The Relationship Between the Expression of Defense-Related Genes and Mildew Development in Barley

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The induction of five defense-related genes in barley was examined in relation to the development of the fungus *Erysiphe graminis* f. sp. *hordei*, the causal agent of powdery mildew. The barley cultivar Midas, carrying the resistance allele *Mla6*, was inoculated with one of two near-isogenic *E. g.* f. sp. *hordei* isolates, CC142 (*Aa6*) and CC143 (*Va6*). The defense genes were not induced by wounding alone, but required the presence of the growing pathogen. The defense genes were induced in response to both isolates, showing similar transcript patterns up to 24 hr after inoculation. It was only after 24 hr that a resistance response to the avirulent isolate was seen. By 30 hr after inoculation a significant difference in the rates of development of the two isolates was observed. The inhibition of the development of the avirulent isolate, CC142, paralleled the continued high levels of transcript seen for three of the five defense genes examined.

Additional keywords: disease resistance, gene-for-gene resistance, *Hordeum vulgare*, race-specific resistance.

Powdery mildew is a major disease of plants in temperate climates. In barley, powdery mildew is caused by the fungus *Erysiphe graminis* f. sp. *hordei*. The genetics of mildew resistance in barley is well understood. Many of the resistance loci follow Flor's gene-for-gene hypothesis (Jørgensen 1988), by which an incompatible response results from the interaction between a plant resistance gene and the corresponding avirulence gene in the pathogen. Nearly half of the known mildew resistance alleles in barley map to a single locus, *Mla* (Jahoor and Fischbeck 1993). However, the occurrence of *E. g.* f. sp. *hordei* isolates able to overcome these resistance alleles reduces the usefulness of gene-for-gene controlled resistance to farmers and breeders.

The mechanism of genetic resistance and the products of these resistance loci are unknown. However, several plant proteins which accumulate in response to pathogen attack have been identified (Bowles 1990; Collinge and Slusarenko 1987). Proteins with known biochemical activity include hydrolytic enzymes, such as chitinases and β-1,3-glucanases; proteins involved in cell wall construction, such as peroxidases and hydroxyproline-rich glycoproteins; enzymes of the phenyl propanoid pathway; ribosome-inactivating proteins (Leah *et al.* 1991); and low molecular weight proteins with toxic properties, such as thionins (Bohlmann *et al.* 1988) and proteins with homology to putative plant lipid-transfer proteins (Molina *et al.* 1993). Other pathogenesis-related (PR) proteins, of unknown activity, are also produced (Bowles 1990). One such group, the PR-R proteins, shares homology with thaumatin (van Kan *et al.* 1989), zeamatin (Vigers *et al.* 1991), and a putative bifunctional α-amylase–trypsin inhibitor from maize (Richardson *et al.* 1987). The role of these proteins in disease resistance is not clear. While most of these proteins belong to multigene families, only certain members of each family are induced by infection (Bowles 1990).

In vitro antifungal activity has been attributed to a number of these defense-related proteins, including chitinases (Toyoda *et al.* 1991; Verburg and Huyuh 1991), β-1,3-glucanases (Ludwig and Boller 1990; Mauch *et al.* 1988), leaf-specific thionins (Bohlmann *et al.* 1988), and PR-R homologous proteins isolated from barley seed (Bryngelsson and Gréen 1989; Hejgaard *et al.* 1991). In transgenic plants, containing chimeric constructs between defense gene promoters and the β-glucuronidase gene (GUS), high levels of GUS expression are seen around the site of pathogen attack (Doerner *et al.* 1990; Roby *et al.* 1990; Samac and Shah 1991). Expression of several defense genes in transgenics has resulted in an increase in resistance (Alexander *et al.* 1993; Broglie *et al.* 1991; Carmona *et al.* 1993; Logemann *et al.* 1992), although particular defense genes appear to be effective against specific sets of pathogens (Alexander *et al.* 1993).

The stages of development of *E. g.* f. sp. *hordei* are well defined, and simultaneously inoculated spores show highly synchronized development (Aist and Bushnell 1991; Carver 1988). Germinating spores produce a primary germ tube (PGT) 1–2 hr after inoculation (hai). The PGT may enter the epidermal cell wall, although this does not always appear to be essential for continued development of the spore (Carver 1988; Carver and Bushnell 1983; Kunoh *et al.* 1978). A second, appressorial germ tube (AGT) is produced shortly after the PGT. The end of the AGT swells to form a mature appressorium some 10–12 hai. The fungus attempts to breach the epidermal cell wall by producing an appressorial infection peg (AIP) from the appressorium. If this fails, secondary, tertiary, and even quaternary lobes may be produced from the appressorium, each of which in turn is capable of producing an infection peg in an attempt to penetrate the cell wall. In response, the plant deposits secondary metabolites, including callose, silicon, calcium, and phenolic-based compounds, in

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papillae beneath the PGT and appressorial lobes (Aist and Bushnell 1991; Carver 1988). If an AIP successfully breaches the cell wall, it swells to form a haustorium, through which the fungus derives nutrients from the plant. The membrane of the epidermal cell is displaced as the haustorium develops, forming digitate processes, which increase the surface area between the fungal wall and the plant cell membrane. Elongating secondary hyphae (ESH) are produced from the appressorial arm, forming a sporulating colony in a compatible interaction, and completing the asexual cycle.

The interaction between barley and *E. g.* f. sp. *hordei* provides an ideal system for studying defense gene responses to pathogen infection. *E. g.* f. sp. *hordei* grows predominantly on the host's surface, infecting only the epidermal cell layer of the plant. This allows the pathogen's development to be followed microscopically while biochemical changes within the plant are monitored. The well-defined genetics of barley gene-for-gene resistance to mildew enables us to relate biochemical changes involved in resistance both to the resistance allele and to the corresponding pathogen avirulence gene. Resistance to mildew in barley ranges from a nonhypersensitive resistance response, with no visual symptoms (infection type 0, or IT0), through hypersensitive resistance (IT1–IT3), showing chlorosis and necrotic flecking, to complete susceptibility (IT4) (Moseman *et al.* 1965). Here we examine the interaction between *E. g.* f. sp. *hordei* and the barley cultivar Midas, which carries the resistance allele *Mla6,* giving nonhypersensitive resistance (IT0). The growth of two near-isogenic isolates of *E. g.* f. sp. *hordei,* CC142 (*Aa6*) and CC143 (*Va6*), was followed in relation to the induction of five defense genes, representing five major groups of defense-related proteins. In this way, we were able to study the interaction between defense gene induction and the resistance, recognition gene *Mla6.*

RESULTS

Microscopic development of mildew isolates.

The microscopic development of two near-isogenic isolates of *E. g.* f. sp. *hordei,* CC142 (*Aa6*) and CC143 (*Va6*), was examined over a period of 72 hr following inoculation of barley cultivar Midas (*Mla6*). This microscopic study was repeated, although sample times differed in the two experiments (experiments 2 and 3). Except for virulence to *Mla6* (*Va6*), CC142 and CC143 have identical pathogenicity profiles, moderate resistance to the fungicide triadimenol and sensitivity to ethirimol, and identical genetic fingerprints (see Brown *et al.* [1991], who described CC142 as α1MS and CC143 as δ1MS). The virulences of CC142 and CC143 were checked every 3 months and did not change over the period during which these experiments were conducted.

During the early stages of development no difference could be distinguished between the avirulent and the virulent isolates. Both isolates showed similar percentages of germlings at each developmental stage up to 24 hai (Fig. 1). From 30 hai onwards, the development of CC142 appeared to slow com-

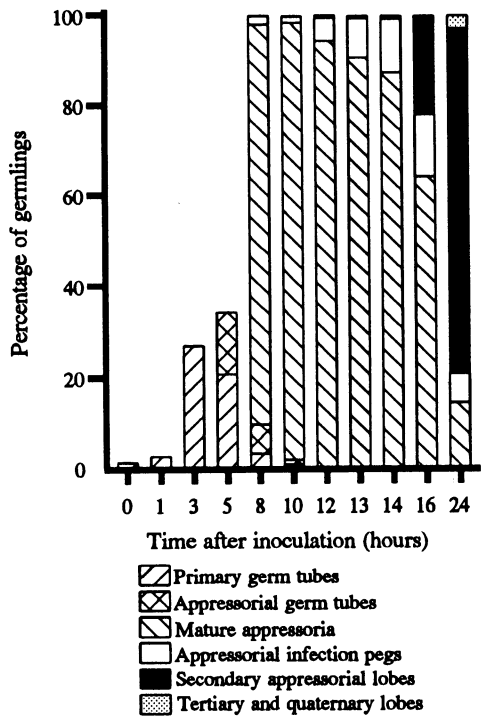

Fig. 1. Percentage of *Erysiphe graminis* f. sp. *hordei* germlings at each stage of development up to 24 hr after inoculation (experiment 2). The barley cultivar Midas (*Mla6*) was inoculated with either of two near-isogenic isolates, CC142 (*Aa6*) or CC143 (*Va6*). No significant differences in the frequencies of germlings of the two isolates at each stage of development were seen up to 24 hr after inoculation. The average of these frequencies for the two isolates are shown.

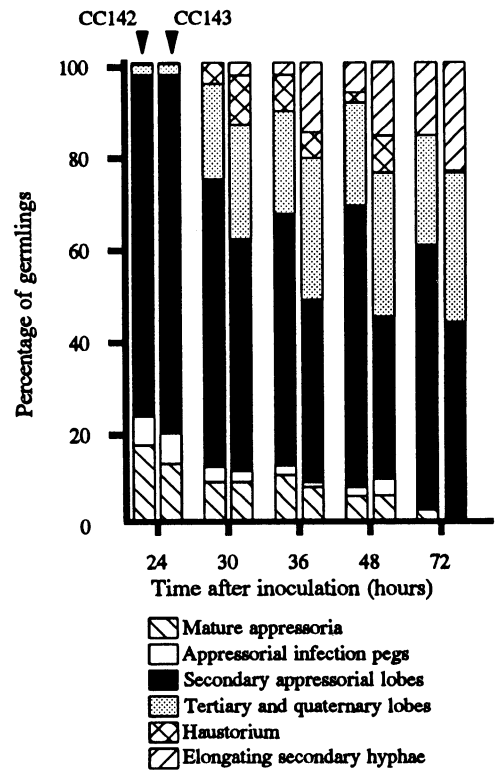

Fig. 2. Percentage of *Erysiphe graminis* f. sp. *hordei* germlings at each stage of development from 24 to 72 hr after inoculation (experiment 2). The barley cultivar Midas (*Mla6*) was inoculated with either of two near-isogenic isolates, CC142 (*Aa6*) or CC143 (*Va6*). Significant differences in the frequencies of germlings of the two isolates at each stage of development were seen only after 24 hr after inoculation.

pared to that of CC143 (Fig. 2 and Tables 1 and 2). Both isolates went on to produce haustoria and ESH, but a larger percentage of CC142 germlings remained at the secondary lobe stage. In experiment 2, approximately 75% of both CC142 and CC143 germlings had reached the secondary lobe stage by 24 hai (Fig. 2), but by 30 hai there was a 10% difference between the two isolates. While the percentage of CC142 germlings at the secondary lobe stage remained around 60%, CC143 germlings continued to develop, reducing the percentage of spores seen at the secondary lobe stage at later times. This arrest in development of the avirulent isolate at the secondary lobe stage was more apparent in experiment 2 (Table 1) than in experiment 3 (Table 2). In experiment 3, more CC142 germlings went on to develop tertiary and quaternary lobes than in experiment 2. Overall, both isolates developed faster in experiment 3, which may have allowed more CC142 germlings to make additional attempts to breach the plant cell wall before the resistance mechanism of the plant took effect. In experiment 3, 3.6% of CC142 and 10.3% of CC143 germlings had produced haustoria by 24 hai, while in experiment 2, only 0.4% of CC142 and 0.2% of CC143 had reached this stage by 24 hai. While significantly more haustoria and ESH were produced by CC143, by 72 hai microscopic colonies of both isolates were present. However, colonies produced by CC143 were twice the size of those of CC142. The mean colony length of CC142 was 292 μm in experiment 2 and 355 μm in experiment 3, and that of CC143 was 540 and 673 μm, respectively. The probability of obtaining, by chance, a difference at least as large as that observed is $P < 0.025$ for experiment 2 and $P < 0.001$ for experiment 3.

Papillae, produced in the leaf beneath PGTs and appressorial lobes, can be detected by epifluorescent microscopy because of the deposition of phenolic-based compounds. Papillae are thought to reduce the chances of an *E. g.* f. sp. *hordei* germling successfully breaching the plant cell wall, a high frequency of papillae being correlated with mildew resistance in barley (Aist and Israel 1986; Bryngelsson and Collinge 1991). However, there were no significant differences in the number of papillae formed in response to inoculation with CC142 and CC143 (data not shown). In experiment 2, fluorescent papillae were seen under approximately 30% of the germlings by 8 hai, the majority of this fluorescence being beneath PGTs. By 16 hai, fluorescent papillae were present beneath 85% of the germlings, while by 24 hai, 60% of the germlings with secondary lobes exhibited fluorescence under both appressorial lobes.

Table 1. Significant differences in development of *Erysiphe graminis* f. sp. *hordei* isolates CC142 and CC143 (experiment 2)[a]

Hours after inoculation	Growth stage[b]	Probability[c]	Direction of significance
30	2° lobes	$P < 0.05$	CC142 > CC143
	HAU	$P < 0.001$	CC142 < CC143
36	2° lobes	$P < 0.001$	CC142 > CC143
	3° and 4° lobes	$P < 0.05$	CC142 < CC143
	ESH	$P < 0.001$	CC142 < CC143
48	2° lobes	$P < 0.001$	CC142 > CC143
	3° and 4° lobes	$P < 0.01$	CC142 < CC143
	HAU	$P < 0.001$	CC142 < CC143
	ESH	$P < 0.001$	CC142 < CC143
72	2° lobes	$P < 0.001$	CC142 > CC143
	3° and 4° lobes	$P < 0.05$	CC142 < CC143
	ESH	$P < 0.05$	CC142 < CC143

[a] The development of two near-isogenic isolates of *E. g.* f. sp. *hordei*, CC142 (*Aa6*) and CC143 (*Va6*), was examined over a 72-hr period after inoculation of the barley cultivar Midas (*Mla6*) (experiment 2). The times at which there was a significant difference between the frequencies of the growth stages of the two isolates are shown.
[b] 2° = Secondary; 3° = tertiary; 4° = quaternary; HAU = haustoria; ESH = elongating secondary hyphae.
[c] Probability of obtaining, by chance, a difference at least as large as that observed.

Temporal induction of defense-related genes.

The induction of five defense-related genes was examined in the barley cultivar Midas, following inoculation with *E. g.* f. sp. *hordei* isolates CC142 and CC143, in three separate experiments. Samples were taken at different times after inoculation in the three experiments. In experiments 2 and 3 gene induction was examined in relation to mildew development. The mRNA levels of a chitinase clone (pD6), a peroxidase clone (pD8), a phenylalanine ammonia lyase (PAL) clone (pP15), a clone showing homology to the PR-R protein from tobacco (pD12) (Green 1991), and a leaf-specific thionin clone (DB4) (Bohlmann and Apel 1987) were measured.

To determine whether viable spores were necessary for defense gene induction, Midas was inoculated with killed spores of isolates CC142 and CC143. Spore viability tests showed zero germination after 5 days. Leaf samples were taken for RNA extraction at 0, 5, 12, 14, 24, 48, and 72 hai. The chitinase, peroxidase, and PR-R homologous clones were hybridized to total RNA, but no hybridization was detected against

Table 2. Significant differences in development of *Erysiphe graminis* f. sp. *hordei* isolates CC142 and CC143 (experiment 3)[a]

Hours after inoculation	Growth stage[b]	Probability[c]	Direction of significance
12	AIP	$P < 0.001$	CC142 < CC143
24	HAU	$P < 0.001$	CC142 < CC143
36	3° and 4° lobes	$P < 0.001$	CC142 > CC143
	ESH	$P < 0.001$	CC142 < CC143
48	2° lobes	$P < 0.001$	CC142 > CC143
	3° and 4° lobes	$P < 0.01$	CC142 < CC143
	ESH	$P < 0.001$	CC142 < CC143
52	ESH	$P < 0.001$	CC142 < CC143
56	3° and 4° lobes	$P < 0.01$	CC142 > CC143
	ESH	$P < 0.001$	CC142 < CC143
58	2° lobes	$P < 0.001$	CC142 > CC143
	ESH	$P < 0.001$	CC142 < CC143
60	2° lobes	$P < 0.001$	CC142 > CC143
	ESH	$P < 0.001$	CC142 < CC143
72	HAU	$P < 0.01$	CC142 > CC143
	ESH	$P < 0.001$	CC142 < CC143

[a] The development of two near-isogenic isolates of *E. g.* f. sp. *hordei*, CC142 (*Aa6*) and CC143 (*Va6*), was examined over a 72-hr period after inoculation of the barley cultivar Midas (*Mla6*) (experiment 3). The times at which there was a significant difference between the frequencies of the growth stages of the two isolates are shown.
[b] 2° = Secondary; 3° = tertiary; 4° = quaternary; AIP = appressorial infection pegs; HAU = haustoria; ESH = elongating secondary hyphae.
[c] Probability of obtaining, by chance, a difference at least as large as that observed.

any of these clones, at any of the sample times (data not shown).

The chitinase, peroxidase, PAL, and PR-R genes were induced in barley following inoculation with either isolate (Figs. 3 and 4), demonstrating a nonspecific induction of transcription by *E. g.* f. sp. *hordei,* irrespective of virulence. At 0 hai, little or no transcript of these four defense genes was detected, except in experiment 3, in which peroxidase mRNA was present (Fig. 4). The reason for the preinduction of the peroxidase gene in this experiment is not known. However, the temporal pattern of peroxidase transcript in experiment 3 was the same as that in experiments 1 (data not shown) and 2 (Fig. 3). The chitinase, peroxidase, and PAL genes were induced at 3–5 hai, coinciding with the development of PGTs, and at 12–14 hai, coinciding with AIP development. Chitinase and PAL transcripts were at higher levels than peroxidase transcripts at 3–5 hai. The PR-R gene did not respond to PGT development, gene induction first being seen at 12–14 hai (Fig. 3). Differences in the induction of chitinase, peroxidase, and PR-R in barley inoculated with CC142 and CC143 were first apparent around 30–36 hai. Higher mRNA levels of these three genes were maintained in barley inoculated with the avirulent isolate than in plants inoculated with the virulent isolate, demonstrating a specific response to the avirulent isolate. These higher mRNA levels persisted for more than 20 hr. At 56–58 hai these transcript levels in CC142- and CC143-inoculated barley were again similar (Fig. 4). Total RNA from experiment 1, probed with the chitinase, peroxidase, and PR-R clones, gave the same nonspecific and specific defense gene responses observed in experiments 2 and 3 (data not shown). With the PAL clone, a specific response to the avirulent isolate was less apparent. In experiment 2, the levels of PAL mRNA differed markedly at only one sample time, 48 hai, when the level was threefold higher in CC142-inoculated barley than in CC143-inoculated barley (Fig. 3). In experiment 3, higher levels of PAL mRNA were again seen in the avirulent inoculation at only one sample time, 36 hai (data not shown).

Total RNA from experiments 2 and 3 was probed with a leaf-specific thionin clone. In both experiments, the levels of thionin mRNA in Midas were already high at 0 hai. In experiment 2, there was a slight increase in thionin mRNA at 3 hai in CC142-inoculated barley (Fig. 5). While the levels of mRNA fluctuated in both the avirulent and the virulent interactions, over the 72-hr sampling period, no specific response to the avirulent isolate was apparent. In experiment 3, there was very little change in thionin mRNA levels above those seen at 0 hai, in both CC142- and CC143-inoculated barley (data not shown).

The quality and quantity of each RNA sample was checked by staining with ethidium bromide after separation on a formaldehyde-agarose gel. To ensure that equal amounts of mRNA had been loaded for each sample, one membrane from each experiment was stripped and reprobed with the wheat ssRubisco gene (Broglie *et al.* 1983). In experiments 1 (data not shown) and 2 (Fig. 6) the ssRubisco gene hybridized equally to total RNA from the earlier samples, but showed slightly less hybridization in the later samples. In experiment 3, this effect was more pronounced, the hybridization signal at all sample times being weaker than that at 0 hai (data not shown).

Induction of defense-related genes following wounding.

The effect of physical damage on defense gene induction was examined in the barley cultivar Midas. In one test, 12-day-old barley plants were grown in a spore-free greenhouse,

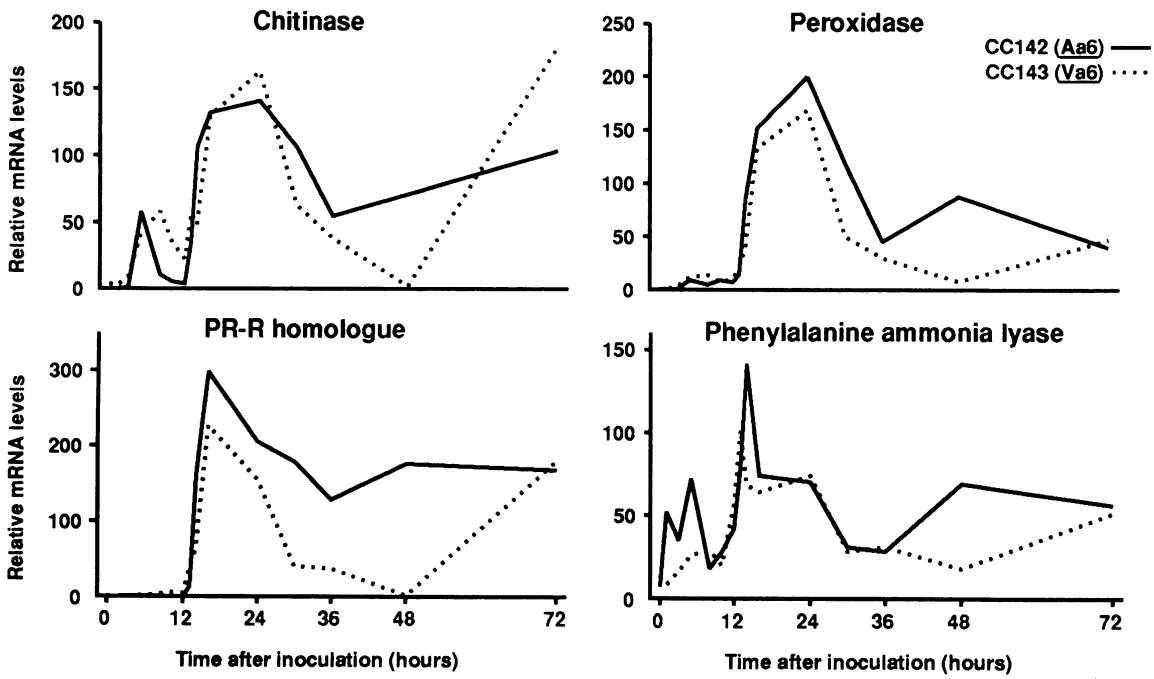

Fig. 3. mRNA levels of four defense-related genes induced in barley following inoculation with *Erysiphe graminis* f. sp. *hordei* (experiment 2). The barley cultivar Midas (*Mla6*) was inoculated with either of two near-isogenic isolates, CC142 (*Aa6*) or CC143 (*Va6*). RNA values were obtained from autoradiograms scanned with a densitometer. PR-R = pathogenesis-related R protein.

while in a second, plants were grown under sterile conditions. Greenhouse-grown barley was wounded, either by perforating the adaxial surface of the leaf with the end of a needle or by rubbing Carborundum over the leaf surface. The plants grown under sterile conditions were wounded with Carborundum. The wounding experiments were repeated, but only the experiments with the most detailed time course are shown here.

In the nonsterile wounding experiment, total RNA was probed with the chitinase, peroxidase, and PR-R clones (Fig. 6). The size of the mRNA transcripts hybridizing to these clones were the same as those seen in barley inoculated with *E. g.* f. sp. *hordei.* Induction of the PR-R gene was not seen until 26–30 hr after wounding (haw), while chitinase and peroxidase were induced as early as 3–8 haw. From repeat experiments, no clear pattern of induction over time was discernible (data not shown), although higher levels of induction were seen at later sample times, and in general more transcript was apparent in the needle-wounded material.

It was considered that induction of these defense genes might be due to opportunistic microorganisms gaining access to the cells of the leaf through wound openings. To eliminate this factor, barley seedlings were grown under sterile conditions. However, growing barley under these conditions tends to reduce the vigor of the plants. Only the more vigorous plants, with a near normal appearance, were wounded with Carborundum. The chitinase, peroxidase, and PR-R clones were hybridized to total RNA. No chitinase or peroxidase mRNA was detected in sterile-grown plants after wounding (data not shown). The PR-R gene however, detected high transcript levels at 0 haw in sterile-grown plants. After wounding, the levels of PR-R mRNA declined, and by 74 haw no PR-R transcript was detected (Fig. 7). The physiological stress imposed on the barley seedlings by growing them under sterile conditions may have induced the expression of a PR-R gene, while wounding eliminated this stress-induced transcription.

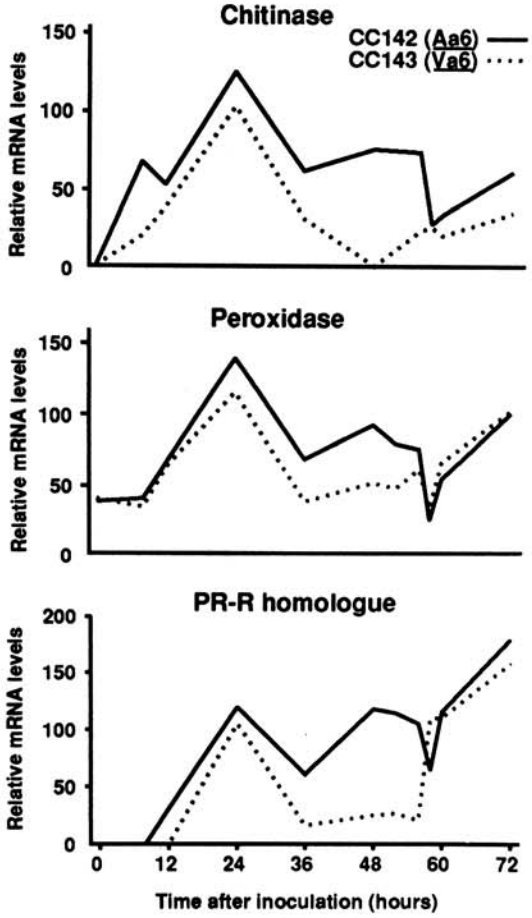

Fig. 4. mRNA levels of three defense-related genes induced in barley following inoculation with *Erysiphe graminis* f. sp. *hordei* (experiment 3). The barley cultivar Midas (*Mla6*) was inoculated with either of two near-isogenic isolates, CC142 (*Aa6*) or CC143 (*Va6*). RNA values were obtained from autoradiograms scanned with a densitometer. PR-R = pathogenesis-related R protein.

DISCUSSION

The response of five barley defense genes to *E. g.* f. sp. *hordei* infection were monitored in relation to the development of the pathogen. The five clones examined included genes for a chitinase, a peroxidase, a PAL, a PR-R homologous protein, and a leaf-specific thionin. Induction of the chitinase, peroxidase, and PR-R genes was not seen following inoculation with killed *E. g.* f. sp. *hordei* spores or after wounding under sterile conditions. Induction of these genes was seen in wounded plants grown under nonsterile conditions and was probably due to opportunistic microorganisms gaining access to leaf tissues through wound sites. Therefore, physical damage to the plant cell, caused by the fungus as it breaches the cell wall, does not appear to be sufficient for defense gene induction; other fungal-specific recognition factors are probably required.

Both the avirulent and the virulent isolates of *E. g.* f. sp. *hordei* were able to induce expression of these genes. The chitinase, peroxidase, and PAL genes showed induction at two points during the development of *E. g.* f. sp. *hordei*, one corresponding to the appearance of PGTs at 3–5 hai, the other to AIP development at 12–14 hai. The PR-R gene, however, only responded to AIP development, showing no expression until 12–14 hai. A differential response to mildew infection was only observed around 30 hai, after haustoria had begun to form. The development of CC142 slowed in comparison to that of CC143, while at the same time a specific response of

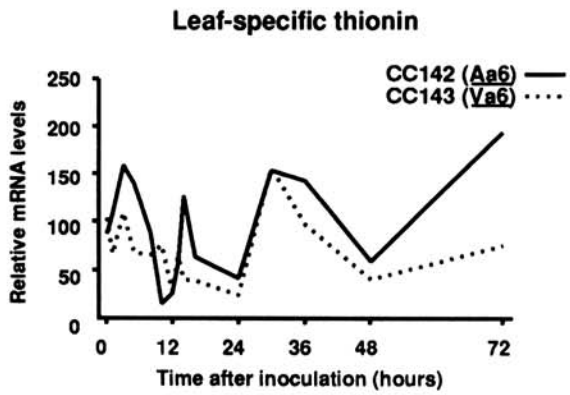

Fig. 5. mRNA levels of a leaf-specific thionin gene, clone DB4, induced in barley following inoculation with *Erysiphe graminis* f. sp. *hordei* (experiment 2). The barley cultivar Midas (*Mla6*) was inoculated with either of two near-isogenic isolates, CC142 (*Aa6*) or CC143 (*Va6*). RNA values were obtained from autoradiograms scanned with a densitometer.

the chitinase, peroxidase, and PR-R genes was observed in barley inoculated with the avirulent isolate. Transcripts of these three genes were maintained at higher levels in CC142-inoculated barley, these higher mRNA levels being apparent until up to 56–58 hai. This maintenance of higher mRNA levels in the avirulent interaction was seen in all three experiments, and it could well be an important contributing factor to the inhibition of the avirulent isolate. However, this specific response to the avirulent isolate was not seen for the PAL and thionin transcripts. There is evidence to suggest that particular host defense genes are effective against particular pathogens (Alexander *et al.* 1993). The chitinase, peroxidase, and PR-R genes looked at in this study may specifically be involved in resistance to *E. g.* f. sp. *hordei,* showing a race-specific response that was not readily apparent with the PAL gene examined. The use of PAL inhibitors in oats reduced adult plant resistance to *E. g.* f. sp. *avenae* (Carver *et al.* 1991) and in barley broke *Mla* but not *mlo* resistance to *E. g.* f. sp. *hordei* (Carver and Zeyen 1993). In barley, thionins present in the cell wall accumulate in the papillae formed beneath avirulent *E. g.* f. sp. *hordei* germlings, but not those of a virulent isolate (Ebrahim-Nesbat *et al.* 1989). However, as in this study, others have found no differences in thionin mRNA levels in barley inoculated with avirulent and virulent isolates (Bohlmann *et al.* 1988). The absence of a specific induction of the thionin gene in barley inoculated with the avirulent isolate may be due to the utilization of thionin transcript already present at 0 hai or to a redistribution of the thionin already present in the cell wall.

It is not known how the gene-for-gene resistance mechanism functions at the cellular or molecular level, nor have the products of the *Mla* alleles or those of the *E. g.* f. sp. *hordei* avirulence genes been identified. Davidson *et al.* (1987, 1988) have postulated that resistance genes may function as regulators of host mRNA expression following infection by *E. g.* f. sp. *hordei.* Macroscopically, *Mla6* gives a nonhypersensitive (IT0) resistance response, with no visible signs of infection after inoculation with an avirulent isolate. However, it is clear from the work reported here that expression of this resistance does not become effective until at least 24 hai. Both isolates are capable of forming ESH and colonies, although colonies formed by CC142 are significantly smaller than those of CC143. The *Mla6* resistance response appears to impede the development of the avirulent isolate, the effect

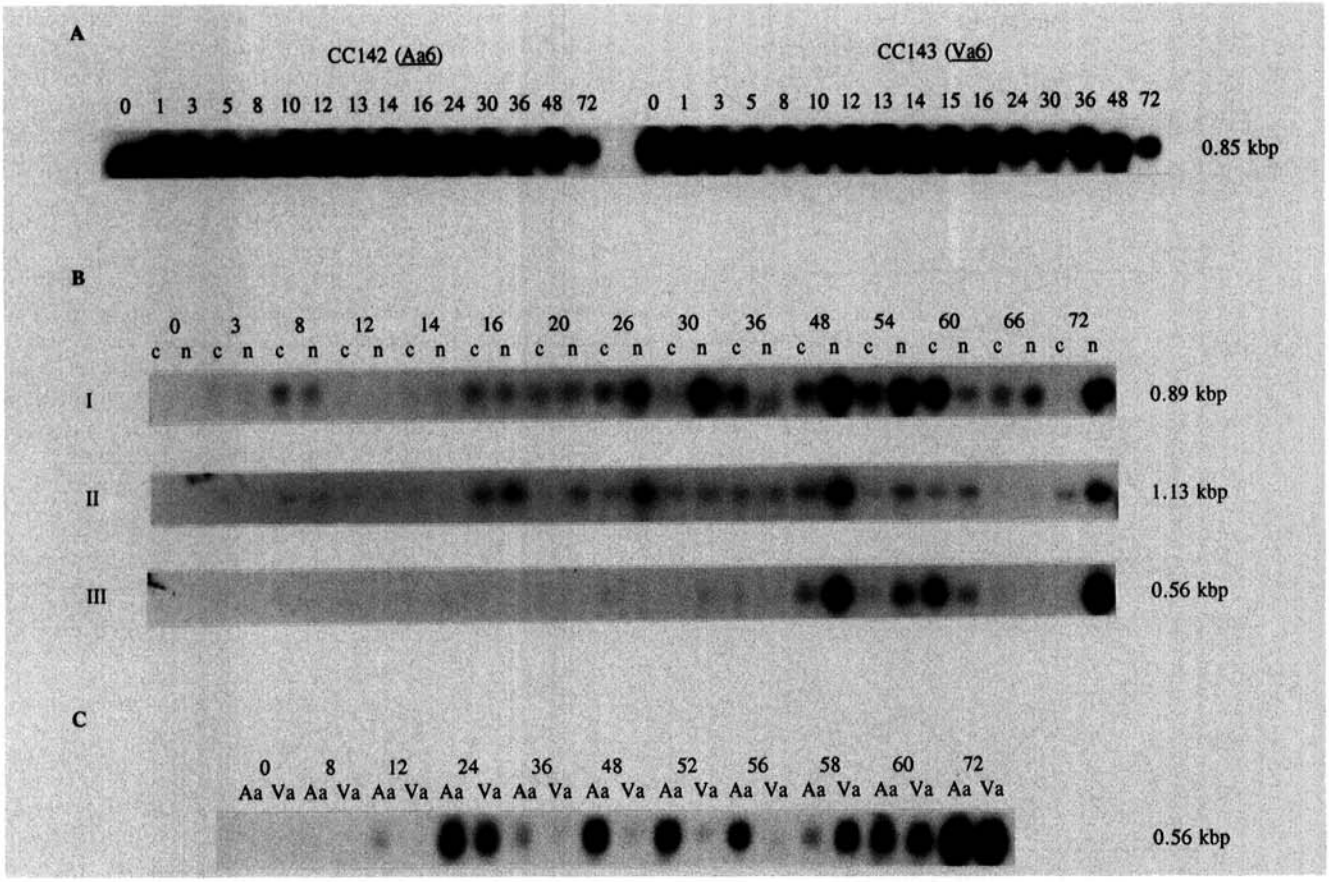

Fig. 6. A, Total RNA probed with the wheat ssRubisco gene (experiment 2). The barley cultivar Midas (*Mla6*) was inoculated with either of two near-isogenic *Erysiphe graminis* f. sp. *hordei* isolates, CC142 (*Aa6*) and CC143 (*Va6*). The sample times, in hours after inoculation, are shown at the top of the figure. **B,** Induction of defense-related genes in wounded barley. Plants of the cultivar Midas, grown in a spore-free greenhouse, were wounded, either by perforating the adaxial leaf surface with a needle (n) or by rubbing with Carborundum (c). The times at which leaf samples were taken, in hours after wounding, are indicated at the top of the figure. Total RNA was probed with a chitinase clone (I), a peroxidase clone (II), and a clone homologous to a pathogenesis-related R protein (PR-R) (III). **C,** Total RNA probed with the PR-R homologous clone (experiment 3). Aa = total RNA from the barley cultivar Midas inoculated with isolate CC142; Va = total RNA from Midas inoculated with isolate CC143. The sample times, in hours after inoculation, are shown at the top of the figure. The sizes of the mRNA transcripts are indicated in kilobase pairs.

first being observed at the secondary lobe stage. The development of CC142, seen before a retardation in its growth becomes apparent, may reflect the point at which the *Mla6* resistance mechanism becomes operational. Direct contact between the pathogen wall and the plant cell membrane is made around 12–14 hai, following AIP ingression. *Mla6* resistance may not be activated until this contact is made, with noticeable inhibition of the avirulent isolate not occurring for another 10 hr or more. Secondary attempts by the avirulent isolate to breach the plant cell wall are then met with resistance, giving a significant interaction at the secondary lobe stage, 30 hai. Others have reported that race-specific, gene-for-gene incompatibility is not observed in barley before the formation of AIPs (Ellingboe and Slesinski 1971; Smedegaard-Petersen and Tolstrup 1985). In the tomato pathogen *Cladosporium fulvum* the *avr9* gene is only expressed *in planta,* allowing extensive pathogen development before race-specific resistance is triggered (van Kan *et al.* 1991). Alternatively, the inhibition of CC142 may be dependent on the establishment of a subtle equilibrium between the resistance mechanism of the plant and the growing germling, before an effect on development is observed. At 30 hai the development of CC142 had slowed, while higher levels of chitinase, peroxidase, and PR-R mRNA were maintained in the host. Similar differences were found for four barley cDNAs induced by *E. g.* f. sp. *hordei,* isolated by Davidson *et al.* (1987). The levels of mRNA were greater in the resistant cultivar (*Mlp*) than in the susceptible (*mlp*). The control of defense genes in resistance may also be operational at levels other than transcription. The localization of the defense gene product, and its access to the pathogen during infection, have also been suggested as factors having a role in resistance (Graham and Graham 1991). The accessibility of the defense proteins to *E. g.* f. sp. *hordei* during infection may differ in compatible and incompatible interactions.

Support for a role of defense genes in resistance to mildew is provided by induced resistance studies (Smedegaard-Petersen *et al.* 1992). Both avirulent and virulent isolates of *E. g.* f. sp. *hordei* are able to induce resistance in barley. Pre-induction of defense genes may increase the resistance of the plant, reducing the amount of infection observed following inoculation with a virulent isolate. While no difference in the effectiveness of avirulent and virulent isolates as inducers of resistance is observed up to 12 hai, avirulent isolates are more effective in inducing resistance after that time, indicating a specific resistance response to the avirulent isolate (Thordal-Christensen and Smedegaard-Petersen 1988).

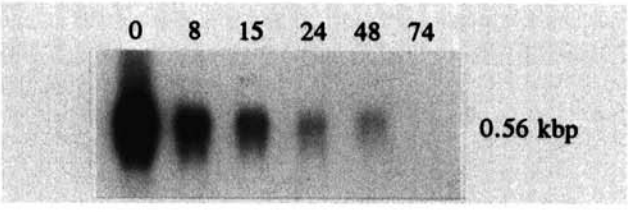

Fig. 7. Total RNA probed with a clone homologous to a pathogenesis-related R protein (PR-R). Plants of the barley cultivar Midas, grown under sterile conditions, were wounded by rubbing the adaxial leaf surface with Carborundum. The times at which leaf samples were taken, in hours after wounding, are indicated at the top of the figure. The size of the mRNA transcript is indicated in kilobase pairs.

In all three experiments, inoculation with *E. g.* f. sp. *hordei* appeared to reduce the amount of ssRubisco mRNA in barley. This response was particularly prominent at later sample times, the levels of ssRubisco mRNA declining with mildew development. This effect of *E. g.* f. sp. *hordei* has been observed in subsequent experiments (L. Boyd, unpublished data) and is consistent with the work of others (Manners *et al.* 1985) in which mildew infection has been shown to reduce the transcript levels of a number of proteins. The reason for the reduction in ssRubisco transcript in barley inoculated with *E. g.* f. sp. *hordei* is not known.

Examination of the temporal and spatial expression of defense genes during pathogen development may not only clarify the role of these genes in defense but help to elucidate how the gene-for-gene interaction results in resistance. The limited physical association between barley and *E. g.* f. sp. *hordei* and the well-defined genetics of gene-for-gene resistance provide a host-pathogen system in which a number of variables important in the defense response can be examined in relation to pathogen development.

MATERIALS AND METHODS

Plant material.

The spring barley cultivar Midas was sown, two seeds per pot, in small peat pots containing John Innes No. 2 compost, by the method of Martinelli *et al* (1993). After coleoptile emergence the trays of seedlings were placed on their sides, so that the adaxial surface of the first leaf (prophyll) would be uppermost when the trays were reinverted, maximizing the area of the adaxial leaf available for spore deposition. Plants were grown in a spore-free greenhouse for 12 days, with a cycle of 16 hr of light and 8 hr of darkness, supplemented with artificial light when necessary, at temperatures of 19° C during the day and 15° C at night. Sterile plants were grown from surface-sterilized seeds in autoclaved vermiculite. Seeds were surface-sterilized by treating them in 20% household bleach (Domestos) for 5 min and 90% ethanol for 10 min and then rinsing them three times in sterile double-distilled water. Seeds were germinated on autoclaved Whatman No. 1 paper for 4 days at 20° C. Germinated seeds were transferred to 2-L glass beakers (approximately 10–12 seedlings per beaker) containing vermiculite soaked in sterile tap water (300 ml). The beakers were re-covered in foil, and the plants grown at 15° C with a cycle of 16 hr of light and 8 hr of darkness for 12 days.

Pathogen material.

Isolates of *E. g.* f. sp. *hordei* were maintained on the susceptible barley cultivar Golden Promise. The first leaves of 10- to 12-day-old barley plants were surface-sterilized by briefly washing in 5% Domestos, immersing in 80% ethanol for 3 min, and then rinsing in sterile double-distilled water. The leaves were cut into 2-cm segments and placed, adaxial side up, on water agar (6 g/L) containing benzimidazole (0.1 g/L). Spores were blown onto the leaf surface and then incubated at 15° C for 9 days to produce sporulating colonies. The virulences of *E. g.* f. sp. *hordei* isolates CC142 and CC143 were checked every 3 months on Pallas and the Pallas near-isogenic lines P-01, P-02, P-03, P-04B, P-08B, P-09, P-10, P-11, P-12, P-13, P-14, P-15, P-17, P-18, P-19, P-20, P-21,

P-23, and P-24 (Kølster *et al.* 1986). Spores were killed by placing them in a freeze drier for 4 days. This method of killing resulted in dry spores that could still be blown onto barley plants. Spore viability was tested by germination on glass slides coated in agar (6 g/L) (Carver and Ingerson 1987).

Inoculations, sampling, and wounding.

Twelve-day-old barley plants were inoculated with *E. g.* f. sp. *hordei* spores blown into an aluminum settling tower, allowing 10 min for spores to settle. Glass slides were distributed evenly in the base of the tower in order to estimate spore density and monitor spore distribution. The largest settling tower available to us (210 cm high and 75 cm in diameter) allowed a maximum of 15 samples to be taken from any one inoculation.

Experiment 1, July 1991. First leaves were taken for RNA extraction at 0, 1, 3, 5, 8, 10, 12, 14, 16, 18, 24, 48, and 72 hai. The inoculation densities of CC142 and CC143 were 24 and 25 spores per square millimeter, respectively.

Experiment 2, November 1991. First leaves were taken for RNA extraction and microscopic analysis at 0, 1, 3, 5, 8, 10, 12, 13, 14, 16, 24, 30, 36, 48, and 72 hai. The inoculation densities of CC142 and CC143 were 75 and 85 spores per square millimeter, respectively.

Experiment 3, May 1992. First leaves were taken for RNA extraction and microscopic analysis at 0, 8, 12, 24, 36, 48, 52, 56, 58, 60, and 72 hai. The inoculation densities of CC142 and CC143 were 40 and 35 spores per square millimeter, respectively.

Physical wounding of the first leaf of 10- to 12-day-old barley plants was either by random perforation of the adaxial surface with a 0.8-mm bore needle or by rubbing with Carborundum. Sterile plants were wounded in a sterile atmosphere, using aseptic techniques, and samples were taken for RNA extraction at 0, 8, 15, 24, 48, and 74 haw. Nonsterile wounded leaves were sampled at 0, 3, 8, 12, 14, 16, 20, 26, 30, 36, 48, 54, 60, 66, and 72 haw.

After treatment, plants inoculated with *E. g.* f. sp. *hordei* and nonsterile wounded plants were covered with a clear plastic propagator lid and placed on the laboratory bench. Samples were taken over the next 72 hr. The temperature in the laboratory varied between 18 and 20° C. Sterile plants, after wounding with Carborundum, were returned to the 15° C incubator. Six leaves, from individual plants, were fixed for microscopic examination, and another six were immediately frozen in liquid nitrogen for later RNA extraction at each sample time.

Microscopy.

Leaves were fixed and cleared by a modification of the method of Carver *et al* (1992). Leaf segments were placed, adaxial side uppermost, on Whatman 3MM paper moistened with a mixture of ethanol and acetic acid (3:1) until the leaves were bleached (approximately 2–3 days). The bleached leaves were placed sequentially on Whatman 3MM paper moistened in 95% ethanol, 90% ethanol, and sterile double-distilled water, each for 24 hr, before finally being placed on tissue paper soaked in lactoglycerol (a 1:1:1 solution of glycerol, lactic acid, and water). Leaves can be stored, in the dark, in lactoglycerol for several months. Between 100 and 200 undamaged spores were scored on each leaf segment. At early sample times (up to 8 hai), leaf segments were observed under a light microscope at a magnification of 200×, without a coverslip, to count germinated and ungerminated spores. To score later stages of development, leaf segments were stained with 0.1% trypan blue in lactoglycerol and examined on a Nikon Microphot S.A. microscope at 400×. Epifluorescence microscopy was used to detect phenolic compounds within the leaf, using a green exciter filter (maximum transmission 560 nm) together with a dichroic mirror (580 nm) and a barrier filter (590 nm). The stages of mildew development scored were 1) ungerminated spores, 2) germinated spores, showing production of a PGT, 3) AGT, 4) mature appressorium, 5) AIP, 6) secondary lobes, 7) tertiary and quaternary lobes, 8) haustoria, and 9) ESH. The number of germlings at each growth stage on the replicate leaf segments was counted for each isolate, and the numbers were compared by generalized linear modeling of contingency tables, using the Genstat 5 package (Numerical Algorithms Group, Oxford). If the χ^2 statistic for the overall interaction between isolate and growth stage was significant, the difference between the frequencies of germlings of each isolate, at each growth stage, was tested by *t* test, using the predicted means and standard errors of the frequencies.

Gene expression analysis.

Total RNA was extracted from leaves by the phenol–sodium dodecyl sulfate (SDS) method and selective LiCl precipitation (Ausubel *et al.* 1987). RNA was quantified by UV spectrophotometry, and 7.0 μg of total RNA was separated on a 1.2% formaldehyde-agarose gel. The RNA was transferred to Hybond-N^+ membrane (Amersham) according to the manufacturer's instructions (Ausubel *et al.* 1987). Great care was taken to ensure that equal amounts of RNA were loaded on each track, and gels were stained with ethidium bromide after transfer, to check for even transfer of all lanes. RNA/DNA hybridizations were done following standard procedures (Sambrook *et al.* 1989). Filters were prehybridized at 65° C for a minimum of 3 hr and hybridized overnight at 42° C in 50% formamide, 5× SSC (20× SSC is 3 M NaCl plus 0.3 M sodium citrate), 5× Denhardt's III (100× Denhardt's III is 2% gelatin, 2% Ficoll 400, 2% polyvinylpyrrolidone, 10% SDS, and 5% sodium pyrophosphate), 0.5% SDS, and denatured salmon sperm DNA (20 μg/ml). All DNA probes were labeled with [α-^{32}P]dCTP by a random primer method (Feinberg and Vogelstein 1983). A 720-bp *Eco*RI-*Acc*I fragment from pD6, an 890-bp *Eco*RI-*Pvu*II fragment from pD8, a 520-bp *Eag*I-*Pst*I fragment from pD12, a 2.3-kbp *Eco*RI fragment from pP15 (Green 1991), a 325-bp *Nco*I-*Pst*I fragment of DB4 (Bohlmann and Apel 1987), and a 1.15-kbp *Eco*RI-*Nco*I fragment from the wheat genomic small subunit of the ribulose-1,5-bisphosphate carboxylase (ssRubisco) gene, clone pWS4.3 (Broglie *et al.* 1983), were used as probes. Filters were washed twice in 2× SSC and 0.1% SDS at room temperature, once in 1× SSC and 0.1% SDS at 42° C, and once in 0.1× SSC and 0.1% SDS at 42° C, for 10 min each. The filters were wrapped in Saran Wrap and exposed to X-OMAT/AR (Kodak) X-ray film at −70° C. The autoradiograms were scanned with a Chromoscan 3 densitometer (Vickers, Malden, MA). RNA levels were adjusted against a concentration calibration curve, obtained from an

RNA dilution series probed with the PR-R clone, to correct the intensity of the autoradiographic image to a linear representation of the amount of radioactivity in the sample. DNA probes were removed from filters by washing in boiling 0.1% SDS.

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