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Identification and fine mapping of *Pi33*, the rice resistance gene corresponding to the Magnaporthe grisea avirulence gene *ACE1*

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Abstract Rice blast disease is a major constraint for rice breeding. Nevertheless, the genetic basis of resistance remains poorly understood for most rice varieties, and new resistance genes remain to be identified. We identified the resistance gene corresponding to the cloned avirulence gene *ACE1* using pairs of isogenic strains of *Magnaporthe grisea* differing only by their *ACE1* allele. This resistance gene was mapped on the short arm of rice chromosome 8 using progenies from the crosses IR64 (resistant) × Azucena (susceptible) and Azucena × Bala (resistant). The isogenic strains also permitted the detection of this resistance gene in several rice varieties, including the differential isogenic line C101LAC. Allelism tests permitted us to distinguish this gene from two other resistance genes [*Pi11* and *Pi-29*(t)] that are present on the short arm of chromosome 8. Segregation analysis in F_2 populations was in agreement with the existence of a single dominant gene, designated as *Pi33*. Finally, *Pi33* was finely mapped between two molecular markers of the rice genetic map that are separated by a distance of 1.6 cM. Detection of *Pi33* in different semi-dwarf indica varieties indicated that this gene could originate from either one or a few varieties.

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Introduction

The rice (*Oryza sativa* L.) – *Magnaporthe grisea* (Hebert) Barr pathosystem is a nice model system to study plant-fungus interactions. Rice blast disease is also a primary concern for rice production worldwide. In this pathosystem, race specific resistance follows the gene-for-gene relationship (Kiyosawa 1971; Silué et al. 1992). Since the seminal works of Sasaki (1922) and Nakatomi (1926) (in Takahashi 1965), performed in order to improve resistance to blast of Japanese rice cultivars, several rice blast resistance genes have been discovered in different rice cultivars, and were later mapped.

In the early sixties, Goto et al. and Yamasaki and Kiyosawa identified the first set of resistance genes *Pia, Pii, Pik, Pik^s*, *Pik^h*, *Pik^m*, *Pik^p*, *Piz*, *Piz^t*, *Pita*, *Pita^2*, *Pib*, *Pit* and *Pish* (Ezuka 1979; Imbe and Matsumoto 1985). Later, Kiyosawa et al. (1986) elaborated a set of differential cultivars, each of them harboring one or two of these resistance genes. Additional resistance genes were identified in *indica* cultivars. This work was started by the introgession of resistance genes from four cultivars (LAC23, 5173, Pai-Kan-Tao and Tetep) into the susceptible cultivar CO39 that led to near-isogenic lines (NILs) harboring one or two resistance gene(s) each. These NILs allowed for the discovery of new resistance genes, namely *Pi1*, *Pi2* (=*Piz^5*), *Pi3* and *Pi4^b* (Yu et al. 1991a, b; Mackill and Bonman 1992; Inukai et al. 1994).

Since McCouch et al. (1988) published the first rice genetic map using RFLP markers, the number of available genetic markers has increased dramatically. Fixed populations useful for genetic mapping were obtained [doubled-haploid (DH) population: Guiderdoni et al. 1992; Zhu et al. 1993; Huang et al. 1997; single-seed descent (SSD) populations: Wang et al. 1994; Price et al. 2000]. These populations were useful for mapping a significant number of rice blast resistance genes (for an updated

synthetic map of resistance genes to blast, see Sallaud et al. 2003). The distribution of rice blast resistance genes throughout the genome is not random. Chromosomes 6, 11 and 12 harbor more than nine resistance genes each, while chromosomes 3 and 9 have none at all. But it is difficult to assess the actual number of resistance genes that are already characterized and mapped, as allelism tests were not always performed for genes mapping in the same area of the genome.

Only two resistance genes have been mapped on chromosome 8: *Pi11* (=*Pizh*) and *Pi29*(t). *Pi11* mapped 15.8 cM from the RFLP marker RZ617 using DH lines from the cross JX17 × ZYQ8 (Zhu et al. 1993). This gene confers resistance to the *M. grisea* strain Zhong 10-8-14, and comes from Zhai-Ye-Qing 8 (ZYQ8). *Pi29*(t) is a resistance gene that comes from the improved *indica* rice cultivar IR64. This gene, together with another IR64 blast resistance gene [*Pi24*(t)], confers resistance to the Colombian *M. grisea* strain CL26 (Sallaud et al. 2003). *Pi29*(t) was mapped near the RFLP marker RZ617 in progeny of 104 IR64 × Azucena DH lines. Because *Pi11* and *Pi29*(t) map in the same area of chromosome 8, it is unclear whether or not they are different genes (Sallaud et al. 2003).

Despite the large number of resistance genes (*R* genes) that have been identified and mapped, and despite the fact the rice blast is a model pathosystem, only two blast resistance genes have already been cloned and characterized: *Pita* (Bryan et al. 2000; Jia et al. 2000) and *Pib* (Wang et al. 1999). More generally, while a significant number of plant resistance genes against bacterial or fungal pathogens have been characterized (for a review see Dangl and Jones 2001; Hulbert et al. 2001), few *R* gene–*avr* gene pairs have been cloned for plant-fungal interaction. The characteristics of the cloned avirulence genes suggest that they encode small proteins secreted during infection and that these are directly recognized by the corresponding resistance gene product. One exception to this model comes from the *M. grisea* avirulence gene *ACE1* (Dioh et al. 2000; Böhnert et al. 2001). This gene encodes a large cytoplasmic enzyme that is unlikely to be recognized by the corresponding *R* gene product. Molecular analysis of this interaction requires the isolation of the *R* gene corresponding to *ACE1*.

We report here the genetic characterization of the resistance gene locus that corresponds to *ACE1*. Several resistant rice cultivars were screened for the presence of this *R* gene. Two of these cultivars had been crossed with a susceptible rice cultivar. Doubled-haploids (DH) or single-seed-descent (SSD) progenies from these crosses were used for the construction of rice genetic maps (IR64 × Azucena and Bala × Azucena). Results from allelism tests and F_2 progeny defined a new resistance gene to blast, *Pi33*, located on the short arm of rice chromosome 8.

Materials and methods

M. grisea strains and culture

Cosmid D31C12 from avirulent isolate Guy11 harbours the avirulence gene *ACE1* (Böhnert et al., unpublished data). This cosmid was introduced by transformation of fungal protoplasts from the virulent strains PH14, PH19 and 2/0/3. Three avirulent isogenic strains were obtained from these experiments: PH14-D31C12, PH19-D31C12 and 2/0/3-D31C12 (Dioh et al., unpublished data). PH14 (=PO6-6) and PH19 (=IK81-25) are from The Philippines and were kindly provided by the International Rice Research Institute (IRRI). 2/0/3 is a laboratory strain obtained by crossing different isolates (Silué et al. 1992). PO6-6 and IK81-25, were used for the characterization of resistance genes *Pi1*(t), *Pi2*(t), *Pi3*(t) and *Pi4*^{b}(t) (Mackill and Bonman 1992). CL6 is a Colombian strain of *M. grisea* that was used for characterization of the resistance genes *Pi24*(t)and *Pi29*(t) (Sallaud et al. 2003).

M. grisea strains were grown for 7 days on rice flour agar medium (20 g of rice flour, 15 g of agar, 2.5 g of yeast extract and 1 l of distilled water) under fluorescent light (12 h a day) at 26 °C. Conidia were harvested by flooding the plate with 10 ml of sterile distilled water.

Rice cultivars and mapping populations

The following rice cultivars were used for the characterization of the resistance gene corresponding to the *ACE1* avirulence gene: Azucena, Bala, BW100, Carreon, CO39, DJ8-341, Hing-Xi 17 (JX17), IR8, IR64, IR1529, IRAT7, Kassalath, LAC23, Maratelli, Nipponbare, Taichung-Native 1 (TN1), Tetep, Tsai-Yuan-Chung (TYC) and Zhai-Ye-Qing 8 (ZYQ8). A set of isogenic rice lines obtained by introgression of different blast resistance genes into the CO39 susceptible rice cultivar (Mackill and Bonman 1992), and a set of differential cultivars established by Kiyosawa (Ezuka 1979; Kiyosawa 1984; Kiyosawa et al. 1986), were also tested for the presence of the resistance gene corresponding to the *ACE1* avirulence gene. Accession numbers in the International Rice Germplasm Center (IRGC numbers) of these rice lines and cultivars are shown in Table 1.

Progeny from the cross between an improved semi-dwarf *indica* cultivar (IR64) developed by IRRI and an upland *japonica* cultivar from The Philippines (Azucena) was used to map the resistance gene corresponding to *ACE1*. We performed this mapping with a first set of 105 DH lines from this cross (Guiderdoni et al. 1992) previously used to map 200 molecular markers (Causse et al. 1994; Huang et al. 1997; Sallaud et al. 2003). An additional set of 501 DH lines and 284 F_7 SSD lines from the F_1 progeny of the cross IR64 × Azucena were obtained from The European comparative gramineae mapping programme (EGRAM) (Filloux et al. 2000). A sample of the F_1 seeds used for the development of DH lines from the cross IR64 × Azucena were kindly provided by the germplasm bank of CIRAD-CA. F_2 seeds were harvested on these plants.

Ninety five F_6 SSD progeny from the cross between Bala (*indica*) and Azucena (Price et al. 2000) were kindly provided by Dr. Adam Price. This population was derived from a F_2 Azucena × Bala progeny obtained by Price and Thomos (1997) and was used to map 101 RFLP and 34 AFLP markers (Price et al. 2000). For allelism tests, 52 DH progeny from the cross ZYQ8 × JX17 used for genetic mapping of the blast resistance gene *Pi11* (also named *Pizh*, Zhu et al. 1993) were kindly provided by Prof. Lihuang Zhu. ZYQ8 is an *indica* cultivar and JX 17 is a *japonica* cultivar.

Rice cultivation and M. grisea inoculation

Rice plants were grown in greenhouse conditions with temperatures between 20 and 30 °C and with supplemental light supplied in winter. Fourteen lines of 10 to 15 seeds were sown in trays of 40 × 29 × 7 cm filled with compost (7/8 Neuhaus compost no. 9, 1/8 pozzolana). Ten to 15 seeds of each cultivar or DH line were sown

in rows in trays containing 14 lines each. Soil was kept moist with water and, once a week, with nutritive solution [1.5 g/l of NPK 17/7/22 fertilizer, 0.25 g/l of Quelado ADDHA Fe (6%), 0.25 g/l of Hortrilon]. Nitrogen fertilization with 8.6 g of nitrogen equivalent per tray was done at 10, 3 and 1 day(s) before inoculation to increase susceptibility to blast.

Inoculations were performed 3 weeks after sowing (4–5 leaf stage) either by injection or by spraying conidial suspensions. Thirty milliliters of a 50,000 conidia per ml suspension (with 0.5% gelatin) were sprayed on each tray. Subsequently, rice plants were incubated for 16 h in a controlled climatic chamber at 24 °C with 95% relative humidity. They were then transferred back to the greenhouse. For the injection method, plants were inoculated by syringe injection of 0.1 ml of a 25,000 conidia per ml suspension into leaf sheaths. For each cultivar, the cross parent, the DH or SSD line, 10 to 15 plants were grown and inoculated at least twice. After 7 days, lesion types on rice leaves were observed and scored 1 (no symptoms) to 6 (typical susceptible lesions) according to a standard reference scale (Silué et al. 1992). Individuals with scores between 1 and 3 were considered to be resistant and individuals with scores from 4 to 6 were considered to be susceptible.

Simple sequence repeat (SSR) amplification

The four mapped SSR markers RM44, RM72, RM404 and RM483 were amplified following published protocols (Wu and Tanksley 1993; Temnykh et al. 2000). Genomic DNA from IR64, Azucena and their progeny were extracted using CTAB (Murray and Thompson 1980) and employed as a template for PCR reactions with oligonucleotide pairs RM44U: CGGGCAATCCGAACAACC, RM44L: TCGGGAAAACCTACCCTACC (RM44, Chen et al. 1997), RM72U: CCGGCGATAAAACAATGAG, RM72L: GCATCGGTCCTAACTAAGGG (RM72, Temnykh et al. 2000), RM404U: CCAATCATTAACCCCTGAGC, RM404L: GCCTTCATGCTTCAGAAGAC (RM404, Temnykh et al. 2001), RM483U: CTTCCACCATAAAACCGGAG, RM483L: ACACCGGTGATCTTGTAGCC (RM483, Temnykh et al. 2001). PCR was performed using the protocol of Chen et al. (1997). PCR products were separated by 4% agarose-gel electrophoresis and stained with ethidium bromide, or by 5% polyacrylamide-gel electrophoresis using 7.5 M urea as a denaturant and radiolabelled oligonucleotides for autoradiographic detection.

RFLP, YAC probes and Southern analysis

Using the sequences of the RFLP probes mapped by Kurata et al. (1994), we designed the following oligonucleotides for PCR amplification of these sequences from rice genomic DNA: C483U: CTTCCACCATAAAACCGGAG, C483L: ACACCGGTGATCTTGTAGCC, [C483, annealing temperature (Ta) = 50 °C]; G1010U: CCAAGTATTCTAGCTCGCTGTC, G1010L: TGCTAGAGATTTGAGAAGATGG, (G1010, Ta = 50 °C); R1813U: TACAATGAGCCTGAGCAGA, R1813L: AACTGGGTGAAGACGGCAA (R1813, Ta = 55 °C); S1633U: TCGCCGCACTTTCTCCA, S1633L: CCGACCCTCTCGCGTACTT (S1633, Ta = 50 °C). PCR products were separated by gel electrophoresis on 2% low-melting agarose. DNA fragments were purified from the gel and used as templates for the synthesis of radiolabelled probes using the Megaprime kit from Amersham Life Science (Saclay, France). Probes from Yeast Artificial Chromosome ends (YAC end probes) were obtained following the protocol of Emmanuelle Bourgeois (personal communication) derived from the walking PCR protocol (Devic et al. 1997). Briefly, YAC DNA was digested and then ligated to an adapter. A nested PCR protocol was then performed to specifically amplify a fragment including a part of the vector and the end of the cloned insert. These probes were obtained using clones Y3140 and Y2643 of the YAC library constructed from Nipponbare (Umehara et al. 1995). Southern transfers and hybridizations were performed according to Hoisington et al. (1994).

Genetic mapping

The resistance gene corresponding to *ACE1*, the four SSR markers RM44, RM72, RM404 and RM483, and the four RFLP markers R1813, G1010, C483 and S1633, were mapped using 105 DH lines from the cross IR64 × Azucena and added to the existing map (Causse et al. 1994). On each side of *Pi33*, the closest SSR marker was chosen. These two closest flanking markers were mapped using an additional set of 501 DH lines and 284 SSD lines from the cross IR64 × Azucena (Filloux et al. 2000). The rice lines that corresponded to recombination events between these two SSR markers, were used to map G1010, R1813, C483 and S1633B. YAC end probes obtained from YAC clones Y2643 and Y3140 were checked for copy number and diversity using IR64 and Azucena DNA digested with several restriction enzymes. Single-copy YAC end probes showing polymorphism between IR64 and Azucena were finely mapped as described above for other RFLP probes.

Results

Identification of rice cultivars resistant to *M. grisea* isolates carrying the avirulence gene *ACE1*

Rice cultivars' resistance to *M. grisea* isolates carrying the avirulence gene *ACE1* was assessed using isogenic *M. grisea* strains that only differ by their *ACE1* allele. Cultivars susceptible to PH14, PH19 or 2/0/3 *M. grisea* isolates (virulent for *ACE1*), were inoculated with isogenic PH14, PH19 or 2/0/3 strains carrying the *ACE1* avirulence allele (PH14-D31C12, PH19-D31C12 or 2/0/3-D31C12). If a cultivar was only resistant to the isogenic avirulent strain, we concluded that it carries the resistance gene corresponding to *ACE1*. The rice cultivars DJ8-341 and IRAT7, that are known to carry the resistance gene corresponding to *ACE1* (Dioh et al. 2000), were used as resistant controls. The rice cultivar Maratelli was employed as a susceptible control, because it does not carry known blast resistance genes (Silué et al. 1992). The rice cultivars Bala, BW100, Carreon, IR64, IR1529, TN1, TYC and ZYQ8 were resistant to at least one isogenic avirulent *M. grisea* strain, while being susceptible to the corresponding virulent isogenic *M. grisea* strain (Table 1), demonstrating that these cultivars carry the *R* gene corresponding to *ACE1*.

To determine whether the resistance gene corresponding to *ACE1* was an already known gene, a set of differential rice cultivars established by Kiyosawa (Ezuka 1979; Kiyosawa 1984; Kiyosawa et al. 1986) were inoculated with the *M. grisea* isogenic strains 2/0/3 (virulent) and 2/0/3-D31C12 (avirulent for *ACE1*). All except K1 showed susceptibility to both strains (Table 1). These results indicate that the resistance gene corresponding to *ACE1* is not any of the following genes: *Pia, Pif, Pik, Pik^m*, *Pik^p*, *Pik^s*, *Pis^h*, *Pit, Pita^2*, *Piz* and *Piz^t*. Since K1 was resistant to both strains, no conclusion could be drawn concerning *Pita*. As the *R* gene corresponding to *ACE1* was shown to be absent from the susceptible rice cultivar CO39, we tested the CO39-derived isogenic rice lines that carry different known resistance genes (*Pi1, Pi2, Pi3, Pita* and *Pi4^b*: Mackill and Bonman 1992). All but one of these isogenic

Table 1 Presence or absence of a specific resistance gene recognizing the *ACE1* avirulence gene in several rice lines and cultivars)

Rice lines and cultivars		Subspecies	IRGC number	R genes harbored	Reaction to inoculation with							Specific resistance to *ACE-1*
					Guy11	PH14	PH14-D31C12	PH19	PH19-D31C12	2/0/3	2/0/3-D31C12	
Parents of crosses used for mapping	IR64	*indica*	66,970	*Pi24* to *Pi32*	1[a]	6	*3*	5	*1*	1	1	Yes
	Azucena	*japonica*	328	–	6	6	6	6	6	6	6	No
	Bala	*indica*	12,884	–	2	6	*2*	6	*3*	5	*2*	Yes
	ZYQ8	*indica*	–	*Pi11*	1	6	*3*	5	*1*	3	2	Yes
	JX17	*japonica*	–	–	–	2	2	–	–	6	6	No
	Nipponbare[b]	*japonica*	–	–	5	5	5	–	–	5	5	No
	Kassalath[b]	*indica*	–	–	4	–	–	–	–	6	6	No
	Maratelli[c]	*japonica*	3,091	–	6	6	6	6	6	6	6	No
	IRAT7[d]	*indica*	–	–	2	4	*1*	3	1	5	*2*	Yes
	DJ8-341[d]	*indica*	–	–	2	–	–	1	1	5	*1*	Yes
	BW100	–	50,657	–	2	–	–	6	*3*	5	*2*	Yes
	Carreon	*indica*	5,993	–	2	–	–	2	1	4	*2*	Yes
	IR1529	*indica*	–	–	2	6	*2*	6	*3*	5	*2*	Yes
	IR8	*indica*	66,395	–	4	6	5	–	–	4	4	No
	DGWG	*indica*	123	–	–	6	6	–	–	5	5	No
	TN1	*indica*	105	–	–	6	*3*	–	–	6	*3*	Yes
	Tetep	*indica*	11,115	–	6	1	1	3	1	6	5	No
	TYC	*indica*	–	–	–	6	*3*	–	–	6	*2*	Yes
	LAC23	*japonica*	14,957	–	–	6	5	3	3	6	5	No
CO39 isogenic lines (Mackill and Bonman 1992)	CO39	*indica*	51,231	–	5	–	–	6	5	5	5	No
	C101LAC	–	–	*Pi1*	2	2	2	6	*3*	5	*2*	Yes
	C104LAC	–	–	*Pi1*	5	–	–	6	5	5	5	No
	C103TTP	–	–	*Pi1*	5	–	–	6	5	5	5	No
	C101A51	–	–	*Pi2*	5	–	–	3	3	6	6	No
	C102A51	–	–	*Pi2*	5	–	–	–	–	5	6	No
	C104PKT	–	–	*Pi3*	4	–	–	–	–	6	6	No
	C101PKT	–	–	*Pita*	4	–	–	–	–	6	6	No
	C101TTP-3	–	–	*Pita*	5	–	–	–	–	6	6	No
	C105TTP-1	–	–	*Pita*	5	–	–	–	–	5	5	No
	C105TTP-2 (L-9)	–	–	*Pita*	5	–	–	–	–	6	6	No
	C105TTP-2 (L23)	–	–	*Pita*	5	–	–	–	–	5	6	No
	C105TTP-4 (L23)	–	–	*Pita* + *Pi4*[b]	3	–	–	–	–	3	3	–
Japanese differential cultivars (Kiyosawa 1984; Kiyosawa et al. 1986) (Ezuka 1979)	Fukunishiki	*japonica*	40,257	*Piz* + *Pish*	5	–	–	–	–	4	4	No
	K1	*japonica*	40,254	*Pita*	3	–	–	–	–	3	3	–
	K59	*japonica*	40,261	*Pit*	6	–	–	–	–	4	3	No
	K60	*japonica*	36,112	Pik^p	6	–	–	–	–	5	5	No
	Kanto51	*japonica*	484	*Pik*	6	–	–	–	–	5	6	No
	Pi-n°4	*japonica*	6,733	$Pita^2$	6	–	–	–	–	5	4	No
	Shin 2	*japonica*	7,661	Pik^s + *Pish*	6	–	–	–	–	5	5	No
	Toride 1	*japonica*	40,260	Piz^t	6	–	–	–	–	5	5	No
	Tsuyuake	*japonica*	40,262	Pik^m	6	–	–	–	–	5	5	No
	Zenith	*japonica*	–	*Piz* + *Pia*	6	–	–	–	–	5	4	No
	Norin 22	*japonica*	10,905	*Pish*	4	–	–	–	–	4	4	No
	Reïho	*japonica*	40,025	*Pia* + $Pita^2$	6	–	–	–	–	5	6	No
	St1	*japonica*	30,329	*Pif*	5	–	–	–	–	5	5	No

[a] Disease score. Scoring was performed according to a standard reference scale (Silué et al. 1992). Cultivars with scores 1 to 3, and 4 to 6, were considered to be resistant and susceptible respectively [b] Parent of cross used by Kurata et al. (1994) [c] Susceptible control [d] Resistant cultivars used for the identification and mapping of *ACE1* (Dioh et al. 2000; Böhnert et al. 2001)

differential rice lines were susceptible to the *ACE1* avirulent *M. grisea* strains, suggesting that they do not carry the *R* gene corresponding to *ACE1* (Table 1). The isogenic differential rice line C101LAC, carrying the resistance gene *Pi1* (Mackill and Bonman 1992), was resistant to the *ACE1* avirulent *M. grisea* strains PH19-D31C12 and 2/0/3-D31C12, while being susceptible to the PH19 and 2/0/3 virulent isolates. These results indicate that C101LAC carries the *R* gene corresponding to *ACE1* and that it could be *Pi1*. However, the isogenic differential rice lines C104LAC and C103TTP, which also carry *Pi1*, were susceptible to the *ACE1* avirulent *M. grisea* strains. Since the differential rice lines that carry *Pi1* react differently to the *ACE1* avirulent strains, we considered that *Pi1* is not the *R* gene corresponding to *ACE1* and that C101LAC must carry at least two resistance genes, *Pi1* and the *R* gene corresponding to *ACE1*.

Surprisingly, the two parents of C101LAC, CO39 and LAC 23, were susceptible to the *ACE1* avirulent *M. grisea* strains, indicating that they do not carry the resistance gene corresponding to *ACE1*. To explain this unexpected result, we hypothesized that the LAC23 seed stock used in this study could be different from the stock used as the resistant parent for C101LAC. LAC23 was phenotypically characterized as conferring resistance to a set of *M. grisea* strains including PO6-6 (=PH14) (Mackill and Bonman 1992). In this study, LAC23 was susceptible to PH14. This result confirms the hypothesis of non-conformity of LAC23 seed lots. No other seed lot was tested since the lot tested in this study was received directly from the institute that developed C101LAC (Genetic Resources Center, International Rice Research Institute) and, thus, was likely to be the best source of LAC23.

Genetic mapping of the *R* gene corresponding to *ACE1*

IR64 × Azucena cross

The rice cultivar Azucena is susceptible to the *ACE1* avirulent *M. grisea*-strain PH14-D31C12, demonstrating that it does not carry the *R* gene corresponding to *ACE1* (Table 1). Since this *R* gene was detected in the rice cultivars IR64 and Bala (Table 1), we used progenies from crosses involving Azucena as the susceptible parent and IR64 or Bala as the resistant parent, to map the *R* gene corresponding to *ACE1*. The 105 DH progeny lines from the cross IR64 × Azucena were inoculated with the virulent strain PH14 and its *ACE1* avirulent isogenic-strain PH14-D31C12. We observed a 1:1 segregation for resistance:susceptibility to PH14-D31C12 (avirulent), whereas all DH lines were susceptible to PH14 (virulent). These results demonstrate that the resistance of IR64 corresponding to the avirulence gene *ACE1* is controlled by a single gene.

We mapped the three molecular markers RM72 (SSR marker), R1813 and G1010 (RFLP markers) in the IR64 × Azucena cross, and showed that they co-segregated and were closely linked (2 ± 1 cM) to the resistance-gene locus. The two molecular markers G104 (RFLP marker) and RM44 (SSR marker) were located on the opposite side of the resistance gene locus (at 4.7 ± 1 and 9.3 ± 1 cM, respectively). Consequently, the resistance gene corresponding to *ACE1* must be on the short arm of rice Chromosome 8 (Fig. 1), where all these SSR and RFLP markers are located (Kurata et al. 1994; Temnykh et al. 2001; Sallaud et al. 2003).

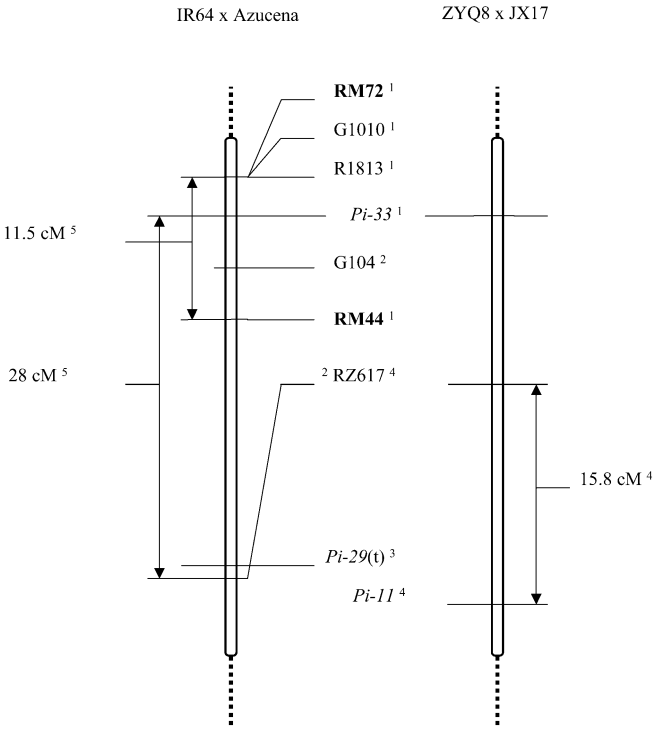

Fig. 1 Mapping of the resistance gene corresponding to the *ACE1 M. grisea* avirulence gene in the IR64 × Azucena and ZYQ8 × JX17 crosses: [1]Mapped during this study, [2]Mapped during the EGRAM project (Filloux et al. 2000). For a complete map and details, see Sallaud et al. 2003, [3]Mapped by Sallaud et al. 2003, [4]Mapped by Zhu et al. (1993) [5]Distance calculated in this study

Azucena × Bala cross

To confirm the position of the resistance gene corresponding to *ACE1*, 95 F_6 SSD lines from the cross Azucena × Bala (Price et al. 2000) were inoculated with the *ACE1* virulent/avirulent isogenic strains 2/0/3 and 2/0/3-D31C12. All the SSD progeny were susceptible to 2/0/3, whereas a 1:1 (resistant:susceptible) segregation was observed with 2/0/3-D31C12, demonstrating the segregation of a single resistance gene from Bala. This gene mapped on chromosome 8 at 3.6 ± 1.8 cM from the RFLP marker G1010, confirming the position determined with the IR64 × Azucena cross.

Characterization of *Pi-33*, a new blast resistance gene

Two known rice blast resistance genes are located on chromosome 8. *Pi11* from the ZYQ8 *indica* cultivar, mapped at 14.9 cM from RFLP marker BP127 (Zhu et al. 1993), and *Pi29*(t) from the IR64 *indica* cultivar was mapped close to RZ617 (Sallaud et al. 2003). Since we showed that these two rice cultivars carry the *R* gene corresponding to *ACE1* (Table 1), we performed allelism tests between *Pi11*, *Pi29*(t) and the *R* gene corresponding to *ACE1*. For *Pi11*, parents and 52 DH progeny from the cross ZYQ8 × JX17 (Zhu et al. 1993), were inoculated with *ACE1* virulent/avirulent isogenic strains PH14/PH14-D31C12. ZYQ8 was susceptible to the virulent strain PH14 (Table 1), but JX 17 and half of the 52 DH lines were resistant to PH14, demonstrating that JX17 carries another resistance gene that recognizes an avirulence factor from the PH14 strain that differs from *ACE1*. Among the 23 DH lines from this cross that were susceptible to PH14, and that showed a clear phenotype for *Pi11*, five DH lines had recombinant phenotypes with regards to the resistance conferred by *Pi11* and by the *R* gene corresponding to *ACE1*. Two DH lines were susceptible to the *ACE1* avirulent strain PH14-D31C12, while carrying a *Pi11* resistant allele, and three were resistant to the *ACE1* avirulent strain PH14-D31C12, while carrying a *Pi11* susceptible allele. Therefore, these two *R* genes are different and separated by 22 ± 4 cM (Fig. 1).

The resistance gene *Pi29*(t) located on chromosome 8 was mapped using *M. grisea* isolate CL6, and 104 of the 105 DH progeny lines were also used to map the resistance gene corresponding to *ACE1* (Sallaud et al. 2003). Resistance of IR64 to CL6 is controlled by two genes, *Pi29*(t) located on chromosome 8 and *Pi24*(t) located on chromosome 1 (Sallaud et al. 2003). Therefore, DH lines resistant to CL6, can carry either *Pi29*(t), *Pi24*(t) or both *R* genes. Among the 20 DH lines that were susceptible to CL6, four were susceptible to the virulent isolate PH14 and resistant to the *ACE1* avirulent isogenic strain PH14-D31C12. The existence of recombinant lines demonstrates that *Pi29*(t) differs from the resistance gene corresponding to *ACE1*. The distance between the two genes was evaluated to be 20 cM (Fig. 1). This result fits with the work of Sallaud et al. (2003), who mapped *Pi29*(t) close to the RFLP marker RZ617. In this study, RZ617 was located 28 cM from the *R* gene corresponding to *ACE1*.

On the basis of the differential reactions of resistant cultivars to *M. grisea* isogenic strains differing only by their *ACE1* allele and of the genetic characterization and mapping of the *R* gene corresponding to *ACE1*, we conclude that we have identified a new resistance gene we named *Pi33*, according to the international rules in use for blast resistance genes (Kinoshita 1998).

To determine whether *Pi33* is a recessive or dominant gene, 207 F_2 individuals from the IR64 × Azucena cross were inoculated with the *ACE1* avirulent isogenic strain PH14-D31C12. One hundred and sixty two plants showed resistance and 45 susceptibility to PH14-D31C12 (3R:1S hypothesis: $\chi^2 = 1.17$, $P = 0.28$). All the 39 IR64 plants showed resistance symptoms, while 26 of 28 (93%) Azucena plants showed susceptibility. The two Azucena plants that did not show susceptibility symptoms were considered to have escaped inoculation. F_2 data were then corrected using the fact that 7% of the susceptible Azucena plants did not show susceptible symptoms. The data obtained after correction were 159 resistant and 48 susceptible plants (3R:1S hypothesis: $\chi^2 = 0.32$, $P = 0.57$). Thus, *Pi33* was considered to be dominant.

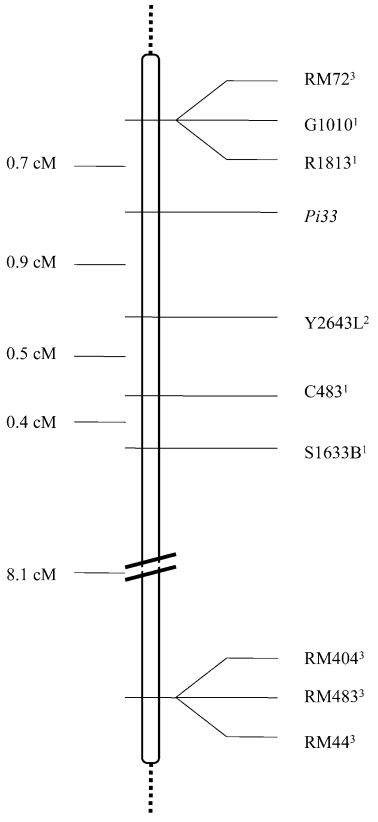

Fig. 2 Fine mapping of *Pi33* using a population of recombinant lines from the IR64 × Azucena cross. All markers were mapped in this study: [1]RFLP markers from Kurata et al. (1994), [2]YAC end marker elaborated in this study using YAC clones from Umehara et al. (1995), [3]SSR markers from Temnykh et al. (2000)

Fine mapping of *Pi33*

Eight-hundred and fifty one IR64 × Azucena DH lines were characterized using the RM44 and RM72 SSR markers located on both sides of the *Pi33* locus. These two markers are at a distance of 10.6 ± 0.1 cM from one another. Additional molecular markers were mapped between RM72 and RM44 using 51 DH and 22 SSD lines that had a recombinant genotype for these two markers. The four RFLP markers G1010, R1813, C483 and S1633B (Kurata et al. 1994) were located between RM44 and RM72 (Fig. 2). *Pi33* was mapped between the

two markers RM72 and C483 that are at a distance of 2.1 ± 0.2 cM from each other. Saji et al. (2001) showed that the Nipponbare YAC clones Y2643 and Y3140 (Umehara et al. 1995) hybridized with markers R1813 and C483, respectively. One of the four YAC end probes, Y2643L, was shown to be a monolocus probe polymorphic between IR64 and Azucena. Y2643L was mapped between *Pi33* and C483, at a distance of 0.5 ± 0.2 cM from C483 and 0.9 ± 0.2 cM from *Pi33* (Fig. 2). Thus, *Pi33* is located in a 1.6 ± 0.2 cM interval between RM72 and Y2643L (Fig. 2).

Discussion

Pi33, a new rice blast resistance gene interacting with *M. grisea* avirulence gene *ACE1*

In this study, we identified and mapped a new rice blast resistance gene, *Pi33*, on the short arm of chromosome 8. Differentiation of a new resistance gene from known *R* genes is difficult due to the uncharacterized avirulence genes in any given rice blast isolate used to identify the *R* gene, and because rice cultivars often carry several resistance genes that provide overlapped resistance phenotypes. Pairs of isogenic *M. grisea* strains that only differ by their allele of the *ACE1* avirulence gene were used to unambiguously distinguish *Pi33* from the other *R* genes. We cannot exclude that *ACE1* could be involved in the recognition of different resistance genes but, to-date, such interactions were seldom reported (Dixon et al. 1998; Van der Hoorn et al. 2001). In these few examples the resistance genes were found at the same locus and shared high-sequence homology. To our knowledge, this is the first report of the use of a cloned fungal avirulence gene to predict the corresponding *R* gene.

We analyzed resistant cultivars for their differential reaction to *M. grisea* isogenic strains differing only by their *ACE1* allele; mapped the *Pi33* gene on the rice genetic map and performed allelism tests with known resistance genes mapping to the same chromosome. These experiments demonstrated that *Pi33* is different from all other known rice blast resistance genes.

Putative origin of *Pi33*

Pi33 was detected in several semi-dwarf *indica* rice cultivars such as Bala, DJ8-341, IR64, IR1529, IRAT7, Taichung Native 1 (TN1) and Zhai-Ye-Qing 8 (ZYQ8).

Two different hypotheses can be proposed for the wide distribution of *Pi33*. First, *Pi33* originates from a common ancestor of these cultivars; and second, *Pi33* is present in several traditional cultivars and has been independently selected from different sources.

In the first hypothesis, the only common parent of most of these cultivars (Bala, BW100, DJ8-341, IR64 and IRAT7) is TN1 (IRRI 2002). This cultivar harbors *Pi33* and was derived from the cross between Tsai-Yuan-Chung (TYC) and Dee-Geo-Woo-Gen (DGWG). DGWG is the spontaneous mutant used for the introgression of the semi-dwarfism gene (*sd1*) into the green revolution *indica* cultivars (Monna et al. 2002). DGWG did not carry *Pi33* whereas TYC did. TN1 was used as a secondary source for semi-dwarfism in many crosses and, thus, is likely to have transmitted *Pi33* in semi-dwarf cultivars.

In the second hypothesis, *Pi33* was introgressed into the semi-dwarf indica cultivars from different sources. This hypothesis is supported by the fact that Carreon (a traditional *indica* cultivar) and ZYQ8 (an improved semi-dwarf cultivar) carry *Pi33*, but do not have TYC nor TN1 in their genealogy.

Whatever the origin of *Pi33*, this gene has been selected several times in different independent crosses. It has been selected for in DGWG × TYC progeny (TN1 cultivar) in Taiwan, TN1 × Ebandioulaye progeny (IRAT7 cultivar) in West Africa and in the successive crosses that led to IR64 in The Philippines. These independent selection events in different breeding programs and geographic areas, may reflect some selective advantage of *Pi33*. We could only detect a few isolates virulent to *Pi33* in our worldwide collection of more than 2,000 isolates originating from 55 countries (data not shown). This broad-spectrum resistance to blast is likely to have favored the selection of *Pi33* in different countries.

Mapping of *Pi33*

The location of the resistance gene was easily determined in two independent crosses. A small but significant number of DH lines (12 among 105) from the IR64 × Azucena cross showed a susceptible phenotype when inoculated with *ACE1* avirulent isogenic strains, although they inherited the allele from the resistant parent IR64 for all markers surrounding the *Pi33* locus. With or without these 12 lines, *Pi33* mapped exactly at the same position on chromosome 8 in the IR64 × Azucena cross. This position was confirmed by the results of the Azucena × Bala cross and none of these "suppressed" lines was observed. Consequently, we considered that these lines harbored *Pi33*, but that one or several additional segregating gene(s) could suppress the expression of this *R* gene. If one independent gene suppresses the expression of *Pi33*, a 1:1 S:R segregation is expected in the lines carrying the IR64 allele for the markers surrounding *Pi33*. Only 12 lines over 54 showed this suppressed phenotype. This result indicated that either more than one gene is involved in this suppressing process, and either the suppressor gene is linked to *Pi33*. When testing this second hypothesis, the suppressor gene was located at the same locus as *Pi33*. At this stage, based on the results obtained, it is not possible to choose between these two hypotheses. Moreover, other hypotheses could be proposed but cannot be tested without additional crosses.

Although rice blast is a model pathosystem, the molecular mechanisms underlying the interaction between avirulence and resistance genes remains poorly understood. Therefore, the characterization of new resistance genes together with the fungal signals they recognize is of importance to understand how plants resist microbial pathogens. This importance is strengthened by the fact that *ACE1* does not share the typical characteristics of *AVR* genes from other fungi. The fine genetic map of *Pi33* described in this study will be very useful in constructing a physical map of this locus and in isolating this resistance gene. Positioning of *Pi33* will also help distinguish it from other *R* genes that could be mapped on chromosome 8. Allelism tests are often difficult to perform. Fine mapping is an alternative way to try to compare *R* genes and may be sufficient to identify a new gene in some cases.

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