SCIENCE CHINA
Life Sciences

• RESEARCH PAPERS •

April 2011 Vol.54 No.4: 372–378
doi: 10.1007/s11427-011-4154-1

Characterization and fine mapping of the rice blast resistance gene *Pia*

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Received October 11, 2010; accepted December 8, 2010; published online March 4, 2011

Blast, caused by *Magnaporthe oryzae*, is one of the most widespread and destructive diseases of rice. Breeding durable resistant cultivars (cvs) can be achieved by pyramiding of various resistance (*R*) genes. *Pia*, carried by cv. Aichi Asahi, was evaluated against 612 isolates of *M. oryzae* collected from 10 Chinese provinces. The *Pia* gene expresses weak resistance in all the provinces except for Jiangsu. Genomic position-ready marker-based linkage analysis was carried out in a mapping population consisting of 800 F_2 plants derived from a cross of Aichi Asahi×Kasalath. The locus was defined in an interval of approximately 90 kb, flanked by markers A16 and A21. Four candidate genes (*Pia-1*, *Pia-2*, *Pia-3*, and *Pia-4*), all having the *R* gene conserved structure, were predicted in the interval using the cv. Nipponbare genomic sequence. Four candidate resistance gene (CRG) markers (A17, A25, A26, and A27), derived from the four candidates, were subjected to genotyping with the recombinants detected at the flanking markers. The first three markers completely co-segregated with the *Pia* locus, and the fourth was absent in the Aichi Asahi genome and disordered with the *Pia* locus and its flanking markers, indicating that the fourth candidate gene, *Pia-4*, could be excluded. Co-segregation marker-based genotyping of the three sets of differentials with known *R* gene genotypes revealed that the genotype of A26 (*Pia-3*) perfectly matched the *R* gene genotype of *Pia*, indicating that *Pia-3* is the strongest candidate gene for *Pia*.

rice blast, resistance gene, *Pia*, fine mapping, genotype

Citation: Zeng X S, Yang X F, Zhao Z H, *et al*. Characterization and fine mapping of the rice blast resistance gene *Pia*. Sci China Life Sci, 2011, 54: 372–378, doi: 10.1007/s11427-011-4154-1

Rice blast disease, caused by *Magnaporthe oryzae* (T. T. Hebert) M. E. Barr (anamorph *Pyricularia grisea* Sacc.), threatens the sustainable production of rice [1]. The use of resistant cultivars (cvs) is one of the most effective and economical ways to mitigate disease losses [1,2]. However, genetic resistance of rice cvs imparted by a single resistance (*R*) gene is commonly short-lived because of the occurrence of new virulent race(s) adopting the *R* gene [3,4]. By contrast, stacking of multiple *R* genes into individual cvs has been advocated to achieve durable blast resistance [5–8]. On the other hand, the majority of plant *R* genes belong to the large NBS-LRR (nucleotide-binding site-leucine-rich repeat) gene family, which aids the search for candidate *R* gene(s) in defined genomic regions [1,9]. This type of *R* gene can be subdivided into stronger and weaker effect *R* genes [6,10]. Combinations of stronger and weaker effect *R* genes would favor a broader resistance spectrum and a higher level of resistance [7,10,11]. Therefore, the identification and utilization of weaker effect *R* genes will signifi-

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cantly improve the durability of genetic resistance in new cvs.

More than 80 major blast *R* genes have been identified to date [12,13], and 13 were identified in Japanese differential lines. Among these, *Pik-s*, *Pia*, and *Pii* are weaker effect *R* genes carried by the native Japanese cvs Shin2, Aichi Asahi, and Fujisaka 5, respectively [6,14]. *Pia*, which displays a narrow spectrum resistance and is widely present in Japanese rice cvs [6,15,16], is located on the short arm of rice chromosome 11 [17–19]. For better utilization of the *Pia* gene in China, its resistance to the fungal pathogen populations collected from various regions in China was characterized and its locus was finely mapped in a larger mapping population using genomic position-ready markers.

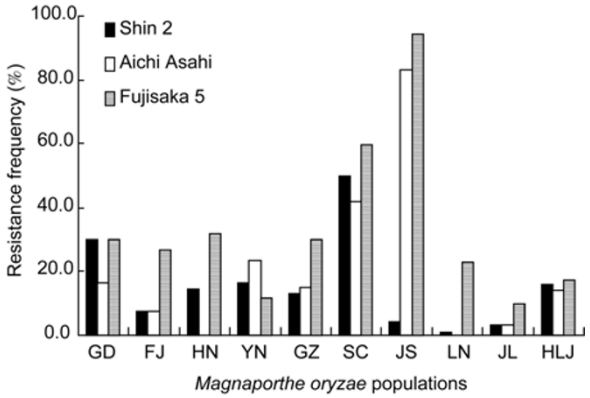

Figure 1 Resistance characterization of three Japanese differential cultivars in the 10 *Magnaporthe oryzae* populations collected in China.

1 Materials and methods

1.1 Isolates

A total of 612 Chinese blast isolates were collected from Guangdong (GD, 60 isolates), Fujian (FJ, 40 isolates), Hunan (HN, 40 isolates), Guizhou (GZ, 60 isolates), Yunnan (YN, 43isolates), Sichuan (SC, 66 isolates), Jiangsu (JS, 72 isolates), Liaoning (LN, 108 isolates), Jilin (JL, 60 isolates), and Heilongjiang (HLJ, 63 isolates), and used to compare the specific reaction patterns and resistance frequencies. F_2 plants derived from a cross of Aichi Asahi×Kasalath were inoculated with a stable *Magnaporthe grisea* isolate, CHL272, which is avirulent on Aichi Asahi and virulent on Kasalath. Inoculations and disease evaluations were conducted in greenhouses, as previously described by Pan *et al*. [20].

1.2 Resistance spectrum analysis

Resistance performance of three Japanese differential cvs, Shin2, Aichi Asahi, and Fujisaka 5, was assessed using the 10 populations mentioned above. Resistance evaluation was represented by the resistance frequency: the number of avirulent isolates compared with the total number of isolates examined for a rice cultivar in every population. The resistance frequencies were generated after statistical analysis in Microsoft Excel (Figure 1).

1.3 Mapping population construction

The F_2 plants derived from a cross of Aichi Asahi×Kasalath were used for resistance segregation analysis and gene mapping. Seedlings of the parents and their progeny were grown in a greenhouse and then challenged with the isolate CHL272 at the four- to five-leaf stage. The inoculation and disease evaluation were conducted in accordance with the method outlined in Pan *et al.* [20]. The leaves of seedlings were sampled and frozen.

1.4 DNA extraction and resistant and susceptible pool construction

Total DNA was extracted from frozen leaves of the seedlings by the CTAB method. Contrast DNA pools were assembled by mixing equimolar amounts of DNA from either 10 resistant or 10 susceptible F_2 individuals. The bulked-segregant analysis (BSA) was carried out to identify molecular markers putatively associated with the *R* gene locus.

1.5 Linkage analysis

The *Pia* locus was mapped in three steps: recombinant screening, fine mapping, and co-segregation analysis [21]. First, simple sequence repeat (SSR) markers on the short arm of rice chromosome 11 were used for BSA analysis [22]. Those markers that produced a differential banding pattern from the resistant and susceptible pools were then genotyped in the whole mapping population by screening for recombinants occurred at the respective marker loci linked to the locus. For constructing the linkage map of the locus, sequences of the linked markers were downloaded from GRAMENE (http://www.gramene.org) and placed on the reference sequence of cv. Nipponbare (http://www.ncbi.nlm.nih.gov/blast). Second, an additional set of SSR markers, in addition to sequence-tagged site (STS) markers, were developed in the defined region for further linkage analysis [23]. Primer pairs were designed using Primer Premier 5.0 (http://primerbiosoft.com). The new markers, which showed clear polymorphisms between the parents, were then applied to a set of recombinant progeny. The physical map covering *Pia* gene locus was constructed, *in silico*, using the genomic position-ready markers [21,23]. Finally, a set of candidate resistance gene (CRG) markers was developed in the target region on the basis of open reading fragments of candidate *R* genes with an NBS-LRR structure, as annotated by the RiceGAAS (http://rgp.dna.affrc.go.jp) software. CRG markers that co-segregated with the *Pia* locus were identified by the recombinants detected at the

flanking marker loci. All the markers are listed in Table 1.

1.6 Co-segregation markers-based genotyping

To further determine candidate gene(s) for *Pia*, three sets of differential lines (mostly carrying known *R* genes; Table 2), developed in Japan, China, and the International Rice Research Institute (IRRI) were genotyped with the co-segregation markers.

2 Results

2.1 Resistance characterization

Resistance performances of three Japanese differential cvs, Shin2, Aichi Asahi and Fujisaka 5 in the 10 *M. grisea* populations are shown in Figure 1. All the three cvs showed moderate levels of resistance to the SC population, and both Aichi Asahi and Fujisaka 5 showed a higher level of resistance to the JS population. For the other eight populations, all the three cvs showed lower levels of resistance.

2.2 Mapping population construction

The segregation between resistance and susceptibility of a total of 924 F_2 individuals was consistent with 3R:1S (resistant/susceptible, 686/238 (χ_c^2=0.24, P>0.6)), indicating that Aichi Asahi carries a monogenic dominant *R* gene, which confers resistance to the isolate CHL272. Therefore, a mapping population, consisting of 585 resistant and 215 susceptible individuals with extreme phenotypes selected from the 924 F_2 progeny, were carried forward (some susceptible plants died).

2.3 Confirmation of the *Pia* gene locus

Pia, carried by Aichi Asahi, was previously mapped on the

Table 1 Experimental details of PCR-based markers used for linkage analysis of *Pia*

Marker[a)]	Type	Primer sequence (5′–3′)[b)]	Genomic position (bp)[c)]	Annealing temperature (°C)[d)]	Expected size (bp)
Recombinants screening					
RM332	SSR	F: GCGAAGGCGAAGGTGAAG R: CATGAGTGATCTCACTCACCC	2827519-2827536 2827669-2827689	55	171
RM167	SSR	F: GATCCAGCGTGAGGAACACGT R: AGTCCGACCACAAGGTGCGTTGTC	4060683-4060463 4060360-4060337	58	147
RM441	SSR	F: CGATGACACACAATTCACACA R: CACATAGGCAAGTCATTCT	6068480-6068461 6068339-6068321	58	160
A6	SSR	F: TCCATCGCTAAGGGAAAGA R: GGTAATTAGTATACTGGAGC	7574264-7574282 7574350-7574369	55	106
RM536	SSR	F: TCTCTCCTCTTGTTTGGCTC R: ACACACCAACACGACCACAC	9035072-9035091 9035295-9035314	55	243
Fine mapping					
A7	SSR	F: GAATAGTCTTACCCCCCCC R: CCTTGTAAATTCCCCTTTGTTG	6245047-6246065 6246170-6246191	60	145
A15	SSR	F: CTCCTCCATTTTTTCCCATCC R: GTGGAGGAGCCAAGAACAG	6391668-6391688 6391775-6391793	64	126
A16	SSR	F: CCACCACTTTTCTTTTTAGG R: TATCCCAGTACAATAAAAG	6491264-6491283 6491407-6491425	52	162
A21	STS	F: CAGACGGTTTCAGAACGAGG R: CAGAACAAGACAGCATAACCC	6583046-6583062 6584044-6584064	58	1022
A9	SSR	F: ATAGGGCCCACAAATCACATC R: GGCCCAATGGACAAATTCTTA	6651922-6651942 6652065-6652085	62	164
Co-segregation analysis					
A17	CRG	F: GCAACGGATACGGAGGCAAT R: GCTTTTCTTAGCAATGTCTGTG	6518740-6518721 6517729-6517708	58	1033
A25	CRG	F: TAAAAATGAGGTTGGGAGTC R: GTTCTTAGCAATGATGTCCTC	6523045-6523064 6523934-6523954	58	908
A26	CRG	F: CTGAAGAGGATGGTGGAGGA R: ATTGTAAACATCCCCATCTG	6528288-6528269 6527480-6527461	58	828
A27[e)]	CRG	F: CTGGGAGTATCTACAGAAAAAG R: CTCTGGAAAAGCACTGGTAAT	6538262-6538283 6539453-6539473	58	1212

a) Markers used for recombinant screening, fine mapping, and co-segregation analysis are grouped. Markers prefixed with RM were adopted from GRAMENE (http://www.gramene.org) and those prefixed with A were developed in the present study. b) F, forward; R, reverse. c) The genomic position of each marker on the short arm of rice chromosome 11 was determined by BLASTN analysis against the reference sequence of cv. Nipponbare (http://ncbi.nlm.nih.gov/blast). d) All PCR runs began with one cycle at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 55–64°C for 30 s, and 72°C for 1–1.5 min; with a final extension at 72°C for 7 min. Amplicons were separated by electrophoresis on 3% and 6% polyacrylamide gels, separately. e) A27 was a dominate marker absent in Aichi Asahi and did not exhibit linkage with markers flanking *Pia* (Figure 2).

short arm of rice chromosome 11 [17–19]; therefore, nine SSR markers (RM286, RM558B, RM332, RM167, RM552, RM441, RM479, RM202, and RM536), which are evenly distributed on the short arm, were selected for BSA analysis. Four polymorphic markers were used for linkage analysis in the whole mapping population. The results showed that there were 145, 102, and 15 recombinants which overlapped with loci RM332, RM167, and RM441, respectively, indicating that these three markers lie on the same side of the *Pia* locus. On the other side, 116 distinct recombinants, except for #290, were detected at RM536. Thus, the *Pia* locus was flanked by these four markers (on the telomeric side, RM332, RM167, and RM441; on the centromeric side, RM536) (Figure 2(B)). To further confirm the recombinants on the centromeric side, a new SSR marker, A6, was developed for screening the recombinants in the whole mapping population. As expected 29 recombinants overlapping with RM536 were detected for this marker. Thus, *Pia* was mapped to a segment flanked by RM441 and A6, which covers 1050 kb (Figure 2(B)). A total of 44 recombinants detected at RM441 and A6 were subjected to fine mapping.

2.4 Fine mapping of the *Pia* gene locus

To narrow the region spanning *Pia* locus, four SSR markers (A7, A9, A15, and A16) and one STS marker (A21) were developed in the region flanked by RM441 and A6. On the telomeric side, 11, eight, and three recombinants derived from RM441 were detected at A7, A15, and A16, respectively. Five and one recombinant(s) originating from A6 were detected at A9 and A21, respectively, on the centromeric side. The location of *Pia* was thereby narrowed to a 90 kb region flanked by A16 and A21 (Figure 2(B)). The correlation between phenotypes and genotypes of the three

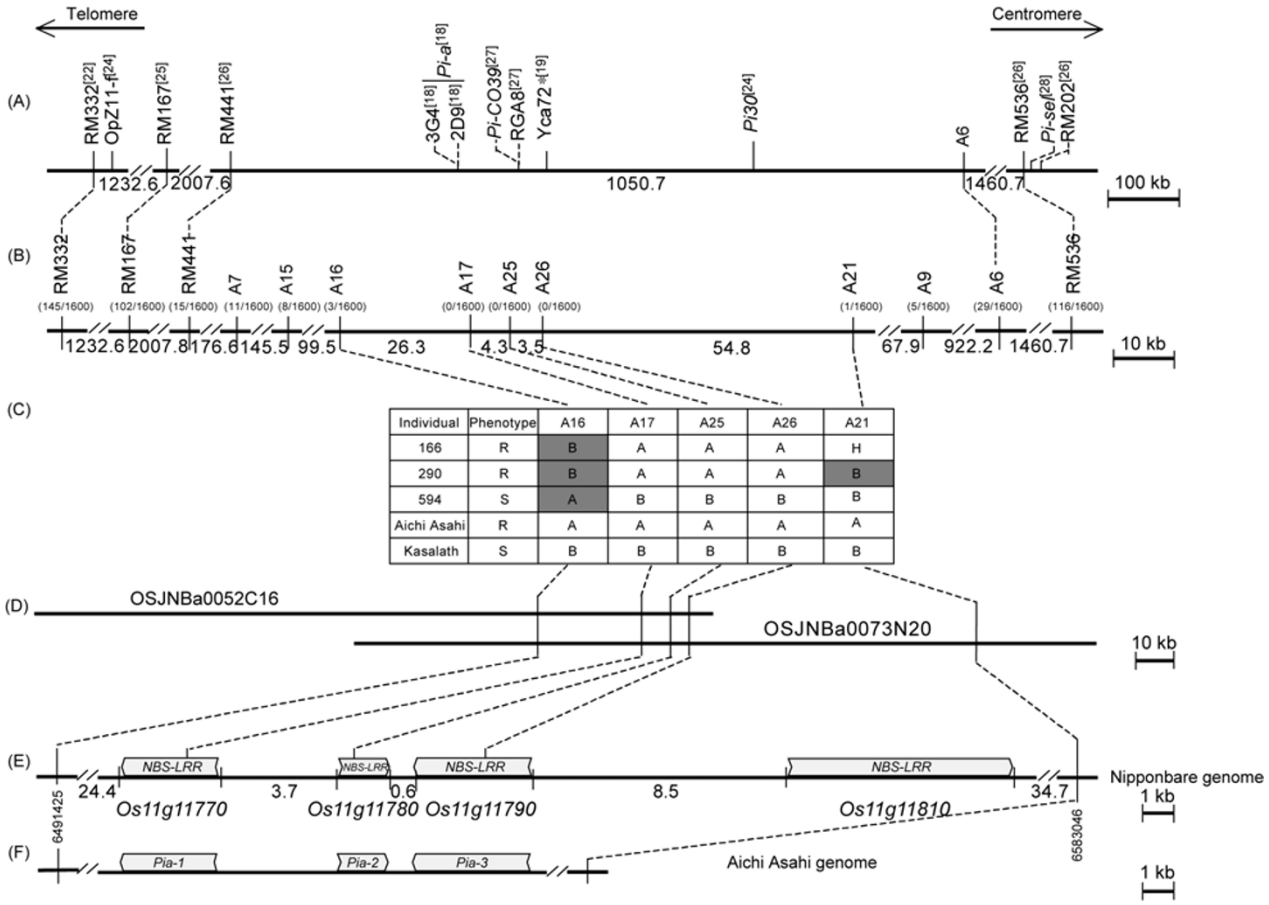

Figure 2 High resolution physical map of the *Pia* locus. (A) An integrated physical map of *Pia*, including three blast resistance genes neighbored to *Pia*, i.e., *Pi30*, *Pi-CO39*, and *Pi-sel*. Map positions were inferred from previous investigations [18,19,22,24–28]. (B) An *in silico* localized physical map of *Pia* based on this study. The numbers below the map represent physical distance of the cv. Nipponbare genomic sequence. The numbers in parentheses are the numbers of recombinants detected between marker loci and *Pia*. (C) Correlation between phenotypes and genotypes of key recombinants and parents at the loci co-segregated with *Pia*. R, resistance; S, susceptible; A, homozygous allele of resistance parent Aichi Asahi; B, homozygous allele of susceptible parent Kasalath; H, heterozygous alleles of both parents. Genotypes with shadow represent recombinants. (D) Contig map spanning the *Pia* locus. The short horizontal lines represent BAC clones of cv. Nipponbare. The vertical lines denote the positions of the respective markers. (E) Four candidate genes predicted as NBS-LRR in cv. Nipponbare genome were identified in the defined region by RiceGAAS (http://ricegaas.dna.affrc.go.jp). The numbers below the map are physical distances between genes. The numbers on both ends of the map denote the position of markers flanking *Pia* locus on Nipponbare genomic sequence. (F) The structural features and candidate genes at the *Pia* locus in the cv. Aichi Asahi genome.

key recombinants and parents at the loci of A16 and A21 are listed in Figure 2(C).

2.5 Co-segregation analysis of candidate genes for *Pia*

The target region defined by A16 and A21 was located on two BAC clones, OSJNBa0052C16 and OSJNBa0073N20, of reference cv. Nipponbare, which cover 6491425–6583046 bp (Figure 2(D)). According to the annotation of these two BAC clones by RiceGAAS, four genes, *Os11g11770*, *Os11g11780*, *Os11g11790*, and *Os11g11810* encoding NBS-LRR domains, were identified as candidate genes of *Pia* in the target region of the cv. Nipponbare genome. To obtain additional markers that completely co-segregate with the *Pia* locus, additional CRG markers based on the coding sequence of every candidate gene were developed for the final round of linkage analysis. Three CRG markers (A17, A25 and A26) derived from *Os11g11770*, *Os11g11780*, and *Os11g11790*, respectively, completely co-segregated with the *Pia* locus (Figure 2(C) and (E)). However, A27, derived from *Os11g11810*, which was dominant, is present in the susceptible cvs. Kasalath and Nipponbare and absent in the resistant parent cv. Aichi Asahi. Recombinant genotyping showed that the linkage relationship between A27 and the *Pia* loci was disordered. To carefully rule out *os11g11810* in the cv. Aichi Asahi genome, three additional markers developed on both sides of *os11g11810* were used for linkage analysis. The results indicated that recombinants detected on these three marker loci were also disordered (data not shown). Thus, the data indicate that there are two genome types in the *Pia*-related region between Nipponbare and Aichi Asahi, and in the latter, the fourth candidate gene *Os11g11810* has been lost and/or shifted (Figure 2(E) and (F)).

Table 2 Genotyping of three candidate genes with the co-segregating markers to the *Pia* locus in three sets of rice blast differential lines

Differential	Resistance gene[a)]	Subspecies	Marker[b)]		
			A17	A25	A26
Japanese differentials					
Shin 2	*Pik-s*, *Pish*	*Japonica*	B	A	B
Aichi Asahi	*Pia*	*Japonica*	A	A	A
Fujisaka 5	*Pii*, *Pik-s*	*Japonica*	B	A	B
Kusabue	*Pik*, *Pish*	*Japonica*	B	A	B
Tsuyuake	*Pik-m*	*Japonica*	B	A	B
Fukunishiki	*Piz*, *Pish*	*Japonica*	B	A	B
K 1	*Pita*	*Japonica*	B	A	B
Pi No.4	*Pita-2*, *Pish*	*Japonica*	B	A	B
Toride 1	*Piz-t*, *Pish*	*Japonica*	A	A	A
K 60	*Pik-p*, *Pish*	*Japonica*	B	A	B
BL 1	*Pib*, *Pish*	*Japonica*	B	A	B
K 59	*Pit*, *Pik-s*	*Japonica*	B	A	B
Chinese differentials					
Tetep	*Pi1*, *Pi5*, *Pi4-b*	*Indica*	A	A	A
Zhenglong 13	Unknown	*Indica*	B	A	B
Sifeng 43	*Pia*, *Pit/Pib*	*Indica*	A	A	A
Dongnong 363	*Pia*, *Pik*	*Japonica*	A	A	A
Kando 51	*Pik*	*Japonica*	A	A	B
Huojiang 18	*Pia*, *Pii*	*Japonica*	A	A	A
LTH	None	*Japonica*	B	A	B
IRRI differentials					
CO39	*Pia*, *PiCO39*	*Indica*	A	A	A
C101LAC	*Pia*, *Pi1*	*Indica*	A	A	A
C101A51	*Pia*, *Pi2*	*Indica*	A	A	A
C101PKT	*Pia*, *Pi4-a*	*Indica*	A	A	A
C104PKT	*Pia*, *Pi3*	*Indica*	A	A	A
C105TTP-4L23	*Pia*, *Pi4-b*	*Indica*	A	A	A
L754	*Pia*, *Pi12*	*Indica*	A	A	A

a) Resistance genes in Japanese, Chinese, and IRRI differential lines were quoted from Imbe *et al.* [14], Kiyosawa and Ling [29], Inukai *et al.* [30], and Kobayashi *et al.* [31].

b) The co-dominant markers A17, A25, and A26 corresponded to the candidate genes *Pia-1*, *Pia-2*, and *Pia-3*, respectively (also see Figure 2). "A" denotes the genotype of the resistant parental cv. Aichi Asahi; "B" denotes the susceptible parental cv. Kasalath. Genotypes in the box were used to deduce *Pia-3* as the most promising candidate gene for *Pia*.

2.6 Genotyping of differential lines

To further confirm candidate gene(s) for *Pia*, three sets of rice blast differentials, mostly carrying known *R* gene(s), were used to genotype with the co-segregation markers derived from each of the three candidate genes (Table 2, Figure 2). The genotype of marker A25 was identical with cv. Aichi Asahi in all the differentials tested, indicating that *Os11g11780* might not be the functional *Pia* gene. As for the other two candidate genes, genotypes of marker A26 completely corresponded to the genotypes of resistance, and those of marker A17 did not. When we focused on the four differentials in the box (Figure 2), it became clear that only the genotypes of marker A26 were consistent with the genotypes of their *R* genes (Table 2). It could be concluded that *Os11g11790* (A26) is the most promising candidate gene for *Pia*, rather than *Os11g11770* (A17). This was supported by the identical genotype of marker A26 in the IRRI differential lines, because the recurrent parental cv. CO39 for these near-isogenic lines was confirmed to carry the *Pia* gene [31]. Intriguingly, two differentials, Toride 1 and Tetep, which were not found to have the *Pia* gene in previous studies, should also carry the *Pia* gene, based on the genotyping results obtained in the present study (Table 2).

3 Discussion

Rice blast is a tricky disease in rice production, because genetic resistance of newly developed cvs is commonly rendered ineffective within a few years after release [5,32]. One long-term strategy for the control of this disease may depend on the durable genetic resistance conferred by *R* gene stacking [6–8,32]. The resistance of *R* genes can be divided into stronger and weaker effects [6,10]. The *R* genes carried by the Japanese differentials, *Pik*, *Pik-m*, and *Pik-p*, exerted stronger effects in most southern regions of China [21]; however, *Pik-s*, *Pish*, *Pia*, and *Pii* displayed weaker effects in these regions (Figure 1). Results from earlier studies indicated that these last four *R* genes, which are ignored by breeders, could have important roles after pyramiding with other *R* genes, whether weaker or stronger *R* genes [6,7,10,11]. It is necessary to assemble better combinations of *R* genes that possess economic, effective, and durable resistance without damaging the other agronomic traits of the crop.

Rice is a model crop with two available reference sequences of two subspecies. The genomic region of a target gene, in which the target gene is isolated via an approach called map-based cloning *in silico*, can be rapidly defined through chromosome-walking with genomic position-ready markers, developed from the available reference sequences. Such a strategy could save a great deal of time and labor because there is no need to construct an artificial chromosome library, and it also could overcome the mapping difficulty because of any complex genetic exchange and genomic structure dynamics (if any) that have occurred in the target region [2,23,33,34]. On the other hand, genomic regions of *R* genes are particularly dynamic and complex because the long-term co-evolution with *AVR* genes of pathogens [2,4,35–37]. A greater genomic difference was also detected in the *Pia*-surrounding region between the resistance donor cv. Aichi Asahi and the susceptible reference cv. Nipponbare, which resulted in the loss of the fourth candidate gene *Os11g11810* in the Aichi Asahi genome (Figure 2). The isolation of *Pia* will also provide insight into the molecular evolution of *R* genes.

Many plant *R* genes are clustered in special genomic regions, and encode highly conserved motifs and structures, such as the NBS-LRR proteins [4,9,33,34,38]. To date, 15 major blast *R* genes have been cloned, 13 of which belong to the large NBS-LRR gene family [2,38]. In the present study, the *Pia* locus was defined into a region with a cluster of four NBS-LRR genes, based on the reference sequence of cv. Nipponbare (Figure 2(E)). Among these four candidate genes, *Pia-3* (*Os11g11790*) was identified as the most promising candidate for *Pia* via CRG marker-based genotyping (Table 2). Sequence alignment analysis revealed that *Pia-3* has a high degree of similarity to the *R* gene *RPM1* of *Arabidopsis thaliana* (data not shown). Interestingly, two ESTs (expressed sequence tags; 2D9 and 3G4; Figure 2(A)), generated from a double haploid (DH) mapping population derived from the cross of IR64 and Azucena, are tightly linked to *Pia* locus [18]. Furthermore, the coding sequences of both ESTs are also highly similar to *RPM1*. The present study also validates the approach of candidate gene(s) by CRG marker-based genotyping [2,33,34]. We have embarked on the identification of the functional *Pia* gene via forward genetic complementation.

This work was supported by the National Transgenic Research Projects (Grant No. 2009ZX08009-023B), the National Basic Research Program of China (Grant No. 2011CB1007), and the National Commonweal Specialized Research Project (Grant No. 200803008). Materials used in the present research were obtained from our own collection, as well as by exchange of materials with colleagues. We thank our colleagues for their enthusiasm and help.

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