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ORIGINAL PAPER

Identification of the blast resistance gene *Pit* in rice cultivars using functional markers

K. Hayashi · N. Yasuda · Y. Fujita · S. Koizumi · H. Yoshida

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Abstract DNA markers that allow for identification of resistance genes in rice germplasm have a great advantage in resistance breeding because they can assess the existence of the genes without laborious inoculation tests. Functional markers (FMs), which are designed from functional polymorphisms within the sequence of genes, are unaffected by nonfunctional allelic variation and make it possible to identify an individual gene. We previously showed that the resistance function of the rice blast resistance gene *Pit* in a resistant cultivar, K59, was mainly acquired by up-regulated promoter activity through the insertion of a long terminal repeat (LTR) retrotransposon upstream of *Pit*. Here, we developed PCR-based DNA markers derived from the LTR-retrotransposon sequence and used these markers to screen worldwide accessions of rice germplasm. We identified 5 cultivars with the LTR-retrotransposon insertion out of 68 rice accessions. The sequence and expression pattern of *Pit* in the five cultivars were the same as those in K59 and all showed *Pit*-mediated blast resistance. The results suggest that the functional *Pit* identified using the markers was derived from a common progenitor. Additionally, comparison of the *Pit* coding sequences between K59 and susceptible cultivars revealed that one nucleotide polymorphism, which caused an amino acid substitution, offered another target for a FM. These results indicate that our DNA markers should enhance prediction of *Pit* function and be applicable to a range of rice varieties/landraces cultivated in various regions worldwide and belonging to the *temperate japonica*, *tropical japonica*, and *indica* groups.

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K. Hayashi (✉) · N. Yasuda · Y. Fujita · S. Koizumi
National Agricultural Research Center,
National Agriculture and Food Research Organization (NARO),
3-1-1 Kannondai, Tsukuba 305-8666, Japan
e-mail: keko1@affrc.go.jp

H. Yoshida
National Agricultural Research Center,
National Agriculture and Food Research Organization (NARO),
1-2-1 Inada, Joetsu 943-0193, Japan

H. Yoshida
National Institute of Crop Science,
National Agriculture and Food Research Organization (NARO),
2-1-18 Kannondai, Tsukuba 305-8518, Japan

Introduction

Blast disease, caused by *Magnaporthe oryzae*, is a major constraint on rice production (Bonman et al. 1992; Babujee and Gnanamanickam 2000). Cultivating varieties with resistance genes is one of the most effective strategies to control blast disease, and various cultivars have been bred worldwide. To support blast resistance breeding, molecular genetics of blast resistance has been extensively studied and many useful DNA markers corresponding to major genes conferring resistance have been identified (Collard et al. 2008; Jena and Mackill 2008). DNA markers for resistance genes are now essential for mapping and contribute to marker-assisted breeding.

For efficient blast resistance breeding, it is often necessary to evaluate the genetic potential for blast resistance in parent cultivars, especially when using exotic germplasm collections. Identification of resistance genes in rice germplasm is generally assessed by inoculation tests. However,

monitoring the presence of individual resistance genes in cultivars is sometimes difficult using this traditional phenotypic assay because the action of one resistance gene masks the actions of others (Noda et al. 1999; Hittalmani et al. 2000; Ebron et al. 2004).

In this case, DNA markers provide efficient and reliable ways to identify resistance genes without inoculation tests. DNA markers for rice blast resistance are typically derived from DNA sites that are genetically linked to the resistance genes and show sequence polymorphisms between resistance and susceptible cultivars; however, several developed DNA markers do not necessarily show tight association to the resistance function. In a previous study, we developed allele-specific PCR markers for nine rice blast resistance genes, each of which is tightly linked to the target resistance gene (Hayashi et al. 2006). Some allele-specific PCR markers amplified nonspecific PCR products from cultivars that did not retain the target resistance genes. These results showed that the use of the allele-specific markers is limited as a diagnostic tool.

To overcome such problems, the application of functional markers (FMs) has been proposed (Andersen and Lübberstedt 2003). FMs are derived from functional polymorphisms, which are polymorphic sites within genes that causally affect the function of interest and never show discrepancy between functional traits and nucleotide polymorphisms in alleles, regardless of genetic background. Therefore, FMs should provide a reliable alternative to inoculation tests for identifying resistance genes.

The challenge of FM development lies in the need to clarify the association between sequence polymorphisms and resistance phenotypic variation. The largest family of resistance-gene structure (class1) is the NBS-LRR gene family; numerous NBS-LRR genes have been identified as resistance genes in plants (Ingvardsen et al. 2008). The rice genome contains approximately 500 NBS-LRR genes, many of which are found in tightly linked clusters (Monosi et al. 2004; Zhou et al. 2004). Clustering is a well-known phenomenon and usually results from tandem duplications of paralogous sequences (Meyers et al. 2005). These duplicated sequences have diverged through accumulated mutation, increasing the complexity of resistance gene sequences. Therefore, extensive intra- and inter-specific genetic variations exist within NBS-LRR genes, making it difficult to identify functional polymorphisms of NBS-LRR alleles based on sequence homology (Ingvardsen et al. 2008).

Many resistance genes have been isolated from various crops (Ingvardsen et al. 2008), and several genes, e.g., rice *Pita* conferring blast resistance (Bryan et al. 2000; Jia et al. 2002, 2004; Wang et al. 2007), wheat *Pm3* conferring powdery mildew (Yahiaoui et al. 2004; Tommasini et al. 2006), and rice *xa5* conferring bacterial leaf blight (Iyer and McCouch 2004; Iyer and McCouch 2007), were targeted for FMs. However, in many other resistance genes, lack of information about the functional polymorphisms that are responsible for resistance is an obstacle in applying FMs for disease resistance breeding.

The rice blast resistance gene *Pit*, which was identified in an Indonesian *indica* rice variety, Tjahaja, confers broad-spectrum resistance (Kiyosawa 1972). In a previous study, we isolated *Pit* and revealed that *Pit* belongs to the CC-NBS-LRR family of resistance genes (Hayashi and Yoshida 2009). Sequence comparison of *Pit* alleles between a resistant cultivar, K59, and a susceptible cultivar, Nipponbare, revealed that the resistance-conferring allele contains four amino acid substitutions, a DNA transposon *dDart*, and a long terminal repeat (LTR)-retrotransposon, named *Renovator*, both inserted in the promoter region. Interestingly, transgenic analyses indicated that the effect of the LTR-retrotransposon on *Pit* promoter activity was greater than that of the amino acid substitutions.

In this study, we present the development of functional PCR-based DNA markers to detect *Pit*. The markers designed from the LTR-retrotransposon sequence were used to screen 68 rice germplasm accessions belonging to *temperate japonica*, *tropical japonica* and *indica* groups for the functional *Pit* sequences. Five cultivars were confirmed to have *Pit* function by inoculating with an *M. oryzae* strain containing *AVR-Pit*. These results show that our markers can be used for functional diagnosis of *Pit*.

Materials and methods

Plant materials

To assess the sequence variation among susceptible cultivars, we used Nipponbare, Koshihikari, Takanari, Yumeaoba, Kusanohoshi, Kusahonami, and Hoshiaoba rice cultivars. Except for Nipponbare and Koshihikari, they were bred from *indica* cultivars or *japonica–indica* crosses. There were only a few cultivars diagnostically identified as *Pit* donors (Kiyosawa et al. 1986). Therefore, these cultivars were inoculated with the *M. oryzae* isolate TH87-06-1 (race 337.3) to confirm that they did not possess the functional *Pit* resistance gene. Senshou and Tadukan cultivars were also used, but no inoculation tests were performed for these cultivars whether they possessed *Pit* function or not.

Sixty-eight germplasm accessions from the National Institute of Agrobiological Sciences (NIAS) global rice core collection (Kojima et al. 2005) was screened for *Pit*. Background information on the varieties (e.g., origin) was obtained and is referenced from the NIAS Genebank database (http://www.gene.affrc.go.jp/index_j.php).

Inoculation tests

To determine whether cultivars possess *Pit* function, disease reactions of cultivars were evaluated as follows (Yasuda et al. 2008). Six seeds of each cultivar were grown in a greenhouse (20–30°C) for 4–5 weeks until the four- to five-leaf stage. These seedlings were inoculated by spraying 30 ml of conidial suspension (1×10^5 conidia/ml in water containing 0.02% Tween 20). The inoculated seedlings were immediately placed in a dark chamber at 100% relative humidity with a moisture-saturated atmosphere at 25°C for 18 h and then transferred to a greenhouse. After 7 days the seedlings were evaluated for the degree of resistance to blast isolates on six scales by scoring disease reactions (Hayashi et al. 2009). Race numbers of the *M. oryzae* isolates were estimated according to the reaction pattern of differential varieties established by Kiyosawa (1984).

RT-PCR

Total cellular RNA was extracted from leaf tissue using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The RNA samples were treated with DNase I and converted to cDNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) prior to RT-PCR. The primers and conditions for RT-PCR have been described by Hayashi et al. (2009).

DNA sequences

Nucleotide sequences of the target gene from the examined cultivars were determined by direct sequencing of PCR-amplified fragments as described by Hayashi et al. (2004). To design PCR primers, we used the *Pit* sequence of K59 (accession no. AB379815) and Nipponbare.

DNA extraction

A rapid method was used to isolate DNA as described by Hayashi et al. (2004). The tips of rice leaves (~0.02 g) were soaked in 0.45 ml isolation buffer (100 mM Tris–HCl, 10 mM EDTA, 1 M KCl, pH 8.0) and ground with a TissueLyser II (QIAGEN, Hilden, Germany) for 10 s at room temperature. After rice leaf debris was precipitated by centrifugation, the supernatant was collected. DNA in the supernatant was precipitated by adding an equal volume of isopropyl alcohol, recovered by centrifugation for 15 min, and finally dissolved in 60 µl of TE buffer (pH 8.0).

Genotyping with PCR markers

For the PCR-based DNA markers used to evaluate the *Renovator* and *dDart* insertions, the reactions (volume 20 µl) each contained 3 µl of genomic rice DNA isolated by the rapid method, 0.5 units of AmpliTaq Gold DNA polymerase (PE Applied Biosystems, CA, USA), 2 µl 10× PCR buffer, 2 µl of 2.0 mM dNTPs, and 0.5 µl each of two or three 10 mM primers. The amplification program parameters were 95°C for 10 min for initial denaturation followed by 36 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s. For SNP genotyping, an allele-specific PCR marker assay described by Hayashi et al. (2004) was used. All PCR reactions were performed in a PCR Thermal Cycler SP (Takara, Japan). PCR products were visualized by gel electrophoresis on a 2% (w/v) agarose gel. The sequence of primers and sizes of the PCR-amplified products with the markers are listed in Table 1.

Results

Sequence variations in the *Pit* gene

Allele sequences of the motifs affecting the *Pit* function are important information for development of FMs. Therefore, we first compared the coding sequences of ten cultivars, i.e., K59, Nipponbare, Koshihikari, Hoshiaoba, Takanari, Yumeaoba, Kusanohoshi, Kusahonami, Senshou, and Tadukan, to investigate the sequence variation of the *Pit* allele (Fig. 1a). The coding sequences were divided into five types: K59-type (K59), Nipponbare-type (Nipponbare), Koshihikari-type (Koshihikari and Hoshiaoba), Takanari-type (Takanari, Yumeaoba, Kusanohoshi, and Kusahonami), and Senshou-type (Senshou and Tadukan). Eight polymorphic sites were identified between the K59- and Senshou-type sequences. One of them, positioned at the 1284th nucleotide (counted from the A of the ATG start codon), is a deletion mutation (Fig. 1a; Supplemental Fig. 1). This deletion in the Senshou-type caused a frameshift mutation with a stop codon leading to a truncated protein, indicating the presence of a null allele of *Pit* in Senshou-type cultivars (Fig. 1b). Except for Senshou and Tadukan, the cultivars that belonged to Nipponbare-, Koshihikari-, and Takanari-type were all susceptible (as detailed in "Materials and methods"). In their coding sequences, six nucleotide substitutions were identified between the Nipponbare- and K59-types, and between the Koshihikari- and K59-types, respectively. Between the Takanari- and K59-types, five nucleotide substitutions were identified. Among them, three substitutions, positioned at the 438, 1080, and 2338 positions, were unique to the *Pit* allele of K59 (Fig. 1a). Among these, only the substitution at the 2338th nucleotide (A/G), which was located in the LRR region, was associated with an amino acid substitution (methionine to valine) at codon 780 (the M780V mutation) (Fig. 1b).

Table 1 Sequence of primer sets for *Pit* used in this study

Marker name	Primer name	Primer sequence	Primer name	Primer sequence	PCR product size (bp)
tNpb	tN11	ATGATAACCTCATCCTCAATAAGT	tN10	AAGCACCAAGTGGCGCCA	590
tK59[a]	tN11	ATGATAACCTCATCCTCAATAAGT	tRn1	GTTGGAGCTACGGTTGTTCAG	733
			tRn2	CCAAGGGATTAGGTCCTAGTG	530
tdDN	tN40	GGAAAAATAGAGTCAAACCGCC	tN401	CCTTTCGATGTTTTTTCTATATAAGC	142
tdDK	tD3	GTGCCACGTGTCGCCTTCCCGTTG	tN401	CCTTTCGATGTTTTTTCTATATAAGC	362
st780A[b]	tT8	TCGAGAATTGTGGCTCGGT	780VMN	GGCCTGCTGGTGGAAGAAC	254
			780VMK	GGCCTGCTGGTGGAAGAAT	254
st780B[b]	tT9	GCCAAGCTTACTGTATATATTTCTTGTC	780VMN	GGCCTGCTGGTGGAAGAAC	150
			780VMK	GGCCTGCTGGTGGAAGAAT	150

[a] Three primers are necessary for genotyping

[b] Strategy of allele-specific PCR markers as described by Hayashi et al. (2004)

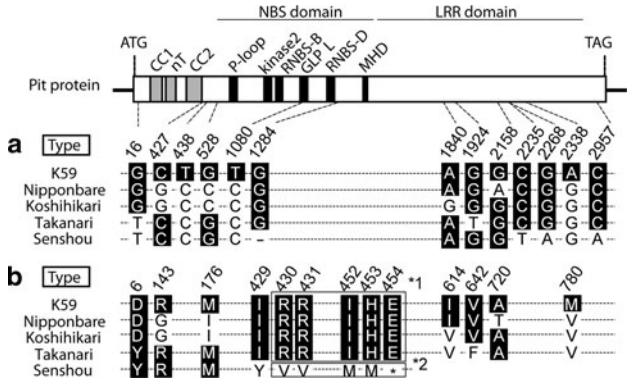

Fig. 1 DNA polymorphisms (**a**) and amino acid changes (**b**) in the *Pit* coding region of susceptible cultivars. *Open box* at the *top* shows the coding region of *Pit*. The structural signatures of Pit are shown in the *upper region* of the figure. Ten cultivars are classified into five types: K59, Nipponbare, Koshihikari (Koshihikari and Hoshiaoba), Takanari (Takanari, Yumeaoba, Kusanohoshi and Kusahonami), and Senshou (Senshou and Tadukan) types. *Numbers* in the *upper parts* in **a**, **b** indicate the positions of nucleotide and amino acid substitutions, respectively. A *hyphen* positioned at the 1284th nucleotide in **a** shows a deletion mutation. An *asterisk* positioned at codon 454 in **b** shows a stop codon. *Open boxes* with *1 and *2 show amino acid sequences "RRDAVAYWWVAEGFVTEVHGYSIHE" and "VVMLSLTGGLLK VSIYATYVSAMM," respectively

Development of markers for *Renovator*

As we previously reported, an LTR-retrotransposon, named *Renovator*, inserted in the promoter region of *Pit* is essential for *Pit* function (Hayashi and Yoshida 2009). The structure of the *Pit* locus in K59 is almost identical to that in Nipponbare. However, the LTR-retrotransposon and a *dDart* transposon (Tsugane et al. 2006) are inserted the upstream region of *Pit* (Fig. 2a). To reveal the relationship between the susceptible phenotypes and the insertion of *Renovator* in the upstream regions of *Pit*, we developed two dominant DNA markers to examine the *Renovator-Pit* structure. To design primers for detecting *Renovator* upstream of *Pit*, we utilized the 3′ sequence of *Renovator* and the 5′ sequence of the *Pit* coding region. However, polymorphisms may be found in the *Renovator* sequence between K59 and another variety because LTR-retrotransposons are extremely polymorphic within species (Kumar and Bennetzen 1999; Pearce et al. 1999). Therefore, to avoid potential failure of amplification caused by mismatch between a primer and genome sequences, two forward primers, tRn1 and tRn2, were designed in the 3′ side of *Renovator* sequence, and one reverse primer, tN11, was designed from the N-terminal region of *Pit* (Fig. 2a). This dominant marker consisting of three primers was named tK59. For detecting the sequences of Nipponbare-type, tNpb was designed by combining a primer, tN10, in the upstream region of the *Renovator* insertion site and the reverse primer of tN11 (Fig. 2a). tNpb and tK59 sheared the reverse primer tN11 because sequence comparison of *Pit* alleles among susceptible cultivars suggested that the polymorphism level of the *Pit* coding region is relatively low.

We genotyped the *Renovator-Pit* structure in K59 and seven susceptible cultivars, Nipponbare, Koshihikari, Hoshiaoba, Takanari, Yumeaoba, Kusanohoshi, and Kusahonami, using these markers (Fig. 2b). Two predicted PCR products of tK59 were amplified only from the genome of K59, but no amplification was obtained from the other cultivars. The amplification patterns of tNpb were opposite to those of tK59. PCR products of tNpb were amplified from the genome of the seven susceptible cultivars, but not from that of K59. These results indicated that tNpb, which was developed from information for the Nipponbare sequence, could genotype the upstream sequence of *Pit* in other susceptible cultivars and revealed that no *Renovator* insertion was found in these cultivars. In previous research,

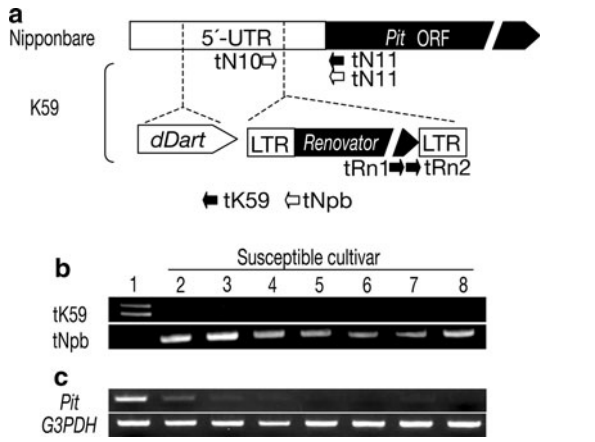

Fig. 2 *Renovator* insertion and expression patterns of *Pit* in the resistant and susceptible cultivars. **a** The genome structures of the *Pit* locus in Nipponbare (*top*) and K59 (*bottom*) are shown. Genes are shown as *boxes* bearing the direction of transcription. Positions of primers for dominant PCR-based DNA markers, tK59 and tNpb, are indicated by *black* and *white arrows*, respectively. **b** tK59 and tNpb markers amplified specific fragments in K59 and susceptible cultivars. The templates were from K59 (*lane 1*), Nipponbare (*2*), Koshihikari (*3*), Hoshiaoba (*4*), Takanari (*5*), Yumeaoba (*6*), Kusanohoshi (*7*), and Kusahonami (*8*). **c** Expression analyses of *Pit* alleles in K59 (*lane 1*) and the susceptible cultivars (*2–8*). Transcript levels of *Pit* genes were examined by RT-PCR. *G3PDH* was used as an endogenous reference gene

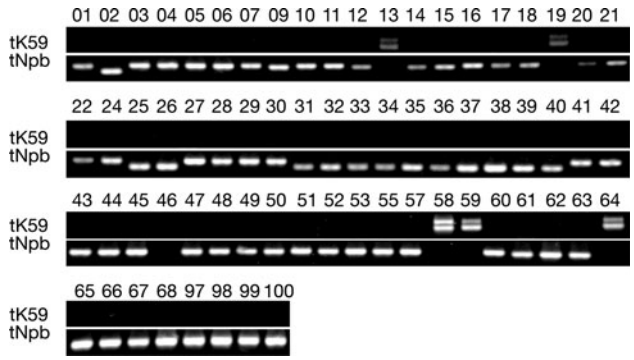

Fig. 3 Identification of functional *Pit* allele from 68 cultivars of the NIAS global rice core collection using tK59 (*upper lanes*) and tNpb (*lower lanes*). *Numbers* above the gel image indicate the world rice collection (WRC) numbers. Information on the cultivars is summarized in Table 2

we showed that high *Pit* expression was necessary for the *Pit* function (Hayashi and Yoshida 2009). Therefore, we checked the expression of *Pit* alleles by RT-PCR to investigate the relationship between the susceptible phenotypes and *Renovator* insertion (Fig. 2c). In all susceptible cultivars, expression of *Pit* allele sequences was significantly suppressed compared to that in K59.

The results suggest that the level of *Pit* mRNA is up-regulated only through the insertion of *Renovator* in these bred cultivars. Accordingly, these results suggest that the marker detecting *Renovator* may be an optimal choice for identifying the functional *Pit* gene.

To assess *Renovator* insertion into the upstream region of *Pit* in various cultivars, we genotyped the *Renovator-Pit* structure in 68 cultivars of the NIAS global rice core collection using the tK59 and tNpb markers (Fig. 3; Table 2). Among the 68 varieties, PCR products of tK59 were amplified in five cultivars, Asu (WRC13), Deng Pao Zhai (WRC19), Neang Menh (WRC58), Neang Phtong (WRC59), and Padi Kuning (WRC64). In five cultivars, no PCR products were amplified by tNpb. In the other 63 cultivars, except for Khao Nok (WRC46), PCR products were amplified by tNpb but not by tK59. Sequence analysis of the *Pit* upstream region in several WRC cultivars indicate that the variation of tNpb PCR products is due to insertion/deletion polymorphisms existing upstream of *Pit*, and no PCR amplification by tNpb in Khao Nok (WRC46) due to a nucleotide substitution existed at the tN10 site (data not shown). These genotyping results indicate that only the five cultivars possess *Renovator* in the upstream of *Pit*. Interestingly, sequencing the *Pit* coding region of five alleles revealed that the five coding sequences were completely identical to that of K59 (Fig. 4a). To evaluate the promoter activity from the *Renovator* sequence, expression of *Pit* mRNA in the five cultivars was compared to that in K59 and Nipponbare (Fig. 4b). The expression levels of *Pit* in these five cultivars were significantly higher than that in Nipponbare and were almost the same as that in K59. The results indicate that the *Renovator* sequence strongly affects the promoter activity of *Pit* in Asu, Deng Pao Zhai, Neang Menh, Neang Phtong, and Padi Kuning.

Development of markers for *dDart*

The characteristic structure of the *Pit* locus in K59 is not only the insertion of *Renovator* but also a *dDart* transposon in the upstream region of *Pit*. To examine the surrounding structure of *Pit* in the 68 cultivars, we evaluated the insertion of *dDart* using two dominant markers, tdDK and tdDN (Fig. 5). tdDK was designed to detect the *dDart* sequence by combining a reverse primer, tD3, in the *dDart* sequence and a forward primer, tN401, in the upstream region of *Pit*. tdDN, which was designed to detect the Nipponbare-type genome, was composed of two primers, tN40 and tN401. We then genotyped the *dDart-Pit* structure in the 68 cultivars using these markers. The PCR products were amplified by tdDK or tdDN in all cultivars and there was no discrepancy between the genotype judged from the PCR patterns of tdDK/tdDN and those of tK59/tNpb except for in Khao Nok (WRC46) (Table 2). The results of PCR genotyping of the *Pit* locus indicated that the five cultivars possessed not only *Renovator* but also *dDart* sequences in the upstream region of *Pit*. The structures of the *Pit* locus in the five cultivars were hypothesized to be same as that of the *Pit* locus in K59.

Table 2 Genotyping for the diagnosis of the functional *Pit* in 68 cultivars of NIAS global rice core collection

WRC no.	Origin	Name	*dDart*			*Renovator*			SNP(M780V)		
			tdDN	tdDK	Genotype[a]	tNpb	tK59	Genotype[a]	st780A	st780B	Genotype[a]
	Japan	K59	−	+	K59	−	+	K59	K	K	K59
1	Japan	Nipponbare	+	−	Npb	+	−	Npb	N	N	Npb
2	India	Kasalath	+	−	Npb	+	−	Npb	−	N	Npb
3	Cambodia	Bei khe	+	−	Npb	+	−	Npb	N	ND	Npb
4	Nepal	Jena 035	+	−	Npb	+	−	Npb	N	ND	Npb
5	India	Naba	+	−	Npb	+	−	Npb	N	ND	Npb
6	Indonesia	Puluik Arang	+	−	Npb	+	−	Npb	N	ND	Npb
7	Philippines	Davao 1	+	−	Npb	+	−	Npb	N	ND	Npb
9	China	Ryou Suisan Koumai	+	−	Npb	+	−	Npb	−	N	Npb
10	China	Shuusoushu	+	−	Npb	+	−	Npb	N	ND	Npb
11	China	Jinguoyin	+	−	Npb	+	−	Npb	N	ND	Npb
12	China	Dahonggu	+	−	Npb	+	−	Npb	−	N	Npb
13	Bhutan	Asu	−	+	K59	−	+	K59	K	ND	K59
14	Philippines	IR 58	+	−	Npb	+	−	Npb	N	ND	Npb
15	India	Co 13	+	−	Npb	+	−	Npb	−	N	Npb
16	Madagascar	Vary Futsi	+	−	Npb	+	−	Npb	−	N	Npb
17	China	Keiboba	+	−	Npb	+	−	Npb	N	ND	Npb
18	Taiwan	Qingyu (Seiyu)	+	−	Npb	+	−	Npb	N	ND	Npb
19	China	Deng Pao Zhai	−	+	K59	−	+	K59	K	ND	K59
20	Philippines	Tadukan	+	−	Npb	+	−	Npb	N	ND	Npb
21	Myanmar	Shwe Nang Gyi	+	−	Npb	+	−	Npb	N	ND	Npb
22	Philippines	Calotoc	+	−	Npb	+	−	Npb	N	ND	Npb
24	Philippines	Pinulupot 1	+	−	Npb	+	−	Npb	N	ND	Npb
25	India	Muha	+	−	Npb	+	−	Npb	−	N	Npb
26	India	Jhona 2	+	−	Npb	+	−	Npb	N	ND	Npb
27	Nepal	Nepal 8	+	−	Npb	+	−	Npb	N	ND	Npb
28	Bhutan	Jarjan	+	−	Npb	+	−	Npb	N	ND	Npb
29	Nepal	Kalo Dhan	+	−	Npb	+	−	Npb	N	ND	Npb
30	Nepal	Anjana Dhan	+	−	Npb	+	−	Npb	N	ND	Npb
31	Bangladesh	Shoni	+	−	Npb	+	−	Npb	−	N	Npb
32	Bangladesh	Tupa 121-3	+	−	Npb	+	−	Npb	−	N	Npb
33	India	Surjamukhi	+	−	Npb	+	−	Npb	−	N	Npb
34	India	ARC 7291	+	−	Npb	+	−	Npb	−	N	Npb
35	India	ARC 5955	+	−	Npb	+	−	Npb	N	ND	Npb
36	India	Ratul	+	−	Npb	+	−	Npb	N	ND	Npb
37	India	ARC 7047	+	−	Npb	+	−	Npb	−	N	Npb
38	India	ARC 11094	+	−	Npb	+	−	Npb	−	N	Npb
39	Nepal	Badari Dhan	+	−	Npb	+	−	Npb	−	N	Npb
40	India	Nepal 555	+	−	Npb	+	−	Npb	−	N	Npb
41	Srilanka	Kaluheenati	+	−	Npb	+	−	Npb	N	ND	Npb
42	India	Local Basmati	+	−	Npb	+	−	Npb	N	ND	Npb
43	China	Dianyu 1	+	−	Npb	+	−	Npb	N	ND	Npb
44	Philippines	Basilanon	+	−	Npb	+	−	Npb	N	ND	Npb
45	Myanmar	Ma Sho	+	−	Npb	+	−	Npb	N	ND	Npb
46	Laos	Khao Nok	+	−	Npb	−	−	UN	N	ND	Npb
47	Brazil	Jaguary	+	−	Npb	+	−	Npb	N	ND	Npb

Table 2 continued

WRC no.	Origin	Name	*dDart*			*Renovator*			SNP(M780V)		
			tdDN	tdDK	Genotype[a]	tNpb	tK59	Genotype[a]	st780A	st780B	Genotype[a]
48	Vietnam	Khau Mac Kho	+	–	Npb	+	–	Npb	N	ND	Npb
49	Indonesia	Padi Perak	+	–	Npb	+	–	Npb	N	ND	Npb
50	USA	Rexmont	+	–	Npb	+	–	Npb	N	ND	Npb
51	Japan	Urasan 1	+	–	Npb	+	–	Npb	–	N	Npb
52	Vietnam	Khau Tan Chiem	+	–	Npb	+	–	Npb	N	ND	Npb
53	Bhutan	Tima	+	–	Npb	+	–	Npb	N	ND	Npb
55	Bangladesh	Tupa 729	+	–	Npb	+	–	Npb	N	ND	Npb
57	Korea	Milyang 23	+	–	Npb	+	–	Npb	N	ND	Npb
58	Cambodia	Neang Menh	–	+	K59	–	+	K59	K	ND	K59
59	Cambodia	Neang Phtong	–	+	K59	–	+	K59	K	ND	K59
60	Laos	Hakphaynhay	+	–	Npb	+	–	Npb	N	ND	Npb
61	Malaysia	Radin Goi Sesat	+	–	Npb	+	–	Npb	–	N	Npb
62	Malaysia	Kemasin	+	–	Npb	+	–	Npb	–	N	Npb
63	Thailand	Bleiyo	+	–	Npb	+	–	Npb	–	N	Npb
64	Indonesia	Padi Kuning	–	+	K59	–	+	K59	K	ND	K59
65	Indonesia	Rambhog	+	–	Npb	+	–	Npb	N	ND	Npb
66	Myanmar (Burma)	Bingala	+	–	Npb	+	–	Npb	N	ND	Npb
67	India	Phulba	+	–	Npb	+	–	Npb	N	ND	Npb
68	Laos	Khao Nam Jen	+	–	Npb	+	–	Npb	N	ND	Npb
97	Myanmar (Burma)	Chin Galay	+	–	Npb	+	–	Npb	N	ND	Npb
98	China	Deejiaohualuo	+	–	Npb	+	–	Npb	N	ND	Npb
99	China	Hong Cheuh Zai	+	–	Npb	+	–	Npb	N	ND	Npb
100	Srilanka	Vandaran	+	–	Npb	+	–	Npb	N	ND	Npb

Three sets of markers were designed to evaluate the insertion of *dDart* and *Renovator* in the upstream region of *Pit*, and a nucleotide substitution positioned at 2338 (A/G)

"+" shows a PCR band amplified by tdDN or tdDK and by tNpb or tK59; "K" and "N" show K59- and Nipponbare-specific PCR bands, respectively; "–" shows no amplification

ND No amplification experiment

[a] K59 and Npb represent the result of genotyping that was K59 and Nipponbare, respectively. UN indicated that a genotype was unconfirmed by tNpb

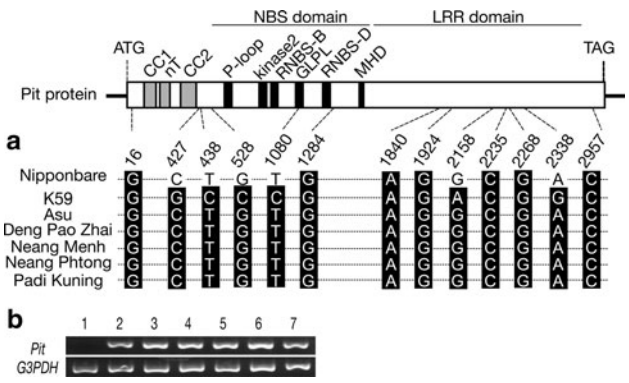

Fig. 4 Sequence variation (**a**) and expression pattern (**b**) of *Pit* alleles in Nipponbare (*lane 1*), K59 (*2*), Asu (*3*), Deng Pao Zhai (*4*), Neang Menh (*5*), Neang Phtong (*6*), and Padi Kuning (*7*). The *numbers* of *upper parts* in **a** indicate the positions of nucleotide substitutions

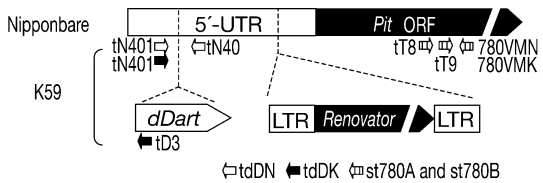

Fig. 5 Position of primers for dominant PCR-based DNA markers, tdDK and tdDN, and dominant allele-specific PCR markers, st780A and st780B. *Black* and *white arrows* indicate positions of primers for tdDK and tdDN, which were developed to evaluate the *dDart-Pit* structure. *Arrows* with *vertical lines* indicate the primers of st780A and st780B for detecting a nucleotide substitution positioned at 2338 (the M780V mutation)

Development of markers for the M780V mutation

As shown in Fig. 1b, sequence comparison of the *Pit* coding sequences among the susceptible cultivars suggested

that methionine at codon 780 may be specific to the functional *Pit*. To evaluate whether this substitution correlates with the insertion of *Renovator* in the upstream region of *Pit*, we developed two allele-specific PCR markers to genotype the polymorphism at the 2338th nucleotide (A/G) in other cultivars (Fig. 5): the two markers, st780A and st780B, shared common allele-specific primer sets, 780VMN (susceptible cultivar-specific primer) and 780VMK (K59-specific primer), but different forward primers, tT8 (for st780A) or tT9 (for st780B). First, we genotyped the polymorphism at codon 780 in the 68 cultivars using st780A; no PCR products of st780A were obtained in 18 of the 68 cultivars. These 18 cultivars were re-genotyped using st780B (Table 2). The results of genotyping revealed that the five K59-type cultivars showed K59-type polymorphism and the others showed Nipponbare-type polymorphism. These results indicated that methionine at codon 780 was unique to the K59-type cultivars.

Disease reaction of the five K59-type cultivars

The sequence and expression analysis strongly suggested that the disease reaction of the five cultivars is identical to that in K59. To confirm whether Asu, Deng Pao Zhai, Neang Menh, Neang Phtong, and Padi Kuning showed *Pit*-specific resistance, the five cultivars were evaluated for disease reactions to five *M. oryzae* races (037.3, 777.3, 337.1, 006.4 and 106.4). The former three races (037.3, 777.3, and 337.1) were avirulent to K59. The latter two races (006.4 and 106.4) were virulent to K59. Their virulence on Nipponbare, K59, Asu, Deng Pao Zhai, Neang Menh, Neang Phtong, and Padi Kuning is shown in Table 3. As expected, the disease reactions in the five cultivars were consistent with that in K59. These results provide pathological evidence that the five cultivars carry the functional *Pit* gene.

Discussion

The starting point of FM development is identification of the sequence(s) that affect gene function. Association studies sometimes contribute to estimating functional motifs within genes that affect phenotypic characters (Thornsberry et al. 2001; Osterberg et al. 2002; Sweeney et al. 2007). However, application of association study to identify functional motifs in NBS-LRRs might be hampered because many NBS-LRRs show a high rate of sequence divergence (Ingvardsen et al. 2008). To overcome this problem, we used a distinctive resistance mechanism of *Pit* to develop FMs: The main mechanism of *Pit* function is not amino acid substitutions but the up-regulated promoter activity conferred by the *Renovator* sequence (Hayashi and Yoshida 2009). Based on this mechanism, we developed the PCR-based markers tK59 and tNpb to detect whether *Renovator* is inserted in the upstream region of *Pit*.

tK59 and tNpb identified five cultivars possessing the functional *Pit* from the NIAS global rice core collection (Fig. 3). The isolates were *indica* varieties but derived from different countries (Table 2): Asu (Bhutan), Deng Pao Zhai (China), Neang Menh (Cambodia), Neang Phtong (Cambodia), and Padi Kuning (Indonesia). Despite the diversity of their geographical distribution, no polymorphism was found in *Pit* of the five cultivars (Fig. 4a). Additionally, *dDart* insertion in the upstream region of *Pit* and the methionine at codon 780 in the *Pit* coding sequences were unique to the K59-type cultivars (Table 2). These results suggest that not only the coding sequences but also the upstream sequences of *Pit* in the five cultivars were identical to those in K59. Thus, the origin of the five genome sequences in the *Pit* locus should be common, and the *Pit* locus may have spread from one progenitor to various cultivars. These evolutionary backgrounds provide positive evidence that tK59 is a reliable marker to isolate cultivars carrying the functional *Pit* gene from rice germplasm worldwide.

Table 3 Disease reaction of Asu, Deng Pao Zhai, Neang Menh, Neang Phtong, and Padi Kuning to *M. oryzae* isolates

Isolates	Ai79-142	Spr777.3	Ao92-06-2-2	THI77-4-2-1	IS72
Race no.	037.3	777.3	337.1	006.4	106.4
Nipponbare	5	5	5	2	1
K59	1	1	2	5	5
Asu	1	1	1	5	5
Deng Pao Zhai	1	1	2	5	5
Neang Menh	1	1	1	5	5
Neang Phtong	1	1	1	5	5
Padi Kuning	1	1	1	5	5

Disease reaction scores are 0: no visible evidence of infection, 1: brown specks <0.5 mm in diameter, 2: roundish lesions <1 mm in diameter surrounded by brown margins, 3: small eyespot lesions <1.5 mm in diameter surrounded by dark brown margin, 4: intermediate size eyespot lesions <2 mm in diameter with necrotic gray center and water-soaked or brown margins, and 5: large eyespot lesions more than 2 mm in diameter

In some NBS-LRRs, amino acid substitutions are involved in resistance and race specificities (Michelmore and Meyers 1998; DeYoung and Innes 2006). The nucleotide substitutions, which are responsible for pathogen recognition, provide candidate polymorphisms for developing FMs. A pioneering work was carried out to produce FMs for the *Pita* gene encoding an NBS-LRR protein. One nonsynonymous change in the C-terminal region was identified as a functional polymorphism (Bryan et al. 2000; Jia et al. 2003). By genotyping the functional nucleotide polymorphism in the coding sequence of *Pita*, Wang et al. (2007) succeeded in identifying cultivars possessing the *Pita* gene from 141 cultivars.

Pit protein encoded by the Nipponbare allele differs from that encoded by the K59 allele at four amino acid positions (Fig. 1b): R143G, M176I, A720T, and M780V. Inoculation tests using chimeric constructs carrying the various regions of the K59 and Nipponbare alleles suggest that R143G and M176I have a potential effect in *Pit* resistance, while A720T and M780V do not (Hayashi and Yoshida 2009). Sequence comparison of *Pit* alleles between K59 and nine susceptible cultivars revealed that the nucleotide polymorphisms, which were correlated with the resistance phenotypes in the transgenic experiments, were not always used for FMs: arginine at position 143 and methionine at position 176, which potentially affected the *Pit* resistance, also existed in the susceptible cultivars of Takanari- and Senshou-types. Methionine at position 780, which had no effect on the *Pit* resistance, was unique in the K59-type cultivars (Fig. 1b). These results show that polymorphic allele sequences, including those of susceptible cultivars, are a prerequisite to evaluating candidate nucleotide polymorphisms for FMs.

In the case of *Pit*, not only the *Renovator* sequence exerting the *Pit* function but also the *dDart* and M780V polymorphisms, which are not responsible for but coincidently associated with the *Pit* function, were available for discrimination between resistance and susceptible cultivars.

The major advantages of FMs in marker-assisted selection (MAS) are (a) FMs allow reliable application of markers in a population without prior mapping, and (b) FMs can be used in mapping a population without the risk of recombination between the marker and the target gene (Andersen and Lübberstedt 2003; Ingvardsen et al. 2008). Therefore, FMs can be useful for pyramiding resistance genes in resistance breeding (Ingvardsen et al. 2008). In particular, the beneficial aspects of FMs that facilitate identification of the target gene even in a collection of diverse genetic backgrounds expand their usage beyond breeding purposes. In our case, FMs of *Pit* could be applied for studies of geographical distribution, evolutionary studies, and population genetics.

Geographical distribution of the functional *Pit* gene

Pit was one of the rice blast resistance genes identified in the 1970s (Kiyosawa 1972), but only a small amount of distributional information was available. In this study, we revealed that 5 out of 68 cultivars, from four Asian countries, possessed the *Pit* function. In the NIAS collection, 61 out of 68 accessions of germplasms are local varieties (Table 2). The results suggest that cultivars possessing *Pit* are being cultivated not only in Indonesia but also in at least several other Asian countries. The markers can be applied to survey the geographical distribution of *Pit*.

Evolutionary study of *Pit*

As far as we know, allelic genes of functional *Pit* have not been identified. In a previous study, we revealed that *Pit* of K59-type was activated by transcriptional alteration caused by the flanking *Renovator* sequence. Although Kiyosawa et al. (1983, 1986) suggested identification of *Pit* in the Tongil variety by inoculation tests, our FMs did not detect *Renovator* upstream of *Pit* in Tongil (Hayashi, unpublished data). This may suggest at least three possibilities: (a) Tongil possesses different mechanisms to activate *Pit* function without the *Renovator-Pit* system; (b) the line of Tongil we used in this study was different from the line of Kiyosawa et al.'s experiment, and the functional *Pit* allele was segregated out in our line; and (c) the inoculation tests were misinterpreted in the previous report. The first possibility is attractive because such kinds of genes would be candidates for the novel *Pit* allelic genes and provide important insights into the evolution of the *Pit* locus. Therefore, the FMs may indirectly contribute to the identification of allelic *Pit* genes and subsequent evolutionary studies of the *Pit* genes.

Impact of *Pit* on pathogen populations

Cultivars carrying a single-locus resistance often break down because of an *M. oryzae* race shift toward strains that escape host recognition by adaptive selection pressure (Bonman et al. 1992; Babujee and Gnanamanickam 2000). Therefore, large-scale cultivation of rice varieties with *Pit* should also affect the diversity of blast fungus races. Although Noda et al. (1999) revealed that 86% of 129 isolates of *M. oryzae* from Vietnam were virulent to *Pit*, our *Pit* genotyping using FMs suggested that two cultivars in Vietnam, Khau Mac Kho (WRC48) and Khau Tan Chiem (WRC52), do not have the functional *Pit* allele. Therefore, it is interesting to pursue further experiments combining monitoring of the Vietnamese cultivar identity using tK59 with inoculation tests to uncover a deeper and strong evolutionary relationship between cultivars and races. FMs

would be a good tool for such population genetics. Our successful application of FMs for the *Pit* gene should accelerate development of blast-resistant cultivars by MAS and also benefit studies using rice cultivars containing the *Pit* gene.

Cloning of resistance genes made it possible to develop FMs. To date, more than 90 resistance genes have been isolated in plants (Ingvardsen et al. 2008) and progress in plant genomics will drastically increase the number of isolated resistance genes over the next decade. As more genes are isolated and more underlying functional polymorphisms are identified, the development and use of FMs will also undoubtedly be promoted. These markers can be applied to reliably identify a functional allele of resistance genes instead of inoculation tests. In the near future, combinatorial use of several FMs could be a systematic diagnosis tool to identify resistance genes for various diseases.

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References

Andersen JR, Lübberstedt T (2003) Functional markers in plants. Trends Plant Sci 8:554–560

Babujee L, Gnanamanickam SS (2000) Molecular tools for characterization of rice blast pathogen (*Magnaporthe grisea*) population and molecular marker-assisted breeding for disease resistance. Curr Sci 78:248–257

Bonman JM, Khush GS, Nelson RJ (1992) Breeding rice for resistance to pests. Annu Rev Phytopathol 30:507–528

Bryan GT, Wu KS, Farrall L, Jia Y, Hershey HP, McAdams SA, Faulk KN, Donaldson GK, Tarchini R, Valent B (2000) A single amino acid difference distinguishes resistant and susceptible alleles of the rice blast resistance gene *Pi-ta*. Plant cell 12:2033–2046

Collard BC, Vera Cruz CM, McNally KL, Virk PS, Mackill DJ (2008) Rice molecular breeding laboratories in the genomics era: current status and future considerations. Int J Plant Genomics 2008:524847. doi:10.1155/2008/524847

DeYoung BJ, Innes RW (2006) Plant NBS-LRR proteins in pathogen sensing and host defense. Nat Immunol 7:1243–1249

Ebron LA, Fukuta Y, Imbe T, Kato H, Yanoria JMT, Tsunematsu H, Khush GS, Yokoo M (2004) Estimation of genes in blast resistance in elite indica-type rice (*Oryza sativa* L.) varieties-bred at the International Rice Research Institute. Breed Sci 54:381–387

Hayashi K, Yoshida H (2009) Refunctionalization of the ancient rice blast disease resistance gene *Pit* by the recruitment of a retrotransposon as a promoter. Plant J 57:413–425

Hayashi K, Hashimoto N, Daigen M, Ashikawa I (2004) Development of PCR-based SNP markers for rice blast resistance genes at the *Piz* locus. Theor Appl Genet 108:1212–1220

Hayashi K, Yoshida H, Ashikawa I (2006) Development of PCR-based allele-specific and InDel marker sets for nine rice blast resistance genes. Theor Appl Genet 113:251–260

Hayashi N, Kobayashi N, Vera Cruz CM, Fukuta Y (2009) Protocols for the sampling of diseased specimens and evaluation of blast disease in rice. JIRCAS Work Rep 63:17–33

Hittalmani S, Parco A, Mew TV, Zeigler RS, Huang N (2000) Fine mapping and DNA marker-assisted pyramiding of the three major genes for blast resistance in rice. Theor Appl Genet 100:1121–1128

Ingvardsen CR, Schejbel B, Lübberstedt T (2008) Functional markers in resistance breeding. In: Lüttge U, Beyschlag W, Murata J (eds) Progress in botany, vol 69. Springer, Berlin, pp 61–87

Iyer AS, McCouch SR (2004) The rice bacterial blight resistance gene *xa5* encodes a novel form of disease resistance. Mol Plant Microbe Interact 17:1348–1354

Iyer AS, McCouch SR (2007) Functional markers for *xa5*-mediated resistance in rice (*Oryza sativa* L.). Mol Breed 19:291–296

Jena KK, Mackill DJ (2008) Molecular markers and their use in marker-assisted selection in rice. Crop Sci 48:1266–1276

Jia Y, Wang Z, Singh P (2002) Development of dominant rice blast *Pi-ta* resistance gene markers. Crop Sci 42:2145–2149

Jia Y, Bryan GT, Farrall L, Valent B (2003) Natural variation at the *Pi-ta* rice blast resistance locus. Phytopathology 93:1452–1459

Jia Y, Redus M, Wang Z, Rutger JN (2004) Development of a SNLP marker from the *Pi-ta* blast resistance gene by tri-primer PCR. Euphytica 138:97–105

Kiyosawa S (1972) The inheritance of blast resistance transferred from some *indica* varieties in rice. Bull Nat Inst Agric Sci D23:69–96

Kiyosawa S (1984) Establishment of differential varieties for pathogenicity test of rice blast fungus. Rice Genet Newsl 1:95–97

Kiyosawa S, Orimoto Y, He YK, Ling ZZ (1983) Estimation of genotypes for blast resistance of rice varieties by testing with differential varieties. Oryza 20:216–222

Kiyosawa S, Mackill DJ, Bonman JM, Tanaka Y, Ling ZZ (1986) An attempt of classification of world's rice varieties based on reaction pattern to blast fungus strains. Bull Nat Inst Agric Resour 2:13–39

Kojima Y, Ebana K, Fukuoka S, Nagamine T, Kawase M (2005) Development of an RFLP-based rice diversity research set of germplasm. Breed Sci 55:431–440

Kumar A, Bennetzen JL (1999) Plant retrotransposons. Annu Rev Genet 33:479–532

Meyers BC, Kaushik S, Nandety RS (2005) Evolving disease resistance genes. Curr Opin Plant Biol 8:129–134

Michelmore RW, Meyers BC (1998) Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res 8:1113–1130

Monosi B, Wisser RJ, Pennill L, Hulbert SH (2004) Full-genome analysis of resistance gene homologues in rice. Theor Appl Genet 109:1434–1447

Noda T, Hayashi N, Du PV, Dinh HD, Lai Van E (1999) Distribution of pathogenic races of rice blast fungus in Vietnam. Ann Phytopathol Soc Jpn 65:526–530

Osterberg MK, Shavorskaya O, Lascoux M, Lagercrantz U (2002) Naturally occurring indel variation in the *Brassica nigra COL1* gene is associated with variation in flowering time. Genetics 161:299–306

Pearce SR, Stuart-Rogers C, Knox MR, Kumar A, Ellis THN, Flavell AJ (1999) Rapid isolation of plant Ty1-*copia* group retrotransposon LTR sequences for molecular marker studies. Plant J 19:711–717

Sweeney MT, Thomson MJ, Cho YG, Park YJ, Williamson SH, Bustamante CD, McCouch SR (2007) Global dissemination of a single mutation conferring white pericarp in rice. PLoS Genet 3:e133

Thornsberry JM, Goodman MM, Doebley J, Kresovich S, Nielsen D, Buckler ES 4th (2001) *Dwarf8* polymorphisms associate with variation in flowering time. Nat Genet 28:286–289

Tommasini L, Yahiaoui N, Srichumpa P, Keller B (2006) Development of functional markers specific for seven *Pm3* resistance alleles and their validation in the bread wheat gene pool. Theor Appl Genet 114:165–175

Tsugane K, Maekawa M, Takagi K, Takahara H, Qian Q, Eun CH, Iida S (2006) An active DNA transposon *nDart* causing leaf

variegation and mutable dwarfism and its related elements in rice. Plant J 45:46–57

Wang Z, Jia Y, Rutger JN, Xia Y (2007) Rapid survey for presence of a blast resistance gene *Pi-ta* in rice cultivars using the dominant DNA markers derived from portions of the *Pi-ta* gene. Plant Breed 126:36–42

Yahiaoui N, Srichumpa P, Dudler R, Keller B (2004) Genome analysis at different ploidy levels allows cloning of the powdery mildew resistance gene *Pm3b* from hexaploid wheat. Plant J 37:528–538

Yasuda N, Noguchi MT, Fujita Y (2008) Induced rice resistance to blast varies as a function of *Magnaporthe grisea* avirulence genes. Plant Dis 92:1144–1149

Zhou T, Wang Y, Chen JQ, Araki H, Jing Z, Jiang K, Shen J, Tian D (2004) Genome-wide identification of NBS genes in *japonica* rice reveals significant expansion of divergent non-TIR NBS-LRR genes. Mol Genet Genomics 271:402–415