Functional Analysis of *Pid3-A4*, an Ortholog of Rice Blast Resistance Gene *Pid3* Revealed by Allele Mining in Common Wild Rice

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Accepted for publication 24 January 2013.

ABSTRACT

Lv, Q., Xu, X., Shang, J., Jiang, G., Pang, Z., Zhou, Z., Wang, J., Liu, Y., Li, T., Li, X., Xu, J., Cheng, Z., Zhao, X., Li, S., and Zhu, L. 2013. Functional analysis of *Pid3-A4*, an ortholog of rice blast resistance gene *Pid3* revealed by allele mining in common wild rice. *Phytopathology* 103:594-599.

The rice blast resistance gene *Pid3* encodes a nucleotide-binding-site leucine-rich repeat (NBS-LRR) protein. This gene was cloned from the rice ‘Digu’ (*indica*) by performing a genome-wide comparison of the NBS-LRR gene family between two genome-sequenced varieties, ‘9311’ (*indica*) and ‘Nipponbare’ (*japonica*). In this study, we performed functional analysis of *Pid3-A4*, an ortholog of *Pid3* revealed by allele

mining in the common wild rice A4 (*Oryza rufipogon*). The predicted protein encoded by *Pid3-A4* shares 99.03% sequence identity with *Pid3*, with only nine amino-acid substitutions. In wild rice plants, *Pid3-A4* is constitutively expressed, and its expression is not induced by *Magnaporthe oryzae* isolate Zhong-10-8-14 infection. Importantly, in transgenic plants, *Pid3-A4*, as compared with *Pid3*, displays a distinct resistance spectrum to a set of *M. oryzae* isolates, including those that prevail in the rice fields of Sichuan Province. Therefore, *Pid3-A4* should be quite useful for the breeding of rice blast resistance, especially in southwestern China.

Additional keywords: broad-spectrum resistance.

Rice blast, caused by the filamentous ascomycete *Magnaporthe oryzae*, is one of the most important diseases of rice. This disease has been found to occur in >85 countries, causing an annual worldwide rice yield loss of 10 to 30% (26). The use of plant varieties with resistance (*R*) genes is, and will continue to be, the most cost-effective and environmentally-friendly way to control this disease (26).

Thus far, >80 rice blast *R* genes have been described at the genetic level (32), 21 of which have been cloned and characterized. With the exception of *Pid2* and the recessive *pi21*, which encode a receptor-like kinase and a proline-rich protein, respectively (5,10), the remaining 19 cloned *R* genes (including *Pib*, *Pi-ta*, *Pi9*, *Piz-t*, *Pi2*, *Pi36*, *Pi37*, *Rbr2*, *Pik-m*, *Pi5*, *Pid3*, *Pit*, *Pb1*, *Pish*, *Pik-p*, *Pik*, *Pia*, *Pi54*, and *Pi25*) (3,4,6,18,31–33) all belong to the nucleotide-binding site leucine-rich repeat (NBS-LRR) family, representing the largest class of *R* genes found in plants (7), although *Pi54* is not a typical NBS-LRR gene (22). Notably, of the 21 cloned rice blast *R* genes, 12 have been found to reside in five independent loci on four chromosomes—*Pi37/Pish* on chromosome 1 (16,27), *Pib/Rbr2* on chromosome 2 (31), *Piz-t/Pi2/Pi9* (34) on chromosome 6 near *Pid3/Pi25* (3), and *Pik/Pik-p/Pik-m* (32,33) on chromosome 11—and each set of allelic or closely linked genes shows a high level of amino acid identity. In addition, at the *Pik*, *Pi5*, and *Pia* locus, their respec-

tive rice blast resistances have been found to require the presence of two adjacent NBS-LRR genes (13,18,33).

With respect to disease resistance, wild rice species are of great importance as genetic resources for rice breeding, and some *R* genes have been transferred from wild rice to cultivars. For example, *Xa21*, *Xa23*, and *Xa27*, known as bacterial blight *R* genes, were integrated into cultivated rice from the wild rice species *Oryza longistaminata*, *O. rufipogon*, and *O. minuta*, respectively (14). The rice blast *R* gene *Pi9*, which is highly resistant to 43 *M. oryzae* isolates collected from 13 countries, was also initially transferred from the wild rice species *O. minuta* (19). The AA-genome common wild rice (*O. rufipogon*) is closely related to cultivated rice, suggesting that this species is an ancestor of cultivated rice (28,35). Indeed, 60 to 80% of polymorphisms have been lost during domestication, leading to a reduced number of alleles in cultivated rice (15). Thus, the exploration of new broad-spectrum resistance alleles or orthologs in wild rice should be feasible.

We previously cloned *Pid3* from the *indica* ‘Digu’ by performing a genome-wide comparison of paired NBS-LRR genes and their pseudogene alleles between two rice genome-sequenced varieties, ‘9311’ (*indica*) and ‘Nipponbare’ (*japonica*) (24). *Pid3* shows complete resistance to a range of *M. oryzae* isolates, thus playing a significant role in both rice breeding and molecular pathology studies. Subsequently, by performing map-based cloning, Chen et al. (3) demonstrated that the known rice blast *R* gene *Pi25* (29) in another *indica*, ‘Gumei’, is actually an allele of *Pid3*, which encodes a putative protein similar to PID3, with the exception of a single nucleotide substitution at position 459. Notably, *Pid3* alleles in most *japonica* varieties were identified as pseudo-

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genes due to the presence of a nonsense mutation; however, this pseudogene mutation did not occur in the *indica* varieties that were tested, in African cultivated rice varieties, or in common wild rice species (24). This suggests that some *Pid3* alleles in these rice resources may also function as rice blast *R* genes. The common wild rice A4 (A4) is a rice blast-resistant accession collected in Dongxiang County, Jiangxi Province, China (24). Dongxiang wild rice is known for its tolerance to very low temperatures (−12.8°C) (28). Thus far, a number of its accessions have been tested for rice blast resistance or susceptibility (2,20) and, to our knowledge, no rice blast *R* gene has been identified in this wild rice. *Pid3-A4*, the ortholog of *Pid3* in A4, was identified using an allele-mining approach, which consists of a full open reading frame with 2,775 nucleotides and lacking introns (23). In the coding sequence, *Pid3-A4* is highly identical to *Pid3* (99.50% identity). The deduced *Pid3-A4* product is a 924-residue polypeptide with the same structure as PID3, belonging to the coiled-

coil NBS-LRR class of *R* proteins. There are only nine amino-acid substitutions in the peptide sequence between PID3-A4 and PID3. Because PID3-A4 shows very high sequence identity with PID3, we presumed that *Pid3-A4* may also function as rice blast *R* genes, like *Pid3*. Here, we report the functional analysis of *Pid3-A4*. Like *Pid3*, this *Pid3* ortholog is a functional race-specific rice blast *R* gene and displays a distinct broad-spectrum resistance to a set of *M. oryzae* isolates.

MATERIALS AND METHODS

Plant materials. A4 is an accession line of the common wild rice *O. rufipogon* collected from Dongxiang County in the Jiangxi Province of China. The susceptible recipient *O. sativa* L. subsp. *japonica* ‘Taipei309’ (TP309) and the highly resistant *O. sativa* L subsp. *indica* Digu are routinely maintained varieties. The species were cultivated in an experimental field of the Institute of Genetics and Developmental Biology in Beijing under normal growing conditions.

Fungal inoculation. *M. oryzae* isolates used in this study are listed in Table 1. Isolates 1 to 18 were collected from rice fields in Sichuan Province of China, and were kindly provided by Dr. Yunliang Peng of Sichuan Academy of Agricultural Sciences (1). Isolates 19 to 30 were collected from other regions of China as well as from Japan, and were kindly provided by Dr. Cailin Lei of Institute of Crop Research, Chinese Academy of Agricultural Sciences (23,24). Isolate Zhong-10-8-14 was used for testing the rice blast resistance of *Pid3-A4*. The other 29 isolates were used to assay the resistance spectra of both *Pid3* and *Pid3-A4*. Fungal spores were collected with distilled water and diluted to a concentration of 2.5×10^5 conidia ml^{−1}. For inoculation, rice seedlings at the tillering stage were injected with 0.1 to 0.2 ml of spore suspension in the field. The disease rating was evaluated 7 days after inoculation, with the susceptible TP309 as a control. The lesion types on rice leaves were observed and scored from level 0 (resistant) to level 5 (susceptible) using a standard reference scale (5,25). In this study, levels 0 and 1 were considered to be resistant; levels 2 and 3 represented medium resistance and medium susceptibility, respectively; and levels 4 and 5 were considered to be susceptible.

Cloning of the *Pid3-A4* promoter region. An ≈2,100-bp-long fragment upstream of the predicted start codon of *Pid3-A4* was amplified from A4 genomic DNA using the forward primer Ppid3F: CGTGTACCGTCCTTCTGATTTGAA and reverse primer Ppid3R: GGTGTCTCGTCGGCGTCCTTGAA. Polymerase chain reaction (PCR) amplification was carried out using the following profile: initial DNA denaturation at 95°C for 4 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 2 min; and final extension at 72°C for 5 min.

Vector construction and rice transformation. Forward primer Pid3F: 5′-TTTCTAGAATGGCGGAGGGTGTGTGGG

TABLE 1. Results of inoculation of *Pid3-A4* and *Pid3* transgenic plants with 30 *Magnaporthe oryzae* isolates^a

Isolate	<i>M. oryzae</i> isolate	‘Taipei309’	<i>Pid3-A4</i> transgenic line	<i>Pid3</i> transgenic line
1	03-10-66-1	S	R	R
2	07-31-1-2	S	R	R
3	03-10-76-3	S	R	R
4	04-8-2-1	S	S	S
5	10-25-1-1	S	R	S
6	10-32-2-1	S	mR	mR
7	10-62-3-1	S	mS	S
8	10-117-17-1	S	S	S
9	03-11-37-1	S	R	S
10	07-55-1-1	S	R	R
11	10-47-9-2	S	R	R
12	10-120-21-2	S	R	S
13	07-21-1-1	S	R	R
14	10-62-2-1	S	R	S
15	07-24-1-1	S	mS	S
16	10-31-2-1	S	S	S
17	07-26-22	S	R	R
18	09-87-2-1	S	R	R
19	91-65-1	S	S	S
20	Sichuan26	S	S	S
21	Y34	S	R	R
22	99-26-2	S	mR	mR
23	CH706	S	R	S
24	Zhong-10-8-14	S	R	R
25	ZB15	S	S	S
26	97-27-2	S	R	R
27	CH45	S	S	S
28	JS2001-108-1	S	R	R
29	99-20-2	S	R	R
30	99-26-1	S	S	S

^a On a 1 to 5 scale, levels 0 and 1 were considered to be resistant (R); levels 2 and 3 represented medium resistance (mR) and medium susceptibility (mS), respectively; and levels 4 and 5 were considered to be susceptible (S).

Fig. 1. Semiquantitative reverse-transcription polymerase chain reaction analysis of *Pid3-A4* expression in A4. Total RNAs were isolated from A4 0, 3, 6, 12, 24, 48, and 72 h after inoculation with **A**, *Magnaporthe oryzae* Zhong-10-8-14 or **B**, water. The rice *Actin* gene was used as a control.

CTC-3' and reverse primer Pid3R: 5'-CTGTCGACTTATTG AATCCTTTCTGCAGCC-3' were used to amplify the full coding sequence of *Pid3-A4* from A4 genomic DNA. An *Xba*I and a *Sal*I recognition site (underlined) with two protecting bases (TT and CT) were added to each 5' end. PCR amplification was carried out using the following profile: initial DNA denaturation at 95°C for 4 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 3 min; and final extension at 72°C for 5 min. The PCR products were purified, sequenced, and inserted into the binary vector pZH01 (30) at the *Xba*I and *Sal*I cloning sites. The final construct was introduced into *Agrobacterium tumefaciens* LBA4404. *Agrobacterium*-mediated transformation was performed using calli derived from mature embryos of susceptible rice TP309 according to Hiei et al. (11). Positive transformants were verified by amplification of the marker gene *HPT* using forward primer HYGF: 5'-GACGGTGTCTGCCATCACAGTTT-3' and reverse primer

HYGR: 5'-ACTCACCGCGACGTCTGTCGAGAA-3'. PCR amplification was carried out using the following profile: initial DNA denaturation at 95°C for 4 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s; and final extension at 72°C for 5 min. In addition, to confirm positive transformants, the cleaved amplified polymorphic sequence (CAPS) marker *Pid3C* was designed. This sequence can distinguish *Pid3-A4* from *Pid3-TP309*, based on the nonsense mutation of *Pid3-TP309*. With the CAPS marker, a 658-bp fragment was amplified using the primer pair 5'-TACTACTCATGGAAGCTAGTTCTC-3' and 5'-ACGTCACAA ATCATTCGCTC-3', followed by digestion with the restriction endoenzyme *Bam*HI. The digested PCR products were resolved on 2% agarose gels. The presence or absence of a 506-bp restriction fragment was considered to represent positive or negative transformants, respectively.

Expression analysis of *Pid3-A4*. The resistant A4 and the susceptible TP309 at the tillering stage were inoculated by injection with fungal spores of *M. oryzae* isolate Zhong-10-8-14; water injections were used as control checks. Total RNAs were isolated from leaf sheath tissue with the TRIzol reagent (Invitrogen, Carlsbad, CA) at 0, 3, 6, 12, 24, 48, and 72 h. cDNA was synthesized from poly(A)⁺ RNA using a cDNA synthesis kit (Transgen, Beijing). Semiquantitative reverse-transcription (RT)-PCR was performed with the *Pid3-A4*-specific primer pair (*Pid3C-F*: 5'-TACTACTCATGGAAGCTAGTTCTC-3' and *Pid3C-R*: 5'-ACGT CACAAATCATTCGCTC-3') for 33 cycles of amplification. Transcription of the *Actin* gene was used to normalize the cDNA levels with the primer pair 5'-CCTCGTCTCGACCTTGCTGGG-3' and 5'-GAGAACAAAGCAGGAGGACGGC-3'. Amplification of the *Actin* gene was conducted for 27 cycles. RT-PCR was initiated with one cycle at 95°C for 2 min followed by 33 or 27 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s, and the reaction was terminated with a final extension at 72°C for 5 min. The PCR products were resolved on 1% agarose gels.

The quantitative (q)RT-PCR method was performed to detect the expression patterns of *Pid3-A4* or *Pid3* in A4, Digu, TP309, and homozygous plants carrying *Pid3-A4* or *Pid3* driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter. Total RNAs were isolated with the TRIzol reagent (Invitrogen) from leaves. The qRT-PCR analysis was conducted using TransStart Green qPCR SuperMix (Transgen). Primers specific to *Pid3-A4* and *Pid3* were designed using the GenScript Real-time PCR Primer Design program (<https://www.genscript.com/ssl-bin/app/primer>) (5'-GAATGCAAATGTTTGGTTCG-3' and 5'-CGCCACATCA TAATTCCTTG-3'); *Actin*-specific primers (5'-AGCAACTGGGA TGATATGGA-3' and 5'-CAGGGCGATGTAGGAAAGC-3') were used as a standard. The data were normalized to the rice *Actin*

Fig. 2. Expression analysis of *Pid3-A4* in primary transgenic plants. The recipient 'TP309' and the primary transgenic lines were inoculated with *Magnaporthe oryzae* Zhong-10-8-14. Expression levels were analyzed using *Pid3*-specific primers *Pid3C*, which could amplify *Pid3-A4* and *Pid3-TP309* at the same time. The *Actin* gene was used as a control.

Fig. 3. Cosegregation of the *Pid3-A4* gene with the resistant phenotype. The selfed progeny (T1) of the primary transgenic line 1 (T0) carrying *Pid3-A4* were inoculated with *Magnaporthe oryzae* Zhong-10-8-14, and the genotypes were analyzed using the *HPT* gene and the *Pid3-A4* cleaved amplified polymorphic sequence (CAPS) marker. Resistant A4 and susceptible 'TP309' were used as controls.

gene using the $2^{-\Delta\Delta CT}$ method. The fold change in the expression level was compared with that of sample TP309. The standard error of the mean was calculated. The dissociation curve program was executed to confirm the specificity of the target amplification product.

RESULTS

Pid3-A4 is constitutively expressed in A4. To compare the predicted promoter region of *Pid3-A4* with that of *Pid3*, we cloned and sequenced an $\approx 2,100$ -bp-long fragment (GenBank accession number KC008606) upstream of the predicted start codon of *Pid3-A4*. The nucleotide identity (96.24%) in the 5' upstream regions of the two genes was found to be lower than that in the coding regions. The 5' upstream region of *Pid3-A4* has an 8- and 9-bp deletion, in addition to many single-nucleotide pro-

teins (SNPs), compared with that of *Pid3*. According to the computer program FGENESH (<http://www.softberry.com>), the two genes should have predicted promoter regions beginning 900 bp from their respective start codons, where the 9-bp deletion and some SNPs exist. To determine whether these changes in the predicted promoter region of *Pid3-A4* would affect the expression profile of this gene, semiquantitative RT-PCR was performed with A4 leaves under normal conditions and with leaves infected with *M. oryzae* Zhong-10-8-14. Like *Pid3* in Digu leaves (23), *Pid3-A4* was constitutively expressed in A4 leaves, and the expression of this gene was not induced by *M. oryzae* infection (Fig. 1).

Pid3-A4 is a functional rice blast resistance gene. Because PID3-A4 has an amino acid sequence that is highly identical to that of PID3, with no substitution in conserved motifs of the NBS domain, we presumed that *Pid3-A4* may function like *Pid3*. To verify this assumption, a construct containing the full length of the *Pid3-A4* coding sequence under the control of the CaMV 35S promoter was introduced into the susceptible TP309, which was the same recipient used for pCaMV35S::*Pid3* in our previous study (24). In total, 21 independent primary transgenic lines (T0) were obtained. Of these, 19 lines were resistant and 2 were susceptible to the *M. oryzae* isolate Zhong-10-8-14, which was also employed as the identification isolate for the *Pid3* assay (Fig. 2). Moreover, *Pid3-A4* transcripts were detected abundantly in the rice blast-resistant lines but not in the two susceptible lines (Fig. 2), indicating that the resistance phenotype is associated with the expression of the transgene. It is noteworthy that *Pid3-TP309*, the ortholog of *Pid3* in TP309, is a constitutively expressed pseudogene (23). In the subsequent inoculation test of the T1 progeny of the resistant transgenic lines, we found that rice blast resistance was strictly linked to the presence of the transgenic selectable marker gene *HPT* and to the specific CAPS marker for *Pid3-A4* as well (Fig. 3). Taken together, these results led us to conclude that *Pid3-A4* is a functional rice blast *R* gene.

The rice blast resistance spectrum of *Pid3-A4* is different from that of *Pid3*. By continuous selfing, we obtained homozygous *Pid3-A4* transgenic plants of the T2 generation, which were all resistant to the isolate Zhong-10-8-14. We then used qRT-PCR

Fig. 4. Quantitative reverse-transcription polymerase chain reaction analysis of the transcript levels of orthologs of *Pid3*. Relative quantity chart showing the expression levels of orthologs of *Pid3* in ‘TP309’, ‘Digu’, A4, and transgenic plants with *Pid3* or *Pid3-A4*, both driven by the promoter *Cauliflower mosaic virus* 35S. Columns: 1, TP309; 2, Digu; 3, A4; 4, transgenic plants with *Pid3*; and 5 to 8, transgenic plants with *Pid3-A4*.

Fig. 5. Phenotyping of rice lines with *Magnaporthe oryzae* isolates. Disease reaction of ‘TP309’, ‘Digu’, A4, and transgenic plants inoculated with *M. oryzae* 10-25-1-1, 03-11-37-1, 10-120-21-2, 10-62-2-1, CH706, CH45, ZB15, and 99-26-1. Columns: 1, TP309; 2, A4; 3, Digu; 4, transgenic plants with *Pid3-A4*; and 5, transgenic plants with *Pid3*.

to quantify the transcript levels of *Pid3-TP309*, *Pid3*, and *Pid3-A4* in the leaves of TP309, Digu, and A4, respectively, and those of pCaMV35S::*Pid3* and pCaMV35S::*Pid3-A4* in transgenic TP309 plants (Fig. 4). We found that the expression levels of pCaMV35S::*Pid3* and pCaMV35S::*Pid3-A4* in their respective transgenic plants were approximately the same, although both levels were 40- to 50-fold higher than those of *Pid3* and *Pid3-A4* in their respective donor plants. The enhanced expression of the two transgenes could be attributed to the effects of the strong promoter CaMV 35S.

To determine whether *Pid3-A4* has the same rice blast resistance spectrum as *Pid3*, we compared the resistance spectra of the two transgenes, both of which showed similar expression levels in the same TP309 background. We used 30 *M. oryzae* isolates, 18 of which were isolated from fields suffering severe rice blast infection in recent years in Sichuan Province of China (isolates 1 to 18) and 12 isolates that were collected from other regions of China as well as from Japan (isolates 19 to 30). All isolates were compatible with the control TP309 (Table 1). *Pid3-A4* transgenic plants showed complete resistance (i.e., race-specific resistance) to 18 isolates and partial resistance to 4 isolates, whereas those of *Pid3* showed complete resistance to 13 isolates and partial resistance to 2 isolates. These results demonstrate that, like *Pid3*, *Pid3-A4* is a race-specific rice blast *R* gene and has a broader resistance spectrum than *Pid3* in the range of the tested isolates. Furthermore, *Pid3-A4* seemed to be more versatile in response to the test isolates collected from Sichuan Province of southwestern China (Table 1, isolates 1 to 18).

To exclude the possibility that the distinct rice blast resistance spectrum of *Pid3-A4* resulted from *Pid3-A4* overexpression, we also inoculated A4 plants with eight *M. oryzae* isolates to which *Pid3* transgenic plants are susceptible (10-25-1-1, 03-11-37-1, 10-120-21-2, 10-62-2-1, CH706, CH45, ZB15, and 99-26-1). Like the *Pid3-A4*-overexpressing transgenic plants, A4 plants showed complete resistance to six isolates and susceptibility to two other isolates (Fig. 5), suggesting that *R* gene overexpression does not change the resistance spectrum, as was the case for *Pid3* (23). It is worthwhile noting that A4 was resistant to CH45 while *Pid3-A4* transgenic plants were susceptible, suggesting that A4 contains additional rice blast *R* genes.

DISCUSSION

Although a number of known rice blast *R* genes have been widely employed in rice breeding, a single *R* gene cannot provide long-term resistance to the disease. Protection from a single rice blast *R* gene often proves short-lived due to the rapid variation in *M. oryzae* isolate populations in the field (26). Therefore, it is necessary to identify more rice blast *R* genes, especially with broad resistance spectrums. Up to now, most of the rice blast *R* genes were isolated using the labor-intensive map-based approach. We previously identified *Pid3-A4* from A4 using an allele-mining approach; in this study, functional analysis of *Pid3-A4* showed that it is a functional rice blast *R* gene and has a broader resistance spectrum than *Pid3* in the range of the tested *M. oryzae* isolates. In addition, recently, the novel rice blast *R* gene *Pi54rh* (an ortholog of *Pi54*) was isolated from the rice-blast-resistant wild rice species *O. rhizomatis* using the allele-mining approach (6). Thus, we believe that allele mining has great potential for revealing new rice blast *R* genes and is a cost-effective approach that can be applied to identify NBS-LRR-type *R* genes in other crop species for the purpose of molecular disease resistance breeding.

Plant NBS-LRR proteins are modular. The activity of these proteins depends on their ability to bind and hydrolyze nucleotides (7,17). The N-termini of many NBS-LRR genes have been implicated in accessory protein binding and signal initiation (9). The NBS domain, responsible for the binding and hydrolysis of

nucleotides, may be essential for activating downstream signal transduction (21). It has been generally accepted that the C-terminal LRR domain is the major determinant of resistance specificity. The earliest proof was provided by research on the flax *L* locus; the chimeric genes *L6-L2* and *L10-L2*, both of which encode the LRR of *L2* in the C-terminal part, confer *L2*-resistance specificity. Thus, the difference in resistance specificity between *L6* and *L11* confirmed the results of amino acid differences between their LRR domains (8). The same situation was also observed for the rice *Piz* locus, where only eight amino acid changes in the LRR domain differentiated PI2 from PIZ-T (34). However, the LRR domain is probably not the only determinant of resistance specificity. For instance, all 11 amino acid differences between *L6* and *L7* were shown to be located within the first 208 amino acids of the N-terminus, and the CC domain was believed to be important for PIK/PIK-M-resistance specificity (12,33). Here, we show that only 14 nucleotide substitutions between *Pid3-A4* and *Pid3* result in a nine-amino-acid difference between their peptide sequences. Most of substitutions between PID3 and PID3-A4 are found to be located within the LRR domain, despite the fact that only D856H is found in the conserved LxxLxLxx motif. Two substitutions are located in the CC domain, and the R44G substitution is found in the nT sequence motif (19). The I259V substitution in the NBS domain is not located within any conserved motif, and both of the residues, isoleucine and valine, are hydrophobic. Therefore, this substitution probably has no influence on the specificity functions of PID3 and PID3-A4. Therefore, the resistance distinction between PID3 and PID3-A4 might be determined by substitutions in the LRR domain or the CC domain. Currently, we are producing a number of *Pid3* orthologs by point mutation and modular recombination, with the aim of clarifying the regions and amino acid residues responsible for blast-resistance specificity.

ACKNOWLEDGMENTS

This work was supported by grants from the National Basic Research Program of China (2011CB100706 and 2012CB114005), the National Transgenic Research Project (2008ZX08009-001, 2008ZX08009-003, and 2009ZX08001-011B), the National Science Fund for Distinguished Young Scholars of China (31025017), and the National Natural Science Foundation of China (31160223). Q. Lv and X. Xu contributed equally to this work. We thank Y. Peng (Sichuan Academy of Agricultural Sciences) and C. Lei (Institute of Crop Research, Chinese Academy of Agricultural Sciences) for kindly providing *M. oryzae* isolates and Y. Xu for his critical reading.

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