

# ***Magnaporthe grisea* interactions with the model grass *Brachypodium distachyon* closely resemble those with rice (*Oryza sativa*)**

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## **SUMMARY**

Germplasm of *Brachypodium distachyon* was inoculated with *Magnaporthe grisea* using either rice- (Guy11) or grass-adapted (FAG1.1.1, PA19w-06, PA31v-01) host-limited forms of the fungus, and interactions with varying degrees of susceptibility and resistance were identified. Ecotype ABR5 was resistant to each *M. grisea* strain whereas ABR1 was susceptible to all but P31vi-01. Mendelian segregation in ABR1 × ABR5 crosses suggested that a single dominant resistance gene conferred resistance to Guy11. Microscopic analyses revealed that the aetiology of Guy11 fungal development and disease progression in ABR1 closely resembled that of rice infections. In ABR5, Guy11 pathogenesis was first suppressed at 48 h post-inoculation, at the secondary hyphal formation stage and was coincident with cytoplasmic granulation. Resistance to strains PA31v-01 and FAG1.1.1 was associated with a localized cell death with little callose deposition. 3,3-Diaminobenzidine staining indicated the elicitation of cell death in *B. distachyon* was preceded by oxidative stress in the interacting epidermal cells and the underlying mesophyll cells. Northern blot hybridization using probes for barley genes (*PR1*, *PR5* and *PAL*) indicated that each was more rapidly expressed in ABR5 challenged with Guy11 although the *B. distachyon* defence genes *BD1* and *BD8* were more quickly induced in ABR1. Such data show that *B. distachyon* is an appropriate host for functional genomic investigations into *M. grisea* pathology and plant responses.

## **INTRODUCTION**

As a result of genome sequencing there is a pressing need to identify the roles of the multitude of genes that are being revealed. This has directly led to the current emphasis on large-scale 'functional genomic' approaches. For instance, with plant–pathogen

interactions large-scale transcript profiling using microarrays is being used to identify co-regulated gene families from which possible functions can be deduced (reviewed by Ramonell and Somerville, 2002). However, relatively few plant or pathogen genomes are likely to be sequenced in the next few years, and therefore important pathogenic interactions will remain genetically difficult to characterize for the foreseeable future. In view of this, comparative approaches based on investigating key model systems are essential to exploit fully genomic data.

When considering phytopathogenic fungi, *Magnaporthe grisea* (anamorph *Pyricularia grisea*) is emerging as a major target for genomic analysis (Martin *et al.*, 2002; Mitchell *et al.*, 2003). *M. grisea* is an economically devastating pathogen (Baker *et al.*, 1997; Dobinson and Hamer, 1993) that is the causal agent of Rice Blast but can also infect temperate cereals and forage grasses (Ou, 1985; Valent and Chumley, 1991). A wide range of pathogenicity and virulence mutants have been isolated, most affecting the formation of a viable appressorium at the hyphal tip (reviewed by Howard and Valent, 1996; Sweigard *et al.*, 1995; Talbot, 2003). Appressorial inflation to achieve phenomenal turgor pressures (as high as 8 MPa, based on the study of Howard *et al.*, 1991) involves the depletion of glycogen and lipids with the concomitant accumulation of glycerol (de Jong *et al.*, 1997; Foster *et al.*, 2003; Thines *et al.*, 2000). Glycerol acts as an osmolyte leading to the net import of water, which is retained within the appressorium by a melanized appressorial layer (de Jong *et al.*, 1997). Metabolite mobilization appears to be initiated by the PMK1 mitogen-activated protein kinase (MAPK) pathway and subsequently requires cAMP-dependent protein kinase A for turgor generation (Thines *et al.*, 2000). Turgor pressure provides the mechanical force to drive a penetration peg through the plant cuticle (Bourett and Howard, 1990) and formation of the latter is dependent on a second MAP kinase, MPS1, which is functionally related to the cell integrity MAPK from Baker's yeast, SLT2 (Xu *et al.*, 1998). Other determinants of penetration peg emergence include PDE1, a P-type ATPase (Balhadère and Talbot, 2001) and PLS, an integral transpanin membrane protein (Clergeot *et al.*, 2001).

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Resistance to *M. grisea* in rice is often dependent on gene-for-gene interactions and a range of *R* genes have been defined (Bryan *et al.*, 2000; Orbach *et al.*, 2000; Wang *et al.*, 1999). One *R* gene, *Pita*, encodes a CC-NBS-LRR class cytoplasmic receptor protein and is one of the few *R* gene products that is known to interact directly with its cognate avirulence protein, Avr-Pita, a putative metalloprotease (Jia *et al.*, 2000). Unlike other fungal-cereal interactions, resistance to *M. grisea* is only rarely associated with papillae formation (Jarosch *et al.*, 1999) but with the elicitation of a limited programmed cell death—the hypersensitive response (Koga, 1989, 1994; Peng and Shishiyama, 1988). The increased resistance exhibited by several rice spontaneous cell death mutants to infection by *M. grisea* suggests that this phenomenon is closely associated with pathogen resistance (Mizobuchi *et al.*, 2002; Takahashi *et al.*, 1999; Yin *et al.*, 2000). In dicots, cell death has been associated with the generation of an oxidative burst (Levine *et al.*, 1994) via a NADPH oxidase complex, which, in mammalian systems, is regulated by Rac-class monomeric GTP-binding proteins (Mehdy, 1994; Torres *et al.*, 2002). Similarly, in rice, a homologue of *gp91*, the cytochrome subunit of the NADPH oxidase, has been isolated (Groom *et al.*, 1996). Transgenic lines over-expressing *OsRac1* demonstrated that the HR and resistance to *M. grisea* correlated with the levels of generated H<sub>2</sub>O<sub>2</sub> (Kawasaki *et al.*, 1999; Ono *et al.*, 2001). Other defensive phenomena seen in both dicots and grasses include phytoalexin accumulation (Christensen *et al.*, 1998) and expression of defence genes such as *PR1* (Kim *et al.*, 2001), proteinase inhibitors (Cordero *et al.*, 1994) and enzymes involved in phenylpropanoid metabolism (Zhang *et al.*, 1997). Further insights into the *M. grisea*/rice interaction are likely to arise following the comprehensive analysis of extensive EST collections that are being used to investigate events occurring within the plant after pathogen penetration (Kim *et al.*, 2001; Rauyaree *et al.*, 2001).

We have recently described the attributes of *Brachypodium distachyon* and suggested it to be an appropriate model for the core-Pooids clade of grasses (Draper *et al.*, 2001), which includes such important cereal species as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). *B. distachyon* is physically small, with a short generation time and has a small genome size (~160 Mbp) with a low degree of repetitive DNA (Shi *et al.*, 1993). Recently, we have also transformed a hexaploid accession by biolistic bombardment (Draper *et al.*, 2001). Crucially, *B. distachyon* is a host

for *M. grisea* as well as other pathogens that cause disease in Pooid cereals, so that their characterization would allow similarities and dissimilarities in the different interactions to be established (Draper *et al.*, 2001). In this paper, we describe various interactions of *M. grisea* with *B. distachyon* ecotypes that could serve as targets for future functional genomic analysis. These include differing resistance mechanisms and disease symptoms that more closely resemble those of rice infections than other alternative hosts for *M. grisea*.

## RESULTS

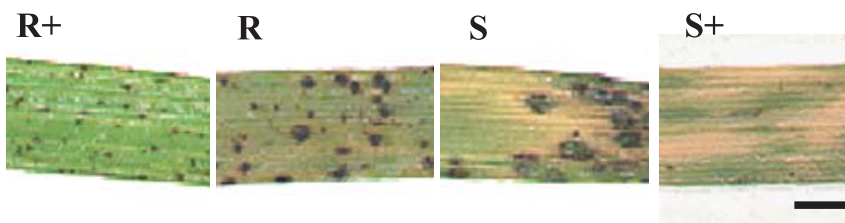
### Variable interactions within the *M. grisea* and *B. distachyon* pathosystem

To explore the range of responses that could be elicited from *B. distachyon* by *M. grisea*, three fungal strains isolated from various grass hosts (FAG1.1.1, PA31v-01 and PA19w-06) were obtained in addition to the rice pathogenic strain Guy11 (Table 1). Challenging 21 *B. distachyon* ecotypes of varying geographical origin and ploidies with Guy11 and the grass-adapted strains resulted in one of four phenotypes (Fig. 1). These phenotypes ranged from highly localized necrotic flecks (R) to varying degrees of spreading necrosis (S/S+) (Table 2). No correlation could be made between lesion phenotype and the geographical origin or ploidy of the ecotypes. Two ecotypes (ABR5 and ABR6) were resistant to all fungal strains whereas only ABR114 was universally susceptible. No *M. grisea* strain elicited either resistance or formed disease across all *B. distachyon* ecotypes.

The interactions of *M. grisea* with two diploid *B. distachyon* ecotypes—ABR1 and ABR5—were selected for further analysis. In susceptible interactions involving ABR1, foci of infection, particularly those elicited by FAG1.1.1, grew over a 4-day period

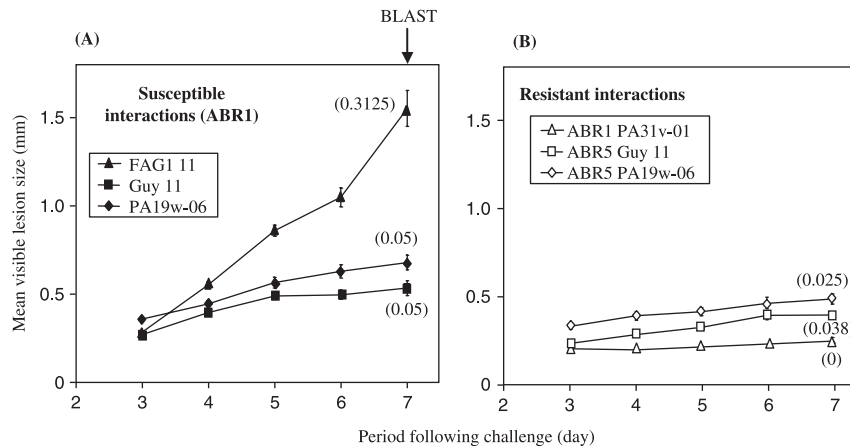
**Table 1** *Magnaporthe grisea* (anamorph = *Pyricularia grisea*) strains used in this study.

Strain	Host	Location
Guy11	Rice ( <i>Oryza sativa</i> )	French Guyana
FAG1.1.1	St. Augustine Grass ( <i>Stenotaphrum secundatum</i> )	FL, USA
PA31v-01	Crabgrass ( <i>Digitaria</i> sp.)	PA, USA
PA19w-06	Perennial Ryegrass ( <i>Lolium perenne</i> )	PA, USA



**Fig. 1** Scoring scheme used to classify the interactions of *Brachypodium distachyon* ecotypes and *Magnaporthe grisea* strains used in this study. Phenotype scores were R+, highly localized necrotic flecks; R, non-spreading lesions; S, spreading lesions; S+, rapidly spreading lesions. Bars = 0.5 cm.

**Fig. 2** Lesion growth in *Brachypodium distachyon* ecotypes ABR1 and ABR5 following challenge with different strains of *Magnaporthe grisea*. Lesion sizes ( $n \geq 200$ ) were measured in (A) susceptible interactions involving ecotype ABR1 and strain FAG1.1.1 ( $\blacktriangle$ ), Guy11 ( $\blacksquare$ ) and PA19w-06 ( $\blacklozenge$ ), and (B) resistant interactions involving ecotype ABR1 and PA31v-01 ( $\triangle$ ) and also ABR5 with Guy11 ( $\square$ ) and PA19w-06 ( $\diamond$ ). Results are given as mean lesion size  $\pm$  SE. Lesion growth rates (mm day<sup>-1</sup>) are given in parentheses.



(3–7 days post-inoculation). After 7 days plants started to exhibit blast symptoms whereupon lesion growth effectively ceased (Fig. 2A). Resistant interactions were so designated based on the failure to exhibit blast symptoms or chlorosis. Visible lesions were never observed in ABR5 challenged with FAG1.1.1 or PA31v-01. In all other interactions, macro-lesion formation was also first observed at 3 days, with no significant difference in initial lesion sizes ( $P = 0.84$ ). However, some lesion growth was observed (Fig. 2B), although this was less than with susceptible interactions. Only in the interaction of PA31v-01 with ABR1 did lesions fail to grow once they became visible.

**Resistance to Guy11 in ABR5 is conferred by a single dominant resistance gene**

To establish the genetic basis of resistance in ABR5 to *M. grisea* strain Guy11, reciprocal crosses were carried out with the susceptible ecotype, ABR1. Successful crosses were detected at the F<sub>1</sub> stage based on an RFLP between the parental lines detected using a *B. distachyon* *BD8* gene probe (see Fig. 6A). Challenging the F<sub>2</sub> progeny of eight segregant families with Guy11 yielded responses that corresponded to either of the parental phenotypes (Table 3). Although the number of crosses was small, no maternal effects

**Table 2** Variable responses by ecotypes of *Brachypodium distachyon* to challenge with strains of *Magnaporthe grisea* (anamorph = *Pyricularia grisea*).

<i>B. distachyon</i> ecotype (ABR)	Chromosome no.	Origin	Responses to challenge with:			
			Guy11	FAG1.1.1	PA19w-06	PA31v-01
1	10	Kaman, Turkey	S	S+	S	R+
2	10	Octon, France	I	n.d.	n.d.	n.d.
3	10	Huesca, Spain	S	I	S	R
4	10	Huesca, Spain	S	S	S	R
5	10	Huesca, Spain	R	R+	R+	R+
6	10	Navarra, Spain	R	R	R+	R
9	10	Ljubljana, Croatia	S	S	S	I
11	10	Soma, Turkey	S	S	I	I
12	10	Iraq	I	I	R+	I
13	10	Iraq	I	R	n.d.	I
14	10	Turkey	S	n.d.	n.d.	n.d.
15	10	Iraq	S	S	S	R
100	30	Kalafabad, Iran	S+	I	n.d.	S
101	30	Darling, South Africa	R	S	R+	I
103	30	Shushtar, Iran	S	n.d.	R+	n.d.
104	30	Pabbi, Pakistan	S	n.d.	n.d.	n.d.
105	30	Ongda, Morocco	S	S	R+	S
106	30	Ex USDA, Uruguay	R	n.d.	I	n.d.
107	30	Ex USDA, Greece	R	n.d.	I	n.d.
108	30	Kaschmar, Iran	S	n.d.	R+	n.d.
109	30	Iraq	S	S	S	R
114	20	Formenterra, Spain	S	S	S	S

Phenotype scores were: R+, highly localized necrotic flecks; R, non-spreading lesions; S, spreading lesions; S+, rapidly spreading lesions; I, no observable response; n.d. = not done.

**Table 3** Responses of F<sub>2</sub> generation ABR1 × ABR5 hybrid families to *Magnaporthe grisea* Guy11.

Family	♂	♀	n	R	I	S
1	ABR5	ABR1	63	44	0	19
2	ABR5	ABR1	32	26	0	6
3	ABR5	ABR1	25	18	1	6
4	ABR5	ABR1	95	69	0	26
5	ABR1	ABR5	24	18	1	5
6	ABR1	ABR5	24	19	1	4

Reciprocal crosses of *Brachypodium distachyon* ecotypes ABR1 and ABR5 were generated as described in the text. F<sub>1</sub> seeds gathered from a single plant were designated hybrid families. These plants were grown and the F<sub>2</sub> seeds were collected. Seedlings grown from F<sub>2</sub> seed were challenged with *M. grisea* Guy11 and scored after 7 days. Abbreviations: R, resistant—phenotype equivalent to that seen in ABR5; S, susceptible—phenotype equivalent to that seen in ABR1; I, intermediate—phenotype like neither ABR1 nor ABR5.

were noted when using ABR1 or ABR5. A number of scenarios based on one or two dominant or recessive alleles were tested statistically (chi square) and the data were consistent with that expected from a single dominant resistance determinant segregating from a homozygous parent ( $\chi^2_{3,1} = 1.22$ ). Tillering this segregant population also allowed the resistance determinants to FAG1.1.1 to be assessed. The results proved to be difficult to interpret but apparently involved multiple elements, none of which was involved in the resistance to Guy11 (G.S., unpublished data).

### Different defence phenotypes are seen in *B. distachyon* ecotypes following challenge by *M. grisea*

Following challenge of both ecotypes with Guy11 approximately 35% conidia germinated and underwent appressorium formation (Fig. 3A). Penetration of epidermal cells and the formation of primary infection hyphae occurred between 24 and 48 h on both ecotypes (Fig. 3B,C). The formation of secondary hyphae at 48 h post-inoculation was the first stage in fungal development at which differences were observed between ABR1 and ABR5 (Fig. 3C,D). With ABR1 only, secondary hyphae filled the first infected epidermal cell prior to penetration of a second plant cell (Fig. 3E). Second cell penetration occurred at ~48 h and was more commonly seen in ABR1 (Fig. 3F). Bulbous hyphal tips (such as seen in Fig. 3E) were frequently observed at the fungal penetration front. In ABR1 infections, macroscopic lesion formation at 72 h was associated with considerable fungal penetration into the surrounding tissue. In ABR5, visible lesion formation seemed to be primarily due to plant cell death and there was no significant further hyphal growth after 48 h. Similar features were observed following challenge with FAG1.1.1, except that in ABR1 fungal colony growth proved to be more extensive (data not shown).

In examining features classically associated with resistance it was noted that plant cell walls in both ecotypes exhibited

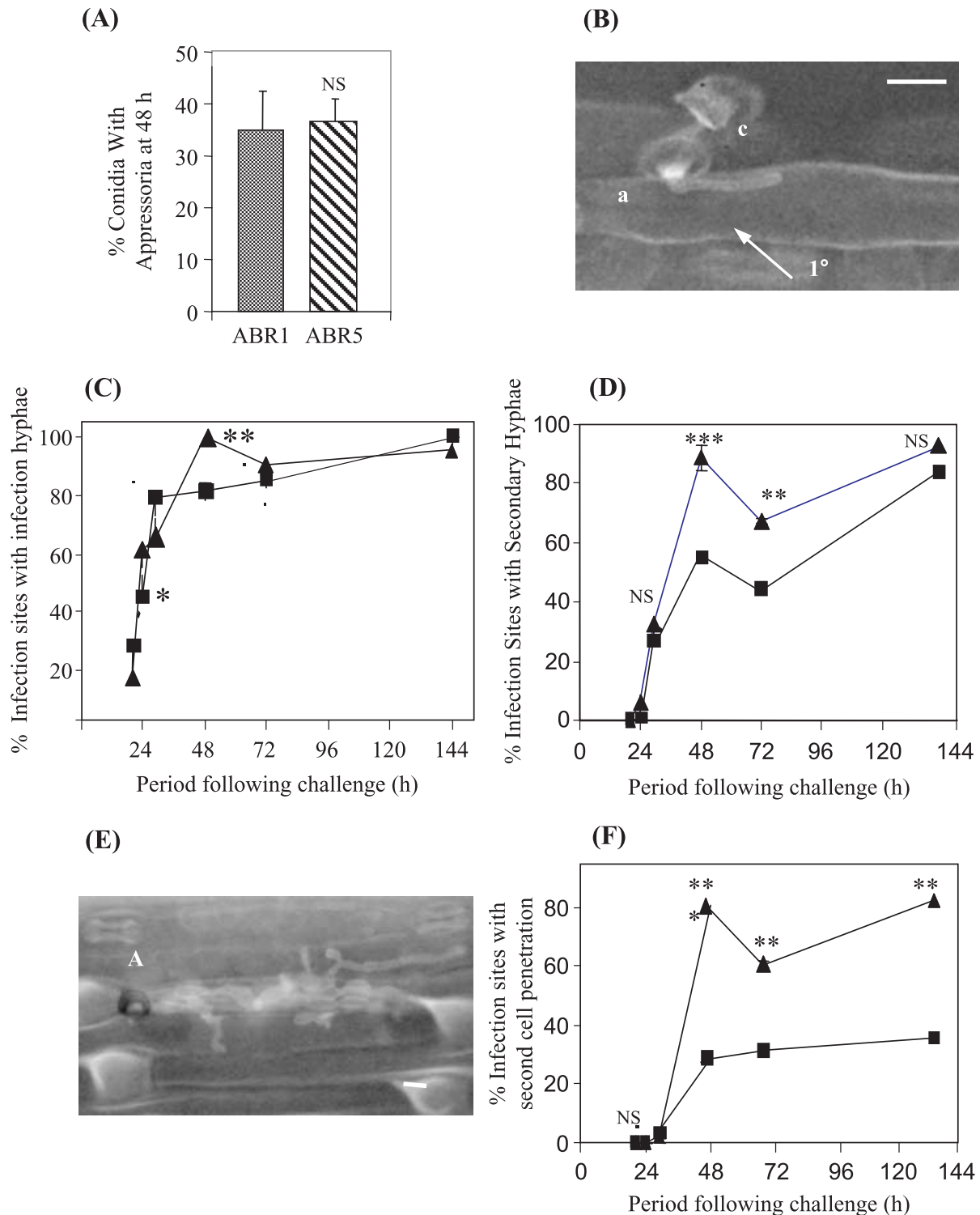
heightened autofluorescence (Fig. 4A,B) following challenge with *M. grisea* Guy11. At 48 h, however, more fungally interacting cells in ABR5 were autofluorescent (Fig. 5A). Cytoplasmic granulation (Fig. 4C), within which secondary hyphal formation could be observed (Fig. 4D), was seen almost exclusively in ABR5 undergoing a resistant response (Fig. 5B). At 48 h transverse sections of interaction sites exhibited chlorophyll loss in both epidermal and the underlying mesophyll cells (Fig. 4E). It is likely that both cytoplasmic granulation and chlorophyll loss are indicative of cell death. Using DAB (3,3-diaminobenzidine) stain to reveal oxidative stress indicated some staining in the mesophyll cells immediately underlying the interacting epidermal cell at 24 h (Fig. 4F). At 48 h only epidermal cells were stained with DAB (Fig. 3G). DAB staining was not observed in developing infections on ABR1 (data not shown).

When challenging ABR5 with FAG1.1.1, cell death occurred much more rapidly than with Guy11. Trypan blue staining indicated that plant cell death was occurring within 24 h of challenge (Fig. 4H), followed by cytoplasmic condensation leading to the formation of rounded necrotic bodies at 48 h (Fig. 4I). Subsequently, limited cell death also occurred in the surrounding epidermal cells and in the underlying mesophyll cells (Fig. 4J).

Examining callose deposition in Guy11-challenged *B. distachyon* ABR5 suggested that this was a feature primarily seen at the later stages of necrotic lesion formation (Fig. 5C). Fluorescent aniline blue staining revealed that in ABR5, necrotic lesion areas correlated with the formation of a distinctive ring of callose in mesophyll cells and in the overlying epidermal cells (Fig. 4K,L). Such coordinated callose deposition was not a feature of lesions forming on the susceptible ABR1 nor resistant interactions involving FAG1.1.1 or PA31v-01 (data not shown).

### Differential defence gene expression in *B. distachyon* challenged *M. grisea*

To further investigate possible resistance mechanisms that could be being deployed at 48 h against Guy11 in ABR5, we examined defence gene expression in both ecotypes. This employed heterologous probes for *PR1*, *PRR* (hereafter referred to by the generic designation, *PR5*) and *PAL* as well as genes isolated from *B. distachyon*. *B. distachyon* defence genes were isolated from a cDNA library constructed from mRNA isolated from ABR5 challenged with *M. grisea* Guy11. This was differentially screened with cDNA derived from mRNA extracted from pathogen-challenged and un-inoculated ABR5. Ten cDNA clones tentatively identified as representing differentially expressed genes were sequenced (Table 4). 'Dot-blot' Northern analysis indicated that the expression of *BD1* (unknown function) and *BD8* (either a xylanase inhibitor protein or a class III chitinase), in particular, was significantly up-regulated upon inoculation with *M. grisea* (data not shown).



**Fig. 3** The *in planta* development of *Magnaporthe grisea* Guy11 in *Brachypodium distachyon* ecotype ABR1 and ABR5. (A) Percentage conidia developing an appressorium at 48 h post-inoculation in ecotypes ABR1 (shaded) and ABR5 (hatched). (B) Plant cell penetration and primary hyphal formation. Indicated are conidia (c), the primary infection hypha (1°) and appressorium (a) (Bar = 10 µm). Percentage sites exhibiting (C) primary and (D) secondary infection hyphae in ecotype ABR1 (▲) and ABR5 (■). (E) Plant cell filling by fungi prior to second cell penetration. Indicated is the appressorium (A) (Bar = 10 µm). (F) Percentage infection sites exhibiting second cell penetration in ecotype ABR1 (▲) and ABR5 (■). Population sizes were always > 50 and results are given as mean percentages. Non-significant differences are indicated by NS ( $P \geq 0.05$ ) and significant observations are indicated by \*\* ( $P \leq 0.01$ ) and \*\*\* ( $P \leq 0.001$ ).

**Table 4** *Brachypodium distachyon* ecotype ABR5 clones isolated from a *Magnaporthe grisea* challenge cDNA library.

Clone	Homology	E value
BD1	<i>Hordeum vulgare</i> ESTs	
	AL511829; AW982536	2e-21; 3e-20
	<i>Triticum aestivum</i> EST Be399572	4e-10
BD7	D30027 <i>Oryza sativa</i> EN448, E2 ubiquitin-conjugating enzyme	7e-49
BD8	CAD19479 <i>Triticum aestivum</i> xylanase inhibitor protein I	3e-10
	JC5845 <i>Oryza sativa</i> Chitinase III (EC 3.2.1.14)	1e-06
BD2	Unknown	
BD4	Unknown	
BD5	Unknown	
BD6	Unknown	
BD9	Unknown	
BD10	Unknown	

cDNAs, designated BD1–10, which represented genes that were up-regulated by *M. grisea* Guy11 in *Brachypodium distachyon* ecotype ABR5, were sequenced. The derived sequence was used in BLAST searches using the NCBI database (<http://www.ncbi.nlm.nih.gov>). The BLAST expectation (*E*) values are indicated for those sequences exhibiting the highest homology to the BD clones. Sequences where no significant matches were found (*E* value > 0.001) are labelled as unknown.

Prior to Northern analyses each gene was used to probe Southern blots of genomic DNA from ABR1 and ABR5 (Fig. 5A). Each *B. distachyon* gene probe yielded a simple banding pattern as shown for *BD8* in Fig. 6A. This was consistent with the simple genomic organization of *B. distachyon* tending towards little gene repetition (Shi *et al.*, 1993). A restriction fragment length polymorphism was detected in *Hind*III-digested and *BD8*-probed genomic DNAs. In order to obtain clear banding patterns for *PR1* and *PR5*, lower stringency hybridization conditions were required. As shown for *PR5* (Fig. 6A), this resulted in a higher degree of background hybridization, although again, a simple banding pattern was suggested. However, hybridization with *PAL* revealed a very complicated banding pattern that suggested the existence of a multigene family (Fig. 6A).

Northern blotting analysis of Guy11-induced gene expression in ABR5 indicated that both *PR5* and *PR1* were expressed, respectively, at around 72 and 96 h. In both cases this proved to be ~24 h quicker than in ABR1. Interpretation of the results obtained when

probing with *PAL* was complicated by the probable existence of a multigene family that could have differential expression patterns. Nevertheless, band intensities suggested higher expression in ABR5 than in ABR1, particularly 72 h following inoculation. However, with both *BD1* and *BD8* more rapid and greater expression was observed with *M. grisea*-challenged ABR1 where disease symptoms were forming.

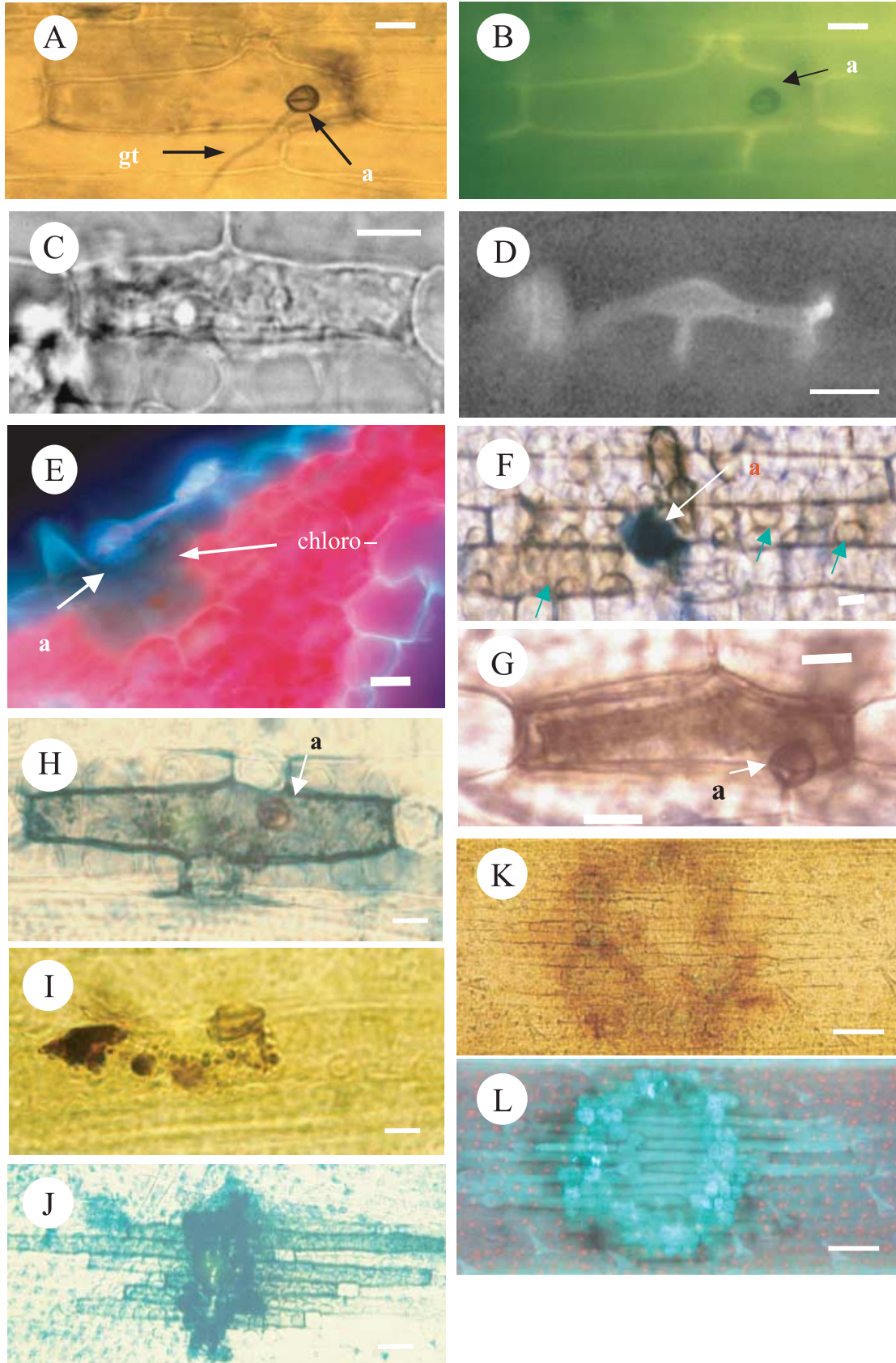
Given that heterologous *PR1* and *PR5* gene probes were utilized, we were concerned that these could be relatively insensitive probes for gene expression. Thus, based on the assumption that amino acid sequences may be more highly conserved, protein extracts from Guy11-challenged ABR1 and ABR5 were probed with antisera raised against tobacco PR proteins. Both anti-*PR1* and *PR5* antibodies detected pathogen-induced proteins of approximately the same size as their putative equivalents in tobacco (Fig. 6C). *PR1* and *PR5* antibody-targeted protein accumulation correlated with the Northern blotting data detected using the heterologous gene probes.

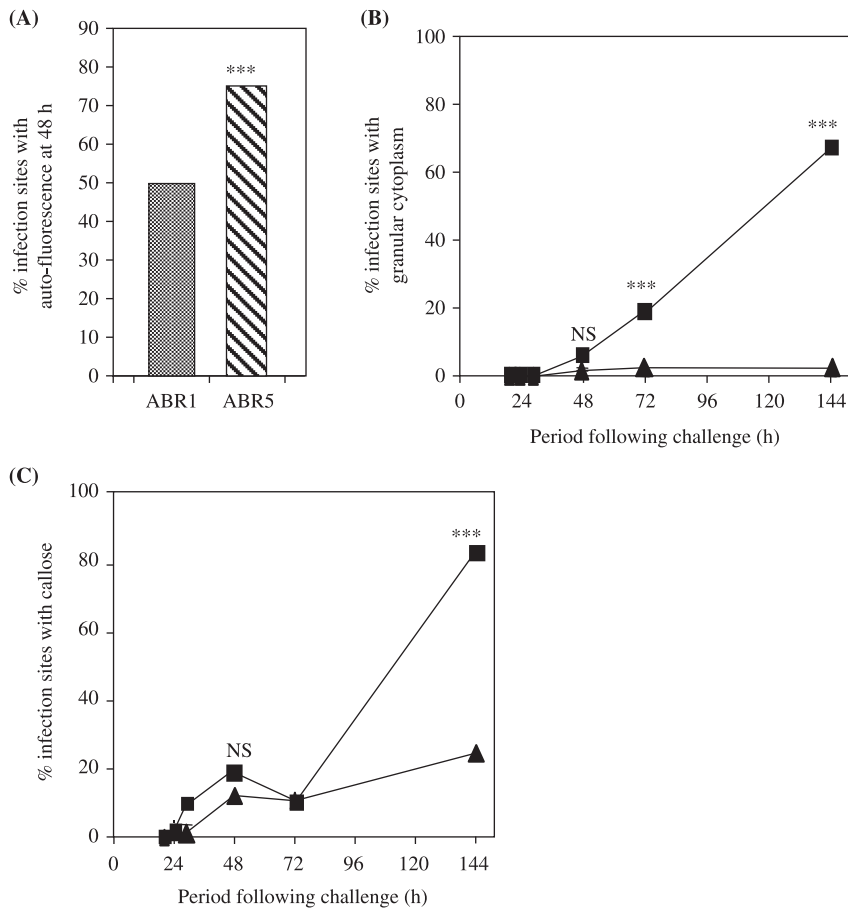
## DISCUSSION

The *M. grisea*/rice pathosystem is perhaps the best characterized fungal–cereal interaction, and with the considerable genomic resources developed for both host and pathogen, further progress will undoubtedly continue to be made (Talbot, 2003). Nevertheless, the observed interactions of *B. distachyon* with *M. grisea* offer additional opportunities for progress in this field (Draper *et al.*, 2001). *B. distachyon* offers an alternative host that, unlike rice, is physically small and easy to grow, which could allow *M. grisea* research projects to be undertaken by a wider number of laboratories. More importantly, *B. distachyon* is a host for a range of pathogens of temperate cereals including *Blumeria graminis* (Braun, 1995), *Puccinia coronata*—which elicits crown rust in forage grasses (J.V.S., unpublished data)—as well as variably interacting with both brown rust (*Puccinia recondita*) and yellow rust (*P. striiformis*) (Draper *et al.*, 2001). This will allow responses of the same plant species to a range of cereal fungal pathogens to be compared and contrasted.

This paper describes the first stages in our characterization of the *B. distachyon*/*M. grisea* interaction. Besides *M. grisea* strain Guy11, our analyses included forage grass-adapted asexual *P. grisea* strains that were isolated from Gray Leaf Spot diseased

**Fig. 4** Microscopic analysis of the fungal development and plant responses during the exhibition of resistance to *Magnaporthe grisea* strains in *Brachypodium distachyon* ecotype ABR5. (A) White-light and (B) UV-light illumination of a cell underlying a developing Guy11 appressorium at 24 h post-challenge. Indicated are the appressorium (a) and germ-tube (gt). (C) Aniline-blue-stained cell exhibiting granular cytoplasm formation at 48 h (D) within which the developing Guy11 hyphum was viewed on UV illumination. (E) A calcofluor-white-stained transverse view of plant cells to Guy11 at 24 h under UV light. Indicated are the fungal appressorium (a) and plant cells with no chlorophyll fluorescence (chloro<sup>-</sup>). 3,3-Diaminobenzidine (DAB) stained Guy11-challenged cells at (F) 24 h and (G) 48 h post-inoculation (appressoria are indicated by white arrows and DAB-stained mesophyll cell with green arrows). Trypan-blue-stained cells following challenge with FAG1.1.1 after (H) 24 h, (I) 48 h and (J) 144 h (bars = 10 µm). Epifluorescent aniline-blue-stained ecotype ABR5 challenged with strain Guy11 illuminated with (K) white and (L) UV light (bars = 100 µm).



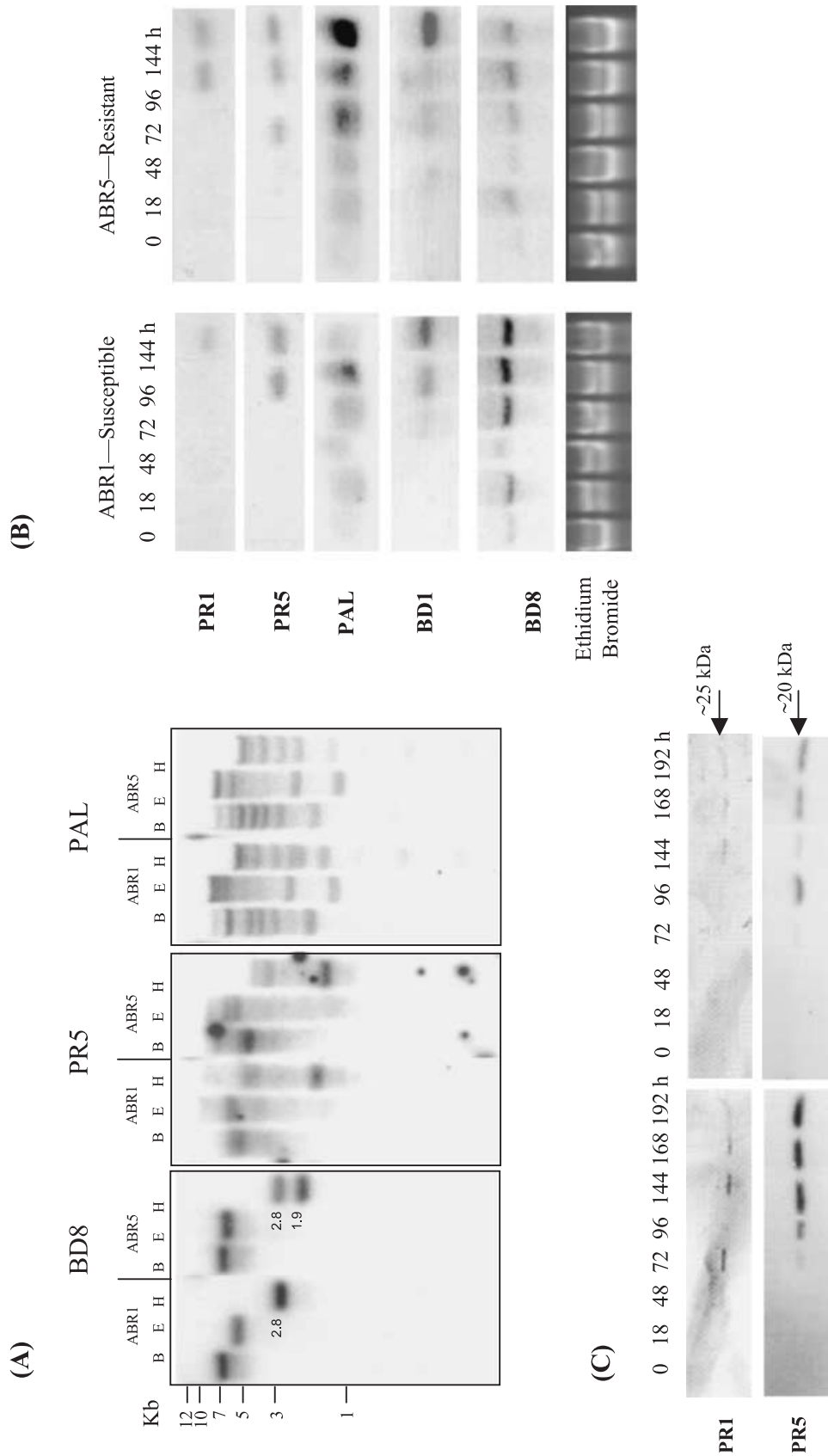


**Fig. 5** Features in the defence response of *Brachypodium distachyon* ecotypes ABR1 and ABR5 to *Magnaporthe grisea* Guy11. Percentage penetration sites displaying (A) autofluorescence at 48 h, (B) granular cytoplasm and (C) callose deposition in ecotype ABR1 (shaded, ▲) and ABR5 (hatched, ■). Population sizes were always > 50 and results are given as mean percentages. Non-significant differences are indicated by NS and significant observations are indicated by \*\*\* ( $P \leq 0.001$ ).

plants (Uddin *et al.*, 1999). However, despite the variation in fungal genotypes, the responses of *B. distachyon* ecotypes could be broadly classified into either resistant or susceptible responses. Crucially, developing fungal infection structures in ABR1 indicated that macro- or microscopic disease development in *B. distachyon* resembled that in rice (Talbot, 1995). These included the formation of bulbous hyphae, which may represent rudimentary haustoria, and the filling of the primary infected cell with haustoria prior to second cell penetration (Talbot, 1995, 2003). The *in planta* events occurring within the fungus are currently obscure but secondary hyphal formation is influenced by the MAPK, PMK1 (Xu and Hamer, 1996). PMK1 exhibits homology to the yeast MAPK proteins—FUS3 and KSS1—that with the transcription factor STE12 contribute towards pseudohyphal growth (reviewed by Gancedo, 2001). An STE12 homologue, MST12, has been found in *M. grisea* the mutation of which suppresses penetration peg emergence from appressoria and subsequent *in planta* hyphal development (Park *et al.*, 2002). Further understanding of the processes involved must be a prime target for functional genomic analyses.

When analysing resistance to *M. grisea* in *B. distachyon*, it became clear that this did not preclude considerable fungal

development prior to its arrest. Microscopic analysis indicated that pre- and early post-penetrative events in Guy11-challenged ABR1 and ABR5 were identical. Only at 48 h was there a suppression of secondary hyphal formation in ABR5 and a range of phenomena were examined as a possible source of this resistance. Cell wall autofluorescence has been associated with resistance to fungal pathogens (Koga, 1994; Lyngkær and Carver, 1999) but a considerable number of cells were autofluorescent in Guy11-challenged ABR1 (Fig. 6A). Instead, the suppression of secondary hyphae in ABR5 more clearly correlated with the formation of granular cytoplasm that has been reported to precede cell death in rice and Weeping Lovegrass infections by *M. grisea* (Heath *et al.*, 1990, 1992; Koga, 1994; Peng and Shishiyama, 1988). However, the kinetics of cell death in response to Guy11 infection may be slow enough to allow fungal penetration of the underlying mesophyll cells with its resulting death. Significantly, in PA31vi-01 and FAG1.1.1, in which cell death in the primary infected cell was rapid, there was initially little cell death seen in the mesophyll cells. In ABR5 challenged with Guy11, a distinctive mesophyll callose ring was only seen > 72 h in ABR5, indicating that this could only play a late role in confining Guy11 to the lesion and preventing blast symptom development. The failure of



**Fig. 6** Genomic structure and *Magnaporthe grisea*-induced expression of homologous and heterologous defence genes in *Brachypodium distachyon*. (A) Southern hybridization of *Bam*HI (B), *Eco*RI (E) and *Hind*III (H) digested genomic DNA from *B. distachyon* ecotypes ABR1 and ABR5 and screened with probes encoding *B. distachyon* *BD8* (a *Hind*III RFLP between the two ecotype DNAs is indicated by labelled hybridizing fragment sizes), barley *PRR* (*PR5*) and phenylalanine ammonia lyase (*PAL*). (B) Northern analysis of barley *PR1*, *PR5*, *PAL* and *B. distachyon* *BD1* and *BD8* transcript accumulation in ecotypes ABR1 and ABR5 following challenge with *M. grisea* Guy11. The ethidium-bromide-stained gels are illustrated as an indicator of equal loadings. (C) Proteins from *M. grisea* Guy11-challenged ABR1 and ABR5 were screened with antisera to tobacco *PR1* and *PR5* following SDS-PAGE and Western blotting. Indicated are the deduced molecular weights of the putative *B. distachyon* pathogenesis-related proteins.

PA31vi-01- and FAG 1.1.1-elicited lesions to exhibit coordinated callose accumulation further suggested that plant cell death was the major determinant of resistance. Similarly, in rice, plant cell death has been correlated with the cessation of *in planta* mycelial growth of *M. grisea* (Koga, 1989).

An alternative/complementary hypothesis is that resistance is dependent on defence gene expression. Our Northern hybridization and Western blotting experiments, however, failed to place defence gene expression within the key 48 h post-inoculation period for resistance. This was the case although these analyses included some key defence genes such as PR protein genes and phenylalanine ammonia lyase (*PAL*) (Dong *et al.*, 1991; Ward *et al.*, 1991). Nevertheless, it may be that defence gene expression localized to the site of attempted infection needs to be assessed before their contribution to overall resistance can be accurately determined.

How far cell-specific events are dependent upon fungal contact or the propagation of plant-derived chemical signals requires further investigation. DAB staining indicated early changes in oxidative stress in the mesophyll cells underlying the infected epidermal cells, which suggested a role for intercellular signalling rather than fungal contact. Vanacker *et al.* (2000) have made similar observations in the mesophyll underlying papillae-forming cells in *B. graminis*-challenged barley, in which there is no possibility of fungal contact. With oxidative events being associated with the initiation of cell death (Levine *et al.*, 1994) the spatial control of H<sub>2</sub>O<sub>2</sub> generation could be an important determinant in resistance to *M. grisea* and other fungal pathogens.

The observation that a single genetic determinant mediated resistance to Guy11 in ecotype ABR5 suggested that it could soon be possible to define gene-for-gene interactions between *B. distachyon* and *M. grisea*. Currently around 20 resistance genes to Blast-eliciting fungal species have been defined (Chao *et al.*, 1999; Yu *et al.*, 1996) and some 15 avirulence genes (Farman *et al.*, 2002; Orbach *et al.*, 2000; Sweigard *et al.*, 1995). Two avirulence genes have been detected in *M. grisea* strain Guy11, with *AVR1-Irat1* conferring resistance to rice cultivars DJ-341, *Irat7* and Carreon (Dioh *et al.*, 2000) and *PWL2* interacting with determinant(s) in the alternative host Weeping Lovegrass (Sweigard *et al.*, 1995). The use of *avr* gene knockouts could allow the nature of the gene-for-gene interaction involving Guy11 and *B. distachyon* ABR5 to be defined.

To conclude, several alternative hosts to rice have been described for *M. grisea* from temperate cereals such as wheat, barley and grasses, for example Weeping Lovegrass (Jarosch *et al.*, 1999; Urashima *et al.*, 1993; Valent *et al.*, 1991). These often display features not seen in rice infection, such as a papilla-based resistance (Heath *et al.*, 1990; Jarosch *et al.*, 1999), exhibition of poor *in planta* hyphal development or delayed cell death (Heath *et al.*, 1990). This was not the case with both compatible and incompatible interactions of *B. distachyon* with *M. grisea*. Thus, it is appropriate to investigate features associated with *M. grisea*

virulence as well as various resistance mechanisms in *B. distachyon*. We are currently developing post-genomic resources, for example microarrays, to aid in these investigations. As already stated, such resources may also be very useful in projects involving other pathogens.

## EXPERIMENTAL PROCEDURES

### Plant growth conditions

All *B. distachyon* ecotypes were grown as described in Draper *et al.* (2001). Following pathogen challenge, plants were grown in Fi-totron 600H environmental cabinets (Fisons, Loughborough, UK) with 6 × 40-W lighting tubes (Osram, Slyvania, Munich, Germany) to give fluence rates of 160 μmol m<sup>2</sup>/s at seedling level.

### *M. grisea* strains, inoculation technique and lesion scoring

*M. grisea* strains FAG1.1.1, PA31v-01 and PA19w-06 were first isolated by Waddar Uddin (Penn State, USA) and are first described in this work. These strains and Guy11 were cultured on Potato Dextrose Agar (Sigma, Poole, UK) in a Gallenkamp Illuminated Cooled Incubator (Loughborough, UK) at 22 °C with a 16-h day length. Spores were harvested into 0.2% (w/v) gelatin, diluted to 10<sup>5</sup> spores/mL and sprayed on to 3–4-week-old *B. distachyon* seedlings to run-off using a artist air-brush (Humbrol Ltd, UK). Inoculated plants were kept in clear polythene bags to maintain high humidity for 18 h, following which the bag was removed.

### Crossing *B. distachyon* ecotypes

Interecotype crosses were produced by hand pollination aided by a Nikon SMZ-10 microscope (Nikon, Tokyo, Japan). Diploid ecotype maternal parent plants were taken 2–3 weeks after vernalization about 1 week prior to anthesis. The pella and lemma were prised apart using watchmakers forceps (Dumont No. 5 extra fine, Agae Scientific, Stanstead, UK) and the inflorescence emasculated. Anthers at the point of anthesis, which had not emerged from within the inflorescence, were removed from the paternal parent 3–4 weeks after vernalization and placed on the stigma of the maternal parent. Any unfertilized inflorescences were removed from the maternal parent. The flower head was then covered with a paper/cellophane bag and mature seed collected after 3–4 weeks.

### Histochemical staining with trypan blue, analine blue, calcafluor white and 3,3-diaminobenzidine

Cell death was identified by trypan blue staining essentially as described by Koch and Slusarenko (1990). Cut samples were immersed in 0.1% (w/v) trypan blue, in lactoglycerol (1 : 1 : 1,

lactic acid–glycerol–H<sub>2</sub>O) and microwaved in a domestic oven at full power for 10 s. The leaves were removed and then cleared in a 2.5 g/mL chloral hydrate solution.

Aniline blue staining was based on the protocol developed by Hood and Shew (1996). Samples were cut into 1.5-cm lengths and autoclaved (Astell Scientific Ltd, Kent, UK) at 121 °C in 1 M KOH for 2 min. Following rinsing three times in sterile distilled water, the samples were mounted on glass slides in several drops of freshly prepared 0.05% water-soluble aniline blue dye (No. 12642 George T. Gurr Ltd, London, UK) in 0.067 M K<sub>2</sub>HPO<sub>4</sub>, pH 9.

Calcofluor white stain was used as described by Rohringer *et al.* (1977). Leaves were sectioned then fixed and cleared by boiling for 1.5 min in lactophenol/ethanol (1 : 2, v/v) and stored overnight at room temperature. Specimens were washed twice for 15 min each with 50% ethanol, then with 0.05 M NaOH and finally equilibrated in 0.1 M Tris-HCl (pH 8.5) for 30 min. The samples were then stained for 5 min with a 0.1% solution of calcofluor white (Sigma, Poole, UK) in 0.1 M Tris-HCl (pH 8.5). The samples washed four times in sterile dH<sub>2</sub>O (10 min each) and finally with 25% aqueous glycerol (30 min) before being mounted in glycerol on a glass slide.

For staining with 3,3'-diaminobenzidine (DAB; Sigma, MO, USA) leaves were excised and placed in a water-filled 50-mL beaker for 4 h to avoid the formation of embolisms. The seedlings were transferred to 50-mL beakers with 1 mg/mL DAB, pH 3.8, and left for a further 1 h. The leaves were then fixed and cleared in (3 : 1, v/v) ethanol–acetic acid. For all stained material, a cover slip was placed over the top and sealed with clear nail varnish.

Mounted samples were examined using an Axioplan epifluorescence microscope (Zeiss, Jena, Germany) with white light or UV illumination.

All presented images of strained material were representative of at least ten similar results.

### cDNA library construction and differential screening

Total RNA was extracted from *M. grisea*-challenged *B. distachyon* ecotype ABR5 leaves sampled after 18, 48, 72, 96 and 144 h as described in Draper *et al.* (1988) and pooled. cDNA and the lambda ZAP II library was constructed as indicated by the manufacturer of the kit used (Stratagene, La Jolla, CA, USA). The cDNA library was differentially screened with <sup>32</sup>P-labelled first-strand cDNA generated using Superscript II (Gibco-BRL, Paisley, UK) from 0.2% gelatin-treated (144 h) plants and subsequently from the pooled RNAs used to construct the library as described by Warner *et al.* (1992).

### Probing and Northern, Southern and Western blots

Southern and Northern blotting and hybridization were carried out as in Draper *et al.* (1988). Probes were prepared by oligonucleotide labelling as stated by the kit manufacturer (Gibco-BRL,

UK). Western blotting experiments were undertaken as stated in Warner *et al.* (1994).

### Statistical analyses

Data were analysed for significance using one-way ANOVA. Percentage data were converted to a parametric distribution by arc sine square root transformation prior to ANOVA. Segregation data were analysed using a chi square test. Analyses were carried out on Minitab v.13 or Excel.

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