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ORIGINAL PAPER

Identification and mapping of *Pi41*, a major gene conferring resistance to rice blast in the *Oryza sativa* subsp. *indica* reference cultivar, 93-11

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Abstract The *Oryza sativa* subsp. *indica* reference cultivar (cv.), 93-11 is completely resistant to many Chinese isolates of the rice blast fungus. Resistance segregated in a 3:1 (resistance/susceptible) ratio in an F_2 population from the cross between 93-11 and the *japonica* reference cv. Nipponbare, when challenged with two independent blast isolates. The chromosomal location of this monogenic resistance was mapped to a region of the long arm of chromosome 12 by bulk segregant analysis, using 180 evenly distributed SSR markers. Five additional SSR loci and nine newly developed PCR-based markers allowed the target region to be reduced to ca. 1.8 cM, equivalent in Nipponbare to about 800 kb. In the reference sequence of Nipponbare, this region includes an NBS-LRR cluster of four genes. The known blast resistance gene *Pi-GD-3* also maps in this region, but the 93-11 resistance was distinguishable from *Pi-GD-3* on the basis of race specificity. We have therefore named the 93-11 resistance *Pi41*. Seven markers completely linked to *Pi41* will facilitate both marker-assisted breeding and gene isolation cloning.

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Introduction

Blast is one of the most destructive diseases of rice worldwide (Ou 1985). The causative agent is the filamentous ascomycete *Magnaporthe oryzae* (Couch and Hohn 2002). Genetic resistance is the most economic, effective and environmentally responsible method for its control, but resistance genes (*R* genes) typically lose their effectiveness after a short period in commercial production. The combining of a spectrum of different *R* genes through marker-aided selection probably represents the best available means to achieve durable control (Hittalmani et al. 2000). For this approach, it is necessary to identify markers closely linked to each *R* gene being incorporated.

The rice-*M. oryzae* pathosystem has been developed as a model system for the study of the molecular events occurring during the host-fungal interaction (Valent 1990). This research has established the prevalence of race-specific resistance governed by gene-for-gene relationships (Silué et al. 1992; Jia et al. 2000). At least 50 major blast resistance genes have been identified to date (Chen et al. 2005; Liu et al. 2005; Deng et al. 2006; Gowda et al. 2006; Nguyen et al. 2006), seven of which (*Pib*, *Pita*, *Pi9*, *Pi2*, *Piz-t*, *Pid2* and *Pi36*) have been positionally cloned and characterized (Wang et al. 1999; Bryan et al. 2000; Qu et al. 2006; Zhou et al. 2006; Chen et al. 2006; Liu et al. 2007). With the exception of *Pi-d2*, which encodes a B-lectin receptor kinase, all belong to the large NBS-LRR gene family (Martin et al. 2003; Monosi et al. 2004). The identification and isolation of additional host *R* genes and pathogen avirulence genes is now required to deepen our understanding of the molecular mechanisms involved in the host-pathogen interaction.

The finished genome sequence of the *japonica* reference cultivar (cv.) Nipponbare (International Rice Genome

Sequencing Project 2005; http://dna.affrc.go.jp), and a whole-genome draft sequence of the *indica* cultivar, 93-11 (Yu et al. 2002; http://rise.genomics.org.cn) facilitate molecular mapping and positional cloning in rice (Sakaki et al. 2005; Xu et al. 2005). 93-11 was grown widely in China, and has been used extensively as a parent in a number of breeding programmes. For example, it acts as the restorer line for the popular hybrid cv. Liang-You-Pei-Jiu (Dai et al. 1997; Yu et al. 2002). Although 93-11 expresses a good level of blast resistance (Dai et al. 1997), the genetic basis of this resistance is poorly understood.

In this report, we describe the identification of *Pi41*, a major gene which contributes to the blast resistance of 93-11. The gene was located with the help of linkage analysis and its race specificity was assessed by pathotesting with a large collection of Chinese blast isolates.

Materials and methods

Plant materials and pathotesting

The genetic basis of the blast resistance carried by 93-11 was elucidated by segregation analysis in an F_2 population derived from the cross between 93-11 (resistant) and Nipponbare (susceptible). The population was challenged with two blast isolates (CHL724 and CHL743) collected from Jilin province, China. Both isolates elicit a differential response on the parents of the cross. Seedling management, inoculum preparation, disease inoculation and evaluation were carried out in a greenhouse, as described elsewhere (Zhu et al. 2004).

Marker development and genetic map construction

Total DNA was extracted by the CTAB method (Murray and Thompson 1980) from frozen rice leaves. Bulk segregant analysis (BSA) (Michelmore et al. 1991) was employed to select markers putatively associated with the resistant phenotype. Two DNA pools were assembled by mixing equimolar amounts of DNA from either ten resistant or ten susceptible F_2 individuals (based on their reaction to inoculation with isolate CHL724). The fine mapping of *Pi41* was achieved by three rounds of linkage analysis (Table 1). Firstly, 180 SSR markers distributed evenly across all 12 rice chromosomes (McCouch et al. 2002; http://www.gramene.org) were used to identify those which produced a differential banding pattern from the resistant and susceptible pools. These markers were then genotyped in the whole mapping population. An additional set of SSR markers, located in the genomic region defined by the initial linkage analysis, was then applied to the set of recombinant progeny, along with some de novo generated sequence-tagged site (STS) and candidate *R* gene (CRG) markers developed from the alignment (using BLAST) within the critical region of the genomic sequences of 93-11 and Nipponbare. The STS markers were developed from InDel polymorphisms in non-genic sequence, while the CRG markers exploited InDel polymorphisms with NBS-LRR sequence open reading frames.

PCR amplification conditions consisted of a denaturing step of 94°C/3 min, followed by 35 cycles of 94°C/30 s, annealing temperature (see Table 1)/30 s, and 72°C/1 min, ending with an extension step of 72°C/7 min. Amplicons were separated by 6% polyacrylamide gel electrophoresis and visualised by silver staining. For STS40-5, the amplicon was digested with *Dra*I, separated by 2% agarose gel electrophoresis and visualised by ethidium bromide staining. Primer sequences and other relevant properties of the marker assays are summarized in Table 1. The recombination frequency between adjacent loci was estimated as $N_r/2N_T$ (N_r being the number of recombinants, and N_T the overall population size, Pan et al. 2003; Gu et al. 2004).

Physical map construction and candidate *R* gene characterization

The physical map in the critical region was based on the Nipponbare contig map (IRGSP 2005). The 93-11 contigs were anchored to this framework using the linked markers. Flanking markers were used to identify candidate NBS-LRR genes, with the help of GENSCAN (http://genes.mit.edu), FGENSH (http://sun1.softberry.com) and RiceGAAS (http://rgp.dna.affrc.go.jp) software. The sequence of each candidate gene was then compared with its Nipponbare homologue. To confirm the functionality of the 93-11 candidate *R* genes, 93-11 and 15 other genotypes harbouring identified major *R* genes were challenged with 543 blast isolates collected from three provinces within China.

Results

Genetic mapping of the *R* gene locus

Altogether, 968 and 668 F_2 individuals were inoculated with isolates CHL724 and CHL743, respectively. The segregation ratio between resistance and susceptibility in both cases was consistent with monogenic, fully dominant inheritance [resistant/susceptible: 735/233 ($\chi^2 = 0.45$) and 489/179 ($\chi^2 = 1.15$), respectively]. The linkage analysis indicated that the same *R* gene was detected by both fungal isolates. As a result, a combined F_2 population, consisting of 341 resistant and 418 susceptible individuals, was taken forward as the mapping population for the genetic and physical mapping of the *R* gene locus. The BSA indicated

Table 1 Experimental details of the PCR markers used for linkage analysis

Marker[a]	Primer sequence (5′–3′)	Genomic position (bp)[b]	Annealing temperature (°C)	Expected size (bp)
First marker set				
RM247	F: TAGTGCCGATCGATGTAACG	3185678–3185659	55	156
	R: CATATGGTTTTGACAAAGCG	3185523–3185542		
RM101	F: GTGAATGGTCAAGTGACTTAGGTGGC	8828464–8828439	55	300
	R: ACACAACATGTTCCCTCCCATGC	8828165–8828187		
RM7102	F: TTGAGAGCGTTTTTAGGATG	13258483–13258464	55	170
	R: TCGGTTTACTTGGTTACTCG	13258314–13258333		
RM519	F: AGAGAGCCCCTAAATTTCCG	19973101–19973120	55	122
	R: AGGTACGCTCACCTGTGGAC	19973222–19973203		
Second marker set				
RM28059	F: TGGCCGGTTAGATTTGATAGAGC	14530317–14530339	55	370
	R: GATGTAATCAACCAAGGGACACG	14530686–14530664		
RM28112	F: TCAGCATCGAATTCACCACTTTGC	16322894–16322917	55	284
	R: CGATCAAACCAACTTGCCAACC	16323177–16323156		
RM28130	F: CAGCAGACGTTCCGGTTCTACTCG	16748253–16748276	55	176
	R: AGGACGGTGGTGGTGATCTGG	16748428–16748408		
RM1261	F: GTCCATGCCCAAGACACAAC	17578053–17578072	55	167
	R: GTTACATCATGGGTGACCCC	17578219–17578200		
RM28204	F: CATTCTACCGATGATTGCAGAGG	18351394–18351416	55	150
	R: CTACATTAAGCGTGAGCGACAGC	18351543–18351521		
Third marker set				
STS40-1	F: TCCACCAGCCACATGTTAGC	16582827–16582808	55	95
	R: GGAAATGTGTGGGGAATGGAG	16582733–16582753		
STS40-2	F: CACCATCAGCATGTTTACCA	16585367–16585348	55	125
	R: ATGGTTAACTGGTCAAGGTGA	16585243–16585263		
CRG40-1	F: TTCCTTGGCACTCAGTTCAG	16589155–16589136	55	814
	R: GGGTTATCTTTGCCTCACAGC	16588342–16588362		
CRG40-2	F: GGTGTATGCCAATTAGGTGCCA	16619814–16619793	55	199
	R: GTGGTCACATGTGGATGGAATG	16619616–16619637		
CRG40-3	F: GCCTTGTTGACCTCGACTTGAC	16636470–16636449	55	152
	R: AAACGTCAGGCATGCCAAATC	16636319–16636339		
CRG40-4	F: CCTATGTGGCACCTACGCTCC	16730106–16730086	55	628
	R: TGTCGCACTGCTCCATCCAC	16729479–16729498		
STS40-5	F: CTACTTTTTCCTTGCGGCGATTG	17032605–17032583	60	2083
	R: CGAGGTGTGCGAGTGTGGTC	17030523–17030542		
STS40-3	F: CCTTCCCTTCCTGACACTTG	17388235–17388254	55	121
	R: GAGTCAAAGACGGATCAAGC	17388355–17388336		
STS40-4	F: CCAAGGGAGCTTAGTACTGTA	17399003–17399023	55	335
	R: AGAGGAAGTGGATTCTGAATC	17399337–17399317		

F forward, *R* reverse

[a] The first and second marker sets included International Rice Microsatellite Initiative SSR markers, and the third included new STS and CRG markers

[b] Genomic position of each marker along chromosome 12 as determined by BLASTN analysis against the Nipponbare genome sequence

that four SSR loci (RM247, RM101, RM7102 and RM519), all located on the long arm of chromosome 12, identified a polymorphism between the two parents and the two pools (Table 1). The recombinational distance from the *R* gene locus was 12.4, 2.7, 1.0 and 13.7 cM, respectively. As the recombinant progenies with respect to RM247,

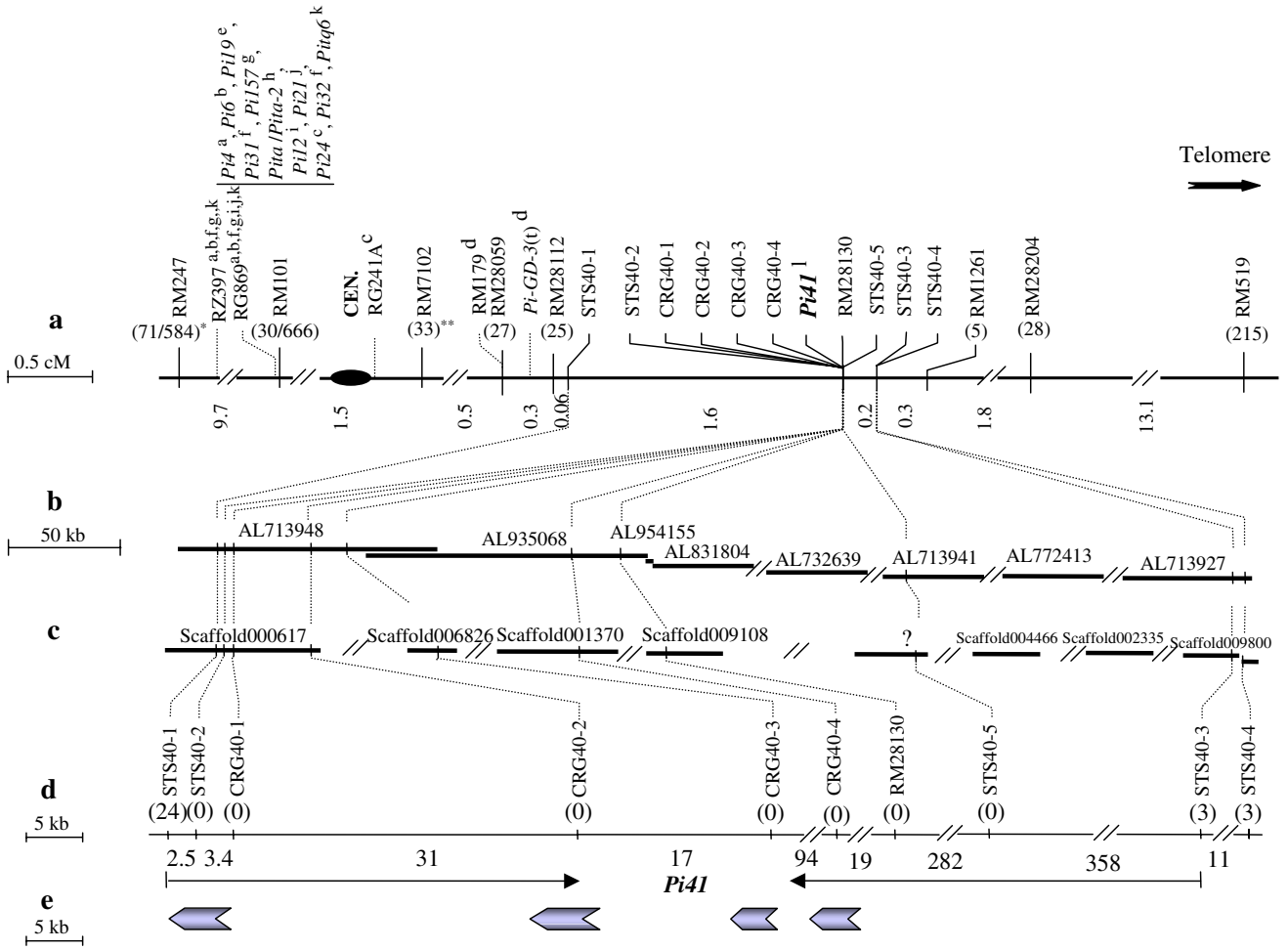

Fig. 1 **a** An integrated genetic map of rice chromosome 12, including 14 blast resistance genes. Map positions were inferred from a: Yu et al. (1991); b: Yu et al. (1996); c: Zhuang et al. (2002); d: Liu et al. (2004); e: Hayashi et al. (1998); f: Sallaud et al. (2003); g: Naqvi and Chattoo (1996); h: Rybka et al. (1997), Bryan et al. (2000); i: Zheng et al. (1996); j: Ahn et al. (2000); k: Tabien et al. (2000); l: this study. *: recombinants/gametes; **: recombinants; *CEN.* centromere. Map distances in cM. **b** Nipponbare contig map around *Pi41*. The *short horizontal lines* represent BAC/PAC clones. The *dashed lines* denote marker positions. **c** 93-11 Contig map around *Pi41*. *Short horizontal lines* marked "?" refer to unanchored BAC/PAC clones. **d** Physical map of the *Pi41* region. The *numbers below* the map are distances in kbp. The *numbers in parentheses* represent the number of recombinants between *Pi41* and the marker locus. **e** Candidate genes for *Pi41*

RM101 and RM7102 were different from those involving RM519, it was concluded that the *R* locus was flanked on the proximal side by RM247, RM101 and RM7102, and on the distal side by RM519 (Fig. 1a). This result allowed the subsequent fine mapping exercise to focus on the 33 recombinants with respect to RM7102 and the 215 recombinants with respect to RM519.

Fine mapping of the *R* gene locus

The RM7102-RM519 interval includes five known SSR loci, which were polymorphic between 93-11 and Nipponbare, and these markers were genotyped in the 248 recombinants described above (Table 1). The respective number of recombination events at RM28059, RM28112, RM28130, RM1261 and RM28204 was 27, 25, 0, 5 and 28 (Fig. 1a). Thus the *R* locus co-segregates with RM28130, and is located at a distance of 1.8 and 1.6 cM, respectively, from RM28059 and RM28112 on the proximal side, and 0.3 and 1.8 cM from RM1261 and RM28204 on the distal side (Fig. 1a). This defines the position of the locus to a ca. 2.0 cM region, flanked by RM28112 and RM1261. The genotyping of the five new STS and four CRG markers in this interval defined 24 recombinant events at STS40-1 on the proximal side, and three at both STS40-3 and STS40-4 on the distal side. STS40-2, STS40-5 and all four CRG markers co-segregated with the resistance (Fig. 1a, d). Thus the *R* locus was located within a ca.1.8 cM interval flanked by STS40-1 and STS40-3, and co-segregates with STS40-2, CRG40-1, CRG40-2, CRG40-3, CRG40-4, RM28130 and STS40-5.

In silico physical mapping of the *R* gene locus

As 93-11 was sequenced using a "whole-genome shotgun" approach, the draft sequence contains several gaps in the target region (Fig. 1c). The physical map of the *R* gene locus region had therefore to be constructed from the Nipponbare sequence (Fig. 1b). Eight Nipponbare BAC/PAC clones were located within the region by a BLASTN analysis based on the sequence of the flanking and co-segregating markers, and the resulting physical map is shown in Fig. 1d. The distance between STS40-1 and STS40-3 is estimated to be about 800 kb (genomic position 16582733–17388355). On the basis of the 93-11 sequence, the distance between STS40-1 and STS40-3 is about 500 kb (genomic position 13208287–13708660).

The annotated Nipponbare chromosome 12 sequence suggests that the region flanked by STS40-1 and STS40-3 contains 122 predicted genes, including 16 known/putative genes, three expressed genes of unknown function, 31 hypothetical genes and 72 transposable element-related genes. A cluster of five NBS-LRR genes (*Os12g28040*, *Os12g28050*, *Os12g28070*, *Os12g28100* and *OS12g28250*) lies between STS40-1 and RM28130 (The Rice Chromosomes 11 and 12 Sequencing Consortia 2005). GENSCAN predicts both *Os12g28040* and *Osg28050* to be intact NBS-LRR genes. Four NBS-LRR genes, each encoding an intact protein, were identified in the target region of 93-11 by RiceGAAS, GENSCAN and FGENSH. Sequence alignment of these four genes showed that they correspond to CRG40-1, CRG40-2, CRG40-3 and CRG40-4, at a homology level of 96.8, 99.3, 99.1 and 98.1% (data not shown), respectively.

Differential analysis of the *R* gene

Thirteen known major blast resistance genes [*Pi4* (Yu et al. 1991), *Pi6* (Yu et al. 1996), *Pi12* (Zheng et al. 1996), *Pi19* (Hayashi et al. 1998), *Pi21* (Ahn et al. 2000), *Pi24* (Zhuang et al. 2002), *Pi31*, *Pi32* (Sallaud et al. 2003), *Pi157* (Naqvi and Chattoo 1996), *Pitq6* (Tabien et al. 2000), *Pita*/*Pita-2* (Rybka et al. 1997; Bryan et al. 2000), *Pi-GD-3* (Liu et al. 2004)] have been mapped to chromosome 12. With the exception of *Pi-GD-3*, all are located on the short arm of the chromosome (Fig. 1a). As to the location of the *Pi-GD-3*, it was roughly mapped based on the flanking markers RM179 (4.8 cM) and NLRinv-5 (23.8 cM) (Liu et al. 2004). Its position was, thus, inferred by the physical distance from the closest marker RM179, which is estimated to be about 1,500 kb based on the average physical/genetic distance ratio of rice, i.e., ~300 kb/cM. The *R* gene in 93-11 maps in the vicinity of the location of *Pi-GD-3*, so the specificities of these two genes were investigated. Since isolate CHL743 elicits a differential response, the 93-11 gene is likely not to be *Pi-GD-3*. Together, the *R* gene identified in 93-11 in the present study seems to be distinct from *Pi-GD-3*, and was designated *Pi41*.

The specificity of *Pi41* was finally assessed against a panel of 15 *R* genes (Table 2). *Pi41* conditions complementary reactions to these genes, and thus represents a useful component for *R* gene-stacking aimed at the breeding of durably blast resistant cvs of rice.

Discussion

Nipponbare and 93-11 are the reference cultivars for the *japonica* and *indica* types. The public availability of their whole-genome sequences has enabled the rapid and effective mapping and isolation of a growing number of functional genes (Gu et al. 2004; Chen et al. 2005; Liu et al. 2005; Xu et al. 2005; Deng et al. 2006; Chen et al. 2006; Liu et al. 2007). We have described here the identification and fine mapping of *Pi41*, delimiting it to a 1.8 cM or ca. 800 kb region. Although it maps to a similar location as *Pi-GD-3* (Liu et al. 2004), it is a distinct gene, since the two genes react differentially when challenged with isolate CHL743. It is well established that most *R* genes are clustered (Michelmore and Meyers 1998; Monosi et al. 2004). Two-thirds of the >50 blast *R* genes identified to date map to chromosomes 6, 11 and 12. Of the 14 mapping to chromosome 12, 12 are either closely linked to the RFLP locus RG869 or are alleles of *Pita,* suggesting the presence of a major *R* gene cluster on the short arm of chromosome 12. Since *R* genes are typically identified in separate cultivars, it is difficult to carry out a classical allelism test between a new gene and others mapping within a cluster (Tabien et al. 2000; Sallaud et al. 2003; Deng et al. 2006). Thus some of the genes mapping to chromosome 12 may be identical to one another (Sallaud et al. 2003). The current alternatives to allelism testing are fine-scale mapping and differential pathotesting.

It has been well documented that the level of recombination frequency along a chromosome varies (Chen et al. 2002; Wu et al. 2003). Several *R* genes are located in regions of low recombination (Chauhan et al. 2002; Chen et al. 2005). In the 800 kb interval defined by STS40-1 and STS40-3, seven markers co-segregated with *Pi41*. This may reflect some localised suppression of recombination, which may be due to its pericentromeric location in the chromosome, where recombination is generally limited (Chen et al. 2002; Wu et al. 2003). An alternative scenario is that some chromosomal rearrangement has affected the region during the diversification of *indica* and *japonica* rice (Chauhan et al. 2002; Wu et al. 2003), resulting in a loss of sequence homology. At least 80% of the 93-11 STS40-1 to STS40-3 sequence is also present in Nipponbare. However,

Table 2 Sixteen rice genotypes, each carrying major genes conditioning specific resistance to blast isolates from Guangdong (GD), Jiangsu (JS), and Jilin (JL) provinces, China

Cultivar (line)	Gene	GD isolates					JS isolates					JL isolates				
		CHL1456	CHL1789	CHL1907	CHL1927	CHL1932	CHL347	CHL348	CHL353	CHL358	CHL403	CHL682	CHL688	CHL692	CHL724	CHL743
A group[a]																
93-11	*Pi41*	S	R	S	R	R	R	R	R	R	S	S	R	R	R	**R**
Nipponbare	?	R	R	S	R	S	S	S	R	S	R	S	S	S	S	S
B group																
SHZ 2	*Pi-GD-3*	R	R	S	R	R	R	R	R	R	R	R	R	R	R	**S**
Kusabue	*Pik*	R	R	R	R	R	S	R	R	S	R	S	S	S	S	S
Tsuyuake	*Pik*-m	R	S	R	R	R	R	R	R	S	R	S	S	S	S	S
Fukunishiki	*Piz*	R	R	R	R	R	R	R	S	S	R	S	S	S	S	S
K1	*Pita*	R	R	S	R	R	S	R	R	S	R	S	S	S	S	S
Pi No. 4	*Pita*-2	R	R	R	R	R	S	S	S	S	R	S	S	S	S	S
Toride 1	*Piz*-t	R	R	R	R	R	S	R	R	S	R	R	S	S	S	R
BL 1	*Pib*	R	R	S	R	R	S	R	R	R	S	R	R	R	S	S
C101LAC	*Pi1*	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S
C101A51	*Pi2*	R	R	R	R	R	R	R	R	R	S	S	S	S	R	R
C104PKT	*Pi3*	S	R	S	S	R	S	S	R	R	S	S	S	S	S	S
C101PKT	*Pi4*	S	R	S	S	R	R	S	R	R	R	S	S	S	S	S
IRBL20	*Pi5*	S	R	S	S	S	S	R	R	R	R	S	S	S	S	S
IRBL22	*Pi9*	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R

The reactions in bold indicate differential reactions between the two resistance genes both were mapped in the same gene cluster on the long arm of chromosome 12

R resistant, *S* susceptible

[a] A group consists of the two parental cultivars, and B group consists of 14 cultivars/lines, each carries the main resistance genes being employed in Chinese breeding programs

the estimated physical length of the interval in 93-11 is 300 kb less than that in Nipponbare, although much of this discrepancy is probably due to the gaps present in the sequence of 93-11. A third possibility relates to the observation that transposon-rich regions characteristically suffer from low levels of genetic recombination (Wu et al. 2003; Arabidopsis Genome Initiative 2000), given that the *Pi41* region is composed of ca. 60% transposon sequence.

Recombination hotspots are commonly concentrated within genic sequence (Dooner and Martinez-Ferez 1997; Inukai et al. 2000; Yao et al. 2002), but recombination hotspots have been identified also in intergenic regions (Wulff et al. 2004; Yao et al. 2002). A potential hotspot is present proximal to *Pi41*, within a 2.5 kb, 1.6 cM interval defined by STS40-1 and STS40-2. In this interval, the physical/genetic ratio (P/G) is >160-fold less than the global mean in rice (Wu and Tanksley 1993). Recombination on both sides of the hotspot was strongly suppressed, suggesting that this hotspot may be specifically active for meiotic recombination, like the well characterized *wx* locus in rice (Inukai et al. 2000). The entire LRR domain, and part of the NBS domain of *Os12g28040* is located within this hotspot. The LRR domains of *R* genes are known to play an important role in pathogen avirulence recognition (Martin et al. 2003; Chisholm et al. 2006), and novel resistance specificities generated by recombination have been documented at the flax rust resistance *L* loci (Elli et al. 2007). The Nipponbare and 93-11 sequences differ at 51 base pair positions in *Os12g28040* (data not shown), so an intriguing possibility is that the recombination hotspot we have identified contributes to the rapid evolution of this region.

Although the delimiting region of *Pi41* spans some 800 kb, only four genes with an intact NBS-LRR structure are present, and these all lie within a ca. 200 kb interval flanked by STS40-1 and RM28130 in Nipponbare. Thus these genes all represent good candidates for *Pi41*, and we are currently using a map-based in silico approach (Liu et al. 2007) as a strategy for gene isolation.

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