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Impaired Fungicide Activity in Plants Blocked in Disease Resistance Signal Transduction

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Fungicide action is generally assumed to be dependent on an antibiotic effect on a target pathogen, although a role for plant defense mechanisms as mediators of fungicide action has not been excluded. Here, we demonstrate that in Arabidopsis, the innate plant defense mechanism contributes to the effectiveness of fungicides. In NahG and *nim1* (for noninducible immunity) Arabidopsis plants, which normally exhibit increased susceptibility to pathogens, the fungicides metalaxyl, fosetyl, and $Cu(OH)_2$ are much less active and fail to control *Peronospora parasitica.* In contrast, the effectiveness of these fungicides is not altered in Arabidopsis mutants defective in the ethylene or jasmonic acid signal transduction pathways. Application of the systemic acquired resistance activator benzothiadiazole (BTH) in combination with these fungicides results in a synergistic effect on pathogen resistance in wild-type plants and an additive effect in NahG and BTH-unresponsive *nim1* plants. Interestingly, BTH treatment normally induces long-lasting pathogen protection; however, in NahG plants, the protection is transient. These observations suggest that BTH treatment can compensate only partially for an impaired signal transduction pathway and support the idea that pathogen defense mechanisms are under positive feedback control. These observations are strikingly reminiscent of the reduced efficacy of antifungal agents in immunocompromised animals.

INTRODUCTION

Plants have evolved complex, integrated dsefense mechanisms against potential pathogenic microorganisms; these mechanisms include preformed physical and chemical barriers as well as inducible defenses, such as strengthening the cell wall and synthesis of antimicrobial molecules (Hammond-Kosack and Jones, 1996). However, some microorganisms are able to overcome the defense system and infect plant tissues. Control of plant diseases caused by these pathogens can be achieved through the selection of resistant varieties or by the use of antimicrobial compounds.

The ability of a resistant plant variety to respond to infection is determined by genetic traits from both the plant and the pathogen, and it is manifested by the development of a hypersensitive response (HR; Bent, 1996; Dangl et al., 1996; Hammond-Kosack and Jones, 1996; Bonas and Van den Ackerveken, 1997). In addition to this gene-for-gene resistance, plants have an inducible resistance called systemic acquired resistance (SAR), which is triggered by necrotic lesions caused by pathogens in both incompatible (HR) and compatible (disease) interactions and is mediated by a distinct signal transduction pathway (Chester, 1933; Hunt and Ryals, 1996; Ryals et al., 1996). The induction of SAR protects the plant against a broad spectrum of pathogens and is correlated with the induction of a well-characterized set of genes (SAR genes) that comprise the so-called pathogenesis-related (PR) proteins (Ward et al., 1991; Uknes et al., 1992). This response seems to modulate or potentiate primary resistance mechanisms, so that activation of SAR can convert a compatible interaction into an incompatible one (Uknes et al., 1992; Cameron et al., 1994; Mauch-Mani and Slusarenko, 1994). Conversely, in plants in which the SAR pathway is incapacitated, defense responses are compromised, and the plant can become susceptible to pathogens that normally would not infect them and more severely susceptible to pathogens that cause disease (Gaffney et al., 1993; Delaney et al., 1994, 1995; Maher et al., 1994; Mauch-Mani and Slusarenko, 1996; Pallas et al., 1996).

Salicylic acid (SA) plays a key role in SAR and also can modulate gene-for-gene resistance. SA levels increase after pathogen infection, and this increase has been shown to correlate with SAR activation (Malamy et al., 1990; Métraux et al., 1990; Rasmussen et al., 1991). Compelling evidence for the role of SA in defense has been obtained from the expression in plants of the bacterial *nahG* gene encoding the enzyme salicylate hydroxylase, which degrades SA to catechol. NahG plants, which are unable to accumulate free SA, are compromised in their defense system and do not induce SAR in response to viral, bacterial, or fungal pathogens

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(Gaffney et al., 1993; Delaney et al., 1994; Bi et al., 1995). Depletion of SA also can cause a breakdown of gene-for-gene resistance (Delaney et al., 1994; Mauch-Mani and Slusarenko, 1996).

In addition to its induction by pathogens, SAR is induced and modulated by the exogenous application of SA or chemically synthesized compounds, such as 2,6-dichloroisonicotinic acid (Métraux et al., 1990; Vernooij et al., 1995) and benzo(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH; Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996). These SAR activators, which are apparent functional analogs of SA, do not increase SA concentration in the plant and activate SAR in both wild-type and NahG plants (Vernooij et al., 1995; Friedrich et al., 1996; Lawton et al., 1996).

A number of mutants altered in plant defense mechanisms have been identified in Arabidopsis, including those affected in SAR (see Hunt and Ryals, 1996; Ryals et al., 1996; Delaney, 1997) and those blocked in ethylene (Bleecker et al., 1988; Guzmán and Ecker, 1990) or jasmonic acid (Staswick et al., 1992) signal transduction pathways. Both SAR-constitutive and SAR-compromised mutants have been obtained (reviewed in Hunt and Ryals, 1996; Ryals et al., 1996). In the first type, SAR is constitutively active, and plants are resistant to virulent isolates of *Pseudomonas syringae* and *Peronospora parasitica* (Lawton et al., 1993; Bowling et al., 1994, 1997; Dietrich et al., 1994; Greenberg et al., 1994; Weymann et al., 1995; Dangl et al., 1996). Conversely, in SAR-compromised mutants, which have the SAR signaling pathway blocked, avirulent isolates of both *P. syringae* and *P. parasitica* become virulent (Cao et al., 1994; Delaney et al., 1994, 1995; Century et al., 1995; Glazebrook et al., 1996; Shah et al., 1997).

nim1 (for noninducible immunity) is one of these SAR-compromised mutants that is able to accumulate SA after pathogen infection but does not respond to SAR activators (Delaney et al., 1995). Several alleles of the *nim1* gene (also called *npr1*) have been identified (Cao et al., 1994; Glazebrook et al., 1996; Ryals et al., 1997; Shah et al., 1997), and recently the gene was cloned (Cao et al., 1997; Ryals et al., 1997). The predicted NIM1 protein shares significant homology with the mammalian IκBα subclass of transcription factor inhibitors, suggesting that the signal transduction pathway may share mechanistic parallels to the mammalian NF-κB signal transduction pathway (Ryals et al., 1997). The NF-κB/IκB regulatory system has been shown to be involved in the activation of pathogen defense systems in mammals and Drosophila (Baeuerle and Baltimore, 1996; Baldwin, 1996; Lemaitre et al., 1996).

Plants with an intact defense system can be infected by virulent pathogens. The use of antimicrobial compounds, such as fungicides, can reduce pathogen infection. Although the activity of these compounds in the plant is generally accepted to be derived from their direct antibiotic action on the invading microorganism, the contribution of plant defense systems to the effectiveness of certain fungicides has been the subject of speculation (Király et al., 1972; Ward et al., 1980; Ward, 1984). In soybean, the effectiveness of metalaxyl, a systemic fungicide active against oomycete fungi (Fisher and Hayes, 1982), has been correlated with the accumulation of glyceollin, a plant antimicrobial phytoalexin (Ward et al., 1980; Lazarovits and Ward, 1982; Börner et al., 1983). Treatment of soybean with the herbicide glyphosate has been shown to reduce glyceollin biosynthesis and metalaxyl effectiveness against *Phytophthora* sp (Keen et al., 1982; Ward, 1984). Furthermore, it has been shown that in potato tubers, metalaxyl is effective against *Fusarium* sp and *Alternaria* sp, fungi toward which metalaxyl shows no direct toxicity (Barak et al., 1984). Similarly, it has been suggested that the fungicide fosetyl and $Cu(OH)_2$, a broad-spectrum fungicide and bactericide, may be involved in the activation of some host defense responses (Guest, 1984; Nemestothy and Guest, 1990; Jones et al., 1991).

We have investigated the role of the plant defense system in the mode of action of various fungicides. Here, we demonstrate that the effectiveness of fungicides in the plant is reduced in SAR-compromised plants. Moreover, combinations of fungicides with the SAR activator BTH synergistically increased their effectiveness in wild-type plants, whereas an additive effect was observed in SAR-compromised plants.

RESULTS

Fungicidal Action Is Reduced in SAR-Defective Mutants

The Arabidopsis–*P. parasitica* pathosystem was chosen to investigate the role of plant defense mechanisms in fungicide action. A specific *P. parasitica* probe (*Pp* probe) was developed to quantify the growth of the fungus in the plant. The probe corresponded to a 555-bp region of the *P. parasitica* 16S-like nuclear rRNA obtained by using polymerase chain reaction, according to White et al. (1990). As shown in Figure 1A, the fungal specificity of the *Pp* probe was tested by using RNA gel blot analysis of the following total RNA samples: *P. parasitica* spores, leaves from uninfected plants, and leaves from infected wild-type and SAR-compromised *nim1* and NahG plants. The plant samples had been previously equilibrated with respect to plant RNA content, using the Arabidopsis β-tubulin probe (Figure 1A). Under our experimental conditions, the *Pp* probe exhibited only weak cross-hybridization with plant RNA that did not interfere with its use to estimate fungal growth.

As previously observed by qualitative visual estimation (Delaney et al., 1994; Dietrich et al., 1994; Ryals et al., 1997), the *Pp* probe showed quantitatively that fungal growth was higher in SAR-compromised plants than in the wild type (Figure 1A). Quantification of the signals by using a PhosphorImager indicated that fungal growth was 2.5- to fivefold higher in SAR-compromised plants than in the wild type. Moreover, NahG plants consistently supported 1.5- to twofold higher degree of infection than did the *nim1* mutant (data not shown).

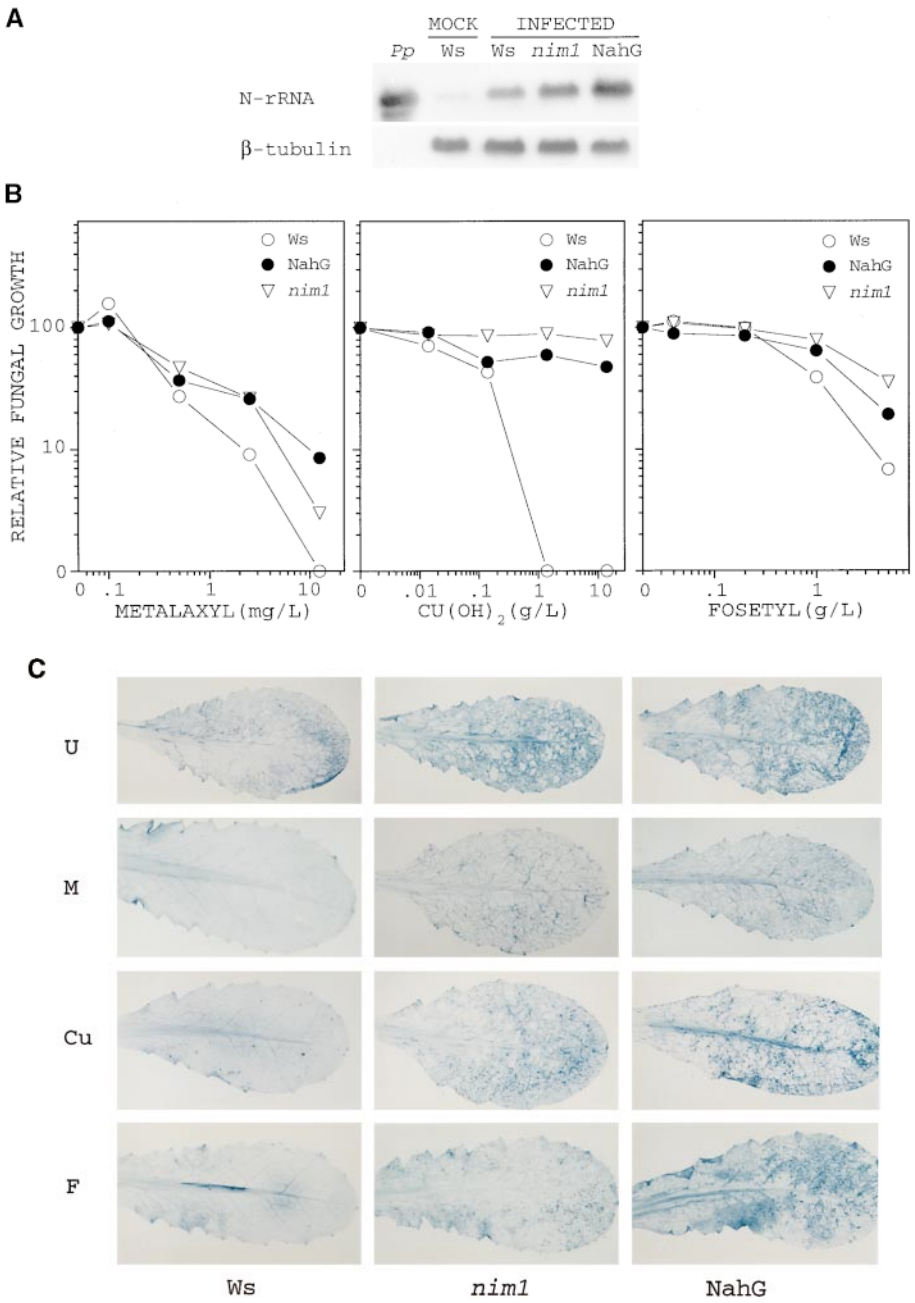

Figure 1. Effectiveness of Fungicides Is Reduced in SAR-Compromised Plants.

(A) A specific *P. parasitica* probe (*Pp* probe) was obtained to quantify fungal growth in the plant. Total RNA (1 μg) from spores of *P. parasitica* isolate Emwa (*Pp*), leaves from uninfected (Mock) Arabidopsis wild-type plants (Wassilewskija [Ws]), and infected leaves (8 days after inoculation) from Ws, *nim1*, and NahG SAR-compromised plants was hybridized with the *Pp* probe corresponding to the 16S-like nuclear rRNA (N-rRNA). Filters had been previously equilibrated for plant RNA content by using a specific Arabidopsis β-tubulin probe.

(B) The effect of metalaxyl, $Cu(OH)_2$, and fosetyl on *P. parasitica* growth in Ws, *nim1*, and NahG plants. Fungal growth in these plants 8 days after inoculation was measured by hybridization of total RNA with the *Pp* probe followed by hybridization with the β-tubulin probe to equilibrate for plant RNA content. Quantification of the hybridization signals was done using a PhosphorImager, as indicated in Methods. Fungal growth in NahG and *nim1* plants, respectively, was four- to fivefold higher and 2.5- to threefold higher than in Ws plants. Fungal growth in the untreated control plants of each genotype was taken as 100%. Statistical analysis of variance (*F* test) indicated that the effectiveness of the three fungicides in Ws plants significantly differed from that in NahG plants ($P > 0.99$ for metalaxyl; $P > 0.95$ for $Cu[OH]_2$; $P > 0.95$ for fosetyl) and *nim1* plants ($P > 0.95$ for metalaxyl; $P > 0.99$ for $Cu[OH]_2$; $P > 0.95$ for fosetyl). In NahG plants, the effectiveness of metalaxyl and fosetyl did not differ significantly from that in *nim1* plants ($P > 0.95$). This is one of three experiments with similar results and significant P values (0.95 to 0.99).

(C) Shown are trypan blue–stained leaves of Ws, *nim1*, and NahG plants 8 days after inoculation with *P. parasitica.* Leaves from untreated plants (U) or plants treated with metalaxyl (M; 2.5 mg/L), $Cu(OH)_2$ (Cu; 1.4 g/L), or fosetyl (F; 5 g/L) are shown. The blue stain intensity correlates with the amount of fungal mycelia.

The ability of three fungicides (metalaxyl, fosetyl, and $Cu[OH]_2$) to control the oomycete fungus *P. parasitica* was investigated in Arabidopsis. Metalaxyl is a phenylamide fungicide that inhibits RNA synthesis apparently by interfering with the RNA polymerase I template complex of sensitive fungi (Davidse, 1984); copper is a broad-spectrum fungicide and bactericide that binds to and modifies sulfhydryl groups of essential amino acids involved in enzyme catalysis (Koller, 1992); and fosetyl degrades in the plant to phosphonic acid, which inhibits oomycete growth by an as yet undetermined mechanism (Fenn and Coffey, 1984; Griffith et al., 1992).

Wild-type, NahG, and *nim1* plants were sprayed with different concentrations of the fungicides 3 days before inoculation, and the degree of infection was estimated 8 days after inoculation both qualitatively, by visual inspection, and quantitatively, by using the *Pp* probe. As demonstrated in Figure 1B, which shows the results obtained using the *Pp* probe, metalaxyl concentrations that contained fungal growth in wild-type plants were not able to do so in NahG or *nim1* plants. Higher fungicide concentrations were needed for complete control of *P. parasitica* in SAR-compromised plants (five- to 20-fold for NahG and five- to 10-fold for *nim1*; Figure 1B and data not shown). The activity of $Cu(OH)_2$ and fosetyl against *P. parasitica* in wild-type plants was lower than that of metalaxyl, and 1000-fold higher concentrations of these fungicides were needed to stop fungal growth in wild-type plants (Figure 1B). As observed with metalaxyl, $Cu(OH)_2$ concentrations that stopped fungal growth in wild-type plants were not effective in SAR-compromised plants (Figure 1B). Fosetyl was less effective than was $Cu(OH)_2$ and was not able to stop completely fungal growth in wild-type plants, even at the highest concentration tested (5 g/L); however, fosetyl was more effective in these plants than in SAR-compromised mutants (Figure 1B). Statistical examination (analysis of variance) of these results confirmed that the effectiveness of the three fungicides in wild-type plants significantly differs from that in NahG and *nim1* plants ($P > 0.95$) and that the effectiveness of metalaxyl and fosetyl in NahG plants does not differ significantly from that in *nim1* plants (Figure 1B).

Evaluation of the infection by counting the number of conidiophores and by visual assessment of trypan-blue stained leaves from fungicide-treated plants inoculated with *P. parasitica* also was consistent with the above conclusion (Figure 1C). As previously described, we observed a clear correlation between the severity of symptoms and the amount of mycelia and number of conidiophores (Figure 1C), which were higher in SAR-compromised plants than in wild-type plants (Delaney et al., 1994, 1995). The highest tested fosetyl concentration (5 g/L), which was only partially effective in wild-type plants, was phytotoxic, as evident by the formation of lesions on leaves, whereas effective concentrations of metalaxyl (2.5 mg/L) and $Cu(OH)_2$ (1.4 g/L) did not have a phytotoxic effect (data not shown).

To investigate possible SAR induction by these fungicides, we analyzed the expression of the SAR marker gene *PR-1* (Uknes et al., 1992) and compared it with that obtained after treatment with the SAR activator BTH. As shown in Figure 2, BTH induced *PR-1* expression in wild-type and NahG plants, whereas only low *PR-1* induction was observed in the *nim1-1* allele mutant used. *nim1-1* mutants are not completely blocked in their ability to respond to SAR activators (Delaney et al., 1995; Lawton et al., 1996; Ryals et al., 1997). Concentrations of metalaxyl (2.5 mg/L) and $Cu(OH)_2$ (1.4 g/L) that control *P. parasitica* did not affect *PR-1* gene expression in any of the genotypes analyzed, whereas the highest fosetyl concentrations tested (5 g/L) did induce transient *PR-1* gene expression in wild-type plants but not in *nim1* and NahG plants (Figure 2). A *PR-1* gene induction pattern similar to that obtained with fosetyl was observed when plants were sprayed with a high concentration of $Cu(OH)_2$ (14 g/L; data not shown). This induction was probably a consequence of the lesions caused by fosetyl and $Cu(OH)_2$ and seemed to be dependent on the SAR signaling pathway, because it was blocked in NahG and *nim1* plants.

It has been shown that *PR-1* gene expression is induced in Arabidopsis plants with pathogen-induced lesions as well as in lesion-simulating disease (*lsd*) and accelerated cell death (*acd*) mutants that show spontaneous lesion formation on leaves (Uknes et al., 1992; Dietrich et al., 1994; Greenberg et al., 1994; Weymann et al., 1995). These results suggested that although the effectiveness of metalaxyl and $Cu(OH)_2$ was dependent on components of the SAR pathway, they were not SAR activators, and that the fungicidal activity of fosetyl in the plant against *P. parasitica* may be partially due to activation of SAR caused by its phytotoxicity.

Fungicidal Action Is Not Altered in Mutants Blocked in Ethylene or Jasmonic Acid Signaling Pathways

Ethylene and jasmonic acid signaling pathways have been implicated in plant defense responses (Ecker and Davis, 1987; Bergey et al., 1996). Arabidopsis mutants altered in

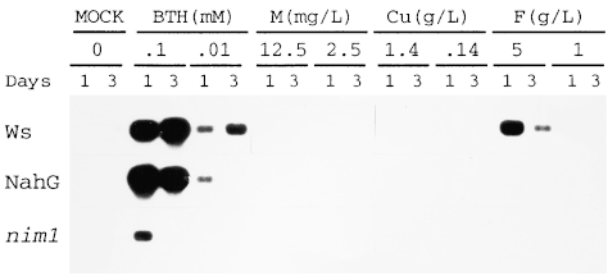

Figure 2. Effect of Fungicides and BTH Treatment on *PR-1* Expression.

Total RNA (7.5 μg per sample) isolated from Ws, NahG, and *nim1* plants treated with wettable powder (Mock) or the indicated concentrations of BTH, metalaxyl (M), $Cu(OH)_2$ (Cu), or fosetyl (F) were blotted and hybridized with the Arabidopsis *PR-1* cDNA probe (Uknes et al., 1992).

these pathways, such as *etr1*, *ein2*, and *jar1*, have been shown to exhibit a normal SAR response (Lawton et al., 1994, 1995). The effectiveness of fungicides to control the compatible Noco2 isolate of *P. parasitica* was investigated in these mutants as well as in wild-type and NahG plants of ecotype Columbia (Col). Growth of *P. parasitica* in *etr1* and *jar1* plants, as estimated with the *Pp* probe, did not differ from that in wild-type plants. In contrast, fungal growth was two- to threefold higher in *ein2* plants and, as expected, five- to sixfold higher in NahG plants with respect to that in wild-type plants (data not shown).

As shown in Figure 3, the effectiveness of metalaxyl was similar in wild-type, *etr1*, and *jar1* plants, but it was reduced in NahG plants. The latter needed 10- to 20-fold higher fungicide concentrations to contain fungal infection (data not shown). Analysis of variance confirmed that these results were significant ($P > 0.95$). Although fungal growth was higher in the *ein2* plants, the concentration of fungicide that was required to completely inhibit the fungal growth was the same as in wild-type plants (Figure 3). Statistical analysis indicated that the slope values of the regression lines corresponding to *ein2* and Col plants did not differ significantly ($P > 0.95$) and therefore that the effectiveness of metalaxyl in *ein2* and Col plants was not significantly different. The same results were obtained with $Cu(OH)_2$ and fosetyl (data not shown). The reduced effectiveness of fungicides in NahG SAR-compromised plants was not dependent on ecotype (Wassilewskija [Ws] versus Col) or fungal isolate (Emwa versus Noco2). These results indicate that the alterations in the ethylene or jasmonic acid signal transduction pathways caused by these mutations have a negligible effect on the effectiveness of the fungicide in the plant.

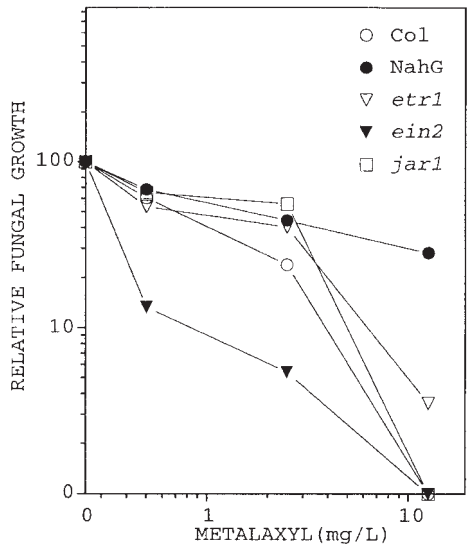

Figure 3. Effectiveness of Metalaxyl Is Not Reduced in Ethylene and Jasmonic Acid Mutants.

Fungal growth in wild-type (Col) and NahG plants and ethylene (*etr1* and *ein2*) and jasmonic acid (*jar1*) mutants 8 days after inoculation with the *P. parasitica* isolate Noco2 was measured. Fungal growth in NahG and *ein2* plants was six- and 2.5-fold, respectively, higher than in Col, *etr1*, and *jar1* plants. *P. parasitica* growth was quantified as indicated in Figure 1. Fungal growth in the untreated control plants of each genotype was taken as 100%. Statistical analysis of variance (*F* test) indicated that the effectiveness of metalaxyl in *etr1*, *ein2*, and *jar1* did not differ significantly from that in Col and that the effectiveness of metalaxyl in NahG plants significantly differed from that in Col ($P > 0.95$), *etr1* ($P > 0.95$), *ein2* ($P > 0.99$), and *jar1* ($P > 0.95$). This is one of two experiments with similar results and significant P values (0.95 to 0.99).

Combinations of Fungicides with the SAR Activator BTH Result in a Synergistic Effect on Wild-Type Plants

To determine if BTH would act synergistically with fungicides, we sprayed wild-type, NahG, and *nim1* plants with different fungicide/BTH combinations 3 days before *P. parasitica* inoculation and measured the degree of infection with the *Pp* probe 8 days after inoculation. Figures 4A and 4B show that concentrations of BTH and metalaxyl that did not substantially limit fungal growth in wild-type or NahG plants when applied separately, effectively controlled fungal infection when applied together. The combined fungicidal effect observed in wild-type plants was more than additive (Figure 4A), whereas it was additive in NahG SAR-compromised plants (Figure 4B). In the *nim1* mutant, BTH treatment was ineffective; therefore, the effect on pathogen infection of the combinatorial treatment did not differ from that observed when plants were treated with metalaxyl alone (data not shown). In ethylene and jasmonic acid mutants, this combined antifungal effect was also synergistic (data not shown).

Similar to the effect observed with metalaxyl, combinations of fosetyl or $Cu(OH)_2$ with BTH resulted in antifungal activities that were synergistic in wild-type plants (Figures 4C and 4D) and additive in NahG plants (data not shown). These results were subjected to an analysis of variance that confirmed that the synergistic and additive effects observed in wild-type and NahG plants, respectively, were statistically significant ($P > 0.95$). These combined antifungal effects resulted in a decrease of the effective concentration of the fungicide or BTH required for pathogen control and allowed the reduction of the chemical dose needed to stop fungal growth. The reduction of the dose of fungicide may mitigate the incidence of foliar damage due to chemical tolerance.

To investigate whether the lack of synergy between fungicides and BTH in NahG plants was due to an alteration in the response to the SAR activator, we studied the effect of BTH on fungal growth. As shown in Figure 5, treatment with 0.1 mM BTH contained fungal growth in wild-type plants. However, the same treatment in NahG plants was not effective; although it contained fungal growth for 10 days, it did not stop the infection completely unless the treatment was

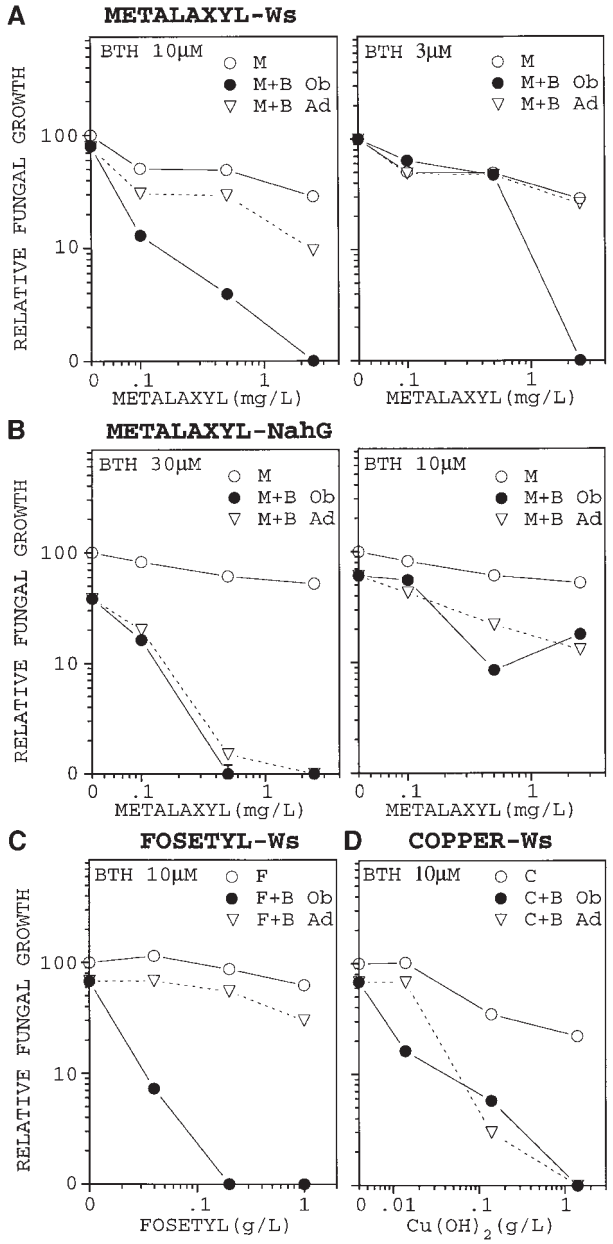

Figure 4. Combinations of Fungicides with BTH Result in a Synergistic Effect on Wild-Type Plants and an Additive Effect on NahG Plants.

(A) and **(B)** Relative fungal growth in Ws and NahG plants, respectively, 8 days after inoculation with *P. parasitica* isolate Emwa. Fungal growth in the untreated Ws and NahG plants was taken as 100% (M, dose 0). Fungal growth values observed in plants treated with the indicated concentrations of metalaxyl (M), BTH (M+B Ob, dose 0), or combinations of metalaxyl with BTH (M+B Ob) are represented. The expected antifungal additive effect (Ad) resulting from the sum of the observed activity of metalaxyl and BTH alone is indicated (M+B Ad). Statistical analysis of variance (*F* test) showed that the synergistic and additive effects were significant ($P > 0.95$). This is one of three experiments with similar results and significant P values (0.95 to 0.99).

(C) and **(D)** Relative fungal growth in Ws plants 8 days after inoculation with *P. parasitica* isolate Emwa. *P. parasitica* infection was estimated as described in Figure 1. Fungal growth in the untreated Ws plants was taken as 100% (C or F, dose 0). Values corresponding to the fungal growth observed in plants treated with the indicated concentrations of fosetyl (F), $Cu(OH)_2$ (C), and 10 μM BTH (F+B Ob or C+B Ob, dose 0) or treated with combinations of 10 μM BTH with fosetyl or copper (F+B Ob or C+B Ob) are represented. The expected antifungal additive effect (Ad) resulting from the sum of the observed antifungal activity of each fungicide and BTH alone is indicated (F+B Ad or C+B Ad). Statistical analysis of variance (*F* tests) showed that the synergistic effects were significant ($P > 0.95$). This is one of two experiments with similar results and significant P values (0.95 to 0.99).

repeated three times at 4-day intervals (Figure 5). Repeated treatments with low concentrations of BTH (10 to 30 μM) were sufficient to control fungal growth in the wild type but had limited effect on NahG plants (data not shown). These results indicate that the response to BTH in NahG plants was transient; thus, the effectiveness of the SAR signaling pathway depends on its continuous reinforcement or potentiation. This potentiation seems to be altered or short circuited in NahG plants, which are unable to accumulate SA.

DISCUSSION

The role of plant defense mechanisms in the mode of action of fungicides has been the subject of some controversy (Király et al., 1972; Cartwright et al., 1977; Ward, 1984). It has been observed that the treatment of infections in plants with concentrations of fungicides or antibiotics that prevent the growth of compatible pathogens results in the expression of a set of phenotypic markers, such as the synthesis of antimicrobial phytoalexins and the formation of necrotic lesions, which resemble those observed in an incompatible interaction (Király et al., 1972; Ward et al., 1980). Although some studies have indicated that these changes are a consequence and not the cause of resistance (Király et al., 1972), others have suggested that these changes directly mediate the action of fungicides and that therefore the plant participates as a component of fungicide effectiveness (Cartwright et al., 1977; Ward, 1984). In support of the latter hypothesis, some fungicides (e.g., fosetyl and copper) that are inactive in vitro against certain pathogen isolates can control them in the plant (Langcake and Wickins, 1975; Guest, 1984; Adaskaveg and Hine, 1985).

Here, we demonstrate that the SAR signal transduction pathway, an SA-dependent plant defense mechanism, mediates fungicide action in the plant. Control of *P. parasitica* infection by three fungicides, metalaxyl, $Cu(OH)_2$, and fosetyl, is reduced in NahG and *nim1* Arabidopsis plants, which are blocked in the signal transduction cascade leading to both SAR and gene-for-gene resistance. Indeed, fungicide concentrations that are effective in wild-type plants do not control fungal infection in these SAR-compromised plants, and substantially higher concentrations (five- to 20-fold) are needed to inhibit fungal growth in these plants. Conversely, the effectiveness of these fungicides was not altered in mutants defective in the ethylene and jasmonic acid signal transduction pathways (Bleecker et al., 1988; Guzmán and Ecker, 1990; Staswick et al., 1992). Both pathways have also been implicated in plant defense (Ecker and Davis, 1987; Ryan, 1992). Because we have previously shown that neither ethylene nor jasmonic acid is required for SAR signal transduction and that SAR is intact in these mutants (Lawton et al., 1994, 1995), we conclude that SAR mediates fungicide effectiveness.

It has been shown previously that *nim1* mutants and plants that cannot accumulate SA do not activate SAR or mount a disease resistance response (Gaffney et al., 1993; Delaney et al., 1994; Maher et al., 1994; Mauch-Mani and Slusarenko, 1996; Pallas et al., 1996). The reduction of metalaxyl effectiveness against *Phytophthora* sp in glyphosate-treated soybean was thought to be due to a decrease in phytoalexin accumulation (Keen et al., 1982; Ward, 1984); however, it is also plausible that this could be due to a decrease in SA biosynthesis. Although a decrease in the synthesis of the phytoalexin glyceollin was observed in these treated plants, that was not sufficient to explain the reduction of metalaxyl activity (Ward, 1984). Glyphosate is an inhibitor of 5-enolpyruvylshikimate 3-phosphate synthase that catalyzes a key step in the biosynthesis of aromatic amino acids, which in turn are the substrates (phenylalanine) for the synthesis of not only phytoalexins and lignin but also for the synthesis of SA. Therefore, it is possible that the glyphosate-dependent reduction of metalaxyl activity could be due to a reduction of SA.

It has been postulated that modulation of the SAR signaling pathway can increase the ability of plants to respond to pathogen infection (Kauss et al., 1992; Weymann et al., 1995; Mur et al., 1996; Shirasu et al., 1997). This control process seems to be SA dependent and allows a faster and stronger defense response (Weymann et al., 1995; Shirasu et al., 1997). Indeed, it has been demonstrated that treatment of plants with SAR activators (e.g., SA, 2,6-dichloroisonicotinic acid, or BTH) before inoculation with fungal elicitors or fungal pathogens can result in an enhanced HR (Kogel et al., 1994; Görlach et al., 1996) and in a synergistic induction of some defense genes, such as those encoding PR proteins and enzymes of the phenylpropanoid pathway (Mur et al., 1996; Kästner et al., 1998; Thulke and Conrath, 1998). NahG plants, which cannot accumulate free SA, are defective in this potentiation process and therefore require repeated stimulation of the pathway by BTH to maintain resistance against pathogens. Our results also provide additional support for a growing body of evidence that the potentiation process is SA dependent (Weymann et al., 1995; Draper, 1997; Shirasu et al., 1997).

In wild-type plants, combinations of fungicides with BTH result in antifungal effects against *P. parasitica* that are synergistic. This combined effect was, by contrast, only additive in NahG and BTH-unresponsive *nim1* plants. Our results suggest that the lack of synergism of the combined treatment in NahG plants can be due to the existence of a defective potentiation mechanism in these plants, which do not respond to the combined treatment with a fast and strong defense response, as observed in wild-type plants. However, the lack of synergism in the NahG plants could be also a consequence of the reduced effectiveness of fungicides in these plants.

Interestingly, the effective concentrations of the fungicides analyzed in our experimental pathosystem are very similar to those used in agricultural practice. This supports

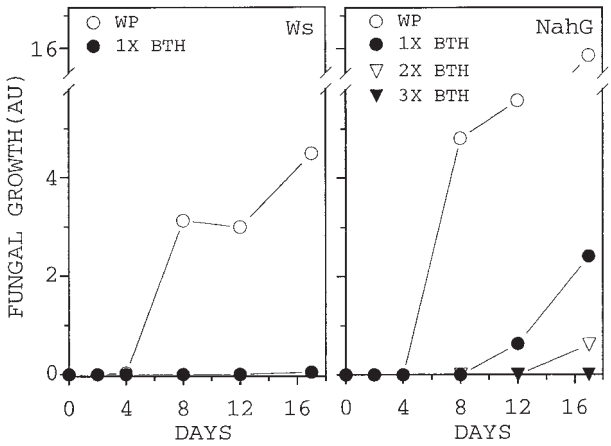

Figure 5. BTH Treatment Induces a Transient Protection in NahG Plants.

Relative fungal growth was measured in Ws and NahG plants inoculated with *P. parasitica* isolate Emwa. Plants were treated with wettable powder (WP) or with 0.1 mM BTH. Treatment was repeated once (1×; day 0), twice (2×; days 0 and 4), or three times (3×; days 0, 4, and 8). Plants were inoculated with *P. parasitica* 2 days after the first treatment. Quantification of the fungal growth (expressed in arbitrary units [AU]) was done as indicated in Figure 1. There were no significant differences ($P > 0.99$) between the fungal growth in plants treated once, twice, or three times with wettable powder. Values corresponding to Ws treated twice and three times with BTH are not represented (no fungal growth observed). At least five plants per genotype, treatment, and time were analyzed. This is one of two experiments with similar results.

the validity of our experimental system and further demonstrates the role of SAR in the mediation of fungicide action in crops. Furthermore, the observed synergistic effect between BTH and fungicides on wild-type plants may facilitate the reduction of the effective concentrations of chemicals and may expand the range of crops on which these chemicals can be used effectively.

Increasing evidence suggests that plant defense responses may be analogous to the "innate" immune responses of vertebrates and insects (Hammond-Kosack and Jones, 1996; Baker et al., 1997; Cao et al., 1997; Parker et al., 1997; Ryals et al., 1997). Indeed, structural similarities between the animal immune system and the plant SA-dependent system have been described (Baker et al., 1997; Wilson et al., 1997). Several plant disease resistance genes, including Arabidopsis *RPP5*, which confers resistance to *P. parasitica*, have significant similarity with both the interleukin-1 and Toll receptors (Baker et al., 1997; Parker et al., 1997). Furthermore, the product of the *nim1* gene has been shown to have structural homology with the transcription factor inhibitor IκBα (Ryals et al., 1997). The interleukin-1 and Toll receptors are involved in the NF-κB/IκB signal transduction pathway that regulates the activation of the innate immune system in animals and the defense system in Drosophila (Baeuerle and Baltimore, 1996; Baldwin, 1996).

In addition to structural similarities, emerging results show that plant and animal defense responses also may exhibit functional similarities. Alterations in the defense signal transduction pathway in mammals and flies result in a dramatic decrease of resistance to microbial infection and increased susceptibility to nonpathogenic microorganisms (Baeuerle and Baltimore, 1996; Baldwin, 1996; Deckert-Schlüter et al., 1996). These findings are reminiscent of those observed in SAR-compromised plants. The defense signal transduction pathway in animals can be induced by numerous stimuli, including pathogens (Baeuerle and Baltimore, 1996; Baldwin, 1996), leading to the synthesis of a number of factors involved in the inflammatory and immune responses, such as interleukins, the granulocyte/macrophage–colony stimulating factors (G-CSF and M-CGF), and interferon-γ (de Martin et al., 1993). Exogenous treatments of mammalian cells with these cytokines reduce the severity of the infection in wild-type animals (Roilides et al., 1995, 1996; Urban et al., 1996).

Interestingly, a reduction in antibiotic activity against infections has been also described in immunocompromised animals (Casadevall, 1996; Georgopapadakou and Walsh, 1996). It also has been observed that combinatorial therapy using antimicrobial compounds and cytokines results in greater controls of infections (Suzuki et al., 1988; Israeleski and Remington, 1990), and it can be particularly relevant in the treatment of infections in immunocompromised patients (Araujo et al., 1997). Strikingly, these observations are similar to those shown here for Arabidopsis and further suggest that SAR, an SA-dependent plant defense mechanism, and the animal immune response may have evolved from a common progenitor pathway.

METHODS

Plant Material

Plants (*Arabidopsis thaliana*) were grown under conditions previously described (Weymann et al., 1995). Plants used in this study were wild-type Wassilewskija (Ws); the *nim1-1* allele mutant (for noninducible immunity; Delaney et al., 1995; Ryals et al., 1997) and NahG transgenic plants (K. Lawton, U. Neuenschwander, and J. Rylas, unpublished data) in the Ws ecotype background; and wild-type Columbia (Col) and NahG transgenic plants (Delaney et al., 1994), ethylene insensitive mutants *etr1* (Bleecker et al., 1988) and *ein2* (Guzmán and Ecker, 1990), and *jar1*, a jasmonate responsiveness mutant (Staswick et al., 1992), in the Col ecotype background.

Plant Treatment and Pathogen Inoculation

The fungicides metalaxyl (*N*-[2,6-dimethylphenyl]-*N*-[methylacetyl]-alanine methyl ester; Ridomil; Novartis Crop Protection, Basel, Switzerland), fosetyl (Aliette; Rhone-Poulenc, Lyon, France), and $Cu(OH)_2$ (Kocide 101; Griffin, Vadosta, GA) and the systemic acquired resistance (SAR) activator BTH (benzo[1,2,3]-thiadiazole-7-carbothioic acid *S*-methyl ester; Novartis Crop Protection), formulated as 25, 80, 70, and 25% active ingredient, respectively, with a wettable powder carrier, were applied as a fine mist to leaves of 3-week-old plants. The wettable powder alone was applied as a control. Three days later, plants were inoculated with a *Peronospora parasitica* conidial suspension as previously described (Delaney et al., 1995). Ws wild-type, *nim1-1*, and Ws-NahG plants were inoculated with the compatible *P. parasitica* isolate Emwa (1 to 2×10^5 spores per mL); Col wild-type, Col-NahG, *etr1*, *ein2*, and *jar1* plants were inoculated with the compatible *P. parasitica* isolate Noco2 (0.5 to 1×10^5 spores per mL). After inoculation, plants were covered to maintain high humidity and placed in a Percival growth chamber at 17°C with a 14-hr-day and 10-hr-night cycle (Uknes et al., 1992). Fungal infection progression was followed for 12 days by viewing under a dissecting microscope to score development of conidiophores, and an infection rate was assigned (Delaney et al., 1994; Dietrich et al., 1994). Lactophenol–trypan blue staining of individual leaves was performed to observe fungal growth within leaf tissue and to confirm the infection rate assigned (Keogh et al., 1980). At least 15 plants per treatment and genotype were sprayed and inoculated. Eight to 10 plants per treatment and genotype were harvested 8 days after *P. parasitica* inoculation, and fungal growth was quantified using the fungal probe as described below.

Analysis of Gene Expression, Fungal Growth Quantification, and Statistical Analysis

Total RNA was purified from frozen tissue by phenol–chloroform extraction followed by lithium chloride precipitation (Lagrimini et al., 1987). In plant samples sprayed with $Cu(OH)_2$, 25 mM EDTA was included in the RNA extraction buffer to avoid RNA degradation. RNA samples were separated by electrophoresis through formaldehyde agarose gels and blotted onto nylon membranes (Hybond N+; Amersham), as described by Ausubel et al. (1994). Fungal growth was determined using a probe corresponding to the 5′ end of the 16S-like nuclear rRNA of *P. parasitica.* The fungal probe was obtained by polymerase chain reaction, according to White et al. (1990), using

primers NS1 and NS2, corresponding to the fungal 16S-like nuclear rRNA and as template DNA from spores of *P. parasitica* Emwa obtained as previously described (Lee and Taylor, 1990). The sequence of the amplified fragment (555 bp long) was almost identical to previously described 16S nuclear rRNA fungal sequences. The Arabidopsis *PR-1* cDNA probe has been previously described (Uknes et al., 1992).

^{32}P-labeled DNA probes were synthesized using a random primer DNA labeling system (Gibco BRL, Gaithersburg, MD). Hybridization and washing were performed as described previously (Church and Gilbert, 1984). Hybridization with the fungal probe was conducted with 100,000 cpm/mL hybridization solution containing 100 μg of herring sperm DNA/mL. Sample loading was normalized for plant RNA content by probing the RNA gel blots with the constitutively expressed β-tubulin cDNA from Arabidopsis. Relative amounts of the transcript were determined by quantification of the signals of the blots by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) following the manufacturer's instructions. Two different RNA gel blots per experiment were used for quantification, and two or three measurements per blot were done (error was $<2\%$).

Data were first subjected to a double logarithmic transformation and subsequently to a model I regression analysis that gave regression lines with significant linear regression values ($P > 0.95$) and y-intercept values that did not differ significantly (Sokal and Rohlf, 1980). The significant linear regression obtained after transformation allowed us to know whether the functional relationships described by the regression equations were the same or different by performing a statistical analysis of variance (*F* test) that tested the significance of the difference between the regression slopes of the different linear regression lines corresponding to the wild type and mutants (Sokal and Rohlf, 1980).

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