High Resolution Mapping of the *Indica*-Derived Rice Blast Resistance Genes II. *Pi-ta*² and *Pi-ta* and a Consideration of Their Origin

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The *indica*-derived rice blast resistance genes *Pi-ta*² and *Pi-ta*, introgressed into a *japonica* background, were mapped on the middle of chromosome 12 with restriction fragment length polymorphism (RFLP)/random amplified polymorphic DNA (RAPD) markers and several nearly isogenic lines (NILs). *Pi-ta*² cosegregated with XNpb 289 and 196, and was flanked by closely linked markers at 0.3 (ta²-1) and 0.7 cM (XNpb 088, 319/079) on the left (up) side, and at 0.3 (RUBss) and 0.7 cM (XNpb316) on the right (down) side of the map, in F₂ analyses of Pi No. 4 × *japonica* crosses, with 160 recessive homozygotic individuals corresponding to 320 chromosomes. CHEF (contour-clamped hexagonal electric field) Southern blotting suggested that XNpb088 and XNpb239-1, both very close to *Pi-ta*², were spanned by a 170-kb band, suggesting about 500 to 750 kb/cM as the physical/genetic distances ratio in the crosses of Pi No. 4 × *japonica*, which was rather low in recombination ratio, in this region. *Pi-ta* was mapped to the position overlapping *Pi-ta*² by graphical genotype analysis of an NIL with a very narrow introgressed region, Shimokita. This result suggested that these functionally related genes are allelic or at least very closely located, and that they may be derived from a common ancestral gene. However, RFLP analyses of the NILs of these two genes indicated that they are derived from rather different genomes, refuting the report that NILs of *Pi-ta* share a common donor with those of *Pi-ta*². This indicates that the two genes differentiated at a rather ancient time. Two other pedigrees, describing spontaneous recent origins of *Pi-ta* in *japonica*, were also refuted and a single *indica* origin of the gene was indicated.

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

Rice blast, caused by *Magnaporthe grisea*, is one of the most threatening diseases against the major staple food upon which the largest portion of the world population depends. True resistance of rice against the disease, which is effective with a single gene, has been studied since the first description by Sasaki (1922), and over 20 true blast resistance genes have been mapped on rice chromosomes (McCouch et al. 1994). In Japan, considerable effort has been made to introgress *indica*-derived resistance genes, which have a wide resistance spectrum against Japanese blast races, into elite *japonica* cultivars. This has resulted in abundant collections of nearly isogenic lines (NILs) (Kiyosawa 1980), providing an excellent environment for mapping the genes. These blast resistance genes were expected to conform to the “gene for gene theory,” and this was confirmed by the isolation of the blast avirulence genes *avr2-YAMO*, *PWLI*, and *PWL2* (Valent et al. 1994).

Rice has various features that facilitate positional cloning of resistance genes: (i) The rice genome is one of the smallest among the major crops (ca. 430 Mb) (Arumuganathan and Earle 1991). (ii) The calculated physical distance corresponding to the unit genetic distance is also small (230 to 300 kb/cM; from Saito et al. [1991] and Causse et al. [1994]). (iii) The transformation system of rice is the most established among those of monocotyledons (Hiei et al. 1994). (iv) Bacterial artificial chromosome (BAC) libraries of considerable insert sizes (125 kb, Wang et al. 1995; 150 kb, Zhang et al. 1996; 155 kb, Nakamura et al., in press) are now available for the rice genome. Therefore, rice can be a model plant for positional cloning in monocotyledons, especially for the economically important genes of cereals. Although Song et al. (1995) recently cloned the rice resistance gene *Xa21* against bacterial blight disease, still the number of examples of cloning is much smaller than is the case in *Arabidopsis* and tomato.

Here, we describe fine mapping of the blast resistance genes *Pi-ta*² and *Pi-ta*, which were reportedly introgressed from a common donor, the *indica* Philippine cultivar Tadukan, into various *japonica* parents (Shigemura and Kitamura 1954). Thus, several developed cultivars are NILs (underlined in Figure 1) in the sense that they carry a narrow, introgressed *indica* region in the genetically rather homogeneous *japonica* background.

The defense spectrum of *Pi-ta*² against blast races includes

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that of *Pi-ta* (Kiyosawa et al. 1986). These genes were inseparable in 1,000 F₂ individuals of a cross of parents with these genes (Kiyosawa 1969), i.e., they are located within about 3 cM. Therefore, cloning one gene or allele may facilitate cloning of the other, and may clarify how these resistance genes differentiated. The origin of these genes has long been controversial because of the paradoxical pedigree indicating that these closely located genes were derived from the same donor (Fig. 1). Moreover, there are two other independent pedigrees of *Pi-ta*, indicating the spontaneous origin from *japonica* cultivars within only a few decades (Fig. 1C) (Kiyosawa 1980), raising the question whether or not resistance genes can arise so frequently.

Pi-ta was suggested to be the same as *Pi-4(t)* (Inukai et al. 1994), which has been mapped at 15.3 cM from the restriction fragment length polymorphism (RFLP) marker RG869 (Yu et al. 1991). This region of chromosome 12 is of further interest because other blast resistance genes, *Pi-6(t)*, *IPi-(t)*, and *IPi-3(t)*, are clustered there (Causse et al. 1994), and because of its close location to the centromere (Singh et al. 1996).

Our fine mapping of *Pi-ta*² and *Pi-ta* on chromosome 12

provides not only the starting point for physical mapping of these genes, but also answers questions regarding their origin.

RESULTS

RFLP analysis of NILs.

Several NILs of *Pi-ta* and *Pi-ta*² were developed by introducing these *indica*-derived genes into a *japonica* background from (reportedly) the same Philippine donor parent, Tadukan (Shigemura and Kitamura 1954) (Fig. 1). We located these genes on an RFLP map with the help of these NILs. Shinoda et al. (1971) indicated that the resistance gene *Pi-ta*ⁿ, also close to *Pi-ta*², is linked to the reciprocal translocation point of chromosomes 9 and 12, and Kinoshita (1990) located *Pi-ta*² to chromosome 9. Therefore, we tested the RFLP markers of chromosomes 9 and 12.

These markers revealed a commonly introgressed *indica* type chromosome region around the middle of chromosome 12, but not of 9, in both series of NILs of *Pi-ta* and *Pi-ta*² (Figs. 2 and 3), which showed non-donor-parental (non-Tadukan type) and donor-parental (Tadukan type) profiles,

Fig. 1. Reported pedigrees of rice cultivars used in this study. Cultivars with resistance genes *Pi-ta* and *Pi-ta*² are indicated by single and double underlines, respectively. A, B, C groups of *Pi-ta* indicate reported independent pedigrees of the gene. Asterisks indicate the reported donor *indica* cultivar. As discussed in the text, the donor of *Pi-ta* in A, B, and C is a common, unknown *indica* parent different from Tadukan. All pedigrees of *Pi-ta* may be wrong in their origin. Linkages doubted by this report are marked by dotted lines. "Okaine" was not available and its features are unclear.

respectively. The narrow, overlapping, introgressed regions in Shimokita (*Pi-ta*, around XNpb289) and Touhoku IL 7 (*Pi-ta*², around XNpb088 and XNpb289), especially around XNpb289, support the observation that these genes are closely located (Kiyosawa 1967, 1969). The region around XNpb289 was the most marker-dense on chromosome 12 (Saito et al. 1991).

NILs of *Pi-ta* showed the non-Tadukan type (third type) of RFLP profiles for four (XNpb248, XNpb088, XNpb289, and XNpb124-1) of the five non-*japonica* type bands. That is, their profiles differed from those of recurrent *japonica* parents, and also from those of NILs of *Pi-ta*² or the present Tadukan (Figs. 2 and 3). This indicates that *Pi-ta* originally belonged to a genome that significantly differed from the present Tadukan type with *Pi-ta*².

We also analyzed the RFLP profiles of other reportedly independent lines of *Pi-ta* originating spontaneously from *japonica* in recent decades (Fig. 1B and C). Group B, originating from Fukei 102, was identical with Shimokita of group A, according to their graphical genotype profile (Fig. 3). Yashimochi, in group C, was also identical with Pi Nos. 1 and 2 and Tosasenbon in group A, except for the absence of the *indica* type right border marker XNpb124-1, which probably recombined to the *japonica* type from Pi No. 1 type. These results indicated that all these NILs of *Pi-ta* share an *indica* donor, different from the present Tadukan, and that they are not derived from a mutation in *japonica*.

RAPD analysis.

To identify markers more tightly linked to the resistance genes, random amplified polymorphic DNA (RAPD) markers were screened from the polymerase chain reaction (PCR) products of 700 primers by comparing the electrophoretic profiles of an NIL (Pi No. 4) and its recurrent *japonica* parent

(Nourin 8). On average, about six major reproducible bands were obtained from a single primer, among which about one was polymorphic between Kasalath (*indica*) and *japonica* cultivars. A second screening for RAPD markers linked to the resistance genes revealed two major bands of similar apparent molecular size that were specific to the resistant cultivars with *Pi-ta*² or *Pi-ta*. These bands, designated *ta*²-1a and *ta*²-1b (Fig. 4), were about 540 (*ta*²-1a) and 520 (*ta*²-1b) bp long and were reproducibly amplified by the primer TCCCCAGCCA. We also found *ta*²-1a and *ta*²-1b in Shimokita, which has a highly restricted *indica* region (Figs. 3 and 4).

F₂ segregation analysis between the DNA markers and *Pi-ta*².

The position of *Pi-ta*² relative to the closely linked RFLP and RAPD markers was determined by analyzing their segregation in F₂ populations. Segregation of resistant and susceptible individuals matched a 3:1 ratio as expected (Table 1). Analysis of 85 susceptible individuals from a cross of Pi No. 4 (*Pi-ta*²) × Nourin 22 (++), which survived after cutting off leaf lesions, showed that the nearby markers *ta*²-1s, XNpb289, and XNpb316 cosegregated with *Pi-ta*², while one recombination with XNpb088 was detected among the 170 chromosomes analyzed (Fig. 5D). Results obtained from the F₂ were verified by analyzing F₃ individuals. Another analysis of 76 susceptible F₂ individuals from a cross between Aichi Asahi (++) × Pi No. 4 (*Pi-ta*²), indicated that *Pi-ta*² cosegregates only with XNpb196 and 289. In this susceptible population there was one recombination between *Pi-ta*² and either XNpb088, 079, or *ta*²-1 on the left (or upper) side of the map, and one or two recombinations between *Pi-ta*² and RUBss or XNpb316 on the right (lower) side, respectively. In these crosses with Pi No. 4, some bands (XNpb 261, 154, 239-1), closely linked to *Pi-ta*² and polymorphic in a previous cross, Kasalath × FL 134 (Fig. 5A, dotted lines) (Saito et al. 1991), showed no polymorphism. These analyses also showed that XNpb 079 and 319 were derived from the same genome fragment. The original data of Saito et al. (1991) were re-examined and XNpb 079(319) was relocated at the revised position shown in Figure 5A.

The recombination ratio was rather low (1.4%) in this region (XNpb088-316) in the above crosses between Pi No. 4 and *japonica* cultivars (161 samples; 322 chromosomes), compared with that in Kasalath × FL134 (5.4%; Fig. 5A). We therefore tested another cross, Kasalath × Koshihikari (Fig. 5E), which included 55 F₂ individuals. The recombination rate in this cross proved to be rather higher (3.3%) than in the crosses with Pi No. 4. This suggested a lower recombination rate in this region in the crosses between the *japonica* cultivars and Tadukan genomes (with *Pi-ta*²), compared with those between *japonica* and Kasalath. A χ -square analysis showed that the difference in the recombination ratio from the cross Kasalath × FL 134 was significant at a risk level below 0.1% for Pi No. 4 crosses, but at about 50% for the cross Kasalath × Koshihikari; this means no significant difference for the latter cross.

The *indica* type chromosome region introgressed into Shimokita (*Pi-ta*) from the unknown donor is indicated in Figure 5B. Therefore, the position of *Pi-ta* was also restricted between XNpb 088 and 316, suggesting that it either is allelic with *Pi-ta*² or lies at a distance no greater than 0.7 cM from *Pi-ta*². This upper limit is significantly lower than that (3 cM) calculated from the result of Kiyosawa (1969).

Fig. 2. Restriction fragment length polymorphism (RFLP) profiles of the marker, XNpb289, cosegregating with *Pi-ta*² and *Pi-ta* genes in nearly isogenic lines (NILs) and related cultivars. Genomic DNAs were digested with *Hind*III. Cultivars with the *Pi-ta*² and *Pi-ta* genes are indicated by single and double underlines, respectively. Parent *japonica* cultivars used to develop the resistant NILs are also included. The *indica* cultivar is marked by an asterisk. Tadukan type (open triangle, open circle), *japonica* type (open triangle, solid circle), and the third type (solid triangle, open circle) are distinguishable by specific sets of two bands, respectively. All the cultivars with *Pi-ta*² were of the Tadukan type and all those with *Pi-ta* were of the third type. There was no *japonica* type cultivar with the resistance genes.

CHEF Southern blotting with closely linked markers.

The genomic DNA of Pi No. 4 was digested with rare-cutting restriction enzymes and resolved by electrophoresis in CHEF (contour-clamped hexagonal electric field) Southern blotting in an attempt to identify bands cohybridizing to markers closely linked to *Pi-ta*² (Fig. 6). XNpb088, 319/079, and 239-1 hybridized to a common *Sse*I 8387 band of 170 kB. Although there were faint bands of 150, 70, 20 kB that seemed to hybridize to both XNpb088 and 319/079, these results were not definitive. A 45-kB *Not*I band seemed to be commonly detected by XNpb088 and 239-1, and similar in size to a band detected by XNpb 289 (52 kB), but the sizes of bands detected by these three markers were slightly different from each other. There were no bands detected in common by XNpb289 and the right side marker RUBss. If the 170-kB

band spans both XNpb088 and 239-1, 0.9 cM distant in the cross of Kasalath × FL134, the physical distance corresponding to 1 cM would be approximately 500 to 750 kb in the Pi No. 4 background, because in this region the recombination ratio in Pi No. 4 crosses is lower than that of Kasalath crosses by about 1/2.5 to 1/4, as mentioned above. There are more uncertainties for this estimate, because the two markers may be more closely located on the 170-kb fragment, or the apparently common band might be due to mere coincidence or repeated sequences.

DISCUSSION

Chromosomal positions of *Pi-ta*² and *Pi-ta*.

As the first step toward positional cloning of the rice blast resistance gene *Pi-ta*², we localized it on an RFLP map and

Fig. 3. Graphical genotype of chromosome 12 of the nearly isogenic lines (NILs) of *Pi-ta* and *Pi-ta*². A map of the restriction fragment length polymorphism (RFLP) markers is on the top. Extent of chromosomal regions of *japonica*, Tadukan, and the third (non-Tadukan, non-*japonica*) types as determined by RFLP analysis is indicated by solid lines, dark bars, and unfilled bars, respectively. All the NILs of *Pi-ta* showed the third type of profile for the band that was not *japonica* type, except for XNpb316, although the third type was never seen in the NILs of *Pi-ta*². All the non-*japonica* type bands of NILs of *Pi-ta*² were of the Tadukan type.

identified closely linked DNA markers. Several NILs, developed in the course of introduction of *Pi-ta* and *Pi-ta²* from *indica* into various *japonica* cultivars (Fig. 1), allowed efficient high resolution mapping of the genes.

Pi-ta² had been classified as a non-located gene in chromosome 9 in the official rice genome map (Kinoshita 1990). However, in their original report, Shinoda et al. (1971) used a series of reciprocal translocation strains, and indicated that translocation points of chromosomes 9 and 12 were closely linked to *Pi-taⁿ*, a gene located close to *Pi-ta* and *Pi-ta²* but with a slightly different resistance spectrum (Shinoda et al. 1971). Our RFLP survey with an array of NILs confirmed the presence of *Pi-ta²* and *Pi-ta* gene on chromosome 12, not on chromosome 9, and located these genes to around 70 cM on the map of Saito et al. (1991) (Fig. 3). This position is similar to that of *Pi-4(t)*, marked by a marker 15.3 cM distant (RG869; Yu et al. 1991). Inukai et al. (1994) indicated that *Pi-4(t)* was in fact close to *Pi-ta²* (<2.4 cM; calculated from their data) and *Pi-ta* (<2.0 cM), but these positions were not mapped.

The very narrow *indica* region introgressed into Shimokita (*Pi-ta*) coincides with the locus of *Pi-ta²*, within 0.7 cM from either side (Fig. 5B). This indicates that these genes are very close, as suggested by Kiyosawa (1967, 1969). The upper limit of the distance between *Pi-ta* and *Pi-ta²* is much closer than the former resolution of <3.1 cM by Kiyosawa (1969), or that by Inukai as mentioned above.

The origin of *Pi-ta* and *Pi-ta²*.

The close distance between *Pi-ta* and *Pi-ta²* re-provoked the question whether these genes were really derived from the same donor parent, the Philippine cultivar Tadukan (Shigemura and Kitamura 1954).

Fig. 4. Common random amplified polymorphic DNA (RAPD) marker (*ta²-1a,b*) close to the blast resistance genes *Pi-ta* and *Pi-ta²*. Polymerase chain reaction products were resolved by electrophoresis in the discontinuous native acrylamide gel system. Nearly isogenic lines of *Pi-ta²* and *Pi-ta* are indicated by single and double underlines, respectively. Arrows indicate the two close bands (*ta²-1a,b*) specifically accompanying all cultivars with the resistance genes *Pi-ta* or *Pi-ta²*.

The results of RFLP analysis of *Pi-ta* and *Pi-ta²* NILs have argued against that report. In NILs of *Pi-ta*, four RFLP markers of the five non-*japonica* type were of the third type (non-*japonica* and non-Tadukan), while in NILs of *Pi-ta²* all three non-*japonica* type were of the Tadukan type (Figs. 2 and 3). This suggests that *Pi-ta* is derived from a genome different from that of the present Tadukan, which has the same profile as the NILs of *Pi-ta²*. The occurrence of two closely located resistance genes with partly common resistance spectra suggests their differentiation from a common ancestral gene. The resistance spectrum of *Pi-ta²* against fungal races includes that of *Pi-ta* (Kiyosawa 1967). Silue et al. (1993) indicated the presence of an avirulence (*avr*) gene common to blast fungi recognized by *Pi-ta* and *Pi-ta²*, and a second *avr* gene specific for *Pi-ta²*, from crosses of blast fungal races with corresponding *avr* genes. This suggests the presence of two very closely linked resistance genes in the apparently single *Pi-ta²* gene locus. Such co-localization of resistance gene families, as seen in the *Pi-k* families (Kiyosawa et al. 1986), may arise from an ancestral gene that multiplied in this region, as seen in the *Pto* locus (Martin et al. 1994). Indeed, this region is rather dense with rice blast resistance genes found in *indica*, such as *Pi-6(t)*, *IPi-(t)*, and *IPi-3(t)* (Causse et al. 1994).

The notion that two other independent pedigrees of *Pi-ta* spontaneously originated from *japonica* parents (Fig. 1) was also refuted by the graphical genotype analysis (Fig. 3). The group B cultivars with *Pi-ta*, reportedly originating from Fukei 102, had the same genotype profile as Shimokita, with the characteristic narrow extent of the *indica* chromosome. The profile of Yashiromochi in group C was common to Pi Nos. 1 and 2 and Tosasenbon, except that one right end marker, XNpb 124-1, of the *indica* region was of a *japonica* type in Yashiromochi. These findings indicate that *Pi-ta* of groups B and C shares the donor with group A, and suggest that resistance genes such as *Pi-ta²* and *Pi-ta* may differentiate over a long period, but not in a few decades as the *Pi-ta* pedigree had suggested.

Although genes with a novel resistance spectrum may arise from crossing over between near allelic genes, as occurs at the *Rp1* complex of maize (Richter et al. 1995), the “parents” of groups B and C of Figure 1 had no resistance genes around the *Pi-ta* region.

Relationship to *avr* genes.

Valent and Chumley (1994) have cloned a rice blast avirulence gene (*avr2-YAMO*) corresponding to a resistance gene in Yashiromochi, a representative cultivar that has only *Pi-ta* as the resistance gene against blast races available in Japan (Kiyosawa et al. 1986). We suggest that *avr2-YAMO* is *avr-ta*, although, to confirm this, crossing between test and differential (panel) races

Table 1. Segregation of resistant and susceptible individuals in the F₂ population of a Pi No. 4 × Nourin 22 cross^a

Crosses	Resistant (%)	Susceptible (%)	Total (%)	χ^2 -fitting to 3:1 segregation	Level of significance
Pi No. 4 × Nourin 22	374 (76.3)	116 (23.7)	490 (100)	0.46	0.50

^a Seedlings at the 5-leaf stage were sprayed with a conidia suspension (10⁵ conidia/ml) of blast race 037.1. After 1 week, symptoms were diagnosed.

is needed, and the latter panel is not yet internationally established. If *avr2-YAMO* is *avr-ta*, cloning of *Pi-ta* would provide another set of matched *avr* and resistance genes isolated, which would help in understanding the mechanism of their interaction and origin of the resistance. Matching sets of *avr* and resistance genes that have been cloned include *avr-9* of *Cladosporium fulvum* (Van den Ackerveken et al. 1992), *Cf-9* of tomato (Jones et al. 1994), *RPS2* of *Arabidopsis*, and *avr-Rpt2* of *Pseudomonas syringae* (Bent et al. 1994; Mindrinos et al. 1994), as well as the N gene of tobacco and the coat protein of tobacco mosaic virus (Whitham et al. 1994).

Fine mapping of the resistance genes.

The use of an NIL (Pi No. 4) with the same *japonica* background (Nourin 8) as the counterpart parents (Aichi Asahi and Nourin 22) for F₂ analysis is important for ease of crossing and homogeneous genetic background, as described for *Pi-b* (Miyamoto et al. 1996). These Japanese cultivars are also well characterized in terms of true (Kiyosawa 1980) and field (Ezuka 1980) resistance against rice blast. Of the other true

resistance genes, only Aichi Asahi had *Pi-a*, and all cultivars used here were m (Pi No. 4, Nourin 22) or s grade (Aichi Asahi and Nourin 8) in the 5-grade scale (rr, r, m, s, and ss) of field (quantitative) resistance (Ezuka 1980). To analyze recombination in the F₂ generation, we used only the susceptible individuals to increase efficiency and reliability of the resistance test (Miyamoto et al. 1996). Rare recombinants near the resistance gene were re-confirmed in their F₃ generation.

Integrating the results of analysis of F₂ and of several NILs, the map positions of *Pi-ta* and *Pi-ta*² were proposed as shown in Figure 5B and C. Although the calculated average physical length corresponding to 0.3 cM, the shortest distance between the markers on Figure 5C, is about 100 kb in rice, the recombination rate between the nearby markers (between XNpb 088 and 316) in Pi No. 4 × *japonica* crosses (1.4%) was lower than that in Kasalath × *japonica* crosses (5.4 or 3.3%), by about 1/2.5 to 1/4. Therefore, 0.3 cM may correspond to 250 to 400 kb in the Pi No. 4 related crosses. This may reflect suppressed recombination between the genomes of the former parents.

Fig. 5. Fine restriction fragment length polymorphism mapping of the *Pi-ta*² and *Pi-ta* genes. **A**, Reference map by Saito et al. (1991). The band 319/079 was revised and integrated into a single band. Markers that were not polymorphic between Pi No. 4 and *japonica* cultivars are indicated in parentheses. **B**, Introgressed *indica* region of Shimokita (*Pi-ta*), the nearly isogenic line with the narrowest *indica* region. **C-E**, Mapping of the nearby markers by F₂ analyses of (**C**) Aichi Asahi × Pi No. 4 with 76 susceptible individuals, (**D**) Pi No. 4 × Nourin 22 with 85 susceptible individuals, (**E**) Koshihikari × Kasalath with 55 individuals. Map B and C are represented by the same scale, and are included in the frame. Region where the resistance gene may be present is shaded.

Prospect for positional cloning of the resistance genes.

CHEF Southern blotting analysis of Pi No. 4 with rare cutter enzymes indicated that XNpb 088 and 239-1 were spanned by a 170-kB band (Fig. 6). As the genetic distance between the markers was smaller in Pi No. 4 crosses than in Kasalath crosses, as described above, the distance between the markers is estimated to be about 0.23 to 0.35 cM, indicating a value of 500 to 750 kb of the length of the unit genetic distance. Although there are many uncertainties for this estimate, it is near to the value 1,000 kb/cM, revealed by a recent BAC contig formation in this region (Nakamura et al., in press). This supports that suppression of recombination is the cause of apparent short genetic distance in Pi No. 4 crosses.

We have also bracketed the blast resistance gene *Pi-b* within close flanking markers (Miyamoto et al. 1996), and developed a rice BAC library with average insert size of 155 kb from Shimokita (*Pi-ta*) (Nakamura et al., in press). The availability of these materials provides a practical starting point to clone rice blast resistance genes.

MATERIALS AND METHODS

Plant materials.

The pedigrees of introgression of *Pi-ta* and *Pi-ta²* genes into *japonica* cultivars are shown in Figure 1 (Shigemura and Kitamura 1954 and Kiyosawa 1980, with some supplements). Most of the seeds were obtained from the gene bank of the NIAR, and the cultivar nomenclature followed that of the gene bank seed catalogue. The seeds of Touhoku IL7, Kyokukei 737, and Sasanishiki were gifts from T. Matsunaga and T. Sasaki of Miyagi Prefectural Furukawa Agricultural Experiment Station (Miyagi, Japan). The seeds of "Tadukan" originally used as a donor to develop Pi Nos. 1 through 5 (Shigemura and Kitamura 1954) were no longer available. Therefore, we used Tadukan seeds in the gene bank collected from the Philippines. The NIL plants were grown in pots in a greenhouse, and DNA was obtained from 3 to 10 g of fully

grown green leaves as described by Rogers and Bendich (1988), with some modifications. All the seeds were checked for the presence of the resistance genes, using the 007 blast fungal race. The types of the resistance genes in Touhoku IL7 (*Pi-ta²*) and Touhoku IL6 (*Pi-ta*) were checked with all the differential races of blast fungi as described by Kiyosawa et al. (1986) to confirm the presence of the designated genes.

RFLP and RAPD analyses.

The RFLP probes of chromosomes 9 and 12 were as described by Saito et al. (1991). RFLP analyses were performed as described (Miyamoto et al. 1996). The genome DNA was cut with the restriction enzymes recommended in the manual describing these RFLP probes (Kuhara and Saito 1992).

Search for nearby markers with RAPD analysis.

RAPD markers close to the resistance genes were screened as described by Williams et al. (1990) with 1.5% agar electrophoresis. The first screening consisted of a search for polymorphisms between an NIL (Pi No. 4) and its recurrent parent (Nourin 8). The second screen consisted of increasing the number of related NILs and their parents to 8 (for *Pi-ta²*) or 6 (for *Pi-ta*).

F₂ segregation analysis for fine mapping.

The location of the resistance genes relative to nearby RFLP and RAPD markers was determined with two sets of F₂ segregation analyses, using about 400 F₂ individuals each from crosses of Aichi Asahi × Pi No. 4 or Pi No. 4 × Nourin 22. Seedlings at the 5- to 6-leaf stage were inoculated by spraying with a conidial suspension (ca. 10⁵ conidia/ml) and resistance was diagnosed as described (Miyamoto et al. 1996). Lesions on susceptible plants were excised and surviving individuals were grown further to harvest leaves (3 to 10 g) for DNA extraction. Their F₃ progeny were again spray inoculated to confirm the absence of the resistance gene. Only DNA of obvious susceptibles (recessive homozygotes for *Pi-ta²*)

Fig. 6. Contour-clamped hexagonal electric field (CHEF) Southern blotting of Pi No. 4 (with *Pi-ta*) probed with the restriction fragment length polymorphism markers. Enzymes: 8 base (1, *Not*I; 2, *Sse*I 8387), 7 base (3, *Cpo*I), and rare 6-base cutters (4, *Nru*I; 5, *Sma*I; 6, *Sp*II). Same-size bands (170 kb) detected by three probes are indicated by filled arrowheads. Bands indicated by diamonds in 088 and 239-1, and open arrowheads in 088 and 319, seem to be the same size but are not definitive. Other probes shown in Figure 5 did not generate clear bands. Data from two membranes are grouped.

was analyzed. The selection of susceptibles doubled the efficiency of detecting recombination between the dominant resistance gene and the nearby markers, since two chromosomes were checked for each F_2 individual.

CHEF Southern blotting.

Nonsheared rice genomic DNA of Pi No. 4 was prepared from protoplasts of pale green leaf seedlings cultured under dim light on 0.6% agar. The DNA was digested with rare-cutting restriction enzymes and processed for Southern blotting (Miyamoto et al. 1996).

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