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Hypersensitive Cell Death and Papilla Formation in Barley Attacked by the Powdery Mildew Fungus Are Associated with Hydrogen Peroxide but Not with Salicylic Acid Accumulation[1]

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We analyzed the pathogenesis-related generation of H_2O_2 using the microscopic detection of 3,3-diaminobenzidine polymerization in near-isogenic barley (*Hordeum vulgare* L.) lines carrying different powdery mildew (*Blumeria graminis* f.sp. *hordei*) resistance genes, and in a line expressing chemically activated resistance after treatment with 2,6-dichloroisonicotinic acid (DCINA). Hypersensitive cell death in *Mla12* and *Mlg* genotypes or after chemical activation by DCINA was associated with H_2O_2 accumulation throughout attacked cells. Formation of cell wall appositions (papillae) mediated in *Mlg* and *mlo5* genotypes and in DCINA-activated plants was paralleled by H_2O_2 accumulation in effective papillae and in cytosolic vesicles of up to 2 μm in diameter near the papillae. H_2O_2 was not detected in ineffective papillae of cells that had been successfully penetrated by the fungus. These findings support the hypothesis that H_2O_2 may play a substantial role in plant defense against the powdery mildew fungus. We did not detect any accumulation of salicylic acid in primary leaves after inoculation of the different barley genotypes, indicating that these defense responses neither relied on nor provoked salicylic acid accumulation in barley.

The role of H_2O_2 and SA in the defense responses of plants against parasites is controversial. Chen et al. (1993) has argued that SA acts via inhibition of a catalase that subsequently results in accumulation of H_2O_2, which may involve cross-linking reactions leading to cell wall toughening (Bradley et al., 1992; Brisson et al., 1994) and/or signaling that results in defense gene activation (Chen et al., 1993; Wu et al., 1997; Chamnongpol et al., 1998). The hypersensitive response, a plant defense reaction that restricts biotrophic and other pathogens (Stakman, 1915), seems to be dependent on the availability of SA (Levine et al., 1994; Shirasu et al., 1997; Tenhaken and Rübel, 1997) and H_2O_2 (Levine et al., 1994; Thordal-Christensen et al., 1997). Bi et al. (1995) and Neuenschwander et al. (1995) have presented data suggesting that systemic acquired resistance does not rely on H_2O_2 accumulation as a downstream event of elevation of SA content. Moreover, van Wees et al. (1997) and Vidal et al. (1998) have provided evidence for SA-independent biological induction of resistance.

We have chosen the interaction of the powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*) with barley (*Hordeum vulgare* L.) to study the role of reactive oxygen species and SA in constitutively expressed and chemically induced resistance. The developmental stages of powdery mildew fungus during its interaction with its host are well defined, and fungal spores show a synchronized growth upon leaf inoculation. After contact of the spore with the cutin layer of a barley leaf, the following fungal structures differentiate within the first 24 hai (Ellingboe, 1972; Aist and Bushnell, 1991): (a) the primary germ tube (1–2 h), (b) the appressorial germ tube with a mature appressorium (10–12 h), and (c) the haustorium initial (16–24 h), which invaginates the epidermal plasma membrane. Formation of aerial mycelium and sporulation are late differentiation events that occur 5 to 7 d post inoculation. The establishment of a mature haustorium represents a key step for successful fungal reproduction, because it is the only organ that serves to feed the pathogen. Quantitative cytological recordings of incompatible interactions have revealed putative host-cell-defense responses conferring arrest of fungal development at distinct stages (Kita et al., 1981; Koga et al., 1988, 1990). Two of these host-cell responses are easily detected: a subcellular, highly localized cell wall reinforcement at sites of attempted penetration (effective papilla) and an active, rapid death of attacked epidermal cells (HR), which can be visualized by whole-cell autofluorescence under UV excitation (Aist and Israel, 1986; Koga et al., 1990; Görg et al., 1993).

In the present study, we used *Bgh*A6, which triggers defense responses in NILs bearing the powdery mildew resistance genes *mlo5*, *Mlg*, and *Mla12* (Hückelhoven and Kogel, 1998). These genes govern fungal arrest at different

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Abbreviations: DAB, 3,3-diaminobenzidine; DCINA, 2,6-dichloroisonicotinic acid; hai, hour(s) after inoculation; HR, hypersensitive cell death response; NIL, near-isogenic backcross line; PR, pathogenesis-related; SA, salicylic acid.

stages of the interaction: (a) at the penetration stage within papillae, leaving the attacked cell alive (*mlo*); (b) within papillae of cells that subsequently undergo HR (*Mlg*); and (c) by HR after penetration of the epidermal host cell (*Mla12*). A previous publication compared the PR generation of the superoxide anion ($O_2^{\cdot -}$) in these NILs (Hückelhoven and Kogel, 1998; Kogel and Hückelhoven, 1999). An $O_2^{\cdot -}$ burst was seen at the interaction sites in epidermal cells that had been penetrated by the fungus (compatible interaction and *Mla12*-mediated resistance), but not at interaction sites showing penetration resistance (*Mlg*- and *mlo5*-mediated resistance). These data suggest that the trigger for $O_2^{\cdot -}$ generation in epidermal cells attacked by powdery mildew fungus was the contact of the parasite with the host plasma membrane after host-cell wall penetration, and that $O_2^{\cdot -}$ accumulation was not required or sufficient for HR elicitation.

We show here that H_2O_2, in contrast to $O_2^{\cdot -}$, is closely associated with HR and the formation of effective papillae, and that these defense responses are not linked to SA accumulation in barley.

MATERIALS AND METHODS

Plants, Pathogens, and Inoculation

The barley (*Hordeum vulgare* L.) cv Pallas and the *mlo5*-, *Mlg*-, and *Mla12*-backcross lines in cv Pallas were obtained from Lisa Munk (Royal Veterinary and Agricultural University, Copenhagen, Denmark). Their generation was described previously (Kølster et al., 1986). Plants were grown in a growth chamber at 16°C, 60% RH, and a photoperiod of 16 h (100 μE). Inoculation was with 20 conidia mm^{-2} from *Blumeria graminis* DC: Fr. f.sp. *hordei*, race A6 (*Bgh*A6) (Wiberg, 1974) on the 7th d after germination.

Chemical Induction

DCINA (Novartis AG, Basel, Switzerland), formulated as a 25% (w/w) active ingredient with a wettable powder carrier (Métraux et al., 1991), was applied to 4-d-old barley seedlings as a soil drench. The final concentration of the compound used was 8 mg L^{-1} soil volume. Controls were treated with wettable powder.

Histochemical Detection of H_2O_2 at Interaction Sites

Detection of H_2O_2 was performed by an endogenous peroxidase-dependent in situ histochemical staining procedure using DAB, as described by Thordal-Christensen et al. (1997). Seven-day-old primary leaves were cut and placed in a solution of 1 mg mL^{-1} DAB for 8 h and subsequently in a clearance solution (0.15% TCA [w/v] in ethyl-alcohol: chloroform [4:1, v/v]) for 24 h. The storage of leaf segments, the staining of fungal structures, and the microscopy were done as described by Hückelhoven and Kogel (1998). Because defense responses of barley epidermal leaf cells vary significantly depending on the cell type, evaluation of cellular interaction phenotypes and H_2O_2 accumulation was restricted to short epidermal cells (A and B cells showing reactions strongly dependent on the genotype) of the adaxial epidermis (Koga et al., 1990). If more than one cellular structure stained with DAB at the same interaction site, we counted the most intensive coloring for statistical evaluation. The autofluorescence of cells undergoing cell death was partly covered by DAB staining, but it was still bright enough to be taken as a reliable measure of HR.

Extraction of SA and HPLC

Eight primary leaves were harvested by freezing in liquid nitrogen and then stored at −80°C. Leaves were homogenized in liquid nitrogen for 3 min, and two portions of 0.3 to 0.5 g fresh weight of frozen material were placed into 2-mL microcentrifuge tubes. Chloroform:methanol (1:2, v/v; 1.3 mL) was added to the samples according to the lipid-extraction method of Bligh and Dyer (1959) before storing the samples for 24 h at −18°C. According to Meuwly and Métraux (1993), the Bligh/Dyer solution contained 300 ng 2-methoxybenzoic acid mL^{-1} as an internal standard. The internal standard was added after confirming that endogenous levels of 2-methoxybenzoic acid did not change after inoculation. After shaking the samples at 200 rpm on a horizontal shaker for 30 min, 400 μL of distilled water was added for separation of solvent phases. Samples were centrifuged at 20,000*g* for 20 min at 4°C. The methanol-water phases were placed in another microcentrifuge tube and shaken again with 350 μL of $CHCl_3$. After a second centrifugation (5 min), the methanol-water phase was concentrated in a vacuum centrifuge.

Residues were resuspended in 400 μL of methanol:water (1:1, v/v), centrifuged for 10 min at 20,000*g*, and placed in HPLC vials for detection of free SA. For hydrolysis of SA-glycosides the concentrated samples were resuspended in 500 μL of 1 m HCl, incubated at 70°C overnight, concentrated again, and resuspended for HPLC in 500 μL of methanol:water (1:1, v:v). The SA content in the extracts was corrected according to the amount of the internal standard. HPLC was performed with a reverse-phase column (LiChroCart 250-4, LiChroSphere C_{18}, 5 μm, Merck, Darmstadt, Germany) linked to a fluorescence photometer (SFM 25, Kontron, Neufarn, Germany) equipped with a 12-μL flow cell. Excitation and emission wavelengths were 298 and 400 nm, respectively. Separation was at 40°C using a flow rate of 1 mL min^{-1}. Elution was carried out with 0.05 m TCA (pH 2.6):methanol (80:20, v/v) for 1 min, followed by a linear 10-min gradient to TCA:methanol (70:30, v/v), isocratic TCA:methanol (70:30, v/v) for 20 min, 5 min washing with methanol (100%), followed by a return to the first step solvent, and equilibration for 10 min before the next run.

RESULTS

Histochemical localization of H_2O_2 using DAB in barley primary leaves attacked by *Bgh*A6 showed an accumulation of this active oxygen species in cell wall appositions, as well as in cells undergoing HR, which is in agreement with the results of Thordal-Christensen et al. (1997). To determine the spatial and temporal profile of H_2O_2 generation in

these prominent defense reactions, we carried out a comparative kinetic analysis of H_2O_2 formation at early developmental stages of the interaction of *Bgh*A6 with NILs bearing the resistance genes *Mla12*, *Mlg*, and *mlo5*. These genes mediate various highly defined defense phenotypes, including the timing and frequency of HR and effective papillae in response to fungal penetration attempts. The quantitatively predominant plant responses governed by these resistance genes were compared with the susceptible phenotype in Figure 1. Figure 1 also displays the defense phenotype mediated by induction of barley with the chemical DCINA. A more detailed quantitative cytological analysis of the interaction phenotypes indicates that the development of *Bgh*A6 on the NILs or DCINA-treated cv Pallas was basically the same as had been observed in earlier studies (Table I; see also Hückelhoven and Kogel, 1998; Kogel and Hückelhoven, 1999).

H_2O_2 in Compatible Interaction

Upon inoculation of the recurrent parent cv Pallas with *Bgh*A6, H_2O_2 accumulated in the epidermal cell wall appositions subjacent to the primary germ tube within 10 hai, as indicated by reddish-brown staining due to DAB polymerization. A low frequency of interaction sites with H_2O_2 in papillae subjacent to the appressorial germ tube was detected by 20 hai. H_2O_2 was detected within papillae not penetrated by the fungus (effective papillae, Fig. 2A). Epidermal cells that successfully repelled fungal attack regularly contained brownish vesicles around the papilla, suggesting that the vesicles targeted to the plasma membrane to deliver cell wall material for papilla toughening contained H_2O_2. Within 22 hai, epidermal cells penetrated by the fungus often showed a local brownish staining of the anticlinal cell wall at approximately 30% of all interaction sites, but ineffective papillae were not stained (shown at 24 hai in Fig. 2B). Local staining of anticlinal cell walls disappeared within 48 hai, when the fungus had developed branched, elongated secondary hyphae. The HR of the attacked epidermal cells, seen at low frequencies (Table I), was always associated with H_2O_2 accumulation in the entire cell wall or the whole cell, beginning 18 hai. Cells undergoing HR and showing whole-cell H_2O_2 accumulation were not invaded by the fungus; successful penetration was not followed by cell death in this compatible interaction. In the mesophyll tissue, H_2O_2 was detected at sites next to epidermal cells undergoing HR. In some incidences, chloroplasts of these cells and of cells in the vicinity of leaf vessels accumulated H_2O_2 (Fig. 2C), although these cells did not die.

H_2O_2 in Resistance Mediated by the *mlo5* Gene

The recessive *mlo5* gene mediates penetration resistance against *Bgh*A6 in BCP*mlo5* due to the formation of effective papillae in approximately 98% of the interaction sites (Table I). The frequency of HR was even lower than in the compatible interaction (<2%). As in all other NILs, unsuccessful penetration was associated with strong H_2O_2 accumulation in papillae and the surrounding vesicles. Thirty hours after inoculation, vesicles staining positively for H_2O_2 reached a diameter of 2 μm. A yellow autofluorescence under UV light excitation suggested that these vesicles contained phenolic compounds in addition to H_2O_2 and peroxidase (Fig. 2D). At the same time, no dark-brown papillae or vesicles were seen in association with ineffective papillae in cv Pallas. The numbers of interaction sites with a stained papilla or staining in the surrounding ves-

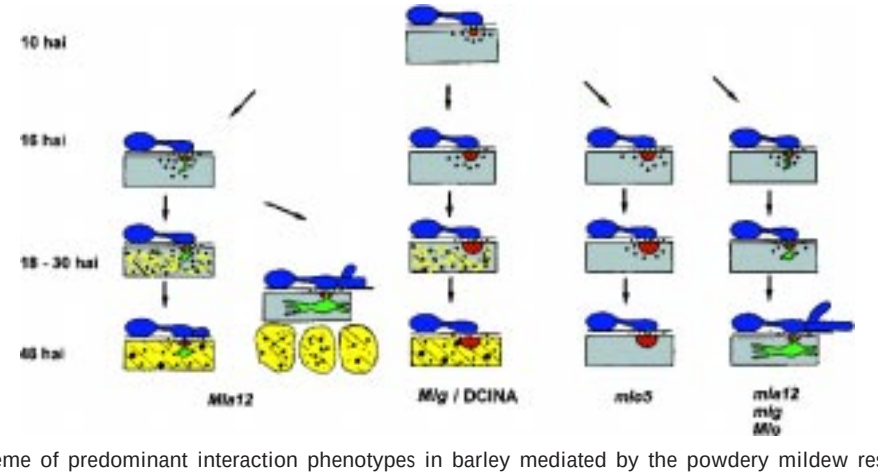

Figure 1. Scheme of predominant interaction phenotypes in barley mediated by the powdery mildew resistance genes *Mla12*, *Mlg*, and *mlo5*, or by the resistance-inducing compound DCINA after inoculation with *Bgh*A6. Beginning at 16 hai, differences in fungal development are evident on the four NILs. Although the fungus penetrated the epidermal cells of susceptible and *Mla12*-resistant barley, effective papillae did impede penetration in the *Mlg*- and the *mlo5*-mediated response. *Mla12* mediated HR in penetrated epidermal cells by 24 to 40 hai. If the fungus was able to establish a differentiated haustorium and branched elongated secondary hyphae, fungal development was arrested by spreading HR of mesophyll cells subjacent to the attacked epidermal cell beginning at 36 hai. The *Mlg* gene governed effective papilla formation and HR of attacked but noninvaded epidermal cells by 18 to 24 hai. The same phenotype is seen in barley treated with DCINA. The recessive *mlo5* gene mediated effective papilla formation, and attacked cells stayed alive. In the compatible interaction, cell wall penetration was followed by formation of a haustorium and elongated secondary hyphae.

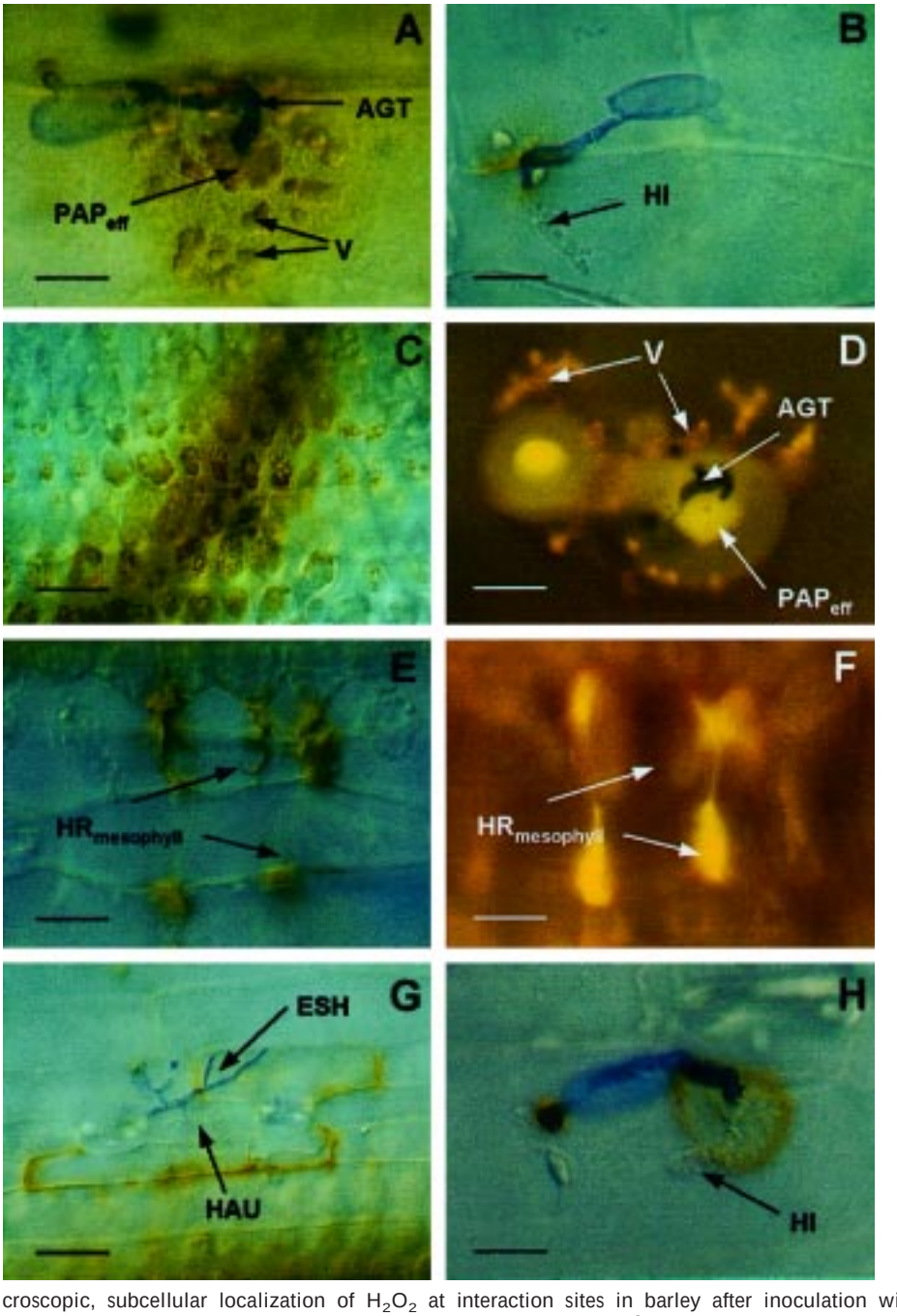

Figure 2. Microscopic, subcellular localization of H_2O_2 at interaction sites in barley after inoculation with *Bgh*A6. Seven-day-old barley primary leaves of NILs were inoculated with 20 conidia mm^{-2} of *Bgh*A6. At different time points after inoculation, leaves were cut off, placed in a solution of 1 mg mL^{-1} DAB, and collected for microscopic analysis 8 h later (at the indicated time points). A, Effective papilla (PAP_{eff}) in formation on cv Pallas (22 hai). Papilla and vesicles (V) with DAB polymers are seen beneath the appressorial germ tube (AGT). Scale bar = 8 μm. B, Successful penetration of cv Pallas (24 hai). The fungus had formed a haustorial initial (HI). Whereas the body of the ineffective papilla shows only a faint staining, the anticlinal cell wall close to the penetration site shows typical brownish DAB polymers. Scale bar = 10 μm. C, DAB staining of chloroplasts in healthy mesophyll cells neighboring a transverse vessel in cv Pallas primary leaves. Scale bar = 40 μm. D, BCP*mlo5* (30 hai). Autofluorescence under UV-light excitation of effective papilla (PAP_{eff}) halos and vesicles beneath primary and AGTs is indicative of the accumulation of phenolic compounds at a repulsed penetration attempt. Scale bar = 10 μm. E, BCP*Mla12* (48 hai): mesophyll HR subjacent to a living penetrated epidermal cell (out of focus). Dead cells show DAB staining. Scale bar = 20 μm. F, Epifluorescence microscopy of the interaction site shown in E. The cells in the center have collapsed. Accumulation of polyphenols is indicated by the yellow autofluorescence. Scale bar = 20 μm. G, BCP*Mla12* (48 hai): epidermal cells surrounding the penetrated, living cell show positive DAB staining,

(Legend continues on facing page.)

icles 22 to 48 hai are shown in Figure 3. Compared with cv Pallas (genotype *Mlo*), BCP*mlo5* showed very high rates of stained papillae, especially at 22 hai (i.e. 14–22 hai, see legend of Fig. 3), a time point that is critical for penetration resistance. In an independent experiment, BCP*mlo5* showed frequencies of DAB staining in papilla clearly higher than in cv Pallas 18 hai (i.e. 10–18 hai).

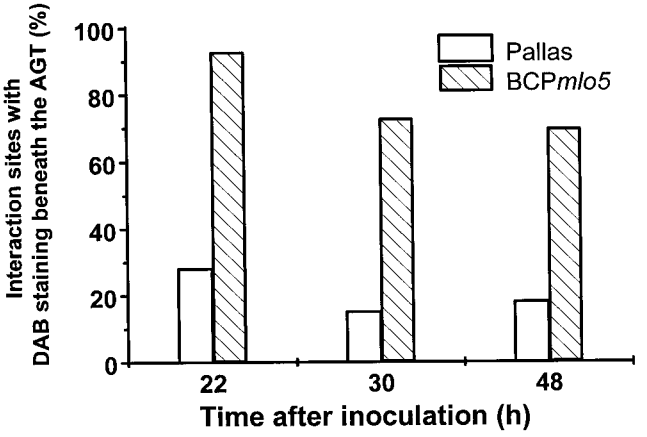

Figure 3. Incidence of interaction sites with H_2O_2 in papillae of attacked cells of the NILs cv Pallas (*Mlo*) and BCP*mlo5* after inoculation with *Bgh*A6. At 14, 22, and 40 hai, leaves were cut off and placed in a solution of 1 mg mL^{-1} DAB. After 8 h (at the indicated time points), leaf segments were analyzed for DAB staining subjacent to appressorial germ tubes (AGT). Each column represents 100 interaction sites per leaf. Three independent experiments gave very similar results.

H_2O_2 in Resistance Mediated by the *Mla12* Gene

Resistance mediated by the *Mla12* gene in BCP*Mla12* against *Bgh*A6 was characterized by a high frequency of interaction sites with HR of penetrated epidermal cells at 22 to 40 hai (50% of interaction sites), resulting in fungal arrest. In 30% of the interaction sites, the fungus succeeded in establishing a compatible single-cell interaction, as indicated by the presence of a fully differentiated haustorium inside the epidermal cell and the formation of branched, elongated, secondary hyphae on the leaf surface. In the latter case, fungal growth was effectively arrested beginning 36 hai by mesophyll cell death just subjacent to attacked epidermal cells (depicted at 48 hai in Fig. 1 and Table I).

The frequency of interaction sites with local staining of anticlinal walls of penetrated cells (Fig. 2B) reached a level of 30% by 22 hai, indicating that there was no difference in H_2O_2 formation at this stage of fungal development between compatible and *Mla12*-incompatible interactions. Later, H_2O_2 accumulated in penetrated cells undergoing HR (Fig. 4; Table I).

Mesophyll cell death that was exclusively detected in BCP*Mla12* with a frequency of 20% of the interaction sites (within 48 hai, see Table I) was closely associated with H_2O_2 accumulation in all of the cells carrying out HR (Fig. 2, E and F). H_2O_2 generation and HR in the mesophyll tissue subjacent to interaction sites was a result of a compatible single-cell interaction in the epidermal layer, leading to successful haustorium formation. By 48 hai, epidermal cells next to cells that had been successfully penetrated by the fungus accumulated H_2O_2 in cell walls, whereas cells containing a haustorium remained essentially free of H_2O_2 (Fig. 2G).

In addition, approximately 10% of all of the interaction sites with successful fungal penetration (cv Pallas and BCP*Mla12*) showed accumulation of H_2O_2 in the cell wall or in the plasma membrane area around the penetration site within 22 hai (seen as a brownish ring in Fig. 2H).

H_2O_2 in Resistance Mediated by the *Mlg* Gene

The resistance response in BCP*Mlg* against *Bgh*A6 was characterized by both the formation of effective papillae and the HR of the attacked epidermal cells. In contrast to the defense phenotype governed by the *Mla12* gene, penetration by the fungus and, consequently, contact of the infection peg and haustorium initial with the host plasma membrane were not required for HR elicitation (Fig. 1).

The frequency of interaction sites with attacked cells undergoing HR reached a level of 76% by 30 hai (Table I). The number of interaction sites with H_2O_2 accumulation in whole cells was strongly correlated with the number of HRs and, compared with cv Pallas, enhanced in all of the evaluated periods (Fig. 4).

H_2O_2 in Resistance Mediated by the Chemical DCINA

Previous work on the mechanism of chemically induced resistance in barley showed that the microscopically defined defense response induced by DCINA was a phenocopy of the response mediated by the *Mlg* gene (Kogel et al., 1994; Kogel and Hückelhoven, 1999) (Fig. 1; Table I).

Soil-drench treatment of cv Pallas seedlings with 8 ppm DCINA enhanced the frequency of interaction sites with HR in attacked epidermal cells compared with control plants (Table I). Whole-cell H_2O_2 accumulation was seen in the cells undergoing HR (Fig. 4). The penetration attempt of the fungus was unsuccessful, and effective papillae were associated with a compact brownish DAB staining (not

Figure 2. (Continued from facing page.)
whereas the attacked cell remained essentially free of dye. ESH, Elongated secondary hyphae; HAU, haustorium. Scale bar = 25 μm. H, BCP*Mla12* (22 hai): the fungus has penetrated the cell and formed a haustorial initial (HI). A brownish halo (in the focus of the cell wall and plasma membrane) is seen around the penetration site, although the body of the ineffective papilla is only weakly stained. Scale bar = 10 μm. All bright-field microscopic pictures were taken using differential interference contrast.

Table I. *Resistance responses of cv Pallas NILs and cv Pallas chemically induced by DCINA upon inoculation with BghA6*

Interaction phenotypes were microscopically analyzed at 22, 30, and 48 h after inoculation. The data represent each 100 interaction sites per leaf. Each of three independent experiments gave very similar results.

Reaction	Interaction Sites				
	Pallas[a]	BCP*Mla12*	BCP*Mlg*	BCP*mlo5*	DCINA-Induced
h			*%*		
Haustorium/elongated secondary hypha					
22	58	62	2	0	29
30	65	54	1	0	35
48	68	5	1	0	26
Effective papilla[b]					
22	32	21	58	99	40
30	17	18	23	99	22
48	22	10	21	98	32
HR (epidermis)[c]					
22	10	17	40	1	31
30	18	28	76	1	43
48	10	64	78	2	42
HR (mesophyll)					
22	0	0	0	0	0
30	0	0	0	0	0
48	0	21	0	0	0

[a] Results shown are from a plant treated with wettable powder as a control for plants treated with DCINA. Wettable powder did not affect the interaction (not shown). [b] Effective papilla in dead cells were evaluated as HR (epidermis). [c] In BCP*Mla12*, HR occurred after penetration, whereas in all other NILs, dead cells did not contain a haustorium.

shown). The pattern of H_2O_2 generation was similar to that found in BCP*Mlg* (Fig. 4).

Determination of SA in Compatible and Incompatible Interactions of NILs with *Bgh*A6

The contents of SA were kinetically analyzed in all of the NILs covering all interaction-relevant time points (Fig. 5). The amount of SA did not differ significantly between Pallas and the corresponding NILs. Upon inoculation with *Bgh*A6, basic levels of total SA did not change in the NILs at any time (0–7 d after inoculation; Fig. 5). This time range covers development of papillae, epidermal HR, multicell mesophyll HR, as well as macroscopically visible necrotic leaf spots (the latter exclusively on BCP*Mla12*). In the same kinetic studies, levels of free SA amounted to one-third of the total SA content and it also did not change after inoculation (data not shown). Each experiment included a positive control from TMV-inoculated tobacco (*Nicotiana tabacum*) cv Xanthi (*NN*). The SA was separated from other compounds by a chromatographic baseline separation using fluorescence detection. SA was detected at 25.2 min, and the internal standard 2-methoxybenzoic acid at 17.1 min after injection (Fig. 6).

Basal levels of SA differed significantly between independent experiments (Fig. 6) but not within an experiment (Fig. 5). Figure 6 shows three representative HPLC traces from independent experiments with SA contents amounting to 101, 1122, and 513 ng g^{-1} fresh weight. The results with the highest contents of SA are shown in Figure 5.

DISCUSSION

H_2O_2 Accumulation Is Associated with Barley Defense Phenotypes

In the present work, we have provided evidence for a host genotype-specific production of H_2O_2 at interaction sites on barley after inoculation with the powdery mildew fungus.

A detailed, cell-type-specific investigation revealed a PR H_2O_2 burst in association with the most prominent barley defense responses toward an attacking pathogen, i.e. the formation of effective papillae and HR. Comparative analysis of NILs bearing the resistance genes *Mlg*, *Mla12*, and *mlo5* demonstrated that these genes affect the frequency, but not the quality, of single-cell defense phenotypes associated with H_2O_2 accumulation. This also applies to the mode of action of the chemical DCINA; i.e. the accumulation pattern of H_2O_2 associated with effective papillae and HR was quantitatively, but not qualitatively, distinguishable from that observed in all interactions of NILs with *Bgh*A6, including the susceptible parent cv Pallas.

Aside from its association with HR, H_2O_2 strongly accumulated in the effective papillae that prevented the attacking pathogen from penetration into the epidermal host cells (Figs. 2, A and D, and 3). All of the papillae were encircled by vesicles delivering material for cell wall toughening at the site of fungal attack (McKeen and Rimmer, 1973; Bushnell and Berquist, 1975). In our experiments, these vesicles did not only stain positively for H_2O_2, but also fluoresced under UV excitation, indicating the presence of phenolic

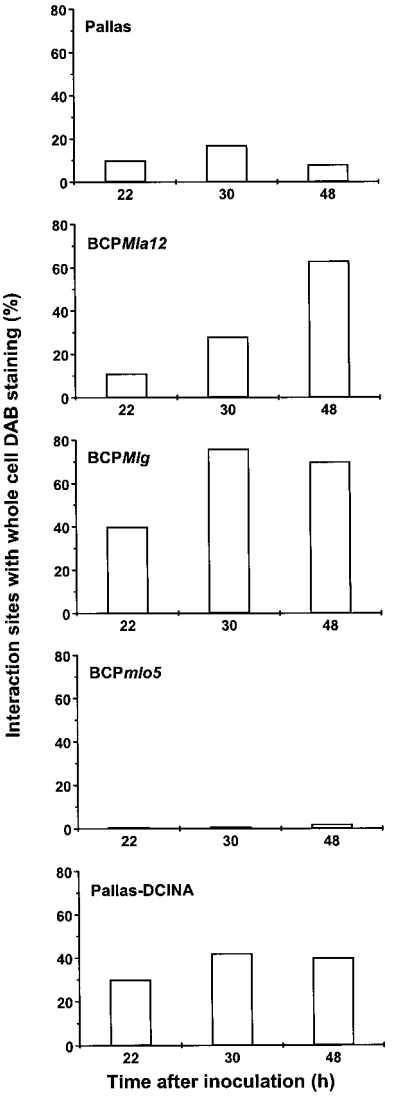

Figure 4. Incidence of interaction sites with whole-cell DAB staining in NILs bearing the resistance genes *Mla12*, *Mlg*, and *mlo5*, and in cv Pallas expressing chemically induced resistance after inoculation with *Bgh*A6. At the indicated time points, leaf segments were microscopically analyzed for positive H_2O_2 staining. Each column represents 100 interaction sites per leaf. Three independent experiments gave very similar results.

material. Because DAB polymerization depends on the presence of H_2O_2 and peroxidase (Thordal-Christensen et al., 1997), our data suggest that the vesicles targeted to effective papillae contained, in addition to H_2O_2, peroxidase, and probably partially polymerized phenolics. Both the papilla-associated H_2O_2 accumulation and the papilla-directed vesicle transport coincided with the arrest of fungal development (Fig. 3; Table I). The absence of H_2O_2 in papillae at early interaction stages correlated with their inefficiency (Figs. 2B and 3; Table I). The unsuccessful penetration attempts by the pathogen may be explained by papilla toughening through cross-linking reactions driven by H_2O_2. Consistently, resistance of papilla-bound phenolics to saponification was found in *mlo*-resistant barley 2 h earlier than in susceptible plants (von Röpenack et al., 1998). H_2O_2 may also have exerted direct toxic effects on the fungus. Thus, successful penetration may have depended on the ability of the fungus to prevent local H_2O_2 accumulation. Garre et al. (1998) have recently suggested that catalases secreted by *Claviceps purpurea* during infection of rye may be suppressors of plant defense.

The PR spatial and temporal patterns of H_2O_2 accumulation were slightly different from those of $O_2^{\bullet-}$ in the same NILs (Hückelhoven and Kogel, 1998; Kogel and Hückelhoven, 1999). $O_2^{\bullet-}$ was localized in a subcellular region where host plasma membrane and fungal haustorium came into contact (genotypes cv Pallas and BCP*Mla12*), but not in association with effective papillae (genotypes BCP*Mlg* and BCP*mlo5*). Thus, $O_2^{\bullet-}$ generation in attacked cells was elic-

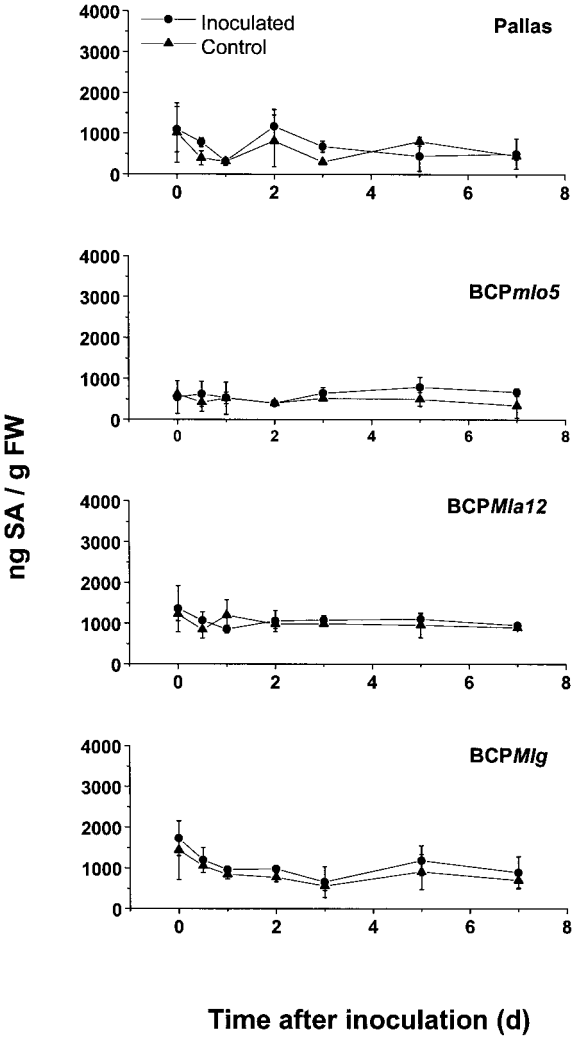

Figure 5. Endogenous levels of total SA in NILs at different time points after inoculation with *Bgh*A6. Seven-day-old primary leaves of the barley lines were inoculated with 20 conidia mm^{-2}. At the indicated time points, leaves were cut off and levels of total SA were determined by HPLC and fluorescence detection. Each point represents the average of duplicates or triplicates of five leaves. Error bars show the SD of the triplicates. Repetition of the experiments led to similar results. FW, Fresh weight.

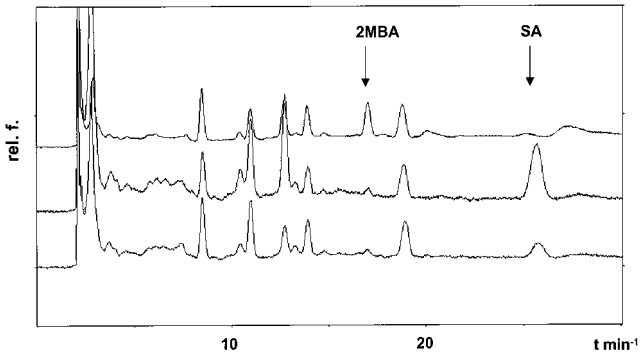

Figure 6. Exemplary HPLC chromatograms of three analog leaf extracts of BCP*mlo5* from independent experiments (different plant batches). Noninoculated samples were taken on the 8th d after seed germination for determination of total SA. For unknown reasons, extracts showed differing contents of SA, although the contents of other compounds did not differ. The middle and the lower chromatogram show low contents of endogenous 2-methoxybenzoic acid (2MBA). The upper line represents an extract containing 2MBA as an internal standard. rel. f., Relative fluorescence.

ited by the cellular contact of host and pathogen, whereas this was not a prerequisite for H_2O_2 accumulation.

The fact that $O_2^{\cdot-}$ and H_2O_2 accumulation could be spatially distinguished in the same defense phenotype also suggests that H_2O_2 in the cell walls and cytosolic vesicles of attacked epidermal cells did not depend upon preceding $O_2^{\cdot-}$ generation driven by an NADPH oxidase (Mehdy, 1994) but might have been the product of alternative H_2O_2-generating sources. Candidate enzymes for PR H_2O_2 production in the epidermis of barley are peroxidase (Gross et al., 1977; Kerby and Somerville, 1992; Kogel et al., 1994; Scott-Craig et al., 1995), which was inhibited in the $O_2^{\cdot-}$ assay (Hückelhoven and Kogel, 1998), oxalate oxidase (Zhou et al., 1998), and an oxalate-oxidase-like protein. Wei et al. (1998) have recently shown the latter to be involved in papilla resistance to powdery mildew fungus. PR H_2O accumulation in the mesophyll tissue was seen especially in BCP*Mla12* (Fig. 2E), and it was accompanied by mesophyll cell death occurring exclusively in this line. In contrast, a strong infection-related and partly light-dependent $O_2^{\cdot-}$ burst was found in both BCP*Mlg* and BCP*Mla12* (Hückelhoven and Kogel, 1998). The $O_2^{\cdot-}$ burst was essentially associated with chloroplasts, whereas H_2O_2 was rarely observed in these organelles. Therefore, PR chloroplastic $O_2^{\cdot-}$ generation did not result in detectable amounts of H_2O_2 at interaction sites. These data indicate that $O_2^{\cdot-}$ and H_2O_2 in the mesophyll tissue were not generated by the same source. We therefore speculate that the H_2O_2 in the mesophyll was generated by an oxalate oxidase recently localized in this tissue of *Mla*-resistant barley (Zhou et al., 1998).

A transient accumulation of H_2O_2 in cv Pallas and BCP*Mla12* was also seen at the sites of successful penetration near the plasma membranes around papillae (Fig. 2H). Because $O_2^{\cdot-}$ was detected at the same subcellular region of these NILs (Hückelhoven and Kogel, 1998), H_2O_2 at this site may have stemmed from $O_2^{\cdot-}$ that was generated in response to the cellular contact of host and pathogen. This local H_2O_2 burst was not sufficient to trigger HR. Xu and Heath (1998) recently reported that a Ca^{2+} influx in cowpea cells attacked by the cowpea rust fungus seemed to be elicited by penetration.

Differences in the pattern of H_2O_2 accumulation between BCP*Mla12* and cv Pallas were detected when epidermal and mesophyll cells of BCP*Mla12* carried out HR with a higher frequency from 30 and 36 hai onward (Figs. 2E and 4). The mesophyll tissue was not directly attacked by the fungus, implying that a signal spreading from the fungus or the attacked cell was essential for this response.

Because H_2O_2 generation was rarely seen before plant responses became microscopically visible, we suggest that active oxygen species generation in the early stages of incompatible interactions may change the cellular redox state before it becomes detectable by histochemical methods. This interpretation is supported by data from Vanacker et al. (1998), who reported that foliar and apoplastic glutathione contents were increased in resistant barley after inoculation with powdery mildew fungus, although apoplastic glutathione was reduced in susceptible barley. The same study showed that enhanced foliar catalase activity was associated with susceptibility. Our data show that haustorium-invaded cells, even on *Mla12*-resistant plants (compatible single-cell interaction), were essentially free of H_2O_2, whereas cells encircling the penetrated cell showed an H_2O_2 burst (Fig. 2G). This may indicate that single-cell compatibility was the cause or the consequence of enhanced antioxidative status of the host cell. Because haustorium establishment on the susceptible line cv Pallas was never followed by HR and/or strong H_2O_2 accumulation, we suggest that this was the key event for the achievement of the antioxidative state in the compatible interaction of barley and powdery mildew fungus.

SA Accumulation Is Not Associated with Defense Responses

A newly developed protocol for SA extraction and its separation by HPLC allowed rapid, cheap, and reliable determination of the SA content in barley leaves. Many reports suggest that SA is involved in HR (Levine et al., 1994; Shirasu et al., 1997; Tenhaken and Rübel, 1997). However, in our experiments, SA did not accumulate during the interaction of the NILs with the powdery mildew fungus. This result is supported by the fact that activity of an SA-sensitive salicylate-2-*O*-glucosyltransferase was not induced during HR and papilla formation after inoculation of BCP*Mlg* with *Bgh*A6 (R. Biermann and K.-H. Kogel, unpublished data). Therefore, in barley, SA accumulation was not required for H_2O_2 accumulation, formation of effective papillae, HR, or the development of necrotic spots (nor did these plant responses elevate the SA content). Pathogen-induced necrotic spots on BCP*Mla12* covered approximately 10% of the total leaf area at 7 d after inoculation. Thus, it can be excluded that a potential, highly localized PR accumulation of SA was masked by the basal SA content, because the whole leaf, not just the attacked epidermal layer, responded to inoculation. Nevertheless, our data do

not exclude that basal SA concentrations were required for the defense responses to occur.

Basal levels of SA differed significantly between independent experiments, but not within the same kinetic analysis. We avoided analytical mistakes by comparing chromatographs of analog samples, clearly demonstrating that the basal content of SA, but not of the other detected compounds, differed from experiment to experiment (Fig. 6). This may explain the greatly differing data for basal SA levels in barley: Vallélian-Bindschedler et al. (1998) found levels of <150 ng g^{-1} fresh weight, and Raskin et al. (1990) found levels of 2130 ng g^{-1} fresh weight.

It is well known that PR gene transcripts accumulate upon inoculation of barley (including the NILs) with powdery mildew fungus (Freialdenhoven et al., 1994; Kogel et al., 1994; Gregersen et al., 1997). Therefore, PR-gene activation is not dependent on elevated SA concentrations regardless of the introgressed resistance genes. This agrees with a study by Vallélian-Bindschedler et al. (1998), who showed that PR gene expression in barley after inoculation with *B. graminis* f.sp. *hordei* and *B. graminis* f.sp. *tritici* was independent of the accumulation of SA. Furthermore, it was shown that biological induction of resistance in dicotyledonous plants did not necessarily depend on SA accumulation (van Wees et al., 1997; Vidal et al., 1998).

Evidence for an SA-sensitive defense pathway comes from experiments with the synthetic SA derivative 3,5-dichlorosalicylic acid, which induced resistance to powdery mildew fungus in cv Pallas (Kogel et al., 1995); and such evidence also comes from experiments with barley *mlo*-double mutants (genotype *mlo ror1*), which are highly sensitive to chemical inducers and show enhanced resistance after treatment with SA (B. Jarosch, P. Schulze-Lefert, and K-H. Kogel, unpublished data). Therefore, as in dicotyledonous plants, there may be SA-dependent as well as SA-independent pathways leading to pathogen resistance in barley.

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