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Transcription of the defense response genes chitinase IIb, PAL and peroxidase is induced by the barley powdery mildew fungus and is only indirectly modulated by R genes

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Abstract

Barley powdery mildew resistance (PM-R) genes control different infection phenotypes against avirulent strains of *Blumeria graminis* f. sp. *hordei* (*Bgh*). A subset of seven Pallas isolines, containing the PM-R genes *Mla1*, *Mla12*, *Mlg*, *Mlk*, *Mlp* and *mlo5*, revealed fast-, intermediate- and slow-acting infection phenotypes. Scanning electron microscopy revealed the extent of *Bgh* development on each genotype through 72 hai. Quantitative RNA blot analysis of chitinase IIb, phenylalanine ammonia lyase and peroxidase transcription at 0–24 hai revealed similar patterns and levels of transcripts in all isolines including the susceptible parent Pallas. At 36–72 hai transcript accumulation was suppressed on the susceptible parent Pallas, where *Bgh* grew unimpeded. In resistant isolines transcript accumulation varied according to whether their PM-R genes were fast, intermediate or slow-acting. Transcript accumulation decreased at 36–72 hai in isolines with fast-acting PM-R genes (*Mla1*, *Mlg*, and *mlo5*), and this corresponded with arrested *Bgh* development. Transcript accumulation at 36–72 hai in isolines with intermediate or slow-acting PM-R genes (*Mla12* and *Mlk*, *Mlp*) remained elevated and correlated with continued *Bgh* development and contact. These results suggest that defense response genes are transcriptionally activated by *Bgh* contact, which probably involves a general elicitor(s) from *Bgh*. Thus, PM-R genes appear to only modulate defense response gene transcription indirectly by limiting fungal development and contact. Fast-acting PM-R genes halt *Bgh* development before 24 hai, while slow-acting R genes allow *Bgh* development throughout 72 hai.

Differences in infection phenotypes due to differing PM-R genes may be due to temporal differences in interactions between R genes and avirulence gene product(s); alternatively, slow-acting PM-R genes or required partner genes may be non-constitutive and need time to be induced.

Keywords: Barley; Defense response gene; PM-R gene

1. Introduction

Powdery mildew of barley is caused by the fungus *Blumeria graminis* DC Speer f. sp. *hordei* EM. Marchal (*Bgh*). This fungus is an obligate, biotrophic parasite of barley (*Hordeum vulgare* L.) [1,7]. Resistance, as judged by phenotypic infection types is controlled by various resistance genes (PM-R genes). These PM-R genes have been widely deployed in barley varieties with variable success [26,27]. Most known R genes control the specificity and timing of hypersensitive cell death (HR) through signaling pathways [4,15]. In barley, different PM-R genes control different infection phenotypes; these are fast acting, intermediate acting or slow acting with respect to time after inoculation [31,43]. In wheat, Slesinski and Ellingboe [55] and Hyde and Colhoun [25] were among the first to note that differences in the timing of resistance associated with PM-R genes correlate with differences in infection phenotype and that at the microscopic level PM-R genes controlled the extent of *Bg* fungal development. Thus, the HR that

Abbreviations: PGT, Primary germ tube; *Bgh*, *Blumeria graminis* f. sp. *hordei*; barley, *Hordeum vulgare*; hai, hours after inoculation; HR, Hypersensitive cell death; PAL, phenylalanine ammonia lyase; PM, powdery mildew disease; R, gene, resistance gene.

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occurred with fast-acting R genes gave low infection phenotype rankings, and there was little if any fungal development after conidial germination and attempted epidermal cell infection. Conversely, slow-acting R genes allowed fairly extensive fungal development and had higher infection phenotype rankings. The mechanism(s) that give rise to differences in timing between fast-acting, intermediate acting, and slow-acting PM-R genes and alleles remain a mystery.

The induction of general defense response gene transcription in barley during attempted penetration by *Bgh* is well documented [6,11–13,20]. Boyd et al. [5,6] and Davidson et al. [16] on the basis of blotting techniques found that defense response gene transcription occurred only slightly earlier in resistant than in susceptible barley lines and suggested that defense response gene transcription may be under the control of R-genes. Later experimentation by Clark et al. [11–13] using quantitative northern blots could not detect large differences in defense response gene transcription between resistant and susceptible barley lines during the first 0–24 hai.

Recently, a carbohydrate elicitor located on the conidial surface of *B. graminis* f. sp. *tritici* was demonstrated to induce defense response gene transcription. This general elicitor was not the product of a PM avirulence gene because it did not cause HR in wheat containing R genes, and neither did it cause HR in barley, oat, rice, rye or maize [51]. Thus, this molecule(s) is a general elicitor of defense response gene transcription, and its finding led us to again question the relationship between PM-R gene activity and defense response gene transcription.

Most studies into defense response gene transcription focus on one or two PM-R genes and this limits the interpretation of what constitutes a resistance phenotype. Our objective was to have a more comprehensive view of resistance, and to examine how defense response gene transcription changes across a set of differentially resistant barley isolines containing 'fast', 'intermediate', and 'slow'-acting PM-R genes. If PM-R genes controlled defense response gene transcription, we would expect that fast acting PM-R genes (low infection phenotypes) would result in earlier and/or greater levels of transcript accumulation than in isolines with slower acting PM-R genes (higher infection phenotypes). Conversely, similarity in the timing and levels of defense response gene transcription between resistant and susceptible isolines would suggest that defense response genes are not under the direct control of PM-R genes.

A subset of Pallas isolines [29] containing the PM-R genes *Mla1* (P01), *Mla12* (P10), *Mlg* (P21), *Mlk* (P17), *Mlp* (P19) and *mlo5* (P22) and the susceptible Pallas parent (P00) were selected. These had infection phenotypes ranging from completely resistant with no visible infection phenotype (Type 0) to completely susceptible with abundantly sporulating colonies (Type 4; see Table 1). The Pallas isoline set was created to study PM-R genes in a near identical genetic background, by crossing donor lines, containing different PM-R genes, with Pallas and then by backcrossing the resistant progeny with Pallas [29]. We confirmed the effects of differing infection phenotypes on *Bgh* fungal development using visible responses (Table 1), and by scanning electron microscopy (SEM; Fig. 1).

Table 1
Visual ratings of infection phenotype for barley isolines Pallas, P01, P10, P17, P19, P21, and P22 inoculated with *Bgh* CR3

Isoline	Isolate CR3 (a)	Isolate CR3 (a)	Kølster et al. 1986 (b)
Susceptible			
Pallas	4	4	4
Fast			
P22 (*mlo5*)	0 c	0	0/4
P01 (*Mla1*)	0 c, n	1	0
P21 (*Mlg*)	0 n	1	0, 2–3 n
Intermediate			
P10 (*Mla12*)	1 c	1	0 n
Slow			
P17 (*Mlk*)	3 n	2	1 n
P19 (*Mlp*)	2 n	2	2 nc

Seven to eight day old seedlings were inoculated with *Bgh* CR3 and incubated for 7–8 days before being rated. c: chlorosis; n: necrosis. (a) Independent ratings taken of isolate of *Bgh* CR3. (b) Published disease severity ratings for Pallas isolines using an avirulent race of *Bgh*.

We used quantitative Northern blotting to determine if temporal and quantitative differences in the steady state levels of three well-known barley, *Bgh* inducible, defense response genes corresponded to infection phenotype for a susceptible and a set of differentially resistant barley isolines. We used a *Bgh* inducible barley phenylalanine ammonia lyase (PAL) probe (first isolated by Green [18]), a *Bgh*-induced barley peroxidase (Genbank accession number: AJ003141) [30], and a previously unknown *Bgh*-inducible response gene RP5, which encodes barley class IIb chitinase (Genbank accession number: X78672) [6,11–13,16,20]. We independently cloned and confirmed that clone RP5 encodes a class IIb chitinase (data not shown). This RP5 fragment was *Bgh* induced in three different barley lines containing the PM-R genes *Mla1*, *mlo5* and *Mlp* [11–13,16].

These defense response genes were selected because they represent parts of different physiological host responses to *Bgh*. PAL activity is required for biosynthesis of phenylpropanoids, which are required for penetration and cell death responses to *Bgh* in barley and in wheat to stem rust [7,41,42,53]. Peroxidase activity produces the oxidative power for cross-linking of proteins and phenylpropanoid radicals resulting in reinforcement of cell walls against attempted fungal penetration [23,30]. High levels of H_2O_2 trigger HR, while lower levels induce the accumulation of transcripts encoding antioxidant enzymes [34]. Since high levels of H_2O_2 also preclude HR, peroxidase activity may modulate HR by altering the concentration of available

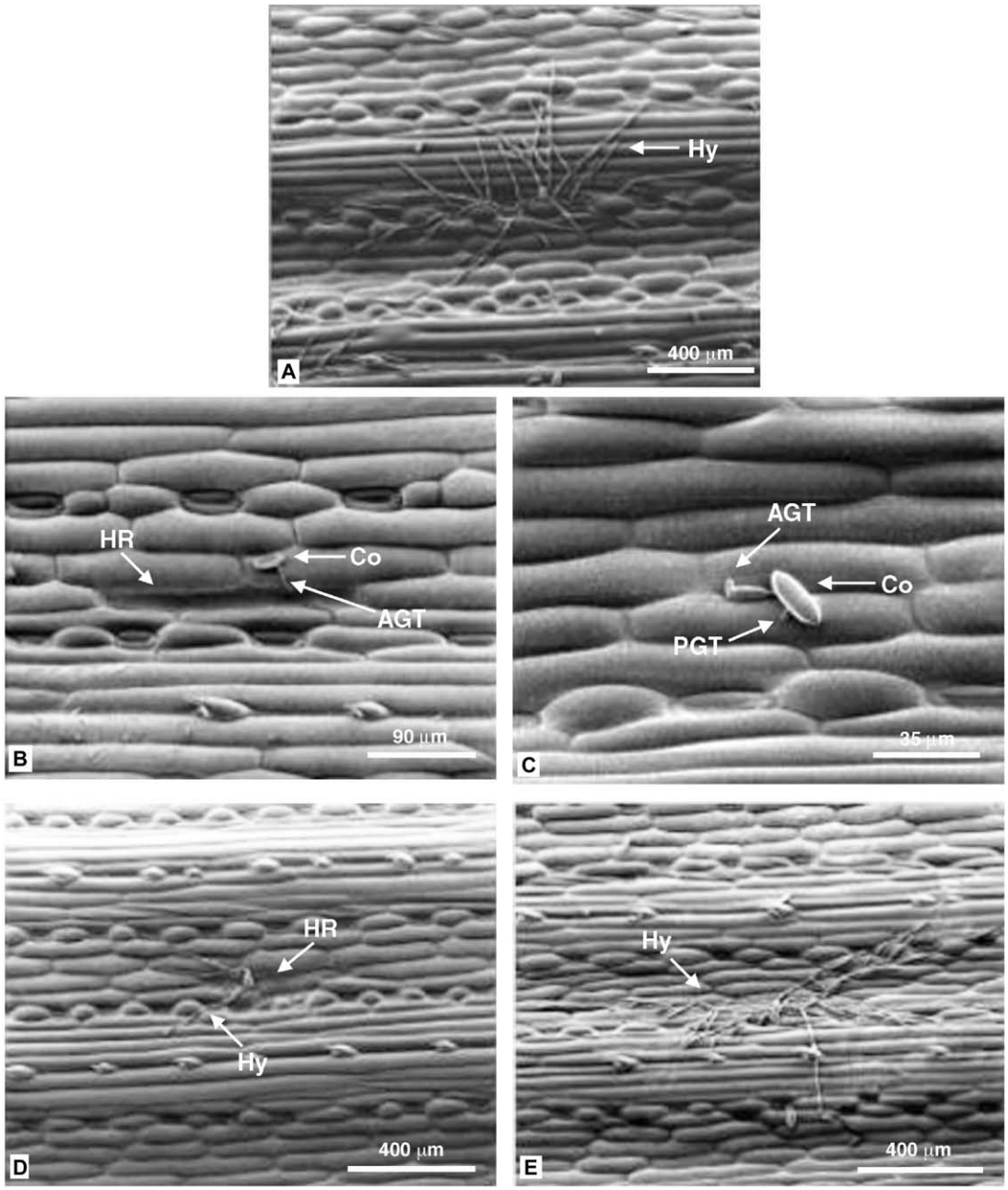

Fig. 1. Scanning electron micrographs of germinated *B. graminis* f. sp. *hordei* conidia at 72 hai. Specimens were frozen-hydrated, uncoated, and a 1.5 kV beam current was used. At 72 hai the development of *Bgh* and epidermal cell death (collapsed cells) can be seen. (A) The susceptible parent cultivar Pallas (P00), (B) P01 contained the fast acting *Mla1* R gene with rapid single epidermal cell HR resulting in arrested fungal development after conidia germination; (C) P22 containing the fast acting *mlo5* R gene where strong penetration resistance prohibits infection and arrests fungal development after conidia germination; (D) P10 contained the intermediate acting *Mla12* R gene, with some later HR that allows limited hyphal development at 72 hai; and, (E) P19 contained the slow-acting *Mlp* R gene which allowed extensive hyphal development and no HR was seen at 72 hai. AGT: appressorial germ tube, Co: conidiospore, HR: hypersensitive cell death, Hy; hyphae, PGT: primary germ tube.

H_2O_2 [23,30,33,57,60]. Chitinase activity has a direct inhibitory effect on *Bgh* because it hydrolyzes chitin in *Bgh* cell walls and impedes its growth [59]. Furthermore, the peroxidase clone that was used is expressed predominantly in the epidermis [30], whereas the PAL and chitinase clones are expressed in the epidermis and mesophyll.

2. Materials and methods

2.1. Barley isolines

Six resistant lines (P01, P10, P17, P19, P21 and P22) from the Pallas isogenic set containing the resistance genes

Mla1, *Mla12*, *Mlk*, *Mlp*, *Mlg* and *mlo5*, respectively, were selected [29]. Pallas, the backcross parent, was included as the susceptible control.

Plants were potted in University of California soil mixture C [40] and grown in growth chambers for 8–9 days at 20 ± 1 °C with 90 ± 10% relative humidity. Continuous white, fluorescent light with a photon flux density at leaf level of 150 μmol m^{-2} s^{-1} was provided.

2.2. Powdery mildew fungus

A single isolate of the *Bgh* (*B. graminis* DC f. sp. *hordei* Marchal) race 3, designated CR3, was used. This isolate was tested for visible phenotype and it was found to be avirulent to the powdery mildew resistance genes in the selected of Pallas isolines, but it was virulent on the Pallas parent (Table 1). The CR3 isolate was provided by W.R. Bushnell (Cereal Disease Laboratory, USDA-ARS, St Paul, MN, USA), and was maintained on Algerian/4* Man (S) barley.

2.3. Inoculation and sampling

Two replicate planting, inoculation and sampling experiments were done. Eight-day-old plants were inoculated with *Bgh* CR3 from infected Algerian/4 (f14) Man S source plants at an average conidial density of about 300 spores cm^{-2}.

First formed leaves were sampled at 0, 2, 4, 6, 8, 10, 12, 15, 18, 24, 36, 48 and 72 h after inoculation. A 10 cm apical segment from approximately 30 leaves was harvested per sample. Uninoculated, control leaves were sampled at 0, 15, 36 and 72 h.

2.4. Infection phenotype

Two 8 in. pots were sown with four seeds of an isoline and were grown under the same conditions. After 9-day old seedlings were inoculated with CR3 and then rated for disease development after 12–15 days using a 0–4 scale [39] where: 0 = no visible symptoms; 1 = small mycelial colonies without sporulation; 2 = small colonies with limited sporulation; 3 = intermediate colonies with moderate sporulation; and, 4 = large colonies with abundant sporulation. In addition, the occurrence of leaf necrosis (n) and chlorosis (c) was noted.

2.5. Scanning electron microscopy

Leaf tissue was inoculated with *Bgh* conidia in a settling tower and was sampled at 72 hai. Leaf segments of about 5 mm were cut and were prepared as frozen-hydrated on a −80 °C cryostage. Specimens were not coated with metals, and a 1.5 kV beam current was used to produce secondary electron images. A Philips 500X SEM was used throughout [13,61].

2.6. RNA extraction

Primary leaves from 10 seedlings were pooled for each of the 238 RNA extractions required for all time points and all barley genotypes. Extractions were performed using a modified protocol of Giroux and Pauls [17]. Leaf tissue was ground in liquid nitrogen using a mortar and pestle, and extraction buffer (200 mM Tris–HCl, pH 8.2; 100 mM LiCl; 5 mM EDTA and 1%, v/v SDS) was added to the ground powdered leaf material. The ground slurry was incubated at 65 °C for 15 min and then extracted with TLE-buffered phenol (TLE: 200 mM Tris–HCl, pH 8.0; 100 mM LiCl; 4.5 mM EDTA) and chloroform-isoamyl alcohol (24:1, v/v). After mixing, samples were centrifuged (Beckman J-21B Centrifuge, Palo Alto, CA, USA) at 10,000g for 20 min at 4 °C and the aqueous phase was extracted with phenol and chloroform-isoamyl alcohol. A final extraction with chloroform-isoamyl alcohol was done to remove traces of phenol. LiCl was added to a final concentration of 2 M and samples were placed at −20 °C overnight and then centrifuged at 9000*g* for 30 min at 4 °C. The RNA pellets were washed in 70% ethanol and resuspended in 0.5 ml DEPC water.

2.7. cDNA library preparation

Messenger RNA (mRNA) was purified from total RNA extracted from inoculated leaf tissue of P22 by oligo-dT affinity chromatography using the mRNA Easy™ purification kit (Stratagene, La Jolla, CA, USA). Seven micrograms of mRNA, from *Bgh*-inoculated Pallas 22, was used for the cDNA synthesis with ZAP-cDNA® Synthesis kit (Stratagene, La Jolla, CA, USA). The resultant cDNA was ligated into λ vector arms and packaged in λ phage using Gigapack® III Gold packaging extract (Stratagene, La Jolla, CA, USA). Positively hybridized clones were released as Bluescript plasmids from phage by coinfecting SOLR™ cells with ExAssist™ helper phage (Stratagene, La Jolla, CA, USA).

2.8. DNA sequencing and analysis

Automated DNA sequencing of selected cDNA clones was done by the Advanced Genetic Analysis Center (University of Minnesota, St Paul MN) on an ABI PRISM® 377 DNA Sequencer (PE Biosystems, Foster City, CA). Clones were provided as cDNA inserts in Bluescript plasmids and sequencing was primed with T7 and T3 oligonucleotides.

2.9. RNA blot preparation and hybridization

Fifteen micrograms of total RNA per lane was run in a 1.2% denaturing agarose gel and transferred onto nylon membranes (Hybond™N +, Amersham Pharmacia Biotech, Uppsala, Sweden) overnight using 20 × SSC [19].

Prehybridization of the RNA membranes was carried out for 4 h at 42 °C in 50% formamide, 5 × SSPE, 5 × Denhardt's, 0.1% SDS and 150 μg/ml salmon sperm DNA. Hybridization was carried out in fresh buffer containing 10^6 cpm/ml ^{32}P-labeled cDNA probes at 42 °C overnight. The cDNA probes were labeled with ^{32}P using Random Primers DNA Labeling System (Gibco BRL Life Technologies, Gaithersburg, MA, USA). Membranes were washed in 1 × SSPE, 0.1% SDS at 42 °C for 1 h, and then in 0.1 × SSPE, 0.1% SDS at 60 °C for another hour. Radioactive hybridization intensity was detected using a Storm™ 850 phosphor screen imaging system (Molecular Dynamics, Sunnyvale, CA, USA) and quantified using ImageQuant software (supplied by Molecular Dynamics). Background hybridization was measured by sampling membrane areas outside loaded lanes. Background radiation counts were then subtracted using ImageQuant. After background subtraction the net signal from each lane was normalized for the amount of total leaf RNA in each lane.

An initial normalization was performed with two sets of RNA blots, containing 15 and 1.5 μg total RNA, using a 28S tomato ribosomal cDNA probe supplied by A. Smith, Department of Horticulture, University of Minnesota, St Paul [46]. By normalizing RNA blots using two different quantities of RNA we were able to obtain highly reproducible hybridization intensities between experiments. The blots for each experiment were first hybridized with the chitinase probe. They were then stripped in boiling 0.1% SDS before being rehybridized with PAL and then stripped again before being hybridized with peroxidase. The blots were then stripped a final time and hybridized with the 28S tomato ribosomal cDNA to check normalization.

Hybridization intensity data is presented as bar graphs. The bars represent the average values of the hybridization intensity (× 10^3) for each sample from two experiments, plus standard deviation. Dashed lines in Figs. 2–4 show the standard deviation above and below average hybridization intensities for uninoculated controls.

3. Results

3.1. Infection phenotype

We identified four classes of infection phenotypes in our Pallas subset using a visual 0–4 scale (Table 1). Abundant hyphal colonies and chains of conidia were visible on the susceptible parent, Pallas. Only Pallas had an infection phenotype '4'. The 'fast-acting' resistant class had an infection phenotype '0' because no visible fungal growth occurred. However, on some of the plants necrotic and/or chlorotic responses were visible. The isolines P01 (*Mla1*), P21 (*Mlg*) and P22 (*mlo5*) were in class 0. An 'intermediate-acting' class had an infection type '1' because there was some visible hyphal development. Some leaf chlorosis was also noted. The P10 (*Mla12*) isoline was the only member of this class. The 'slow-acting' resistant class contained P17 (*Mlk*) and P19 (*Mlp*), and had infection type '2' or '3' as colony growth and slight sporulation was visible. Necrotic lesions were also visible (Table 1).

3.2. Fungal development on barley leaves 72 hai

The development of *Bgh* CR3 germlings on barley lines from each of the infection phenotype classes was examined 72 hai with SEM (Fig. 1). On the susceptible Pallas isoline fungal hyphae developed radially outward from the germinated, infecting conidia and spread over the leaf surface (Fig. 1A). Epidermal cells in contact with or close to hyphae were turgid and did not differ from epidermal cells distant from hyphae. On the fast-acting PM-R gene containing isolines there were no hyphae; germinated *Bgh* conidia did not develop beyond the matured appressorial germ tube stage. In P01 (*Mla1*) fungal growth arrest was associated with HR of the attacked epidermal cells, which had collapsed (Fig. 1B). HR cell collapse was also observed with fast-acting P21 (*Mlg*). In P22 (*mlo5*) germinated conidia were arrested without any accompanying HR, no collapsed epidermal cells were evident (Fig. 1C). On the intermediate-acting isoline P10 (*Mla12*) germinated conidia penetrated and infected, and limited *Bgh* hyphal growth occurred. Often, epidermal cells that were initially infected collapsed, but the other epidermal cells appeared 'normal' (Fig. 1D). On the slow-acting resistant isolines P19 (*Mlp*) and P17 (*Mlk*) *Bgh* hyphal development at 72 hai was similar to that on the susceptible Pallas isoline (Fig. 1E). There was no HR of initially infected epidermal cells.

3.3. Defense response gene mRNA accumulation

3.3.1. Time course of chitinase transcript accumulation

In the first 24 hai there were two peaks of chitinase transcript accumulation. These occurred at 4–6 hai and 15–24 hai, in the susceptible Pallas parent as well as in all other isolines i.e. those with fast-, intermediate- and slow-acting PM-R genes (Fig. 2). The peaks of transcript accumulation coincided with the timing of attempted penetration from *Bgh* primary and appressorial germ tubes, respectively. In contrast, chitinase transcripts in uninoculated leaves did not vary with time and were maintained at low steady state levels. Relative to uninoculated leaves, *Bgh* inoculated leaves had a 2-fold rise in the level of chitinase transcripts at 4–6 hai and an 8–10-fold induction at 15–24 hai. This occurred in the susceptible and all resistant isolines (Fig. 2).

At 36 and 48 hai, the steady state levels of chitinase in the susceptible parent Pallas declined and by 72 hai they were near those of uninoculated controls (Fig. 2a). Isolines with fast-acting PM-R genes, P01 (*Mla1*), P21 (*Mlg*) and P22 (*mlo5*) had similar declines, although the decreases in P01 (*Mla1*) were somewhat slower to occur (Fig. 2b, d and f). By

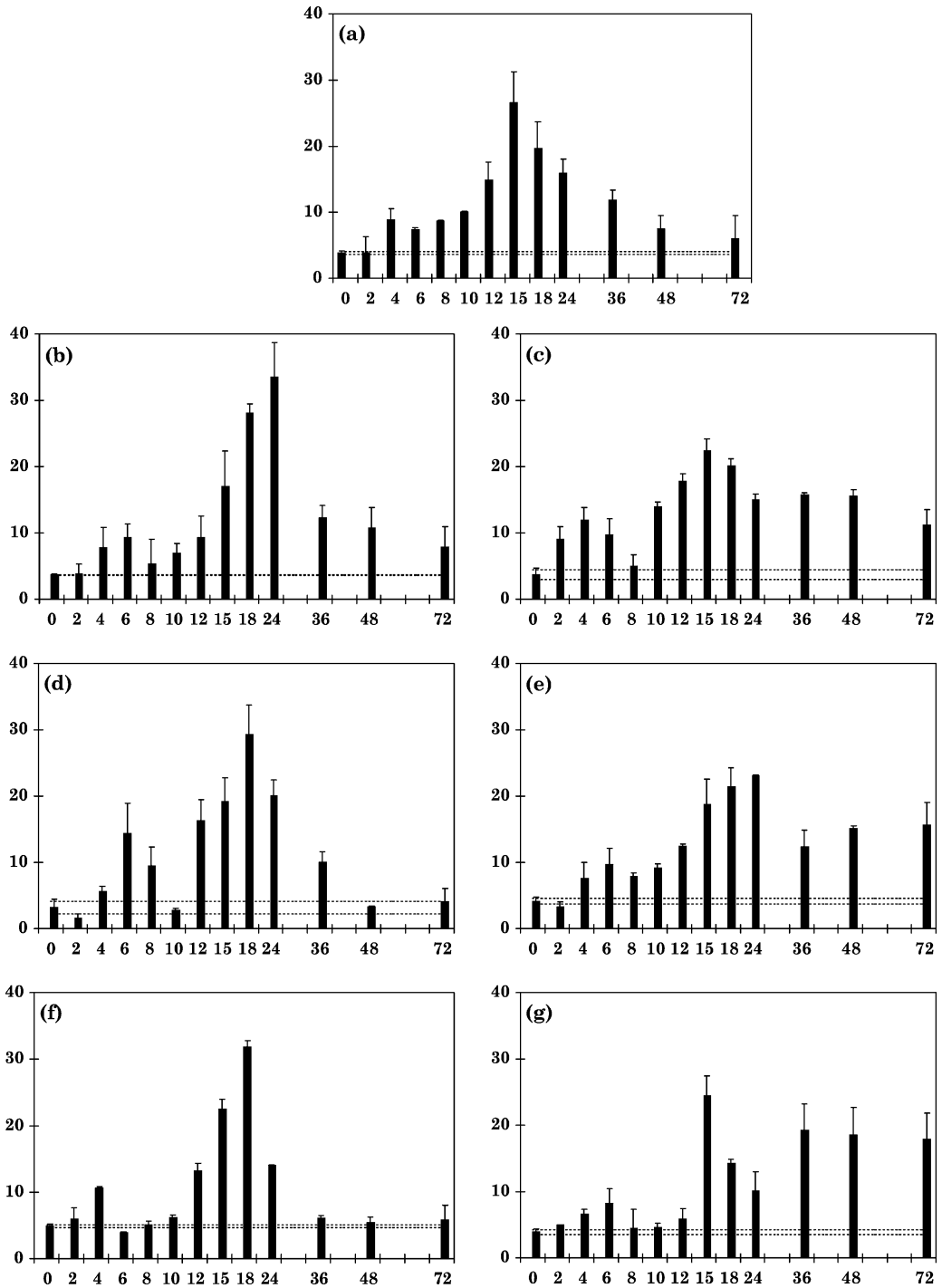

Fig. 2. Levels of chitinase transcripts. Temporal changes in the steady state level of defense response gene transcripts for (a) Pallas, (b) P01 containing *Mla1*, (c) P10 containing *Mla12*, (d) P21 containing *Mlg*, (e) P17 containing *Mlk*, (f) P22 containing *mlo5*, and (g) P19 containing *Mlp*. The bars represent the average values of the hybridization intensity ($\times 10^3$) for each sample from two experiments, plus standard deviation. The dashed lines represent one standard deviation above and below the average hybridization intensity for the uninoculated controls, which were sampled at 0, 15, 36 and 72 h. The average for the uninoculated controls is not shown.

contrast the levels of chitinase transcripts at 36, 48 and 72 hai were maintained above uninoculated levels in the intermediate- P10 (*Mla12*) and slow-acting P17 (*Mlk*) and P19 (*Mlp*) PM-R gene containing isolines (Fig. 2c, e and g). The maintenance of high levels of chitinase transcript in these isolines coincided with hyphal growth, which contacted previously uncontacted epidermal cells; this phenomenon was observed by SEM at 72 hai (Fig. 1).

3.3.2. Time course of PAL transcript accumulation

During the first 24 hai, there were also two peaks in the steady state level of PAL transcripts. These occurred around

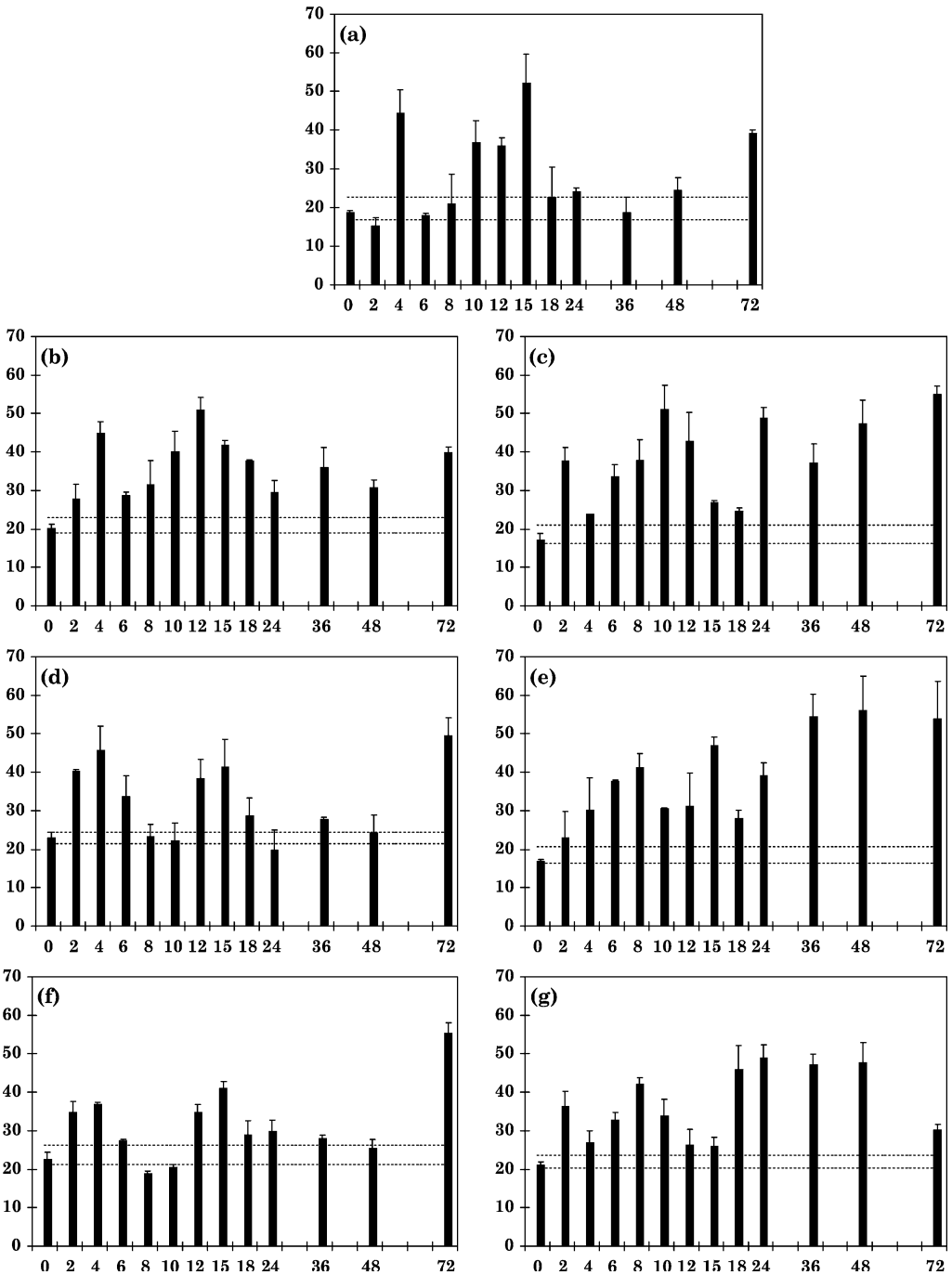

Fig. 3. Levels of PAL transcripts. Temporal changes in the steady state level of defense response gene transcripts for (a) Pallas, (b) P01 containing *Mla1*, (c) P10 containing *Mla12*, (d) P21 containing *Mlg*, (e) P17 containing *Mlk*, (f) P22 containing *mlo5*, and (g) P19 containing *Mlp*. The bars represent the average values of the hybridization intensity ($\times 10^3$) for each sample from two experiments, plus standard deviation. The dashed lines represent one standard deviation above and below the average hybridization intensity for the uninoculated controls, which were sampled at 0, 15, 36 and 72 h. The average for the uninoculated controls is not shown.

4 and at 10–15 hai in the susceptible Pallas and in all PM-R gene containing, resistant Pallas isolines (Fig. 3). In uninoculated control plants the levels of PAL transcripts remained relatively constant. When compared to uninoculated leaves, there was a 1.5–2-fold induction in PAL transcripts during the initial *Bgh* induced peak and a 2–2.5-fold induction during the second peak.

At 36 and 48 hai the steady state level of PAL transcripts in the susceptible parent Pallas declined and approached levels found in uninoculated controls (Fig. 3a). A decrease also occurred in PM-R gene containing fast-acting isolines P01 (*Mla1*), P21 (*Mlg*) and P22 (*mlo5*) (Fig. 3b, d and f). Like chitinase, this decline took a little longer in the P01 with the *Mla1* PM-R gene. In the intermediate- P10 (*Mla12*)

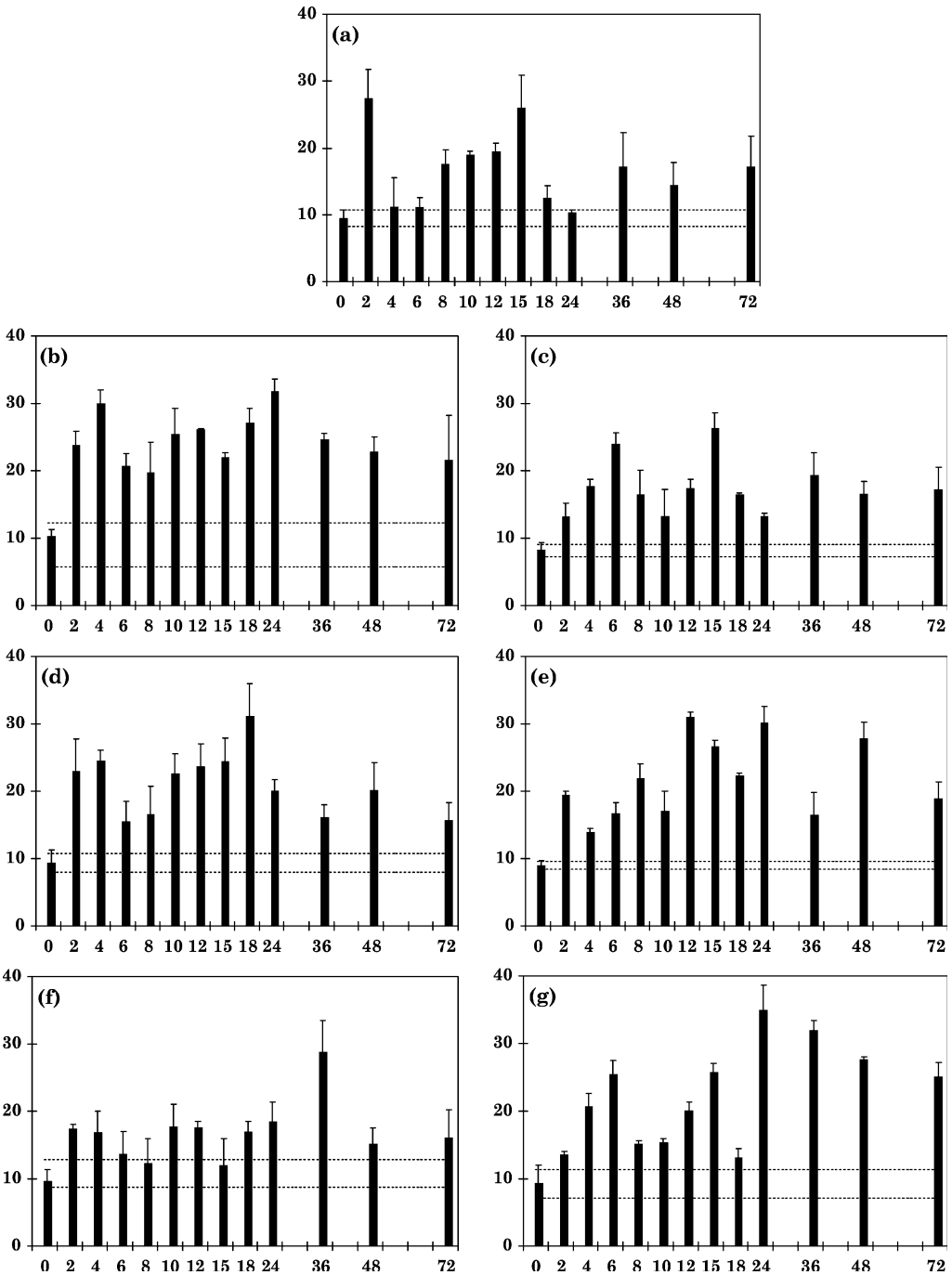

Fig. 4. Levels of peroxidase transcripts. Temporal changes in the steady state level of defense response gene transcripts for (a) Pallas, (b) P01 containing *Mla1*, (c) P10 containing *Mla12*, (d) P21 containing *Mlg*, (e) P17 containing *Mlk*, (f) P22 containing *mlo5*, and (g) P19 containing *Mlp*. The bars represent the average values of the hybridization intensity ($\times 10^3$) for each sample from two experiments, plus standard deviation. The dashed lines represent one standard deviation above and below the average hybridization intensity for the uninoculated controls, which were sampled at 0, 15, 36 and 72 h. The average for the uninoculated controls is not shown.

and slow-acting resistant isolines P17 (*Mlk*) and P19 (*Mlp*) levels of PAL transcripts at 36 and 48 hai were higher than in the susceptible parent Pallas and in the isolines with fast-acting PM-R genes (Fig. 3c, e and g). Furthermore, at 36 and 48 hai, levels of PAL transcripts were greater than or equal to peaks at 10–15 hai (Fig. 3).

At 72 hai PAL transcripts accumulated at higher levels than in uninoculated leaves. This 72 hai increase occurred in all isolines, except P19 containing *Mlp*. This rise in PAL transcripts at 72 hai appeared to be independent of continued *Bgh* growth and development as observed by SEM (Fig. 1).

3.3.3. Time course of peroxidase transcript accumulation

As with chitinase and PAL there were two peaks in the steady state levels of peroxidase transcripts in the first 24 hai in Pallas and in all PM-R containing isolines (Fig. 4). These occurred at 2–6 and at 15–24 hai. There was approximately a 2.5–3-fold increase in peroxidase transcripts in Pallas and in all PM-R containing isolines, except P22 containing *mlo5*. In P22 there was only a 1.5-fold increase compared to levels in uninoculated control leaves.

At 36, 48 and 72 hai levels of peroxidase transcripts in the susceptible parent Pallas decreased from its highest peak at 15 hai until it reached levels in uninoculated control leaves. In P01 with the fast-acting *Mla1* PM-R gene allele, peroxidase transcripts were higher than in uninoculated leaves. In the P21 with the fast-acting PM-R gene *Mlg*, peroxidase transcripts were just barely higher than those in uninoculated controls at 36, 48 and 72 hai. The level of peroxidase transcripts was highest at 36 hai in P22 with is fast acting penetration resistance allele *mlo5*, but at 48 and 72 hai levels were at or slightly greater than in uninoculated leaves.

In P10 with the intermediate acting PM-R gene allele *Mla12*, peroxidase transcripts at 36, 48 and 72 hai remained consistently higher than in uninoculated controls. In the slow-acting PM-R genes in P17 (*Mlk*) and P19 (*Mlp*) peroxidase levels were consistently greater than uninoculated steady state levels at 36, 48 and 72 hai (Fig. 4).

4. Discussion

The selected subset of Pallas isolines were classed as susceptible, fast-, intermediate- and slow-acting resistant, based on their infection phenotypes. Dramatic differences in *Bgh* development on leaf epidermis of Pallas barley isolines containing differing PM-R genes were easily distinguishable by 72 hai using SEM (Fig. 1). The extent of fungal development observed with SEM on each isoline was consistent with the visible infection phenotype (Table 1; Fig. 1).

Fast acting PM-R genes in P01 (*Mla1*), P21 (*Mlg*), and P22 (*mlo5*) allowed no *Bgh* hyphal development (Fig. 1). Hyphal development, which requires establishment of a haustorium, was prevented either by penetration resistance and rapid HR in the cases of P01 (*Mla1*) (Fig. 1B) and P21 (*Mlg*), or penetration resistance alone in the case of P22 (*mlo5*) (Fig. 1C). The results with these fast acting PM-R genes agreed with previous studies involving these particular R genes [1,8–13,24,29,31,38,50,61,62]. The intermediate acting P10 (*Mla12*) isoline limited hyphal development was observed and cell death of the initially infected epidermal cells occurred sometime before 72 hai, apparently slowing *Bgh* growth (Fig. 1D). In the barley line Sultan5, which also contains *Mla12* and was the donor parent in P10, HR was reported as early as 24 hai and the frequency of HR increased at 30 and 48 hai [50]. Slow acting PM-R genes in the isolines P17 (*Mlk*) and P19 (*Mlp*) allowed robust hyphal growth by 72 hai (Fig. 1E). In fact at 72 hai it was not possible to distinguish between the slow acting P17 and P19 isolines and the susceptible parent Pallas (Fig. 1A and 1E). Thus, HR in the isolines P17 (*Mlk*) and P19 (*Mlp*) occurs after 72 hai allowing visible colonies to form (Table 1).

We examined defense response gene transcription at 0–72 hai. In the time span between 0 and 24 hai our quantitative RNA blot data revealed no major temporal or quantitative differences in the steady state levels of the Class IIb chitinase, PAL or peroxidase transcripts between the susceptible parent Pallas and any of the PM-R gene containing resistant isolines (Figs. 2–4). The 0–24 hai time interval coincides with conidial germination, attempted penetration, and occurs on all isolines [31]. Our data strongly suggests that accumulation of the three defense response gene transcripts is a general response of barley to infection attempts by germinating *Bgh* conidia. These data suggest that *Bgh* or its attempted penetration activities, produces a general elicitor(s) that is sensed by a general receptor(s) leading to defense response gene transcription the barley Pallas background. Our results are also consistent with previous quantitative northern blot studies of defense response gene transcription caused by *Bgh* attack on other barley lines at 0–24 hai [11–13]. The idea that *Bgh* may produce a general elicitor of defense response gene transcription is supported by the results of Schweizer et al. [51] using *B. graminis* f. sp. *tritici* (*Bgt*). They showed that an elicitor from *Bgt* conidia caused transcription of defense response genes in wheat and in non-hosts like barley, rice and maize. The existence of a similar general elicitor(s) in germinating *Bgh* conidia would explain the general induction of defense response gene transcripts that we observed between 0 and 24 h. At 0–24 hai there was no evidence that PM-R genes were in any way involved with the transcription of the class IIb chitinase, PAL or specific inducible peroxidase defense response genes. Thus, we have strong evidence supporting the idea of Collinge et al. [14] that defense response genes are transcriptionally activated in a coordinated manner in all barley genotypes contacted by *Bgh*. Our 0–24 h time period also supports the idea of a general elicitor(s), general receptor(s) model of inducing defense response gene transcription put forward by Schweizer et al. [51].

At 36, 48 and 72 hai the level of defense response genes transcripts in isolines with PM-R genes were consistent with the extent of hyphal development. This also suggests the continued presence of a general elicitor(s). However, as will be discussed later, the susceptible parent Pallas differed in defense response gene transcript accumulation at these later time periods, suggesting suppression of defense response gene transcription.

Of the three defense response genes investigated, chitinase had the clearest patterns of transcript accumulation

in response to *Bgh* at 36, 48 and 72 hai. In addition, steady state levels of chitinase transcripts in uninoculated plants were less than those of PAL and peroxidase. This is probably because chitinase has a very specific role in defense. There are isoforms of PAL and peroxidase involved in other metabolic functions and their transcription is independent of the defense response, and it is possible that our cDNA probes may have hybridized slightly with conserved sequences of other PAL and peroxidase homologs. On the susceptible parent Pallas and on isolines with fast acting *Mlg* and *mlo5* PM-R genes the 36–72 hai levels of defense response gene transcripts were lower than on the isolines with intermediate- and slow acting PM-R genes. This was generally the case, albeit less apparent, for the fast acting *Mla1* PM-R gene. Thus, where penetration resistance or rapid HR occurred there was no hyphal development and the continued elicitation of defense response gene transcription like that occurring earlier at the appressorial contact peaks (15–18 hai) was not evident. In contrast, the intermediate- and slow-acting isolines had slower rates of cell death and continued hyphal growth, during 36–72 hai. The continued hyphal contact, brought about by lack of penetration resistance and a slower rate of HR, appears responsible for additional *Bgh* contact and defense response gene transcription at 36, 48 and 72 hai (Figs. 2–4). Thus, our data indicates that PM-R genes exert only indirect control of the accumulation of defense response gene transcripts by influencing fungal development and hyphal growth. It is the continued growth and contact of *Bgh* hyphae, and their attempted penetrations, that causes continued defense response gene transcription—presumably due to a general elicitor(s) sensed by a general receptor(s) in the barley isolines.

It is unclear why there are differences in the timing of HR associated with individual PM-R genes. Recently R gene alleles from the *Mla* locus were found to encode for NBS-LRR type proteins [22]. Some alleles at this locus (e.g. *Mla12*) as well as some unlinked PM-R genes require the participation of other down stream genes like *Rar1* for full expression of resistance [47,54]. However, there are other PM-R genes, including alleles at the *Mla* locus, which do not require *Rar1* [48]. Thus, in barley, race specific PM resistance is mediated through RAR1-dependent and RAR1-independent signaling pathways, and while *Rar1* is a convergence point in PM-R gene signaling leading to HR its function is not required by all PM-R genes [48]. Homologues of barley *Rar1* in *Arabidopsis thaliana* and *Nicotiana benthamiana* also function in some, but not all resistance gene signaling pathways, which indicates that the function of *Rar1* in regulating resistance is conserved in monocots and dicots [35,44,58]. The RAR1 protein interacts with subunits of the SCF (Skp1-Cullin-F-box) ubiquitin ligase complex, which is believed to result in the degradation of regulatory proteins resulting in programmed cell death, HR [2,3].

Differences in the timing of HR presumably involve signaling pathways operating at different rates. However, since the intermediate-acting R gene *Mla12* and the slow-acting R gene *Mlk* are both RAR1-dependent it suggests that a degree of control may occur upstream of RAR1 and involve differences in *Bgh* avirulence gene product-R gene interaction. If this were the case then differences in the timing of HR may occur because fast acting PM-R genes recognize *Bgh* avirulence factors produced at early stages of attempted infection while slow acting PM-R genes recognize different avirulent gene products produced at later stages of fungal development. An alternative explanation may involve the need for higher levels avirulence gene product because intermediate and slow acting R gene products are not as efficient at signal reception or are not as abundantly produced. Perhaps intermediate and slow acting PM-R gene proteins are not constitutive and they or their required partner genes need time to be induced and their products expressed. Whatever the explanation for fast, intermediate and slow acting PM-R genes involves, the ability to clone avirulence genes from *Bgh* and to accurately measure the steady state levels of avirulence and R gene products should help design experiments in which various hypotheses can be tested.

The susceptible parent Pallas did not maintain induced levels of defense response gene transcripts at 36, 48 and 72 hai despite prolific *Bgh* hyphal growth and colony development. We believe that this may be due to suppression of defense response gene transcription by *Bgh*. Suppression of defenses occurs in several pathogenic and in symbiotic plant–fungal interactions [21,52]. Although a suppressor from *Bgh* has not yet been identified, there is evidence to support the idea that the suppression of barley defenses leads to an increase in the frequency of successful *Bgh* penetration. Physiological studies have shown that general penetration resistance and HR of barley containing *Mla1* are suppressed by inhibition of phenylpropanoid synthesis [31,62]. Furthermore, the phenomenon of induced accessibility of barley to avirulent races of *Bgh* may also be due to suppression of defense responses [36–38].

The availability of genetic maps and ESTs should provide greater understanding of *Bgh* obligate parasitism and facilitate the identification and cloning of potential *Bgh* suppressors [45,56]. Transcript profiling experiments and comparative bioinformatics, which have been applied in other pathosystems, may make it possible to screen the barley transcriptome for all genes elicited or suppressed by *Bgh* [28,32,49].

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