The Plant Cell, Vol. 10, 2103–2113, December 1998, www.plantcell.org © 1998 American Society of Plant Physiologists

Concomitant Activation of Jasmonate and Ethylene Response Pathways Is Required for Induction of a Plant Defensin Gene in Arabidopsis

Iris A. M. A. Penninckx,[a] Bart P. H. J. Thomma,[a] Antony Buchala,[b] Jean-Pierre Métraux,[b] and Willem F. Broekaert[a,1]

[a]F.A. Janssens Laboratory of Genetics, Katholieke Universiteit Leuven, Kardinaal Mercierlaan 92, B-3001 Heverlee-Leuven, Belgium
[b]Institut de Biologie Végétale, Université de Fribourg, Rue Albert-Gockel 3, CH-1700 Fribourg, Switzerland

Activation of the plant defensin gene *PDF1.2* in Arabidopsis by pathogens has been shown previously to be blocked in the ethylene response mutant *ein2-1* and the jasmonate response mutant *coi1-1.* In this work, we have further investigated the interactions between the ethylene and jasmonate signal pathways for the induction of this defense response. Inoculation of wild-type Arabidopsis plants with the fungus *Alternaria brassicicola* led to a marked increase in production of jasmonic acid, and this response was not blocked in the *ein2-1* mutant. Likewise, *A. brassicicola* infection caused stimulated emission of ethylene both in wild-type plants and in *coi1-1* mutants. However, treatment of either *ein2-1* or *coi1-1* mutants with methyl jasmonate or ethylene did not induce *PDF1.2*, as it did in wild-type plants. We conclude from these experiments that both the ethylene and jasmonate signaling pathways need to be triggered concomitantly, and not sequentially, to activate *PDF1.2* upon pathogen infection. In support of this idea, we observed a marked synergy between ethylene and methyl jasmonate for the induction of *PDF1.2* in plants grown under sterile conditions. In contrast to the clear interdependence of the ethylene and jasmonate pathways for pathogen-induced activation of *PDF1.2*, functional ethylene and jasmonate signaling pathways are not required for growth responses induced by jasmonate and ethylene, respectively.

INTRODUCTION

Higher plants induce various defense responses when they are attacked by microbial pathogens, such as fungi, bacteria, or viruses. These defense responses include suicide of the attacked host cell (the so-called hypersensitive response); the production of antimicrobial secondary metabolites (called phytoalexins); the production of pathogenesis-related (PR) proteins, of which many exert antimicrobial properties; and the production and oxidative cross-linking of cell wall polymers. The efficacy of these defense responses often determines whether plants are susceptible to infection by a pathogen.

Elicitors secreted by or released from microbial invaders are the primary signal for induction of plant defense responses (Ebel and Cosio, 1994). Each pathogen produces a particular mixture of elicitors, which are sometimes accompanied by suppressors, and these molecules interact with receptors on the host cells that further translate the primary signal into particular events in the plasma membrane, the cytosol, and/or the nucleus (Shirasu et al., 1996). Induction of some defense genes requires the generation of secondary endogenous signal molecules (stress hormones) by the challenged cells in the infection site. The secondary signal molecules in turn set in motion signal transduction cascades in receiving cells, eventually leading to activation of pathogen-responsive genes. Secondary signal molecules thus serve to amplify and spread the response of the host after initial recognition of the pathogen. Several secondary signal molecules whose synthesis is increased in response to elicitor recognition and that are involved in the activation of defense genes have been identified. These include H_2O_2 or other active oxygen species (reviewed in Lamb and Dixon, 1997), salicylic acid (SA; reviewed in Dürner et al., 1997), ethylene (reviewed in Boller, 1991), jasmonic acid (JA; reviewed in Creelman and Mullet, 1997), and abscisic acid (reviewed in Zeevaart, 1988), but it is likely that still others remain to be discovered.

Some of these molecules, such as H_2O_2, have a high turnover rate and can only activate genes in a limited area immediately surrounding the infection site (Levine et al., 1994). Others, such as SA, are transported over a longer distance and can be spread via the vascular system to organs that are distant from the infection site (Shulaev et al., 1995). Defense genes responding to signal molecules with limited mobility are said to be locally induced, whereas those responding to mobile signals are said to be systemically induced. The

[1]To whom correspondence should be addressed. E-mail willem.broekaert@agr.kuleuven.ac.be; fax 32 16 32 19 66.

response of a pathogen-responsive gene to a particular signal molecule(s) depends primarily on the presence and relative position of binding sites for transcription factors in its promoter (Somssich, 1994).

It is well documented that particular sets of pathogen-responsive antimicrobial protein genes require SA accumulation for their induction by pathogens. Transgenic tobacco plants expressing a bacterial gene (*nahG* from *Pseudomonas putida*) for an SA-converting enzyme were unable to accumulate SA when infected, and they failed to induce genes for extracellular PR-1, PR-2, and PR-3 proteins (Friedrich et al., 1995). These plants also had reduced susceptibility to tobacco mosaic virus, *Pseudomonas syringae* pv *tabaci*, *Phytophthora parasitica*, and *Cercospora nicotianae* (Gaffney et al., 1993; Delaney et al., 1994). Similarly, *nahG*-expressing Arabidopsis plants infected with pathogens had drastically reduced levels of SA and transcripts for extracellular PR-1 proteins compared with infected control plants (Lawton et al., 1995). These plants were more susceptible to infection by *P. syringae* pv *tomato* and *Peronospora parasitica* (Delaney et al., 1994). The genes for extracellular PR proteins in tobacco and Arabidopsis are strongly induced when SA is applied externally to leaves (Brederode et al., 1991; Ward et al., 1991; Uknes et al., 1992).

Evidence is now accumulating that not all pathogen-responsive antimicrobial protein genes are dependent on SA for their induction. For instance, the induction of tobacco genes for vacuolar PR-2 and PR-3 proteins after infection by *Erwinia carotovora* was similar in *nahG*-expressing plants and wild-type plants (Vidal et al., 1997). Pathogen-responsive tobacco genes for vacuolar PR-2, PR-3, and PR-5 proteins are known to be induced much more efficiently by the external application of ethylene than SA (Brederode et al., 1991; Xu et al., 1994; Beffa et al., 1995), and elicitor-induced induction of a vacuolar *PR-5* gene in tobacco could be blocked by norbornadiene, which is a blocker of ethylene action (Chang et al., 1995). In Arabidopsis, we have recently identified a gene (*PDF1.2*) encoding an antifungal peptide belonging to the family of plant defensins (Penninckx et al., 1996; see Broekaert et al., 1995, for a review on plant defensins). This gene is induced by exogenous treatment with either ethylene or methyl jasmonate (MeJA) but not by SA, whereas the opposite was observed for induction of the Arabidopsis *PR-1* gene. Inoculation of either wild-type or *nahG*-expressing Arabidopsis plants with the fungus *Alternaria brassicicola* caused systemic accumulation of PDF1.2, indicating that pathogen-induced accumulation of this protein is SA independent. On the other hand, both an ethylene-insensitive mutant, called *ein2-1*, and a jasmonate-insensitive mutant, *coi1-1*, were unable to accumulate substantial amounts of PDF1.2 in inoculated leaves as well as in noninoculated leaves of inoculated plants (Penninckx et al., 1996).

Our finding that both a component of the ethylene signal transduction pathway (EIN2) and one of the jasmonate signal transduction pathway (COI1) are involved in pathogen-induced activation of the Arabidopsis *PDF1.2* gene raises questions about the possible interaction between the ethylene and jasmonate response pathways in this defense response. In this study, we present evidence that concomitant triggering of the ethylene and jasmonate pathways is required for *PDF1.2* induction to occur.

RESULTS

ETR1 Is Required for Pathogen-Induced Activation of *PDF1.2*

It was previously observed that the activation of *PDF1.2* in response to challenge with *A. brassicicola* is abolished in the ethylene-insensitive mutant *ein2-1* but only partially in another ethylene-insensitive mutant, *etr1-3* (Penninckx et al., 1996). *ETR1* encodes an ethylene receptor, whereas *EIN2* codes for a downstream ethylene signal transduction component (Kieber, 1997). The weak influence of the *etr1-3* mutation on *PDF1.2* expression casts some doubts on the involvement of the entire ethylene signal transduction chain and thus on that of ethylene itself.

To clarify this point, Arabidopsis plants carrying the *etr1-1* mutation, conferring stronger ethylene insensitivity than the allelic *etr1-3* mutation, were tested for their ability to induce *PDF1.2* when infected with *A. brassicicola.* Pathogen-induced expression of *PDF1.2* in *etr1-1* was apparently blocked at the transcriptional level, because no plant defensin mRNA could be detected in leaves of pathogen-treated plants (Figure 1A). Consistent with this observation, the levels of plant defensins in pathogen-treated and nontreated systemic leaves of *etr1-1* mutants after inoculation with *A. brassicicola* were found to be at least 12-fold lower than the levels in corresponding leaf samples of pathogen-treated wild-type plants (Figure 1B). In a similar test, it was found that pathogen-induced accumulation of plant defensins in *etr1-3* mutants was only weakly affected compared with that of wild-type plants (results not shown). This result is consistent with our previous findings (Penninckx et al., 1996). These results point to the requirement of a functional ethylene receptor and thus most likely of ethylene production itself in pathogen-induced activation of *PDF1.2.*

Ethylene and JA Activate the *PDF1.2* Gene via Parallel Signaling Pathways

The observation that pathogen-induced expression of *PDF1.2* is blocked both by mutants affected in the ethylene response (*ein2-1* and *etr1-1*) as well as by a mutant affected in its response to jasmonate (*coi1-1*) implies that ethylene and JA somehow interact with each other to affect expression of this gene (Penninckx et al., 1996). Conceptually, three different models for the interaction between ethylene and JA can be conceived (Figure 2). The first model implies that patho-

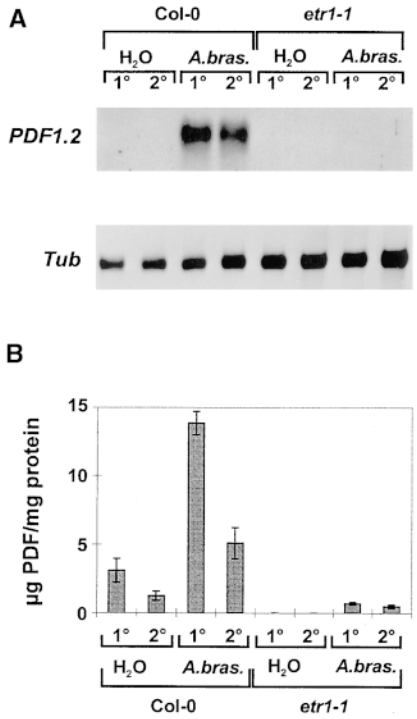

Figure 1. Induction of Plant Defensins in Arabidopsis Wild-Type Plants (Col-0) and in the Ethylene Receptor Mutant *etr1-1.*

(A) RNA gel blots probed with either *PDF1.2* or Tubulin β-1 (*Tub*) riboprobes. The samples represent 4 μg of total RNA.

(B) Plant defensin (PDF) contents as determined by ELISA, using an antigen affinity-purified anti–Rs-AFP1 (*Raphanus sativus*–antifungal protein 1) antiserum. Values are means (±SE) of three independent determinations.

Arabidopsis plants were inoculated with *A. brassicicola* (*A.bras.*) by applying 5-μL drops of a spore suspension (5×10^5 spores per mL) on four lower rosette leaves (five drops per leaf). Control plants were treated identically with 5-μL drops of water (H_2O). Pathogen-treated leaves (1°) and nontreated leaves of the same plants (2°) were collected 2 days after inoculation for RNA extractions and 3 days after inoculation for protein extractions. Total RNA and proteins were extracted as described in Methods. The experiment was repeated twice with similar results.

gen recognition leads to increased ethylene production, which in turn results in stimulated JA production and subsequent *PDF1.2* activation. The second model is identical to the first, except that the hierarchy between the ethylene and JA signals is switched. In the third model, we propose that ethylene and JA do not act in a sequential manner but rather via parallel pathways, both of which need to be activated for induction of the *PDF1.2* gene upon pathogen recognition.

It was previously shown that inoculation of Arabidopsis wild-type plants (ecotype Columbia [Col-0]) with *A. brassicicola* results in a remarkable increase in the levels of JA in the leaves (Penninckx et al., 1996). The first model predicts that such a pathogen-triggered rise in JA level would not occur in the ethylene-insensitive mutant *ein2-1*, whereas according to models 2 and 3, the *ein2-1* mutation would not affect pathogen-stimulated JA production. To test these divergent predictions, *ein2-1* and wild-type plants (Col-0) were inoculated with the pathogen *A. brassicicola.* After inoculation, JA levels were monitored at different time points. As shown in Figure 3, JA levels in fungus-inoculated wild-type plants started to increase 24 hr after inoculation. This increase was more dramatic 48 hr after inoculation, and JA levels peaked ~72 hr after inoculation. In the *ein2-1* mutant, pathogen-stimulated production was clearly not abolished but was even more pronounced relative to that in wild-type plants. When wild-type plants were exposed to 50 ppm ethylene for different periods (0.5, 2, 4, 8, 12, 24, or 48 hr), *PDF1.2* transcript levels increased from 8 hr after treatment, whereas no increase in endogenous JA above background levels in air-treated plants occurred until 24 hr after treatment, reaching 10-fold higher levels at 48 hr (results not shown). Hence, all these results are in conflict with the predictions made based on model 1.

To further discriminate between models 2 and 3, ethylene production by wild-type plants and *coi1-1* mutants was measured in response to inoculation with *A. brassicicola.* Model 2 predicts that the *coi1-1* mutant would be blocked in its ability to stimulate ethylene production when attacked by a pathogen, whereas model 3 implies that the ethylene response in the *coi1-1* mutants would not be reduced versus that of wild-type plants. Inoculation of wild-type plants with *A. brassicicola* resulted in ethylene production levels that were approximately three times higher than those in mock-inoculated plants 36 hr after inoculation (Figure 4). An increase in ethylene production levels was also observed in the *coi1-1* mutant plants 36 hr after inoculation with *A. brassicicola*, reaching levels that were approximately fivefold higher compared with levels in mock-inoculated *coi1-1* mutants. Because pathogen-stimulated ethylene production clearly was not abolished in the *coi1-1* mutants, we conclude that model 2 is not valid. Also in conflict with model 2 is our observation that application of drops containing 50 μM MeJA on leaves of Arabidopsis plants does not result in increased ethylene emission relative to control plants whether measured 0.5, 2, 4, 8, 12, 24, or 48 hr after treatment, whereas *PDF1.2* transcript levels were already increased 4 hr after treatment (results not shown).

An alternative way to verify the validity of the three different models is to check whether the *PDF1.2* gene can be induced in MeJA-treated *ein2-1* mutants or in ethylene-treated *coi1-1* mutants. Treatment of wild-type plants with 50 μM MeJA resulted in a marked elevation of plant defensins and *PDF1.2* transcripts relative to those in untreated wild-type plants. In contrast, the content of plant defensins and their transcripts in *ein2-1* mutants was not elevated relative to those in untreated plants (Figures 5A and 5B). This observation is in conflict with model 1, which predicts that jasmonate-induced PDF1.2 accumulation would not be affected by the *ein2-1* mutation. Exposure of wild-type plants

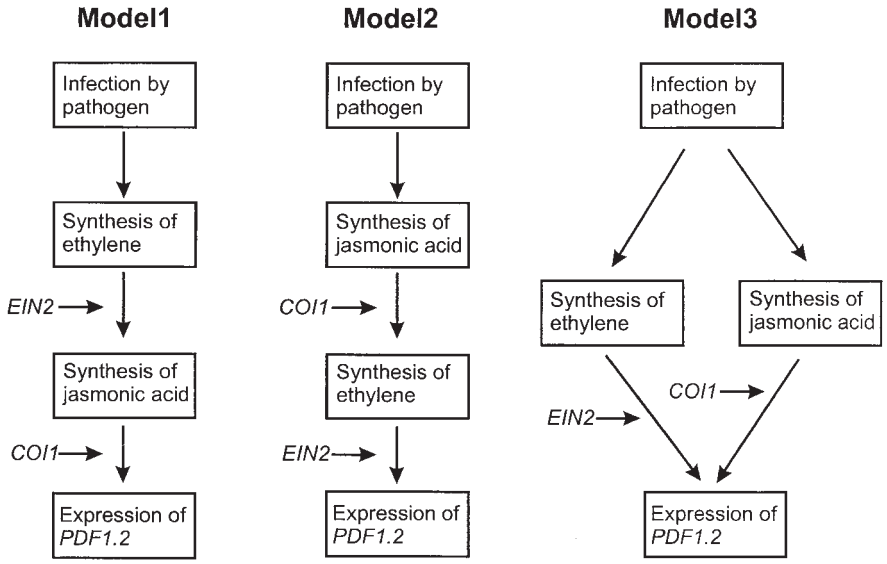

Figure 2. Three Alternative Models for the Interaction between Ethylene and Jasmonate Signals during Activation of the *PDF1.2* Gene in Pathogen-Challenged Arabidopsis Plants.

Model 1 implies that pathogen infection initially stimulates production of ethylene, which subsequently stimulates production of jasmonates, which in turn activates *PDF1.2.* Model 2 implies that pathogen infection initially leads to enhanced production of jasmonates, which subsequently triggers elevated production of ethylene, which in turn controls *PDF1.2* expression. Model 3 predicts that pathogen infection results in simultaneous production of ethylene and jasmonates, which are both required for induction of *PDF1.2.* The positions of the gene products EIN2 and COI in the different pathways are indicated by short arrows.

to ethylene boosted protein and mRNA levels of plant defensins compared with air-exposed plants. However, no accumulation of plant defensins could be observed in ethylene-treated *coi1-1* mutants (Figures 5A and 5B), which contradicts the predictions of model 2. Hence, model 3, proposing parallel ethylene and jasmonate signaling pathways, is the only model that is in agreement with all observations.

Synergistic Induction of *PDF1.2* by Ethylene and Jasmonate

Our finding that JA and ethylene act via parallel pathways to activate the *PDF1.2* gene suggests that combinations of JA and ethylene may have a synergistic effect on the activation of this gene. It was observed that treatment of Arabidopsis plants, grown under sterile conditions in agar, with either ethylene or MeJA resulted in a much weaker induction of *PDF1.2* compared with the same treatments applied to plants grown on nonsterile potting soil (Figures 6A and 6B). For instance, treatment of sterile plants with 5 ppm of ethylene did not cause a detectable induction of *PDF1.2*, whereas induction was clearly observed in nonsterile plants. However, combined application of 5 ppm of ethylene and 0.05 μM MeJA boosted the level of *PDF1.2* transcripts in sterile plants to approximately the same level as that in sterile plants treated with 50 μM MeJA alone. When applied in combination to sterile plants, 5 ppm of ethylene and 5 μM MeJA caused a rise in *PDF1.2* transcripts that was significantly higher relative to separate treatments with 50 ppm of ethylene or 50 μM MeJA and that was of the same order of magnitude as that observed for nonsterile plants treated with either 50 ppm of ethylene or 50 μM MeJA alone (Figure 6A). Similar observations were made using ELISA assays to detect PDF1.2 (Figure 6B). Treatment of nonsterile plants with combinations of ethylene and MeJA only marginally increased PDF1.2 levels compared with treatments with either hormone applied separately (results not shown).

Activation of *PDF1.2* by Superoxide Anion–Generating Paraquat Requires Functional Ethylene and Jasmonate Response Pathways

One of the most rapid events observed during plant–pathogen interactions is the production of reactive oxygen species, which are initially in the form of superoxide anion, by an NADPH oxidase complex (Lamb and Dixon, 1997). We have previously shown that treatment of Arabidopsis leaves with subherbicidal levels of the superoxide-generating compound paraquat can induce expression of *PDF1.2*, whereas no activation of the SA-dependent *PR-1* gene was observed at the doses used (Penninckx et al., 1996). Therefore, we investigated whether signal components of the ethylene and JA

response pathways are required for paraquat-induced activation of *PDF1.2.* As shown in Figures 5A and 5B, accumulation of plant defensins and their transcripts was strongly reduced in paraquat-treated *ein2-1* mutants compared with similarly treated wild-type plants, whereas this accumulation was below the level of detection in paraquat-treated *coi1-1* mutants. Hence, these results strongly suggest that reactive oxygen species act upstream of ethylene and JA.

EIN2 and COI1 Are Not Involved in Developmental Cues Influenced by Jasmonate and Ethylene, Respectively

In addition to being involved in defense responses, ethylene and JA are also implicated in developmental and growth processes of plants (Creelman and Mullet, 1997; Kende and Zeevaart, 1997). Growth of Arabidopsis seedlings in the presence of ethylene causes drastic inhibition of hypocotyl elongation, radial swelling of hypocotyls, and exaggeration of the curvature of the apical hook (Kieber, 1997), whereas MeJA causes drastic reduction of root elongation in Arabidopsis seedlings (Staswick et al., 1992). We investigated whether the ethylene-insensitive *ein2-1* mutant was insensitive to MeJA-induced inhibition of root elongation, as is *coi1-1.* We also investigated whether the JA-insensitive *coi1-1* mutant was insensitive to inhibition of hypocotyl elongation caused by ethylene, as is *ein2-1.* Root elongation of MeJA-treated *ein2-1* seedlings and hypocotyl elongation of ethylene-treated *coi1-1* seedlings were inhibited to the same extent as they were for similarly treated wild-type seedlings (Figure 7). Hence, EIN2 is implicated in growth processes influenced by ethylene but not in those influenced by JA, with the reverse being true for COI1.

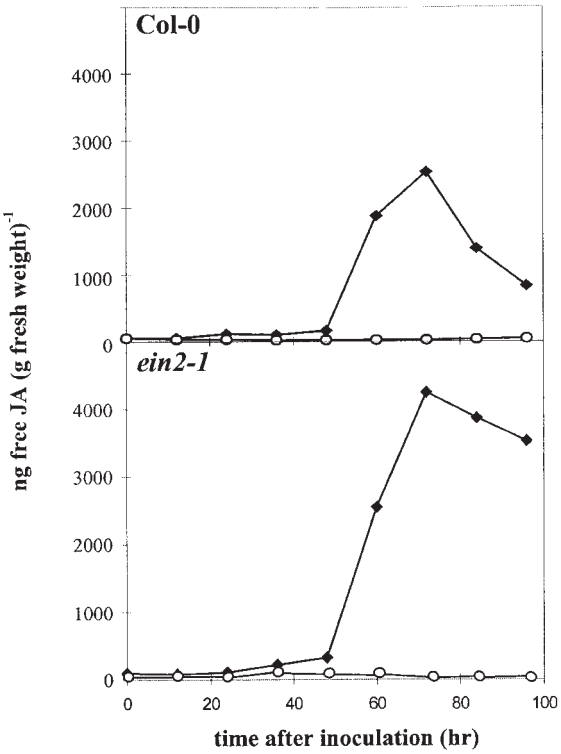

Figure 3. Time Course of Endogenous JA Content in Arabidopsis Wild-Type Plants (Col-0) and Ethylene-Insensitive Mutants (*ein2-1*) When Inoculated with *A. brassicicola* (Closed Symbols) or Mock Inoculated with Water (Open Symbols).

JA levels, expressed as nanograms of free JA per gram fresh weight of tissue, were determined by HPLC with extracts from Arabidopsis leaves collected 0, 12, 24, 36, 48, 60, 72, 84, and 96 hr after inoculation with 5-μL drops (five drops per leaf) of either an *A. brassicicola* spore suspension at 5×10^5 spores per milliliter or distilled water. Each data point is the average of measurements on two separate sets of three plants each. The experiment was repeated once with similar results.

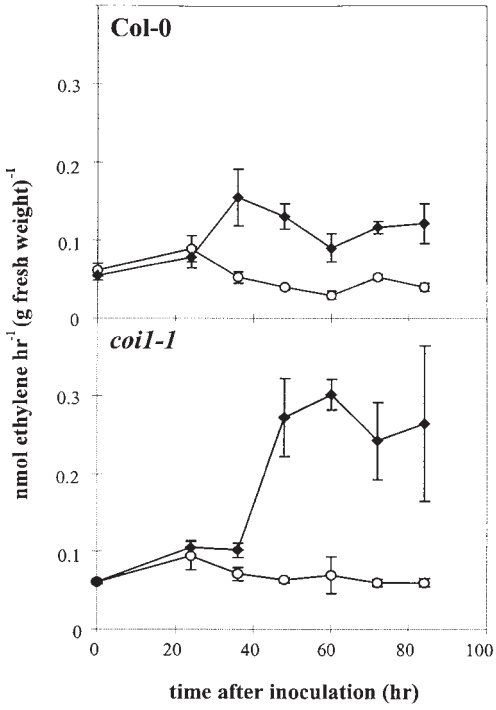

Figure 4. Time Course of Ethylene Production Levels in Arabidopsis Wild-Type Plants (Col-0) and Jasmonate-Insensitive Mutants (*coi1-1*) When Inoculated with *A. brassicicola* (Closed Symbols) or Mock Inoculated with Water (Open Symbols).

Ethylene production, expressed as nanomoles of ethylene per hour per gram fresh weight of tissue, was determined 0, 12, 24, 36, 48, 60, 72, 84, and 96 hr after inoculation with 5-μL drops (five drops per leaf) of either an *A. brassicicola* spore suspension at 5×10^5 spores per milliliter or distilled water. Each data point is the average (±SD) of measurements on two separate sets of two plants each. The experiment was repeated twice with similar results.

DISCUSSION

We demonstrated previously that pathogen-induced activation of the Arabidopsis *PDF1.2* gene, which encodes an antifungal plant defensin, is abolished in a mutant carrying the *ein2-1* mutation. EIN2 is a signal transduction component involved in the response to ethylene and acts downstream of the ethylene receptor, of which ETR1 forms a part (Kieber, 1997). Our finding that *PDF1.2* induction is also abolished in the disfunctional *etr1-1* allele implies that not only downstream response elements, such as EIN2, but also the ethylene receptor and hence most probably the entire signal transduction chain are involved in *PDF1.2* activation. It should be noted that the impact of the *etr1-3* mutation on pathogen-induced *PDF1.2* activation is much weaker compared with that of the *etr1-1* or *ein2-1* mutations. This observation has some implications for the interpretation of the results of Bent et al. (1992), who showed that the development of chlorotic symptoms in Arabidopsis plants inoculated with either *Pseudomonas* or *Xanthomonas* pathogens was severely reduced in *ein2-1* mutants compared with wild-type plants but not in *etr1-3* (previously called *ein1-1*) mutants. Because the *etr1-3* mutation has only a weak impact on the induction of pathogen-responsive genes, these experiments might be inconclusive regarding the involvement of ETR1 in the induced response to pathogens. Nevertheless, the experiments of Bent et al. (1992) have been translated by several authors into a model in which ETR1 is involved in the ethylene response but not in the pathogen response, whereas EIN2 is involved in both responses (Ecker, 1995; Kieber, 1997). Obviously, our data call this model into question.

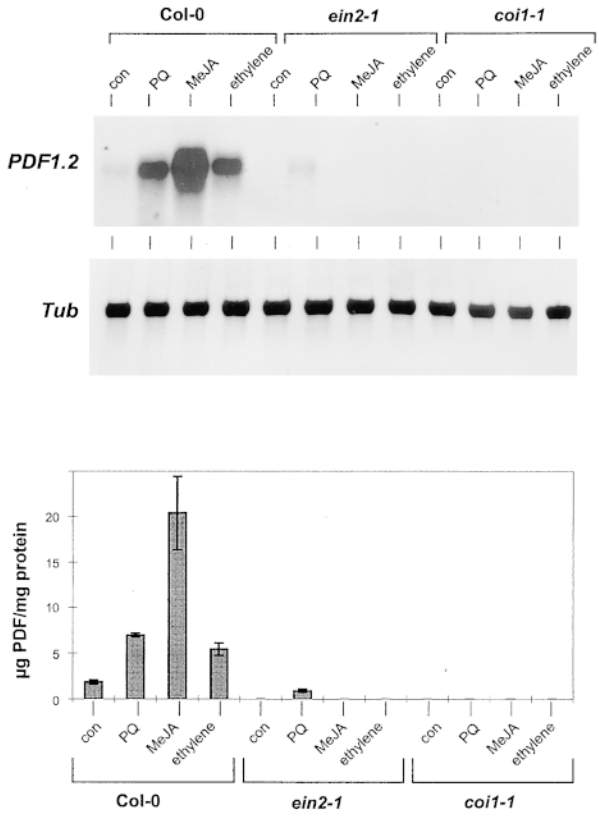

Figure 5. Induction of Plant Defensins in Arabidopsis Wild-Type Plants (Col-0), Ethylene-Insensitive Mutants (*ein2-1*), and Jasmonate-Insensitive Mutants (*coi1-1*).

(A) RNA gel blots probed with either *PDF1.2* or Tubulin β-1 (*Tub*) riboprobes. The samples represent 4 μg of total RNA.

(B) Plant defensin (PDF) contents as determined by ELISA, using an antigen affinity-purified anti–Rs-AFP1 antiserum. Values are means (±SE) of three independent determinations.

Arabidopsis leaves were inoculated with 5-μL drops (five drops per leaf) of water (con), paraquat (PQ; 25 μM), and MeJA (45 μM in 0.1% [v/v] ethanol). Ethylene treatment was performed by placing plants in an airtight chamber with an ethylene concentration of 50 ppm. All leaf samples were collected 48 hr after the initiation of treatment. The experiment was repeated two times with similar results.

The lack of pathogen-induced *PDF1.2* activation in the jasmonate perception mutant *coi1-1* implies that not only the ethylene response pathway but also a jasmonate-based signaling pathway is required for the induction of this gene. The fact that *PDF1.2* induction could not be triggered by ethylene treatment of the *coi1-1* mutant or by jasmonate treatment of the *ein2-1* mutant clearly points to the conclusion that the ethylene and jasmonate signaling pathways are triggered independently and concomitantly and rules out the possibility of sequential activation of the jasmonate pathway by ethylene, or vice versa. We did not observe any elevated ethylene production after treatment of Arabidopsis plants with exogenous MeJA. This is in clear contrast with the situation in tomato leaves, where application of JA results in a rapid but transient increase in ethylene emission (O'Donnell et al., 1996). On the other hand, we observed that treatment of Arabidopsis plants with exogenous ethylene resulted in a rise in JA levels, as previously reported by Laudert and Weiler (1998) for treatment of Arabidopsis with the ethylene-releasing compound ethephon. Increased JA production triggered by ethylene treatment, however, was a rather late response and clearly occurred after *PDF1.2* induction upon ethylene exposure, thus arguing against ethylene-stimulated JA production being the primary stimulus for *PDF1.2* induction. Ethylene-stimulated JA production may nevertheless contribute to and enhance *PDF1.2* activation in the late stages of infection by a pathogen. Although triggering of both the ethylene and jasmonate signaling pathways appears to be the main cause of pathogen-induced expression of *PDF1.2*, our experiments do not show at which point both pathways converge to affect expression of this gene. It is possible that transcription factors activated by the ethylene response pathway cooperate with transcription factors activated independently by the jasmonate response pathway to

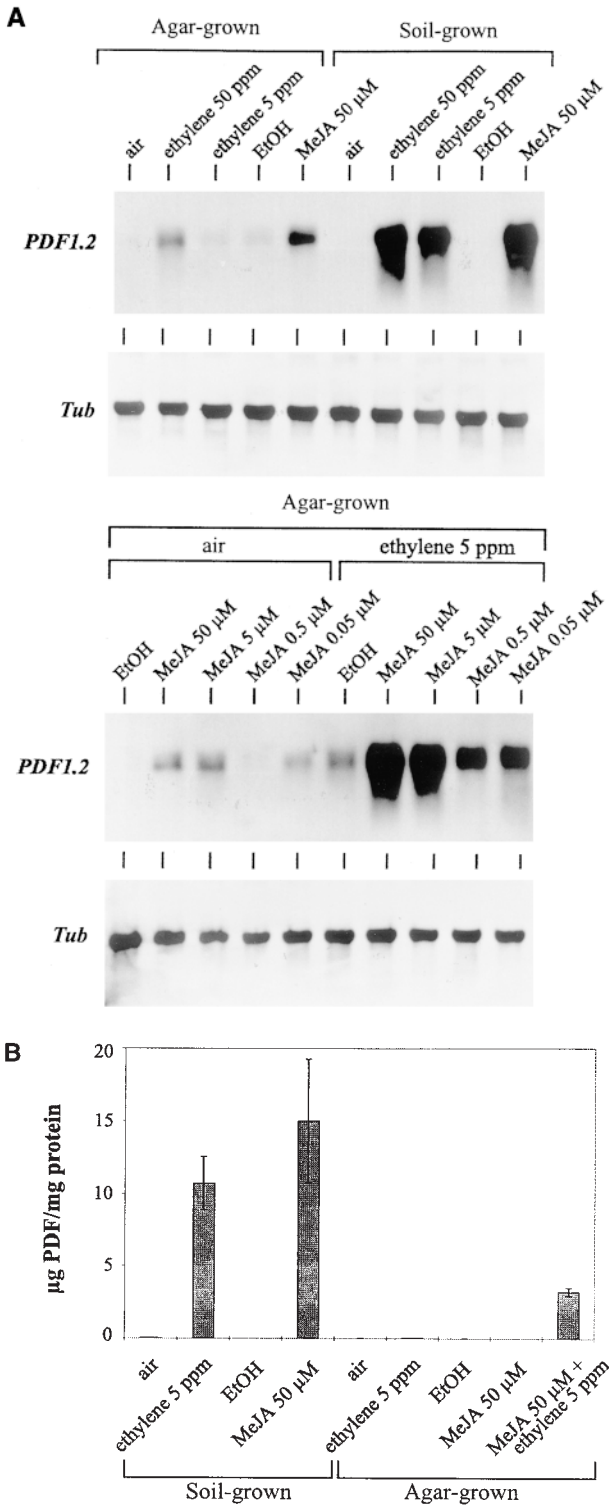

Figure 6. Synergistic Induction of Plant Defensins by Ethylene and Jasmonate.

(A) RNA gel blots probed with either *PDF1.2* or Tubulin β-1 (*Tub*) riboprobes. The samples represent 4 μg of total RNA.

(B) Plant defensin (PDF) contents as determined by ELISA, using an antigen affinity-purified anti–Rs-AFP1 antiserum. Values are means (±SE) of three independent determinations.

Arabidopsis leaves from either agar-grown sterile plants or soil-grown nonsterile plants were inoculated with 5-μL drops (five drops per leaf) of MeJA (at the indicated concentration in 0.1% [v/v] ethanol) or 0.1% (v/v) ethanol (EtOH) either in the presence or the absence of ethylene (at the concentration indicated) in the atmosphere of an airtight chamber. All leaf samples were collected 48 hr after the initiation of treatment. The experiment was repeated three times with similar results.

form a functional transcription initiation complex. Alternatively, signal transduction components activated upon jasmonate perception may alter the sensitivity of cells to the ethylene signal input or vice versa. It is known, for instance, that *EREBP1*, a gene encoding a transcription factor that is involved in the transcription of ethylene-activated genes, is induced upon treatment of tobacco plants with jasmonate (Horvath et al., 1998). A jasmonate-stimulated increase in EREBP1 levels might increase the responsiveness of the cell to the ethylene signal input.

The fact that treatment of plants with either ethylene or MeJA results in *PDF1.2* induction (Penninckx et al., 1996) is in apparent conflict with our model proposing concomitant and cooperative ethylene and jasmonate signaling events. Indeed, this model (Figure 2) implies that only treatment with a combination of ethylene and jasmonate can effectively trigger *PDF1.2* expression. Consistent with the model, however, we found that plants grown under sterile conditions and treated with either jasmonate or ethylene alone show very weak *PDF1.2* induction, whereas application of both hormones simultaneously leads to strong synergistic *PDF1.2* induction. We do not know why plants grown under nonsterile conditions behave differently in this respect. Obviously, the environmental conditions of plants grown on agar in a closed container and plants grown in potting soil differ in many parameters, including mineral nutrition, water availability, and gas exchange. Any of these parameters might influence the physiological response of plants to applied hormones. One other difference to be considered is the lack of microorganisms in the rizosphere of plants grown on agar under sterile conditions. It has previously been observed that the interaction between Arabidopsis roots and particular soil-borne *Pseudomonas* species leads to a systemic change in the resistance of the plants to pathogens such as *Fusarium oxysporum* f sp *raphani* (Pieterse et al., 1996). Such an interaction might alter the sensitivity of the plants to both jasmonate and ethylene, or alternatively, it might alter the endogenous levels of these hormones. In support of the latter

idea, we found that plants grown in potting soil have basal ethylene emission levels that are at least fivefold higher relative to those of plants of the same age grown axenically (I.A.M.A. Penninckx, unpublished results). Higher basal ethylene production levels in soil-grown plants may act synergistically with externally applied MeJA to boost PDF1.2 levels.

Synergistic interactions between ethylene and MeJA have previously been observed for the induction of the gene encoding pathogen- and wound-responsive osmotin (vacuolar PR-5) in tobacco (Xu et al., 1994). The regulation of this gene may turn out to be very similar to that of *PDF1.2* in Arabidopsis, although the latter is not induced by wounding (Penninckx et al., 1996). In tomato, induction of the wound-responsive proteinase inhibitor *Pin2* gene occurs when plants are treated with JA but not with ethylene. However, treatment of plants with inhibitors of ethylene biosynthesis or ethylene perception abolished the responsiveness of this gene to both wounding and JA treatment (O'Donnell et al., 1996). Hence, the ethylene signal pathway appears to cooperate somehow with the JA response pathway to effect expression of a stress-related gene in tomato.

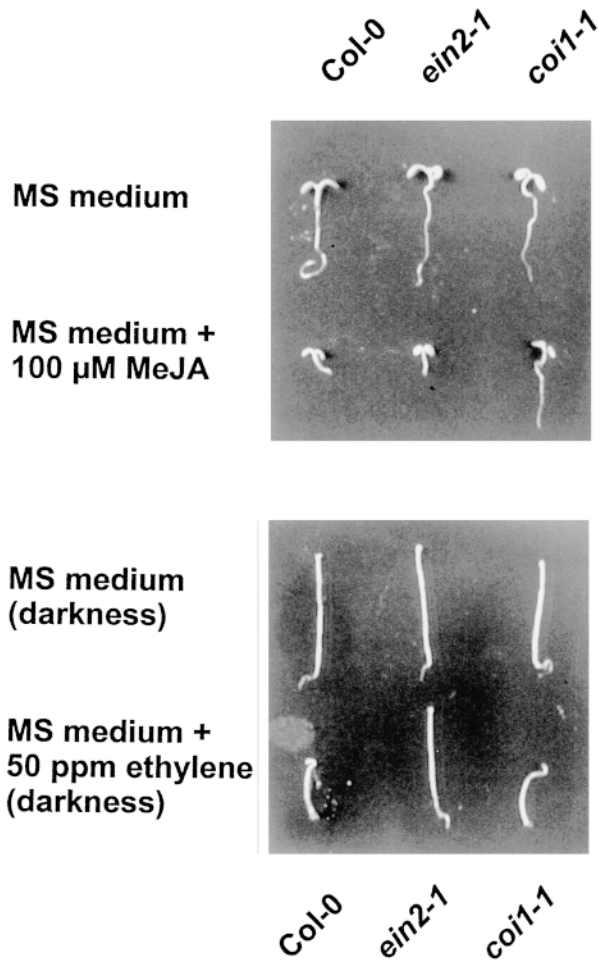

Figure 7. *ein2-1* and *coi1-1* Plants Are Not Affected in Developmental Cues Influenced by Jasmonate and Ethylene, Respectively.

Wild-type (Col-0), *ein2-1*, and *coi1-1* plants were grown under sterile conditions with a 12-hr photoperiod on Murashige and Skoog (MS) medium in the absence or presence of 100 µM MeJA (top). The same ecotypes were grown under sterile conditions in darkness in airtight containers in the absence or presence of 50 ppm ethylene in the atmosphere (bottom).

Ethylene has also been shown to affect the sensitivity of Arabidopsis plants to SA. When Arabidopsis plants were treated with ethylene, lower concentrations of SA were required to induce the *PR-1* gene (Lawton et al., 1994). However, in this case, the requirement for ethylene appears to be less strict because *etr1-1* and *ein2-1* mutants still induce the *PR-1* gene when challenged by a pathogen or treated with SA (Lawton et al., 1994, 1995; Penninckx et al., 1996).

In this study, we present genetic evidence for the strict requirement of both the jasmonate and the ethylene signaling pathways for the induction of a pathogen-responsive gene. Interestingly, we found that the JA and ethylene response pathways do not affect ethylene- and JA-mediated developmental processes, respectively, such as hypocotyl and root elongation. Our observations in this respect are in line with those of Berger et al. (1996), who reported that Arabidopsis JA response mutants other than *coi1-1* did not show an altered sensitivity to ethylene-induced hypocotyl growth reduction. Hence, ethylene and JA regulate at least three distinct responses in Arabidopsis, depending on whether they act alone or in combination (Figure 8). The effect of ethylene on hypocotyl elongation has been suggested to involve interaction with auxins, possibly based on inhibition of polar auxin transport by ethylene (Hobbie, 1998). Cooperative interactions between signaling response pathways may be seen as means developed by plant species to increase the number of distinct gene repertoires that can be controlled by a limited set of signaling molecules and hence to increase behavioral plasticity.

METHODS

Biological Material

The ethylene response mutants *ein2-1* (Guzmán and Ecker, 1990) and *etr1-1* (Bleecker et al., 1988; Chang et al., 1993) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH; accession numbers CS3071 and CS237, respectively). The jasmonate response mutant *coi1-1* (Feys et al., 1994) was obtained from J. Turner (University of East Anglia, Norwich, UK). Because this mutation is recessive and causes male sterility, we identified *coi1* mutants in F_2 plants grown from seed from selfed *COI1*/*coi1* hemizy-

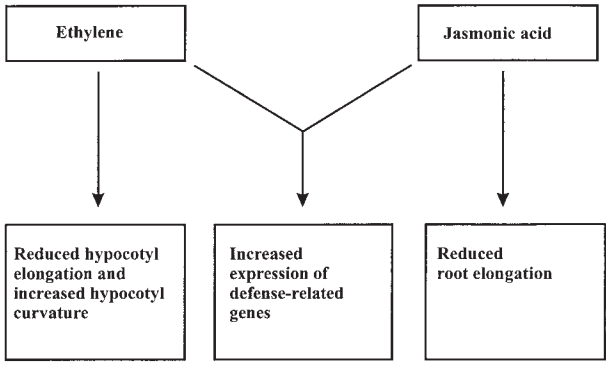

Figure 8. Three Distinct Responses Controlled by Ethylene and Jasmonate in Arabidopsis.

Ethylene is involved in the control of hypocotyl elongation and curvature and possibly in interactions with other hormones such as auxins, but not with jasmonates. Ethylene and jasmonates interact to regulate the expression of particular defense-related genes such as *PDF1.2* upon pathogen perception. Jasmonates are involved in the control of root elongation, possibly in interaction with other hormones excluding ethylene.

gous plants. The F_2 population was therefore subjected to different treatments as follows. Leaves from each individual plant were collected separately, and the plants were then grown until seed set. Individuals that did not form siliques were identified as having the *coi1*/*coi1* genotype. All mutant and transgenic lines listed above were derived from the *Arabidopsis thaliana* Columbia (Col-0) ecotype. Growth and spore harvesting of the fungus *Alternaria brassicicola* (MUCL 20297; Mycothèque Université Catholique de Louvain, Louvain-la-Neuve, Belgium) were done as described previously (Broekaert et al., 1990).

Plant Growth Conditions, Chemical Application, and Inoculation

Arabidopsis seeds were sown on flower potting compost containing a macronutrient supplement (Asef, Didam, The Netherlands) in Petri dishes. Seeds were vernalized for 2 days at 4°C after sowing. After 5 days of incubation in a growth chamber (20°C daytime temperature and 18°C nighttime temperature, with a 12-hr photoperiod at a photon flux density of 100 μE m^{-2} sec^{-1}), seedlings were transferred to pots (5 $\times$ 4 $\times$ 4 cm) containing potting compost supplemented with macronutrients and grown under the same conditions as given above. Irrigation was with tap water via the trays carrying the pots. Surface-sterilized seeds were sown on sterile Murashige and Skoog medium (Sigma Chemical Co.), in some cases including methyl jasmonate (MeJA), as indicated in Results, in sterile translucent polypropylene pots. Seeds were vernalized for 2 days at 4°C after sowing and incubated under the same conditions as soil-grown plants. Plants were 4 weeks old when treated.

Paraquat (25 μM), MeJA (45 μM in 0.1% [v/v] ethanol), and 0.1% (v/v) ethanol were applied at the concentrations indicated as 5-μL droplets on leaves (five drops per leaf for soil-grown plants; one drop per leaf for sterile plants). The stock solution of MeJA was 45 mM in ethanol. Ethylene treatment was performed by placing pots in an airtight translucent chamber in which gaseous ethylene was injected via a silicon rubber septum. The ethylene concentration in the chamber was verified by gas chromatography. Control plants for the ethylene experiment were placed in an identical chamber without ethylene.

Inoculation with *A. brassicicola* was done by applying 5-μL drops of a spore suspension (density of 5 $\times$ 10^5 spores per mL in distilled water) on four lower rosette leaves (five drops per leaf). Control plants were treated identically with water droplets. The plants with drops of spore suspension or water were placed randomly (if different genotypes were treated simultaneously) in a propagator flat with a clear polystyrene lid and kept at high humidity for 2 days to stimulate infection by hyphal germlings. Thereafter, lids were removed, and the plants were incubated further until the harvesting of leaf material. The treated leaves (cotyledons and rosette leaf numbers 1 to 4) were collected separately from the nontreated leaves (rosette leaf numbers 5 to 8) of the same plants. The isolate of *A. brassicicola* and inoculation conditions used here caused limited brown necrotic lesions under the drops of spore suspension within 48 hr after inoculation, and these lesions failed to spread further.

RNA Gel Blot Analysis

RNA was extracted by the phenol–LiCl method according to Eggermont et al. (1996) from tissues frozen in liquid nitrogen and stored at −80°C. RNA gel blotting was performed as described previously (Penninckx et al., 1996). Blots were prehybridized, hybridized with digoxigenin-labeled antisense RNA probes (either derived from *PDF1.2*- or tubulin β-1–encoding expressed sequence tags), and developed by immunochemiluminescence, as described previously (Penninckx et al., 1996).

ELISA

Proteins were isolated from frozen leaf material, as described by Penninckx et al. (1996). Protein concentrations were determined in the crude extracts according to Bradford (1976) by using BSA as a standard. After heat treatment (10 min at 80°C) of the extract, the heat-stable soluble protein fraction was analyzed in a competition ELISA, as outlined previously (Penninckx et al., 1996).

Quantitative Analysis of Jasmonic Acid and Ethylene Production

The extraction and quantitative analysis of jasmonic acid (JA) were performed as previously described (Penninckx et al., 1996). For measurements of ethylene emission, 4-week-old soil-grown Arabidopsis plants were either inoculated with *A. brassicicola* as described above or mock inoculated with water. After 0, 12, 24, 36, 48, 60, 72, 84, and 96 hr of incubation, the plants were removed from their soil substrate, their roots were washed with tap water, and each plant was placed in a 20-mL glass bottle closed with a rubber cap. After 3 hr, a 1-mL gas sample was withdrawn from the headspace with a syringe and analyzed by gas chromatography on a Delsi Nermag 200 (Delsi, Paris, France) instrument equipped with a Poropack R (vinylpyrolidone) column and a flame ionization detector.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO) and in part by a grant from the Swiss National Science Foundation for the jasmonic acid measurements. I.A.M.A.P. is the recipient of a predoctoral fellowship of the Vlaams Instituut voor Bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie (IWT). B.P.H.J.T. is a Research Assistant of the FWO. We thank Dr. John Turner for providing seed from Arabidopsis mutants and Dr. Maurice De Proft for advice on ethylene emission measurements.

Received May 7, 1998; accepted October 12, 1998.

REFERENCES

Beffa, R., Szell, M., Meuwly, P., Pay, A., Vögeli-Lange, R., Métraux, J.-P., Neuhaus, G., Meins, F., and Nagy, F. (1995). Cholera toxin elevates pathogen resistance and induces pathogenesis-related gene expression in tobacco. EMBO J. **14,** 5753–5761.

Bent, A.F., Innes, R.W., Ecker, J.R., and Staskawicz, B.J. (1992). Disease development in ethylene-insensitive *Arabidopsis thaliana* infected with virulent and avirulent *Pseudomonas* and *Xanthomonas* pathogens. Mol. Plant-Microbe Interact. **5,** 372–378.

Berger, S., Bell, E., and Mullet, J.E. (1996). Two methyl jasmonate–insensitive mutants show altered expression of AtVsp in response to methyl jasmonate and wounding. Plant Physiol. **111,** 525–531.

Bleecker, A.B., Estelle, M.A., Somerville, C., and Kende, H. (1988). Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana.* Science **241,** 1086–1089.

Boller, T. (1991). Ethylene in pathogenesis and disease resistance. In The Plant Hormone Ethylene, A.K. Mattoo and J.C. Suttle, eds (Boca Raton, FL: CRC Press), pp. 293–314.

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. **72,** 248–254.

Brederode, F.T., Linthorst, H.J.M., and Bol, J.F. (1991). Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV light and wounding. Plant Mol. Biol. **17,** 1117–1125.

Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A., and Vanderleyden, J. (1990). An automated quantitative assay for fungal growth. FEMS Microbiol. Lett. **69,** 55–60.

Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A., and Osborn, R. (1995). Plant defensins: Novel antimicrobial peptides as components of the host defense system. Plant Physiol. **108,** 1353–1358.

Chang, C., Kwok, S.F., Bleecker, A.B., and Meyerowitz, E.M. (1993). Arabidopsis ethylene-response gene *etr1*: Similarity of product to two-component regulators. Science **262,** 539–544.

Chang, P.F.L., Cheah, K.T., Narasimka, M.L., Hasegawa, P.M., and Bressan, R.A. (1995). Osmotin gene expression is controlled by elicitor synergism. Physiol. Plant. **95,** 620–626.

Creelman, R.A., and Mullet, J.E. (1997). Biosynthesis and action of jasmonates in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48,** 355–381.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E., and Ryals, J. (1994). A central role of salicylic acid in plant disease resistance. Science **266,** 1247–1250.

Dürner, J., Shah, J., and Klessig, D.F. (1997). Salicylic acid and disease resistance in plants. Trends Plant Sci. **2,** 266–274.

Ebel, J., and Cosio, E. (1994). Elicitors of plant defence responses. Int. Rev. Cytol. **148,** 1–36.

Ecker, J.R. (1995). The ethylene signal transduction pathway in plants. Science **268,** 667–675.

Eggermont, K., Goderis, I.J., and Broekaert, W.F. (1996). High-throughput RNA extraction from plant samples based on homogenisation by reciprocal shaking in the presence of a mixture of sand and glass beads. Plant Mol. Biol. Rep. **14,** 273–279.

Feys, B.J.F., Benedetti, C.E., Penfold, C.N., and Turner, J.G. (1994). Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell **6,** 751–759.

Friedrich, L., Vernooij, B., Gaffney, T., Morse, A., and Ryals, J. (1995). Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. Plant Mol. Biol. **29,** 959–968.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessman, H., and Ryals, J. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261,** 754–756.

Guzmán, P., and Ecker, J.R. (1990). Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. Plant Cell **2,** 513–523.

Hobbie, L.J. (1998). Auxin: Molecular genetic approaches in Arabidopsis. Plant Physiol. Biochem. **36,** 91–102.

Horvath, D.M., Huang, D.J., and Chua, N.-M. (1998). Four classes of salicylate-induced tobacco genes. Mol. Plant-Microbe Interact. **11,** 895–905.

Kende, H., and Zeevaart, J.A.D. (1997). The five "classical" plant hormones. Plant Cell **9,** 1197–1210.

Kieber, J.J. (1997). The ethylene response pathway in Arabidopsis. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48,** 277–296.

Lamb, C., and Dixon, R.A. (1997). The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48,** 251–275.

Laudert, D., and Weiler, E.W. (1998). Allene oxide synthase: A major control point in *Arabidopsis thaliana* octadecanoid signaling. Plant J. **15,** 675–684.

Lawton, K.A., Potter, S.L., Uknes, S., and Ryals, J. (1994). Acquired resistance signal transduction in Arabidopsis is ethylene independent. Plant Cell **6,** 581–588.

Lawton, K.A., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals, J. (1995). Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. Mol. Plant-Microbe Interact. **8,** 863–870.

Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. (1994). H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell **79,** 583–593.

O'Donnell, P.J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H.M.O., and Bowles, D.J. (1996). Ethylene as a signal mediating the wound response of tomato plants. Science **274,** 1914–1917.

Penninckx, I.A.M.A., Eggermont, K., Terras, F.R.G., Thomma, B.P.H.J., De Samblanx, G.W., Buchala, A., Métraux, J.-P., Manners, J.M., and Broekaert, W.F. (1996). Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid–independent pathway. Plant Cell **8,** 2309–2323.

Pieterse, C.M.J., van Wees, S.C.M., Hoffland, E., van Pelt, J.A., and van Loon, L.C. (1996). Systemic resistance in Arabidopsis induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. Plant Cell **8,** 1225–1237.

Shirasu, K., Dixon, R.A., and Lamb, C. (1996). Signal transduction in plant immunity. Curr. Opin. Immunol. **8,** 3–7.

Shulaev, V., Léon, J., and Raskin, I. (1995). Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? Plant Cell **7,** 1691–1701.

Somssich, I.E. (1994). Regulatory elements governing pathogenesis-related (PR) gene expression. In Plant Promoters and Transcription Factors, L. Nover, ed (Berlin: Springer-Verlag), pp. 163–179.

Staswick, P.E., Su, W., and Howell, S.H. (1992). Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc. Natl. Acad. Sci. USA **89,** 6837–6840.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. (1992). Acquired resistance in Arabidopsis. Plant Cell **4,** 645–656.

Vidal, S., Ponce de Leon, I., Denecke, J., and Palva, E.T. (1997). Salicylic acid and the plant pathogen *Erwinia carotovora* induce defence genes via antagonistic pathways. Plant J. **11,** 115–123.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Métraux, J.-P., and Ryals, J.A. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3,** 1085–1094.

Xu, Y., Chang, P.-F.L., Liu, D., Narasimhan, M.L., Raghothama, K.G., Hasegawa, P.M., and Bressan, R.A. (1994). Plant defense genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell **6,** 1077–1085.

Zeevaart, J.A.D. (1988). Metabolism and physiology of abscisic acid. Annu. Rev. Plant Physiol. Plant Mol. Biol. **39,** 439–473.