Early H_2O_2 Accumulation in Mesophyll Cells Leads to Induction of Glutathione during the Hyper-Sensitive Response in the Barley-Powdery Mildew Interaction[1]

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H_2O_2 production and changes in glutathione, catalase, and peroxidase were followed in whole-leaf extracts from the susceptible (AlgS [Algerian/4* (F14) Man.(S)]; *ml-a1* allele) and resistant (AlgR [Algerian/4* (F14) Man.(R)]; *Ml-a1* allele) barley (*Hordeum vulgare*) isolines between 12 and 24 h after inoculation with powdery mildew (*Blumeria graminis* [DC]. Speer [syn. *Erysiphe graminis* DC] f.sp *hordei* Marchal). Localized papilla responses and cell death hypersensitive responses were not observed within the same cell. In hypersensitive response sites, H_2O_2 accumulation first occurred in the mesophyll underlying the attacked epidermal cell. Subsequently, H_2O_2 disappeared from the mesophyll and accumulated around attacked epidermal cells. In AlgR, transient glutathione oxidation coincided with H_2O_2 accumulation in the mesophyll. Subsequently, total foliar glutathione and catalase activities transiently increased in AlgR. These changes, absent from AlgS, preceded inoculation-dependent increases in peroxidase activity that were observed in both AlgR and AlgS at 18 h. An early intercellular signal precedes H_2O_2, and this elicits anti-oxidant responses in leaves prior to events leading to death of attacked cells.

Plants exhibit a wide array of defense strategies against pathogen attack. They possess preformed physical barriers (e.g. the cuticle and cell wall) and biochemical defenses (e.g. antimicrobial toxins). In addition rapidly inducible defenses may be activated by pathogen attack. One of the most important of these is the hypersensitive response (HR) where plant cell death due to host-pathogen incompatibility prevents further pathogen infection (Mehdy, 1994).

HR involves the induction of an oxidative burst at the plasma membrane that produces active oxygen species (AOS) such as superoxide that is rapidly dismutated to hydrogen peroxide (Wojtaszek, 1997). The rapid generation of AOS is a very early response to pathogen infection. It has been described in many plant-pathogen interactions and is now considered a characteristic and common feature of HR leading to programmed cell death (for review, see Sutherland, 1991; Mehdy, 1994; Baker and Orlandi, 1995; Tenhaken et al., 1995; Low and Merida, 1996; Lamb and Dixon, 1997; Wojtaszek, 1997).

The present study describes in situ studies on rapidly inducible responses, including cell death in barley (*Hordeum vulgare*), elicited as a response to attack by the powdery mildew fungus *Blumeria graminis* [DC]. Speer [syn. *Erysiphe graminis* DC] f.sp *hordei* Marchal. This biotrophic fungus causes powdery mildew, one of the most important diseases of temperate cereals. Following germination on the leaf surface, spores form a specialized infection structure, the appressorium, by approximately 10 to 12 h after inoculation. A penetration peg emerges from the appressorium and attempts to penetrate the host leaf epidermal cell directly (approximately 12–20 h). If penetration succeeds and the host cell remains alive, a feeding structure, the haustorium, develops within the epidermal cell. The haustorium extracts nutrients to supply the development of superficial hyphae that ramify over the leaf surface and form a colony. Host cells respond to attempted penetration by depositing wall appositions, papillae, directly beneath appressoria. Papilla deposition occurs irrespective of the specific compatibility or incompatibility of the host-pathogen interaction. Papillae consist of a callose matrix enriched in proteins, various elements, and autofluorogenic phenolic compounds (e.g. Lyngkjaer and Carver, 1999) that are thought to convey resistance to penetration. If penetration fails, further fungal development is prevented, although the host cell remains alive. This defensive response is effective in some cells of even nominally "susceptible" host genotypes attacked by virulent, compatible fungal isolates (Carver et al., 1994). However, where plants possess specific genes for resistance to incompatible fungal isolates, HR leading to plant cell death is a common response (Johnson et al., 1979; Zeyen and Bushnell, 1979; Koga et al., 1990; Hippe-Sanwald et al., 1992). In this case, cells adjudged dead by their inability to take up neutral red or plasmolyse, also

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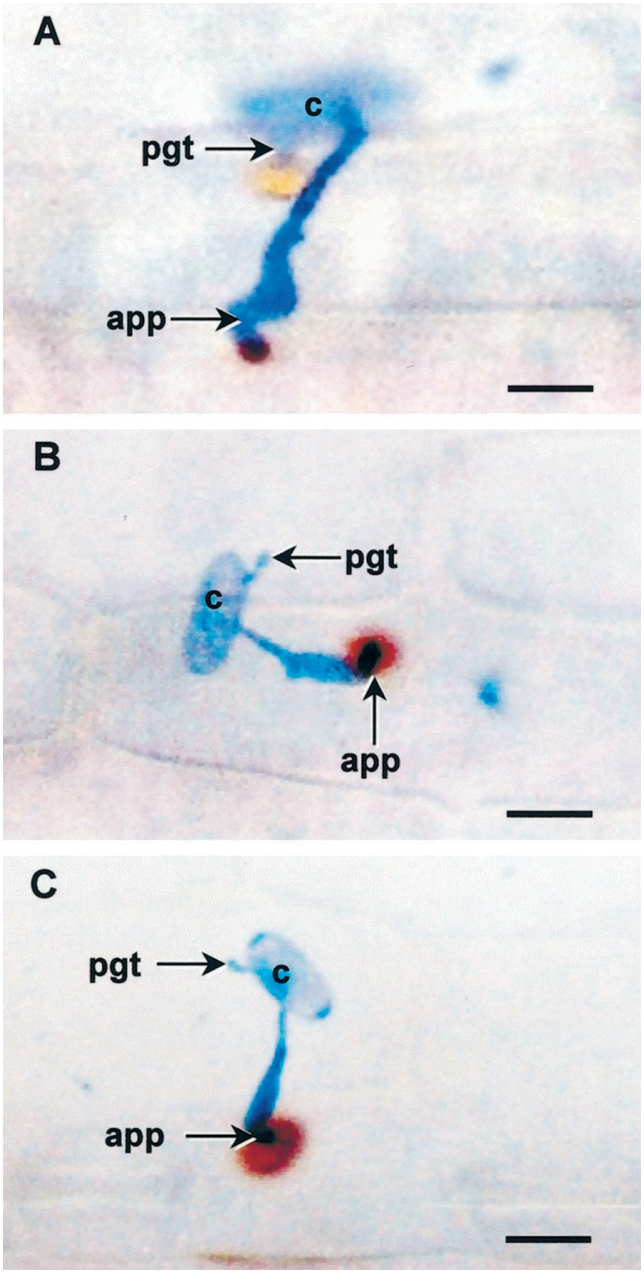

Figure 1. The time course, between 12 and 16 h after inoculation, of the appearance of brown coloration due to DAB staining of hydrogen peroxide production in localized sites of response of AlgS barley epidermal cells attacked by powdery mildew. Specimens were stained post-fixation with aniline blue to show fungal structures. Micrographs were obtained using transmitted white light. A, Twelve hours after inoculation. A small area of intense browning is evident in the epidermal cell wall directly associated with the fungal appressorium. B, Fourteen hours after inoculation. The diameter of brown staining has increased and remains intense at the center of response. C, Sixteen hours after inoculation. The diameter of browning has increased further. app, Appressorium; c, conidium; pgt, primary germ tube. These structures are sometimes not clearly in focus, being out of the focal plane. Bar = 25 μm. Where localized epidermal cell responses were seen in AlgR barley, they were indistinguishable from those illustrated here for AlgS. When these same specimens were viewed by incident blue light, no autofluorescence was detectable in the response sites. No browning or autofluorescence was detectable in mesophyll cells underlying the attacked epidermal cell.

show whole-cell autofluorescence under UV or blue light excitation, and this fluorescence can be taken as an objective and convenient indicator of cell death (Koga et al., 1988; Zeyen et al., 1995).

The damage caused to pathogen-attacked plant cells is influenced by the efficiency of the endogenous anti-oxidant defense system. Highly efficient anti-oxidative defense systems, composed of both nonenzymic and enzymic constituents, minimize damage caused by H_2O_2 and other AOS (Foyer et al., 1994; Noctor and Foyer, 1998). In the present study we have examined the time course of H_2O_2 accumulation and coincident changes in the activities of the enzymes peroxidase and catalase and in the glutathione pool in whole-leaf extracts taken from leaves of susceptible and resistant barley isolines during the critical period of 12 to 24 h after inoculation with powdery mildew. This covers the period when mature fungal appressoria initiate their attack on leaf epidermal cells and plant responses determine the ultimate success or failure of attempted infections.

Two near-isogenic lines of barley, developed by J. G. Moseman and differing at the *Ml-a* locus were used. Algerian/4* (F14) Man. (R) (hereafter referred to as AlgR), has single gene controlled, race-specific resistance to powdery mildew conferred by the dominant *Ml-a1* allele (Johnson et al., 1979; Zeyen and Bushnell, 1979; Koga et al., 1990; Hippe-Sanwald et al., 1992). Algerian/4* (F14) Man. (S) (hereafter referred to as AlgS), carries the recessive *ml-a1* allele for susceptibility to powdery mildew. In AlgR attacked by an avirulent powdery mildew isolate, approximately 50% of attacked epidermal cells express HR and show whole-cell autofluorescence (Zeyen et al., 1995). By contrast, AlgS shows cell death at a very low frequency (approximately 1% of attacked cells).

RESULTS

Temporal and Spatial Relationships between Fungal Development and Host Cell Responses

The fungal germlings developed somewhat asynchronously so that not all attacks from appressoria or related host cell responses were initiated at precisely the same time. We know, however, from previous work using identical conditions that the outcome of virtually all attempted infections is determined within the first 24 h after inoculation (Vanacker et al., 1998). The data reported here are not intended to give a full quantitative description of pathogen development and host response but rather to describe the predominant stages seen at different sample times allowing us to define spatial and temporal relationships of the following processes.

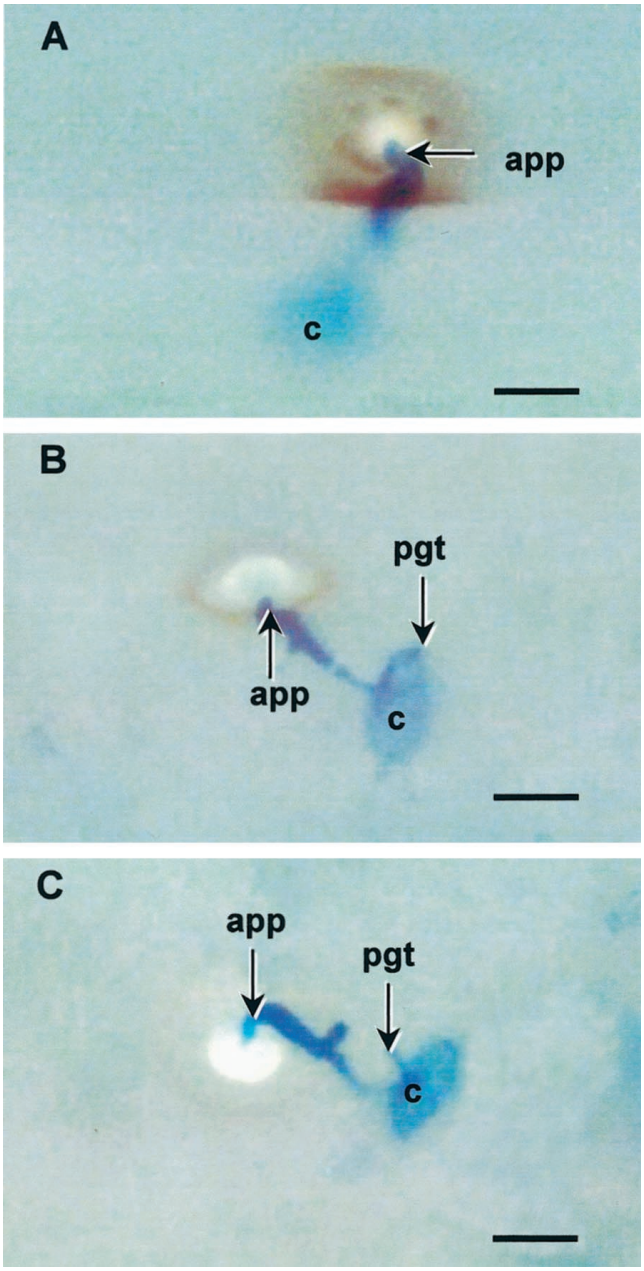

Figure 2. The time course between 18 and 24 h after inoculation of changes in brown coloration due to DAB staining of hydrogen peroxide and the appearance of autofluorogenic phenolic compounds in localized sites of response of AlgS barley epidermal cells attacked by powdery mildew. Specimens were stained post-fixation with aniline blue to show fungal structures. Micrographs were obtained using incident blue-violet light to reveal both the brown coloration and autofluorogenic compounds. A, Eighteen hours after inoculation. Autofluorescence is evident in the epidermal cell wall directly beneath the fungal appressorium in an area corresponding to the papilla deposited by the epidermal cell. The diameter of the brown area has increased compared with earlier samples but is no longer evident in the center of the response site occupied by the autofluorogenic compounds. B, Twenty-two hours after inoculation. The diameter and intensity of the autofluorescence response has increased, and brown staining remains evident only as a ring encircling the autofluorogenic area. C, Twenty-four hours after inoculation. The diameter of the autofluorescence response has increased with a central zone outlining the papilla site and surrounding zone indicating a "halo" in the epidermal cell wall region surrounding the papilla. Browning remains but as a barely detectable ring encircling the autofluorogenic area. Fungal structures are sometimes not clearly in focus, being out of the focal plane. Bar = 25 μm. Where localized epidermal cell responses were seen in AlgR barley, they were indistinguishable from those illustrated here. Viewing by transmitted white light (not shown) indicated that brown coloration was truly absent from autofluorogenic areas and was not simply masked by autofluorogens revealed under incident blue light. No browning or autofluorescence was detectable in mesophyll cells underlying the attacked epidermal cell.

Localized H_2O_2 Production, Papilla Deposition, and Localized Autofluorescence Responses in Living Epidermal Cells of AlgS and AlgR Barley

In both barley isolines, localized brown staining indicative of localized H_2O_2 production was first evident 12 h after inoculation in leaves supplied with 3,3′-diamino benzidine (DAB; Fig. 1A). At this time, small areas of browning were apparently localized in the host cell wall immediately beneath some appressoria. The area and intensity of the browning increased up to 16 h after inoculation (Fig. 1, B and C) when it was associated with approximately 50% of appressoria. From 16 h it was possible to see the first evidence of papilla deposition beneath some appressoria. These papillae were first evident as small refractive bodies at the center of the dark staining region.

Up to 18 h after inoculation, examination under incident blue light failed to reveal autofluorogenic materials present in epidermal cells beneath appressoria. However, from 18 h after inoculation, autofluorogenic material was evident in response sites, and this material coincided with the papilla structure (Fig. 2A). It was noticeable at this time that, although the diameter of brown-stained areas was increased compared with earlier samples, the intensity of the brown coloration was reduced compared with earlier samples. Further, there was no longer any sign of brown staining in the center of the response site coinciding with the autofluorescent papilla. Except where penetration succeeded and haustoria were formed (see below) little change was seen over the next 2 h, but by 22 h the brown staining had faded and remained only as a faint ring encircling the increasingly large area of autofluorescence (Fig. 2B). By 24 h, where haustoria were absent, and it can therefore be assumed that attempted penetration had failed, most sites of attack were brightly fluorescent, and the ring of encircling brown stain had virtually disappeared (Fig. 2C).

It is important to note that epidermal cells that showed localized brown staining followed by localized accumulation of autofluorogenic material showed no whole-cell autofluorescence. This indicated that these cells remained alive until fixation. It is also important to note that the staining reaction was closely associated with the eventual deposition

of the papilla and that no reaction was seen in any underlying mesophyll cells.

H_2O_2 Production and Whole-Cell Autofluorescence Indicating Hypersensitive Death of Epidermal Cells in AlgR Barley

Our previous experiments showed that death of attacked epidermal cells, the most important factor limiting successful infection of AlgR, was maximized by 24 h (Vanacker et al., 1998). In the current study of AlgR a sequence of histological events associated with the eventual death of attacked epidermal cells was seen to be engaged as early as 14 h after inoculation.

The first sign of events unique to the AlgR cell death response involved the appearance of brown staining, indicative of H_2O_2 production, in mesophyll cells underlying some attacked epidermal cells. At 14 h the staining was obvious but faint (Fig. 3A). It was impossible to be sure of the exact location of the stain, but it appeared to be in the wall of mesophyll cells where they made contact with the attacked epidermal cell. Mesophyll that had no contact with the attacked epidermal cell did not show any brown staining. At the time that this reaction was first seen, the epidermal cell under attack showed no brown coloration at all (Fig. 3A). By 16 h the browning in mesophyll cells had intensified, and in many cases there was now faint but extensive browning in the walls of the attacked epidermal cell itself (Fig. 3B). Two hours later the browning of the underlying mesophyll appeared to have faded somewhat, whereas the attacked mesophyll cell showed intensified staining throughout the cell walls and cytoplasm (Fig. 3C). Up to this time (18 h), observation with incident blue light failed to detect any signs of autofluorescence either in the attacked epidermal cell or in the underlying mesophyll.

The first evidence of whole-cell autofluorescence was seen 20 h after inoculation. By now, brown coloration was obvious throughout the attacked epidermal cell cytoplasm and cell walls (Fig. 4A), and faint autofluorescence was detectable in the cell walls (Fig. 4A). It is important to note that by this time there was no browning of the underlying mesophyll and that the underlying mesophyll cells showed no autofluorescence, indicating that they were alive at the time of fixation. At 22 h, the brown coloration had faded considerably in attacked epidermal cells, although they were now showing distinct whole-cell autofluorescence indicative of cell death (Fig. 4B). By 24 h, many attacked epidermal cells showed bright whole-cell autofluorescence indicating that they were dead at the time of fixation, and brown coloration, where detectable, was extremely faint (Fig. 4C). Mesophyll cells underlying the dead epidermal cells were neither autofluorescent nor brown and appeared no different from mesophyll cells distant from attack sites.

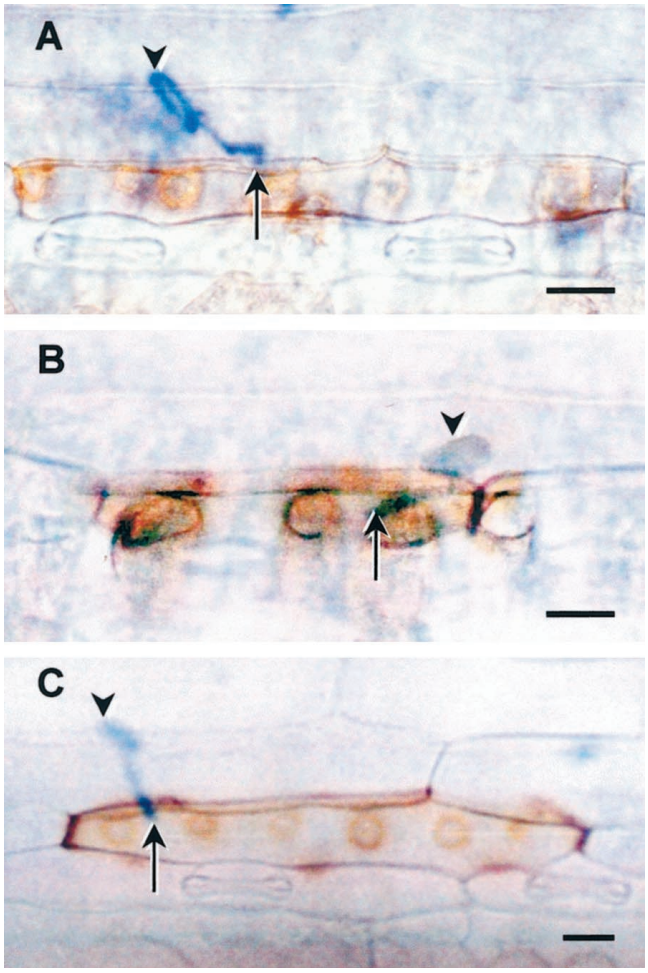

Figure 3. The time course of the change in brown coloration due to DAB staining of hydrogen peroxide, in epidermal cells, and underlying mesophyll cells of AlgR barley between 14 and 18 h after inoculation with powdery mildew. Specimens were stained post-fixation with aniline blue to show fungal structures. Micrographs were obtained using transmitted white light. A, Fourteen hours after inoculation. Faint but distinct brown coloration is evident in mesophyll cells having direct contact with the attacked epidermal cell. The attacked epidermal cell shows no browning. B, Sixteen hours after inoculation. Intense browning is now evident in mesophyll cells having direct contact with the attacked epidermal cell, which now shows faint browing in the cell wall. C, Eighteen hours after inoculation. Browning of mesophyll cells having direct contact with the attacked epidermal cell is less obvious than in the previous sample, but the wall of the attacked epidermal cell now shows distinct brown coloration. Arrow indicates appressorium; arrowhead indicates conidium. These fungal structures are sometimes not clearly in focus, being out of the focal plane. Bar = 30 μm. When these same specimens were viewed using incident blue-violet light, no autofluorescence was detectable in either the attacked epidermal cell or in the underlying mesophyll cells.

Responses in Cells Containing Haustoria

Successful penetrations, indicated by the presence of haustoria beneath appressoria, were not seen in either isoline until 22 h after inoculation. It should be noted that before this time it was impossible to be

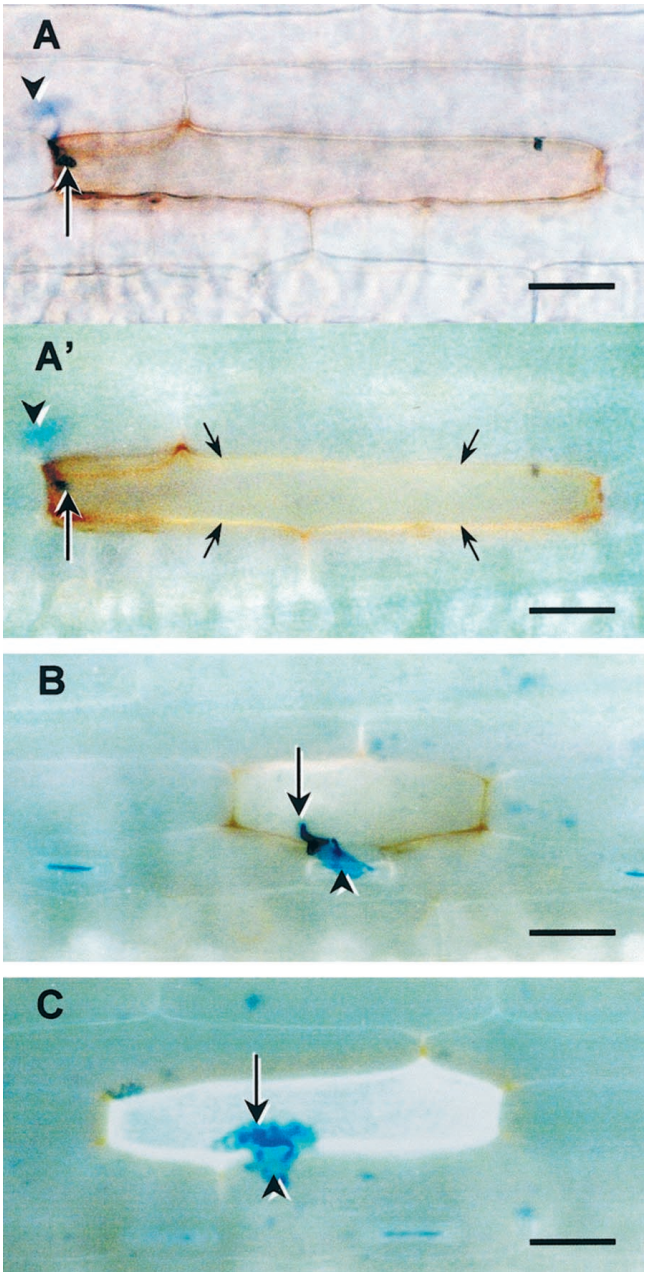

Figure 4. The time course of the change in brown coloration due to DAB staining of hydrogen peroxide and the appearance of autofluorogenic phenolic compounds in epidermal cells of AlgR barley between 18 and 24 h after inoculation with powdery mildew. Specimens were stained post-fixation with aniline blue to show fungal structures. Micrographs were obtained using transmitted white light (A) or incident blue-violet light (A′, B, and C) to reveal both the brown coloration and autofluorogenic compounds. A, Twenty hours after inoculation. Attacked epidermal cell viewed with transmitted white light. Distinct brown coloration is evident in the wall of the attacked epidermal cell. Unlike in earlier samples (Fig. 3) the mesophyll underlying the attacked epidermal cell shows no browning. A′, Twenty hours after inoculation. The same attacked epidermal cell as shown in A above. Browning remains evident under incident blue light, but for the first time faint autofluorogenesis can also be detected in the anticlinal walls of the long axis of the cell (small arrows). Note, the mesophyll underlying the attacked epidermal cell shows no autofluorescence. B, Twenty-two hours after inoculation. Brown coloration of the attacked epidermal cell wall remains distinct, but whole-cell autofluorescence due to the accumulation of autofluorogens in the cell cytoplasm is now evident, indicating death of the attacked cell. The mesophyll underlying the attacked epidermal cell shows neither browning nor autofluorescence. C, Twenty-four hours after inoculation. Only very faint brown coloration can be seen in the epidermal cell wall. Bright, whole-cell autofluorescence indicates hypersensitive death of the cell resulting from attack by the pathogen. The mesophyll underlying the attacked epidermal cell shows neither browning nor autofluorescence. Arrow indicates appressorium; arrowhead indicates conidium. These fungal structures are sometimes not clearly in focus, being out of the focal plane. Bar = 50 μm.

sure whether appressoria were destined to fail or succeed in attempted penetration of epidermal cells. The first appearance of haustoria at 22 h was a little later than in others' comparable studies where haustoria were first seen as early as 15 h (Clark et al., 1993). However, confirming earlier studies (Carver et al., 1994; e.g. Zeyen et al., 1995), in AlgR, less than 5% of appressoria formed haustoria by 24 h, whereas in AlgS approximately 20% of appressoria had done so. In all cases haustoria were rudimentary, having formed few, if any, digitate processes by the time of fixation.

All epidermal cells of AlgR that contained haustoria showed faint to moderate brown coloration due to DAB staining, and faint whole-cell autofluorescence was evident throughout the cell walls and cytoplasm. There was, however, no evidence of brown coloration or autofluorescence in the underlying mesophyll. By contrast, where AlgS epidermal cells contained a haustorium, there was no evidence of brown coloration either in the cell cytoplasm or in the host cell wall, even at the point of penetration. As in previous studies (Carver et al., 1994) there was sometimes slight localized autofluorescent host cell response in the host cell wall close to the penetration site but not in all cases.

Anti-Oxidant Activity in Barley Leaves during Powdery Mildew Fungus Attack

Peroxidase activity was measured in control (noninoculated) and inoculated AlgR (resistant, Fig. 5A) and AlgS (susceptible; Fig. 5B) barley leaves sampled between 12 to 24 h after inoculation. In both AlgR and AlgS, no significant changes in peroxidase activity was observed from 12 to 16 h after inoculation in inoculated whole-leaf extracts when compared with controls (Fig. 5). However, inoculation caused significant increases in peroxidase activity in both AlgR and AlgS at 18 h (42% and 77%, respectively), 20 h (48% and 104%, respectively), 22 h (34% and 20%, respectively), and also 24 h (25% and 44%, respectively; Fig. 5).

Catalase activity was measured in control (noninoculated) and inoculated AlgR (resistant; Fig. 6A) and AlgS (susceptible; Fig. 6B) barley leaves sampled

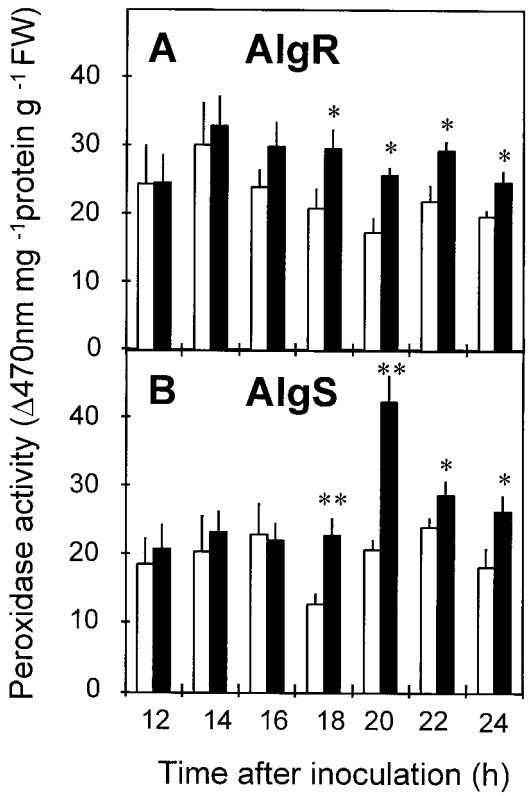

Figure 5. The effect of powdery mildew attack on peroxidase activity of AlgR (A) and AlgS (B) barley leaves from 12 to 24 h after inoculation. Black columns, Inoculated; white columns, noninoculated controls. Bars represent SE of means ($n = 4$). * and ** indicate that values differ significantly from control at $P < 0.05$ and $P < 0.01$, respectively.

between 12 to 24 h after inoculation. In AlgS, no significant change in catalase activity was found from 12 to 22 h after inoculation in inoculated whole-leaf extracts when compared with controls (Fig. 6B). At 24 h, a significant inoculation-dependent increase (87.5%) in catalase activity was observed in AlgS. In marked contrast, a transient significant increase in foliar catalase was observed between 14 and 18 h after inoculation in AlgR (Fig. 6A).

The total glutathione pool (Fig. 7) and the redox state of the pool (Fig. 8) were measured in healthy and inoculated AlgR (Figs. 7A and 8A) and AlgS (Figs. 7B and 8B) leaves sampled from 12 to 24 h after inoculation. In AlgS leaves no significant inoculation-dependent changes in either the total amount of glutathione (Fig. 7B) or in the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) in the leaves (Fig. 8B) were observed from 12–24 h after inoculation.

In AlgR leaves, however, large inoculation-dependent changes in both the total amount of glutathione and in the redox state of the glutathione pool were observed. The total amount of glutathione decreased between 14 and 16 h after inoculation. A decrease of 35% was observed 16 h after inoculation. This initial decrease in total foliar glutathione was transient, however, and was rapidly followed by a large increase in the total glutathione pool 18 h after inoculation (Fig. 7A). Inoculation-dependent enhancement of the total glutathione pool was persistent, being still evident in AlgR even 24 h after inoculation (Fig. 7A).

Clear inoculation-dependent changes in the redox state of the glutathione pool in AlgR were also observed (Fig. 8A). The total glutathione pool was present largely in the GSH form (>97% reduced) both in the healthy controls and in the inoculated AlgR leaves for up to 12 h after inoculation. After this point, however, a change in the GSH to GSSG ratio was observed in the inoculated AlgR leaves so that 14 h after inoculation 94% of the pool was present as GSH, whereas at 16 h this had fallen to only 82% (Fig. 8A). Recovery of the GSH to GSSG ratio was then observed so that 18 h after inoculation 95% of the pool was present as GSH. By 24 h the GSH to GSSG ratio was comparable with controls (Fig. 8A).

DISCUSSION

H_2O_2 production is an early response in plant-pathogen interactions. Current concepts of signal transduction involve calcium influx (Price et al.,

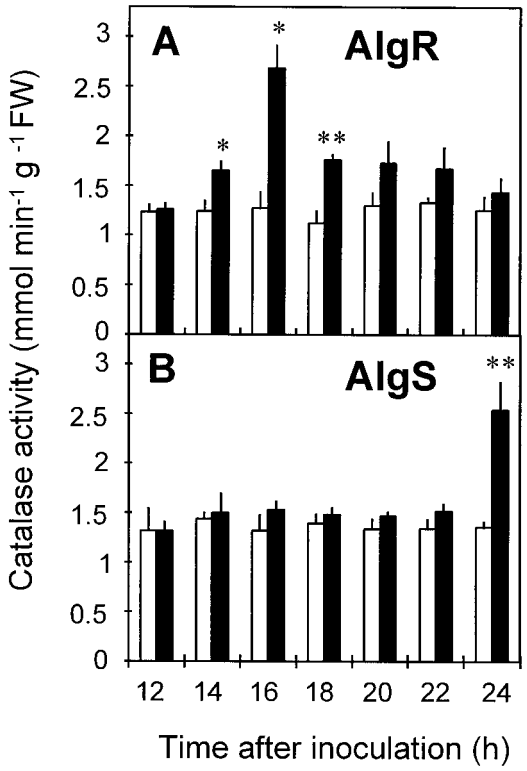

Figure 6. The effect of powdery mildew attack on catalase activity of AlgR (A) and AlgS (B) barley leaves from 12 to 24 h after inoculation. Black columns, Inoculated; white columns, noninoculated controls. Bars represent SE of means ($n = 4$). * and ** indicate that values differ significantly from control at $P < 0.05$ and $P < 0.01$, respectively.

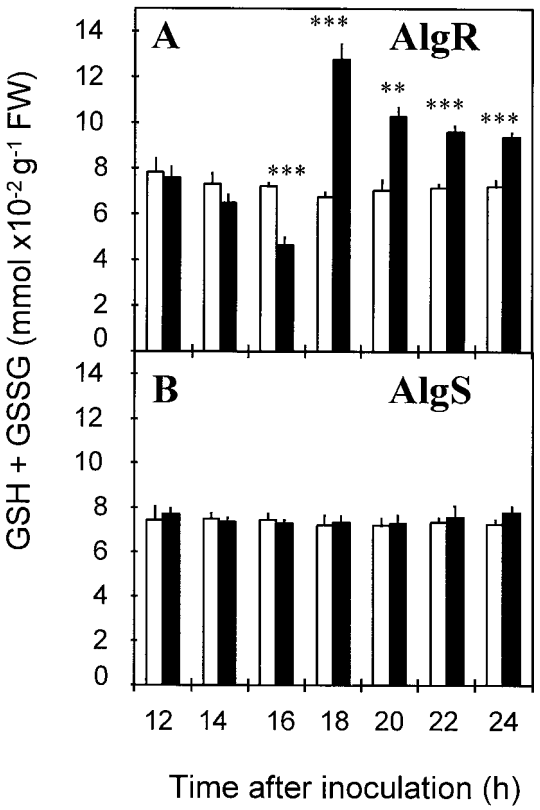

Figure 7. The effect of powdery mildew attack on the total glutathione pool of AlgR (A) and AlgS (B) barley leaves from 12 to 24 h after inoculation. Black columns, Inoculated; white columns, noninoculated controls. Bars represent SE of means ($n = 4$). ** and *** indicate that values differ significantly from control at $P < 0.01$ and $P < 0.001$, respectively.

1994) and protein kinase activation in attacked cells. Little is known about cell-to-cell communication during this response. The results presented here suggest that early in the HR response, cells other than the attacked cell respond by transient H_2O_2 production. This response precedes H_2O_2 accumulation, and subsequent accumulation of autofluorescent compounds in attacked epidermal cells. Foliar glutathione accumulation and transient increase in catalase activity are coincident with the mesophyll response and not with the subsequent production of H_2O_2 by the attacked epidermal cell. These increases were largely specific to AlgR and hence HR and were not observed in AlgS. It is interesting that changes in peroxidase activity occurred later in the response and were observed both in AlgS and AlgR. In relation to HR, the critical period for H_2O_2 production by the mesophyll cells underlying the attacked cell was from 14 to 18 h after inoculation, whereas that in the attacked cell occurred later between 20 and 24 h after inoculation.

Prior to 12 h there was no brown coloration of attacked leaf areas except at the tips of the primary germ tubes. This was shown to be initiated as early as 6 h after inoculation (Thordal-Christensen et al., 1997). The appearance of localized DAB staining beneath appressoria was an early response to attack, occurring at around the time of attempted penetration of the cell wall by the fungal penetration peg. This was reported to coincide with elevated transcription of a putative peroxidase gene induced by powdery mildew attack in barley (Thordal-Christensen et al., 1992), which, it was suggested, may be implicated in papilla deposition. Thordal-Christensen et al. (1997) speculated that the H_2O_2 generator might be catalyzed by an extracellular oxalate oxidase-like protein present in the region of papilla deposition. However, the substrate for this protein is unknown (Wei et al., 1998). Further studies

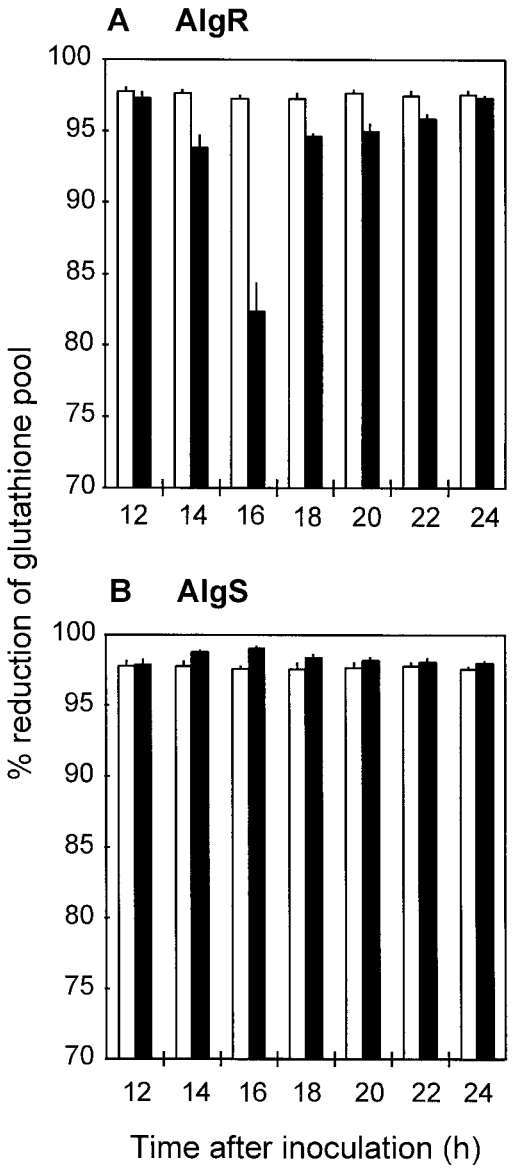

Figure 8. The effect of powdery mildew attack on the redox state of the glutathione pool of AlgR (A) and AlgS (B) barley leaves from 12 to 24 h after inoculation. Black columns, Inoculated; white columns, controls.

(Huckelhoven and Kogel, 1998) using nitroblue tetrazolium staining to visualize superoxide radical anions (O_2^-) suggested that O_2^- generation in powdery mildew-attacked barley was associated with successful penetration of attacked cells; O_2^- accumulation was not associated with the deposition of effective papillae that prevented penetration. Kogel and Huckelhoven (1999) speculated that because O_2^- does not accumulate in effective papillae, the source for H_2O_2 generation in papillae is not likely to be a plasma membrane-bound NAD(P)H oxidase.

The period that was studied in detail here (12–24 h) covers the critical phases when mature fungal appressoria initiate their attack on leaf epidermal cells and when plant responses determine the ultimate success or failure of attempted infection. Visible local H_2O_2-dependent color formation associated with appressoria was identified as early as 12 h after inoculation in both isolines. In AlgS the coloration remained localized throughout the time course of the experiment and preceded the deposition of papillae by living epidermal cells. It was followed by localized accumulation of autofluorogenic material from 18–20 h onwards. At this point, brown coloration became increasingly faint, indicating the absence of H_2O_2 (Fig. 2). Therefore, the current findings differ from Thordal-Christensen et al. (1997) where browning intensified until the end of the time course (in barley with the *Ml-a3*). It is important to point out that the current studies demonstrate that epidermal cells showing localized reactions did not then progress to the stage of whole-cell autofluorescence, indicating that these cells remained alive until fixation.

A similar localized response was also observed in AlgR, but in this case there were also concommittent, unique patterns of DAB staining associated with the cell death response. It is important to note that both patterns of response were not seen within the same cell and hence appeared to be mutually exclusive. Bearing in mind that the fungal population developed and stimulated host responses somewhat asynchronously, it is nevertheless possible to draw the following conclusions.

Production of H_2O_2 in Attacked, Dying Epidermal Cells Occurs after Accumulation in the Underlying Mesophyll Cells

Certain responses were unique to AlgR (*Ml-a1* allele for resistance) and preceded death of epidermal cells. The first unique, visible response was the appearance of circles of brown staining at the points of contact between the attacked cell and the immediately subadjacent mesophyll cells (Fig. 3). Again this agrees with observations of cell death in a barley containing the *Ml-a3* allele for resistance (Thordal-Christensen et al., 1997), suggesting that H_2O_2 production was first initiated around the mesophyll cells, which had points of contact with each attacked epidermal cell. This response was detectable as early as 14 h after inoculation. It is interesting to note that at this stage no visible H_2O_2 accumulation was observed around the attacked epidermal cell. These observations suggest the possibility that an early signal, preceding the activation of hydrogen peroxide production, is transmitted from the attacked epidermal cell to underlying mesophyll cells with which it has contact. This implies that although, as shown in other systems (Wojtaszek, 1997), H_2O_2 production is an early response in this plant-pathogen interaction, it may not be the first signal(s). In mammalian cells, signaling via AOS is integrated with a second signaling pathway involving active nitrogen species such as nitric oxide (NO) which induce GMP and calcium signaling cascades (Poderoso et al., 1996). NO is produced in plants where it has been found to fulfill similar functions to those observed in animals (Cueto et al., 1996; Millar and Day, 1996; Beligni and Lamattina, 1999). Most importantly the simultaneous generation of NO and AOS in the same cell appears to be required for cell death in plant-pathogen interactions (Delledonne et al., 1998). We are currently developing a microelectrode-based system to measure NO production in these tissues.

In the present study the speed with which the signal of pathogen attack is transmitted from the epidermal cell to the underlying mesophyll suggests that the signal is unlikely to be a protein, since de novo synthesis, transport, and action would preclude rapid signal transduction. This signal may be NO or an electrical signal or both; NO and depolarization of the plasmamembrane (Keppler and Novacky, 1986) lead to calcium mobilization (Levine et al., 1994; Poderoso et al., 1996). This response clearly involves the concerted activation of contiguous subadjacent cells that do not undergo cell death in the process.

H_2O_2 accumulation in the mesophyll cells ceased between 20 to 24 h after inoculation. Moreover, the underlying mesophyll cells showed no autofluorescence, suggesting that they remained alive throughout this period and were not harmed by the oxidative stress to which they were subjected. It is possible to conclude, therefore, that H_2O_2 produced in these mesophyll cells does not trigger their death and that cell death is not an inevitable consequence of H_2O_2 production. It is clear that the temporal and spatial orchestration of H_2O_2 production in intact leaves differs from that observed in isolated cells or in single epidermal cell layers (Bushnell, 1981). In isolated epidermal cells HR occurs at a high frequency despite the complete absence of mesophyll cells (Bushnell, 1981).

In the present study H_2O_2-dependent DAB staining disappeared from the dying epidermal cells between 20 and 24 h after inoculation. Two explanations are possible. First, H_2O_2 production may cease after 20 h. Alternatively the capacity of the anti-oxidant systems in the attacked cells may be increased. Anti-oxidative

defenses in the vicinity of H_2O_2 production may be overwhelmed temporarily during the initial phase of the oxidative burst as suggested by Lamb and Dixon (1997) and Wojtaszek (1997).

Transient Inoculation-Dependent Changes in the Glutathione Pool and in Catalase Activity Precede Changes in Peroxidase Activity

Since peroxidase activity was induced irrespective of the presence of an effective allele for disease resistance, this enzyme does not seem to be related to the expression of *Ml-a1*-conditioned defense responses. In contrast, differential responses in catalase activity and in foliar glutathione were observed in AlgR and AlgS. In AlgR, catalase activity transiently increased to over double control values between 14 and 18 h after inoculation (Fig. 6). The increase in catalase activity observed in AlgR coincided with the appearance of H_2O_2 accumulation in the mesophyll underlying attacked epidermal cells (Fig. 3). Catalase activity may be triggered by accumulation of H_2O_2 in the mesophyll cells, allowing them to survive. In AlgS, increased catalase activity was first seen only at 24 h after inoculation. It is possible that the late induction of catalase activity in AlgS is associated with establishment of biotrophy.

In AlgR, transient oxidation of the glutathione pool was also associated with accumulation of H_2O_2 in the mesophyll cells and not in the epidermal cells. Following the onset of glutathione oxidation a large increase in the total glutathione pool was observed. Although the amount of GSSG was increased at 18 h, an increase in the GSH to GSSG ratio was observed. Stimulation of GSH synthesis must be occurring in this period (Noctor et al., 1997; Noctor and Foyer, 1998). H_2O_2 is known to influence cellular GSH accumulation by causing derepression of translation of existing mRNA encoding the enzymes of the GSH biosynthetic pathway (Xiang and Oliver, 1998). Jasmonic acid has been found to increase the abundance of transcripts of the biosynthetic enzymes, but translation requires an oxidative signal (Xiang and Oliver, 1998). Since resistance against powdery mildew in barley appears not to be associated with enhanced endogenous jasmonate concentrations (Kogel et al., 1995), other unknown signals may operate to induce enhanced expression of the genes coding for enzymes of GSH biosynthesis in AlgR.

Accumulation of glutathione following inoculation was observed only in the resistant isoline. Moreover, in a previous study (Vanacker et al., 1999) GSH has also been found to accumulate only in resistant oat lines but not in susceptible lines during powdery mildew attack. These oat lines expressed race nonspecific resistance to this fungal pathogen. GSH accumulation occurred, therefore, independently of the nature of the resistance, whether it was race specific resistance (barley) or race non-specific resistance (oat).

Papilla-Based Resistance to Penetration and Hypersensitive Cell Death May Involve Independent Oxidative Processes

In common with many other investigations (e.g. Zeyen et al., 1995), our results support the view that failure of powdery mildew to establish a biotrophic relationship may be due to at least two separate phenomena, both of which involve oxidative processes.

The first involves responses localized within the attacked plant epidermal in the region subtending the appressorial contact site. Here, a localized oxidative burst occurs directly beneath the region of attempted penetration leading to the rapid accumulation of H_2O_2 at the site of eventual papilla deposition. In our experiments this occurred before any increase in peroxidase enzyme activity was detectable. It may well be that this local accumulation is involved in oxidative cross-linking of components such as phenolics, proteins, and elemental constituents into the papilla and associated cell wall region. Papilla deposition occurs even in the most susceptible host genotypes and is probably a non-specific "background" form of resistance (Carver et al., 1991, 1994). However, where penetration succeeded in cells of the susceptible genotype (AlgS), no such accumulation was detectable. In these cells, processes leading to H_2O_2 accumulation may either have failed so that papillae were incomplete or their components were deposited too slowly for effective resistance to be expressed, or anti-oxidative activity of the fungus may have overwhelmed the plant's response.

The second response is observed only in AlgR and this involves HR. When HR was expressed in AlgR, death of the attacked cell was preceded by accumulation of H_2O_2 in the underlying mesophyll. However, the attacked cell itself showed no local accumulation of H_2O_2, suggesting that the early stages of papilla deposition were dysfunctional. Thus this may represent a case where failure of the papilla response (which would lead to successful penetration in the suscept) allowed the transmission of signals from the avirulent fungal isolate to engage processes leading to HR. Therefore, in this case HR acts as a second line of defense to contain infection when the papilla defense fails. Hence we suggest that while papilla deposition may be independent of the HR response in AlgR, the failure of the papilla response may be a prerequisite for the expression of HR.

MATERIALS AND METHODS

Plant and Pathogen Material

Seedlings of AlgR (resistant; *Ml-a1* allele) and AlgS (susceptible; *ml-a1* allele) were grown under a 16-h photoperiod with irradiance at 340 μmol m^{-2} s^{-1} supplied by white fluorescent tubes, 20°C day/15°C night, and with constant (70%) relative humidity.

Inoculation and Incubation of Experimental Material

An isolate of powdery mildew fungus (*Blumeria graminis* [DC]. Speer [syn. *Erysiphe graminis* DC] f.sp *hordei* Marchal) (isolate CC1; avirulent to *Ml-a1*) was maintained on susceptible barley (*Hordeum vulgare*) seedlings in a spore-proof greenhouse. One day before inoculum was required for experimentation, heavily sporulating plants were shaken to remove older conidia and to ensure a supply of vigorous young spores.

Fungal spores were applied to leaves of intact plants. Inoculation was performed using a settling tower. Test plants were taken to the laboratory where their leaves were laid adaxial surface up under the spore-settling tower and inoculated. Spores from a donor plant carrying inoculum were blown directly into the tower using an air gun and allowed to settle down the tower. A slide placed under the tower was used to monitor inoculum density, which was adjusted to give 20 spores mm^{-2}. Plants were then returned to standard incubation conditions.

In Vivo Detection of H_2O_2

The in vivo detection of H_2O_2 during the barley-powdery mildew interaction was carried out using DAB according to Thordal-Christensen et al. (1997). DAB polymerizes locally as soon as it comes into contact with H_2O_2 in the presence of peroxidase, giving a reddish-brown polymer. DAB is taken up by living plant tissue and can be used to show H_2O_2 production when peroxidase activity is present (Thordal-Christensen et al., 1997).

Eight hours before the time due for sampling (fixation), the leaves were excised, and the cut end was immersed in water where an additional 10 mm was cut from the base of the excised shoot. This effectively removed air embolisms formed during the initial excision that may have blocked vascular tissues. The cut ends of leaves were then immersed in a solution containing 1 mg mL^{-1} DAB dissolved in water to which HCl was added to bring the pH to 3.8 to solubilize the DAB. Leaves were then incubated in the growth chamber for an additional 8-h period to allow DAB uptake and reaction with H_2O_2 and peroxidase. At specific time points after inoculation the DAB reactions were examined on three replicate leaves of the resistant (AlgR) and susceptible (AlgS) barley lines. To allow resolution of fungal structures and host cell responses, leaves were fixed and cleared as described below. H_2O_2 was visualized as a reddish-brown coloration in DAB-treated leaves.

The central 3-cm segment of leaves was used for microscopy while the tip and the basal segments were weighed, immersed in liquid nitrogen, and stored at −80°C for subsequent assay of peroxidase activity. At the same time-points, noninoculated and inoculated leaves were harvested in a similar way for analysis of catalase and peroxidase activities and glutathione content.

Sampling, Fixation, and Clearing of Leaf Tissue for Microscopy

Three inoculated leaves of each line were fixed for light microscopy at two hourly intervals from 12 to 24 h after inoculation for assessment of epidermal host cell responses to attack.

Leaves were fixed and prepared for microscopy by a procedure that avoids displacement of the fungus (Carver et al., 1991). For fixation, 3-cm segments cut from the center of inoculated leaves were laid adaxial surface up on filter paper moistened with an ethanol:glacial acetic acid mixture (3:1, v/v) for 24 to 48 h until the chlorophyll had been removed. When bleached, they were transferred to water-soaked filter paper for at least 4 h to relax leaf tissue and finally transferred to papers soaked with lacto-glycerol (1:1:1, lactic acid:glycerol:water, v/v) for at least 24 h. Segments were stored on lacto-glycerol.

For microscopy, cleared leaf segment was mounted on a microscope slide without a coverslip and observed using a microscope (BH-2, Olympus, Tokyo) with a "no coverslip" 40× objective lens (Carver et al., 1991). For data collection, specimens were observed without staining, but to stain fungal structures for micrography, a drop of aniline blue (0.1% [v/v] in lactoglycerol) was pipetted onto leaf surfaces immediately before they were photographed. To assess the success of attempted primary infection by powdery mildew, 50 germlings with appressoria were examined on each leaf segment by transmitted light microscopy to determine whether or not they had penetrated the host epidermal cell successfully to form a primary haustorium. The microscope was also fitted with a reflected light fluorescence attachment (BH2-RFC, Olympus), and for each germling, autofluorescent responses of epidermal cells to attack by powdery mildew were visualized using incident fluorescence microscopy (using a blue-violet excitation filter with a maximum transmittance of 400 nm; dichroic mirror and barrier filter with a transmittance range of 500–800 nm). Autofluorescence was seen as a bright blue-white fluorescence associated with localized responses of host epidermal cells to appressorium contact. When present, whole-cell autofluorescence, indicative of hypersensitive epidermal cell death (Koga et al., 1988), was also clearly visible.

Anti-Oxidant Analysis

Peroxidase

Freshly cut leaves (0.15 g) were immersed in liquid nitrogen and ground to a fine powder in 1 mL of 50 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) buffer, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 0.1% (v/v) Triton X-100, 1 M NaCl, 5 mM dithiothreitol, and 0.5 mg mL^{-1} bovine serum albumin. When the mixtures had thawed, they were ground again and kept on ice until assay. One molar NaCl was included in some samples to allow the release of cell wall-bound peroxidase. Peroxidase activity was assayed as described by Hammerschmidt et al. (1982). The reaction mixtures (1 mL) consisted of 0.25% (v/v) guaiacol in 0.01 M sodium phosphate buffer, pH 6.0, and 0.1 M H_2O_2. Extract was added to initiate the reaction, which was followed at 470 nm. Activity was expressed as the increase in A_{470} min^{-1} mg^{-1} protein.

Catalase

Freshly cut leaves (0.15 g) were immersed in liquid nitrogen and ground to a fine powder in a 1-mL mixture containing 0.1 M KH_2PO_4/KOH buffer, pH 7.4, and 30 mM dithiothreitol. Samples were centrifuged at 13,000 rpm for 3 min and kept on ice until assay. Catalase was assayed polarographically at 20°C in a liquid phase oxygen electrode (Hansatech, King's Lynn, UK). Total extractable catalase activity was measured via O_2 evolution upon the addition of 0.5 M H_2O_2 to a reaction medium (1 mL) containing 100 mM HEPES/KOH (pH 7.4) and extract (Clairborne, 1985). One unit of catalase activity was defined as the quantity of catalase that would liberate 1 μmol O_2 min^{-1} under these conditions.

Glutathione

Glutathione was determined as described by Vanacker et al. (1998).

Protein Determination

Protein content of the extracts was estimated spectrophotometrically by the method of Bradford (1976).

Statistical Analysis

The significance of differences between mean values obtained from four inoculated and noninoculated (controls) samples produced in two experiments was determined by one-way analysis of variance.

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