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ORIGINAL ARTICLE

Artificial introgression of a large chromosome fragment around the rice blast resistance gene *Pi-ta* in backcross progeny and several elite rice cultivars

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Studying the size of genomic introgressions should lead to a better understanding of linkage disequilibrium in crop breeding. In this study, progeny of the cross between a tropical japonica rice cultivar Katy containing the rice blast resistance gene *Pi-ta* and a temperate japonica cultivar M202 (*Pi-ta*) were inoculated with the race IB49 of *Magnaporthe oryzae* that recognizes *Pi-ta*. The resistant progeny were identified during backcrossing for five generations. Two progeny of each of the 22 BC_5F_1 were genotyped using 12 simple sequence repeat markers around the *Pi-ta* genomic region on chromosome 12. Unlinked DNA in 43 BC_5F_2 individuals was found primarily from the recurrent parent M202 as expected. However, unexpectedly, various sizes of genomic fragments around *Pi-ta* ranging from half (14 Mbp) to the entire chromosome (27 Mbp) were found from the donor. Similarly, large segments of comparable sizes of the *Pi-ta* genomic region originating from a landrace indica variety Tetep from Vietnam were also identified in *Pi-ta* containing US rice cultivars, Katy, Madison, Kaybonnet, and Drew. It was also determined that Tetep had an identical chromosome 12 to another landrace cultivar Tadukan from the Philippines. The most widely grown *indica* cultivar IR64 was found to contain the same 6.4 Mbp around *Pi-ta*. This study demonstrates that a large portion of the chromosome was maintained by artificial selection for blast resistance during crop breeding.
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Introduction

In plant breeding, to add traits from sexually compatible donors, novel genes are routinely introduced to cultivars being improved by artificial selection after controlled crossing. In backcrossing, it is widely accepted that in the absence of linkage drag and selection, the expected proportion of the donor to the genome is $(1/2)^{n+1}$, where n is the number of backcrosses (Stam and Zeven, 1981). One common breeding strategy hence is to introduce genes into elite cultivars by the use of backcross breeding. Through repeated backcrossing, it is anticipated that the trait under selection will be maintained, and the remaining genome will be replaced by the genome of the recurrent parent. In practice, at least five backcrosses are needed to introduce novel genes into elite cultivars (Briggs and Knowles, 1967). During this process, other genes linked to the gene of interest may also be brought into the elite cultivar. This is referred to as linkage drag (Hanson, 1959; Brinkman and Frey, 1977). Linkage drag has been often observed in crop breeding and genetic studies; however, molecular mechanisms of linkage drag are still poorly understood. Thus far, it is known that linkage drag can be influenced by the location on the chromosome, the method of selection, and the factors that affect recombination (Naveira and Barbadilla, 1992; Remington *et al*., 2001; Nordborg and Tavare, 2002; Jain *et al*., 2004, 2008; Metkar *et al*., 2004).

Understanding the molecular mechanisms of linkage drag is extremely important for crop breeding and genetics studies. Sizes of linkage drag have rarely been measured although predicted earlier (Young and Tanksley, 1989; Jena *et al*., 1992; Naveira and Barbadilla, 1992; Ballini *et al*., 2007). Rice (*Oryza sativa* L.) is an ideal organism to measure the size of linkage drag because the genome has been sequenced (Goff *et al*., 2002; IRGSP, 2005). Abundant codominant simple sequence repeat (SSR) markers are publicly available (Chen *et al*., 1997; Temnykh *et al*., 2000; www.gramene.org). Physical differences between wild relatives, landrace and cultivated rice can be easily determined with as few as 25 simple sequence (SSR) markers (Ram *et al*., 2007). In rice, blast disease caused by the filamentous ascomycete fungus *Magnaporthe oryzae* (formerly *Magnaporthe grisea* (Hebert) Barr) has been a limiting factor for production, and blast resistance (*R*) genes have been commonly bred into diverse elite rice cultivars from landrace varieties and wild relatives (Shigemura and Kitamura, 1954; Plucknett *et al*., 1983; Brar and Khush, 1997; Tanksley and McCouch, 1997). In the Southern US, blast epidemics occurred in the 1980s due to the widespread deployment

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of the susceptible cultivar Newbonnet (Lee, 1994). The blast *R* gene, *Pi-ta* is known to prevent infections by races of *M. oryzae* that contain *AVR-Pita* (Bryan *et al*., 2000; Orbach *et al*., 2000; Jia *et al*., 2004; Zhou *et al*., 2007). The resistant cultivar Katy released in the 1990s inherited the major blast *R* gene *Pi-ta* from a Vietnamese landrace *indica* variety Tetep (Moldenhauer *et al*., 1990). Since then, Katy has been used as the *Pi-ta* donor in the development of the resistant cultivars Kaybonnet, Drew, and Madison (Moldenhauer *et al*., 1990; Gravois *et al*., 1995; Moldenhauer *et al*., 1998; McClung *et al*., 1999). In Japan, *indica* landrace Tadukan from the Philippines was used as the *Pi-ta* donor (Shigemura and Kitamura, 1954). Both Tetep and Tadukan were the progenitors for the development of IR64, the world's most popular *indica* cultivar, but their genomic relations have not been documented.

To understand the process of introgression around the *Pi-ta* gene, we performed repeated backcrosses for five generations. For each generation, the resistant progeny was selected using a fungal race that contains *AVR-Pita*, and crossed with the susceptible recurrent parent M202. We examined the size of introgression in 43 BC_5F_2 progeny in *Pi-ta* containing landraces and cultivated varieties using SSR markers. The results demonstrated that a wide range of sizes of linked donor DNA was introgressed into recurrent parent and elite cultivars, and unlinked donor DNA was eliminated in BC_5F_2 individuals. The possible mechanism of this linkage drag and its impact on genes on chromosome 12 is discussed.

Materials and methods

Plant material, growth, crossing, fungal isolate and pathogenicity assay

Rice cultivars Tetep, Katy, Tadukan, Drew, Madison, IR64, and M202 (Johnson *et al*., 1986) were provided by Drs Karen Moldenhauer (University of Arkansas, Rice Research and Extension Center, Stuttgart, Arkansas), Anna McClung (USDA-ARS DB NRRC, Stuttgart, Arkansas), and Harold Rockleman (USDA-ARS, Aberdeen, Idaho). For each cultivar, 5–8 seedlings were soaked, and grown to the 3–4 leaf stage in a greenhouse with a temperature of 24 °C for DNA preparation and pathogen inoculation. Standard methods of crossing were used for all genetic crosses. Susceptible cultivar M202 was used as the female for the initial cross, a tropical japonica cultivar Katy, known to contain the *Pi-ta* gene, was used as the male parent in the cross. The cross between M202 and Katy produced F_1 and F_2. Resistant F_2 individuals were identified and crossed with M202, and only resistant progeny were selected for subsequent backcrossing for five generations. A total of 50 resistant BC_5F_1 were identified (Table 1), and 22 BC_5F_1 of which were advanced to produce 22 BC_5F_2 families. A total of 880 BC_5F_2 individuals (40 BC_5F_2 individuals/each BC_5F_1) were inoculated, and a ratio of 3:1 resistant:susceptible was observed for each of the BC_5F_2 family as expected from a single dominant gene. Forty-four resistant progeny of BC_5F_2 (two from each BC_5F_2 family) were used for determining the genotypes, and genotype results of 43 progeny (one plant was lost) were analyzed and presented in Figure 1.

The blast race IB49 (isolate ZN61) containing the *AVR-Pita* gene was used to evaluate the disease reactions using the standard pathogenicity assay (Moldenhauer *et al*., 1992; Correll *et al*., 2000). The fungal spores for pathogenicity assays were produced using earlier described procedures (Valent *et al*., 1991). Plants were inoculated with an airbrush with 20 ml of the spore suspensions (5×10^5 to 1×10^6 spores/ml). After inoculation, plants were sealed in a plastic bag to maintain high humidity. Plants were moved out after 24 h of inoculation, and then were returned to the greenhouse for an additional 6 days. Disease reactions were determined 7 days after inoculation using a rating scale described by Valent (1997).

Table 1 Segregation of resistance in crossing and backcrossing progeny of the cross of M202 with Katy

Pedigrees	*Number of plants inoculated with pathogen*					
	Total	R	S	R:S	χ^2	$P_{(0.05)}$
F_1 (M202/Katy)	35	35	0			
F_2	40	29	11	3:1	0.067	0.825
BC_1F_1	40	21	19	1:1	0.100	0.775
BC_2F_1	38	18	20	1:1	0.105	0.767
BC_3F_1	30	17	13	1:1	0.533	0.476
BC_4F_1	5	3	2	1:1	0.200	0.671
BC_5F_1	50	27	23	1:1	0.320	0.594

Notes: The susceptible cultivar M202 was used as female for all cross and backcross so that the presence of resistant progeny verified the true cross. *R* indicates resistance and *S* indicates susceptibility. Only five BC_4F_1 were survived to the greenhouse failure and were inoculated.

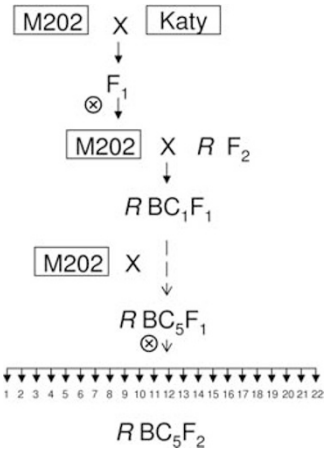

Figure 1 Schematic presentation of backcross scheme showing the procedure of progeny selection and backcrossing. *R* indicates resistance as determined using pathogenicity assays by a race IB49 of *M. oryzae* that recognizes the *Pi-ta* gene.

The *Pi-ta* single nucleotide length polymorphism and SSR marker analysis

DNA was isolated using a Qiagen kit following the manufacturer's instruction. To confirm the presence of the *Pi-ta* gene, the single nucleotide length polymorphism codominant marker for *Pi-ta* (Jia *et al*., 2004) was used to genotype all rice materials following the methods described by Jia *et al*. (2004). A total of 200 SSR markers selected from (http://www.gramene.org) were analyzed on parental materials, and 12 SSR markers were

identified to evaluate the genomic contents on chromosome 12 (Table 1). Two SSR markers on both arms of each chromosome were identified to determine the genomic contents from chromosome 1 to 11 in BC_5F_2 individuals with the exception of chromosomes 1 and 4. On chromosome one, five markers were run and on chromosome 4, only three were run because of low polymorphism between Katy and M202 (Supplementary Table S1). For each marker, forward primers were labeled with fluorescent dyes (6FAM, NED, and Hex) from Applied Biosystems (Foster City, CA, USA) or Integrated DNA Technologies (Coralville, IA, USA). DNA was amplified with MJ Research Tetrad thermocyclers (Waltham, MA, USA) under the following PCR conditions: (1) initial denaturation at 94 °C for 5 min; (2) 35 cycles of 94 °C for 1 min, 55–67 °C (marker dependent) for 1 min, 72 °C for 2 min; (3) 5 min final extension at 72 °C. PCR products were pooled based on color and size range of amplified PCR products (typically three markers per run along with ROX-labeled with size standard), and the DNA was denatured by heating at 94 °C for 5 min. The samples were separated on an ABI Prism 3730 DNA analyzer according to the manufacturer's instructions (Applied Biosystems). The size of the SSR fragment was estimated and the alleles were binned using GeneMapper (Applied Biosystems). Analyzed alleles were exported into a Microsoft Excel spreadsheet.

Results

Resistance to blast was segregated as a single locus from BC_1 to BC_5

Initially, a resistant F_2 individual was identified from F_1 of the cross of M202 with Katy. Identified F_2 individuals were used to cross with the recurrent parent M202 for an additional five backcrosses (Figure 1). All cross and backcrossing hybrids were examined with race IB49, which recognizes resistance provided by the *Pi-ta* gene (Table 1). The segregation ratio of F_2 was 3:1 resistant:susceptible, indicating the presence of a single dominant *R* gene. Consistently, a ratio of 1:1 resistance:susceptibility was observed in all five backcross hybrids, indicating that resistance to IB49 was also inherited as a single locus for each backcross generation (Table 1).

Large linkage block on chromosome 12 in BC_5F_2 Progeny

A total of 43 resistant BC_5F_2 progeny were produced as described in Figure 1. The presence of *Pi-ta* in all backcross progeny was then determined using a codominant marker from *Pi-ta* located on 10.6 Mbp near the centromere, and the resistant reaction was verified using a *M. oryzae* race that recognizes the *Pi-ta* gene (Supplementary Table S2; Figure 2). Results of the genotyping using 12 selected SSR markers showed that a few recombinations had occurred at the distal ends of chromosome 12, and the majority of the central portion of the chromosome 12 had not recombined (Supplementary Table S2). Results of these SSR alleles and the *Pi-ta* marker revealed that large fragments estimated to be half of the chromosome (14 Mbp) to the entire chromosome (27 Mbp, www.gramene.org) were from the donor parent Katy (Supplementary Table S2; Figure 2). The genomic compositions on chromosomes 1–11 were summarized in Supplementary Table S1. The results show that the majority of the genome in all 43 BC_5F_2 individuals was from the recurrent parent M202, suggesting successful elimination of the donor parent segment as predicted in the fifth backcross progeny (Supplementary Table S1). Genotype profiles of all backcross progeny were in agreement with their F_2 generations evidenced by percentages of homozygous and heterozygous SSR alleles. These findings indicate that there are large linkage blocks on chromosome 12 in these backcross progeny (Figure 3).

The same linkage block was shared among two landraces and *Pi-ta* containing cultivars from the US and the Philippines

We next examined three cultivars, Madison, Kaybonnet, and Drew, where Katy was used as parent, to determine whether the same linkage block was also shared. Landrace indica variety, Tetep, the donor for *Pi-ta* in Katy, was also genotyped as control. The results showed that the same linkage block from 1.6 Mbp (RM3483) to 13.2 Mbp (RM7102) originating from Tetep was present in these US cultivars (Figure 3; Supplementary Table S2). The landrace *indica* variety Tadukan from the Philippines was the donor for *Pi-ta* in a number of cultivars used in Japan and was also genotyped. All SSR alleles in Tadukan were identical to those in Tetep from 1.6 to 26 Mbp (Figure 3; Supplementary Table S2), suggesting a common origin on chromosome 12 in both landraces. The cultivar IR64, for which both Tetep and Tadukan were used as progenitors, was also genotyped. These results showed that the genomic region from 6.8 Mbp (RM7003) to 13.2 Mbp (RM7102) on chromosome 12 was shared by both landraces and also with IR64 (Figure 3).

Discussion

Backcrossing is one of the most common practices for plant breeding. It was hoped that backcrossing would remove donor parent chromosomes both linked and unlinked to the target gene (Young and Tanksley, 1989); however, this study identified one of the largest example of linkage drag ever found. Such a phenomenon is statistically improbable because the proportion of the donor to the genome in the crossing employed. In this study, a resistant F_2 individual was selected to for backcrossing, and resistant progeny of BC_5 were used for genotyping. Not considering recombination that occurred during selfing, the proportion of donor in a BC_5 progeny can be 37 cM (Naveira and Barbadilla, 1992). Rice chromosome 12 is about 110 cM in size with an average recombination rate of 1 cM for every 244 Kb, thus the maximum donor fragment expected in a BC_5 progeny would be 9 Mbp (Feltus *et al*., 2004). On the other hand, in the absence of selection and linkage drag, the proportion of donor genome was predicted to be only 1.56% in BC_5 progeny (Stam and Zeven, 1981).

The size and importance of genome introgression in crop plants has been poorly documented. To date, the only other large introgression whose size was measured in rice was recently reported by Ballini *et al*. (2007). Baillini and colleagues reported that at least 5.23 and 7.6 Mbp of *O. rufipogon* IRGC101508 around the blast resistance gene *Pi33* was found on chromosome 8 in cultivars IR64 and IR36, respectively (Ballini *et al*., 2007).

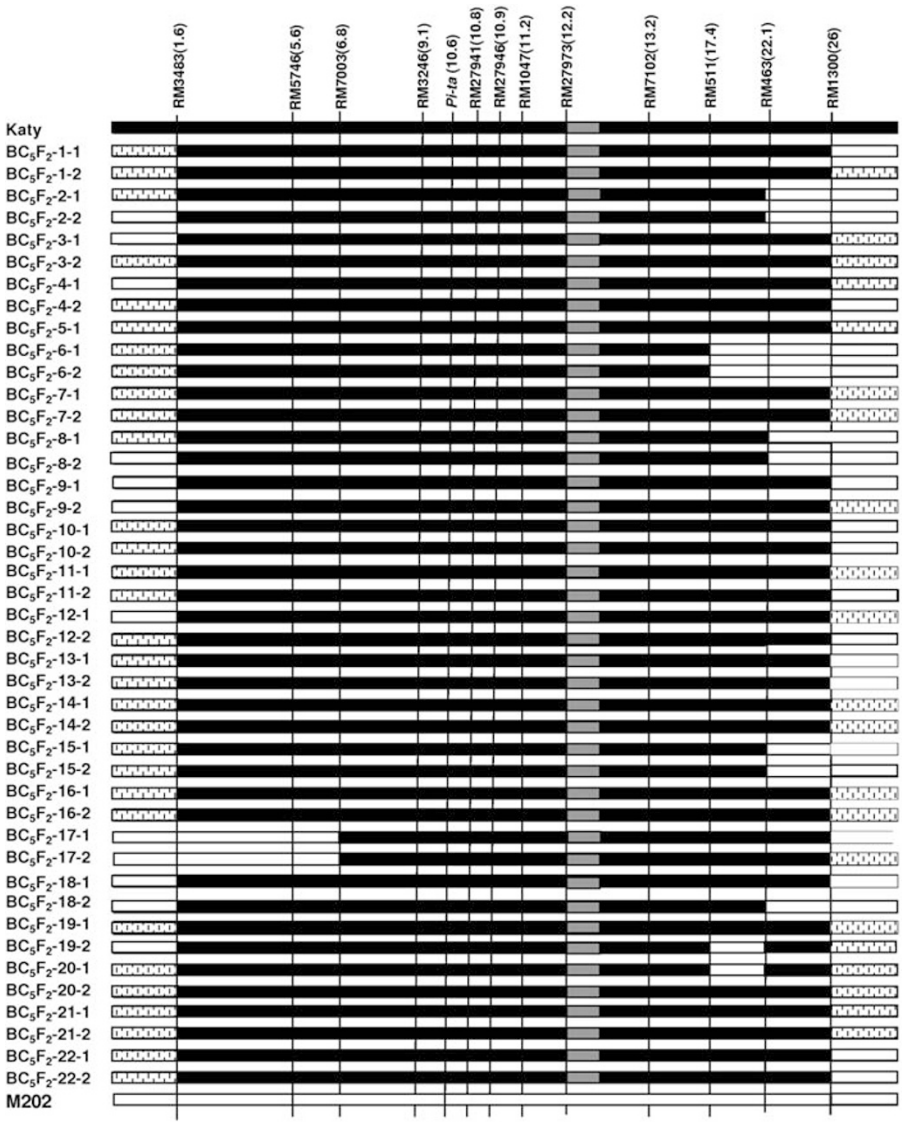

Figure 2 Graphic genotypes of backcross progeny of the cross of Katy and M202 showing the estimated sizes of the introgressed segments around *Pi-ta*. White region shows the graphical genotype for recurrent parent, M202. The approximate location of the centromere was indicated using grey-shaded region. Unknown genotypes at both ends were indicated with broken lines. Physical locations of markers were given in mega bases.

In their study, selection for blast resistance was one of the most important factors for the observed large introgression (Ballini *et al*., 2007). In tomato, using restriction fragment length polymorphism markers, the size of introgressed fragments around the tomato mosaic virus resistance gene *TM-2* was determined (Young and Tanksley, 1989). In one tomato variety, an introgression of 51 cM representing the entire short arm of tomato chromosome 9 was found after 11 backcrosses. In their study, the cultivar was developed by backcrossing breeding with selection for resistance to the tomato mosaic virus resistance gene *TM-2*. In soybean, although the sizes were undetermined, it was estimated that relatively large introgressed fragments in both linked and unlinked to the selected traits were identified in a cultivar that had been developed by pedigree breeding (Sharpe and Lydiate, 2003). In this study, unlinked segments from donor Katy in the backcross progeny were removed by the fifth backcross generation as expected. However, unexpectedly, a large chromosomal fragment around *Pi-ta* and in some cases the entire chromosome from Katy was found in BC_5F_2 individuals. A large segment of Tetep was also identified in several elite rice cultivars. By selecting for blast resistance, the introgressed fragment revealed in backcrossing progeny represented approximately half and in some cases, the entire rice chromosome 12. Additionally, the large introgression of similar genomic fragments was found in cultivars Katy, Madison, Drew, and Kaybonnet all of which were selected to contain resistance to the race IB49 of *M. oryzae* that have *AVR-Pita* during breeding (Moldenhauer *et al*., 1990, 1998; Gravois *et al*., 1995; McClung *et al*., 1999). All resistant backcrossing lines were found to harbor the *Pi-ta* locus, suggesting that *Pi-ta*-mediated resistance was selected although it was unclear how many other blast *R* genes might have been selected. Recently, *Pi-ta*-mediated blast resistance was found to require an additional nuclear gene *Ptr(t)* (Jia

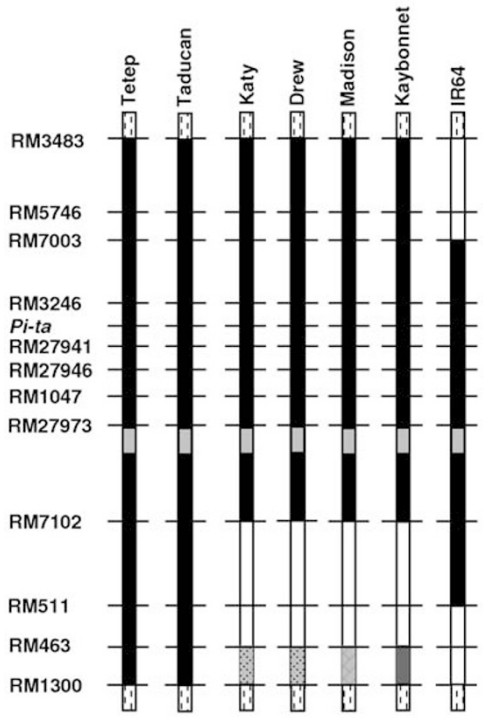

Figure 3 Graphic genotypes of *Pi-ta* containing landraces and cultivars. Approximate location of SSR markers on Figure 2 was shown. Tetep allele was shown as dark-shaded region and non-Tetep is shown in the white region. Unknown genotypes at both ends were indicated with broken lines. Different color shading was used for different genotype.

and Martin, 2008). *Ptr(t)* was located within 9 Mbp of the *Pi-ta* region that was in all of the introgressed segments in all backcrossing progeny. A cluster of *R* genes including *Pi-ta*^{2} was also mapped at the same region, and both of them were inseparable in 4000 F_2 individuals of a cross involving Katy with a breeding line RU9101001 (Chao *et al*., 1999; Jia, unpublished data). In addition, two blast *R* genes, *Pi20(t)* and *Pi39(t)* were recently mapped near the *Pi-ta*/*Pi-ta*^{2}/*Ptr(t)* genes although their allelic relationship to *Pi-ta*/*Pi-ta*^{2}/*Ptr(t)* is unclear (Liu *et al*., 2007; Li *et al*., 2008). It is also unknown if *Pi20(t)* and *Pi39(t)* confer resistance to the race IB49 of *M. oryzae*. Further studies of the above-mentioned subjects may help to clarify if *Pi20(t)* and/or *Pi39(t)* were also selected during backcrosses and cultivar development in this study. Taken together, these findings suggest that a 'super-locus' compromised of multiple genes required for *Pi-ta*-mediated resistance responses might be selected in concert during breeding for blast resistance.

It is important to note that the following possibilities may also explain the large introgressed segments observed in this study: (1) Additional plant components for *Pi-ta*-mediated resistance were embedded within chromosomal rearrangements and (2) genes involved in recombination might have an unknown role in maintaining a large segment during crosses. There are two types of the *DMC1* gene, which is a major homologous recombination gene in rice: type A and type B (Katerir-esan *et al*., 2002; Jain *et al*., 2004; Metkar *et al*., 2004). One copy of the type A is on chromosome 12 and might have a role in maintaining the segment during crosses (Jain *et al*., 2008). The large introgressions observed in this study perhaps resulted because the chromosome 12 in Katy may not be similar to that of the cultivar M202, and recombination of the entire and portions of chromosome 12 had not occurred during meiosis resulting in such large introgressions.

The presence of a large Tetep segment on chromosome 12 in IR64 determined in this study was consistent with the fact that both Tetep and Tadukan were the progenitors for the development of IR64 (Ballini *et al*., 2007). Similarly, the presence of a large Tetep segment on chromosome 12 was consistent with the fact that Tetep was the progenitor for Katy, and the presence of a large Katy segment on chromosome 12 in Madison, Drew, and Kaybonnet was consistent with the fact that Katy was the progenitor for them (Moldenhauer *et al*., 1990, 1998; Gravois *et al*., 1995; McClung *et al*., 1999). In fact, these cultivars were developed by different breeding methods at different times. The presence of such a large physical size of the linkage block was unexpected. However, knowledge of the size of a linkage block in modern cultivars is indeed useful for studies on linkage disequilibrium because the level of linkage disequilibrium controls the resolution and practicability of association-mapping studies (Remington *et al*., 2001; Nordborg and Tavare, 2002). SSR markers in rice can also be easily used to measure the size of linkage drag. In this study, it was demonstrated that a large segment can be maintained at a linked chromosomal region but not in unlinked chromosomal regions during repeated back-crosses. On the other hand, linkage drag and large introgression segments can be reduced by selecting the presence of SSR DNA markers associated with the target gene of the genome of the recurrent parent in back-crossing (Young and Tanksley, 1989; Jena *et al*., 1992; Naveira and Barbadilla, 1992).

Landrace species of rice were traditionally identified by farmers before the technology of breeding was available. The surviving progeny with superiority on yield after disease epidemics were selected, and seeds were saved for next year. Thus, landraces have been a choice of breeding because they were selected from the field after years of adaptations, and can therefore offer a good package of disease resistance (Plucknett *et al*., 1983). The landraces, Tetep from Vietnam and Tadukan from the Philippines, have been used as donors for blast resistance worldwide. Although Tadukan was genotypically different on other chromosomes from that of Tetep (Y Jia *et al*., data not shown), the finding that both Tetep and Tadukan shared chromosome 12 suggests that a common genomic region for disease resistance exists on chromosome 12, and further detailed analysis using single nucleotide polymorphism markers should shed light on details of these important genomic regions.

A 6.4 Mbp segment around the *Pi-ta* gene from Tetep or Tadukan was found in IR64. A large fragment around the blast *R* gene *Pi33* from a wild rice was also found in IR64 (Ballini *et al*., 2007). These observations may explain in part why IR64 has been and still is the most widely grown rice cultivar in the world. Similarly, the US tropical japonica cultivar Katy has been effective in preventing blast disease since its deployment in the 1990s. The *Pi-ta* gene in Katy rice confers resistance to *M. oryzae* containing the corresponding avirulence gene

AVR-Pita, and both *Pi-ta* and *AVR-Pita* were cloned and their interaction and evolutions have been well characterized (Bryan *et al*., 2000; Orbach *et al*., 2000; Jia *et al*., 2004; Zhou *et al*., 2007). Evidently, half of chromosome 12 originating from Tetep was found in several US *Pi-ta* containing rice cultivars, and these cultivars are still effective in preventing blast disease in the Southern US. These observations suggest that genes on different regions on chromosome 12 may be needed to prevent the infections by a wide range of races of the blast pathogen.

In conclusion, this study reveals that a large segment of the entire chromosome 12 can be inherited in five backcross generations, and linked genes on chromosome 12 have not been recombined from early crop domestication from landraces into several modern elite rice cultivars. These findings suggest that the entire chromosome can evolve together as a large linkage block, and rice plants containing recombination near the resistance gene *Pi-ta* were rarely selected during backcross and cultivar development.

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Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)