Investigation of *Pid3* Rice Blast Resistant Gene in Northern Upland Rice Varieties (*Oryza sativa* L.), Thailand Using Molecular Markers

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***Abstract*—Rice blast disease caused by *Pyricularia oryzae* Cavara is the most devastating rice diseases causing major yield losses worldwide. The presence of *Pid3* gene, one of major blast resistant genes, was determined in 86 upland rice varieties from the northern region of Thailand using two deriver cleaved amplified polymorphic sequences (dCAPS) markers, Pid3-dCAPS 1 and Pid3-dCAPS 2. The PCR products were able to be classified into two alleles (resistant and susceptible alleles). Forty-four varieties showed the resistant *Pid3* alleles of rice blast disease whereas 13 varieties revealed the susceptible alleles and 23 of them showed the heterozygous alleles. Nevertheless, these two markers could not distinguish between resistant and susceptible alleles in six varieties. The results from this study could be used in future development of rice blast disease resistance.**

***Index Terms*—upland rice, *Pid3* gene, *R* gene, NBS-LLR, rice blast disease**

I. Introduction

Rice blast disease caused by *Pyricularia oryzae* Cavara is the most serious and widespread of rice disease worldwide. It causes significantly yield lost (10-30%) in rice production [1], [2]. Although the farmers control the out breaks of rice blast disease by using fungicides, the residues of chemical can contaminate the environment as well as grains. Thus, many rice blast disease resistant varieties have been studied especially in genetic characteristics to use as alternative way to control and protect the rice from blast disease [3].

Approximately 100 blast resistant genes (*R* gene) have been identified and mapped on both *Oryza sativa* L. *indica* and *Oryza sativa* L. *japonica* [4], [5]. Many blast *R* proteins contain a nucleotide-binding site (NBS) and a leucine-rich repeat (LRR) domain at the carboxyl terminus having a binding ATP and/or hydrolysis. The nucleotide binding site-leucine rich repeat (NBS–LRR) protein can be divided into two subclasses. The subclass I is Toll and mammalian interleukin-1 receptor (TIR) domain consisted of an N-terminal TIR domain, a central NBS domain, and a C-terminal LRR region. The TIR has homology to the intracellular domain of the *Drosophila Toll* and mammalian interleukin-1 receptors in their N-terminus which observed only in the dicot plant species [6]-[9]. The subclass II contains a coiled-coil (CC) structure (CC-NBS-LRR) on the basis of their N-terminal sequence. This subclass II is a unique gene and found only in cereal or rice.

Twenty-one of rice blast *R* genes have been successfully cloned and named *Pib* [10], *Pi-ta* [11], *Pi9* [12], *Piz-t* [13], *Pi2* [14], *Pi36* [15], *Pi37* [16], *Rbr2* [17], *Pik-m* [18], *Pi5* [19], *Pid3* [20], *Pit* [21], *Pb1* [22], *Pish* [23], *Pik-p* [24], *Pik* [25], *Pia* [26], *Pi54* [27], *Pid-2* [28], *pi-2(t)* [29] and *Pi25* [30] and all of these belong to the NBS-LRR gene family. Except for *Pid-2* gene which encodes a B-lectin receptor that has a serine/threonine kinase domain combination. Many of *R* genes are clustered and located on chromosome 6 and 11.

The *Pid3* gene (GenBank Accession No.: FJ745364.1) also known as *Pi25* gene was mapped and identified in an *indica* variety (Digu) by performing *R* gene family as NRS-LRR [20]. In 2014, Xiao reported the gene *Pid3* ortholog found in *indica* verities and wild rice. This gene also was described as CC-NBS-LLR and contained 924 amino acids. The total of 2,775 nucleotides at the position of 1-300, 481-1560 and 1621-2670 are a gene that encodes a CC, NBS and LRR, respectively. But the nucleotide sequences from w14 and w9 of *japonica* varieties have a premature transcription termination at the nucleotide position 770 and 635, respectively. Similarly, the nucleotide at position 737 of w11 and w17 wild rice varieties showed the same patterns of premature transcription termination. The *Pid3* ortholog gene showed highly similarity from 99.0% to 100 % to *Pid3* gene [31]. In 2013, Lv found the *Pid3*-A4 in common wild rice A4 (*oryza rufipogon*), which related to ortholog of *Pid3* and showed high identity of 277 nucleotide (99.50%) at the DNA level. The *R* gene family encodes a CC-NBS-LRR with 924 amino acids, which Pid3 gene has only 9 amino

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acids substitution. The wild rice species are respected to rice blast disease resistance based on genetically resources [32].

Currently, the rice blast resistant gene *Pid3* displays broad spectrum resistance of *M. oryzae* strains [20], [31], [32]. This gene has been found in several subspecies of rice such as *indica* and *japonica*. Thus in this study, the *Pid3* gene was investigated in Thai northern upland rice varieties using deriver cleaved amplified polymorphic sequences (dCAPS) markers.

II. Materials and Methods

A. Plant and Materials

One hundred upland rice varieties collected from the Northern part of Thailand were used to investigate for the presence of gene *Pid3* and three rice varieties, Nippon bare, Khao Dawk Mali 105 and Hom Nil varieties received from Kasetsart University, Thailand were used as controls. The details of the source of 86 varieties are listed in supporting information, Supplemental Table I.

TABLE I. Sequences of dCAPS Markers

Markers	*Sequence (5'-3')*	*Ta (°C)*	*Enz.*	*Expected size (bp)*	
				Resistant allele	**Susceptible allele**
Pid3-dCAPS1	F: 5'-TACTACTCATGG-AAGCTAGTTCTC-3' R: 5'-GCAGCACTTCTT-GACTACTGTCTGT-3'	58	*Bam*HI	153	178
Pid3-dCAPS2	F: 5'-TACTACTCATGGA-AGCTAGTTCTC-3' R: 5'-AGCACTTCTTGAC-TACTGTCTGCCT-3'	56	*Xba*I	178	153

B. DNA Extraction

The seeds of all varieties were geminated and genomic DNA was isolated from the bulk of young fresh leaves of each variety following the CTAB method previously described by Doyle and Doyle (1990) [33]. DNA was quantified by NanoDrop 1000 spectrophoto-meter (Thermo Scientific).

C. Identification of Pid3 Gene

The *Pid3* cleaved amplified polymorphic sequence (dCAPS) markers; Pid3-dCAPS1 and Pid3-dCAPS2 (Table I) were used for amplification of genomic DNA to examine the presence of gene Pid3. Each PCR reaction containing 100 ng of genomic DNA, 1.25 mM MgCl2, 1x PCR buffer, 200 µM dNTP, 5 pmol/ 20 µl forward and reverse primer, 1 Unit of Taq Polymerase (Vivantis, Malaysia), 1 µl DMSO and 10.3 µl distilled water. The thermocycling program was 5 min at 94°C for initial denaturation followed by 35 cycles of 30 sec at 94°C, 1 min at 58°C for Pid3-dCAPS1 marker and at 56°C for Pid3-dCAPS2 marker, 1 min at 72°C and final extension at 72°C for 10 min. Both markers amplified a 178-bp PCR fragment and then PCR products of Pid3-dCAPS-1 and Pid3-dCAPS-2 markers were digested with *Bam*HI and *Xba*I, respectively. Digested fragments were separated on 2% agarose gels, staining with SYBR safe (Invitrogen Corporation, CA) and visualized by UV light.

D. Sequence Analysis

The digested fragments of both dCAPS markers were sequenced by Macrogen Inc., Korea. The sequences were analyzed and compared between resistant and susceptible alleles of *Pid3* gene using BLASTN search program (http://www.ncbi.nlm.nih.gov/BLAST/).

III. Results and Discussion

A. Identification of Pid3 Gene

The *Pid3* alleles were performed using two deriver cleaved amplified polymorphic sequence (dCAPS) markers [20]. Both Pid3-dCAPS1 and Pid3-dCAPS2 markers amplified a 178-bp fragment. The initial position of Pid3-dCAPS1 marker is located on the 2070 nucleotide position of *Pid3* gene and the terminal position is 2226 (Fig. 1). PCR products of Pid3-dCAPS1 were digested with *Xba*I. The results showed 24 varieties revealing a 178-bp fragment meaning that these varieties contain a susceptible allele of rice blast disease resistant (*Pid3*) gene and 48 varieties appeared to have a 153-bp fragment suggesting that all 48 varieties consist of a resistant allele of rice blast disease resistant gene. Fourteen varieties showed both 153-bp and 178-bp fragments indicating the resistant and susceptible Pid3 and these alleles were found in heterozygous form (Fig. 2). Similarly, the initial position of Pid3-dCAPS2 marker is located at 2070 nucleotide position of Pid3 gene and the termination site is at the nucleotide position of 2224. PCR products from Pid3-dCAPS2 marker were digested with *Bam*HI. Fifty-three varieties of upland rice appeared to have the resistant *Pid3* alleles (178-bp fragment) and 13 rice varieties had the susceptible *Pid3* alleles (153-bp fragment) and the rest of upland rice showed both resistant and susceptible *Pid3* alleles of rice blast resistant gene. Rice variety Nipponbare containing the *Pid3* gene was used as the positive control and Khao Dawk Mali 105 and Jao Hawm Nil with no *Pid3* gene were used as the negative control.

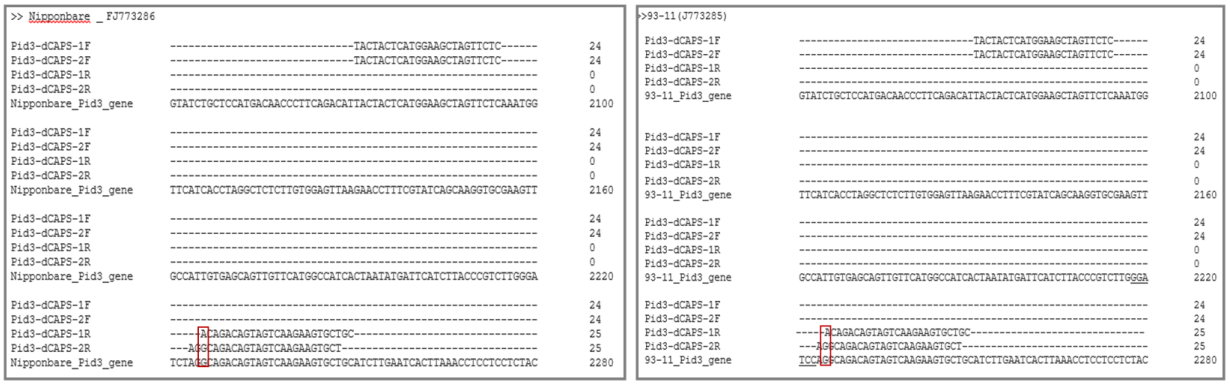


Figure 1. Aliment of Pid3-dCAPS1 and Pid3-dCAPS2 markers with Pid3 gene of Nipponbare (fj773286) and 93-11 (j773285).

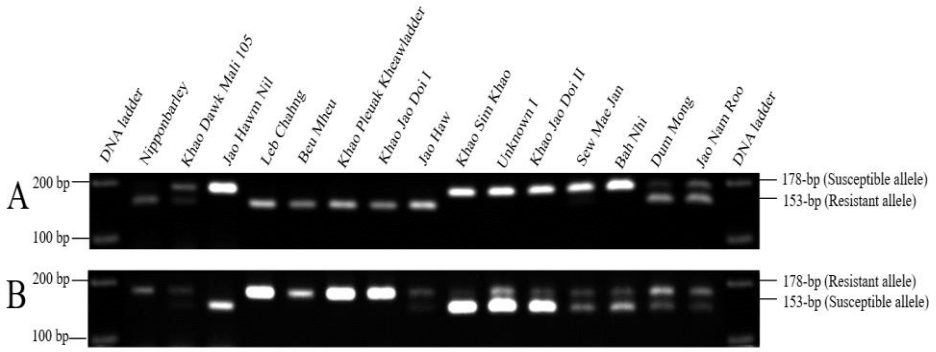


Figure 2. Identification the *Pid3* gene in upland rice varieties using the Pid3-dCAPS1 and Pid3-dCAPS2 markers. (A) Pid3-dCAPS1 marker showed resistant allele at 153-bp and susceptible allele at 178-bp (B) Pid3-dCAPS2 marker showed resistant allele at 178-bp and susceptible allele at 153-bp.

As a result of both markers, 44 varieties contained a resistant allele at Pid3 gene. Thirteen and 23 varieties had susceptible and heterozygous alleles, respectively. Furthermore, six varieties of 86 varieties displayed ambiguous results between these two markers (Table II).

B. Sequence Analysis

The nucleotide sequences of *Pid3* gene located on chromosome 6 consist of 3224-bp. DNA sequencings of Khao Pleuak Kheaw, Khao Jao Doi II, Ka Moo and Dum Mong were aligned with the *Pid3* gene sequences of Nipponbare (FJ773286; *japonica* variety) and 93-11 (J773285; *indica* variety) using Clustal Omega. The pairwise sequence alignment showed that all samples had highly homologous with an average identity from 99% to 100%. These conserved regions encoded a LLR protein and contained a premature stop codon due to change the C to T (nonsense mutation) at the nucleotide position 2223, leading to the disruption of LLR region (Fig. 3).

TABLE II. ANALYSIS OF PID3-DCAP MARKERS FOR RICE BLAST RESISTANT GENE (*PID3*) IN 86 UPLAND RICE VARIETIES

No.	Name	Pid3 dCAPS1	Pid3 dCAPS2	No.	Name	Pid3 dCAPS1	Pid3 dCAPS2
1	Leb Chahng	R	R	44	O-Sa	S	H
2	Khao Sim Khao	S	S	45	Chae Sah Ma	R	R
3	Beu Mheu	R	R	46	Che Ba Ma	R	R
4	Pi Ai Zoo	R	R	47	U-Mah Na	R	R
5	Hawm Doi	S	S	48	Che Bah Jui	R	R
6	Khao Fam	R	R	49	Chae Sa	H	H
7	Khao Daeng	R	R	50	Ka Moo	R	R
8	Khao Kam	R	R	51	Unknown VI	H	R
9	Khao Kam	R	R	52	Khao Kum	R	R
10	Khao Pleuak Kheaw	R	R	53	Dum Mong	H	H
11	Unknown I	S	H	54	Ma Li Rai	H	H
12	Khao Jao Doi I	R	R	55	Sew Mae Jan	H	S
13	Khao Jao Doi II	S	H	56	Jao Nam Roo	H	H
14	Unknown 2	R	R	57	Lao Teak	R	R

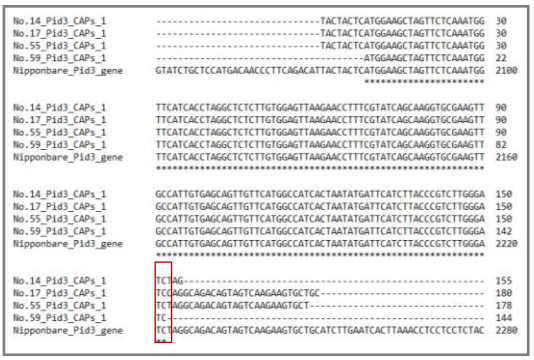
15	A-Kha Ja Bue	H	H	58	Blay Kleua	R	H
16	La Hae	R	R	59	Jao Haw	H	R
17	Unknown III	R	R	60	Beu Po Lo	S	S
18	Chaw Miae Chae	R	R	61	Leum Pua	R	R
19	La Hae	R	R	62	San Pah Tawng	H	H
20	Unknown IV	R	R	63	Bah Nhi	S	H
21	Khao' Khao Chae Bah	R	R	64	Pa-Yah Leum Gaeng	R	R
22	Chae Mew	R	R	65	Khao Kon Jud	H	H
23	Chae Yah Yaw Ti	R	R	66	La Aoob	S	H
24	Chae Yah Yaw Heu	H	H	67	Khao Tah Nhong	S	S
25	Jar Lo Mah	R	R	68	Hang Pla Lhai	R	R
26	Kha Pah Chae Ne	R	R	69	Man Pu	H	H
27	Daw Choo	R	R	70	Khao' Sew	S	R
28	Chair Miaw Rae	H	R	71	Lai San	R	R
29	Unknown V	R	R	72	Pah Baw	S	S
30	Mae Suay	R	R	73	Na Khoo	R	R
31	Ja Naw Vuey	R	R	74	Khao Leuang	R	S
32	Kaw Rue Sue	S	H	75	Khao Phee	S	S
33	Kaw How	S	H	76	Khao Rai Lhaeng	S	R
34	Ja Beu Mah	R	R	77	Lai San	S	S
35	Khaw Mah Gam	R	R	78	Ja Nhe	S	R
36	Ja Hae	R	R	79	Beu Saw Mee	S	S
37	Ja Seu Hae	R	R	80	Feung Kam	R	S
38	Ja Ber Ger	S	H	81	Khao E-Noi	S	S
39	Khao Pueng Luang	H	R	82	Kra Lheang	S	S
40	Kaw Mah Hah	S	R	83	Ya Foo Thow	S	H
41	Ja Swu Mah	R	R	84	Jao Hawm Nil	S	S
42	Khao Pleuak Dum	R	R	85	Khao Dawk Mali 105	R	R
43	Kaw Mah Hah Ja Chi	R	R	86	Nipponbarley	R	R

*R: Resistant alleles
S: Susceptible alleles
H: Heterozygous alleles

For Pid3-dCAPS1 marker, DNA sequencing of all varieties showed the restriction sites of *Xba*I which the last six nucleotide sequences of resistant *Pid3* allele (153-bp fragment) were 5'-TCTAGA-3'and 5'-TCCAGA-3' for susceptible *Pid3* allele (178-bp fragment). In contrast, Pid3-dCAPS2 marker, the last sex nucleotide sequences of susceptible (153-bp fragment) and resistant (178-bp fragment) *Pid3* alleles were 5'-GGATCC-3' and 5'-GGATCT-3', respectively. Because the 153-bp fragment of both markers at the last six nucleotide had the nucleotide substitution which leaded to the change of restriction site of *Xba*I and *Bam*HI, T^CTAGA-3' and 5'-G^GATCC-3', respectively (Fig. 1).

The analysis of *Pid3* gene revealed that this gene co-localizes between NBS-LRR gene and resistant loci on the 6th chromosome. The LRR motifs are protein-protein interactions to recognize the special pathogens, for promoting plant resistant responses [34]. Among *R* protein family, the LLR domain has highly variability nucleotide substitution in sequences to adaptive evolution [35], [36]. From this study, a premature stop codon sequence disrupted the structure of LLR which was characterized by differentiation of *Pid3/pid3* gene expression, resistance and susceptibility, respectively [20].

A



B

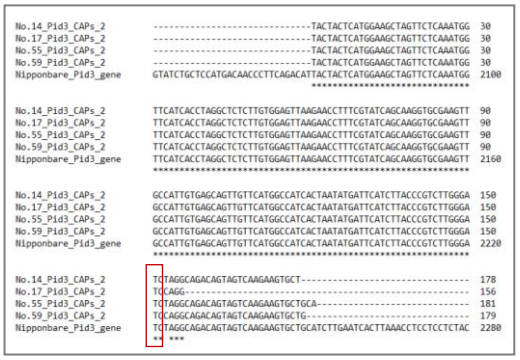


Figure 3. Sequence alignment between Nipponbare allele (*Pid3*) and PCR products of Pid3-dCAPS1 (A) and Pid3-dCAPS2 (B) markers. In the box show the nucleotide sequences of resistant and susceptible alleles from upland rice varieties aligned with the *Pid3* gene sequences of Nipponbare, showed a single nucleotide substitution C/T presented in the recognition site of *Xba*I and *Bam*HI.

Previously, some studies about *Pid3* gene [20], [31], and [32] only used Pid3-dCAPS2 marker to identify rice blast resistant alleles [20], [37]. To determine whether *Pid3 gene* was able to distinguish the heterozygous alleles, in this experiment both Pid3-dCAPS 1 and Pid3-dCAPS 2 were used to serve that purpose. The results showed that Pid3-dCAPS 2 could detect the heterozygous alleles. Further investigations are required to identify other *R* genes for more effective way to screen for the rice blast resistant varieties and inoculation of rice blast disease.

IV. Conclusion

According to this study, the resistant *Pid3* alleles of rice blast disease were found in 44 varieties of upland rice and 13 varieties had the susceptible alleles whereas 23 varieties showed the heterozygous alleles. However, these two markers could not differentiate the differences between the resistant and susceptible alleles of *Pid3* gene. This study is significant in future development of rice blast disease resistant varieties.

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