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Characterization of four rice mutants with alterations in the defence response pathway

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SUMMARY

A fast-neutron mutagenized population of rice seedlings was screened with *Magnaporthe grisea*, the causal agent of rice blast disease, to identify mutants with alterations in the defence response. Three mutant lines, ebr1, ebr2 and ebr3 (enhanced blast resistance) were identified that display enhanced resistance to *M. grisea*. ebr1 and ebr3 also confer enhanced resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). ebr3 develops a lesion mimic (LM) phenotype upon inoculation with *M. grisea*, and the phenotype is also induced by a shift in environmental conditions. The fourth mutant line, ncr1 (necrosis in rice), has an LM phenotype under all conditions tested and lacks enhanced resistance to either *M. grisea* or *Xoo*. Complementation testing using the mutant lines ebr3 and ncr1 indicates that the *ebr3* and *ncr1* loci are nonallelic and recessive. ebr1 and ebr2 display no alterations in expression of the rice pathogenesis-related (PR) genes *PBZ1* and *PR1*, compared to wild-type CO39. ebr3 has an elevated expression of *PBZ1* and *PR1* only in tissue displaying the LM phenotype. ncr1 strongly expresses *PBZ1* in tissue displaying the LM phenotype, whereas *PR1* expression in this tissue is similar to wild-type CO39.

INTRODUCTION

Plants defend themselves against pathogen challenge by the activation of defence response pathways that result in coordinated defence gene expression and the subsequent containment of the pathogen (Staskawicz *et al.*, 1997). One feature of a host resistance reaction is the hypersensitive response (HR), which is characterized by rapid plant cell death immediately at the point of pathogen ingress (Dangl *et al.*, 1996). This localized HR response is a specialized version of programmed cell death (PCD) (Greenberg, 1996). The HR is correlated with a transient burst of active oxygen species, activation of specific defence related genes, accumulation of antimicrobial compounds, and alterations in of the cell wall (Yin *et al.*, 2000). In some plant species, the HR leads to a systemic response that confers enhanced resistance to subsequent pathogen infections (Ryals *et al.*, 1996). This secondary response has been termed systemic acquired resistance (SAR) and is accompanied by an elevated expression of pathogenesis-related (PR) genes (Uknes *et al.*, 1992).

Genetic screens have led to the isolation of mutants that develop an HR-like response in the absence of pathogen challenge (Dangl *et al.*, 1996; Greenberg *et al.*, 1994; Walbot *et al.*, 1983; Wolter *et al.*, 1993; Yin *et al.*, 2000). It has been proposed that these mutants carry defects in PCD pathways, making them unable to contain the boundaries of cell death once an HR has been initiated (Dangl *et al.*, 1996). This mutant class is commonly termed 'lesion mimics' (LM) (Walbot, 1983). The LM phenotype may occur in response to various stimuli that include alterations in light regimens or temperature or pathogen challenge (Dietrich *et al.*, 1994; Yamanouchi *et al.*, 2002; Yin *et al.*, 2000). Mutations in key steps of metabolism have also been identified in maize LM mutants that lead to the toxic or deleterious accumulation of intermediates (Gray *et al.*, 1997; Hu *et al.*, 1998; Mach *et al.*, 2001). The plethora of LM mutants identified in a broad range of plant species suggests that multiple and independent pathways are required for the proper initiation and containment of the HR (Lorrain *et al.*, 2003).

The appearance of the LM phenotype can occur with a simultaneous activation of the defence response (Dangl *et al.*, 1996). Defence response activation phenotypically manifests itself as enhanced resistance to pathogens, the induction of SAR, and the elevated expression of PR genes (Ryals *et al.*, 1996). Enhanced resistance LM mutants have been shown for either a single pathogen species or broad spectrum, non-species specific resistance, similar to SAR (Buschges *et al.*, 1997; Dietrich *et al.*, 1994; Takahashi *et al.*, 1999). Conversely, LM mutants have been identified that have elevated PR gene expression without the concomitant enhanced resistance to pathogens (Greenberg *et al.*, 1994; Yin *et al.*, 2000). This uncoupling of defence response

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activation and PCD suggests that there exist PCD signalling pathways that are independent of the defence response (Lorrain *et al.*, 2003). Further characterization of the LM mutant class will highlight their unique role in the defence response as well as the regulatory and metabolic steps essential for initiation and containment of PCD.

Three collections of LM mutants have recently been identified and characterized in rice. The 13 *spl* (*spotted leaf*) and three *cdr* (*cell death and resistance*) LM mutants were all found to spontaneously develop HR-like lesions and all had an elevated expression of the rice PR genes *PBZ1* and *PR1* in tissue displaying the LM phenotype (Misobuchi *et al.* 2002a; Takahashi *et al.*, 1999; Yin *et al.*, 2000). Eight *spl* mutants and all three *cdr* mutants display enhanced resistance to the fungal pathogen *Magnaporthe grisea* (*M. grisea*), which is the causal agent of rice blast disease (Ou, 1985; Takahashi *et al.*, 1999; Yin *et al.*, 2000; Mizobouchi *et al.*, 2002b). The five remaining *spl* mutants have no enhanced resistance to *M. grisea*, even though they have an elevated expression of *PBZ1* and *PR1* (Yin *et al.*, 2000). Further characterization of these mutants will be critical to the identification of the stimuli that initiate the HR, the mechanisms by which rice maintains the boundaries of an HR, and the relationship between the pathways for defence response activation and PCD.

In order to identify new loci involved in the defence response of rice, a genetic screen of the rice cultivar CO39 was performed using *M. grisea*. CO39 is partially susceptible to the CA-1 isolate of *M. grisea* that was used in this screen (Greer and Webster, 2001). Three mutant lines were identified as having an enhanced resistance phenotype to *M. grisea.* These lines are designated as ebr1 (enhanced blast resistance 1), ebr2 and ebr3. ebr1 and ebr3 also have enhanced resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). ebr3 has an LM phenotype that resembles an HR and is induced by *M. grisea* and *Xoo* inoculation This LM phenotype can also be induced in the growth chamber in the absence of pathogen inoculation. The ebr1 and ebr2 mutant lines do not display an LM phenotype and therefore belong to a newly identified class of enhanced resistance rice mutants (Zhang *et al.*, 2003). A fourth mutant line, ncr1 (necrosis in rice 1) has an LM phenotype that spontaneously appears on seedlings at 2 weeks post-germination. In ncr1, the lesions appear as patches that spread across the leaf as it emerges. ncr1 mutants have no resistance to *M. grisea* on leaves lacking the LM phenotype, and ncr1 lacks enhanced resistance to *Xoo*.

RESULTS

Initial characterization of ebr1, ebr2, ebr3 and ncr1 with the CA-1 isolate of *M. grisea*

Three putative mutant lines were found to have enhanced resistance to *M. grisea* and were grouped as ebr lines. Wild-type CO39 develops type 3 lesions at 7-days post-inoculation with the CA-1 isolate of *M. grisea* (Fig. 1A). ebr1 develops type 0 and 1 lesions at 7-days post-inoculation, with CA-1 reflecting a complete resistance phenotype (Fig. 1B) whereas the ebr2 mutant develops type 1 and 2 lesions at 7 days post-inoculation reflecting an enhanced resistance phenotype (Fig. 1C). The degree of enhanced resistance in ebr2 is less than that of ebr1, but this mutant has a noticeable reduction in size and frequency of CA-1 lesions when compared with CO39. ebr3 does not develop characteristic *M. grisea* lesions at 7 days post-inoculation. In contrast, ebr3 displays an LM phenotype on all leaves at 7 days post-inoculation

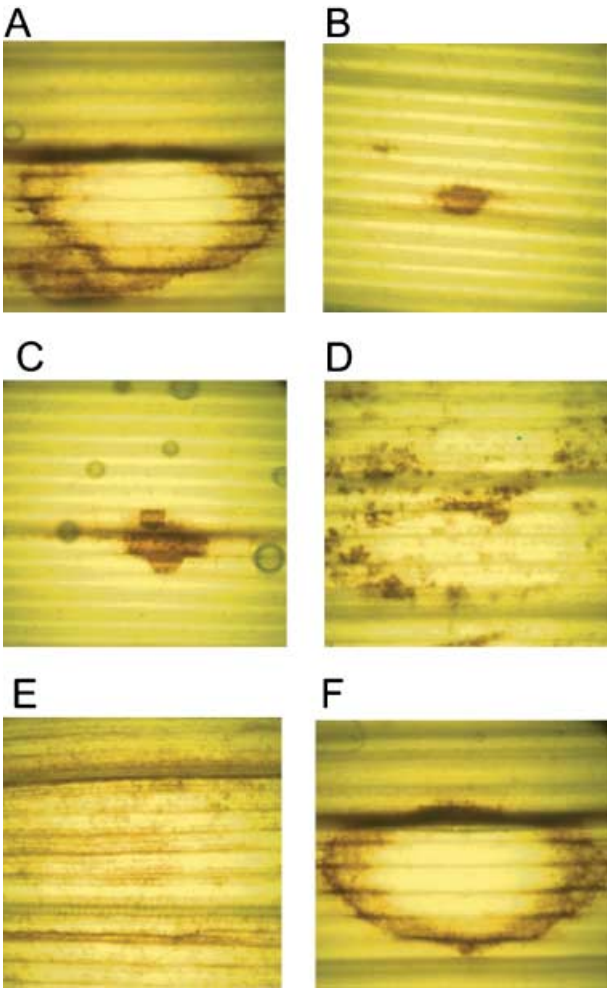

Fig. 1 Brightfield microscopy (40× magnification) of inoculated tissue observed 7 days post-inoculation with the CA-1 isolate of *M. grisea* for the four mutants and wild-type CO39. All images utilize cleared leaf tissue. Seedlings were inoculated at 3 weeks. (A) Wild-type CO39 cultivar displaying a type 3 lesion. (B) ebr1 leaf tissue displaying a type 1 lesion. (C) ebr2 leaf tissue displaying a type 2 lesion. (D) ebr3 leaf tissue displaying the LM phenotype. (E) ncr1 leaf tissue displaying the necrotic LM phenotype. (F) ncr1 leaf tissue lacking the LM phenotype displaying a type 3 *M. grisea* lesion. The size marker (black bar) represents 0.5 mm.

Table 1 *M. grisea* reaction scores over 10 days post-inoculation using the CA-1 isolate*

Mutant line	Day 0	Day 2	Day 3	Day 4	Day 5	Day 7	Day 10
CO39	0	1	1 & 2	1 & 2	2	2 & 3	3
ebr1	0	0	0	0 & 1	0 & 1	0 & 1	1
ebr2	0	0	0 & 1	0 & 1	1	1 & 2	1 & 2
ebr3	0	0	1	LM[b]	LM[b]	LM†	Dead
ncr1‡	0	0	1	1 & 2	2	2 & 3	3

*Reaction scores follow a 0–5 numerical rating described in the Experimental procedures (Mackill and Bonman, 1992).
†Indicates the appearance of a LM phenotype. These observed lesions are not characteristic of *M. grisea* infection.
‡The lesion scores for the ncr1 line are for leaf 5 only.

Table 2 Leaf phenotype of ebr3 seedlings over 10 days under mock-inoculation conditions*

Leaf†	Day 0	Day 2	Day 3	Day 4	Day 5	Day 7	Day 10
1	WT‡	WT	WT	LM§	LM	LM	LM
2	WT	WT	WT	WT	LM	LM	LM
3	WT	WT	WT	WT	WT	LM	LM
4	WT	WT	WT	WT	WT	WT	WT
5	WT	WT	WT	WT	WT	WT	WT

*Mock-inoculation conditions are described in the Experimental procedures.
†Leaf stage is from 1 (basal, oldest) to 5 (apical, youngest) (Century *et al.*, 1999).
‡WT indicates a wild-type phenotype.
§LM indicates the appearance of LM phenotype.

(Fig. 1D). ebr3 seedlings grown under MI conditions also develop the LM phenotype on older leaves (see below). The fourth mutant line, ncr1, develops a necrotic LM phenotype at 2 weeks post-germination on leaves 1 and 2. By 4 weeks, the LM phenotype is present on leaves 1–4 on 4 week-old ncr1 seedlings (Fig. 1E). No *M. grisea* lesions were observed on tissue displaying the ncr1 LM phenotype, suggesting that the LM phenotype triggers enhanced resistance to *M. grisea*. On leaf 5 of 4 week-old inoculated ncr1 seedlings, type 3 *M. grisea* lesions develop (Fig. 1F). The ebr3 LM phenotype has clusters of necrosis that are distinct from the ncr1 necrotic LM phenotype that spreads along the parallel vascular bundles (Fig. 1D,F).

Characterization of ebr1, ebr2, ebr3 and ncr1 over a 10 day span post-inoculation with the *M. grisea* isolate CA-1

A 10-day evaluation of *M. grisea* lesion development was made for each of the mutants and wild-type CO39 using the CA-1 isolate on 4-week-old seedlings (Table 1). CO39 wild-type seedlings developed type 2 and 3 lesions by 7 days post-inoculation. At 10 days post-inoculation, all lesions developed as type 3. ebr1 had lesion scores of 0 and 1 over the 10 days post-inoculation indicating complete resistance. ebr2 had delayed development of *M. grisea* lesions and a reduction in lesion size (types 1 and 2) and frequency. ebr3 appears phenotypically normal during the first 3 days post-inoculation when compared with CO39. However, on the fourth day post-inoculation, the ebr3 LM phenotype appears in patches across all leaves. On the seventh day post-inoculation, the ebr3 LM phenotype has spread across the leaves. On the tenth day post-inoculation, the inoculated leaves are covered with the LM phenotype and have died. ncr1 develops type 3 *M. grisea* lesions by day 7 only on the leaf 5 (not displaying the necrotic LM phenotype). ncr1 leaves 1 through 4 display the necrotic LM phenotype and lack observable *M. grisea* lesions. Inoculation with CA-1 did not induce the necrotic LM phenotype for ncr1.

The LM phenotype was observed on ebr3 mutants under mock-inoculated, but not greenhouse, conditions

The four mutants and CO39 3-week-old seedlings were subjected to mock-inoculation (MI) conditions and were evaluated over a 10-day period. ebr1 and ebr2 MI seedlings were phenotypically identical when compared with the CO39 wild-type seedlings. The ebr3 MI seedlings developed the LM phenotype on leaves 1, 2 and 3 (Table 2). The MI ebr3 LM phenotype appeared in an age-dependent fashion, with leaf 1 developing the phenotype at day 4. Leaves 2 and 3 developed the LM phenotype at days 5 and 7, respectively, and ebr3 leaf 5 did not display the LM phenotype after 10 days under MI conditions. In contrast, *M. grisea*-inoculated ebr3 mutants develop the LM phenotype on all five leaves of the seedling by day 4 post-inoculation. Greenhouse grown ebr3 4-week-old seedlings never developed the LM phenotype in repeated assays.

Unlike the inducible ebr3 LM phenotype, ncr1 mutants under MI conditions are not phenotypically different from greenhouse grown ncr1 mutants of the same age, indicating that a shift in environmental conditions neither exacerbated nor reduced the necrotic LM phenotype (data not shown). Figure 2 shows the age-dependent progression of the ncr1 LM phenotype on leaves 1–5 of 4-week-old seedlings.

ebr1 and ebr3 confer enhanced resistance to the PXO99 isolate of Xoo

To assess if the ebr1, ebr2 and ebr3 mutants also conferred resistance to a bacterial pathogen, these mutants were inoculated with the PXO99 isolate of *Xoo* (Hopkins *et al.* 1992). Compared to the CO39 control, ebr1 and ebr3 restrict the progression of the *Xoo*-induced water soaked lesions (Fig. 3). Inoculation with *Xoo* induces the ebr3 LM phenotype at the point of inoculation (Fig. 3). All plants were grown in the greenhouse prior to inoculation (greenhouse grown ebr3 mutant lines do not display the

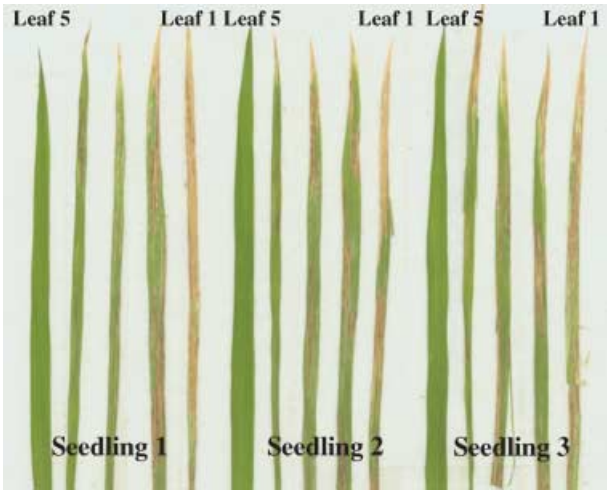

Fig. 2 Macroscopic visualization of the necrotic LM phenotype of ncr1 greenhouse-grown seedlings at 4 weeks old. Five leaves are shown from three separate seedlings. For each seedling leaf series, the leftmost leaf is leaf 5 and the rightmost leaf is leaf 1.

Fig. 3 Lesion length analysis for the PXO99 isolate of *Xoo* on 6-week-old plants. Representative leaves displaying water-soaked lesions at are shown at 14-days post-inoculation for wild-type CO39 and the ebr1 and ebr3 lines.

LM phenotype). A quantitative *Xoo* growth curve analysis was performed for ebr1 and ebr3 using PXO99, with the wild-type CO39 as a control. The 14-day growth curve analysis indicated that both ebr1 and ebr3 have a nearly 10-fold reduction of bacterial titre at 10-, 12-, and 14-days post-inoculation when compared with wild-type CO39 (Fig. 4). PXO99 lesion length analysis for ebr2 showed no significant reduction in lesion lengths, and a bacterial growth curve analysis did not show a significant difference in bacterial titre when compared with CO39 (data not shown).

At 6 weeks, all expanded leaves of ncr1 displayed an LM phenotype that obscured the progression of *Xoo*-induced water

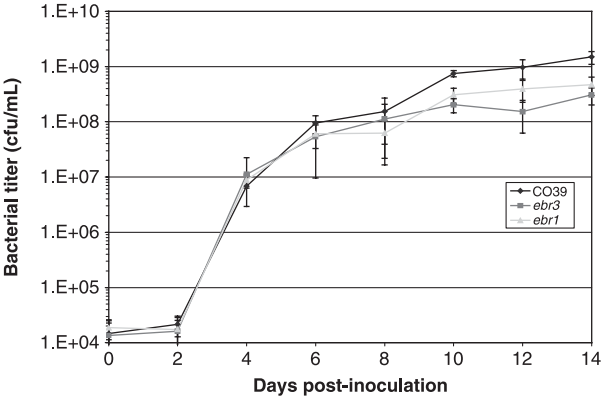

Fig. 4 Growth curve analysis for CO39 and the ebr1 and ebr3 lines using the PXO99 isolate of *Xoo*. All data points over the 14 day assay period are the average of the titre of five independently inoculated leaves. Error bars indicate the standard deviation of the average.

soaked lesions; therefore lesion length analysis could not be performed on the ncr1 mutants. Instead, a growth curve assay was performed using the IRBB21 line carrying the dominant *Xa21* resistance gene as a resistant control. *Xa21* confers complete resistance to the PXO99 isolate of *Xoo* (Song *et al.*, 1995). CO39, having the *xa21* allele, is fully susceptible to PXO99. If ncr1 has enhanced resistance in leaves displaying the LM phenotype, the reduction in bacterial titre can be compared with the complete resistance conferred by *Xa21*. An F_2 population was developed from a cross between ncr1 and the Indica cultivar IRBB21. F_3 families representing the four possible homozygous combinations were identified (Fig. 5). From this cross, the *ncr1* locus was found to have a recessive mode of inheritance (see 'Mode of inheritance' below). The two homozygous F_3 families with the *xa21* susceptibility allele, i.e. *NCR1/NCR1 xa21/xa21* (no LM phenotype) and *ncr1/ncr1 xa21/xa21* (LM phenotype), had similar bacterial titres ($\sim 1 \times 10^9$ cfu/mL) at 14-days post-inoculation (Fig. 6). By contrast, both F3 families with the dominant *Xa21* resistance gene, i.e. *NCR1/NCR1 Xa21/Xa21* (no LM phenotype) and *ncr1/ncr1 Xa21/Xa21* (LM phenotype), had a 10-fold reduction in the bacterial titre ($\sim 1 \times 10^8$ cfu/mL). These results indicate that the ncr1 LM phenotype conferred by the *ncr1/ncr1* genotype did not restrict bacterial growth. Furthermore, *Xa21*-mediated signalling was not affected in plants having the *ncr1*/*ncr1* genotype, indicating that these loci do not interact epistatically.

The ncr1 and ebr1 mutants display a short stature

During characterization of the mutants with *Xoo*, both the ebr1 and ncr1 lines were observed to have a reduction in stature. To further investigate this phenotype, 30 six week-old CO39 were evaluated for their height. All 150 plants were measured at the same time and were grown under identical greenhouse

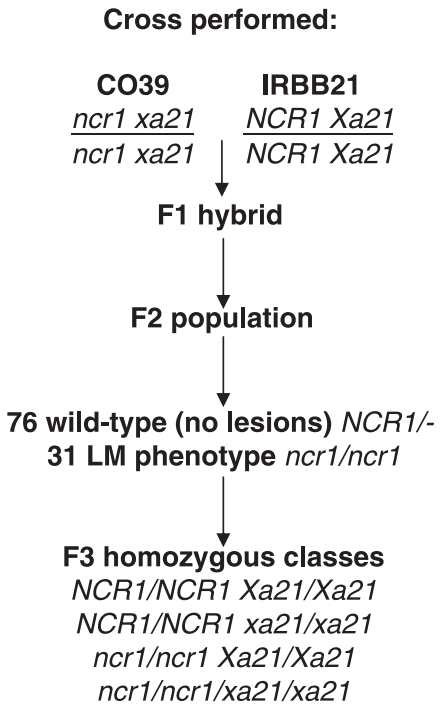

Fig. 5 Scheme for developing the four F_3 homozygous lines from a cross between IRBB21 and the *ncr1/ncr1* genotype.

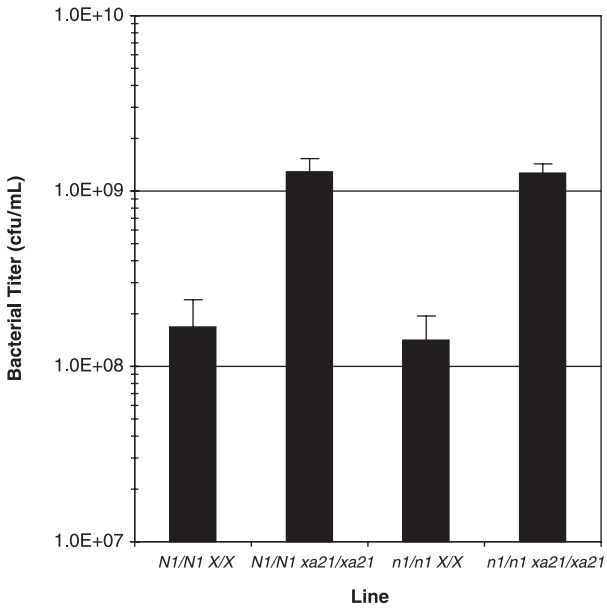

Fig. 6 Bacterial titres at 14 days post-inoculation for the four F_3 homozygous lines using the PXO99 isolate of *Xoo*. Each bar represents the average of five independent inoculations. The error bars are the standard deviation of the mean. *N1* = *NCR1* (from IRBB21), *n1* = *ncr1*, *X* = *Xa21* (PXO99 resistance gene), and *xa21* = CO39 susceptibility allele.

conditions for 6 weeks. The height was measured after extending the youngest, emerging leaf vertically. CO39 was found to have an average height of 75 cm (± 4.1 cm). Both ebr2 (77 cm (± 4.0 cm)) and ebr3 (81 cm (± 3.9 cm)) were not significantly different from CO39. In contrast, both the ebr1 and ncr1 lines displayed significant reductions in stature with measured heights of 63 cm (± 5.2 cm) and 64 cm (± 3.1 cm), respectively.

PR gene expression analysis of the four mutants

The four mutants were analysed for their expression of the rice PR genes *PR1* and *PBZ1*. All analyses took place on 4-week-old seedlings. For the CO39, ebr1, ebr2 and ebr3 seedlings grown under GH conditions, no expression of *PR1* and *PBZ1* was detected (data not shown). When grown under MI conditions, ebr1 and ebr2 and CO39 had a very low expression of both PR1 and PBZ1, whereas ebr3 seedlings had an elevated expression of both PR genes (Fig. 7A). ebr3 shows the LM phenotype under these conditions (Table 2). Next, leaves 1, 2 and 3 of ebr3 showing the LM phenotype were sampled separately from leaves 4 and 5 that displayed no lesions. As a control, the same leaves were sampled from 4-week-old ebr3 seedlings grown under GH conditions that do not display the LM phenotype. *PR1* and *PBZ1* only have elevated expression in leaves 1, 2, and 3 displaying the LM phenotype that is induced by MI conditions (Fig. 7B). When grown under GH conditions, these same leaves from ebr3 do not display an elevated expression (or the LM phenotype)

The ncr1 LM phenotype is apparent on leaves 1–4 but not on leaf 5 for 4-week-old seedlings. These leaves were sampled separately for RNA expression analysis. In the ncr1 line, an elevated expression of *PBZ1* was observed for leaves 1–4 with the necrotic LM phenotype and no *PBZ1* expression was observed in leaf 5, which is similar to wild-type CO39 (Fig. 7C). In contrast to *PBZ1*, *PR1* did not have an elevated expression in either set of ncr1 leaves (Fig. 7C).

Mode of inheritance for ncr1 and ebr3

The segregation analysis of an F_2 population derived from the ncr1 mutant line and IRBB21 cross support the hypothesis that the *ncr1* locus has a recessive Mendelian mode of inheritance (Table 3). Chi-square analysis of the F_2 phenotypic segregation data supports this hypothesis. For the F_2 populations derived from reciprocal crosses of the ebr3 and ncr1 mutant lines, segregation data and χ^2 analysis support the hypothesis that both the *ebr3* and *ncr1* mutants have a recessive mode of inheritance (Table 4). A cross between two recessive mutants should phenotypically segregate 9 : 3 : 3 : 1 by Mendelian laws of inheritance. In the phenotypic score of the F_2 populations, all lines displaying the ncr1 LM phenotype were identified visually in 3-week-old greenhouse grown seedlings and were tagged. The F_2 populations were

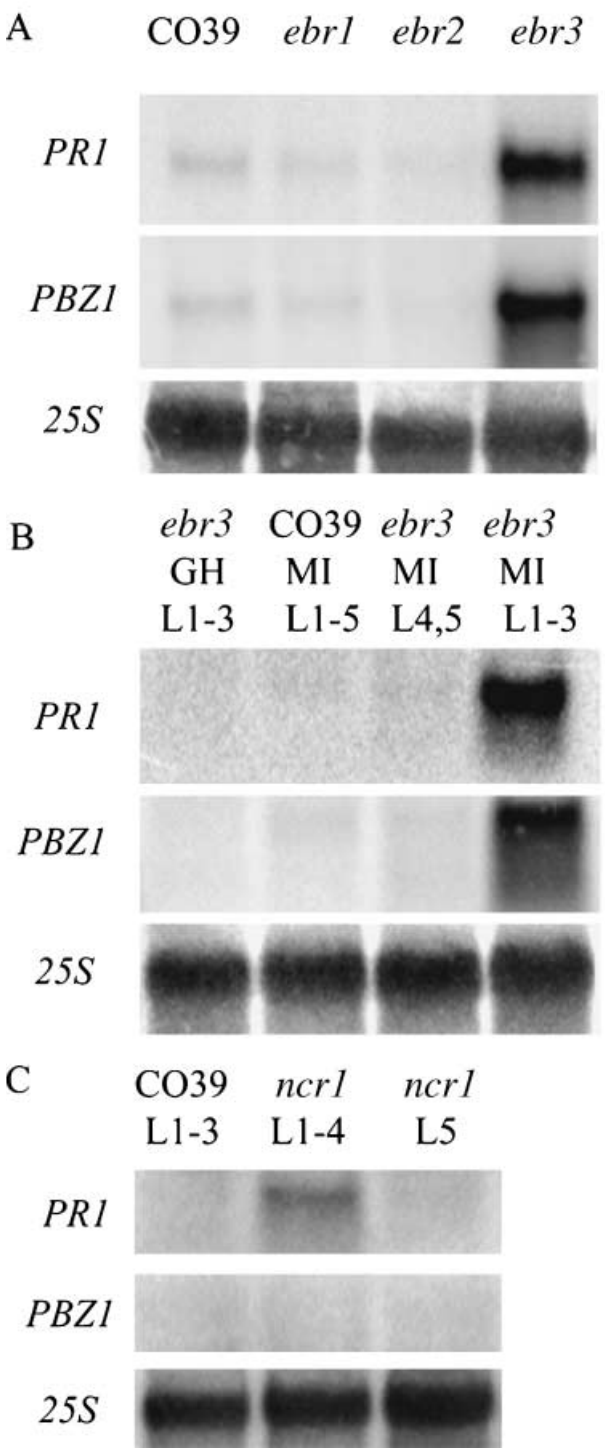

Fig. 7 Expression analysis for *PBZ1* and *PR1* in 4-week-old ebr1, ebr2, ebr3, and ncr1 seedlings. (A) *PR1* and *PBZ1* expression analysis for ebr1, ebr2 and ebr3 lines. RNA was isolated from whole seedling samples grown under mock-inoculation (MI) conditions. A *25S* rDNA probe was used as a control to evaluate equal transfer. (B) Expression analysis of *PR1* and *PBZ1* in 4-week-old ebr3 seedlings. Lane 1 has ebr3 tissue that was grown under greenhouse conditions (GH) and lacks the LM phenotype (L1–3). Lane 2 is wild-type CO39 grown under MI conditions. Lane 3 is ebr3 tissue collected from leaves 4 and 5 (L4,5) grown under MI conditions and lacks the LM phenotype. Lane 4 is ebr3 tissue collected from leaves 1, 2 and 3 (L1–3) grown under MI conditions and display the LM phenotype. 25S rDNA probe was used as a control to evaluate equal transfer. (C) Expression analysis of *PR1* and *PBZ1* in four week-old ncr1 seedlings. All tissue sampled was grown under greenhouse conditions. Lane 1 is tissue sampled from leaves 1 through 5 for CO39. Lane 2 is ncr1 tissue sampled from leaves 1, 2, 3 and 4 that display the LM phenotype. Lane 3 is ncr1 tissue sampled from leaf 5 that lacks the necrotic LM phenotype. *25S* rDNA probe was used as a control to evaluate equal transfer.

then subjected to MI conditions to induce the ebr3 HR-like phenotype. After 7 days, the ebr3 LM phenotype was readily apparent on leaves 1 and 2 of the F_2 seedlings. Any tagged individual displaying both the ncr1 necrotic LM phenotype with the ebr3 HR-like LM phenotype was scored as a double mutant. For these double mutants, the ncr1 necrotic LM phenotype was not sufficient to induce the ebr3 HR-like LM phenotype under greenhouse conditions. The ebr3 LM phenotype was only induced in the double mutants in response to the shift to MI conditions. The double mutants with the *ebr3/ebr3 ncr1/ncr1* genotype displayed both of their respective LM phenotypes simultaneously. The *ncr1* and *ebr3* loci do not interact epistatically since the χ^2 analysis supports the 9 : 3 : 3 : 1 segregation ratio. The mode of inheritance for the mutant loci in the ebr1 and ebr2 lines has not yet been determined.

DISCUSSION

This genetic screen successfully identified four mutant lines having disparate phenotypes in response to inoculation with the CA-1 isolate of *M. grisea*. The lines include two with enhanced resistance to CA-1 (ebr1 and ebr2), one with enhanced resistance to CA-1 and an inducible lesion mimic phenotype (ebr3), and a lesion mimic mutant (ncr1) that spontaneously developed necrotic patches. These mutants were further characterized with the bacterial pathogen *Xoo* for variation in stature and for expression of the rice PR genes *PBZ1* and *PR1*. In addition, the mode of inheritance was determined for the *ebr3* and *ncr1* mutations that cause lesion mimic phenotypes.

The ebr1 line displays complete resistance to the CA-1 phenotype and also confers enhanced resistance to the bacterial pathogen *Xoo.* A lesion length assay with PXO99 showed a reduction in water-soaked lesion symptoms that was verified with the quantitative growth curve assay. Average PXO99 titres were significantly reduced at the 10-, 12- and 14-day time points with a nearly 10-fold reduction at the 14-day time point. This reduction in average titre is comparable to the complete resistance affected by the *Xa21* resistance gene. Expression analysis indicated that ebr1 does not display an elevated expression of rice PR genes. In addition, a lesion mimic phenotype was not observed under any of the described environmental conditions

Table 3 F_2 segregation analysis to determine the mode of inheritance for *ncr1*

Genotype	F_1 phenotype	F_2 total	Phenotype		Ratio*	χ^2
			Wild-type	*ncr1*		
ncr1/*ncr1*×*NCR1*/*NCR1*	Wild-type	107	76	31	3 : 1	0.9 ($P > 0.34$)

*Indicates the expected F_2 Mendelian ratio for a single recessive trait.

Table 4 F_2 segregation analysis to determine the mode of inheritance for ebr3 and *ncr1*

Genotype	F_1*	F_2 total	Phenotype				Ratio‡	χ^2
			WT	*ncr1*	*ebr3*	*ncr1 & ebr3*†		
ebr3/*ebr3*×*ncr1*/*ncr1*	WT	165	95	27	37	6	9 : 3 : 3 : 1	3.54 ($P > 0.34$)
ncr1/*ncr1*×*ebr3*/*ebr3*	WT	392	217	82	75	18	9 : 3 : 3 : 1	2.79 ($P > 0.42$)

*WT = lack of LM phenotype for either *ncr1* or *ebr3*.

†The phenotype of the double mutants has both the patches of necrosis for *ncr1* and the brown, specking lesions characteristic of *ebr3*.

‡The expected F_2 Mendelian ratio for a cross between two recessive, non-allelic, and unlinked loci.

for ebr1. Based upon this characterization, ebr1 is a novel addition to the collection of rice enhanced resistance mutants and mutant lines (Zhang *et al*., 2003).

The rice ebr1 mutant shares many characteristics with *Arabidopsis* lines carrying the *edr1* mutation. *edr1* was identified from a genetic screen using the bacterial pathogen *Pseudomonas syringae* and was subsequently determined to have enhanced resistance to the fungal pathogen *Erysiphe cichoracearum* (Frye and Innes, 1998). Similar to rice ebr1, *Arabidopsis edr1* does not display an elevated expression of the *Arabidopsis* PR genes *PR1*, PR2 and PR5, and does not display a lesion mimic phenotype (Frye and Innes, 1998). *EDR1* encodes a MAPKK kinase and is postulated to negatively regulate the inducible expression of the defence response (Frye *et al*., 2001). A putative orthologue of *EDR1* is present in rice in the dbEST database (accession no. D41138). Subsequent analysis will be needed to determine if the ebr1 carries a genomic alteration in this *EDR1* orthologue.

The ebr2 has enhanced resistance to the CA-1 isolate when compared to wild-type CO39. The 10-day CA-1 inoculation assay revealed that ebr2, with type 1 and 2 lesions, has a reduction in lesion severity. This mutant, unlike ebr1, has no enhanced resistance to *Xoo*. Rice PR gene expression analysis indicates that the ebr2 line does not constitutively express *PR1* or *PBZ1*, nor does it display a lesion mimic phenotype under any of the environmental conditions tested.

Because ebr1 and ebr2 both display enhanced resistance to the CA-1 isolate, they may also confer resistance to other *M. grisea* strains. Previous research has shown that CO39 is highly susceptible to a wide range of *M. grisea* isolates, and these strains would therefore be logical candidates for testing on ebr1 and ebr2 (Inukai *et al*., 1994; Mackill and Bonman, 1992). However, USDA restrictions prevent the import of these isolates into California, so these tests will need to be conducted at other locations.

Recently, the *pmr* class of mutants from *Arabidopsis* has been characterized for resistance to the obligate biotroph *Erysiphe cichoracearum* (powdery mildew) (Vogel and Sommerville, 2000; Vogel *et al*., 2002). The enhanced resistance in these mutants was not broad spectrum. All *pmr* mutants were susceptible to the bacterial pathogen *Pseudomonas syringae*. The loci identified in this *Arabidopsis* screen are postulated to encode susceptibility factors resulting from fungal species evolving the ability to utilize host genes for its own needs. This adaptation is further supported by the narrow spectrum of enhanced resistance in the *pmr* mutants (Vogel and Sommerville, 2000; Vogel *et al*., 2002). The ebr2 line, having enhanced resistance to only CA-1, may represent a mutation in a susceptibility factor for *M. grisea*, whereas the ebr1 line, having broad-spectrum resistance *M. grisea* and *Xoo*, does not fit the phenotypic characteristics for a susceptibility factor. An import restriction for *M. grisea* into California has prevented a comprehensive screening of ebr1 and ebr2 with a broad range of isolates. Subsequent BLAST screening elsewhere will reveal more phenotypic data on the type and degree of *M. grisea* resistance, and will be of particular use in the further characterization of ebr2.

The *M. grisea* enhanced resistance in the ebr3 mutant line is quite different from either ebr1 or ebr2. Inoculation with CA-1 induced a LM phenotype that eventually led to the death of the leaf over the course of a 10-day inoculation assay. The LM phenotype was also induced by MI and *Xoo* inoculation. ebr3 confers enhanced resistance to *Xoo* in both lesion length and growth curve analyses, and the resistance is similar to that conferred by *Xa21*. Expression of *PR1* and *PBZ1* are elevated in leaves that display the LM phenotype but not in CO39 or ebr3 leaves lacking the LM phenotype. This broad-spectrum resistance to both a fungal and bacterial pathogens share features with plants that are undergoing SAR. For example, ebr3 shares many similarities with

the *Arabidopsis lsd1* (*lesions simulating disease 1*) mutant (Dietrich *et al.*, 1994). The *lsd1* lesion mimic phenotype is induced by both a shift in lighting regimen and pathogen challenge. Similar to ebr3, the pathogen-induced LM phenotype spreads to consume the leaf. *Arabidopsis* PR genes are strongly expressed in leaves displaying the lesion mimic phenotype, and *lsd1* has enhanced resistance to both bacterial and oomycete pathogens (Dietrich *et al.*, 1994; Dietrich *et al.*, 1997). Similarly, a rice lesion mimic mutant, *spl11*, shares many of the phenotypic characteristics with the ebr3 line and *lsd1*, including enhanced resistance to both fungal and bacterial pathogens, elevated PR gene expression in lesion mimic affected tissue, and induction of the lesion mimic phenotype by pathogen inoculation. Unlike either ebr3 or *lsd1*, the *spl11* mutant has not been reported to have induction of the lesion mimic phenotype under an alteration of environmental conditions. In contrast to all rice lesion mimic mutants reported to date, the ebr3 line develops the HR-like lesion mimic phenotypes spontaneously under greenhouse conditions (Takahashi *et al.*, 1999; Yin *et al.*, 2000). Therefore, ebr3, with the inducible LM phenotype, represents a new class of rice lesion mimic mutants.

The ncr1 mutant line develops a patchy LM phenotype at 2 weeks post-germination. Leaves lacking the LM phenotype display type 3 lesions in response to *M. grisea.* LM unaffected tissue did not display obvious *M. grisea* lesions although the susceptible phenotype may have been obscured by the LM phenotype. Unlike ebr3, the LM phenotype conferred by the *ncr1/ncr1* genotype has neither enhanced resistance to *Xoo* nor was the LM phenotype induced by *M. grisea*. Similarly, five previously described spl mutants, *spl2*, *spl3*, *spl4*, *spl6* and *spl7*, have no enhanced resistance to four different isolates of *M. grisea*. Moreover, inoculation with these isolates did not induce the LM phenotype on any of the five mutants in a way that was consistent with the *ncr1* mutant (Yin *et al.*, 2000). The *ncr1* mutation does not confer resistance to *Xoo*. The *ncr1/ncr1 xa21/xa21* and *NCR1/NCR1 xa21/xa21* F_3 lines had similar bacterial titres of $\sim 1 \times 10^9$ cfu/mL. By comparison, the presence of the *Xoo* resistance gene *Xa21* reduced bacterial titre 10-fold in both the *NCR1/NCR1 Xa21/Xa21* and *ncr1/ncr1 Xa21/Xa21* genotypes. For these five *spl* mutants, no characterizations with *Xoo* were described (Yin *et al.*, 2000). The ncr1 mutant line has a differential expression of PR genes, in contrast to the *spl* mutants that strongly express both *PBZ1* and *PR1* in LM affected tissue. Given that the ncr1 line only expresses *PBZ1* in LM affected tissue, this mutant is unusual among rice LM mutants in its differential expression of *PR1*. This mutant will need to undergo complementation analysis testing with the *cdr* and *spl* mutants in order evaluate if these mutants are allelic

Two mutants having an LM phenotype were identified in this screen. These two additions bring the total number of LM mutants characterized for alterations in the defence response to 14 (Takahashi *et al.*, 1999; Yin *et al.*, 2000). Only one of these LM mutants, *spl7*, has been cloned and was found to share sequence similarity with heat stress transcription factors (Yamanouchi *et al.*, 2002). Subsequent mapping and identification of the mutant alleles for the *ebr3*, *ncr1*, *spl* and *cdr* mutants will prove useful in uncovering the components involved in the regulation and control of the HR. As with *Arabidopsis* and maize, the large number of rice LM mutants obtained thus far indicate that multiple, independent pathways are involved in PCD, and only a subset of these are also involved in the defence response. Subsequent genetic analysis of these rice LM mutants will uncover the degree of conservation among PCD pathways in higher plants.

EXPERIMENTAL PROCEDURES

M. grisea inoculum preparation

The *M. grisea* CA-1 isolate was obtained from R. Webster at UC-Davis and maintained at –20 °C (Greer and Webster, 2001). Cultures were grown at RT on oatmeal agar (OA). OA was prepared by mixing 100 g of rolled oats in 1 L of water and holding the mixture at 70 °C for 1-h. The oats were filtered out and the solution was brought to 2 L with water. Bacto Agar (13.5 g/L) was added and the solution was autoclaved. The oatmeal agar was then cooled to 60 °C and ampicillin was added to a final concentration of 25 μg/L. The plates were then poured. Plate cultures were seeded from the master culture and were grown for 14 days in darkness at RT. Conidiation was induced by scraping the mycelia with a sterile spoon and continuously illuminating the scraped plate cultures for 5 days at RT. Ten millilitres of water was then added to the plate and the surface scraped to harvest the conidia. The conidial suspension was filtered through Miracloth and adjusted to 5×10^4 conidia/mL using a haemacytometer.

M. grisea disease evaluation

Seedlings were grown for 3 weeks in the greenhouse. Seedlings were then moved to the dew chamber (100% r.h.) for spray inoculation. Seedlings remained in the dew chamber for 16 h in complete darkness. Inoculated seedlings were then removed from the dew chamber and grown in a controlled environmental chamber for 6 days under 12 h light at 28 °C and 12 h darkness at 20 °C. The relative humidity was maintained at 85%. The chamber was equipped with metal halide and incandescent lights providing a PAR of 103 μmol photons/m^2/s^1. PAR reflects the total light intensity between 400 nm and 700 nm. By comparison, PAR for a sunny day in the greenhouse is ~670 μmol photon/m^2/s (Fitzgerald *et al.*, 2004). Total light abundance and quality were monitored using a LiCor 1800 spectroradiometer. Disease reactions were scored at 7 days post-inoculation according to a standardized protocol (Mackill and Bonman, 1992). The lesions were scored from 0 to 5 as follows: 0 = no evidence of infection, 1 = brown specking indicative of an HR response

(< 0.5 mm in diameter), 2 = brown lesions having a 0.5–1 mm diameter, 3 = round to elliptical lesion 1–3 mm in diameter (or length) with a grey centre, 4 = spindle shaped lesions with a grey centre, and 5 = coalesced type 4 lesions across the majority of the leaf. Plants with lesion scores of 0–2 are resistant, 3 are partially susceptible, and 4–5 are susceptible.

Creation of the mutant population and initial screening

Three thousand wild-type CO39 seeds were irradiated with 15 Gy of fast-neutron irradiation at the International Atomic Energy Commission in Austria. The M_1 plants were grown in the greenhouse. M_2 seeds from 2700 M_1 parents were harvested and maintained separately. M_2 seeds from each M_1 parent were sterilized in a 10% bleach solution for 30 min at room temperature (RT). The sterilized M_2 seeds were washed three times in sterile water. Twenty to 40 M_2 seeds from each M_1 parent were germinated in Petri dishes with 10 mL distilled water at RT until both the radicle and epicotyl had emerged. All germinated seeds were grown in the greenhouse for 3 weeks. Aberrations in chlorophyll deposition (i.e. zebra mutants (Kinoshita, 1998)), albinos, seedling lethals, and morphological variants were scored for the first 20 000 M_2 seedlings representing progeny from 800 M_1 parents. A mutational rate of 1.2% was collectively observed for these phenotypes. The seedlings were then moved to the dew chamber for inoculation as described above.

Definition of leaf stages

Four-week-old greenhouse grown CO39 seedlings have five leaves. These leaves are numbered according to the order of emergence (Century *et al.*, 1999). Leaf 1 is basal and is the oldest leaf and leaf 5 is apical and is the youngest leaf.

Putative mutant identification

Over 70 000 M_2 individuals were scored for their reaction with CA-1. One hundred and twenty-one individuals displayed alterations in the defence response. These 121 M_2 individuals were derived from 54 separate M_1 parents. These 121 M_2 individuals were isolated and selfed to generate M_3 progeny. The M_1 line 1902 gave rise to three M_2 progeny with complete resistance to CA-1 and 22 partially susceptible progeny. The three M_2 individuals from line 1902 with complete resistance were selfed. Twenty M_3 seedlings from each of the three M_2 parents with complete resistance were re-screened with *M. grisea.* All M_3 progeny also displayed complete resistance. These M_3 progeny with enhanced resistance were pooled, and the line renamed ebr1. The M_1 line 1946 gave rise to two M_2 progeny with enhanced resistance to CA-1 and 22 partially susceptible progeny. The two enhanced resistance M_2 individuals were selfed. Twenty M_3 seedlings from the two enhanced resistance M_2 parents were re-screened with *M. grisea.* All M_3 progeny displayed enhanced resistance. These M_3 progeny were pooled and the line renamed ebr2. The M_1 line 803 gave rise to one M_2 progeny with a LM phenotype and enhanced resistance to CA-1 and 24 partially susceptible progeny. The M_2 parent with the LM phenotype and enhanced resistance was selfed. Twenty M_3 progeny all displayed the LM phenotype and enhanced resistance to *M. grisea.* These M_3 progeny were pooled and the line was renamed ebr3. The M_1 line 2099 gave rise to two M_2 progeny that displayed an LM phenotype at 2 weeks post-germination and 23 wild-type progeny. The two M_2 parents with the LM phenotype were selfed. Twenty M_3 progeny from both M_2 parents all displayed the LM phenotype. These M_3 progeny were pooled and the line renamed ncr1.

For the four lines (1902, 1946, 803 and 2099), the M_2 siblings that displayed the partially susceptible phenotype were also selfed and the resulting M3 progeny were re-screened. These M_3 progeny all displayed the wild-type phenotype.

Histochemical analysis

Leaves were cleared using a lactophenol solution (50% of 95% ethanol and 5% water solution, 25% water-saturated phenol, 25% lactic acid). Detached leaves were immersed in lactophenol and incubated at 65 °C overnight. The lactophenol solution was replaced and leaves were cleared for an additional 24-h at 65 °C. The leaves were then equilibrated in 70% glycerol solution for microscopy.

Xoo inoculum preparation, inoculation and growth curve protocol

Lesion length assays on 6-week-old plants were performed as described previously (Song *et al.*, 1995). A 14-day growth curve analysis on 6-week-old plants was performed as described previously using the PXO99 isolate of *Xoo* (Song *et al.*, 1995). All plants were grown for 6 weeks in the greenhouse and moved to the controlled environmental chamber for the 14-day inoculation assays with *Xoo*. Inoculations were performed using a *Xoo* suspension of OD = 600 nm. *Xoo in vitro* culture methods have been previously described (Song *et al.*, 1995).

Rice crosses

The ncr1 mutant line was crossed with IRBB21 (pollen donor) in the greenhouse. F1 hybrid seed was dehusked and surface sterilized with 10% bleach before germination. True F_1 hybrids were verified by scoring for the presence of the *Xa21* resistance gene using a primer set derived from the *Xa21* genomic sequence (GenBank no. U72723). The forward primer was 5′-ATAGCAACTGATTGCTTGG and the reverse primer 5′-CGATCGGTATAACAGCAAAAC. The conditions for PCR amplification were 94 °C for 30 s, 55 °C for

45 s, and 72 °C for 90 s for 35 cycles. This primer set detects a 1377 bp amplicon from the IRBB21 *Xa21* allele and an ~1300 bp amplicon from the CO39 *xa21* allele that were resolved on a 1% agarose gel. Three true F_1 hybrids were identified, and F_2 seed was collected. F_2 individuals homozygous for the *Xa21* resistance gene were determined by PCR. Based upon phenotypic screening of the segregating F_2 population, *ncr1* was found to have a recessive mode of inheritance (see 'Mode of inheritance' below). The F_3 progeny were screened with the *Xa21* primer set to identify the presence of the alleles and were also visually screened for the ncr1 LM phenotype. Reciprocal crosses were performed for the ebr3 and ncr1 lines. All crosses were performed in the greenhouse.

Tissue collection protocols for RNA gel blot analysis

Tissue was collected from 4-week-old ebr1, ebr2, ebr3 and CO39 seedlings under two separate environmental conditions for RNA gel blot analysis. Greenhouse conditions (GH) were seedlings grown for 4 weeks in the greenhouse. For mock inoculation (MI) conditions, the seedlings were grown for 3 weeks in the greenhouse, moved to the dew chamber for 16 h, and then grown under controlled environmental conditions (in the same chamber used for inoculations) for 7 days. Whole seedlings were taken for tissue sampling. For ebr3, MI conditions were sufficient to induce the LM phenotype on leaves 1, 2 and 3. ebr3 tissue showing the LM phenotype (leaves 1, 2 and 3) was sampled separately from ebr3 tissue lacking the LM phenotype (leaves 4 and 5) for subsequent RNA gel blot analysis. ncr1 mutants were grown under GH conditions for 4 weeks. Leaves 1, 2, 3 and 4 displayed the LM phenotype and were sampled separately from leaf 5 lacking the LM phenotype.

RNA gel blot analysis

Total RNA was isolated with TRIZOL Reagent (Life Technologies, Gaithersburg, MD). Ten to 15 μg of RNA per lane was separated on 1% formaldehyde–agarose gels and transferred to Hybond-N+ membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) using capillary transfer with 20× SSPE. ^{32}P-labelled DNA probes were generated using Ready-to-Go DNA Labeling Beads (Amersham Biosciences, Piscataway, NJ). The hybridization solution was (per 100 mL): 50 mL of formamide, 30 mL 20× SSPE, 10 mL 50× Denhardt's solution, 5 mL 10% SDS and 5 mL of water. Ten grams of dextran sulphate were dissolved in 100 mL of hybridization solution prior to use. All hybridizations took place at 42 °C. All hybridized blots were washed with 2× SSPE, 0.1% SDS twice and then washed once with 1× SSPE, 0.2% SDS. All washing steps were 15 min in duration at 42 °C. Membranes were stripped using boiling 0.5% SDS and left to cool for 3 h. All RNA blots were reprobed with a rice *25S* rDNA probe as a control to evaluate equal transfer. *PR1* was obtained from M. Yoshikawa (Department of Biology, San Francisco State University) as a 174 bp insert in the plasmid pCRII (Invitrogen, Carlsbad, CA) (unpublished data). *PR1* probe DNA was obtained by PCR amplification using the M13 forward and reverse primers. *PBZ1* (GenBank accession no. D31870) was isolated by PCR amplification using the sequence specific primers PBZ1-5 and PBZ1-6 (PBZ1-5 = 5′CATGCTACTGCTCACCTTTGA and PBZ1-6 = 5′TCATCTAGGTGGGGATATACT). PCR products were purified from agarose gels using the Geneclean Spin Kit (Bio101, Carlsbad, CA).

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