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ORIGINAL PAPER

The identification of *Pi50*(t), a new member of the rice blast resistance *Pi2/Pi9* multigene family

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Abstract The deployment of broad-spectrum resistance genes is the most effective and economic means of controlling blast in rice. The cultivar Er-Ba-Zhan (EBZ) is a widely used donor of blast resistance in South China, with many cultivars derived from it displaying broad-spectrum resistance against blast. Mapping in a set of recombinant inbred lines bred from the cross between EBZ and the highly blast-susceptible cultivar Liangjiangxintuanheigu (LTH) identified in EBZ a blast resistance gene on each of chromosomes 1 (*Pish*), 6 (*Pi2/Pi9*) and 12 (*Pita/Pita-2*). The resistance spectrum and race specificity of the allele at *Pi2/Pi9* were both different from those present in other known *Pi2/Pi9* carriers. Fine-scale mapping based on a large number of susceptible EBZ × LTH F_2 and EBZ × LTH BC_1F_2 segregants placed the gene within a 53-kb segment, which includes *Pi2/Pi9*. Sequence comparisons of the LRR motifs of the four functional NBS-LRR genes within *Pi2/Pi9* revealed that the EBZ allele is distinct from other known *Pi2/Pi9* alleles. As a result, the gene has been given the designation *Pi50*(t).

X. Zhu and S. Chen contributed equally to this work.

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Introduction

The rice disease blast, caused by the fungus *Magnaporthe oryzae*, is one of the most devastating diseases afflicting the rice crop (Ou 1985) and is best combated by the deployment of genetic resistance (Bonman et al. 1992). Cultivars (cvs) whose resistance is determined by a single gene tend to retain an effective level of resistance for only a short time (Bonman et al. 1992; Babujee and Gnanamanickam 2000), especially when they are grown over a large acreage. Reliance on broad-spectrum resistance is a more sustainable strategy, and much attention has therefore been focused on genetically characterizing cultivars which show this behavior (Deng et al. 2006; Liu et al. 2007; Jeung et al. 2007; Huang et al. 2011). The evidence suggests that frequently (but not exclusively), broad-spectrum resistance requires the simultaneous presence of several race-specific resistance (*R*) genes. Examples of this include the West African cv. Moreberekan and the Vietnamese cv. Tetep, both of which carry at least five *R* genes (Inukai et al. 1994; Wang et al. 1994; Chen et al. 1999; Barman et al. 2004), and also the widely cultivated cvs IR64 and Sanhuangzhan 2, both of which carry at least three *R* genes (Sallaud et al. 2003; Liu et al. 2004). To date, over 80 distinct blast *R* genes have been identified (Zhang 2007; Ballini et al. 2008; Huang et al. 2011), but of these, only *Pi1* (Yu et al. 1991), *Pi2* (Chen et al. 1996), *Pi3/Pi5* (Jeon et al. 2003) and *Pi9* (Liu et al. 2002) appear to confer a broad spectrum of resistance.

In total, 17 blast *R* genes have now been isolated, and the majority of these have been shown to encode NBS-LRR type of proteins (see a review in Ballini et al. 2008; and Ashikawa et al. 2008; Lee et al. 2009; Takahashi et al. 2010; Okuyama et al. 2011; Yuan et al. 2011; Zhai et al. 2011).

Since the 1990s, the *indica* cv. Er-Ba-Zhan (EBZ) has represented an important source of blast resistance in rice breeding programs based in South China (Zhu et al. 2003). EBZ is known to harbor at least one broad-spectrum *R* gene, because most of its derivatives have exhibited a broad spectrum of resistance (Zhu et al. 2006). Here, we have used a series of defined segregating populations in order to elucidate the genetic basis of its blast resistance in EBZ. We show that EBZ carries an *R* gene on each of chromosomes 1, 6 and 12, and that the gene on chromosome 6 is associated with broad-spectrum resistance. Fine-scale mapping and sequence analysis were then used to show that this gene, designated *Pi50*(t), is a member of the *Pi2*/*Pi9* multigene family.

Materials and methods

Plant materials

The three sets of segregating material were bred from the cross between EBZ and the highly blast-susceptible cv Liangjiangxintuanheigu (LTH). The first of these was a population of 297 F_8 recombinant inbred lines (RILs), obtained by single seed descent, which was used for the coarse-scale mapping of the blast *R* genes present in EBZ. The second comprised 652 susceptible EBZ × LTH F_2 and (EBZ × LTH) × LTH BC_1F_2 segregants, and the third two resistant near isogenic BC_6F_4 lines (NILs) in a genetic background of LTH, selected on the basis of their reaction to the broadly virulent blast isolate GD98288. The latter two materials were combined for fine-scale mapping and candidate gene identification.

Blast inoculation and disease evaluation

Seven rows of seedlings, each with 15–20 plants, were planted in a greenhouse in 30 cm x 20 cm × 5 cm trays. Inoculation of the RIL population was performed at the 3.5–4 leaf stage using a set of 20 blast isolates selected to show a diverse spectrum of virulence. EBZ was resistant and LTH was susceptible to all 20 of these isolates. A 20-ml spore suspension (10^5 spores/ml) was applied to each tray using an airbrush connected to a source of compressed air. Each isolate–host combination was assessed in two replications. After inoculation, the trays were held in the dark for 24 h at 95–100% relative humidity and 25°C, after which they were transferred to a greenhouse where the ambient temperature was maintained at 25–28°C. Six days later, disease symptoms were evaluated on a standard 0–9 scale, as described elsewhere (International Rice Research Institute 1996). Individuals assigned a score of >3 were considered susceptible.

Genotyping and mapping

Genomic DNA was extracted from frozen leaf material using the CTAB method (Murray and Thompson 1980). For the purposes of a modified bulk segregation analysis (BSA) (Michelmore et al. 1991), the DNA of 16 RILs susceptible to all 20 isolates was pooled. The parental cvs were initially screened with a panel of 520 microsatellite (SSR) primer pairs (http://www.gramene.org; McCouch et al. 2002), and those which were informative were then tested on the pooled DNA; where this screen suggested possible linkage with susceptibility, the constituent individual DNAs forming the bulk were assayed separately (Fig. 1). To prepare a fine-scale genetic map of *Pi50*(t), F_2 and BC_1F_2 individuals were challenged with isolate GD98288, and 652 fully susceptible individuals were selected to form a targeted mapping population. Based on the location of *Pi50*(t) predicted from the mapping of the full RIL population, 50 de novo markers (including SSRs, InDels and CAPS) were designed from the local sequences of cvs. Nipponbare (http://rgp.dna.affrc.go.jp) and 93–11 (http://www.genomics.org.cn). FASTPCR v4.0 software (http://www.biocenter.helsinki.Fi/bi/programs/fastpcr.htm) was used for primer design.

Resistance spectrum comparison

Six RILs and two NILs, each containing one of the *R* genes present in EBZ (Fig. 2), were inoculated with a set of 523 blast isolates, originating from various parts of China. Twelve monogenic stocks and NILs for various blast *R* genes (Inukai et al. 1994; Kobayashi et al. 2007) were included as a control. Similarity matrices generated according to the simple matching coefficient were used to carry out a cluster analysis using NTSYS-pc, v1.7 software (Exeter Software, Seatauket, New York) and the unweighted pair group method with arithmetic averaging algorithms (UPGMA).

Sequence comparison of LRR motifs of the *Pi2/9* multigene family

The annotation given for the cv. Nipponbare genome indicates the presence of seven NBS-LRR genes at the *Pi2*/*Pi9* locus. Of these, NBS-LRR3 appears to be non-functional due to the presence of several premature stop codons within the coding region, while both NBS-LRR5

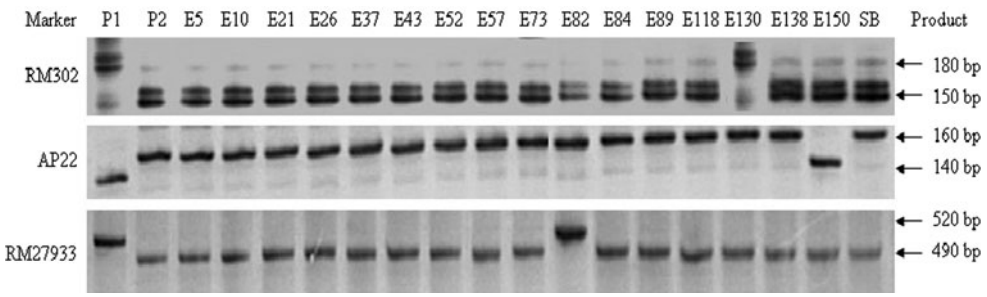

Fig. 1 Modified BSA analysis identified the presence in EBZ of an *R* gene on each of chromosomes 1, 6, and 12. P1: EBZ, P2: LTH, E5-E150: blast-susceptible RILs used to form the susceptible bulk DNA, *SB* the susceptible bulk

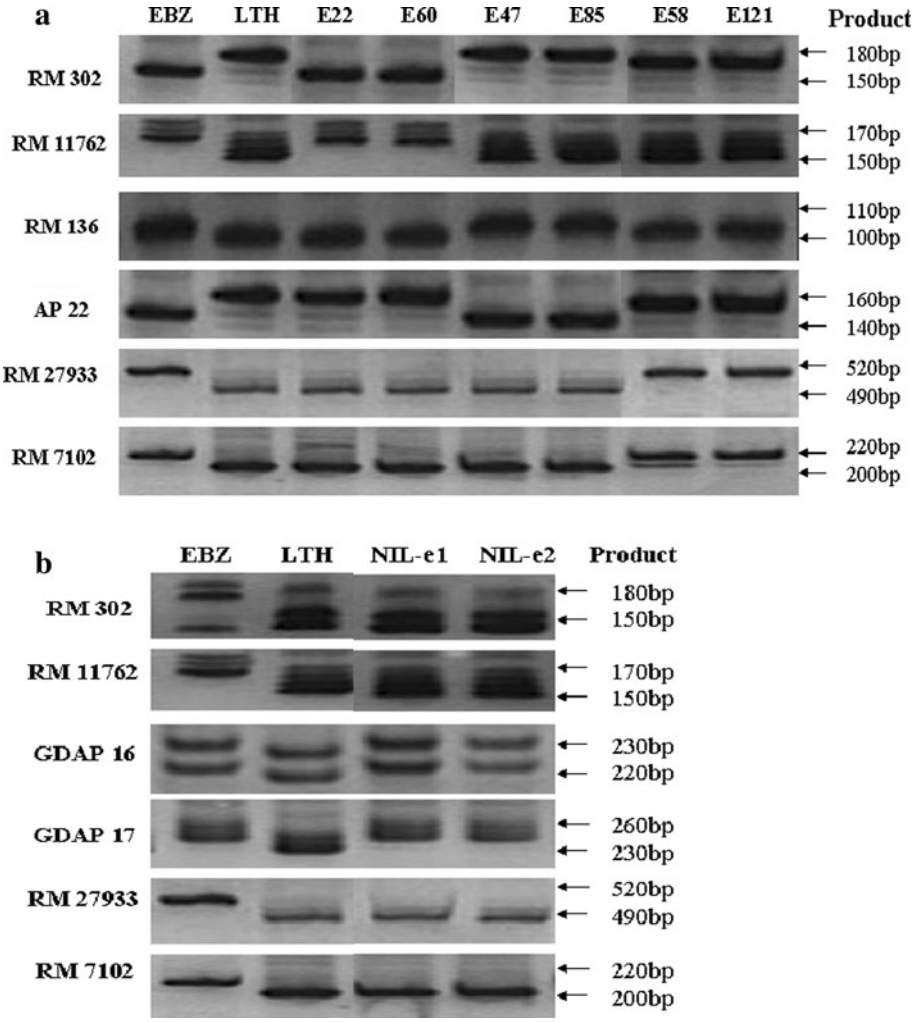

Fig. 2 Genotyping single *R* gene RILs and NILs using markers identified by the modified BSA. **a** RILs E22 and E60 carrying an *R* gene in the interval RM302-RM11762 on chromosome 1, RILs E47 and E85 carrying an *R* gene in the interval RM136-AP22 on chromosome 6, and RILs E58 and E121 carrying an *R* gene in the interval RM27933-RM7102 on chromosome 12. **b** NIL-e1 and NIL-e2 carrying the chromosome 6 *R* gene *Pi50*(t)

and NBS-LRR6 resemble pseudogenes due to deletions in their coding region (http://www.ncbi.nlm.nih.gov/nuccore/AP005659; Zhou et al. 2006; Fig. 4). The remaining four were therefore taken forward for a sequence-based analysis of the LRR motifs present in EBZ and other cultivars carrying various *Pi2/9* alleles. LRR motifs were predicted by applying the simple modular architecture research tool (http://smart.embl-heidelberg.de/). The amplified product of each of these LRR motifs was purified by agarose gel electrophoresis and directly sequenced. Sequence comparisons were achieved using a combination of FGENESH (http://linux1.softberry.com/berry.phtml), ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and DNAMAN (http://www.lynnon.com/) software.

Results

Segregation for blast resistance in the RIL population

The segregation for blast resistance/susceptibility among a subset of 150 of the RILs fitted either a ratio of 1:1, 3:1 or

Table 1 Segregation among RILs derived from the cross EBZ × LTH to inoculation with 20 isolates of *M. oryzae*

Blast isolates	No. of RIL lines		Segregation ration	χ^2	*P*
	Resistant	Susceptible			
GD98288	150	147	1:1	0.03	0.87
GD00193	150	147	1:1	0.03	0.87
GD9559	112	38	3:1	0.01	0.93
GD93286	76	74	1:1	0.03	0.87
GD0712	76	74	1:1	0.03	0.87
GX0608	76	74	1:1	0.03	0.87
GX0613	76	74	1:1	0.03	0.87
HB0407	76	74	1:1	0.03	0.87
HB0412	133	17	7:1	0.19	0.67
HN0007	76	74	1:1	0.03	0.87
HN0011	76	74	1:1	0.03	0.87
FJ0601	76	74	1:1	0.03	0.87
FJ0609	112	38	3:1	0.01	0.92
JL0802	118	32	3:1	1.08	0.30
JL0807	76	74	1:1	0.03	0.87
SC0602	108	42	3:1	0.72	0.40
SC0609	76	74	1:1	0.03	0.87
HL-72-1	112	38	3:1	0.01	0.92
TJ-0802	129	21	7:1	0.31	0.58
ZJ-0010	108	42	3:1	0.72	0.40

Twenty isolates were selected from Guangdong (GD), Guangxi (GX), Hubei (HB), Hunan (HN), Fujian (FJ), Jilin (JL), Sichuan (SC), Heilongjiang (HL), Tianjin (TJ) and Zhejiang (ZJ) provinces in South and North China

7:1, depending on the choice of the isolate (Table 1). Twelve of the isolates detected the presence of just one *R* gene in EBZ, six detected two *R* genes, and the other two detected three *R* genes. A total of 76 of the RILs were resistant to every isolate, consistent with the notion that EBZ carries a broad-spectrum *R* gene. When the isolates GD00193 and GD98288 were used to inoculate the full RIL population, a monogenic segregation was evident (Table 1).

Identification of the candidate *R* genes

The parental screen (EBZ vs. LTH) of the 520 SSRs identified 350 informative loci, and these were taken forward into the modified BSA. (A resistant bulk was not included because of the segregation of more than one *R* gene in the population.) The informative loci were distributed across all 12 rice chromosomes (37 on chromosome 1, 29 on chromosome 2, 26 on chromosome 3, 21 on chromosome 4, 23 on chromosome 5, 34 on chromosome 6, 22 on chromosome 7, 25 on chromosome 8, 29 on chromosome 9, 20 on chromosome 10, 46 on chromosome 11 and 38 on chromosome 12). Presumptive linkage with an *R* locus was displayed by RM302 and RM11762 (chromosome 1), RM136 and AP22 (chromosome 6), and RM27933 and RM7102 (chromosome 12) (Figs. 1, 2a). These regions coincide with the location of, respectively, *Pish*, *Pi2/9* and *Pita*/*Pita-2* (Bryan et al. 2000; Qu et al. 2006; Zhou et al. 2006; Takahashi et al. 2010). RILs E22 and E60 carried only one *R* gene, which mapped within the chromosome 1 interval, while RILs E47 and E85 (as well as the NILs Nil-e1 and Nil-e2) also were monogenic carriers (in the chromosome 6 interval), as were RILs E58 and E121 (in the chromosome 12 interval) (Fig. 2).

Characterization of *R* gene race specificity

The eight single *R* gene carriers referred to above, along with a set of 12 lines carrying known *R* genes, were challenged with a set of 523 blast isolates (Fig. 3). The response of RILs E22 and E60 was similar to that of the *Pish* monogenic line IRBLsh-S, while that of RILs E58 and E121 mirrored the response of the *Pita-2* monogenic line IRBLta2-Re. Thus, two of the *R* genes present in EBZ are likely to be alleles of either *Pish* or *Pita-2* (Fig. 3). However, the response of RILs E47 and E85 (as well as of the NILs Nil-e1 and Nil-e2) differed markedly from that of lines carrying either *Piz*, *Piz-t*, *Pi9* or *Pi2* (Table 2; Fig. 3). While the NILs were resistant to 97.7% of the isolates, the proportion of incompatible isolates on the *Pi9*, *Pi2*, *Piz* and *Piz-t* lines was, respectively, 93.7, 92.2, 68.4 and 54.5% (Table 3). Thus, along with *Pish* and *Pita-2*, EBZ also carries a new, broad-spectrum allele at the *Pi2*/*Pi9* locus.

Fine mapping of the broad-spectrum *R* gene

When the full RIL population was assayed with the pair of presumptively linked SSR loci RM136 and AP22, 39 recombinants (i.e., the resistant RILs that showed the marker genotype of the susceptible parent and vice versa) between the resistance and RM136, and 16 between the resistance and AP22 were identified. These frequencies correspond to a separation between the resistance locus and RM136 of 6.6 cM, and of 2.7 cM between the resistance locus and AP22. Fine-scale mapping of the resistance locus was based on the sample of 652 fully susceptible EBZ × LTH F_2 and EBZ × LTH^2 BC_1F_2 segregants, of which 60 showed recombination between the resistance locus and RM136, and 27 between the resistance locus and AP22 (Fig. 4). Of the set of 50 de novo markers developed to saturate the target region, 13 were informative between EBZ and LTH (Table 3), and thus could be applied to the 60 recombinants between the *R* gene and RM136 and to the 27 between the *R* gene and AP22. GDAP26, GDAP39, GDAP41, GDAP33, GDAP32, GDAP17 and GDAP51

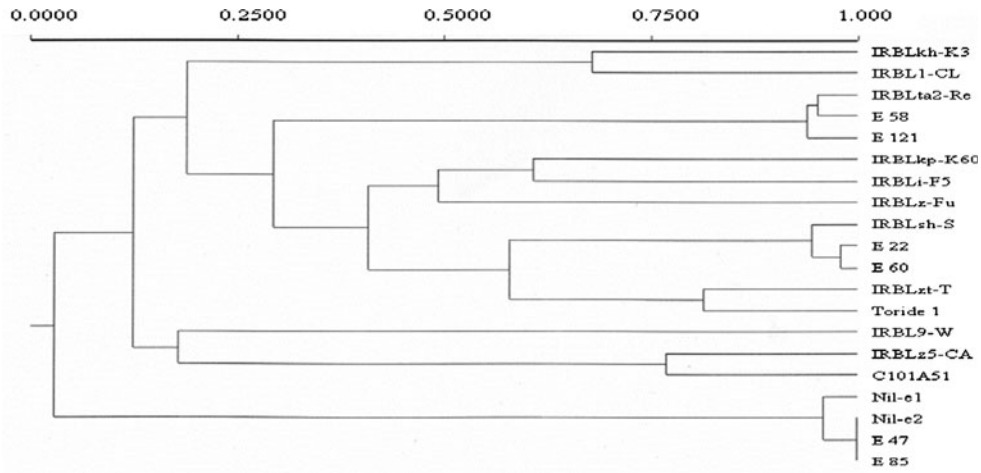

Fig. 3 Cluster analysis of the race-specific resistance of EBZ-derived RILs and NILs, based on their reaction to inoculation with 523 Chinese *Magnaporthe oryzae* isolates. The known *R* gene carriers are IRBLkh-K3 (*Pik-h*), IRBL1-CL (*Pi1*), IRBLta2-Re (*Pita-2*), IRBLkp-K60 (*Pik-p*), IRBLi-F5 (*Pii*), IRBLz-Fu (*Piz*), IRBLsh-S (*Pish*), IRBLzt-T (*Piz-t*), Toride (*Piz-t*, *Pish*), IRBL9-W (*Pi9*), IRBLz5-CA (*Pi2*) and C101A51 (*Pi2*, *Pia*; see Inukai et al. 1994; Kobayashi et al. 2007; Zeng et al. 2011)

Table 2 Reaction of single *R* gene RILs and NILs to 23 isolates of *M. oryzae,* and the resistance spectrum following inoculation with 523 Chinese blast isolates

Isolate	Origin	Reactions[a]							
		Nil-e1 *Pi50*(t)	Nil-e2 *Pi50*(t)	IRBLz5-CA *Pi2*	C1O1A51 *Pi2+Pia*	IRBL9-W *Pi9*	IRBLz-Fu *Piz*	IRBLzt-T *Piz-t*	Toride *Piz-t+Pish*
08-2045a	Gungdong	8	8	1	1	1	1	1	1
09-044a	Gungdong	8	8	1	1	1	6	9	1
09-137a	Gungdong	8	8	8	7	3	3	9	6
W08-037a	Hubei	8	8	1	1	1	3	3	3
W08-059a	Hubei	8	8	1	2	3	3	1	2
09-3041a	Hubei	1	1	6	4	3	1	8	8
10-026a	Guangdong	1	1	7	6	1	3	1	1
10-149a	Guangdong	1	1	7	7	1	5	7	7
SC0602	Sichuan	1	1	8	7	1	7	1	1
SC-RB14	Sichuan	1	1	9	7	1	3	6	7
05-006a	Guangdong	1	1	1	1	8	7	1	2
10-109a	Guangdong	1	1	1	1	5	3	9	7
HN0102	Hunan	1	1	1	1	7	1	8	6
W06-18a	Guangxi	1	1	1	1	6	3	1	1
05-161a	Guangdong	1	1	2	1	1	8	1	1
HLJ07-80-1	Heilongjiang	1	1	1	1	1	8	1	1
W08-011a	Fujian	1	1	1	1	3	7	3	1
W08-061	Hubei	1	1	1	2	1	8	1	2
FJ0601	Fujian	1	1	1	1	1	1	7	7
HN0007	Hunan	1	1	2	1	1	1	8	8
JL0807	Jilin	1	1	1	2	1	1	7	7
SC4-5-2-2	Sichuan	1	1	3	1	1	3	7	7
ZJ0109a	Zhejiang	1	1	1	3	1	1	8	7
Resistance spectrum (%)[b]		97.7	97.7	92.2	93.0	93.7	68.4	54.5	66.5

[a] Reactions were scored according to system devised by IRRI (1996). The *Pi* gene content of cvs. C1O1A51 and Toride was taken from Zeng et al. (2011)

[b] Resistance spectrum calculated from the ratio of the number of incompatible isolates and the total number of isolates tested (523)

detected, respectively, 36, 21, 17, 13, five, two and one recombinant(s) with respect to the *R* gene among the former set of plants, while GDAP23, GDAP37 and GDAP16 detected, respectively, nine, five and one recombinant(s) among the latter. The three markers GDCP1, GDG4-3 and GDAP47 co-segregated with the *R* gene (Fig. 4).

Table 3 Sequences of the PCR primers used in the modified BSA, the fine-scale mapping and the re-sequencing of LRR motifs

Marker[a]	Marker type	Forward primer (5′–3′)	Reverse primer (5′–3′)	Annealing temperature (°C)
BSA analysis				
RM302	SSR	TGCAGGTAGAAACTTGAAGC	AGTGGATGTTAGGTGTAACAGG	55
RM11762	SSR	AAGCGACAACTGAAGGAAACTCG	TTTGAAAGTCCACTGCCAAGTGC	55
RM7102	SSR	GGGCGTTCGGTTTACTTGGTTACTCG	GGCGGCATAGGAGTGTTTAGAGTGC	55
RM27933	SSR	TCCTCTGTCATATGGCTGTAAACG	GGACAAGGAGGAACTATTGATTGG	55
RM136	SSR	GAGAGCTCAGCTGCTGCCTCTAGC	GAGGAGCGCCACGGTGTACGCC	55
AP22	SSR	GTGCATGAGTCCAGCTCAAA	GTGTACTCCCATGGCTGCTC	55
Linkage analysis				
GDAP16	SSR	TGTAGGAGTTGCGGCACATCAC	AGCATCCCGTTGACTCGCGATAGC	60
GDAP17	SSR	AGATCGGCCGCCAATTCACG	CGCATGATGACTTCCAAACG	60
GDAP23	InDel	ACTACTGCGGGACACGCCAC	TAACCTTGTGCAGTTGGAGC	60
GDAP26	InDel	ATTAGGATACGGCTTCTAGG	ACCTCATCGACAGGCCTTTAGC	55
GDAP32	InDel	CCGACGATTGGCGCACACGGTC	ACACAGAATGCAGCCTAACG	55
GDAP33	InDel	AGATAGCTTATCCTGTGGAGCTC	AGCACTGTTGTGTGGCTGTTGAC	55
GDAP37	SSR	AGGTGCCCCAAGCCCAACGT	ACTGGTCTTACTATAGCCAGC	60
GDAP39	InDel	CCCTACAGCCGAGTCCATC	AGGCCAAAGGGGAAGGAGGG	55
GDAP41	SSR	CAATGACCTCCTCCCCACCT	CCACGTCAGCACCCTCATTC	60
GDAP47	SSR	CGAGCTCTGCCACGCAATGC	GTGGGAGCATGCGCGGAAGT	55
GDAP51	SSR	TGTAACTCCATACCACATCGGTG	GAAGCGTGAATAGGCTCTCC	60
GDCP1	CAPS	CAACCACCTACCATCCCATC	GTGACTGATCCTTCGGCATC	60
GDG4-3	CAPS	TCTCGGGTACAACCATGG	ATGAGCCTCCACCAACGA	60
LRR analysis				
FS1	N/A	GGAGAAGATCACTGTTGTAGCGGA	GGACATGCAATCGAATTGAGCCAC	60
FS2	N/A	CCTACTGAGGACACTCGGGTTG	CGTGATCACCTGCGACTGCGA	60
FS4	N/A	CTCAGATGGTATTGGAACAC	TAGGCAACCGAAGGTGACGA	60
FS7	N/A	TGATGGCTCCAATCTAGCACAGG	CCCTCAGCATCCACACGGAGAG	60

[a] RM markers taken from McCouch et al. (2002), AP22 from Wu et al. (2005). The remainder are de novo markers. GDCP1 and GDG4-3 are CAPS markers requiring post-PCR digestion with, respectively, *Eco*RI and *Hin*cII. The PCR comprised an initial denaturing step (94°C/4 min), followed by 35 cycles of 94°C/45 s, 55°C or 60°C/45 s, 72°C/60 s, and a final extension step (72°C/5 min)

The closest flanking markers GDAP51 and GDAP16 defined a 53-kb portion of cv. Nipponbare PAC clone P0649C11 (AP005659), which also contained *Pi2*/*Pi9* (Fig. 4).

LRR diversity within the Pi2/Pi9 gene family

The *Pi2*/*Pi9* locus in cv. Nipponbare genome contains seven NBS-LRR genes (Fig. 4), but NBS-LRR3, NBS-LRR5 and NBS-LRR6 are either non-functional or pseudogenes. We focused therefore on NBS-LRR1, NBS-LRR2, NBS-LRR4 and NBS-LRR7 in the subsequent comparison of LRR motif sequences among EBZ and seven cultivars carrying known *Pi2*/*Pi9* alleles. The NBS-LRR2 comparison was based on cv. 75-1-27 (*Pi9*), the NBS-LRR4 comparison on cvs. C101A51 (*Pi2*) and IRBLzt-T (*Piz-t*), while cvs. IRBLz-Fu (*Piz*), Gumei4 (*Pigm*), Gumei2 (*Pi26*) and IR65482-4-13 (*Pi40*) related to as yet undetermined NBS-LRRs. Amplicons of length ~1,000 bp (NBS-LRR1 and NBS-LRR7) and ~700 bp (NBS-LRR2 and NBS-LRR4) were used for sequence comparisons. The NBS-LRR1 and NBS-LRR7 sequences of the eight cultivars were rather well conserved, but both the NBS-LRR2 and NBS-LRR4 sequences were quite variable (Figs. 5 and S1). With respect to NBS-LRR2, EBZ differed from the other seven cultivars at between three and 21 residues, while a similar comparison focusing on NBS-LRR4 generated 13–41 polymorphisms (Fig. S1). We concluded on this basis that EBZ carries an as yet undocumented member of the *Pi2*/*Pi9* multigene family, conferring a broad-spectrum resistance to Chinese *M. oryzae* isolates. It has therefore been tentatively designated *Pi50*(t).

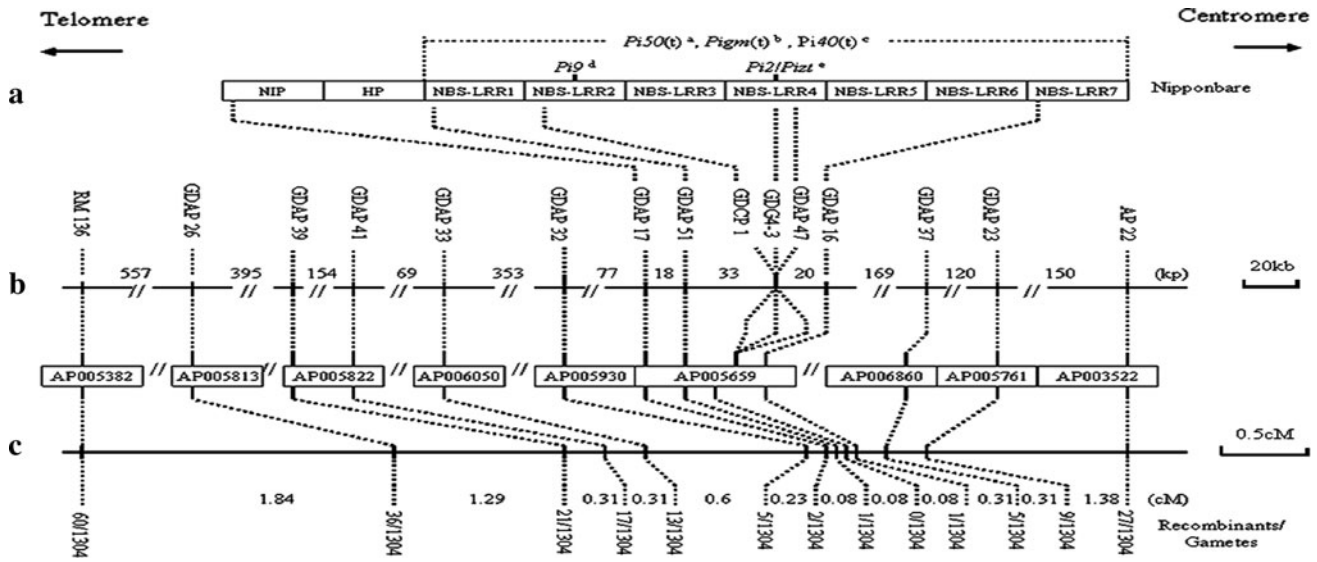

Fig. 4 An integrated map of the *Pi2*/*Pi9* NBS-LRR cluster on chromosome 6, including *Pi50*(t). **a** The cluster present in cv. Nipponbare. Positions of the various *R* genes and markers obtained from this study (*a*), Deng et al. (2006) (*b*), Jeung et al. (2007) (*c*), Qu et al. (2006) (*d*) and Zhou et al. (2006) (*e*). **b** A physical map of the *Pi50*(t) locus. The *numbers* above the map refer to kbp. The accession numbers in the *rectangles* below the map represent cv. Nipponbare BAC or PAC clones. **c** A genetic map of the *Pi50*(t) locus. The *numbers* between the *vertical* and *dashed lines* denote marker positions. The *numbers* underneath the *vertical lines* refer to the number of recombinants/gametes

Discussion

The deployment of broad-spectrum resistance is probably the most effective means available to the breeder for reducing the risk of resistance breakdown in the face of a pathogen as genetically flexible as *M. oryzae*. Here, we have detected the presence of three *R* genes in the broad-spectrum resistant cv EBZ. An analysis of the resistance mediated by the chromosome 6 *R* gene against a large collection of *M. oryzae* isolates has shown that this gene is largely responsible for the broad-spectrum resistance displayed by EBZ. Fine-scale mapping of the gene showed that it lies within the *Pi2/9* cluster, and its race specificity and LRR motifs indicate it as a new member of the *Pi2*/*Pi9* multigene family.

The *Pi2*/*Pi9* family consists of genes of the NBS-LRR type (Zhou et al. 2006). The function of the LRR motif is thought to be concerned with pathogen recognition (Ellis et al. 1999; Jia et al. 2000). The solvent-exposed positions appear to be particularly variable and subject to positive selection, reflecting their need to respond to the rapid evolution of pathogen ligands (Ellis et al. 2000; Dodds et al. 2001). The LRR region is known to be an important determinant of the race specificity of individual rice blast *R* genes (Zhou et al. 2006; Cao et al. 2007). Here, we obtained the LRR motif sequence of the four functional NBS-LRR genes at the *Pi2*/*Pi9* locus in a set of eight cvs carrying various members of the gene family and showed that, while two of them (NBS-LRR1 and NBS-LRR7) were highly conserved, both NBS-LRR2 (*Pi9*) and NBS-LRR4 (Pi2/*Piz-t*) were rather diverse. It is unclear why certain of the NBS-LRR paralogs at the *Pi2*/*Pi9* locus are so stable, while others are rapidly evolving, and why NBS-LRR2 and NBS-LRR4 appear to be the most important components of this gene family.

The genetic basis of broad-spectrum resistance is not clear-cut. In cvs. 5173 and 75-1-27, broad-spectrum resistance is thought to rely on the presence of a single *R* gene (respectively, *Pi2* and *Pi9*) (Chen et al. 1996; Liu et al. 2002). However, in both cv. Moroberekan (containing *Pi5*, *Pi7* and *Pi12*) and cv. SHZ-2 (*Pi-GD-1*, *Pi-GD-2* and *Pi-GD-3*), resistance is based on the presence of several *R* genes, each showing a limited resistance spectrum (Wang et al. 1994; Liu et al. 2004). A third possibility has been documented in cv. Tetep, in which broad-spectrum resistance is delivered by the presence of a single broad-spectrum *R* gene (*Pi1*) in combination with certain limited spectrum *R* genes (*Pi4* and *Pi5*) (Inukai et al. 1994; Yi et al. 2004). The EBZ resistance appears to be similar to that in Tetep, involving a broad-spectrum *R* gene (*Pi50*(t)) plus *Pish* and *Pita-2*.

EBZ has been extensively exploited as a source of blast resistance in a number of rice breeding programs in South China (Zhu et al. 2003). Now that the major component of its resistance has been defined and genetically tagged, it should be rather straightforward to move this apparently durable source of blast resistance into elite cvs. A research priority for the near future will be to determine which of

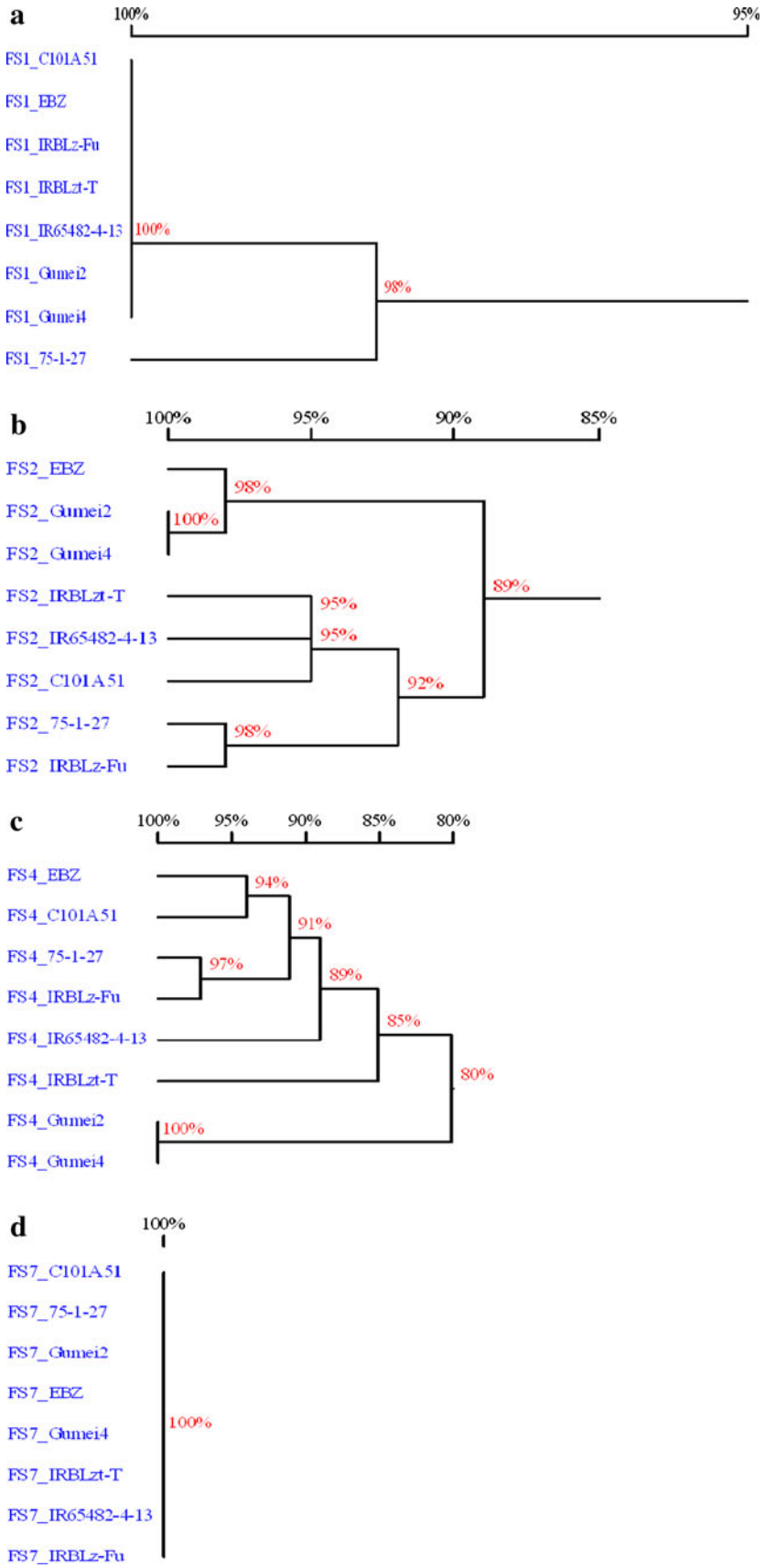

Fig. 5 Cladistic analysis of the nucleotide sequence of the LRR motifs present in the blast *R* genes *Pi2* (C101A51), *Pi9* (75-1-27), *Piz* (IRBLz-Fu), *Piz-t* (IRBLzt-T), *Pi26*(t) (Gumei2), *Pigm*(t) (Gumei4), *Pi40*(t) (IR65482-4-13) and *Pi50*(t) (EBZ). **a**, **b**, **c**, **d** NBS-LRR1, NBS-LRR2 (*Pi9* ortholog), NBS-LRR4 (*Pi2* ortholog) and NBS-LRR7, respectively

the NBS-LRR2 and/or NBS-LRR4 are required for the expression of broad-spectrum resistance. This effort will require a combination of forward and reverse genetic approaches (Yuan et al. 2011; Zhai et al. 2011), and should provide useful insights into the molecular basis of broad-spectrum disease resistance.

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