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High Resolution Mapping of the *Indica*-Derived Rice Blast Resistance Genes. I. *Pi-b*

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Rice is a good material for studying positional cloning of disease resistance genes because its ratio of physical to genetic distance is small (100 to 300 kb/cM), and abundant genetic information is available. As a first step in cloning, the rice blast resistance gene *Pi-b* was finely mapped near the terminal region of chromosome 2 using restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers. Various near-isogenic lines (NILs) developed by the introgression of the *indica*-derived *Pi-b* gene into *japonica* recurrent parents facilitated its rapid and accurate localization and also the F_2 analyses. Although *Pi-b* was introgressed independently from four cultivars, all the donors showed the same RFLP profile for all the nearby probes used, whereas there was some polymorphism among *japonica* recurrent parents, suggesting a single origin of the resistance gene. *Pi-b* cosegregated with RAPD (b-1) and RFLP (RZ123) markers, and it was bracketed between flanking RFLP markers at 0.5 cM on the centromeric side, and 1.9 cM on the telomeric side. The centromeric and telomeric limits of the *indica* regions of NILs BL-1 and BL-7 were closer to *Pi-b* than the flanking nucleic markers on each side, respectively, providing better (<0.5 and <1.9 cM) delineating markers. A long-range Southern blot with rare-cutter restriction enzymes revealed an 85-kb common band between the two 0-cM markers. These markers provide an excellent environment for the positional cloning of *Pi-b*.

Additional keywords: Magnaporthe grisea, Oryza sativa.

Resistance conforming to the "gene for gene theory" (for a review, see Flor 1971), also known as true or qualitative resistance, is an organized plant defense mechanism against pathogens. It specifically recognizes invading pathogens at a very early stage of infection and confines them and the defense reaction to a small, limited region (as a review see Keen 1990). Although several avirulence genes of pathogens (e.g., Staskawicz et al. 1984; Van den Ackerveken et al. 1992) and, recently, some plant resistance genes have been cloned (Martin et al. 1993; see also Stascawicz et al. 1995), still, the mechanism of recognition of pathogen and induction of the concerted defense reactions remains obscure, and more case studies are needed.

Rice blast (caused by *Magnaporthe grisea*) is one of the most devastating diseases of this crop, especially in the region near the northern limit of cultivation, and there is a long history of resistance gene study in Japan, since the first description of the single gene resistance by Sasaki (1922), (Kiyosawa 1981; Yamazaki and Kozaka 1980). Recently, international cooperation for the study of blast resistance has also been extensively carried out (see Zeigler et al. 1994). Various *indica*-derived resistance genes have been introgressed to *japonica* backgrounds to develop an array of blast-resistant cultivars. This has provided excellent near-isogenic line (NIL) materials for the restriction fragment length polymorphism (RFLP) analysis, simplifying the genetic analysis by being removed from other interfering resistance genes against the Japanese blast races, and making the crossing easy for F_2 analyses.

Rice is an excellent model plant for genome analysis, because (i) the genome is one of the smallest among the major crops (200 Mb/haploid, Nishibayashi 1991; 420 Mb, Arumuganathan and Earle 1991), only two to five times that of *Arabidopsis*; (ii) the physical length corresponding to the unit genetic distance is also small (100 to 300 kb/cM), comparable to that of *Arabidopsis,* calculated from the genome size as in (i) and the map length (Saito et al. 1991; or Causse et al. 1994); (iii) the transformation system is the most established among monocotyledons (Hiei 1994; Christou 1991; Shimamoto 1988); and (iv) abundant materials, genetic information, and RFLP probes are available (Kinoshita 1990; Saito et al. 1991; Causse et al. 1994). Furthermore, rice will provide a representative system for monocotyledonous crops that are important as food staples and are one of the best sources of information about disease-resistant lines, but hitherto these merits are not exploited sufficiently. The molecular analysis of the corresponding avirulence genes in blast fungus and its genome analysis are also among the most advanced in the pathogenic fungi (Valent and Chumley 1994; Skinner et al. 1993).

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Pi-b is an *indica*-derived resistance gene, introgressed independently from two Indonesian and two Malaysian cultivars (Yokoo et al. 1978) into various *japonica* cultivars, developing several NILs (Fig. 1). This gene is highly resistant for most Japanese blast fungal races. The position on the rice genome map is roughly located near the 0-cM end of chromosome 2 by the analysis of reciprocal translocation lines (Shinoda et al. 1971) on the map of Kinoshita (1990). In the following section, we describe the fine mapping of the gene as the first step for its positional cloning.

RESULTS

RFLP analysis of NILs of *Pi-b*.

The RFLPs of various NILs of *Pi-b*, derived by backcrossing of *indica* donor cultivars to *japonica* recurrent parents (Fig. 1), were analyzed in the region near the 0-cM end of chromosome 2 (on the map of Saito et al. 1991). Most of the NILs showed *indica*-type profiles with the markers close to the 0-cM terminal of chromosome 2 (GN1234, cDK440), while those on the right (centromeric) sides on the map showed more *japonica*-type profiles (LN181, XNpbs; Figs. 2 and 3). These findings suggested that *Pi-b* is near the former two markers. However, BL-1 showed all *japonica* profiles with the NIAR-developed RFLP probes mentioned above (Figs. 2 and 3). Therefore, some Cornell markers, located in the more telomeric region (Xiao et al. 1992), were tested and found to be of the *indica* type in BL-1 (RZ213, RZ123; Figs. 2 and 3), indicating localization of *Pi-b* close to RZ123. It was noticeable that RZ213 showed a *japonica*-type profile on BL-7, indicating that this cross-over point from *indica* to *japonica* is nearer *Pi-b* than RZ213 itself (Fig. 3).

There were no polymorphisms among the four *indica* donor parents with any of the RFLP probes studied, while some *japonica* cultivars showed polymorphism among them (cDK440; Fig. 2). This indicates that these *indica* donor parents are mutually closely related, considering that the *indica* cultivars have much more genetic diversity among them than do the *japonica* groups (Nakano et al. 1992).

Survey of RAPD markers.

To find markers closer to the *Pi-b* gene than the above, random amplified polymorphic DNA (RAPD) markers (bands) specifically common to the NILs of *Pi-b* and their donor parents were searched using 800 random primers. In a preliminary experiment using 70 decanucleotides primers, on average, about seven bands were amplified from the rice genome per primer, among which about one band/primer showed polymorphism between *indica* (Kasalath) and *japonica* (Koshihikari) cultivars. As we used 800 primers, this means

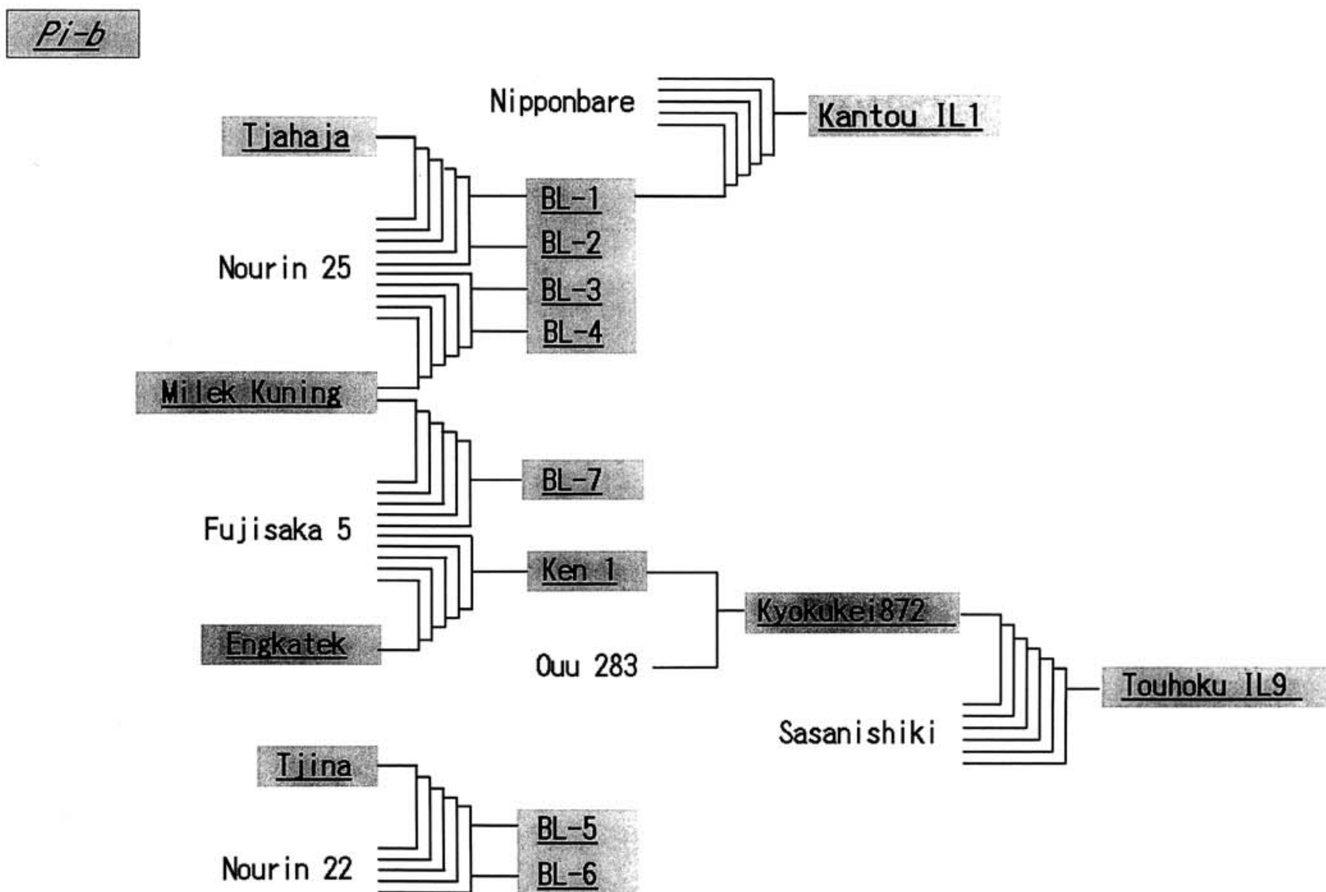

Fig. 1. Pedigree for the introgression of the rice blast resistance gene *Pi-b* into *japonica* cultivars. The cultivars retaining *Pi-b* are underlined and shaded. Four *indica* donors, two Indonesian cultivars, Tjahaja and Tjina, and two Malaysian cultivars, Engkatek and Milek Kuning, were backcrossed with elite *japonica* cultivars to produce several near-isogenic lines of the *Pi-b*.

that about the same number of polymorphic bands were screened in the rice genome. Sixteen prospective candidates that showed polymorphism on the first screening with the pair of NIL (BL-1) and recurrent parent (Nourin 22) were further tested by increasing the number of NILs-related cultivars to 18. After all such screening using 800 primers, only one marker band remained as stably cosegregating with the *Pi-b* in all 13 tested resistant lines; nine NILs, and four donor *indica* parents mamong 18 cultivars. We named this band b-1 (Fig. 4). This band (1.0 kb) was derived from the primer sequence of GTGATCG CAG. It was quite stably reproducible and absent in *japonica* parents. No other band was common to more than three among the nine NILs.

F_2 analysis to locate *Pi-b* among nearby markers.

Genetic distances between the *Pi-b* gene and nearby markers were determined by F_2 analyses. DNA was prepared from 78 susceptible (recessive homo on *Pi-b*) individuals that have survived from 94 susceptibles among 409 total F_2 individuals of the crossing BL-1 (*Pi-b*) × Aichi Asahi (++) (Table 1). Similarly, 44 susceptibles survived from 406 F_2s of BL-1 × Nourin 22. These F_2 segregation ratios fitted 3:1 with a sufficient significance level, confirming the functioning of a single resistance gene (Table 1). We checked these 122 individuals (244 chromosomes) for the presence of recombination with the nearby markers, and b-1 was found to completely cosegregate *Pi-b*.

Use of only the susceptibles lowers the risk of misdiagnosis because the apparently resistant often include "escaped" individuals from inoculation. Furthermore, the homozygotes make the DNA analysis more efficient.

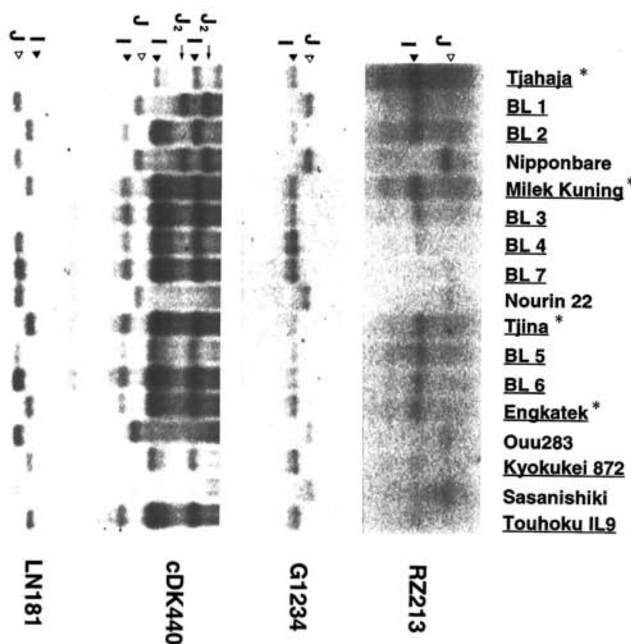

Fig. 2. Restriction fragment length polymorphism profile of the near-isogenic lines of *Pi-b* and their donor and recurrent parents. The cultivars with *Pi-b* are underlined. Donor parent (*indica*) cultivars are indicated by asterisks on the shoulder. Bands corresponding to *japonica* and *indica* are indicated by solid (J) and open arrowheads (I), respectively at the left. Small arrows (J_2) on the left of cDK440 indicate polymorphic bands between *japonica* cultivars.

The Cornell University markers near the terminals of chromosome 2 were tested for their polymorphism between BL-1 and Aichi Asahi and markers RG520, RG322, RZ213, RZ531, and RZ123 were polymorphic. An analysis of the above 78 F_2 individuals of BL-1 × Aichi Asahi showed that RZ123 cosegregated with *Pi-b* and the other markers were mapped as described in Figure 5. The recombination rates between RZ123 and the other markers in the BL-1 × Aichi Asahi crossing were smaller in about 25% of the cases of *indica* × *O. longisterminata* (Causse et al. 1994) on average, suggesting some recombination difficulties in the former crossing.

There are no *indica*-typed RFLP markers in the right (centromeric) side of *Pi-b* for BL-1 (Fig. 3). Therefore, the map distances between *Pi-b*-cosegregating marker b-1 and the right side RFLP markers (G1234, cDK440, XNpb275) were determined with another cross of Koshihikari (*japonica*) × Kasalath (*indica*) at hand. Polymerase chain reaction (PCR) band b-1 was isolated from an agarose gel after electrophoresis and used as the RFLP marker. It worked well as a single polymorphic band between F_2 parents upon digestion with *Eco*RV. G1234 and cDK440 were found at 0.5 and 1.1 cM, respectively, from b-1. This means that they are on the opposite sides of the RZ/RG markers that showed *indica*-type profiles on BL-1. Considering all the above information, the fine marker map around *Pi-b* was determined as in Figure 5. As a result, the cross-over point from *indica* to *japonica* of BL-1 and BL-7 served as the closest delineating marker on the right (centromeric, <0.5 cM) and left (telomeric, <1.9 cM) sides of *Pi-b*, respectively.

Long fragment Southern blots of the closest markers.

The physical distances of the closest markers were estimated by Southern blotting with various rare cutters and by contour-cramped hexagonal electric field (CHEF) electrophoresis. RZ123 and b-1 showed 85-kb common bands but G1234 did not (Fig. 6), indicating that the distance between these 0-cM markers is within this limit. As no band over 150 kb was found among the bands detected by the three probes, no longer-range estimation was possible.

DISCUSSION

The origin of the *Pi-b* gene.

The blast resistance gene *Pi-b* has been independently introgressed into *japonica* backgrounds from four *indica* cultivars (Yokoo et al. 1978), which provides an excellent system for analyzing the locus of the gene. The *indica*-type RFLP or RAPD patterns of NILs of *Pi-b* were concentrated near the 0-cM terminal of chromosome 2 (Fig. 3). These band profiles were identical among the four donor *indica* lines, whereas the bands probed by cDK440 showed a polymorphism in Nipponbare different from that of the other *japonica* cultivars (Fig. 2). The rather close geographic origin of these donor parents and their identical profiles in nearby RFLP or RAPD markers suggest a single origin of the *Pi-b* gene. This will be finally proved by cloning and sequencing the gene.

The nearby markers of *Pi-b*.

The locus of *Pi-b* was first suggested to be near the terminal of the second chromosome, as *Pi-s*, by Shinoda et al.

(1971), from a study of reciprocal translocation lines. *Pi-s* was later shown to be the same as *Pi-b* by Kiyosawa (1978). This helped to limit the number of testing RFLP probes, and the presence of numerous NILs helped pinpoint the locus of the gene (Fig. 3). F_2 recombination analysis with a crossing of BL-1 × Aichi Asahi/Nourin 22 including 244 chromosomes revealed that the RAPD marker b-1 cosegregated with *Pi-b*. Similarly, the RFLP marker RZ123 cosegregated with *Pi-b* in

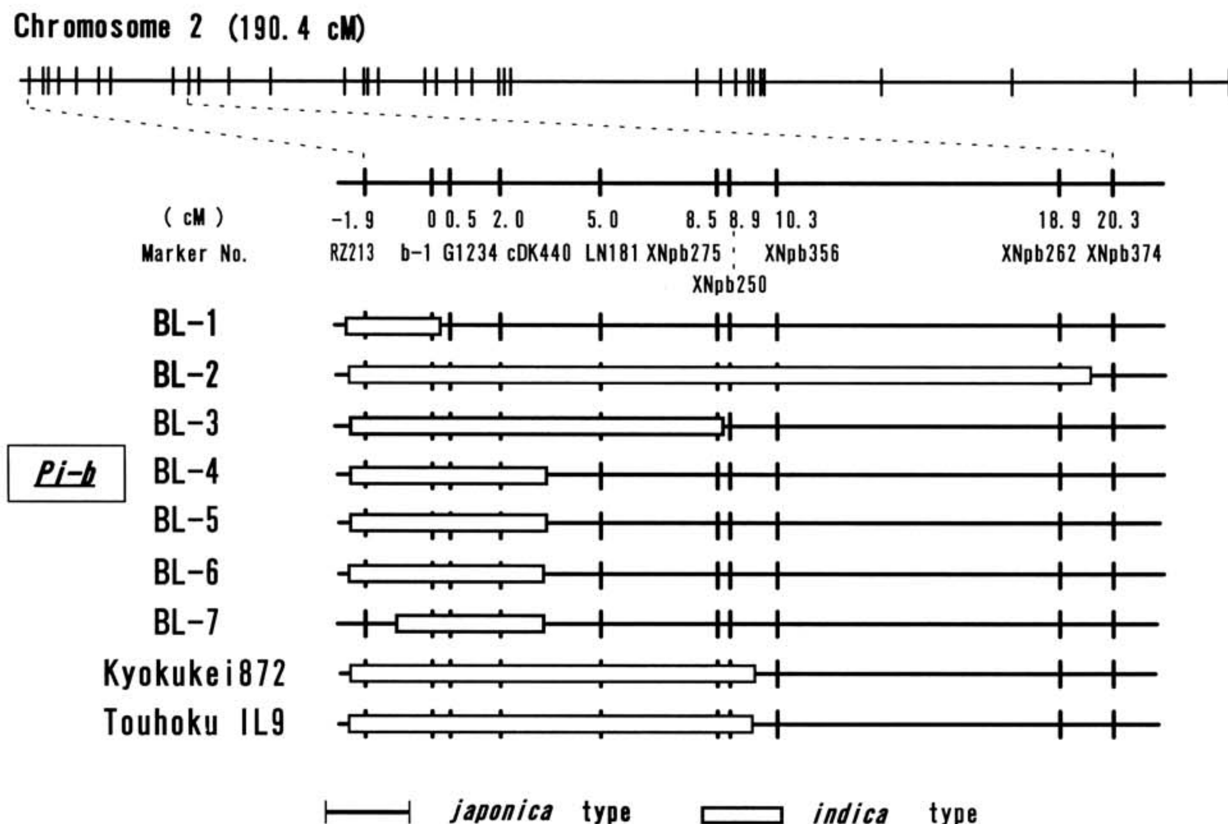

Fig. 3. Graphical genotype of the restriction fragment length polymorphism markers at the initiating terminal of chromosome 2. The terminal map in the second row is composed from the reports of Saito et al. (1991), Kurata et al. (1992), and Causse et al. (1994). The position of b-1 was added from the data of F_2 analysis and set as the 0-cM starting point. The *indica*-type region on the chromosomes is indicated by columns.

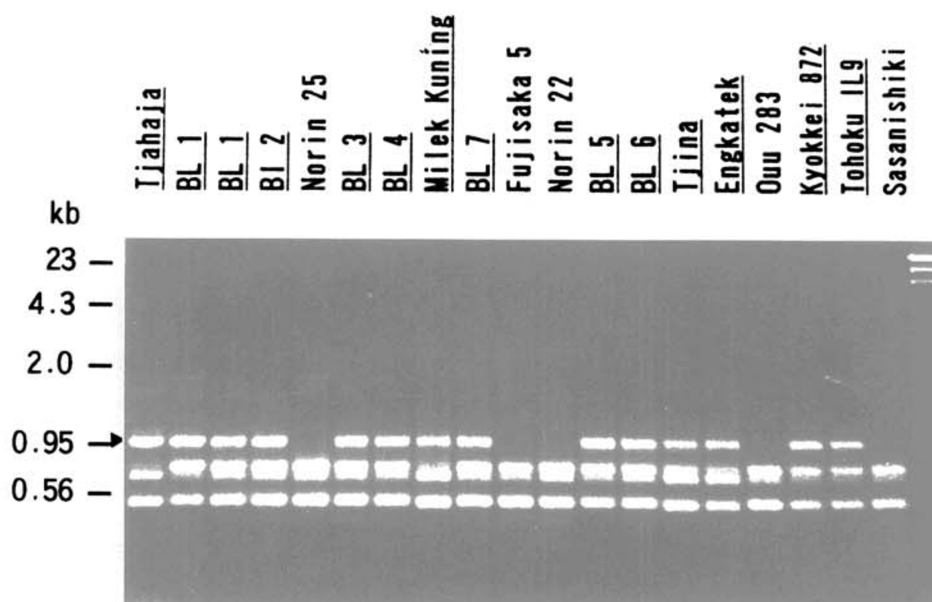

Fig. 4. The random amplified polymorphic DNA marker b-1 showing the cosegregation with *Pi-b* among the near-isogenic lines and their parents. The cultivars with *Pi-b* are underlined. The position of b-1 is indicated by the arrowhead on the left.

156 chromosomes and three RZ or RG markers were located at 1.9 cM on the left (telomeric) side. Because BL-1 has no *indica*-type RFLP marker on the right (centromeric) side of the *Pi-b* on the chromosome, the distance from b-1 to the right-side markers was determined in place of that from *Pi-b* using an *indica* × *japonica* crossing, and a 0.5-cM marker (G1234) was found on this side (Fig. 5). However, the right and left ends of the *indica* regions of the BL-1 and BL-7 chromosomes were closer to *Pi-b* than G1234 and RZ213, respectively, and will serve as better markers to delineate the gene than the RFLP markers (Fig. 5).

Prospects of contig formation.

These four close markers (b-1, RZ123, and the right and left ends of the *indica* regions of BL-1 and BL-7, respectively) should be very useful in cloning the *Pi-b* gene, because the calculated average size of 0.5 cM in the rice genome is 50 to 150 kb from genome sizes of about 200 (Nishibayashi 1991) or 420 Mb (Arumuganathan and Earle 1991), and the total genetic length of RFLP maps (1,836 cM for *indica* (Kasalath) × *japonica*; Saito et al. 1991: or 1,491 cM for *Oryza sativa* × *O. longisterminata*; Causse et al. 1994). Southern blotting with rare-cutter restriction enzymes (Fig. 6) indicated that b-1 and RZ123 are within a fragment of 85 kb, and, at least, this does not contradict the above estimate that 0.5 cM corresponds to 50 to 150 kB. Therefore, if this estimate holds, a BAC library with an average insert size of about 150 (Shizuya et al. 1992; Woo et al. 1994) or 125 kb (Wang et al. 1995), or even a cosmid library (Evans and Wahl 1987) with an average insert size of 40 kb should be sufficient to make a contig spanning the 1-cM region over the *Pi-b* gene of the chromosome. Thermal asymmetric interlaced (TAIL)–PCR (Liu et al. 1994) or inverse PCR (Triglia et al. 1988) will help to facilitate walking steps. We constructed more than five genome sizes of a rice cosmid library and a rice BAC library, with average inserts of approximately 40 and 180 kb, respectively. We are now constructing contigs from these libraries.

Application of the markers to gene pyramiding.

As an application of these close markers for breeding, development of a multiresistant line against rice blast by accumulating resistance genes is prospective, because we also obtained very close markers for two other *indica*-derived resistance genes $Pi\text{-}ta^2$ and $Pi\text{-}z^t$. All of them have wide resistance spectra against Japanese rice blast races. By accumulating these three resistance genes with wide defense ranges, cultivars will also become recalcitrant to the "breakdown of the resistances," because, for most Japanese blast races, mutations of all the three corresponding avirulence genes are needed to survive on the developed line. As the mutation of avirulence genes to virulent type generally decreases the competitive viability of the races (Kiyosawa et al. 1993), the possibility that a race will accumulate these three mutations is very small. Such gene pyramiding has been difficult, because the corresponding virulent races were needed to determine the presence of the resistance genes by the traditional method. The need of the infecting races is a contradiction for developing strong multiresistant lines. No combination of the Japa-

Table 1. Segregation of resistant and susceptible individuals in F_2 population of BL-1 × Aichi Asahi and BL-1 × Nourin 22 crosses[a]

Crosses	Resistant	Susceptible	Total	χ^2-fitting to 3:1 segregation	Level of significance
BL-1 × Nourin 22	306	100	406	0.03	ca. 0.90
Ratio (%)	75.4	24.6	100	...	...
BL-1 × Aichi Asahi	315	94	409	0.89	ca. 0.35
Ratio (%)	77.0	23.0	100	...	...

[a] Seedlings at the five-leaf stage were sprayed with a conidial suspension (10^5/ml) of blast race 037.1. After 1 week, symptoms were diagnosed.

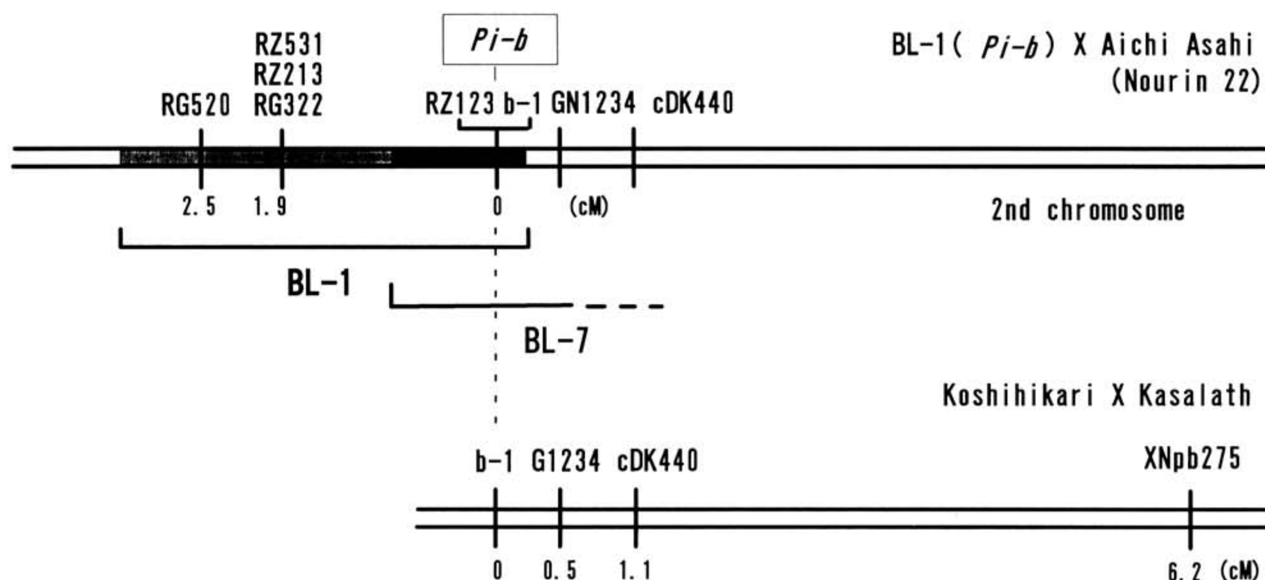

Fig. 5. High resolution map close to *Pi-b* integrated from F_2 analyses of crossings of BL-1 (*Pi-b*) × Aichi Asahi or Nourin 22 and Koshihikari (*japonica*) × Kasalath (*indica*). The *indica* region on the BL-1 chromosome is indicated by shading, and the common *indica* region as BL-7 is further hatched.

nese blast races can differentiate these accumulated genes. The presence of close or cosegregating nucleic acid markers clears this difficulty, and the resultant multiresistant line will provide a good experimental case to test the degree to which it can withstand breakdown by accumulating the new resistance genes. We crossed the NILs with these resistant genes and are now selecting the triple homozygotes of these resistance genes with the markers. In future, transformation will become the most efficient method to accumulate them in a desirable cultivar.

Comparison with other systems.

Wang et al. (1994) extensively surveyed several blast resistance genes on an RFLP map with a quantitative trait loci (QTL) analysis, but no resistance gene was mapped on chromosome 2. Yu et al. (1991) mapped *Pi-2(t)* and *P-4(t)* on chromosomes 6 and 12, respectively, and indicated a marker at 2.8 cM from *Pi-2(t)*. However, these markers were too far apart for cloning of the resistance genes. Although Ronald et al. (1992) have obtained four cosegregating markers for the *Xa21* bacterial blight resistance gene in an NIL developed from *O. longisterminata,* the long distances (2.7 or 7.4 cM) between them and the flanking markers on both sides may suggest the presence of non-homologous regions between the donor and recurrent parent species, making recombination analysis difficult. There is a higher frequency (65%) of polymorphism between *O. sativa* and *O. longisterminata* among RAPD bands than between the Kasalath (*indica*) and *japonica* (approximately 15%). This intermediate phylogenic distance between Kasalath and *japonica* makes crossing rather easy. However, the apparent recombination rate between the Cornell markers was somewhat smaller in the crossing of BL-1 × Aichi Asahi than in the case of *O. sativa* × *O. longisterminata.* This may indicate difficulty in recombination around the resistance gene *Pi-b,* as in *Xa21.*

Future prospects.

Although the fungal avirulence factor (*avr-b*) corresponding to *Pi-b* has not yet been cloned, it may be cloned rather efficiently, as the blast fungus has now the most established RFLP map among the pathogenic fungi (Valent and Chumley 1994, Skinner et al. 1993). This will also greatly facilitate understanding the process of interaction between the plant resistance gene and the avirulence gene of the pathogen.

Cloning the blast resistance gene, and its subsequent manipulation, will provide a means of counteracting the frequent mutation of blast fungi, which causes breakdown in resistances. This may be analogous to the development of antibiotic derivatives against antibiotic-resistant bacterial strains. The resistance gene will also become a powerful tool with which to investigate the chains of signal transduction from the starting side.

MATERIALS AND METHODS

Plant materials.

Seeds of rice cultivars were primarily supplied from the NIAR gene bank and we followed its nomenclature system. Cultivars related to the Touhoku IL series (Sasanishiki, Touhoku IL 9, and Kyokukei 872) were gifts from K. Matsunaga and T. Sasaki of the Miyagi Prefectural Furukawa Agricultural Experiment Station (MPFAES, Furukawa, Japan). Donor parents of *Pi-b* (Tjina, Tjahaja, Milek Kuning) were gifts from S. Maruyama of NARC. The pedigree of the NILs of *Pi-b* (Fig. 1) was constructed from the report by Yokoo et al. (1978) and supplemented from a review by Kiyosawa (1980), as well as reports from MPFAES and NARC. The presence of the resistance gene in the seeds was checked for by inoculating them with the blast line 86F-15 (race 007). Blast fungal lines were those kept in the laboratory of Ando, NARC.

RFLP probes.

RFLP probes of XNpb series, GN/cDK/L series, and RZ/RG series were supplied by A. Saito (Kyushu Agricultural Experiment Station: Saito et al. 1991), the Rice Genome Project (Tsukuba, Japan; Kurata et al. 1992), and Cornell University (Ithaca, NY; Causse et al. 1994), respectively. These plasmids were transformed into DH10B by electroporation with the electro cell manipulator 600 (BTX, San Diego, TX) as described in the manual. Amplified plasmids were prepared using an automatic plasmid isolation system (Kurabo PI-100 Sigma, Tokyo). Inserts were excised from the electrophoresed gel and isolated by digesting the gel with agarase (Nippon Gene, Tokyo).

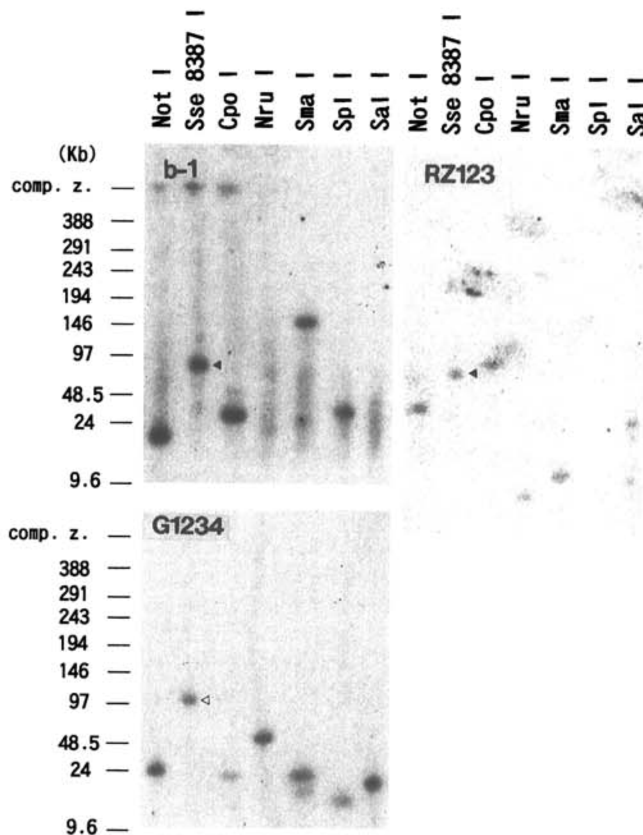

Fig. 6. Long fragment Southern blotting using the close markers b-1, RZ123, and GN1234 with rare-cutter restriction enzymes and CHEF electrophoresis. Solid arrowheads indicates the common band of 85 kb between b-1 and RZ123. The band for GN1234 (open arrowhead) did not overlap the above band. Genome DNA of BL-1 was cut with two 8-base (*Not*I, *Sse* 8387 I), one 7-base (*Cpo*I), and four rare 6-base cutters (*Nru*I, *Sma*I, *Spl*I, *Sal*I).

Preparation of rice DNA and RFLP and RAPD analyses.

Rice plants were cultivated for a few months in pots or vats. Fresh green leaves (3 to 10 g, fresh weight) were frozen in liquid nitrogen, preserved in a deep freezer, then processed as described by Rogers and Bendich (1988). DNA preparations were digested with various enzymes and 2 µg/lane was electrophoresed in 1% agarose in 40 mM Tris acetate, 1 mM EDTA pH 8.0 (TAE) at 6 V/cm for 4 h. The gel was blotted onto nylon membranes (Hybond N^+, Amersham, UK) as described by Sambrook et al. (1989). Hybridizing probes were labeled with ^{32}P-dCTP by random priming and the hybridization profile was visualized using a laser-excited radio fluorescence imaging system (Fuji BioImage Analyzer BAS 2000, Fuji, Tokyo).

Search for nearby markers with RAPD analysis.

Nearby markers of the resistance gene were searched by means of RAPD analysis as described by Williams et al. (1990) using 10-mer primers (Operon, Alameda, CA) and 1.5% agar electrophoresis. The first screening was achieved by searching for polymorphism between an NIL (BL-1) and its recurrent parent (Nourin 22). Second screening proceeded by increasing the number of related NILs and parents to 18 (Fig. 1).

F_2 segregation analysis for fine mapping.

The map position of the resistance gene and an RAPD marker among the RFLP markers were determined by means of F_2 segregation analysis with RFLP markers. Approximately 400 seedlings of F_2 for each crossing of BL-1 (*Pi-b*) × Nourin 22 or Aichi Asahi, at the five- to six-leaf stage, were inoculated with a spray of conidial suspension (1×10^5/ml) of the blast fungus strain Ina 83-23B (race 037.1). After placing them in a humid chamber (25°C, 100% relative humidity) for 24 h, the seedlings were allowed to develop symptoms in a greenhouse and were diagnosed after 7 days. Seedlings diagnosed as susceptible, with leaves showing progressive lesions, were removed and further grown for 1 month in bread containers to prepare DNA. Only susceptible individuals (recessive homo) were selected for DNA preparation, because of the reliable diagnostic symptoms and high efficiency in analyzing chromosomal recombination.

Long fragment Southern blotting analysis.

Nonsheared rice genome DNA was prepared from protoplasts of pale-green leaf seedlings cultured under a dim light on 0.6% agar, as described by Nomura and Kawasaki (1992), with some modification. Leaves cultured for 2 weeks (about 10 g) were cut into 2- to 3-mm sections and incubated in 5 vol (vol/wt) of enzyme solution consisting of 1% Cellulase RS (Yakult, Tokyo), 0.02% Pectolyase Y23 (Shinshin Pharmaceutical, Tokyo) and 0.6 M Mannitol, pH 5.7 for 2 to 3 h at 32°C. Protoplasts were gently squeezed through Miracloth, and the leaf debris remaining on the cloth was resuspended in 0.6 M Mannitol and squeezed repeatedly a few times. Protoplasts were collected by centrifugation onto a sucrose (30% wt/wt) interface by centrifugation (100 × *g*, 5 min), and mixed with low-melting-point agar at a density of 10^8/ml. The agar block was processed as described by Shizuya et al. (1992), digested with rare-cutter restriction enzymes, then electrophoresed using the CHEF system (BIO-RAD DR-II) in 0.5× TBE buffer (tris-borate 45 mM pH 8.0, 1 mM EDTA) at 200 V, and at a ramping interval of current direction changes for 1 to 30 s over 15 h. Southern blotting proceeded as described above.

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