virulent group were able to cause neck blast with disease score of 7 – 9. The fungi were then divided into 2 groups; group 1 consisted of 2 isolates, CCO56003 and UBN2010 7384, which can

cause only neck blast with disease score of 7 – 9 (from 0 – 9 of neck blast disease score) and group 2 consisted of 23 isolates which can cause both leaf and neck blast with disease score of 5 – 6 (from 0 – 6 of leaf blast disease score) and 7 – 9 (from 0 – 9 of neck blast disease score), respectively (Table 4). Each isolate of the two fungal groups were inoculated on 31 NILs to determine avirulence.

Table 3. Group of *Pyricularia oryzae* classified based on severity of leaf blast symptom on KDML105

Group of fungi	Isolates
Avirulent	BKK55001, CCO56003, CRI59005, NKI2010 47181, PL2, PNB60001, PRE59006.2, UBN2009 11308, UBN2010 11351, UBN2010 195171 and UBN2010 7384
Moderately virulent	CCO55002, CCO56004, Chiangrai34.1, Phitsanulok40.4, PNB59003.3 and RBR55001
Virulent	BKK55003, BRM60012.2, CRI59004.1, CRI59004.2, KKN2008 7357, KKN2009 61067, LBR59003.2, MSN60013, MSN60014, MSN60018, NBP60001, NBP60003.1, NBP60002, PNB59001.1, PNB59001.2, PNB59001.3, PNB59001.4, PRE59006.1, RBR55003, SKN2008 60867, SRN54005, UBN2010 195167 and UDN60001.1

Table 4. Group of *Pyricularia oryzae* classified according to their ability to cause blast symptoms on KDML105 in the seedling and the booting stages

Group of fungi	Isolates
Causing only neck blast	CCO56003 and UBN2010 7384
Causing both leaf and neck blast	BKK55003, BRM60012.2, CRI59004.1, CRI59004.2, KKN2008 7357, KKN2009 61067, LBR59003.2, MSN60013, MSN60014, MSN60018, NBP60001, NBP60003.1, NBP60002, PNB59001.1, PNB59001.2, PNB59001.3, PNB59001.4, PRE59006.1, RBR55003, SKN2008 60867, SRN54005, UBN2010 195167 and UDN60001.1

Pathotype of blast fungi on near-isogenic lines (NILs)

The study on the pathotype pattern can indicate the diversity of fungi associated with their avirulence (*Avr*) genes. Examination of the *Avr* gene relied on the reaction between the resistance (*R*) gene in the host and *Avr* gene in the pathogen. The fungi group 1 and group 2, a total of 25 isolates were tested on 31 NILs. The examination tests showed that the number of *Avr* genes found in

the fungal member of group 1 were less than the group 2. The isolates CCO56003 and UBN2010 7384 were found to contain 7 and 9 *Avr* genes, respectively and the isolate UDN60001.1 in group 2 contained up to 23 *Avr* genes (Figure 1). The *Avr* genes including *Avr-Pik* group (*Avr-Pik, Avr-Pikm, Avr-Pikp*), *Avr-Pi1, Avr-Pi3, Avr-Pi5(t), Avr-Pi7(t), Avr-Pi9, Avr-Pi20, Avr-Pii* and *Avr-Pita* were founded in both groups, while *Avr-Pi11, Avr-Pi12, Avr-Pi19, Avr-Pia, Avr-Pib, Avr-Pikh, Avr-Piks, Avr-Pish, Avr-Pita, Avr-Piz, Avr-Piz-5* and *Avr-Piz-t* were found only in the group 2 (Figure 2).

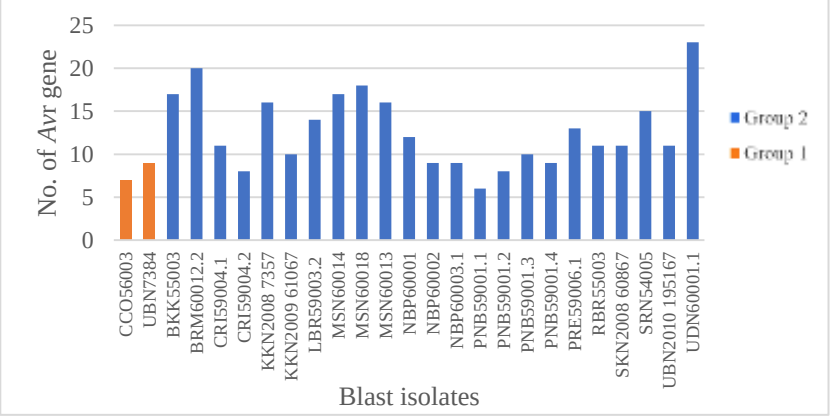

Figure 1. Distribution of 23 *Avr* genes in 25 blast isolates based on disease reaction appeared on 31 NILs

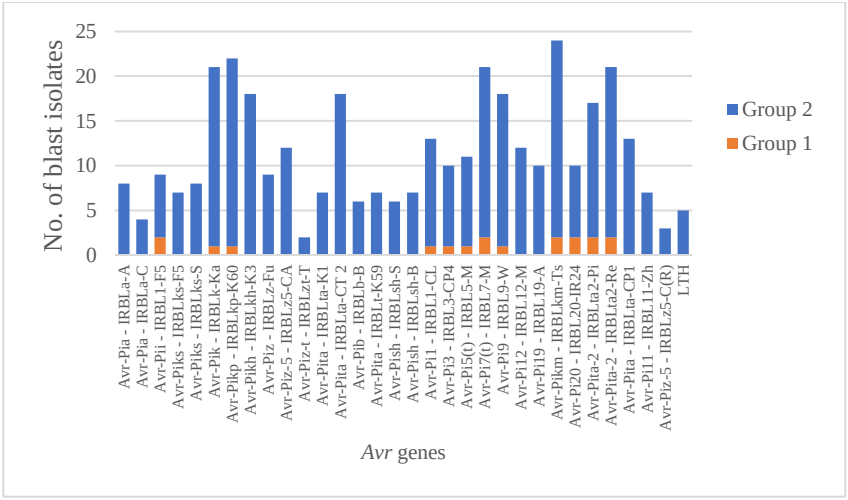

Figure 2. Number of 23 *Avr* genes of *Pyricularia oryzae* found in group 1 and group 2 based on disease reaction appeared on 31 NILs

Discussion

The pathogenicity of 40 blast isolates collected from rice samples infected by *P. oryzae* from rice fields in Thailand was examined. The disease activity was tested on KDML105 at the seedling (leaf blast) and booting (neck blast) stages. The results of this study indicated that 2 out of the 11 isolates were avirulent to leaf blast at the seedling stage and were virulent to neck blast at the booting stage, while all 23 isolates that were virulent to leaf blast were also virulent to neck blast. Similar results have been reported that some blast fungi were avirulent on indica rice variety Zhong 156 at seedling stage, but virulent to neck blast (Zhuang *et al*., 2002). Puri *et al*. (2009) reported the reaction of rice lines to both leaf and neck blast. Avirulent isolates were avirulent to both leaf and neck blast and virulent isolates were virulent to both leaf and neck blast, which similar to this study. The mechanisms of the rice blast infections at different development stages of rice plant might be different in fungi.

Evaluation of the *Avr* gene of each fungal group was tested on 31 NILs. The *Avr* genes including *Avr-Pi11, Avr-Pi12, Avr-Pi19, Avr-Pia, Avr-Pib, Avr-Pikh, Avr-Piks, Avr-Pish, Avr-Pita, Avr-Piz, Avr-Piz-5* and *Avr-Piz-t* were founded only in the group 2. These *Avr* genes might be important for fungi to cause rice blast disease. The number of *Avr* genes found in the fungal member of group 2 were more than the group 1, which might result in the ability of group 2 members to cause blast symptom at both the seedling and booting stages. Moreover, the *Avr* genes including *Avr-Pik* group (*Avr-Pik, Avr-Pikm, Avr-Pikp*), *Avr-Pi1, Avr-Pi3, Avr-Pi5(t), Avr-Pi7(t), Avr-Pi9, Avr-Pi20, Avr-Pii and Avr-Pita* were found in both groups, while the ability of pathogenesis of both groups were different. Thus, the success of blast fungi to cause disease on rice plants might result from the presence of not only avirulence gene but also depends on other factors that influence fungal pathogenesis such as environment, plant growth stage and varietal resistance.

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