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Multiple functional polymorphisms in a single disease resistance gene in rice enhance durable resistance to blast

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The improvement of resistance to blast, a devastating fungal disease of rice, would support the sustainable production of one of the world's staple foods, yet the identification of genes for durable resistance in rice is a challenge owing to their complicated genetic control. Here we show that map-based cloning of *Pi35* identifies multiple functional polymorphisms that allow effective control of the disease, and that *Pi35* is allelic to *Pish*, which mediates race-specific resistance to blast and encodes a protein containing a nucleotide-binding site (NBS) and leucine-rich repeats (LRRs). Analysis using *Pish–Pi35* chimeric genes demonstrated that multiple functional polymorphisms cumulatively enhance resistance, and that an amino acid residue in a LRR of *Pi35* is strongly associated with the gene's mediation of quantitative but consistent resistance to pathogen isolates in Japan, in contrast to *Pish*, which mediates resistance to only a single isolate. Our results reinforce the substantial importance of mining allelic variation for crop breeding.

Rice blast caused by the fungal pathogen *Magnaporthe oryzae* is a destructive disease of rice (*Oryza sativa* L.)[1]. The use of resistance (*R*) genes is an effective way to control it. Extensive studies have identified more than 90 *R* genes for resistance to *M. oryzae* from diverse genetic resources[2]. Most of them are race specific, and all of those genes for race-specific resistance that have been cloned so far encode proteins containing a nucleotide-binding site (NBS) and leucine-rich repeats (LRRs), constituting a major class of *R* genes in plants[3,4]; an exception is *Pid2*, which encodes a receptor-like kinase[5]. Race-specific resistance induces strong defense responses that are characterized by a hypersensitivity reaction (HR)[6] and are explained by the gene-for-gene theory[7], yet this resistance is rapidly overcome by the pathogen[8,9]. Plant breeders are thus obliged to find further *R* genes and introduce them to cultivars over and over, resulting in an evolutionary "arms race" between the crop and the pathogen[10].

In contrast, resistance conferred by quantitative trait loci (QTLs) is characterized by a susceptible infection type, usually without race specificity or gene-for-gene interaction[11]. In general, cultivars carrying resistance QTLs maintain their resistance for a long time. Therefore, QTL-mediated resistance is considered to be more durable than race-specific resistance, possibly because of decreased selection pressure against the pathogen. Intensive genetic mapping has highlighted several chromosomal regions harboring beneficial QTLs[12,13]. From these QTLs, *Pi21* is the first gene for resistance to *M. oryzae* cloned in rice[14]. However, on account of their small phenotypic effects, identification of the genes underlying QTLs is still a challenge. For example, *qBR4-2*, a gene complex comprising 3 loci that cumulatively enhance resistance to *M. oryzae*, presents difficulties in cloning of individual resistance alleles[15]. This complexity underlies the complicated genetic control of QTL-mediated resistance to *M. oryzae*[16,17]. In addition, the low resolution of genetic maps makes it difficult for plant breeders to introduce resistance QTL alleles from a donor without penalty, on account of linkage drag: the co-introduction of undesirable agricultural traits with the resistance[14].

Pi35 is a QTL found in the Japanese breeding line Hokkai 188. As this line has maintained consistent resistance in natural field conditions since 1961[18], it has been used as a source of QTL-mediated resistance in the Japanese rice breeding program. *Pi35* has been roughly mapped to a region on chromosome 1, where race-specific resistance genes *Pi37* and *Pish* are located[19,20]. These 2 genes are typical *R* genes that encode NBS-LRR proteins. Their tandem arrangement implies a duplication event in this region, as in other resistance gene complexes[21–23] and in the *Pi9* and *Pik* gene complexes on chromosomes 6 and 11, respectively[24–28]. These examples indicate that

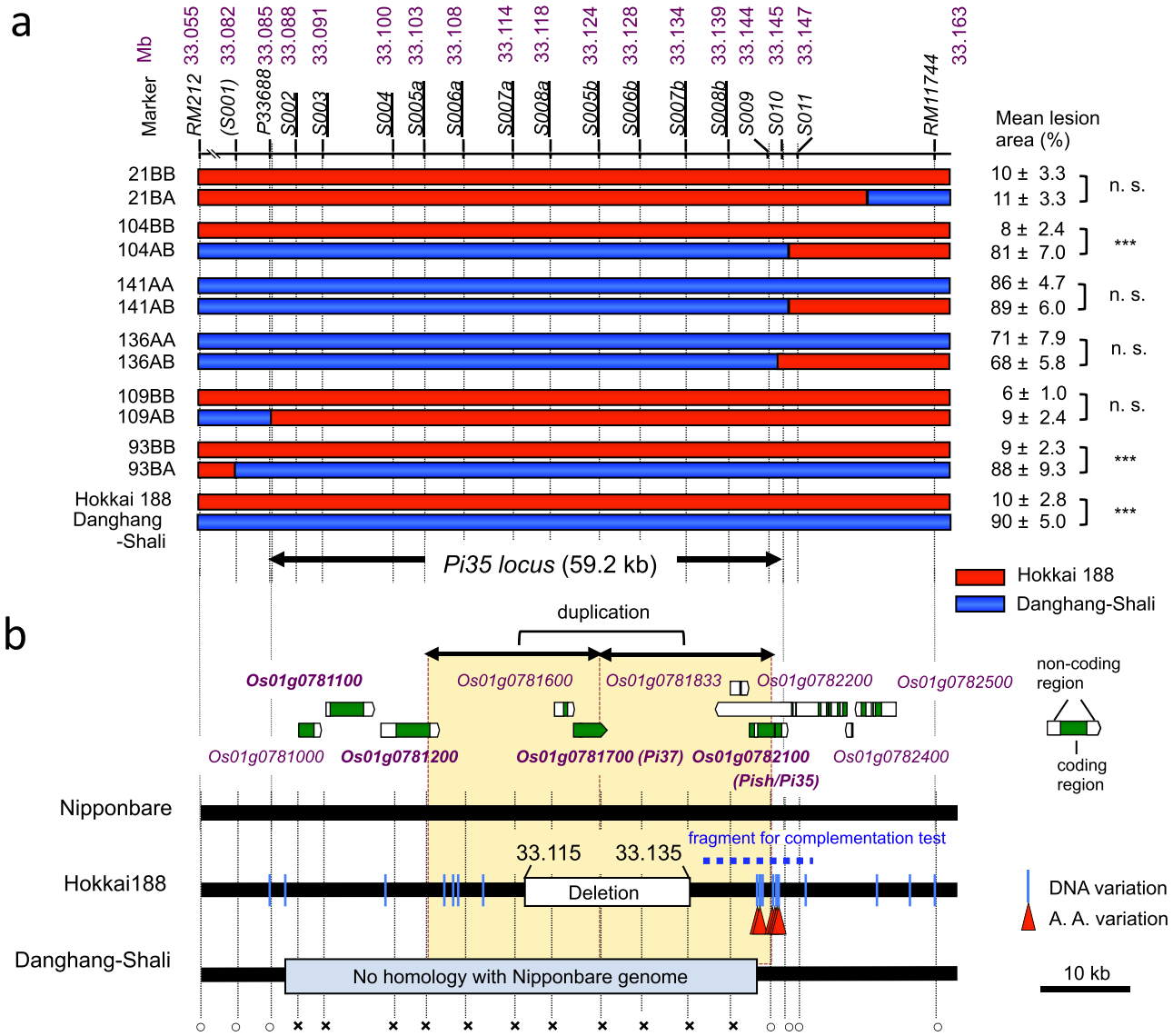

Figure 1 | Genetic mapping and physical maps of *Pi35*. (a) Graphical genotypes of recombinant inbred lines used to delimit the *Pi35* locus. Red bars indicate chromosomes derived from the resistant cultivar Hokkai 188; blue, from the susceptible cultivar Danghang-Shali. Positions are based on the International Rice Genome Sequencing Project 1.0 pseudomolecules of the Nipponbare genome. Underlined markers are dominant; the marker in parentheses is not polymorphic, and is used for confirming the presence of amplified product. The location of the *Pi35* locus, indicated at the bottom, is based on phenotypic data obtained by field test, tabulated on the right. Data are means ± SD. Asterisks illustrate results of 2-tailed *t*-tests: ***$P < 0.001$; n.s., not significant, $P > 0.05$. (b) Sequence comparison of the *Pi35* region between the susceptible cultivar Nipponbare and the resistant cultivar Hokkai 188. Positions of gene loci described in RAP-DB are indicated by boxes: white boxes, non-coding regions; green boxes, coding regions. The names of the genes that encode proteins containing a nucleotide-binding site (NBS) and leucine-rich repeats (LRRs) are in bold. Blue vertical bars indicate DNA sequence variations between Hokkai 188 (resistant) and Nipponbare (susceptible); red triangles indicate those that cause amino acid changes. The similarity of the Danghang-Shali genome to Nipponbare is determined from the presence of PCR products at 18 loci obtained by using 14 primer pairs listed in Supplementary Table S1, and the results are indicated at the bottom; circles, the presence of PCR products; crosses, the absence of PCR products.

NBS-LRR genes are an important component in the evolution of race-specificity in plant resistance. However, direct evidence that explains the linkage between NBS-LRRs and QTL-mediated blast resistance is limited.

We cloned *Pi35* by a map-based cloning strategy to enhance our understanding of the molecular basis for QTL-mediated resistance and to clarify the relationship between the QTL and race-specific genes in the chromosomal region.

Results

Fine-scale genetic mapping of *Pi35* locus. To delimit the *Pi35* locus, we screened recombinants in a mapping population in which the locus segregated. In field tests, we evaluated 56 pairs of inbred lines from 3112 F_5 plants, which include lines with recombination events within or around *Pi35* and non-recombinant control lines. Data for the lines that delimit the position of the *Pi35* locus are presented (Fig. 1a). The mean lesion area of the lines carrying the resistance allele was <12%, similar to that of the donor parent, Hokkai 188, whereas the mean lesion area of the lines carrying the susceptible allele was >60%, similar to that of the susceptible parent, Danghang-Shali. We concluded that *Pi35* lies in the 59.2-kb region between markers *P33688* and *S010* (Fig. 1a).

Candidate gene for *Pi35*. In the new Os-Nipponbare-Reference-IRGSP-1.0 genome assembly[29], we delimited the *Pi35* locus to a

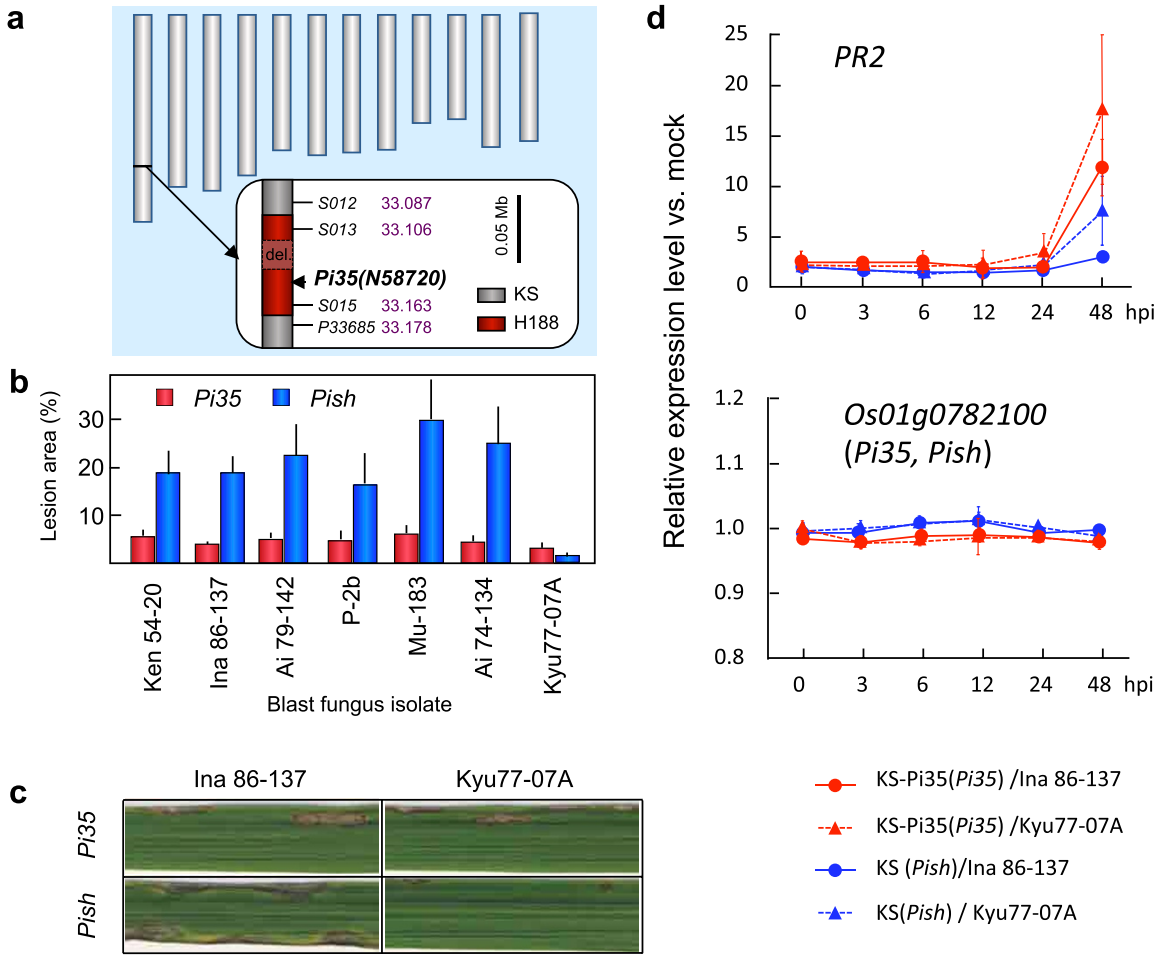

Figure 2 | Characterization of the resistant *Pi35* allele. (a) Chromosome map of near-isogenic line KS-Pi35. Gray boxes, chromosomes from the susceptible cultivar Koshihikari (KS); red box, from the resistant donor cultivar Hokkai 188 (H188). Positions are based on the International Rice Genome Sequencing Project 1.0 pseudomolecules of the Nipponbare genome. "Del." represents a 20.1-kb deletion compared with Nipponbare. (b) Lesion area of 30-day–old plants inoculated with each of 7 *M. oryzae* isolates. *Pi35* allele is from H188. The *Pish* allele from KS shows resistant infection type accompanying hypersensitive reaction only against isolate Kyu77-07A. (c) Representative diseased leaves of KS-Pi35 and KS that were challenged by either Ina86-137 or Kyu77-07A. (d) Expression profiles of *PR2* (AK070677) and *Os01g0782100* after inoculation of isolates Ina86-137 (solid) and Kyu77-07A (dashed) into 30-day–old KS (blue) and KS-Pi35 (red) plants. Leaves were sampled at 0, 3, 6, 12, 24, or 48 h after inoculation (hpi). Expression of *PR2* and *Pi35* was standardized to that of mock inoculation. Data are means ± SEM (n = 3). Total RNA was extracted from leaves and reverse-transcribed, and qPCR was performed. Primer pairs are listed in Supplementary Table S2.

region where the Rice Annotation Project[30] identified 8 gene loci (Fig. 1b). Four of these loci (*Os01g0781100*, *Os01g0781200*, *Os01g0781700*, and *Os01g0782100*) show similarity to previously reported disease resistance proteins containing NBS-LRRs. Comparison of the candidate region between Hokkai 188 with Nipponbare revealed overall similarity, but a 20.1-kb deletion in Hokkai 188 resulted in the loss of 2 loci, including *Os01g0781700*, which corresponds to *Pi37*[19] (Fig. 1b). We found 17 sequence variations, of which only 6 in *Os01g0782100*, which corresponds to *Pish*[20], showed variation in the deduced amino acid sequence; this left *Os01g0782100* as the candidate for *Pi35* (Fig. 1b). PCR analysis identified that the candidate region of the susceptible cultivar, Danghang-Shali, had no similarity with those of cultivars, Hokkai 188 or Nipponbare except around the *P33688* locus and downstream of the *S009* locus (Fig. 1b).

Characterization of *Pi35* allele. Leaves of KS-Pi35, a near-isogenic line carrying *Pi35* in the genetic background of a susceptible cultivar (KS) (Fig. 2a), had a smaller lesion area than KS in a greenhouse inoculation test (Fig. 2b). *Pi35* had a consistent effect against all 6 isolates, which represent widely distributed races of *M. oryzae*, and against isolate Kyu77-07A avirulent to KS carrying *Pish*[31], although the *Pi35*-triggered resistance was incomplete compared with that triggered by *Pish* (Fig. 2b, c). The results confirm empirical observations: plants with *Pish* show HR-inducing resistance to Kyu77-07A, but are highly susceptible in field tests and in inoculation tests to other Japanese isolates. In contrast, plants with *Pi35* do not show HR-inducing resistance against any of the isolates tested, but lesions against virulent races were smaller than in plants carrying *Pish*.

The expression of *pathogenesis-related gene 2* (*PR2*, AK070677) was increased in plants carrying *Pi35* at 48 h after inoculation with the avirulent isolate Kyu77-07A or the virulent isolate Ina86-137 and in plants carrying *Pish* challenged by Kyu77-07A but not by Ina86-137 (Fig. 2d). As the level of *Pi35* expression was the same as that of *Pish* before and after inoculation (Fig. 2d), we speculate that genetic variation in the coding region of *Os01g0782100* controls the differences in lesion area and in the level of *PR2* expression.

Natural variations at *Os01g0782100*. Variations were found in the NBS-domain-containing region and in the LRR region between the *Pish* allele from Nipponbare and the *Pi35* allele from Hokkai 188 (Fig. 3a). Sequence analysis of the region of *Os01g0782100*

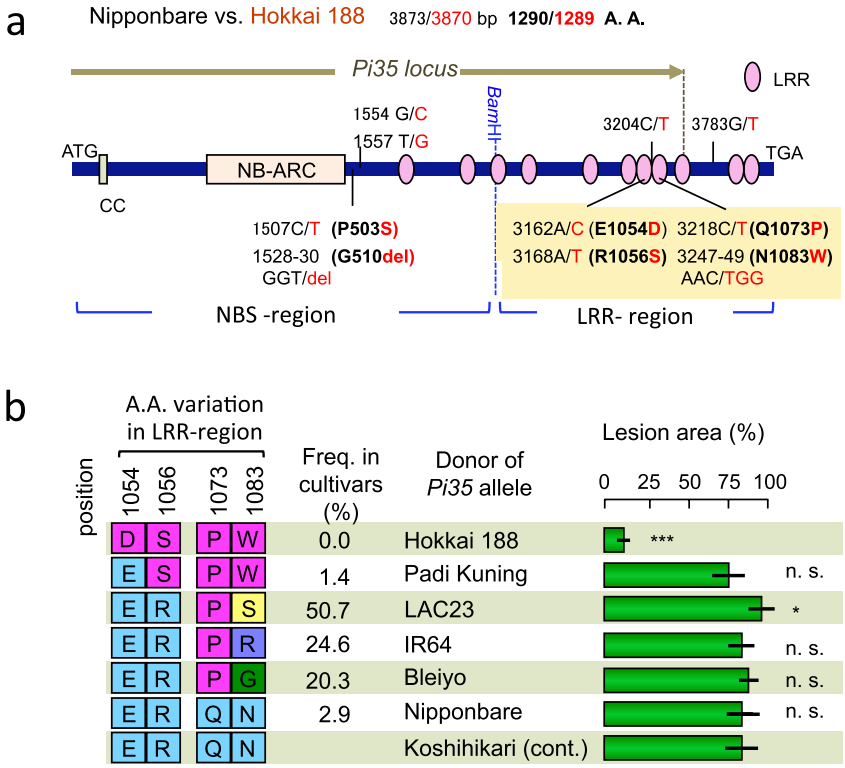

Figure 3 | Natural variations at the *Os01g0782100* locus. (a) Differences in DNA sequences (and amino acid sequences in parentheses) between the *Pish* allele from Nipponbare (black) and the *Pi35* allele from Hokkai 188 (red). Positions of respective variations from the start codon are indicated. The gene is divided into 2 regions by the *Bam*HI site: the NBS region and the LRR region. The NBS region contains coiled-coil (CC), nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4 (NB-ARC) and two LRRs; the LRR region contains 9 LRRs. (b) Sequence analysis of the LRR region of *Os01g0782100* identifies 6 haplotypes on the basis of 4 amino acid variations (boxed letters) among rice accessions. The Hokkai 188 type is absent in a core collection of 69 cultivars that represent the genetic diversity of Asian cultivated rice. Bars indicate the average lesion areas of backcrossed lines (n = 3) carrying the respective *Os01g0782100* haplotypes (from indicated donor cultivars) evaluated in a field test. Asterisks illustrate the results of two-tailed *t*-tests: ***$P < 0.001$; * $P \leq 0.05$; n.s., not significant, $P > 0.05$. Error bars indicate SD.

downstream of the *Bam*HI site identified 6 haplotypes on the basis of 4 amino acid variations in LRRs among Asian cultivated rice[32] (Fig. 3b, Supplementary Fig. S1). In field tests, only the Hokkai 188 type had a significantly smaller average lesion area than the control among backcrossed lines carrying the respective *Os01g0782100* haplotypes in the genetic background of a susceptible cultivar (Fig. 3b).

Complementation testing and molecular characterization of *Pi35*. Transforming a 13.2-kb genomic fragment containing *Os01g0782100* from Hokkai 188 (Fig. 1b) into a susceptible cultivar conferred resistance to multiple isolates in T_0 plants (Fig. 4a) and in progeny tests (Fig. 4b, "HH-Pi35"). To clarify whether variation in the NBS region or in the LRR region alters resistance to *M. oryzae*, we produced chimeric gene constructs between *Pish* and *Pi35*, introduced them into the susceptible cultivar, and inoculated plants with virulent Ina 86-137 or with Kyu77-07A, avirulent to plants carrying *Pish*. Replacement of the LRR region of *Pish* with that of *Pi35* resulted in the loss of race-specific resistance to the avirulent isolate, but conferred QTL-mediated resistance to both avirulent and virulent isolates (Fig. 4b, "NH-Pi35"). Out of 4 amino acid residues in the LRR region of *Pi35* that differ from those in *Pish*, an Asp residue at position 1054 from the start codon significantly reduced the lesion area against Ina 86-137, (Fig. 4b, "HN1054D-Pi35"). This Glu to Asp substitution (E1054D) was still not sufficient to confer the full effect of *Pi35* against Ina 86-137; however, it had a deleterious effect with Kyu77-07A. The Asp residue was strongly associated with the level of resistance to *M. oryzae* in the test with introgression lines (Fig. 3b). Plants carrying the chimeric Pi35-NBS/Pish-LRR gene (HN-Pi35) showed a non-significantly smaller lesion area than those carrying *Pish* (NN-Pi35) (Fig. 4b).

Discussion

We found that *Pi35* is involved in QTL-mediated resistance and is a member of the NBS-LRR class, a major class of *R* genes[3,4]. A weak allele of an *R* gene is considered to be a component of plant defense[10,12,33], yet direct evidence that links NBS-LRRs and QTL-mediated resistance is limited. On account of the small gene effects, isolating genes associated with QTL-mediated resistance is time-consuming and more difficult than isolating genes for race-specific resistance. We identified *Pi35* at the *Os01g0782100* locus by fine-scale genetic mapping, complementation testing, and progeny testing, and showed that *Pi35* is allelic to *Pish*, which is responsible for race-specific resistance to *M. oryzae*[20].

The important finding of our study is that a combination of multiple functional polymorphisms in the gene confers a practical level of resistance. Although the effects of individual polymorphisms other than E1054D appeared to be non-significant, this substitution confers only about half of the effect of the *Pi35* allele, suggesting that the rest is due to a cumulative effect of other polymorphisms. Race-specific resistance is easily overcome by rapid evolution of the fungal pathogen[8,9]. Enhancing resistance by combining multiple variations in a gene might confer a more durable form of resistance, owing to decreased selection pressure against the pathogen than by a single functional polymorphism that confers race-specific resistance, and might allow us to avoid an evolutionary arms-race between a crop and its pathogens[10].

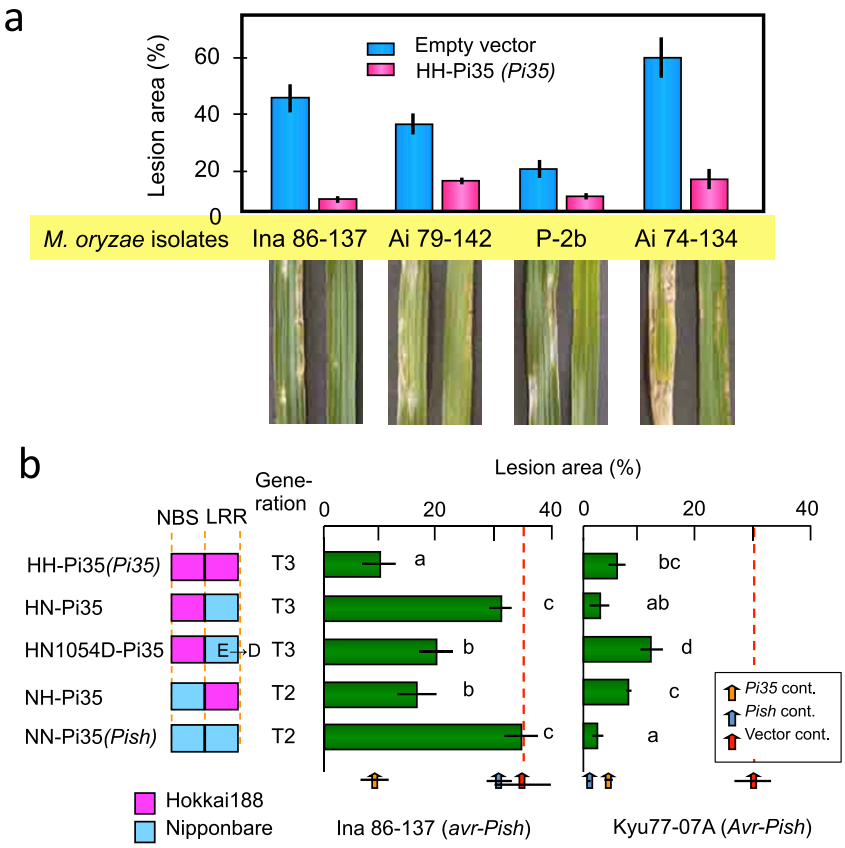

Figure 4 | Complementation testing and molecular characterization of *Pi35*. (a) Lesions of T_0 Aichiasahi plants carrying a *Pi35* construct (HH-Pi35) or an empty vector, 7 d after inoculation with 4 *M. oryzae* isolates: Ina 86-137, Ai 79-142, P-2b, or Ai 74-134. Data are means ± SD (n = 5). The photos below the graph show the phenotypic differences between the two. (b) Lesions on T_2 or T_3 Aichiasahi plants carrying *Pi35*, *Pish*, or chimeric gene constructs, 7 d after inoculation with Ina 86-137 or Kyu77-07A. "E → D" indicates substitution of a single amino acid residue Glu with Asp at position 1054 in the 7th LRR. Five single-copy lines derived from independent T_0 plants were used; inoculation tests were performed 3 times. Controls: *Pi35*, near-isogenic line for *Pi35* (KS-Pi35); *Pish*, Koshihikari (KS); Vector, T_3 Aichiasahi plants carrying empty vector. Data are means ± SEM. Green bars followed by the same letters are not significantly different according to Tukey's HSD test at the 5% level.

Molecular characterization of the *Pi35* and *Pish* alleles allowed us to dissect sequence variations associated with QTL-mediated and race-specific resistance, respectively. The NBS domain is considered to be involved in signal transduction[34,35], while the LRR region plays a critical role in the determination of race specificity[36,37]. Recent evidence shows that the NBS domain also determines race specificity[19]. Our data confirm that the LRR region is the primary determinant of *Pish*-mediated resistance. In addition, the LRR region of *Pi35* significantly reduced the lesion area provoked by isolates both avirulent and virulent to cultivars with *Pish*. Among the 4 amino acid residues of *Pi35* that differ from *Pish* in the LRR region, Asp at position 1054 significantly reduced the lesion area. The high susceptibility of an introgression line with the same amino acid residues in the LRR region as in *Pi35* except for the Asp (Padi Kuning allele in Fig. 3b) supports the hypothesis that this residue is important to the defense response to widely distributed pathogen races in Japan. The low frequency of accessions with Asp at this position among Asian cultivated rice accessions implies that the mutation occurred recently. Our experimental data suggest that a single mutation event can offer the opportunity to generate a beneficial QTL allele.

Dynamic structural changes in genomic regions containing *R* genes are considered to be the basis for the evolution of disease resistance in plants in the context of the co-evolution of plants and their pathogens[38–41]. Previous studies identified an array of *R* genes and uncovered their evolutionary processes on the basis of sequence comparison among genotypes in several species. *Pi35* is located in a cluster of 4 *R* gene loci, *Os01g0781100*, *Os01g0781200*, *Os01g0781700*, and *Os01g0782100*. It is proposed that *Os01g0781100* was the ancestral form, *Os01g0781200* was generated next, and either *Os01g0781700* or *Os01g0782100* was generated last[19,20]. Since Hokkai 188 is likely to carry a genotype without the final gene duplication and lacks *Os01g0781700*, our results support the hypothesis that *Os01g0781700* is the most recent locus[20]. The observations from the previous studies and here suggest that the 2 newer loci contribute to resistance to *M. oryzae*, but that the older 2 do not. *Pi35*, a rare allele at *Os01g0782100*, might be more adaptive in regions where rice is severely damaged by blast. Therefore, the *R* gene cluster on rice chromosome 1 strongly supports the idea that multiple duplication and substitution events generate new *R* genes[38–40].

Our results show that an allele of a typical *R* gene can confer a more durable form of resistance to *M. oryzae*, reminding us of the importance of mining allelic variation for use in crop breeding[42,43]. The fact that even a single amino acid substitution in an R protein can alter disease resistance in a quantitative manner might explain the continuous phenotypic variations that researchers find in diverse genetic resources or mapping populations[16,17]. QTL-mediated resistance is often undetectable on account of the race-specific resistance that is induced by pathogen races that are avirulent to cultivars carrying some *R* genes. As well as differential fungal isolates[44,45], advanced-backcrossed lines[46,47] are useful for the identification and fine-scale mapping of beneficial QTLs from diverse resources, on account of their efficient incorporation into elite genetic backgrounds without linkage drag[14]. A near-isogenic line for *Pi35* (KS-Pi35) is being evaluated in field trials to confirm the absence of undesirable agronomic

traits; monitoring of this line will confirm or deny the durability of *Pi35*-mediated resistance. Such research projects allow us to develop more durable crops for sustainable food production by combining multiple QTLs into elite genetic backgrounds.

Methods

Fine-scale genetic mapping and development of near-isogenic line for *Pi35*. The resistance QTL *Pi35* was located around the marker locus *RM1216* on chromosome 1 by an analysis using F_3 lines of the cross between the susceptible *indica* cultivar Danghang-Shali and the resistant *japonica* breeding line Hokkai 188[18]. A preliminary genetic mapping using progeny lines from a previous study and the addition of genetic markers allowed us to delimit the *Pi35* locus to an 892-kb region between marker loci *P31065* and *RM1003* on the rice reference genome. We used F_5 plants in which resistance was segregated at this marker interval for fine-scale genetic mapping of *Pi35*. From 3112 F_5 plants, we selected 56 fertile, normally growing plants among 124 recombinants between marker loci *P31035* and *RM1003*, and evaluated their progeny lines (F_6) by phenotype to determine the genotype at the *Pi35* locus. A near-isogenic line for *Pi35* was developed by 4 rounds of marker-assisted backcrossing using the susceptible *japonica* cultivar Koshihikari as the recurrent parent and the selection of minimum introgression in the BC_4F_2 and BC_4F_3 generations from nearly 800 individuals. Supplementary Table S1 shows PCR primers.

Construction of bacterial artificial chromosome library, sequencing, and gene prediction. Megabase-size DNA was prepared from young leaves of Hokkai 188 as described[48]. A bacterial artificial chromosome (BAC) library was constructed by ligation of the megabase DNA with the pIndigoBAC vector (Epicentre) and transformation of BACs into DH10B *Escherichia coli* cells (Invitrogen)[49]. The library consisted of 19082 clones with an average insert size of 115 kb. Clones containing the *Pi35* locus were screened with DNA markers *P33688* and *RM11744* (Supplementary Table S1), and a positive clone (HO188-07L09) was shotgun sequenced[50].

Comparison of genome structure. A sequence of the BAC clone HO188-07L09 containing a 183-kb insert including *Pi35* was aligned with the reference genome of *japonica* cultivar Nipponbare (IRGSP)[29] by using the SEQUENCHER software (Gene Codes Co.). The structure of the susceptible Danghang-Shali's genome was analyzed with a set of 14 PCR primers designed from the Nipponbare genome (Supplementary Table S1) to identify Danghang-Shali genomic regions with similarity to the Nipponbare genome on the basis of the presence or absence of amplicons.

Detection of natural variation in *Pi35*. DNAs from 69 cultivars that represent the genetic diversity of Asian cultivated rice_ENREF_35[32] were amplified and sequenced with the primer pairs listed (Supplementary Table S1). Deduced amino acid sequences were aligned with CLUSTALW software[51]. To confirm the effect of each haplotype on resistance to *M. oryzae*, we used 6 backcrossed lines, each carrying the target chromosome from 1 of 6 donors: Hokkai 188 (BC_4F_4), Padi Kuning (BC_2F_2), LAC23 (BC_4F_4), IR64 (BC_4F_4), Bleiyo (BC_4F_3), or Nipponbare (BC_4F_4). The lines for LAC23 (SL2207), IR64 (SL2003), and Nipponbare (SL602) were used in previous studies[52–54].

Complementation test. A 13.2-kb *Spe*I–*Xho*I fragment containing the resistant *Pi35* allele from the Hokkai 188 BAC clone HO188-07L09 (HH-Pi35) was inserted into the multiple cloning site of the binary vector pPZP2H-lac[55] to create pPZ-HH-Pi35. pPZ-HH-Pi35 was introduced into the susceptible cultivar Aichiasahi by *Agrobacterium*-mediated transformation[56]. The presence of the transgene was confirmed by the presence of *Bam*HI fragments obtained from the resistant *Pi35* allele but not from susceptible allele (primer pair 5′-AAGCGTAAAAGACTCTAGATTC-3′/5′-GACTCTAGATTCTCAAAATAGG-3′). We inoculated 131 T_0 plants carrying HH-Pi35 and 60 T_0 plants carrying the empty vector with each one of 4 *M. oryzae* isolates (Ina 86-137, Ai 79-142, P-2b, or Ai 74-134).

Chimeric gene analysis. The region between the start codon and a unique *Bam*HI site (which we designate as NBS) encodes the NBS domain and 2 LRRs, whereas the region between the *Bam*HI site and the stop codon (which we designate as LRR) encodes 9 LRRs. The NBS region contains 2 amino acid variations, and the LRR region contains 4 variations between the *Pish* allele from the susceptible Nipponbare and the *Pi35* allele from the resistant Hokkai 188. To test the effect of the respective regions on resistance to *M. oryzae*, we produced 4 additional types of constructs: NN-Pi35 (both NBS and LRR regions from Nipponbare), HN-Pi35 (NBS from Hokkai 188 + LRR from Nipponbare), NH1054D-Pi35 (a substitution of the 1054th amino acid residue Glu in HN-Pi35 with the Hokkai 188–specific Asp by PCR-based site-directed mutagenesis[57]), and NH-Pi35 (NBS from Nipponbare + LRR from Hokkai 188). The chimeric genes were obtained by digestion with *Bam*HI followed by ligation. The same procedure as used for the complementation test was used to create transgenic plants. Five T_2 or T_3 lines that originated from independent T_0 plants and carried a single-copy transgene were selected on the basis of the relative amounts of the *hygromycin phosphotransferase* (*HPT*, K01193) and *Pi35* genes, normalized to *rice ubiquitin 2* (*RUBQ2*, AF184280)[58], by using TaqMan probe–based quantitative real-time PCR (qPCR) (Supplementary Table S2). qPCR was performed with qPCR MasterMix (Eurogentec) in a 7900HT Fast Real-time PCR System (Applied Biosystems) according to the manufacturers' instructions. Five T_3 lines that carried a single copy of HH-Pi35 were used for reference. Three controls were used for lesion area measurement: *Pi35*, near-isogenic line for *Pi35* (KS-Pi35); *Pish*, Koshihikari (KS); vector, T_3 Aichiasahi plants carrying the empty vector.

Expression analysis. Total RNA was isolated from 30-day–old plants at 0 (just after inoculation), 3, 6, 12, 24, and 48 h after inoculation by using an RNeasy Plant Mini Kit (Qiagen), and total RNA was reverse-transcribed with Oligo-dT18 primer and a First-Strand cDNA Synthesis Kit (GE Healthcare) according to the manufacturer's instructions. qPCR was performed as above with the primers listed in Supplementary Table S2. The expression level of *RUBQ2* was used to standardize the RNA sample for each analysis. Expression of *PR2* and *Os01g0782100* was standardized to that of mock inoculation. RT-PCR and qPCR were performed at least 3 times for 3 independent samples per line.

Assessment of resistance to *M. oryzae*. Danghang-Shali and Hokkai 188 show a susceptible infection type, lacking HR, in inoculation tests using pathogenic races of *M. oryzae* widely distributed in Japan[59]. In fine-scale genetic mapping, we evaluated the resistance to *M. oryzae* of homozygous progeny of the recombinant plants (F_6 lines) in an experimental field at the National Institute of Agrobiological Sciences (Tsukuba, Ibaraki), where the predominant fungal race is 037.3, and gained susceptible lesions on our plants. Plants were grown from 50 seeds per line in each of 3 independent experiments. The lesion area of 60- to 70-day–old plants was scored against a published reference scale[14]. The susceptible Aichiasahi was grown on either side of each line. In complementation testing and chimeric gene analysis, 30-day–old plants grown in the greenhouse and inoculated as described previously[14] were used to assess resistance. The percentage of lesion area per leaf at 7 d after inoculation was scored against the reference scale. In chimeric gene analysis, 24 plants per line were used, and the experiments were performed 3 times.

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Author contributions

S.F. designed the experiments and wrote the manuscript. T.T.T.N. and S.K. developed the mapping population. S.-I.Y., K.O. and K.S. performed transformation experiments. S.-I.Y., M.R. and S.F. developed transformant lines. S.F., N.Y. and Y.F. performed phenotyping in glasshouse inoculation tests. M.R. and S.F. performed phenotyping in field tests. S.-I.Y. and S.F. performed genotyping for cloning *Pi35*. T.M. performed BAC clone analysis. S.-I.Y., K.N. and S.F. performed sequencing analysis. Y.U. performed expression analysis and copy number measurement in transformants. M.Y. provided advice on the experiments and the manuscript.

Additional information

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