Genetic and Physical Mapping of the Partial Resistance Gene, *Pi34*, to Blast in Rice

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ABSTRACT

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Partial resistance to rice blast in the *Oryza sativa japonica* group cv. Chubu 32 is controlled by *Pi34*, a major quantitative trait locus (QTL) on chromosome 11, and several uncharacterized QTLs. The objectives of the study were (i) high-resolution genetic and physical mapping of *Pi34* and (ii) identification of new QTL imparting resistance to rice blast. Chubu 32 was crossed with a susceptible chromosomal segment substitution line (CSSL) of cv. Koshihikari. From 4,012 of segregating individuals, 213 recombinants in the *Pi34* region were screened by using polymerase chain reaction-based markers and tested resistance in the field and greenhouse. The *Pi34* locus is located in the 54.1-kb region on the genomic sequence of cv. Nipponbare. We constructed a bacterial artificial chromosome (BAC) library of Chubu 32, selected the clone containing *Pi34*, and sequenced it. The *Pi34* locus consequently was located on an interval of 65.3 kb containing 10 predicted open reading frames (ORFs). Two of these ORFs were predicted only in Chubu 32 and encoded transposable elements. The other eight ORFs were found in both Chubu 32 and Nipponbare and one of them, which encoded an unknown protein, showed significantly different amino acid sequences between two cultivars. The new QTL, *Piq6*(t), was detected on the short arm of chromosome 6 and the genetic distance of flanking markers was 16.9 centimorgans in Nipponbare. *Pi34* and *Piq6*(t) acted additively on resistance to rice blast but the effect of *Piq6*(t) was relatively small compared with *Pi34*.

Additional keywords: *Magnaporthe grisea*, *Oryza sativa*.

Blast disease caused by *Magnaporthe grisea* (Herbert) Barr (anamorph *Pyricularia grisea*) (23) is one of the most important damaging diseases of rice (*Oryza sativa* L.). Dozens of major resistance (*R*) genes to blast have been identified and each of them has been introduced to various commercial rice cultivar. However, after only a few years in cultivation, new races or pathotypes of the blast pathogen have appeared and caused the collapse of resistance in these cultivars (14,22). Thus, resistance controlled by a single major gene is not generally durable.

Blast resistance in rice is categorized into complete (qualitative) and partial (quantitative) resistance (3,16). Complete resistance is characterized by the prevention of infection in incompatible combinations of hosts and parasites, and generally is controlled by a single major gene. This resistance gene, in turn, is under the control of the "gene-for-gene relationship" (8) with an avirulence gene in the blast pathogen (25); therefore, it is not durable. Partial resistance is considered to be a quantitative trait characterized by the reduction of the extent of pathogen reproduction in compatible combinations. Most partial resistance is regarded as non-race specific and controlled by polygenes (3,16), except for some partial resistance controlled by a single major gene such as *Pif* (26) and *Pb1* (9). The important point is that the blast pathogen has all the mechanisms to break down, in time, most combinations of several major genes. Here, we suggest that the presence of a major quantitative trait locus (QTL) controlling partial resistance can reduce the potential development of a blast epidemic causing severe yield losses.

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Recent progress in molecular genomics has allowed the cloning of complete resistance genes to blast, such as *Pita* (4) and *Pib* (27), both of which encoded the nuclear binding site (NBS) leucine-rich region (LRR) domains. Jia et al. (13) reported that the avirulence (AVR)-Pita protein, metalloprotease, binds directly to the LRR of the cytoplasmic receptor encoded by *Pita* inside the plant cell to initiate a Pita-mediated defense response. Some other *R* genes and associated avirulence genes have been mapped on genetic or physical maps (2,5). In contrast, although many QTL analyses have been performed on partial and field resistance to blast, there are few reports on precise map positions of partial resistance genes. Genetic information on partial resistance genes is very important for their efficient introduction into new cultivars.

We previously have reported that partial resistance in the *O. japonica* rice cv. Chubu 32 is controlled by a single gene (29). Chubu 32 shows a high level of partial resistance, which allows the development of only a few susceptible lesions on leaves infected with compatible *M. grisea* strains. In segregation analysis of partial resistance in recombinant inbred lines (RILs), resistance (with few lesions) and susceptible (with many lesions) RILs could be discriminated easily. We regarded this partial resistance as a quantitative trait, designated the involved gene as *Pi34*, and mapped it between restriction fragment length polymorphism (RFLP) markers C1172 and C189 on chromosome 11 (30). In the following research project, however, we observed that the levels of partial resistance in several F_3 and F_4 recombinants were inconsistent with the genotypes of the *Pi34* region. This suggested that Chubu 32 has some other QTLs for partial resistance, in addition to *Pi34*.

The objectives of this study were (i) high-resolution genetic and physical mapping of *Pi34* for identification of a candidate DNA fragment and (ii) identification of the new QTL, which is different from *Pi34* and affects the partial resistance to rice blast.

MATERIALS AND METHODS

Rice cultivars and mapping population. The *O. sativa japonica* group rice cv. Chubu 32, with a high level of partial resistance, was crossed with 98G-98-5, a chromosomal segment substitution line (CSSL) of *O. sativa japonica* group rice cv. Koshihikari developed by the Rice Genome Research Program (RGP) (6). Neither Chubu32 nor 98G-98-5 has complete resistance genes effective to the field strains of *M. grisea* (race 007.0) prevailing in Akita prefecture, Japan. We crossed Chubu 32 with CSSL and developed F_2, F_3, F_4, and F_5 populations.

Marker-assisted selection. A small amount of the total DNA was extracted from a large number of F_3 and F_4 populations following Aljanabi et al. (1) with slight modifications, and a large amount of the purified DNA was extracted from recombinant individuals using the cetyltrimethylammonium bromide method (19). Four types of markers were used in this study. Simple-sequence repeat (SSR) markers with the prefix 'RM' were developed by McCouch et al. (18) and released by the International Rice Microsatellite Initiative (IRMI). Cleaved amplified polymorphic sequence (CAPS) markers with the prefix 'C' or 'E' were developed from RFLP or sequence-tagged site (STS) markers provided by RGP (11,15). Other STS and single-strand conformation polymorphism markers were designed by using the rice genome sequences determined by the International Rice Genome Sequence Project.

Pi34 has been mapped between RFLP markers C1172 (1.6 centimorgans [cM] proximal to *Pi34*) and C189 (3.2 cM distal to *Pi34*) on a genetic map (30). In all, 6 F_2 individuals heterozygous in the region containing *Pi34* were selected from 32 F_2 individuals using the RFLP marker E51178, which mapped more proximal to C1172 on a high-density map constructed by RGP (11), and C189 in 2001. Out of 3,150 F_3 individuals, those with recombination between marker E51178 and C189 were selected as recombinants in 2002 and 2003. In 2004, recombinants were selected again from 866 F_4 individuals. Some F_3 and F_4 recombinants genetically fixed at the *Pi34* region also were selected.

Partial resistance assay. Two types of assays were performed to evaluate partial resistance precisely in the mapping population. One was an upland nursery trial (29) and the other was a greenhouse assay (28). Partial resistance of F_4 lines generated from F_3 recombinants was evaluated in 2003 and 2004, and F_5 lines generated from F_4 were evaluated in 2005. The lines were classified into "resistant" (slightly infected, such as Chubu 32), "susceptible" (severely damaged, such as susceptible control cv. Koshihikari), and "heterozygous" (segregating the disease severity).

A search for new QTLs in Chubu 32. To detect the other QTLs which were different from *Pi34* and affected partial resistance in Chubu 32, we selected 9 recombinants that carry the Kasalath allele at the *Pi34* locus but show high partial resistance, and 11 recombinants that carry the Chubu 32 allele but show relatively low partial resistance, and called them "discrepantly resistant individuals" (DRIs) and "discrepantly susceptible individuals" (DSIs), respectively. The genotype of the QTL was anticipated in each individual; that is, the DRIs will have the QTLs and the DSIs will not. The genomic regions which showed predicted genotypes were surveyed in them with 30 SSR markers showing polymorphisms between Chubu 32 and CSSL. To evaluate the influence of QTL on partial resistance, the four genotype groups derived from the combination of *Pi34* and QTL loci were investigated in an upland nursery trial. The effect of the QTL was evaluated by the multiple comparison procedure within the genotype of *Pi34* and QTL groups using the test of Steel-Dwass.

Fine mapping of the *Pi34* locus. We first selected individuals or lines that do not carry the new QTL, and then used them for fine mapping of *Pi34*. To identify the recombination points close to the *Pi34* locus more precisely, six markers were designed between the flanking makers RM5961 and 35K (Fig. 1B). We used five recombinants (RKC, RCH, RKH-1, RKH-2, and RHK) to narrow down the region of *Pi34*. The lines 29-8 and 16-82 were used as resistant and susceptible controls (control-r and control-s), respectively.

Construction of a bacterial artificial chromosome library and sequencing. Megabase-size rice DNA was prepared as described by Zhang et al. (31) using young leaves of Chubu 32. The bacterial artificial chromosome (BAC) library was constructed by ligation of the megabase DNA with pIndigoBACS (Epicentre) and transformation of BACs into DH10B (Invitrogen Corporation) (21). After preparing the library, the clones containing the *Pi34* locus were screened using two markers, RM2596 and Z82. Two positive clones (Ch46F14 and Ch41M22) were obtained, and Ch46F14 was sequenced by shotgun sequencing (7).

Gene prediction and annotation. Open reading frames (ORFs) were predicted by a rice genome automated annotation system, RICEGAAS (24).

RESULTS AND DISCUSSION

Detection of a new QTL for partial resistance in Chubu 32. The graphical genotypes flanked by the marker RM3034 and RM2615 on chromosome 6 were consistent with the phenotypes in 18 of 21 individuals whose genotypes in the *Pi34* region were inconsistent with phenotypes (Table 1). Genetic distance between these markers was 16.9 cM (1.71 Mbp) on the IRMI 2003 map in Gramene Genome Browser. In the other three individuals, crossing-over occurred between these markers (Table 1). This result suggests that a new QTL associated with partial resistance to blast is located between RM3034 and RM2615 on chromosome 6 in Chubu 32. We designated the locus as *Piq6*(t). In 2003, percent mean diseased leaf area (%MDLA) of the four groups, which classified with the combination of the genotypes of *Pi34* and *Piq6*(t), completely depended on the genotypes at the *Pi34* locus, and the efficacy of the *Piq6*(t) locus was not clear (Table 2). In 2004, the effect of *Piq6*(t) on partial resistance was not significant. In 2005, *Piq6*(t) conferred obvious resistance on the groups without *Pi34* (Table 2). *Piq6*(t) had not been identified in our previous analysis (29), probably because (i) both parental cultivars used were *japonica* group; therefore, we could not construct a map with enough high-density markers to detect *Piq6*(t), or (ii) the phenotypic variance attributable to *Piq6*(t) was very changeable according to the disease progress in the field. The position of *Pi34* on a genetic map was distal to marker C1172 in our previous report, but was proximal in the present study. This inconsistency of the location of *Pi34* may be because we had left *Piq6*(t) out of consideration and evaluated the phenotype of the recombinants incorrectly.

Fine mapping of the *Pi34* locus. Using 213 selected recombinants, *Pi34* was mapped between the SSR marker RM5961 and CAPS marker 35K (Fig. 1A). The genetic distance between them was 0.25 cM. The resistance of RKC was equivalent to control-r and that of RCH segregated in the progeny. In contrast, the resistance of RKH-1 and RKH-2 was significantly lower than that of control-r, while that of RHK was moderately high and segregated in the progeny. These results indicate that *Pi34* is located between Z77 and z150-5 (Fig. 1B).

Physical mapping of the *Pi34* locus. We found that the region between Z77 and z150-5 is covered by two Nipponbare BAC clones, OSJNBa0019A16 (accession no. AC108223) and OSJNBa0038F07 (accession no. AC108224). The physical distance between the two markers was 54.1 kb. We then constructed a BAC library of Chubu 32, which consisted of 15,975 clones with an average insert size of 150 kb, corresponding to a sixfold coverage of the *O. sativa* genome. A screening of the library led to the detection of two clones, Ch46F14 and Ch41M22, which carried the *Pi34* locus. The DNA sequencing of Ch46F14 revealed

that the physical distance between Z77 and z150-5 in Chubu 32 is 65.3 kb (Fig. 1C).

Comparison of genome sequences and predicted genes. Ten and eight ORFs were predicted between Z77 and z150-5 in Chubu 32 and Nipponbare, respectively (Fig. 1C). The clone from Chubu 32 had a 11.2-kb insertion, which contained a transposon protein (ORF6) and a retrotransposon protein (ORF7). The remaining eight ORFs were found in both Chubu 32 and Nipponbare. ORF1, ORF2, and ORF3 had no hits to expressed sequence tags (ESTs), and their functions were unknown (Table 3). ORF5 had a hit to ESTs but its function was unknown. ORF8, ORF9, and ORF10 had hits to ESTs, and encoded amino acid sequences similar to nuclear transportin, fiber protein, and alpha/beta hydrolase, respectively. In contrast, the amino acid sequence of ORF4 was significantly different between Chubu 32 and Nipponbare, and its spliced DNA sequence did not hit to ESTs.

The blast resistance genes characterized previously encode common sequence motifs, such as LRR, NBS, and kinase domains,

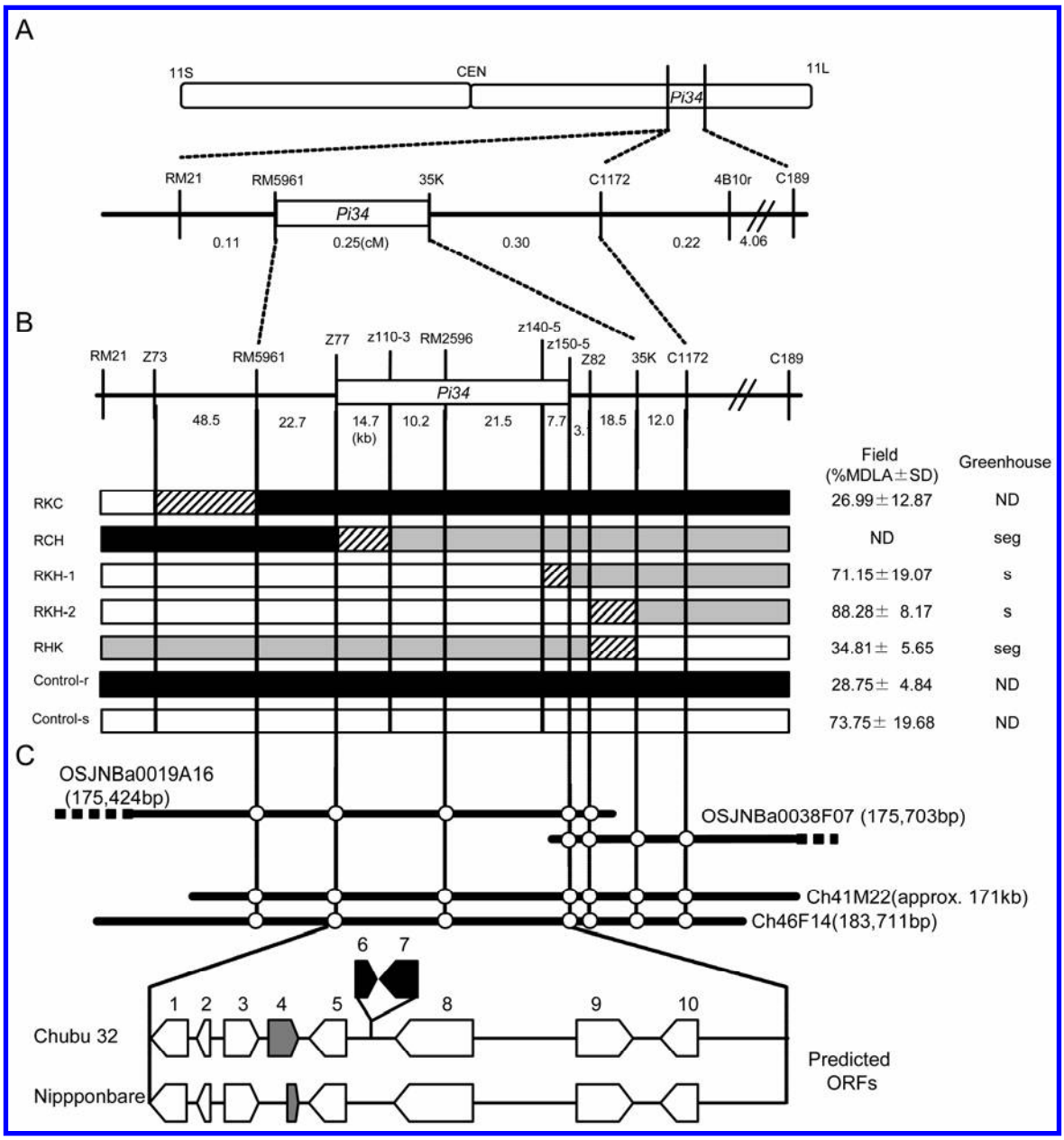

Fig. 1. Genetic and physical maps of the region of the *Pi34* locus, graphical genotypes of recombinant lines, and partial resistance. **A,** Chromosomal location of the centromere (CEN), quantitative trait loci for partial resistance gene to blast (*Pi34*), simple sequence repeats (SSR) markers (RM21 and RM5961), cleaved amplified polymorphic sequence markers (35K, C1172, and 4B10r), and sequence tagged site (STS) marker (C189) on the genetic map. The numbers below the map are genetic distances in centimorgans. **B,** Physical map of the *Pi34* locus. Additional STS markers (Z73, Z77, and Z82), single-strand conformation polymorphism markers (z110-3, z140-5, and z150-5), and SSR marker (RM5961) are denoted to narrow down the *Pi34*. The numbers below the map are physical distance in kilobases based on the sequence of BAC clone Ch46F14 of cv. Chubu 32. Graphical genotypes of the recombinants determine the location of *Pi34*, the control line with *Pi34* (Control-r) and the control line without *Pi34* (Control-s). Black bar and white bar intervals are homozygous for a Chubu 32 allele and a Kasalath allele, respectively; gray bars indicate heterozygous intervals; hatched bar interval in which a recombination occurred. The phenotype of each line was evaluated by the assay in the field and in the greenhouse. The word seg indicates that the partial resistance is segregated in the progeny, and s indicates the progeny shows susceptibility. **C,** Contig map spanning the *Pi34* locus. OSJNBa0019A16 and OSJNBa0038F07 are BAC clones of cv. Nipponbare that are released by the IRGSP (International Rice Genome Sequencing Project). Ch46F14 is a BAC clone selected from the BAC library of Chubu 32. These clones are anchored by the corresponding markers linked to the *Pi34* locus. Open reading frames (ORFs) are genes predicted in the *Pi34* region by RiceGAAS (Rice Genome Automated Annotation System); white arrows indicate ORFs that predicted almost the same (>95% similarity) amino acid sequences in Chubu 32 and Nipponbare; gray arrows indicate ORFs that predicted different amino acids in them; and black arrows show ORFs only predicted in Chubu 32.

TABLE 1. Detection of simple sequence repeat (SSR) markers linked to a quantitative trait loci (QTL) controlling partial resistance other than *Pi34*

Groups[a]/cultivar	Individual no.	% MDLA ± SD[b]	Predicted genotype of the QTL	Marker genotype		
				Flanking marker of *Pi34*[c]	RM3034 (chr.6)	RM2615 (Chr.6)
DRIs	1	38.80 ± 5.65	Resistant homozygous	*Ka*[d]	*C*	*C*
	2	41.34 ± 0.00		*Ka*	*C*	*C*
	3	41.34 ± 0.00		*Ka*	*C*	*C*
	4	38.82 ± 5.80		*Ka*	*C*	*Ko*
	5	41.58 ± 5.00		*Ka*	*C*	*Ko*
	6	31.55 ± 0.00		*Ka*	*C*	*C*
	7	44.85 ± 6.07		*Ka*	*C*	*Ko*
	8	38.08 ± 5.60		*Ka*	*C*	*C*
	9	41.58 ± 10.16		*Ka*	*C*	*C*
DSIs	1	32.73 ± 16.57	Susceptible homozygous	*C*	*Ko*	*Ko*
	2	41.58 ± 10.16		*C*	*Ko*	*Ko*
	3	38.79 ± 14.52		*C*	*Ko*	*Ko*
	4	43.13 ± 27.59		*C*	*Ko*	*Ko*
	5	32.02 ± 9.10		*C*	*Ko*	*Ko*
	6	41.58 ± 10.16		*C*	*Ko*	*Ko*
	7	35.28 ± 10.49		*C*	*Ko*	*Ko*
	8	32.02 ± 9.09		*C*	*Ko*	*Ko*
	9	36.56 ± 18.17		*C*	*Ko*	*Ko*
	10	32.02 ± 9.10		*C*	*Ko*	*Ko*
	11	35.81 ± 5.65		*C*	*Ko*	*Ko*
Chubu 32 (resistant control)		17.70 ± 2.54	*C*	*C*	*C*	*C*
Koshihikari (susceptible control)		83.61 ± 5.60	*Ka*	*Ka*	*Ka*	*Ka*

[a] DRIs and DSIs indicate discrepantly resistant individuals and discrepantly susceptible individuals, respectively.
[b] % MDLA and SD indicate the percentage of mean diseased leaf area and standard deviation, respectively. The resistance assay was performed in 2005 in an upland nursery trial.
[c] Flanking markers of *Pi34* were RM21 and C1172.
[d] *C*, *Ka*, and *Ko* indicate homozygous for Chubu 32, Kasalath, and Koshihikari allele, respectively.

TABLE 2. Difference in percentage of mean diseased leaf area (%MDLA) of leaf blast for different genotype classes between *Pi34* and *Piq6*(t) in F_3 and F_4 recombinants derived from cross between a chromosomal segment substitution line and Chubu 32 in upland nursery trials[a]

Genotype group[b]		%MDLA ± SD[c]		
Pi34	*Piq6(t)*	2003	2004	2005
C	*C*	37.5 ± 7.5 (9)	67.7 ± 8.5 (7)	25.9 ± 2.5 (7)
C	*Ko*	33.1 ± 11.9 (10)	66.7 ± 10.4 (8)	34.2 ± 6.7 (8)
Ka	*C*	79.9 ± 8.6 (4)	78.6 ± 7.1 (7)	39.3 ± 3.4 (8)
Ka	*Ko*	75.1 ± 10.2 (4)	87.6 ± 5.9 (8)	80.6 ± 6.0 (5)
Chubu 32	…	23.9 ± 3.3	51.6 ± 3.7	17.7 ± 2.5
Koshihikari	…	91.4 ± 3.6	93.0 ± 2.3	83.6 ± 5.6

[a] The different recombinants were evaluated in upland nursery trials in each year at Omagari, Akita, Japan.
[b] Genotypes are represented by the nearest simple-sequence repeat marker loci, RM5961 (*Pi34*) and RM3034 (*Piq6(t)*). *C*, *Ka*, and *Ko* indicate homozygous for Chubu 32, Kasalath, and Koshihikari allele, respectively.
[c] SD = standard deviations. Numbers in parentheses indicate the number of tested recombinants.

TABLE 3. Candidate genes at the *Pi34* region in Chubu 32

ORF[b]	Predicted function	Rice ESTs[c]	EST source[a]	
			Cultivars	Tissue
1	Unknown	No hit	…	…
2	Unknown	No hit	…	…
3	Unknown	No hit	…	…
4	Unknown	No hit	…	…
5	Unknown	AK072076	Nipponbare	Shoot
6	Transposon protein	No hit	…	…
7	Retrotransposon	No hit	…	…
8	Nuclear transportin/protein transporter	AK109206	Nipponbare	Unknown
9	Fiber protein	AK066017	Nipponbare	Shoot
	…	AK071393	Nipponbare	Flower
	…	CB633186	IR36	Leaf infected by *Magnaporthe grisea*
10	α/β hydrolase fold	CR287329	Unknown	Unknown
	…	CR283925	…	…

[a] EST = expressed sequence tag.
[b] ORF = open reading frame.
[c] Rice ESTs found in GenBank that are at least 98% identical to predicted ORFs. ESTs with the prefix 'AK' are full-length cDNAs, and other ESTs are partial sequences of mRNA.

and play a role in pathogen recognition and signal transduction (13,20). Because no ORFs had the typical resistance gene structures or encoded genes related to well-known defense pathways in this study, it is difficult to speculate on the function of *Pi34*. Incidentally, studies on key regulators and signaling pathways in basal resistance and systemic acquired resistance (SAR) are now in progress (10,12,17). However, whole mechanisms of such resistance have not been elucidated. It would be interesting to test whether *Pi34* is correlated with basal resistance and SAR, because the manner of *Pi34* has something in common with that of basal resistance and SAR; namely, partial resistance in the cultivar carrying *Pi34* is enhanced with leaf age, and the phenotype is represented not only as the reduction of lesion numbers but also as the suppression of lesion enlargement (data not shown). Expression analyses of the candidate ORFs in parental cultivars and near-isogenic lines (NILs) for *Pi34*, and complementation experiments with the candidate genes, are necessary to identify the *Pi34* gene.

To clone the gene or genes conferring quantitative traits, it is necessary to perform QTL analyses with enough markers to detect minor QTLs, to develop NILs of each QTL, to perform a close linkage analysis of the target locus using NIL, and to establish a method suitable for precise estimation of phenotypes.

The DNA sequence of the partial resistance gene to blast and its genetic information will help us to breed durable resistant cultivars efficiently. Actually, *Pi34* is being introduced into the Japanese elite-cultivars by marker-assisted selection, and these cultivars not only will be available for the reduction of the chemical use in field but also will be useful for analysis of host–parasite interactions involving complex mechanisms such as partial resistance.

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