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Production of Salicylic Acid Precursors Is a Major Function of Phenylalanine Ammonia-Lyase in the Resistance of Arabidopsis to *Peronospora parasitica*

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Arabidopsis ecotype Columbia (Col-0) seedlings, transformed with a phenylalanine ammonia-lyase 1 promoter (*PAL1*)–β-glucuronidase (*GUS*) reporter construct, were inoculated with virulent and avirulent isolates of *Peronospora parasitica*. The *PAL1* promoter was constitutively active in the light in vascular tissue but was induced only in the vicinity of fungal structures in the incompatible interaction. A double-staining procedure was developed to distinguish between GUS activity and fungal structures. The *PAL1* promoter was activated in cells undergoing lignification in the incompatible interaction in response to the pathogen. Pretreatment of the seedlings with 2-aminoindan-2-phosphonic acid (AIP), a highly specific PAL inhibitor, made the plants completely susceptible. Lignification was suppressed after AIP treatment, and surprisingly, pathogen-induced *PAL1* promoter activity could not be detected. Treatment of the seedlings with 2-hydroxyphenyl-aminosulphinyl acetic acid (1,1-dimethyl ester) (OH-PAS), a cinnamyl alcohol dehydrogenase inhibitor specific for the lignification pathway, also caused a shift toward susceptibility, but the effect was not as pronounced as it was with AIP. Significantly, although OH-PAS suppressed pathogen-induced lignification, it did not suppress pathogen-induced *PAL1* promoter activation. Salicylic acid (SA), supplied to AIP-treated plants, restored resistance and both pathogen-induced lignification and activation of the *PAL1* promoter. Endogenous SA levels increased significantly in the incompatible but not in the compatible combination, and this increase was suppressed by AIP but not by OH-PAS. These results provide evidence of the central role of SA in genetically determined plant disease resistance and show that lignification per se, although providing a component of the resistance mechanism, is not the deciding factor between resistance and susceptibility.

INTRODUCTION

Downy mildew, caused by the oomycete fungus *Peronospora parasitica*, occurs on cultivated Brassicas and other crucifers with a worldwide distribution. *P. parasitica* is a very successful obligate biotroph that has coevolved closely with the host species such that, in a compatible interaction, very little damage is apparent in the early stages of infection. However, in wet, cool conditions that favor heavy sporulation, necrosis in the host can be extensive and disease outbreak serious. In nature, primary infection of Arabidopsis with *P. parasitica* at the beginning of the season results from oospores that are set free from plant debris in the soil from the previous season. After germination, the germ tubes penetrate the roots of the host, and the coenocytic mycelium ramifies intercellularly throughout the plant. Numerous haustoria are produced into host cells along the length of the hyphae but predominantly in cortical and mesophyll cells. The haustoria invaginate the host plasmalemma but do not penetrate it and are thought to facilitate transfer of nutrients from the host to the fungus. In compatible interactions, no symptoms are usually visible before conidiophores, carrying the asexual conidia, emerge from the plant through the stomata. The bed of conidiophores can be seen as a white down covering; hence, downy mildew.

P. parasitica exists in a number of host-adapted forms pathogenic on different crucifers (Channon, 1981), including Arabidopsis (Koch and Slusarenko, 1990). Several different pathogen isolates have been characterized as separate races that exist in a gene-for-gene relationship with specific Arabidopsis genotypes (Holub et al., 1994). At least 11 different major host resistance genes have been characterized at the genetic level (Holub et al., 1994), and one of these (RPP5, resistance to *P. parasitica*) has been cloned (J. Parker and J. Jones, personal communication). Recognition of the pathogen by the host is coupled by an as yet undefined signal transduction pathway leading to the activation of defense genes. Resistance in Arabidopsis is associated with a hypersensitive response (HR) involving one or a few host cells locally at the site of

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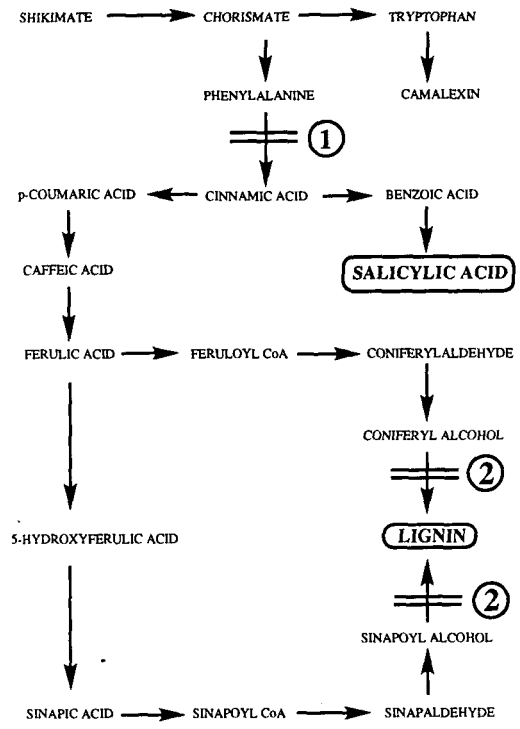

Figure 1. Branches of the Shikimate and Phenylpropanoid Pathways Leading to the Synthesis of Tryptophan and Phenylalanine and of Lignin and SA, respectively.

The inhibition of PAL activity by AIP (1) affects both SA and lignin synthesis, whereas the inhibition of CAD activity by OH-PAS (2) influences only lignin formation.

pathogen ingress (Koch and Slusarenko, 1990; Mauch-Mani et al., 1993) and the accumulation of the antimicrobial substance camalexin, which is an indole compound with a thiazole substituent (Tsuji et al., 1992). The indole ring in camalexin is derived from tryptophan, which is synthesized from chorismate, as is phenylalanine, but on a separate branch of the pathway (Figure 1).

One defense gene activated in the incompatible interaction is phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), which catalyzes the deamination of L-phenylalanine to produce (*E*)-cinnamic acid, a substrate feeding into several biosynthetic routes to various classes of phenylpropanoid-derived secondary plant products (Figure 1; Hahlbrock and Scheel, 1989). Transcripts of *PAL* genes have been shown to accumulate in several different incompatible host–pathogen combinations and in response to elicitors (Edwards et al., 1987; Davis and Ausubel, 1989; Hahlbrock and Scheel, 1989). Early reports that PAL activity in elicited bean cell suspension cultures was not correlated with the accumulation of isoflavonoid phytoalexins in bean (Dixon and Fuller, 1978; Dixon et al., 1981) and reports of PAL activity increases in plants that did not synthesize phenylpropanoid-derived phytoalexins drew attention to other potential roles for PAL in disease resistance.

PAL activity provides precursors for lignin biosynthesis (Henderson and Friend, 1979; Friend, 1985; Davis and Ausubel, 1989; Hahlbrock and Scheel, 1989) and other phenolics that accumulate in response to infection, for example, salicylic acid (SA) (Malamy et al., 1990; Métraux et al., 1990, Ward et al., 1991; Yalpani et al., 1993). SA recently has been shown to be essential for systemic acquired resistance (SAR) (Gaffney et al., 1993; Vernooij et al., 1994) and for the expression of genetically determined primary resistance of Arabidopsis to *P. parasitica* (Delaney et al., 1994). One function of SA might be to inhibit catalase activity, which, by removing H_2O_2, suppresses the oxidative burst necessary for the HR (Chen et al., 1993; Levine et al., 1994).

SA is made from cinnamic acid by decarboxylation and side chain shortening to benzoic acid, followed by hydroxylation (Figure 1; Yalpani et al., 1993; Pierpoint, 1994). The hydroxylated and methoxylated cinnamic acid lignin precursors are synthesized from the CoA esters in a two-step process via cinnamoyl–CoA reductase and cinnamyl alcohol dehydrogenase (CAD) (Figure 1). CAD activity is regarded as specific for lignin synthesis and was reported to increase rapidly in elicitor-treated bean suspension cells (Grand et al., 1987).

The proposal that lignification was a general defense mechanism of plants was made by Hijwegen (1963) after he investigated the infection of cucumber plants by *Cladosporium cucumerinum*. Lignification as an active response to pathogen attack has since been shown in several plants, particularly in graminaceous species in which lignified papillae often form at the sites of attempted fungal penetration (Ride, 1983) and cells undergoing hypersensitive cell collapse become lignified (Beardmore et al., 1983). Enhanced lignification has been reported in response to challenge inoculations with *Colletotrichum lagenarium* or *C. cucumerinum* in immunized cucumber plants in the SAR state (Hammerschmidt and Kuc, 1982). In Arabidopsis, PAL activity is not required for the synthesis of the phytoalexin camalexin, which accumulates in response to *Peronospora* (Slusarenko and Mauch-Mani, 1991) and *Pseudomonas* (Tsuji et al., 1992). We had observed previously that the Arabidopsis *PAL1* promoter was activated at the site of pathogen ingress in the incompatible interaction of Arabidopsis with *P. parasitica* (Mauch-Mani and Slusarenko, 1993). Thus, this model pathosystem is well suited to investigating roles for PAL in defense other than synthesizing phytoalexins. The work reported here investigated the contributions of PAL-dependent lignification and SA accumulation to resistance in this pathosystem.

RESULTS

PAL1 Promoter Activation and Lignification in the Compatible and Incompatible Interaction

Arabidopsis ecotype Columbia (Col-0) plants transformed with the Arabidopsis *PAL1* promoter–β-glucuronidase (*GUS*) reporter gene (*A. thaliana* pSO1) (Ohl et al., 1991) were colonized

rapidly by the virulent NOCO isolate of *P. parasitica*; 5 days after inoculation, the first conidiophores could be observed emerging from the leaves through the stomata. In Magenta-β-D-GlcA (Magenta-Gluc)/trypan blue double-stained preparations, the constitutive activity of the *PAL1* promoter in the vascular tissues in the light was visualized by the magenta color (Figure 2A). No activation of the *PAL1* promoter was observed near the trypan blue–stained fungal hyphae and oospores. The confinement of *PAL1* promoter activity to the vascular system (Figure 2B) correlates with a strong positive reaction of phloroglucinol/HCl reagent with lignified cell wall thickenings of a xylem element (Figure 2C). Thus, in the compatible interaction, there is no apparent activation of the *PAL1* promoter in response to colonization by the pathogen. In the incompatible interaction of *A. thaliana* pSO1 plants with the avirulent EMWA isolate of *P. parasitica*, activation of the *PAL1* promoter around the site of fungal invasion was evident from strong staining with Magenta-Gluc. Thus, where the trailing necrosis type of growth (Mauch-Mani et al., 1993) was observed, a band of *PAL1* promoter activity (magenta) associated with the presence of the hypha (blue) was seen (Figure 2D). When fungal penetration was stopped with a local HR, local activation of the *PAL1* promoter was visible at the HR site (Figure 2F). A correlation between cells expressing *PAL* in an incompatible combination and cells undergoing lignification was observed (Figures 2E and 2F).

When dichloro-isonicotinic acid (INA) is used to induce SAR in Arabidopsis, a normally compatible interaction is converted to a resistant reaction, and a trailing necrosis associated with hyphal growth also is observed often (Uknes et al., 1992). In such cases, cells surrounding the hyphae also turned out to be lignified (Figure 2G). Interestingly, the number of cells showing activation of the *PAL1* promoter is usually greater than the number of cells that react with the phloroglucinol/HCl reagent (Figures 2A and 2C to 2G).

In 8% of the EMWA- and 11% of the WELA-isolate–inoculated plants, occasional hyphae were observed that were not associated with trailing necrosis. In these cases, the fungal hyphae, rather than the adjacent plant cells, reacted positively with the phloroglucinol/HCl reagent (Figure 2H). Interestingly, only the intercellular hyphae, but never the intracellular haustoria, showed an orange coloration with phloroglucinol/HCl reagent (Figure 2H). These hyphae ceased to grow and often appeared light brown in cleared leaves (Figure 2I). When these plants were stained for GUS activity, the normal-looking plant cells in the vicinity of the hyphae showed *PAL1* promoter activity (Figure 2I). However, these plant cells did not collapse and lignify but remained healthy looking.

Inhibition of PAL and CAD Activity

Treatment of pSO1 plants with the PAL inhibitor 2-aminoindan-2-phosphonic acid (AIP) or the CAD inhibitor 2-hydroxyphenyl-aminosulphinyl acetic acid (1,1-dimethyl ester) (OH-PAS) had no influence on the compatible interaction with the virulent isolate NOCO of *P. parasitica*. In both cases, the plants became infected, and the interaction was indistinguishable from nontreated controls (data not shown). However, treatment of pSO1 plants with AIP before infection with EMWA converted an incompatible interaction to a fully compatible one in which both sexual and asexual sporulation was able to occur and *GUS* expression was seen only in the vascular bundle and not in the plant cells near the hyphae (Figures 2J and 3). In contrast, OH-PAS treatment of pSO1 plants, although suppressing EMWA-induced lignification (data not shown) and allowing more pathogen growth than in the controls, did not enable the pathogen to sporulate (Figure 3). In addition, the pathogen-induced activation of the *PAL1* promoter still occurred (data not shown).

Influence of SA on Plant Responses

SA fed to AIP-treated plants, which were subsequently inoculated with EMWA, restored resistance to the level seen in the controls (Figure 3). In addition, measurement of endogenous SA levels in pSO1 plants showed that SA accumulated to relatively high levels in the incompatible combination with EMWA but not in the compatible combination with NOCO (Figure 4). AIP treatment reduced the accumulation of SA, whereas OH-PAS did not suppress SA accumulation significantly (Figure 4). The restoration of resistance by SA in AIP-treated plants restored the activation of the *PAL1* promoter associated with the HR in EMWA-inoculated seedlings.

Influence of AIP and OH-PAS on CAD Activity

Treatment of the plants with AIP and OH-PAS had no influence on overall protein concentration. However, specific activity of CAD was reduced in AIP- and OH-PAS–treated plants compared with untreated controls. Control plants showed a specific CAD activity of 14 nanokatals mg^{-1} protein, whereas the specific CAD activity was inhibited by 51% in AIP-treated and 98% in OH-PAS–treated plants.

DISCUSSION

Arabidopsis Col-0 is resistant to the EMWA and WELA isolates of *P. parasitica*; in these host–isolate combinations, the pathogen has limited growth, and an HR ensues in the host cells adjacent to the hyphae. Frequently, when hyphae grow into the leaf, a trailing necrosis of host cells is seen in their wake; this eventually overtakes the hyphal tip and growth ceases (Mauch-Mani et al., 1993). The interaction phenotypes of WELA and EMWA with Col-0 were graded as FR (flecking, rare sporulation) by Holub et al. (1994). Col-0 is, however, susceptible to the NOCO isolate of *P. parasitica*, and colonization is associated with sexual and asexual sporulation of the pathogen (Parker et al., 1993). The interaction phenotype was graded as EH (early, heavy sporulation) by Holub et al. (1994).

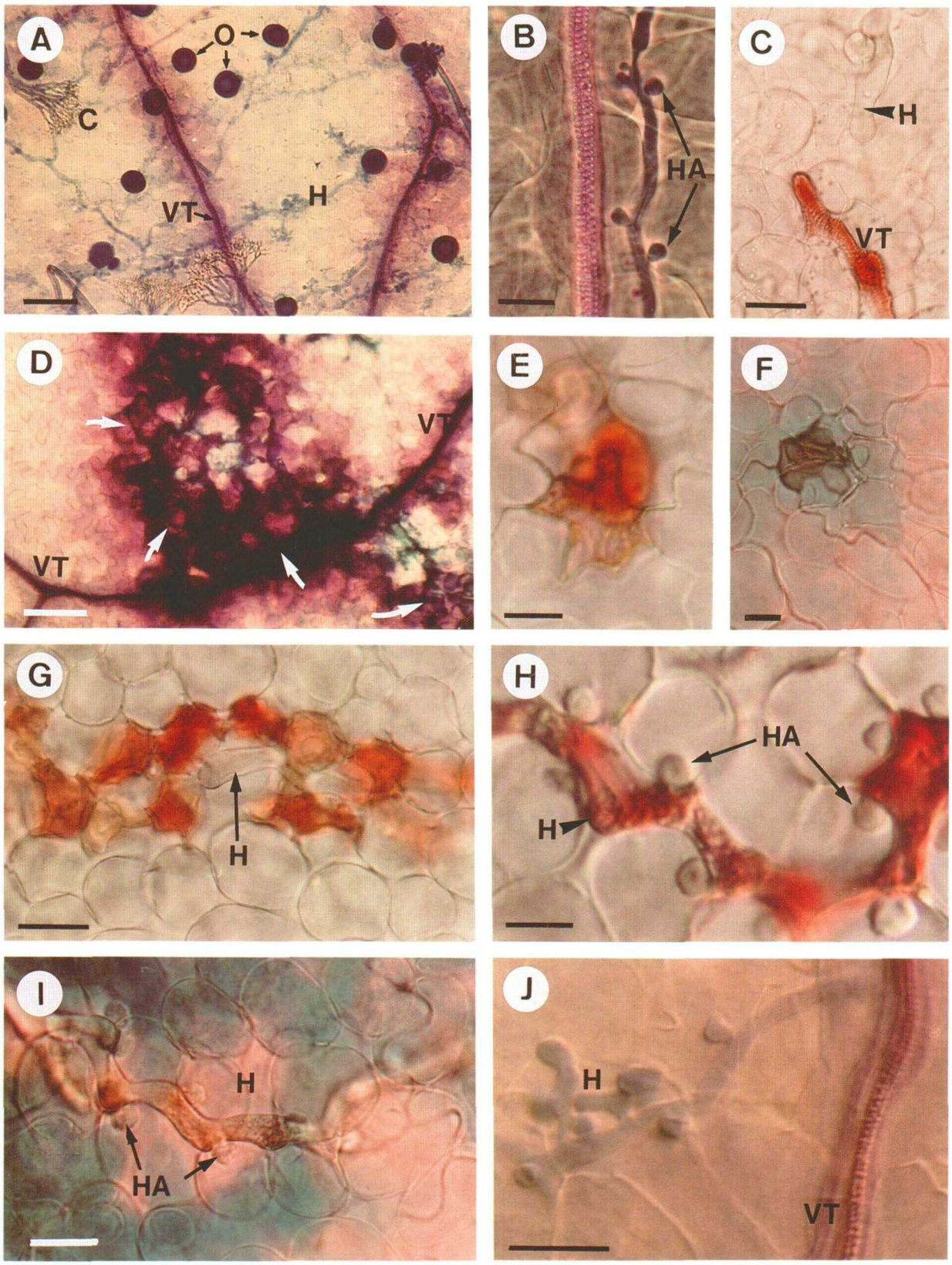

Figure 2. *PAL1* Promoter Activity and Lignification in Compatible and Incompatible Interactions of Arabidopsis Ecotype Columbia (Col-0) (Transformed with an Arabidopsis *PAL1* Promoter–*GUS* Reporter Construct) with *P. parasitica*.

The activation of the Arabidopsis *PAL1* promoter in cells undergoing an HR in the incompatible interaction reflects the putative sites of lignification as indicated by phloroglucinol/HCl coloration. This is compatible with the hypothesis that PAL plays a role in resistance in Arabidopsis by providing precursors necessary for lignification. The Arabidopsis *PAL1* promoter also was reported to be activated after stable transformation into tobacco when the plants were challenged with *Pseudomonas solanacearum* (Huang and McBeath, 1994).

The finding that the number of cells expressing PAL is usually higher than the number of cells actually undergoing lignification could reflect a true activation of the *PAL1* promoter in several cells (whereas lignification only occurs in a limited number of these), or it could be a result of the previously documented "leaky" non-cell-autonomous nature of the *GUS* reporter that has been observed under some circumstances (Ludwig et al., 1990).

It has been suggested that lignification as a resistance response not only might involve toughening the plant cell wall and thus rendering it resistant to attack by pathogen hydrolases but also that the free radical–mediated polymerization of lignin precursors in intercellular spaces might lignify pathogen structures (Ride, 1983). Hammerschmidt and Kuc (1982) reported that a lignin-like substance was deposited on mycelia of *Colletotrichum lagenarium* and *C. cucumerinum* when incubated with coniferyl alcohol, H_2O_2, and a crude cucumber peroxidase preparation. An increase in peroxidase activity in the incompatible interaction of Arabidopsis with *P. parasitica* has been described by Mauch-Mani et al. (1993). Our observations support the hypothesis that lignification of fungal hyphae by the plant might be a part of the defense repertoire of the host. It is particularly interesting that the intracellular haustoria, which invaginate the plasmalemma into the "penetrated" cell, presumably are not exposed to the lignifying milieu in the intercellular space and remain unaffected (Figure 2I).

To assess the contribution of lignification to the resistance of Arabidopsis to *P. parasitica*, we initially decided to suppress PAL activity in vivo using the very potent inhibitor AIP (Zon and Amrhein, 1992). This very specific PAL inhibitor was previously shown to suppress lignification in the vascular tissues of growing plant parts and is thus lethal eventually (Keller et al., 1990).

The fact that inhibition of PAL activity by AIP led to a suppression of the *PAL1* promoter activity was unexpected because initially it was not clear why the *PAL1* promoter activity usually induced in response to the pathogen in the incompatible interaction (Figures 2D and 2E) should be suppressed by inhibiting enzyme activity. The complete conversion of the plants from resistant to susceptible was reminiscent of the same phenomenon observed when the accumulation of SA was suppressed by the expression of the bacterial *nahG* gene in transgenic plants (Delaney et al., 1994). Because PAL activity is necessary for SA synthesis as well as for providing lignin precursors, we decided to try and assess separately the effects of these two components on resistance. To this end, the

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Figure 2. (continued).

(A) Low-magnification microscopy of the compatible interaction with *P. parasitica* isolate NOCO. The plant was double stained with Magenta-Gluc/trypan blue 5 days after inoculation. The constitutive activity of the *PAL1* promoter in the vascular tissue is evidenced by the magenta color. Fungal structures are stained blue. C, conidiophore; H, hypha; O, oospore; VT, vascular tissue. Bar = 100 μm.

(B) Compatible interaction with *P. parasitica* isolate NOCO at high magnification (phase contrast optics). Double staining with Magenta-Gluc/trypan blue 5 days after inoculation clearly demonstrates *PAL1* promoter activity in the vascular tissue only but not in the vicinity of the hypha. HA, haustorium. Bar = 25 μm.

(C) Phloroglucinol/HCl test for lignin in the compatible interaction with *P. parasitica* isolate NOCO. The lignin in the vascular tissue (VT) is stained red-orange by this procedure, and no staining is visible in the cells near the pathogen. H, hypha. Bar = 25 μm.

(D) Incompatible interaction with *P. parasitica* isolate WELA stained with Magenta-Gluc/trypan blue. *PAL1* promoter activity occurs in cells surrounding an invading hypha (white arrows) as well as in the vascular tissue (VT). Bar = 35 μm.

(E) and **(F)** Light microscopy of the hypersensitive reaction of single cells in the incompatible interaction with *P. parasitica* isolate WELA. The site of lignification, seen as red-orange coloration after phloroglucinol/HCl treatment in **(E)**, correlates with the *PAL1* promoter activity, denoted by the blue color after staining with X-Gluc in **(F)**. Bars = 15 μm.

(G) Phloroglucinol/HCl stain of a trailing necrosis resistance phenotype after immunization of a susceptible plant with INA. The cells surrounding the hypha of the normally virulent NOCO isolate are lignified as visualized by their red-orange color. H, hypha. Bar = 25 μm.

(H) Lignin-positive reaction of a hypha of *P. parasitica* isolate WELA in an incompatible interaction. The intercellularly growing hypha became red-orange after exposure to the phloroglucinol/HCl reagent, whereas the intracellular haustoria remained unstained. Note the absence of lignification in the plant cells adjacent to the hypha. H, hypha; HA, haustorium. Bar = 20 μm.

(I) X-Gluc staining of an incompatible interaction with *P. parasitica* isolate WELA as given in **(D)**. *PAL1* promoter activity is visible in the healthy, nonlignified, non-HR plant cells surrounding the hypha. The hypha has a natural brown discoloration, but the haustoria within the plant cells are not discolored. H, hypha; HA, haustorium. Bar = 25 μm.

(J) Nomarski interference contrast microscopy after Magenta-Gluc/trypan blue staining of a genotypically incompatible interaction with *P. parasitica* isolate EMWA made phenotypically compatible by AIP treatment. The constitutive expression of the *PAL1* promoter can be seen in the vascular tissue, but in contrast to **(D)**, there is no *PAL1* promoter activity associated with the hyphae. H, hypha; VT, vascular tissue. Bar = 25 μm.

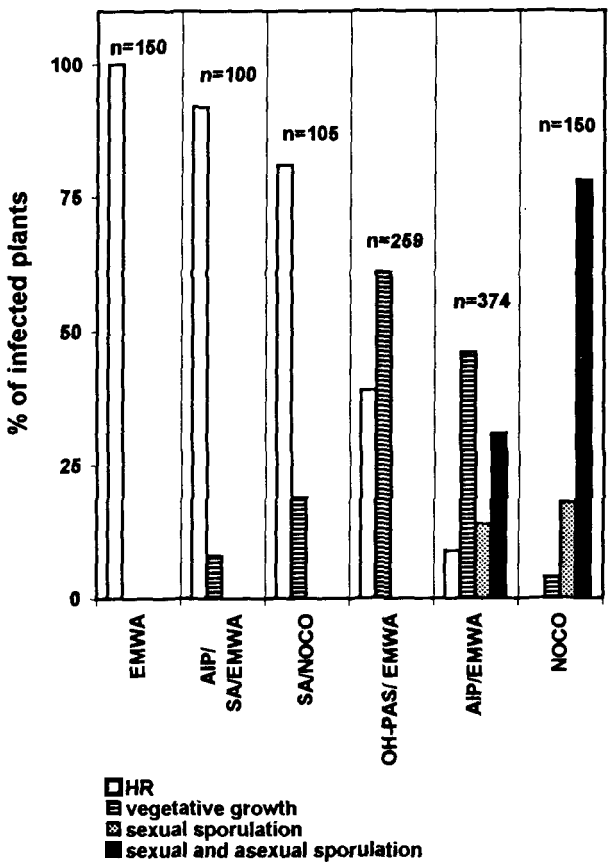

Figure 3. Effect of Inhibitors of PAL (AIP) and CAD (OH-PAS) on Resistance to *P. parasitica* and the Complementary Effect of SA.

The histogram shows the infection phenotypes of pSO1 transgenic Col-0 plants infected with either avirulent EMWA or virulent NOCO and the effects of the various treatments on these phenotypes. The transition from resistant to susceptible after AIP treatment is clear, as is the reversal of this effect by SA. Note the intermediate effect of OH-PAS on resistance of Col-0 to EMWA.

CAD inhibitor OH-PAS was used to suppress lignification specifically without affecting PAL. The much greater reduction of CAD activity in response to OH-PAS compared with AIP suggests that lignification was reduced to a greater extent by OH-PAS than by AIP.

In addition, we investigated changes in endogenous SA levels and the effect of feeding SA in selected experiments with the inhibitors. CAD is regarded as an enzyme specific for the lignin biosynthetic pathway because it synthesizes cinnamyl alcohols that are the immediate precursors of lignin. This role is supported by recent *CAD* promoter studies showing that activity is localized to lignifying vascular tissues of stem, roots, petioles, and leaves and in parenchyma cells surrounding lignified phloem vessels and sclerenchyma fibers (Feuillet et al., 1995). The latter authors speculate that the CAD activity in the nonlignified young parenchyma cells provides lignin precursors to the adjacent lignifying elements.

Lignification has been associated with hypersensitive resistance in wheat (Beardmore et al., 1983). However, HR occurred under circumstances in which lignification was suppressed, and this result does not support the speculation that lignification per se is the mechanism by which host cells might die in the HR (Moerschbacher et al., 1990), at least in Arabidopsis. Experiments with wheat using PAL inhibitors less potent than AIP in vivo (i.e., α-aminooxy acetic acid, [*S*]-2-aminooxy-3-phenylpropionic acid and [*R*]-[1-amino-2-phenyl-ethyl] phosphonic acid) (Zon and Amrhein, 1992), and OH-PAS and NH_2-PAS as CAD inhibitors, were also shown to reduce resistance (Moerschbacher et al., 1990). However, in the wheat/wheat stem rust pathosystem, the CAD inhibitors caused a shift to susceptibility greater than did the PAL inhibitors (Moerschbacher et al., 1990). This result might reflect the difference in the relative importance of defense reactions in cereals.

Thus, although lignification as a component of hypersensitive resistance in cereals is well documented (Ride, 1983), data suggesting a role for SA in induced resistance or the HR in cereals have not yet been published, and indications are that SA does not induce resistance in wheat or other cereals, as was shown recently for barley by Kogel et al. (1995). Indeed, Silverman et al. (1995) show that neither virulent nor avirulent pathogens alter endogenous levels of SA in rice leaves. However, they do find a correlation between horizontal resistance of rice cultivars to *Magnaporthe grisea* and the endogenous levels of SA in leaves. Therefore, it was suggested that SA might play a role as a constitutive defense compound.

Taken together, our results suggest that in the Arabidopsis–*Peronospora* pathosystem, where PAL is not required for the synthesis of the phytoalexin camalexin, PAL is involved in synthesizing SA and precursors for lignification, lignification is associated with the HR but not causally, and SA contributes more to the expression of resistance than lignification. Endogenous SA levels need to rise in Arabidopsis for resistance to be expressed, and the increase depends on PAL activity. Interestingly, the inhibition of SA accumulation caused by the PAL inhibitor prevents the pathogen-induced activation of the *PAL1* promoter. It is not clear whether the observed block of *PAL1* promoter activity by AIP is really due to reduced accumulation of SA or whether it has some other cause.

The elucidation of the relative contributions of these biochemical events, functional in the expression of host resistance in Arabidopsis and downstream of pathogen recognition by classical resistance genes, is helping to build an emerging picture of how plants defend themselves against pathogen attack and provides another example of the usefulness of Arabidopsis as a model in plant pathology.

METHODS

Plant Material

Seed of *Arabidopsis thaliana* ecotype Columbia (Col-0) transformed with a phenylalanine ammonia-lyase 1 promoter (*PAL1*)–β-glucuronidase (*GUS*) reporter construct (Ohl et al., 1991) were surface sterilized

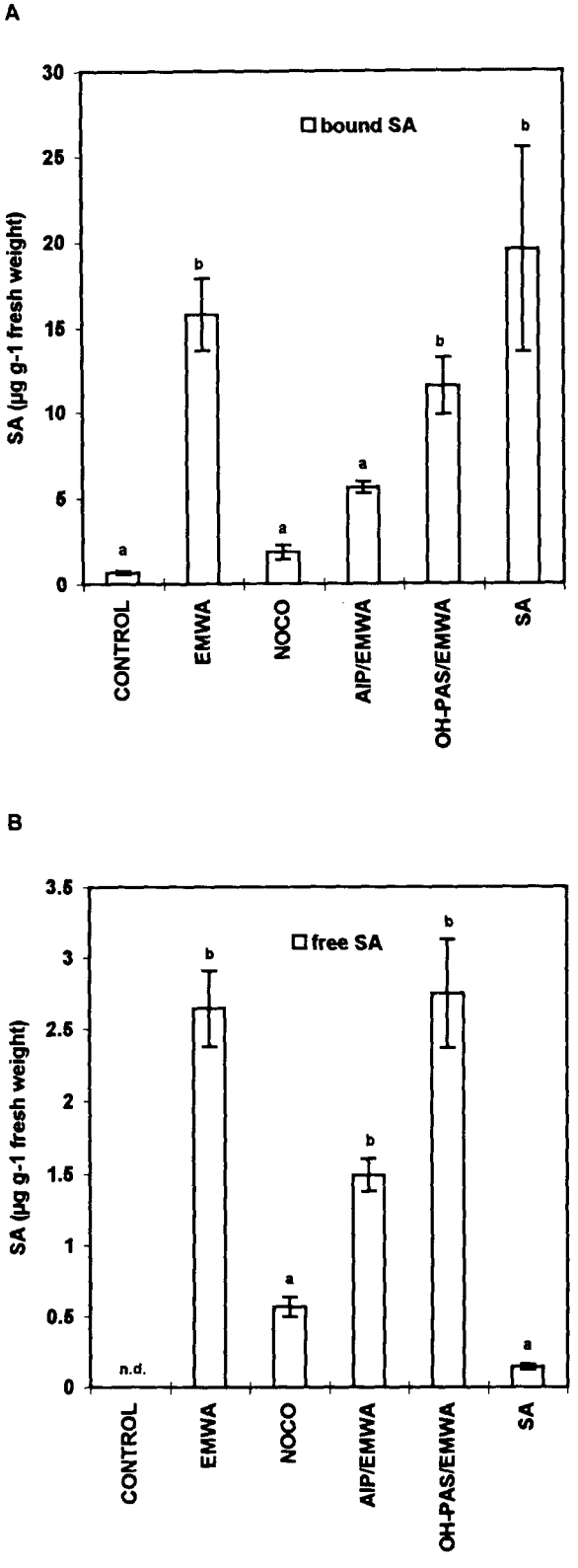

Figure 4. Measurements of SA Levels after Various Treatments.

The data shown are from two independent experiments of three measurements each (i.e., six data values per treatment). The data were analyzed by a one-factor ANOVA with repeated measures, and the Fisher PLSD test for significance was applied. Columns labeled with different letters are significantly different from each other ($P = 0.001$). Bars show SEM.

(A) Bound (conjugated) SA.

(B) Free (nonconjugated) SA. n.d., no difference.

Note the difference in scale between **(A)** and **(B)**, which reflects the high toxicity of free SA to the plant.

in 2.5% bleach for 10 min, followed by several washes with sterile tap-water, before sowing into 30-mL pots containing cactus soil. The seeds were kept for 7 days at 4°C in the dark before being transferred to a growth chamber with a photoperiod of 8 hr of light (150 µE m^{-2} sec^{-1} at plant level) at 22°C and 16 hr of dark at 18°C.

For inhibitor and salicylic acid (SA) experiments, seed were surface sterilized as described above, and the plants were grown aseptically on autoclaved cosmetic cotton wool pads imbibed with sterile liquid Murashige and Skoog medium (Murashige and Skoog, 1962) in Petri dishes. These plants were grown under the same conditions as described for soil-grown seedlings.

Fungal Cultures

Isolates of *Peronospora parasitica* were maintained by subculturing weekly on Arabidopsis ecotype Weiningen (Wei-0) for the WELA isolate (Koch and Slusarenko, 1990), on ecotype Col-0 for the NOCO isolate (Parker et al., 1993), and on ecotype Wassilewskija (WS) for the EMWA isolate (Holub et al., 1994). The NOCO and EMWA isolates are both oospore germlings derived from material supplied by J. Parker (The Sainsbury Laboratory, Norwich, UK) and E. Holub (Horticultural Research International, East Malling, UK), respectively.

Inoculation of Plants

Four-week-old plants (after sowing) were inoculated by spraying with a conidial suspension (~10^5 conidia mL^{-1} sterile tap water) of *P. parasitica* (Mauch-Mani and Slusarenko, 1994) and incubated in a growth chamber under a photoperiod of 8 hr of light (75 µE m^{-2} sec^{-1}) and 16 hr of dark, at a constant temperature of 16°C. The inoculated plants were kept under high air humidity for the whole period of the experiment.

Treatment of the Plants with INA, SA, AIP, and OH-PAS

Dichloro-isonicotinic acid (INA) treatment was performed 3 weeks after sowing, according to Uknes et al. (1992), on soil-grown plants to give a final concentration of 1 mM INA in the soil. SA (Fluka, Buchs, Switzerland) and 2-aminoindan-2-phosphonic acid (AIP) were dissolved in distilled water, and 2-hydroxyphenyl-aminosulphinyl acetic acid (1,1-dimenthyl ester) (OH-PAS) was first moistened in a few drops of ethanol before the water was added. All solutions were filtered through a 0.2-µM filter (Schleicher & Schuell, Keene, NH) before being applied to the cotton pads used to grow the plants on. SA and OH-PAS were applied at a concentration of 10^{-3} M and AIP at a concentration of

10^{-4} M. AIP and OH-PAS solutions were fed to the plants for 7 days and SA for 4 days before inoculation with *P. parasitica*. Controls were treated with sterile water. After 7 days of treatment, AIP- and OH-PAS–treated and control plants were quick-frozen in liquid nitrogen and stored at −70°C to be used for cinnamyl alcohol dehydrogenase (CAD) activity measurements.

Staining for Microscopy

The plants were taken for microscopy 5 days after inoculation. Whole plants had the roots removed and were placed in fixation solution (0.3% formaldehyde, 0.001% Silwet L-77 [Union Carbide, Danbury, CT], 10 mM Mes, pH 5.6, 0.3 M mannitol), vacuum infiltrated until the leaves appeared dark green, and incubated in the fixative for 1 hr at room temperature. The fixed plants were washed five times in 50 mM Na_2PO_4, pH 7.0, and then transferred to the histochemical reagent (10 mg of Magenta-β-D-GlcA [Magenta-Gluc] or 10 mg of X-GlcA CHX [X-Gluc] [Biosynth AG, Staad, Switzerland] in 100 μL of dimethylformamide diluted to 10 mL with 50 mM Na_2PO_4). The plants were incubated in this solution for 5 hr at 37°C. Plants stained with X-Gluc were transferred directly to chloral hydrate (2.5 g mL^{-1} H_2O) for clearing. Plants stained with Magenta-Gluc were washed twice with 50 mM Na_2PO_4 and counterstained overnight with an alcoholic trypan blue solution (Keogh et al., 1980). The double-stained plants also were cleared in chloral hydrate for ~24 hr. Single leaves were mounted in chloral hydrate and viewed under a microscope with bright-field, phase-contrast, or Nomarski interference optics.

Lignified structures were visualized using the phloroglucinol/HCl test. Plants were incubated in a solution of 1% phloroglucinol in 70% ethanol until they were totally cleared. Single leaves were then mounted on slides, a few drops of concentrated hydrochloric acid were added, and the leaves were covered with a coverslip (Gurr, 1965). After ~5 min, lignified structures appeared red-orange, but the color faded within ~30 min.

Determination of SA

Tissues for SA determination were harvested 5 days after inoculation with *P. parasitica*, when plants were stained for microscopic investigation, quick-frozen in liquid nitrogen, and stored at −20°C. Extraction of SA was performed as described by Meuwly and Métraux (1993). HPLC separation was performed on an LKB (LKB Produkter AB, Bromma, Sweden) system (2152 HPLC controller, 2 × 2150 HPLC pumps) equipped with a Nucleosil 100-5 C18 AB–reversed phase column (15 cm × 4.6 mm with 5 μm packing; Macherey-Nagel, Oensingen, Switzerland). The column was preceded by a Nucleosil 100-5 C 18 guard column (8 mm × 4 mm × 5 μm). Flow rate and elution gradient were performed according to Meuwly and Métraux (1993), and SA was detected photometrically at 280 nm using an LKB 2158 Uvicord SD detector. Under the separation conditions used, the SA peak was well separated from other peaks, and the detection limit was equivalent to < 0.01 ng μg^{-1} fresh weight of tissue.

Assay of CAD Activity

CAD activity was determined according to Wyrambrick and Grisebach (1975). Five hundred micrograms of frozen leaf material was ground to powder in a cold mortar, 1 mL of extraction buffer (0.1 M sodium phosphate buffer, pH 6.5, 0.1 mM dithiotreitol, 10 mg mL^{-1} Dowex 1 × 2) was added, and the extract was stirred for 10 min and centrifuged (14,000*g* for 8 min). The resulting supernatant was used as enzyme extract for assessments of CAD activity.

CAD enzyme assays were performed in a volume of 800 μL at 30°C. A typical assay contained 500 μL of Tris-HCl (0.2 M, pH 8.8, 0.3 mM $NADP^+$) and 200 μL enzyme extract. The reaction was started by adding 100 μL of substrate solution (2 mM coniferyl alcohol [Sigma] in 0.2 M Tris-HCl, pH 8.8). Absorbance was monitored at 400 nm over a period of 6 min. Protein concentration in the extracts was determined by the method of Bradford (1976), using BSA as a standard protein. The calculation of enzyme activity was performed as described by Wyrambrick and Grisebach (1975).

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