Histological and molecular analysis of *Rdg2a* barley resistance to leaf stripe

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SUMMARY

Barley (*Hordeum vulgare* L.) leaf stripe is caused by the seed-borne fungus *Pyrenophora graminea*. We investigated microscopically and molecularly the reaction of barley embryos to leaf stripe inoculation. In the resistant genotype NIL3876-*Rdg2a*, fungal growth ceased at the scutellar node of the embryo, while in the susceptible near-isogenic line (NIL) Mirco-*rdg2a* fungal growth continued past the scutellar node and into the embryo. Pathogen-challenged embryos of resistant and susceptible NILs showed different levels of UV autofluorescence and toluidine blue staining, indicating differential accumulation of phenolic compounds. Suppression subtractive hybridization and cDNA amplified fragment-length polymorphism (AFLP) analyses of embryos identified *P. graminea*-induced and *P. graminea*-repressed barley genes. In addition, cDNA-AFLP analysis identified six pathogenicity-associated fungal genes expressed during barley infection but at low to undetectable levels during growth on artificial media. Microarrays representing the entire set of differentially expressed cDNA-AFLP fragments and 100 barley homologues of previously described defence-related genes were used to study gene expression changes at 7 and 14 days after inoculation in the resistant and susceptible NILs. A total of 171 significantly modulated barley genes were identified and assigned to four groups based on timing and genotype dependence of expression. Analysis of the changes in gene expression during the barley resistance response to leaf stripe suggests that the *Rdg2a*-mediated response includes cell-wall reinforcement, signal transduction, generation of reactive oxygen species, cell protection, jasmonate signalling and expression of plant effector genes. The identification of genes showing leaf stripe inoculation or resistance-dependent expression sets the stage for further dissection of the resistance response of barley embryo cells to leaf stripe.

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INTRODUCTION

Seeds are potentially a rich source of food for plant pathogens, and are particularly prone to attack during development and germination. In addition, transmission of a disease through the seed provides a direct route to infect the next generation. Accordingly, seeds are important sites of plant defence. Pathogen-free seeds express genes otherwise known to be associated with pathogen infection in other tissues, which presumably contribute to pre-formed or primed systems for defending the seed against pathogen attack. A transcriptome analysis of pathogen-free barley embryos 12–37 days after flowering identified at particular developmental stages expression of pathogenesis-related (PR) genes encoding enzymes from the shikimate pathway involved in the production of phytoalexins, and genes providing protection against reactive oxygen species (ROS) produced during pathogen defence (Nielsen *et al.*, 2006). During the early stages of barley seed germination, proteins with antifungal activity are released from embryos and surrounding grain tissues (Leah *et al.*, 1991; Swegle *et al.*, 1992). Embryos of germinating grain were also found to express the H_2O_2-generating germin-like oxalate oxidase (Caliskan and Cuming, 1998) and a gene for lipoxygenase 1 known to be involved in the methyl-jasmonate defence pathway (Rouster *et al.*, 1998).

Changes in gene expression have been observed in maize embryos as a result of inoculation with *Fusarium verticillioides* (synonym, *F. moniliforme*). mRNAs encoding a proteinase inhibitor were found to accumulate in germinating maize embryos upon fungal infection (Cordero *et al.*, 1994) and transcripts for a

calcium-dependent protein kinase and the Prm class of PR proteins were shown by *in situ* mRNA hybridization to accumulate in scutellum epithelial cells of maize embryos in response to fungal infection and elicitor treatment (Murillo *et al.*, 2001). Proteome profiling identified 47 proteins in maize embryos showing altered steady-state levels in response to challenge with *F. verticillioides* (Campo *et al.*, 2004). The 36 up-regulated proteins and 11 down-regulated proteins included proteins involved in protection from oxidative stress, protein synthesis and stabilization, sugar metabolism and defence. These represent factors that potentially contribute to induced defences in the seed.

Leaf stripe, caused by the seed-transmitted fungus *Pyrenophora graminea*, is a serious disease of barley which typically causes the most severe problems when low temperatures are experienced during sowing or when organic farming methods are used (Porta-Puglia *et al.*, 1986). The fungal mycelia survive in seeds between the parenchymatic cells of the pericarp. During germination, the growing mycelia penetrate the coleorhiza and, in susceptible genotypes, colonize the plant systemically starting from the root tip. Infection spreads into the young leaves, where it causes longitudinal dark brown stripes that form between the leaf veins. Spores produced on the infected leaves of susceptible plants during flowering spread to infect nearby heads. No secondary cycles are present in this disease. In incompatible interactions controlled by major resistance genes, the hyphae degenerate in the basal part of the coleorhiza (Platenkamp, 1976) and no stripe symptoms appear on the leaves of young or old plants (Bulgarelli *et al.*, 2004). The fungus produces a host-specific glycosyl compound (*Pg* toxin) which, when infiltrated into barley leaves of susceptible or resistant genotypes, induces the formation of lesions resembling those produced by infection with the pathogen (Haegi and Porta-Puglia, 1995). Both polygenic partial resistance (Arru *et al.*, 2002, 2003; Pecchioni *et al.*, 1996) and strong monogenic race-specific resistance (Giese *et al.*, 1993) have been identified and mapped. The major resistance gene *Rdg2a* located distal on the short arm of chromosome 1 (7HS) (Tacconi *et al.*, 2001) has been mapped to fine genetic resolution (Bulgarelli *et al.*, 2004). *Rdg2a* confers complete resistance (immunity) to at least three Italian isolates of the pathogen, including the most virulent Italian isolate (Dg2). In young barley roots of resistant and susceptible genotypes differing for *Rdg2a* alleles, a number of PR genes were found to have altered expression in response to leaf stripe inoculation, encoding thaumatin-like proteins, peroxidase, thionin and β-(1,3)-glucanases (Valè *et al.*, 1994, 1998).

Despite the importance of seed-borne diseases to agriculture and plant defence in general, relatively little research has been done on resistance responses to seed-borne diseases. To date, no detailed gene expression analyses of barley tissues infected with *P. graminea* have been performed. In the present study we seek to gain insights into the relationships between the pathogen *P. graminea* and its host barley by conducting a detailed transcript analysis of embryos from resistant and susceptible (*Rdg2a* and *rdg2a*) near-isogenic barley lines challenged with this pathogen. We complement this analysis with a cytological characterization of the infection process to identify specific tissues and developmental stages critical for host resistance. The results reveal gene classes and molecular mechanisms that may contribute to resistance of barley to this pathogen and perhaps to seed-borne pathogens in general.

RESULTS

Microscopic analysis of barley embryo responses to leaf stripe colonization

For the microscopic analysis, we used the Dg2-GUS isolate of *P. graminea* expressing β-glucuronidase from a transgene (Aragona and Porta-Puglia, 1999) to allow the progression of fungal infection to be followed by GUS staining. Seeds of the susceptible barley cv Mirco-*rdg2a* and its near-isogenic resistant BC line NIL3876-*Rdg2a* were inoculated with Dg2-GUS and embryo sections taken at 7, 11, 14, 18, 20, 24 and 27 days after inoculation. Embryos taken at 7, 14 and 18 dai are shown in Fig. 1. By 7 dai the leaf stripe pathogen was detected in GUS-stained embryo sections examined under bright-field microscopy, but only in the distal part of the provascular tissue of some embryos (upper right side of Fig. 1A). At 14 dai, the pathogen had only colonized a small part of the provascular tissue in the resistant NIL3876-*Rdg2a* (Fig. 1G), while in the susceptible line Mirco-*rdg2a* hyphal growth was more extensive and had begun to appear in the scutellum (Fig. 1J). Closer inspection confirmed the presence of blue-stained hyphae growing intercellularly (Fig. 1V). In susceptible inoculated embryos, no necrosis (browning) was observed in host cells in regions where intercellular hyphae were present, indicating that the pathogen does not induce cell death in its host at this early stage of infection during compatible interactions. In NIL3876-*Rdg2a* the fungus was still confined to the embryo-distal side of the scutellum at 18 dai (Fig. 1M), and by 20, 24 and 27 dai colonization had progressed no further (data not shown). In contrast, Mirco-*rdg2a* showed diffuse colonization of the scutellum at 18 dai (Fig. 1P) and dense colonization of the shoot apex beginning from 20 dai (data not shown). These results indicate that *Rdg2a*-triggered resistance expressed at the base of the young shoot (at the scutellar node) and at the basal region of provascular tissue is responsible for preventing progression of fungal growth in to the growing shoot.

Autofluorescence in inoculated embryos at 7, 14 and 18 dai was observed mainly at the junction of the scutellum and the scutellar node, both in resistant NIL3876-*Rdg2a* (Fig. 1B,H,N, respectively) and in susceptible Mirco-*rdg2a* (Fig. 1E, K,Q, respectively), while the corresponding non-inoculated controls essentially lacked this specific autofluorescence (Fig. 1C,F,I,L,O,R). Although
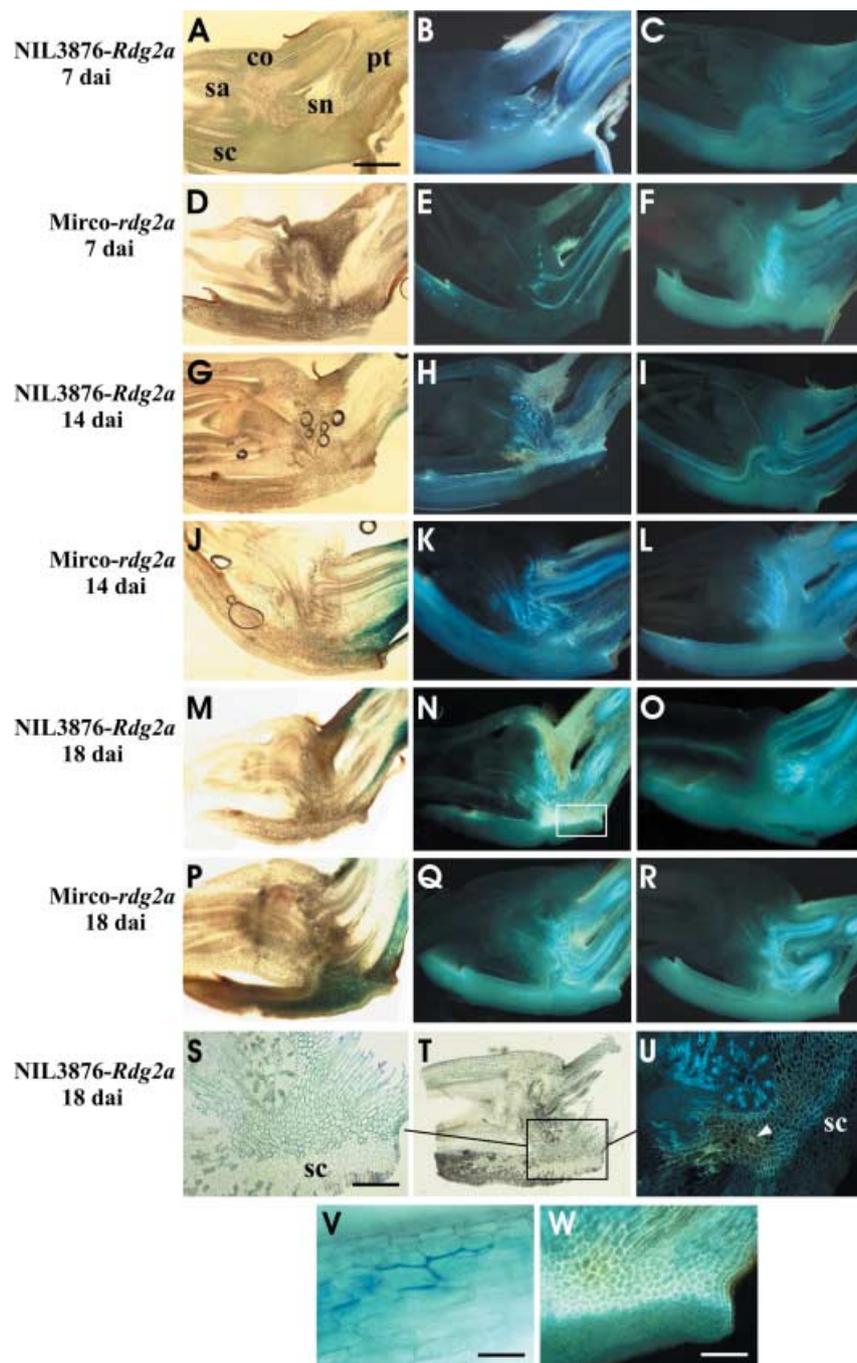
Fig. 1 Barley embryo sections indicating *P. graminea* growth and defence reactions. The transgenic *P. graminea* Dg2-GUS isolate containing the β-glucuronidase transgene was used, allowing detection of fungal colonization by (blue) GUS staining. A, D, G, J, M and P are bright-field views of GUS-stained sections from NIL3876-*Rdg2a* and from Mirco-*rdg2a* at the time points of inoculation indicated. A close-up view of the Mirco-*rdg2a* embryo at 14 dai (V) shows blue GUS staining of fungal mycelium. The same sections were also observed by UV epifluorescence microscopy (B, E, H, K, N, and Q) for NIL3876-*Rdg2a* and for Mirco-*rdg2a* at the time points of inoculation indicated. C, F, I, L, O and R show epifluorescence views of sections of non-inoculated control embryos of NIL3876-*Rdg2a* and Mirco-*rdg2a* harvested at the same time points as the inoculated samples. S and T show high- and low-magnification views of an inoculated NIL3876-*Rdg2a* embryo treated with Toluidine Blue O, which stains phenolic compounds blue-green. U is the same view as in S except observed under UV light, illustrating co-localization of pathogen-induced UV autofluorescence with Toluidine Blue O staining. W is a magnified view of the boxed area in N, illustrating cell-wall localization of autofluorescence. Scale bars represent 1 mm (A), 300 μm (S), 50 μm (V) and 200 μm (W). Arrowhead indicates a cell showing whole-cell autofluorescence. co = coleoptile; pt = provascular tissue; sa = shoot apex; sc = scutellum; sn = scutellar node.

by 7 dai few hyphae were detected in embryo sections by GUS staining (Fig. 1A), at this same time-point NIL3876-*Rdg2a* embryos reacted with autofluorescence (Fig. 1B), indicating that even a small amount of colonization could trigger a defence reaction in the resistant genotype. Autofluorescence at the junction of the scutellum and scutellar node occurred principally in the cell walls (Fig. 1W). Autofluorescence observed at other time points (20, 24 and 27 dai) was also restricted to the cell walls (data not shown).

Whole-cell autofluorescence, which is indicative of hypersensitive cell death (Görg *et al.*, 1993; Hückelhoven *et al.*, 1999), was observed only occasionally (i.e. arrowhead in Fig. 1U) in these embryo regions in response to the pathogen. Hence, resistance triggered by the *Rdg2a* resistance did not appear to involve a hypersensitive response (HR).

To compare the levels of autofluorescence in resistant and susceptible barley genotypes, we examined 48 embryos from
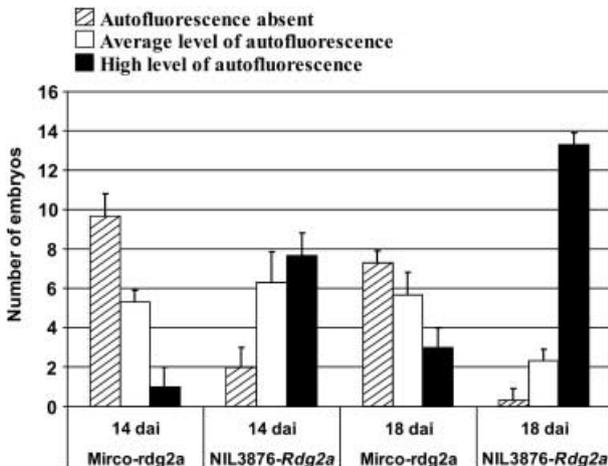
Fig. 2 Quantification of UV autofluorescence of resistant and susceptible embryos inoculated with *P. graminea* isolate Dg2. Embryos of Mirco-*rdg2a* and NIL3876-*Rdg2a* were observed by epifluorescence microscopy at 14 and 18 dai and assigned to categories according to their level of autofluorescence. Shown are mean and SD obtained from three biological replicates comprising 16 embryos each per genotype and time point.

each genotype at 14 and 18 dai (Fig. 2). Embryos were grouped in to three classes: (1) autofluorescence absent; (2) autofluorescence moderate and restricted to small parts of the provascular tissue and/or to the junction between the scutellar node and scutellum (i.e. Fig. 1Q); and (3) autofluorescence high throughout the scutellar node and frequently also in the basal part of the coleoptile (i.e. Fig. 1N). The results, summarized in Fig. 2, revealed a large proportion of NIL3876-*Rdg2a* embryos with high autofluorescence levels (7.6 and 13.3 embryos at 14 and 18 dai, respectively), and only a few Mirco-*rdg2a* embryos with high autofluorescence levels (one and three embryos at 14 and 18 dai, respectively). Hence, the degree of pathogen-induced autofluorescence was positively correlated with host genotype resistance status.

Pathogen-induced cell-wall UV autofluorescence indicates changes in cell-wall composition, which can include accumulation of phenolic compounds (von Röpenhack *et al.*, 1998). To investigate the nature of the fluorescent substances, embryo sections were treated with Toluidine Blue O, which stains phenolic compounds blue/green. Under bright-field illumination, staining was visible at 18 dai in cell walls (Fig. 1T,S) in the same places where pathogen-induced UV autofluorescence appeared (Fig. 1U), while no staining was observed in the adjacent non-autofluorescing tissues. This result suggests that the pathogen-induced UV autofluorescence derives from accumulated phenolic compounds.

Suppression subtractive hybridization and cDNA-AFLP

Based on the results of the microscopic analysis, we reasoned that most of the gene expression changes crucial for resistance

should take place in the embryo between 7 and 14 dai. At this last time point the pathogen had colonized the scutellar node region and part of the scutellum of the embryo in the susceptible Mirco-*rdg2a* genotype (Fig. 1J) while defence reactions had restricted pathogen growth to the provascular tissue in the resistant NIL3876-*Rdg2a* line (Fig. 1G). Therefore, to identify genes potentially contributing to resistance, a subtracted cDNA library was made by suppression subtractive hybridization (SSH) from embryos of the barley leaf stripe-resistant line NIL3876-*Rdg2a* at 7 and 14 dai, subtracting with mRNA from non-inoculated control NIL3876-*Rdg2a* embryos harvested at the corresponding time points. A total of 1800 randomly selected cDNA clones from the subtracted library were subjected to two rounds of reverse-Northern analysis, providing evidence for greater expression in inoculated tissues for only 12 of the cDNA clones. Subsequent Northern analysis of the 12 clones indicated that only five of them were expressed at higher levels in inoculated tissues than non-inoculated tissues (Fig. 3). These five genes are predicted to encode two enzymes from the sulfur reductive assimilation pathway (ATP sulfurilase and ferredoxin-sulfite reductase), glutathione-S-transferase (GST), a cytochrome P450 and the H_2O_2-generating enzyme oxalate oxidase. Their sequences, putative functions and BLAST search results are reported in supplementary Table S1.

Because SSH yielded so few differentially expressed genes, cDNA amplified fragment-length polymorphism (AFLP) analysis was also utilized. This technique was used to examine genes expressed in Mirco-*rdg2a* and NIL3876-*Rdg2a* at 7, 11 and 14 dai. Selective amplification with 32 primer combinations revealed an average of 100–120 bands per primer combination, allowing detection of approximately 3500 cDNA fragments per RNA sample. Genes were identified which appeared to be induced in Mirco-*rdg2a* and constitutive in NIL3876-*Rdg2a* (Fig. 4A), induced in both resistant and susceptible genotypes (Fig. 4B), induced in the resistant genotype only (Fig. 4C), induced in the susceptible genotype only (Fig. 4D) and down-regulated in the resistant genotype (Fig. 4E). For comparison, cDNA-AFLP was also performed in parallel on RNA extracted from fungal mycelium grown on potato dextrose agar (PDA) to identify potentially fungal-derived fragments, such as the fragment indicated in Fig. 4F. About 700 fragments showed differences in intensity between genotype/treatments. These were isolated from the gels, re-amplified using non-labelled primers identical to those employed for selective AFLP, subjected to direct sequencing in one direction and the sequences used to search the GenBank (nr) and EST (dbEST) databases with the BLASTN and BLASTX algorithms (Altschul *et al.*, 1997). Of the 610 sequences obtained, 543 matched entries in the TIGR barley gene index database and 18 were found to be of fungal origin. Of the remaining 49 sequences, 26 showed significant homology with wheat, rice or Arabidopsis sequences while 23 showed no significant homology to any plant or fungal sequence in the databases. Of the 23 that showed no homology to any known plant or fungal

Fig. 3 Northern blot analysis of genes induced in barley embryos by *P. graminea* inoculation. The genes used as probes had been identified by SSH and were those subsequently confirmed by Northern analysis to be induced. Blots contain mRNAs from inoculated (I) or non-inoculated control (C) embryos of susceptible Mirco-*rdg2a* or resistant NIL3876-*Rdg2a*, harvested after 7, 11 or 14 days. Ethidium bromide-stained gel images (bottom panels) show RNA loading.
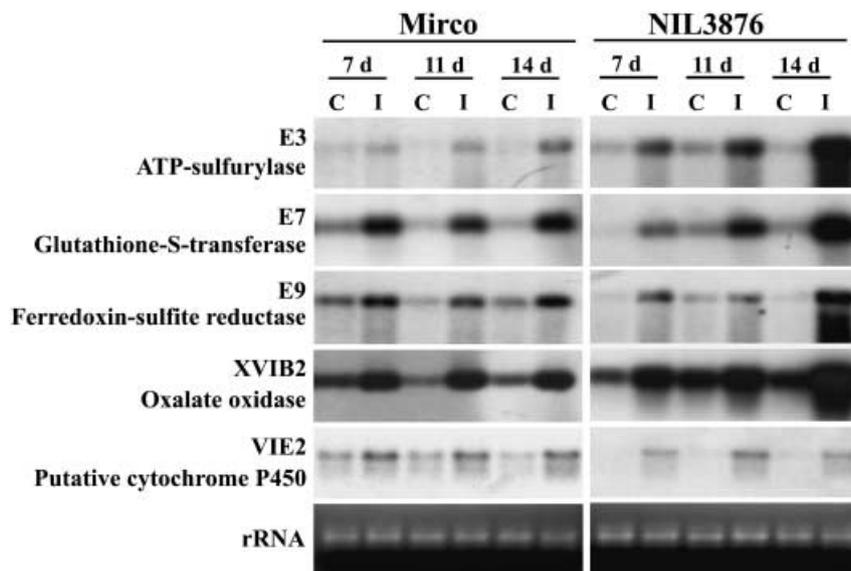

Fig. 4 cDNA-AFLP profiles illustrating expression patterns influenced by barley inoculation with *P. graminea*. Samples were collected from fungal mycelium grown on PDA (0) or from embryos at different time-points after inoculation (7, 11 and 14 days), from NIL3876-Rdg2a inoculated (1) or non inoculated (2) with leaf stripe or from Mirco-*rdg2a* inoculated (3) or non-inoculated (4) with leaf stripe. The six primer combinations (A, B, C, D, E and F) provide examples of different expression patterns as explained in the text. Arrows indicate cDNA-AFLP bands eluted from the gels.
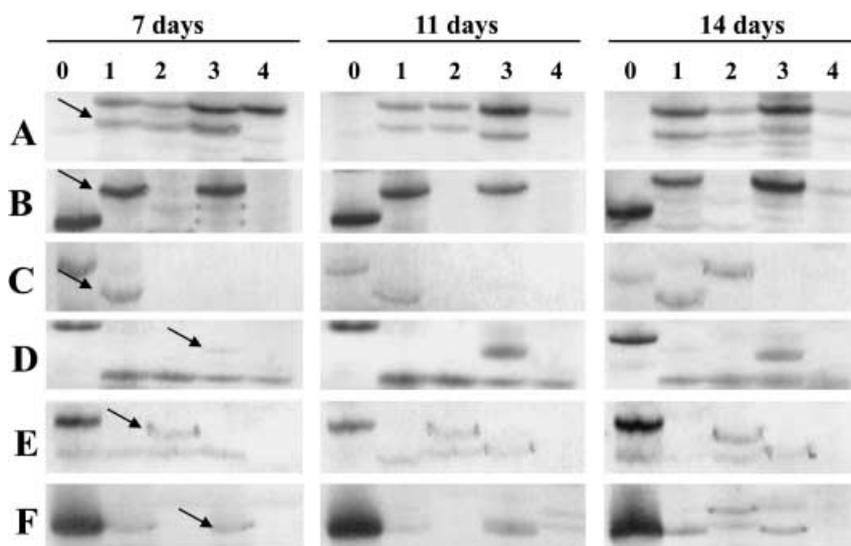
sequences, four were later verified by the microarray analysis to be significantly up- or down-regulated by inoculation in the resistant genotype only, indicating that at least these four sequences were unlikely to originate from contamination of the cDNA with plant genomic DNA and may represent novel pathogen-induced barley genes.

Micro-array analysis

We made a 35–40 mer oligo-array chip containing 2240 unique probes derived from 595 genes identified by cDNA-AFLP as being putatively differentially regulated between treatments (15 of the 610 identified sequences were not suitable for probe design), in addition to barley homologues of 100 genes reported in the literature to have possible or demonstrated roles in pathogen defence in wheat, rice, sorghum and barley (e.g. genes encoding PR proteins, protein kinases, transcription factors, proteins involved in cell-wall modifications, ROS-generating enzymes and enzymes involved in cell detoxification/protection). Differentially expressed genes identified by SSH were not included in the chip as, by this time, these had been tested by Northern analysis.

Microarrays were probed with RNAs harvested from Mirco-*rdg2a* or NIL3876-*Rdg2a* embryos 7 or 14 days after inoculation with the leaf stripe isolate Dg2, or from embryos cultured for the same periods of time in the absence of the pathogen. Of the 695 genes represented on the microarray, 186 were identified as
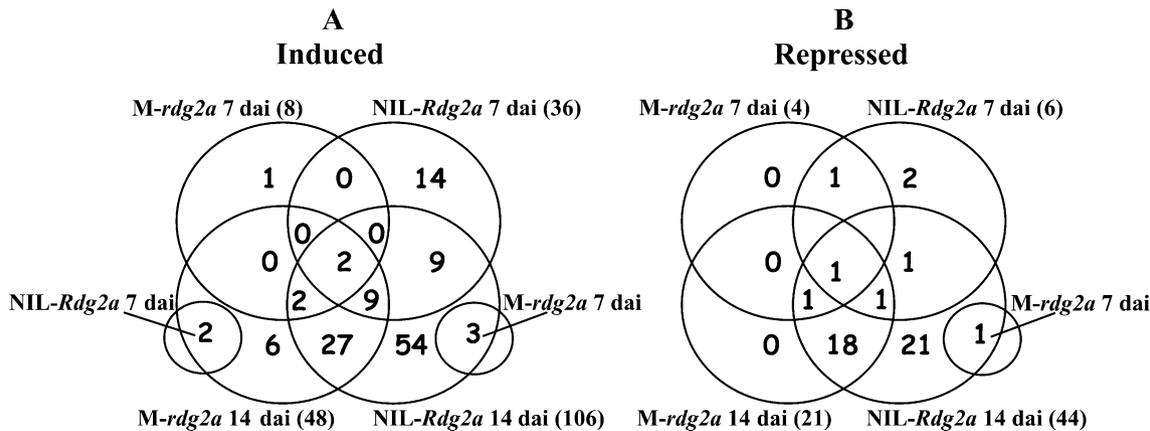
A
Induced

M-*rdg2a* 7 dai (8)   NIL-*Rdg2a* 7 dai (36)

B
Repressed

M-*rdg2a* 7 dai (4)   NIL-*Rdg2a* 7 dai (6)

Fig. 5 Venn diagrams showing numbers of barley genes shown by the microarray analysis to be significantly induced (A) or repressed (B) in barley embryos after inoculation with *P. graminea* for various combinations of genotypes and time-points. The genotypes are Mirco-*rdg2a* (M-*rdg2a*) and NIL3876-*Rdg2a* (NIL-*Rdg2a*), and the time points are 7 and 14 dai. For each individual genotype/time-point combination, the total number of induced/repressed genes is shown in parentheses.

being significantly induced or repressed by inoculation for at least one of the four genotype/time point combinations, including 143 genes first identified through cDNA-AFLP. Of the latter group, 11 genes were known from sequence database searches to be fungus-derived (Fig. 7; supplementary Table S1), while four sequences had shown no homology to any wheat, rice or Arabidopsis sequence in the databases (supplementary Table S1). Hence, the microarray identified 171 pathogen-responsive plant genes. Only 24% (143 out of 595) of the cDNA-AFLP identified sequences were confirmed by the microarray analysis to be differentially regulated.

The numbers of genes induced or repressed for the various combinations of genotypes and time points are shown in Fig. 5. The greatest numbers of induced and repressed genes were observed at 14 dai, when NIL3876-*Rdg2a* also showed more than twice as many induced/repressed genes as Mirco-*rdg2a*. Because pathogen colonization was effectively halted in NIL3876-*Rdg2a* by 14 dai (Fig. 1A), it is possible that some of the genes induced/ repressed in NIL3876-*Rdg2a* but not Mirco-*rdg2a* between 7 and 14 dai may contribute to the resistance.

Coordinated regulation and functional categorization

The 171 significantly regulated host genes were classified into four groups according to their relative expression levels in the two genotypes and at the two time points (Fig. 6) using the *k*-means clustering algorithm (Kaufman and Rousseeuw, 1990; Slonim, 2002). The magnitudes of the expression level changes and functional annotations of the genes assigned to each of the clusters are provided in supplementary Table S2. Genes expressed differently in the resistant and susceptible genotypes (clusters 3 and 4) may include ones that contribute to the *Rdg2a*-triggered resistance. Cluster 3 includes 30 genes that are expressed mostly
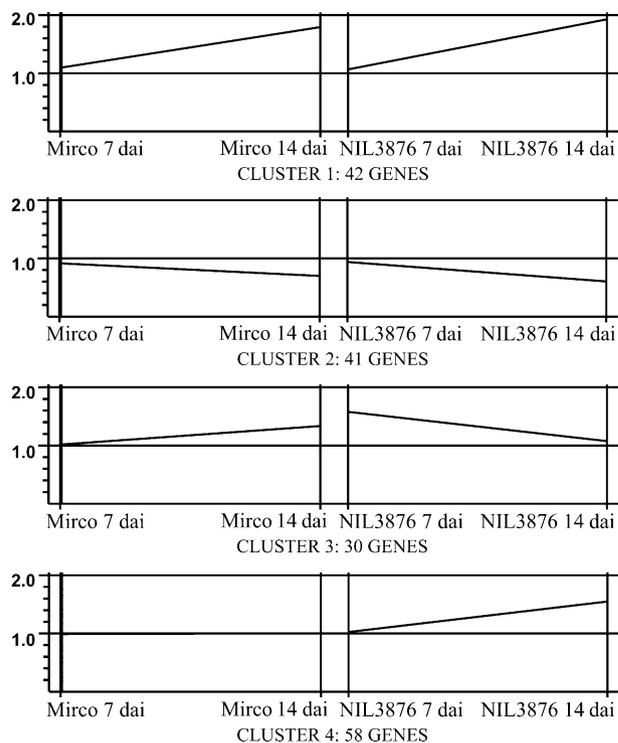

Fig. 6 Cluster analysis. The 171 genes significantly regulated (> 1.3-fold induced relative to untreated controls; *P* < 0.05) in barley embryos were classified into four different expression profiles. The *x*-axes show the genotypes and the infection time-points. The *y*-axes show the normalized signal intensity ratios (global mean fold change) between infected and control samples.

at a higher level in NIL3876-*Rdg2a* than Mirco-*rdg2a* at 7 dai, and includes genes encoding a considerable number of proteins implicated to have roles in defence (e.g. encoding thaumatin-like proteins, thionins and PR1a), cell-wall reinforcement (peroxidase)
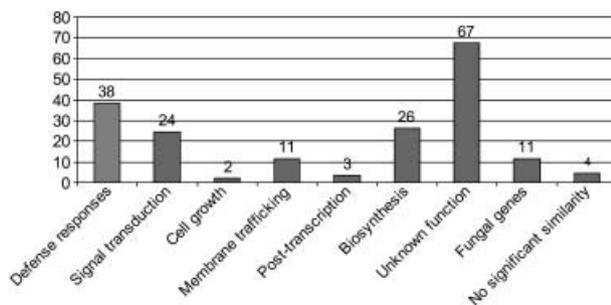
Fig. 7 Functional characterization of significantly modulated genes. Genes showing significant ($P < 0.05$) induction/repression on microarrays based on three biological replicates and a ratio of at least 1.3 relative to untreated controls were assigned to functional classes based on homology (*e* value > 10e-6) to characterized sequences in the GenBank (nr) and EST (dbEST) databases.

and signal transduction (zinc finger protein). Cluster 4, comprising genes induced only in NIL3876-*Rdg2a*, is the largest cluster (58 genes), and contains genes for proteins implicated to have roles related to stress responses (superoxide dismutase, catalase and cyclopropane fatty acid synthase), signalling (protein kinases, transcription factors, calmodulin and NB-ARC genes) and biosynthesis of cell walls (syntaxin, callose synthase genes).

The significantly modulated genes were also arranged into nine groups based on their putative functions (Fig. 7; supplementary Table S1). The largest group contained all the genes that could not be ascribed any function. The second largest group (38 genes) contained all the defence-related genes.

Gene expression analysis by qRT PCR and RT PCR

Table 1 indicates genes selected as having a putative role in defence of barley embryos against the leaf stripe pathogen, based both on differential expression observed in this study and on homology to proteins implicated or shown in the literature to have roles in defence. Twenty-five genes with representatives from each of the four defence-related functional categories of Table 1 were chosen for analysis by quantitative real-time PCR (qRT PCR). This was done both to test the validity of the microarray data interpretation and to characterize further the regulation of these genes during embryo responses to leaf stripe. Transcripts were quantified at 7 and 14 dai in the resistant and susceptible near-isogenic lines (NILs) under control and inoculated conditions using a biological replicate independent from those used for microarray analysis. Four genes did not show differential expression and were eliminated from the list of the significantly regulated genes, whereas up-regulation in response to leaf stripe infection was confirmed for the remaining 21 genes (Table 1). For six genes (CTC3:2, TGC45:1, AY177665, CAA7, AY246906 and CAT2), significant up-regulation was detected by qRT-PCR but not by microarray analysis, probably reflecting the higher sensitivity of
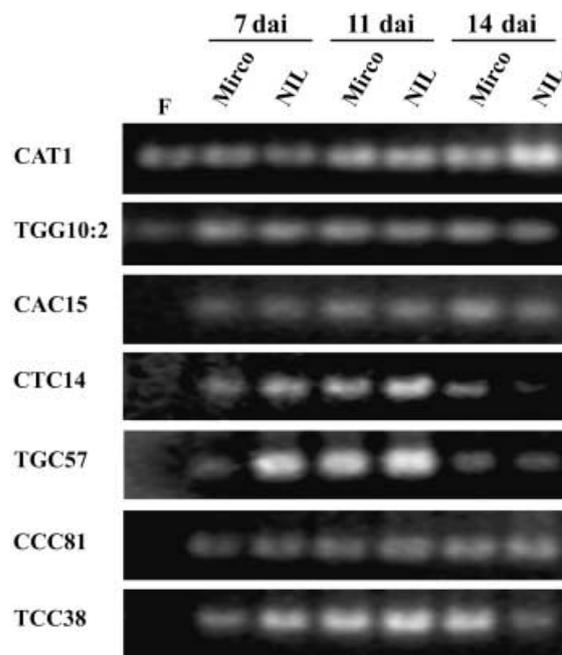

Fig. 8 RT-PCR expression analysis of fungal genes. mRNA was extracted from *P. graminea* Dg2 fungus that was grown on PDA medium (F) and from Mirco-*rdg2a* and NIL3876-*Rdg2a* barley embryos inoculated with Dg2 and collected at 7, 11 and 14 dai. The fungal gene CAT1 (ubiquitin) was used to adjust input template amounts so that templates used for each TGG10:2, CAC15, CTC14, TGC57, CCC81 and TCC38 RT-PCR reaction contained equal amounts of CAT1 transcript (see Experimental procedures).

qRT-PCR for low-abundance transcripts. The partial differences in the outcomes of the two techniques were expected. For example, qRT-PCR and microarrays had previously been found to give similar results for only 55% (Czechowski *et al.*, 2004) to 70–80% (Salzman *et al.*, 2005; Svensson *et al.*, 2006) of genes.

Eighteen cDNA-AFLP-derived sequences of likely fungal origin based on BLAST searches at NCBI and Cogeme (supplementary Table S1) were investigated by RT-PCR. PCR primers were first tested for their ability to amplify from fungal (*P. graminea* isolate Dg2) and plant (Mirco-*rdg2a* and NIL3876-*Rdg2a*) genomic DNA templates. Primer pairs for eight of the genes could not be used because they failed to amplify any product or because they gave rise to multiple products. Primer pairs for the remaining ten genes amplified single products from the fungal DNA only, and were used for RT-PCR on the fungus alone (control) and on embryos of Mirco-*rdg2a* and NIL3876-*Rdg2a*, at 7, 11 and 14 dai. The fungal gene CAT1 was expressed in both PDA- and plant-grown fungus (Fig. 8) and was used for normalization, such that template used in all other RT-PCRs contained equal amounts of CAT1 transcript (see Experimental procedures). The data were then interpreted with the condition that the CAT1 gene encoding ubiquitin was expressed at the same level in PDA-grown fungus and fungus from all inoculated embryos, and that each RT-PCR reaction

Table 1 Expression ratios for selected barley genes significantly up-regulated by *P. graminea* inoculation in barley embryos and having putative functions relating to defence.

	Expression ratios*								Homologue accession no.	Putative function	Cluster
	Mirco				NIL3876						
	7 dai		14 dai		7 dai		14 dai				
Clone	μa	qP	μa	qP	μa	qP	μa	qP			
Oxidative stress and cell protection pathways											
TGA61:1	ns	np	1.30	np	ns	np	1.87	np	AF250935	Germin F	1
Y14203	1.49	1.78	2.47	2.12	ns	0.97	3.94	3.59	Y14203	Oxalate oxidase	1
CK210220	ns	np	ns	np	ns	np	1.36	np	CK210220	Catalase isozyme 2	4
TGG15	ns	np	ns	np	ns	np	1.39	np	U20778	Catalase	4
CTC3:2	ns	0.58	ns	2.44	0.60	2.35	2.05	20.46	Y17386	In2.1 protein	4
CCA5	ns	np	2.17	np	1.73	np	2.00	np	AB207242	Glutathione-S-transferase	1
AJ238745	ns	np	1.39	np	ns	np	2.00	np	AJ238745	Glutathione peroxidase-like	1
TAA17	ns	1.77	1.95	4.01	ns	1.49	1.91	3.94	AF321869	Cytochrome P450	1
BF260610	ns	np	ns	np	ns	np	1.68	np	BF260610	Cu/Zn superoxide dismutase	4
AJ006358	ns	np	1.39	np	1.32	np	1.95	np	AJ006358	Ascorbate peroxidase	1
TTA10	ns	1.16	ns	0.87	ns	1.54	1.31	5.21	ABA97030	Cyclopropane fatty acid synthase	4
TGC45:1	ns	2.79	1.34	2.06	ns	1.40	ns	2.27	CAD59574	PDR-like ABC transporter	3
TAA28	ns	1.56	ns	1.48	ns	1.16	1.40	3.03	AAL79756	Organic solute transporter	4
TTC12	1.48	2.47	ns	1.48	ns	1.17	2.00	3.68	BAD73111	NIC2 protein	1
Cell-wall-related genes and secondary metabolic pathways											
CTC26:1	ns	1.80	1.36	4.04	ns	1.24	1.48	4.58	BAD46391	Cellulose synthase-like protein	1
AY177665	ns	1.55	ns	1.25	ns	2.95	1.34	2.77	AY177665	Callose synthase-like protein	4
CGT19	ns	0.78	1.37	2.06	1.32	1.86	1.36	2.92	ABA96590	Dirigent-like protein	1
M73234	ns	0.84	ns	1.55	1.32	3.36	ns	1.12	M73234	Peroxidase BP1 (Prx5)	3
Z49145	ns	1.95	ns	1.87	ns	1.74	1.43	2.91	Z49145	Phenylalanine ammonia-lyase	4
AY228552	1.32	1.73	1.31	2.91	ns	0.73	1.46	3.25	AY228552	Agmatine coumaroyltransferase	1
CAA7	ns	1.59	ns	2.52	ns	2.17	1.41	5.70	BAD35400	2′-hydroxyisoflavone reductase	4
AY246906	ns	0.57	ns	1.77	ns	2.51	1.32	2.35	AY246906	Ror2 syntaxin	4
CGG37	1.32	2.14	ns	1.69	ns	0.74	1.64	2.50	BAD05209	Clathrin coat assembly protein σ2	1
Ubiquitination and jasmonate pathways											
CCA11:2	ns	0.53	ns	1.67	ns	0.73	1.34	2.19	NP_568264	Ubiquitin-protein ligase	4
L35931	ns	1.70	ns	1.87	ns	1.10	1.38	2.50	L35931	Lipoxygenase 1 (LoxA) gene	4
CAT2	ns	1.20	1.41	11.35	ns	2.14	1.39	2.99	AAY26524	12-oxo-phytodienoic acid reduct.	1
Signal transduction											
AY541586	ns	1.55	ns	1.71	1.32	2.69	1.42	2.26	AY541586	WRKY38 TF	4
BF264537	ns	0.92	ns	1.97	ns	1.12	1.83	2.56	BF264537	Calmodulin protein	4
AY465427	ns	np	ns	np	ns	np	1.48	np	AY465427	C3H2C3 RING-finger protein	4
CAA23	ns	np	ns	np	ns	np	1.59	np	AAR06369	Calmodulin-binding TF	4

*Expression ratios determined from the ratios of the expression level of a particular gene in inoculated tissues to the expression level of that gene in control (non-inoculated) tissues. Microarray (μa) fold-numbers for each gene indicate global mean fold-inductions (relative to untreated controls) from all slides (three replications) in each genotype × time condition. Fold-inductions for qRT PCR data (qP) indicate average $2^{\Delta\Delta Ct}$ ($\Delta\Delta CT$ inoculated/$\Delta\Delta CT$ control) based on two to three replicates. cDNA templates for qRT PCR reactions were made from the same RNA samples used to generate the microarray cDNA probes. dai = days after inoculation, ns = microarray results not significantly differentially expressed, np = qRT PCR were not performed.

therefore contained an equal amount of fungal RNA. The genes CAC37, CAA10 and CGG10 (supplementary Table S1) were expressed at roughly equal levels in PDA- and plant-grown fungus (data not shown). One gene (TGG10:2) was expressed at higher levels in inoculated tissues than in fungus grown on PDA (Fig. 8). The remaining five fungal genes showed detectable expression only in the fungus from inoculated embryos, and expression of these genes did not appear to be influenced by host plant resistance genotype (Fig. 8).

The function of most of the analysed fungal genes could be inferred by homology (supplementary Table S1). The four genes expressed at similar levels in PDA- and plant-grown fungus

showed homology with ubiquitin (CAT1 and CAA10) and with components of the vesicular transport machinery (CAC37 and CGG10). Genes expressed specifically during plant infection encode a xylanase (TGC57) and different dehydrogenases (mannitol, TCC38, or lactic, CTC14). One gene (TGG10:2) shares similarity to an *Alternaria brassicicola* sequence identified from a cDNA library made from a nitrogen-starved culture. No putative functions could be ascribed to CAC15 and CCC81 expressed only during infection.

DISCUSSION

Microscopically visible host responses accompanying *P. graminea* growth arrest in the incompatible interaction

In this study we showed that the resistance of NIL3876-*Rdg2a* conferred by the *Rdg2a* gene arrests *P. graminea* fungal growth at the scutellar node and basal region of provascular tissue of the barley embryo (Fig. 1B,H,N) and, by comparing pathogen colonization in resistant and susceptible near-isogenic backgrounds, it was evident that this arrest was already effective by 14 dai. Therefore, defence responses driven by *Rdg2a* at the base of the young shoot are responsible for the resistance. The resistance was found to be temporally, spatially and genetically associated with UV autofluorescence and Toluidine Blue O staining in the host cells (Figs 1 and 2), suggesting that the resistance involves accumulation of phenolic compounds. UV autofluorescence indicating the presence of phenolic compounds has been observed in pathogen-challenged tissues throughout the cell wall (Mitsuda *et al.*, 2005) and in appositions (papilla) deposited on the cell wall opposite challenge sites during expression of barley powdery mildew *mlo* resistance (von Röpenhack *et al.*, 1998). Accumulation of phenolics has been observed in several plant pathogen interactions and different defence roles have been attributed to this class of chemical compounds, including reinforcement of cell walls by formation of phenolic polymers such as lignin (Nicholson and Hammerschmidt, 1992), and antimicrobial activity, as demonstrated for several phenolic compounds in rice (Padmavati *et al.*, 1997) and for the phenolic polyamine conjugate *p*-coumaroyl-hydroxyagmatine in barley (von Röpenhack *et al.*, 1998).

Whole-cell accumulation of phenolic compounds (and autofluorescence) indicative of whole-cell suicide, termed HR, is characteristic of many resistances conditioned by single dominant resistance genes in barley and other plants (Görg *et al.*, 1993; Hückelhoven *et al.*,1999; Prats *et al.*, 2005). Autofluorescence appearing during *Rdg2a* resistance was essentially confined to the cell walls (Fig. 1W) and was only occasionally observed throughout a whole cell (i.e. arrowhead in Fig. 1U), suggesting that the *Rdg2a* resistance does not involve HR. However, this conclusion must be

treated with some caution, as the reliability of UV autofluorescence as a marker for HR in embryos is not known. Our attempts to test independently for HR using trypan blue staining was not successful. This stain normally also stains fungal structures, but no such staining of hyphae within the embryo slices was observed, even with vacuum infiltration, indicating that the stain did not penetrate the embryo tissues (not shown). As previously described (Platenkamp, 1976), *P. graminea* hyphae grow intercellularly (Fig. 1V) and degrade host cell walls to derive nutrients. Hydrolytic enzymes that may be used for this purpose include polygalacturonase, cellulase and pectin-methylesterase (Hammouda, 1988), and also β-1,4-xylanase as indicated by the expression data obtained in the current study (TGC 57 in Fig. 8). However, our analysis revealed essentially no host cell death in susceptible embryos inoculated with *P. graminea*, indicating that the fungus does not induce cell death as part of its infection process at this early stage. Because the embryo is a small structure critical for plant reproduction, one could imagine that a resistance-associated HR in the embryo, or a cell death in the embryo induced by the fungus during a compatible interaction, might be detrimental for long-term survival of both the host and the pathogen.

Rdg2a controls expression of several defence processes

A strategy combining SSH, cDNA-AFLP and microarray analysis of barley NILs resulted in the identification of five (SSH) and 171 (cDNA-AFLP and microarray) host genes whose expression in barley embryos is up- or down-regulated by leaf stripe inoculation. Early time-points of inoculation (from 7 to 14 dai) were selected for this analysis as by these times, defence responses had already deployed in the resistant line NIL3876-*Rdg2a* (Fig. 1B,H) and halted fungal colonization (Fig. 1G). Failure of the microarrays to confirm regulation of many of the genes identified by cDNA-AFLP (only 24% of the cDNA-AFLP sequences were confirmed as differentially expressed in the microarray analysis) was probably mainly due to the lower sensitivity of the microarray compared with the PCR-based cDNA-AFLP technique. For example, in another study such a limitation resulted in the failure of a microarray to confirm 35% of glucose-regulated yeast genes identified by cDNA-AFLP (Reijans *et al.*, 2003). In part, the difference in outcomes of the two techniques may also derive from amplification artefacts in the cDNA-AFLP.

In the microarray experiments, differences in expression were declared using a 1.3-fold response cutoff and a further filter for significance to retain genes passing the 1.3-fold response cutoff at $P < 0.05$, as indicated in the Experimental procedures. This low cutoff value was chosen because a number of defence-related genes are already expressed in pathogen-free germinating barley seeds and it is possible that pathogen infection increases expression of these genes only subtly. In addition, transcriptional

responses may have been occurring in only a small portion of the tissue, as visible responses to the fungus were limited to only parts of the embryo (Fig. 1). These two factors could explain the low number of differentially expressed genes identified by SSH and the low fold-change observed for several genes in the microarray and qRT PCR analyses. While the 1.3-fold response cutoff may have resulted in some false positives, we reasoned that it was more important at this early phase of the investigation to employ a liberal cutoff to avoid overlooking genuinely regulated genes. Twenty-one of 25 tested genes chosen for validation by qRT PCR were shown by qRT PCR to be genuinely differentially expressed, confirming the validity of our approach.

Microarray analysis identified 23 genes that were significantly pathogen-induced in NIL3876-*Rdg2a* but not in Mirco-*rdg2a* at 7 dai (members of cluster 3 in supplementary Table S2; Fig. 5), plus an additional 54 genes that were induced in NIL3876-*Rdg2a* but not in Mirco-*rdg2a* by 14 dai (Fig. 5). Therefore, *Rdg2a*-triggered processes influence the expression of about 77 (59%) of the 129 pathogen up-regulated genes. Lack of induction of any one of these genes in the susceptible NIL may be due to lack of induction of a specific defence signalling pathway in the first place, and/or an indirect result of greater fungal growth and suppression of defence signalling by fungal-derived effector molecules. Similarly, the 24 genes repressed only in the resistant NIL3876-*Rdg2a* line either by 7 dai or 14 dai (Fig. 5; members of cluster 2 in supplementary Table S2) may also contribute to resistance. Among them we identified genes (xylose isomerase and arabinoxylan arabinofuranohydrolase, AXAH) whose products are involved in the hydrolysis of the complex heteroxylan polysaccharides of the primary cell wall. An extremely high level of AXAH transcript was observed in growing barley coleoptiles and at an early stage of grain development (Farrokhi *et al.*, 2006). Down-regulation of AXAH (and possibly also of xylose isomerase) during leaf stripe inoculation might alter the composition of the cell walls and make them more resistant to breakdown by the fungus.

Genes that are up- or down-regulated by inoculation only in the resistant NIL3876-*Rdg2a* are of particular interest because one or a combination of these genes may contribute to *Rdg2a*-mediated leaf stripe resistance. Among the genes that were pathogen-induced only in the resistant NIL3876-*Rdg2a* line were ten genes for signalling factors such as protein kinases, calmodulin and transcription factors (Table 1 and clusters 3 and 4 in supplementary Table S2). Potentially, these could be involved in *Rdg2a* resistance signalling. Data based mainly on qRT PCR assays demonstrated *P. graminea* induction of the WRKY38 transcription factor gene, particularly at the earlier (7 dai) time point, and induction of the calmodulin protein gene (at 14 dai) in the resistant NIL3876-*Rdg2a* line (Table 1). Rice and Arabidopsis homologues of barley WRKY38 affect levels of disease resistance against bacterial and fungal pathogens (Liu *et al.*, 2006; Xu *et al.*, 2006).

Furthermore, Hv-WRKY38 differs by only one amino acid from a gene called Hv-WRKY1, a repressor of basal defence mechanisms and immune responses determined by some specific resistance genes to the obligate biotroph powdery mildew fungal pathogen *Blumeria graminis* (Shen *et al.*, 2007). Additionally, at least some members of the WRKY gene family are known to regulate PR gene expression positively (Ulker and Somssich, 2004). Clearly, members of the WRKY family of transcription factors can play important roles in disease resistance, and the expression pattern of WRKY38 observed in this study raises the possibility that this WRKY member may contribute to *Rdg2a*-specified *P. graminea* resistance in barley.

Microscopic observations indicated that cell-wall modifications take place during the *Rdg2a* resistance response. Potentially, these and other undetected changes in the cell wall of inoculated tissues could constitute physical or chemical barriers to infection. Some genes found to be induced by *P. graminea* were consistent with activation of cell-wall-modifying processes. Induction of callose synthase and peroxidase genes was observed in NIL3876-*Rdg2a* only (Table 1). Callose forms a major component of papillae deposited on the inner face of the cell wall in response to pathogen challenge (Schulze-Lefert, 2004) and peroxidases are involved in lignification and cross-linking of phenolics, proteins and carbohydrates (Moerschbacher, 1992). We also observed the induction of the *Ror2* gene in NIL3876-*Rdg2a* (Table 1). *Ror2* (named PEN1 in Arabidopsis) helps prevent fungal penetration of the plant cell wall in certain forms of resistance to powdery mildew, and encodes a member of the syntaxin family of proteins normally involved in exocytotic vesicle trafficking (Assaad *et al.*, 2004; Collins *et al.*, 2003). It would be interesting to see if *Ror2* also contributes to resistance to a non-appressoria-forming pathogen such as *P. graminea* and whether *P. graminea* triggers aggregation of ROR2 protein beneath challenge sites, as observed with powdery mildew (Bhat *et al.*, 2005).

Also potentially related to cell-wall reinforcement is the production of ROS. In the present study, genes encoding H_2O_2-generating enzymes (germin F and oxalate oxidase) were found to be induced in both resistant and susceptible barley genotypes but at a higher level in the resistant NIL (Table 1). Northern analysis of uninoculated tissues also revealed a higher basal level of transcription of the oxalate oxidase gene in NIL3876-*Rdg2a* than in Mirco-*rdg2a*. This correlates with the observation that NIL3876-*Rdg2a* embryos under control and inoculated conditions uniformly stain reddish-brown upon histochemical staining of H_2O_2 with DAB (3,3'-diaminobenzidine) (data not shown). Germin F and oxalate oxidase are both located in the cell wall (Cona *et al.*, 2006; Zhou *et al.*, 1998) and can contribute to a local generation of H_2O_2 for both cross-linking of cell-wall components and for defence signalling. Coincident with a possible higher production of H_2O_2 in NIL3876-*Rdg2a* is also the higher expression level in NIL3876-*Rdg2a* of some genes involved in ROS detoxification/

protection such as ATP-sulfurilase (Fig. 3), a GST-like protein (In2.1), superoxide dismutase, ascorbate peroxidase and cyclopropane fatty acid synthase (Table 1). This last enzyme uses S-adenosylmethionine to generate a methylene bridge across the double bonds in unsaturated fatty acids, contributing to the protection of membranes and other cellular components from damage by ROS (Anthony *et al.*, 2005). Perhaps connected with a requirement for ROS detoxification is the higher induction in NIL3876-*Rdg2a* of genes coding for an organic solute transporter and NIC2, a member of the multidrug and toxic compound extrusion (MATE) gene family (Table 1). These transporters can shuttle toxic compound out of the cell or transport glutathione-S-conjugates into the vacuole (Jasinski *et al.*, 2003; Sanchez-Fernandez *et al.*, 2001). In barley embryos they may function in the export or vacuole-sequestration of toxic secondary plant metabolites (such as compounds derived from oxidative stress) and/or pathogen toxins. One such toxin may be the host-specific glycosyl toxin (*Pg* toxin) produced by leaf stripe fungus in infected barley tissues (Haegi and Porta-Puglia, 1995).

In NIL3876-*Rdg2a* we observed induction of genes encoding proteins involved in jasmonate (JA) synthesis, including lipoxigenase and 12-oxo-phytodienoic acid reductase (the latter was also induced in the susceptible NIL) (Table 1). Treatment with methyl jasmonate has been shown to increase expression of genes for phenylalanine ammonia lyase and peroxidase in barley leaves (Cona *et al.*, 2006) and of an oxalate oxidase gene in barley roots (Hurkman and Tanaka, 1996). The first two genes were found to be induced only in NIL3876-*Rdg2a* embryos in response to leaf stripe (Table 1), while a higher basal level of transcription of the oxalate oxidase gene was observed in NIL3876-*Rdg2a* than in Mirco-*rdg2a* (Fig. 3). The JA-responsive genes in barley also include genes for thionins and lipoxygenase (Reinbothe *et al.*, 1997; Rouster *et al.*, 1997), which were found in our study to be induced only in NIL3876-*Rdg2a*. Expression of the thionin gene only in the *Rdg2a* donor genotype, but not in a barley line susceptible to leaf stripe, had been demonstrated previously (Valè *et al.*, 1994). JA-mediated responses also involve the action of components of the ubiquitin/proteasome pathway of protein degradation and we observed in NIL3876-*Rdg2a* induction of a ubiquitin protein ligase (Table 1). These data appear to indicate that *Rdg2a* resistance to *P. graminea* involves production of JA and triggering of JA-dependent defence pathways involving production of ROS, thionins and expression of lipoxygenase. Triggering of JA-related defences would be consistent with the fact that *P. graminea* is a nectrotroph for at least the latter part of its lifecycle (*P. graminea* growth in embryos is biotrophic and only becomes nectrotrophic at later stages of the infection cycle), and that JA-dependent resistance tends to be triggered by, and is effective against, necrotrophic pathogens, at least in Arabidopsis (Hammond-Kosack and Parker, 2003).

In conclusion, by using a genomics approach, we identified a wide variety of genes differentially expressed during the resistance response to leaf stripe. Further functional characterization of these genes will assist in determining molecular mechanisms and cellular processes involved in barley resistance to leaf stripe and perhaps defence pathways deployed by barley embryos in general.

Fungal genes expressed during infection

Fungal genes which were expressed specifically during plant colonization and not during growth on PDA media (Fig. 8) may be involved in pathogenicity. While demonstration of a role of individual genes in virulence determination would ultimately require targeted gene or transcript (silencing) disruption followed by virulence assays, we can presently speculate about the roles of some of these genes. The TGG10:2 *P. graminea* gene expressed at higher levels during infection is homologous to an *Alternaria brassicicola* sequence (GenBank accession number DN477023) derived from a cDNA library made from a nitrogen-starved culture. It is tempting to speculate that fungi may experience nitrogen starvation conditions during growth on the host plant, and that such a gene may be required for growth under these conditions. Lactate (CTC14) and mannitol (TCC38) dehydrogenases are involved in general 'metabolism', as are many fungal genes classified as 'pathogenicity genes' (Baldwin *et al.*, 2006). Perhaps up-regulation of such genes during infection reflects differences in nutritional status between plant tissues and specific artificial growth media used (e.g. PDA). TGC57 encodes a xylanase. Xylanases are cell-wall-degrading enzymes that catalyse the initial breakdown of xylan by hydrolysing β-1,4 bonds in the polymer; as xylans represent a large proportion of the hemicellulosic fraction of cereal cell-wall matrices, these degrading enzymes are expected to be important offensive components of cereal pathogens (Beliën *et al.*, 2006). Several cereal pathogens, including *Septoria nodorum*, *Stagonospora nodorum* and *Claviceps purpurea*, produce xylanases only during colonization of their host, supporting a role for microbial xylanases in pathogenicity. However, targeted disruption of individual xylanases from several pathogens has not revealed any dramatic effects on pathogenicity. These results probbaly reflect a genetic redundancy between the multiple xylanases present in microorganisms (Beliën *et al.*, 2006; Idnurm and Howlett, 2001). *P. graminea* growth in embryos is biotrophic. Enzymatic functions may loosen the structure of the host cell walls enough to assist intercellular mycelium growth but not enough to cause host cell death. No putative function can be assigned to the genes CAC15 and CCC81 identified by cDNA-AFLP. Further investigations will be required to investigate the possible roles of these and other genes in pathogenicity of *P. graminea* in barley.

EXPERIMENTAL PROCEDURES

Plant material, pathogen isolates and inoculation

Studies were performed with the leaf stripe susceptible barley cv. Mirco-*rdg2a* and the corresponding resistant near-isogenic line 3876 (NIL3876-*Rdg2a*) containing the resistant allele of the gene *Rdg2a* conferring complete resistance to the isolate Dg2 of the pathogen. NIL3876-*Rdg2a* was generated by crossing the resistant cv. Thibaut and the susceptible cv. Mirco, backcrossing six times to the susceptible parent, followed by two selfing generations, as described in Tacconi *et al.* (2001). NIL3876 was confirmed to be *Rdg2a* homozygous both by inoculation on progeny and by scoring with *Rdg2a*-linked co-dominant markers. The Dg2 isolate of leaf stripe (*P. graminea*) is the most virulent isolate in a previously described collection of monoconidial isolates (Gatti *et al.*, 1992). For the microscopic analysis, infections were carried out with Dg2 genetically transformed with the β-glucuronidase (GUS) gene (isolate Dg2-GUS; Aragona and Porta-Puglia, 1999). The expression of GUS was used to follow the colonization of the pathogen inside germinating barley seeds. The wild-type and transformed *P. graminea* isolates were also grown on PDA (Liofilchem, Italy), in Petri dishes at 20 °C for 10 days in the dark. Seeds were surface-sterilized in 70% ethanol for 30 s and then in 5% sodium hypochlorite for 20 min prior to inoculation using the 'sandwich' technique (Pecchioni *et al.*, 1996). Uninoculated control seeds were germinated on sterile moist filter paper under the same environmental conditions in the absence of the fungus.

Microscopic analysis

For microscopic observations, barley seeds were inoculated with the Dg2-GUS isolate and the embryos excised from the seeds at 7, 11, 14, 18, 20, 24, 27 dai. Embryo sections were taken by hand with a razor blade, mounted in water and examined with an Olympus BX51 microscope equipped with an epifluorescence filter (335- to 380-nm excitation, > 420-nm barrier filter). Images were recorded using an Olympus DP50 microscope digital camera system. Sections were then immersed in sodium hypochlorite (4% active chlorine) for 10 min, rinsed with water and stained for β-glucuronidase activity as previously described (Aragona and Porta-Puglia, 1999). After staining, sections were mounted in glycerine solution (15%, v/v, in water) and observed with bright-field illumination. Phenolic compounds were stained by treating sections for 2 min with 0.05% (w/v) Toluidine Blue O in 0.1 M phosphate buffer at pH 7.2. Toluidine blue stains phenolic compounds green or bluish green (O'Brien *et al.*, 1964). For the initial microscopic examination, in each of three repeat experiments at least four embryos were taken per genotype/treatment/timepoint, and at least four sections per embryo were

examined. For autofluorescence quantification, three biological replicates of 16 embryos each, per genotype per timepoint, were used.

Identification of differentially expressed sequences by SSH and cDNA-AFLP

For SSH analysis, poly(A)+-enriched RNAs were isolated from frozen embryos as previously described (Baldi *et al.*, 1999). mRNA was isolated from the leaf stripe-resistant line NIL3876-*Rdg2a* germinated under control or inoculation conditions for 7 and 14 days. cDNA synthesis and subtraction was performed with the PCR-select cDNA subtraction kit (Clontech, Palo Alto, CA), as recommended by the manufacturer. A cDNA pool prepared from barley embryos inoculated for 7 and 14 days was used as 'tester' sample and that prepared from the corresponding control was used as 'driver' for the forward subtraction. Fragments remaining after subtraction were cloned into the T/A cloning vectors pGEM-T and pGEM-T Easy (Promega, Madison, WI) using blue-white selection on LB agar plates containing Ampicillin/X-gal/IPTG to identify insert containing colonies. A total of 1800 colonies were individually picked to 150-μL volumes of 'Freezing Broth' (Sambrook *et al.*, 1989) containing 100 μg/mL ampicillin dispensed in to a 96-well culture plate. After incubation overnight at 37 °C, plates were stored at –80 °C. At the same time, clone inserts were individually amplified by colony-PCR, separated on high-density 1% agarose gels and blotted on to Nylon membranes (Millipore Immobilon, Millipore, Bedford, MA) using 0.4 N NaOH as transfer solution. Approximately 100 ng of ^{32}P-dCTP-labelled cDNA representing each of the forward subtracted probe and the reverse subtracted probe was hybridized separately to the filters. Hybridization signal for individual insert fragments was quantified using a Typhoon 9210 phosphorimager (GE Healthcare, Chalfont St. Giles, UK) with a range of screen exposure times.

For cDNA-AFLP-TP analysis, mRNAs were isolated using an RNeasy Midi Kit and Oligotex mRNA Midi Kit (Qiagen, MD) from embryos of NIL3876-*Rdg2a* and Mirco-*rdg2a* grown under control or inoculation conditions for 7, 11 and 14 days. For each genotype/treatment/timepoint 150 embryos were used. These RNA samples were also those used to produce microarray probes. For cDNA-AFLP, mRNA was also obtained from fungal mycelium grown on PDA under the same conditions as those used to inoculate seeds. Synthesis of double-stranded cDNA and cDNA-AFLP procedures were essentially as previously described (Polverari *et al.*, 2003). A total of 32 selective amplifications were carried out with fluorescently labelled *Bst*YI primers containing one extra selective nucleotide per primer. Scanning of the gel bands, excision of bands showing clear variation in intensity, re-amplification and purification of PCR products were as described (Polverari *et al.*, 2003).

Sequencing was carried out using the BigDye sequencing kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems), with an M13 universal primer (SSH clones) or the selective cDNA-AFLP primer (cDNA-AFLP fragments). Sequence homology searches against GenBank (nr) and EST (dbEST) databases were carried out using the BLASTN and BLASTX algorithms (Altschul *et al.*, 1997) at the NCBI and TIGR sites and the Cogeme site (fungal sequences only).

Preparation of microarrays

Genes considered for representation on microarrays included those containing the 610 sequenced cDNA-AFLP fragments showing genotype/treatment differences in band intensity, in addition to barley homologues of 100 genes reported in the literature to have possible or demonstrated roles in pathogen defence in wheat, rice, sorghum and barley. The barley Unigene set (at http://www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=4513) was used to identify extended transcript sequences upon which to design probes and to ensure probe specificity. Probes were designed using the Combimatrix Automated Probe-Design Suite of programs (CombiMatrix Corp., WA) and were selected without secondary structure and with homogeneous melting temperature. For expressed sequence tags (ESTs) that did not cluster with the Unigene set, probes were designed on themselves with the Probe-Design Suite. Only 695 of the 710 considered sequences were used for probe generation due to degeneracies, duplications and the presence of highly repetitive motifs in some of the sequences. For most of the 695 selected sequences, 3–4 probes were designed to different regions, and for the remaining sequences, 1–2 probes could be designed. A total of 2240 35–40 mer probes were synthesized onto each sector of a CustomArray 4 × 2000 microarray slide (CombiMatrix Corp., WA). In addition, negative control probes from viruses and bacteria (CombiMatrix Corp.), and the beta-tubulin housekeeping gene (TC61079, TiGR Database) were included on the array.

Microarray probe labelling, hybridization and scanning

Hybridization probes were made from 24 mRNA preparations, representing three biological replicates each of Mirco-*rdg2a* or NIL3876-*Rdg2a* embryos, inoculated with the leaf stripe isolate Dg2 or left uninoculated, and harvested at 7 or 14 dai. Probes were made using the Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion, USA). Three hundred nanograms of each poly(A) RNA sample was converted to cDNA using T7 oligo(dT) primers and amplification by *in vitro* transcription to synthesize amino allyl-modified aRNA. The aRNA (10 μg per probe) was labelled with Cy-5 dye and Cy5-aRNA (2 μg per probe) and fragmented to lengths of 50–200 bases. The fragmented Cy5-aRNA was mixed with hybridization buffer (6× SSPE, 0.05% Tween-

20, 20 mM EDTA, 25% deionized formamide, 0.1 mg/mL sheared salmon sperm DNA and 0.04% SDS) and hybridized to the Combimatrix 4 × 2000 Chip at 45 °C for 16 h. After hybridization, stringent washes were performed according to the manufacturer's instructions and the slides immediately coated with imaging solution prior to scanning using a ScanArray 4000XL scanner (Perkin-Elmer, USA). Cy5 channel, PMT and laser power settings were kept constant for all hybridizations.

Microarray data extraction and analysis

Microarray data were extracted using Microarray Imager software (CombiMatrix Corp.). The background signal value for each probing of an array was defined as the median fluorescence intensity of all the negative control genes. This value was subtracted from the median fluorescence intensity of each other spot in order to obtain genuine hybridization signal intensities for the latter. Spots showing 'low' intensity values (< 1% of maximal intensity), dust particles and areas on the arrays showing irregular shapes or high background were excluded from the analysis. To avoid making conclusions on artefactual signals, only probes showing a signal intensity of at least 500 fluorescence units (Galbraith, 2003) for all biological replicates were considered for further analysis. Intra-chip data normalization was performed using probes for the tubulin housekeeping gene, and inter-chip data normalization was performed using the entire chip hybridization signal. Genes induced or suppressed by inoculation were identified using a paired Student's *t*-test on the ratios inoculated/control samples. The raw *P* values were adjusted by the Bonferroni correction, which controls the family-wise error rate (FWER). We considered as being significantly differentially expressed the genes with a Bonferroni *P* value of < 5% as described in Lurin *et al.* (2004) and a minimum signal difference of 1.3-fold.

Expression profiles of differentially expressed genes were grouped by *k*-means clustering using the GeneSpring program (Agilent Technologies, Redwood City, CA). The number of clusters was set to 4 and the minimal correlation coefficient (Pearson) was set to 0.7.

Gene expression analysis

For Northern blot analysis using SSH-derived cDNA probes, poly-A+-enriched RNA was extracted from barley embryos of Mirco-*rdg2a* and NIL3876-*Rdg2a* at 7, 11 and 14 days after inoculation with *P. graminea* or from non-inoculated control embryos collected at the same time points. Two micrograms of each mRNA sample was separated by electrophoresis in 1% agarose gels containing formaldehyde and blotted onto positively charged nylon membranes (Sigma, St Louis, MO) using 20× SSC transfer solution (Sambrook *et al.*, 1989). Preparation of ^{32}P-dCTP labelled probes, pre-hybridization, hybridization and washing conditions

were performed using standard procedures (Sambrook *et al.*, 1989). Autoradiography was performed using BioMax MS film (Kodak, New York).

qRT PCRs of plant genes were performed using mRNA samples obtained from a biological replicate independent of the three biological replicates used to make the microarray hybridization probes. First-strand cDNA was prepared from 1 μg of mRNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA), and quantified with a Qubit fluorometer (Invitrogen). qRT PCR was performed using duplicate or triplicate 25-μL reactions in a real-time PCR thermal cycler (7300 Real Time PCR System, Applied Biosystems). PCRs contained 1 ng of cDNA, 12.5 μL of Sybr Green qPCR Master Mix (Applied Biosystems) and forward and reverse primers (final concentration 0.2 μM each). The cycling conditions comprised 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Melting curve analysis was performed after PCR to evaluate the presence of non-specifc PCR products and/or primer dimers. Primer pairs used for the analysis were designed using the software Vector NTI Suite 9 (InforMax, Invitrogen). If possible, full-length cDNA sequences of genes were identified from the database and the 3′ used to design gene-specific primers, but otherwise primers were designed directly on the cloned fragments (primers sequences, accession numbers used for primer design and annotation are provided in supplementary Table S3). The qRT PCR data were plotted as ΔR_n fluorescence signal versus cycle number. The SDS 7300 absolute quantification software (Applied Biosystems) calculates the ΔR_n using the equation $\Delta R_n = (R_n^+) - (R_n^+)$, where R_n^+ is the fluorescence signal of the product at any given time and R_n^- is the fluorescence signal of the baseline emission during cycles 6–13. An arbitrary threshold was set at midpoint of the log ΔR_n versus cycle number at which the ΔR_n crosses the threshold (C_t). The C_t was used to calculate the fold changes (FC) in each infected sample with respect to the expression level detected in the corresponding sample under control conditions at same time point (baseline) with the following formula:

$$FC = 2^{-\Delta\Delta C_t}, \text{ where } \Delta\Delta C_t = (C_{t \text{ target}} - C_{t \text{ βact}})_{\text{infected sample}}$$
$$- (C_{t \text{ target}} - C_{t \text{ βact}})_{\text{uninfected sample}}$$

Normalization was carried out with the β-actin constitutively expressed gene. Standard variation in all samples was lower than 10%.

To test expression of putative fungal genes, primer pairs were designed directly from the sequences of the cloned fragments. To verify origin, the primer pairs were first used to amplify from fungal and plant genomic DNA of Mirco-*rdg2a* and NIL3876-*Rdg2a*. mRNAs isolated from Mirco-*rdg2a* and NIL3876-*Rdg2a* embryos at 7, 11 and 14 dai were used for the analysis. For the control, a bulk of mRNAs obtained from mycelium grown on PDA only at the same stage and conditions of the three inoculation time points was used. Expression of the fungal CAT1 (ubiquitin) gene was used in an attempt to make the amount of fungal input RNA in all the RT-PCR reactions the same (assuming equal CAT1 expression in PDA- and plant- grown fungus). RT-PCRs were performed with 1 ng of PDA-grown fungus mRNA template, while the amounts of inoculated embryo mRNA template were adjusted until the CAT1 amplification intensity appeared almost identical to that obtained from the PDA-grown fungus (obtained using 40–100 ng of inoculated embryo mRNA). At this stage, the amount of CAT1 amplification from inoculated embryos collected at 11 and 14 dai was still a little greater than for the PDA and 7 dai samples (Fig. 8, CAT1). Therefore, the CAT1 products were quantified by densitometric scanning and the 11- and 14-dai mRNA template amounts reduced further to achieve more accurate normalization in amplifications of the other gene fragments. First-strand cDNA was made in 50-μL volumes containing SuperScript II (Invitrogen, Carlsbad, CA), the manufacturer's pre-mix, 0.2 μL Taq Gold (Applera Corp., Norwalk), 0.3 μM each primer (sequences provided in supplementary Table S3) and specific quantities of mRNA template. Thirty-five cycles of amplification were carried out and amplification products were observed on 2% agarose gels.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Table S1 Functional classifications of differentially expressed barley genes.

Table S2 Cluster analysis results.

Table S3 Sequences of the primers utilized for qRT-PCR analysis of the barley embryo induced genes and for the RT-PCR of the fungal genes.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/full/10.1111/j.1364-3703.2008.00479.x
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