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RESEARCH ARTICLE

Acquired Resistance Signal Transduction in Arabidopsis Is Ethylene Independent

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To clarify the role of ethylene in systemic acquired resistance (SAR), we conducted experiments using Arabidopsis ethylene response mutants. Plants that are nonresponsive to ethylene (i.e., *etr1* and *ein2*) showed normal sensitivity to the SAR-inducing chemicals salicylic acid (SA) and 2,6-dichloroisonicotinic acid with respect to SAR gene induction and pathogen resistance. This indicated that chemically induced SAR is not an ethylene-dependent process in Arabidopsis. Ethephon, an ethylene-releasing chemical, induced SAR gene expression in both the wild type and ethylene mutants, whereas ethylene alone did not, suggesting that induction of these genes by ethephon is not due to the action of ethylene. Furthermore, transgenic plants expressing salicylate hydroxylase, a bacterial enzyme that degrades SA to catechol, did not accumulate SAR mRNAs in response to ethephon. Thus, SAR gene induction by ethephon appears to be mediated through SA. Other experiments suggested that ethylene may play a role in SAR by enhancing tissue sensitivity to the action of SA.

INTRODUCTION

Many plants can respond to pathogen infection by inducing long-lasting, broad-spectrum resistance. This phenomenon, referred to as systemic acquired resistance (SAR) (Ross, 1961), has been studied for many years, but the biochemical events leading to maintenance of an induced-resistant state are not clear. Although SAR has been described in many plant species and seems to be ubiquitous, it has been most extensively studied at the biochemical level in tobacco, cucumber, and Arabidopsis (Kuc, 1982; Métraux et al., 1991; Ward et al., 1991; Uknes et al., 1992, 1993). In tobacco, a necrotic lesion formed by a pathogen can induce SAR. Ross demonstrated in the early 1960s that the lesions have to be formed by a pathogen, and treatment of the plant with necrotizing chemicals, such as salts, would not induce resistance (Ross, 1961, 1966). Following pathogen infection, a number of genes are coordinately induced in both the infected and uninfected leaves at a time in which the resistant state is being established (Ward et al., 1991; Uknes et al., 1992, 1993). These genes encode proteins that include the 10 pathogenesis-related (PR) proteins (Van Loon and Van Kammen, 1970; Van Loon, 1975; Gianinazzi and Ahl, 1983) and have been termed SAR genes, because the accumulation of the corresponding mRNAs is tightly correlated with SAR (Ward et al., 1991). A role for these proteins in resistance is supported by the finding that transgenic tobacco plants expressing high levels of PR-1a, one of the SAR-related gene products, have increased tolerance to *Peronospora tabacina* and *Phytophthora parasitica* (Alexander et al., 1993).

Salicylic acid (SA) has been implicated in the signal transduction pathway leading to SAR. SA accumulation correlates with the onset of resistance in tobacco (Malamy et al., 1990) and cucumber (Métraux et al., 1990), and exogenous application of SA results in increased accumulation of the SAR mRNAs and pathogen resistance in tobacco (Ward et al., 1991) and Arabidopsis (Uknes et al., 1992, 1993). The most compelling evidence linking SA to SAR signaling comes from experiments with transgenic tobacco plants that express the bacterial enzyme salicylate hydroxylase (SAH), which degrades SA to catechol (Gaffney et al., 1993). These plants are unable to mount the SAR response, thereby demonstrating that SA is required for establishment of acquired resistance. The possibility that other signal molecules might be involved in SAR is suggested by experiments with cucumber in which resistance is established systemically prior to detectable increases in SA (Rasmussen et al., 1991) and from experiments in tobacco in which SAR is observed in Xanthi-nc scions grafted onto transgenic rootstocks that express the salicylate hydroxylase gene (B. Vernooij, L. Friedrich, A. Morse, R. Reist, R. Kolditz-Jawhar, E. Ward, S. Uknes, H. Kessmann, and J. Ryals, manuscript submitted).

Recently, ethylene has been suggested to act as a signal involved in SAR (Raz and Fluhr, 1992). Van Loon demonstrated that in tobacco, pinpricking leaves with ethephon induces the

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accumulation of PR proteins and enhances resistance to tobacco mosaic virus (Van Loon, 1977). This experiment has been interpreted to indicate that ethylene is involved in PR gene expression and the induction of SAR. Further support for a role for ethylene in disease resistance is based upon the observations that ethylene is produced when cells undergo necrosis resulting from pathogen infection (Ross and Williamson, 1951; Mauch et al., 1984; Spanu and Boller, 1989) and that the exogenous application of ethylene induces the accumulation of defense-related enzymes, such as phenylalanine ammonia-lyase (PAL), chalcone synthase (Ecker and Davis, 1987), and vacuolar hydrolases (e.g., chitinase and glucanase) (Boller and Vogeli, 1984; Mauch and Staehelin, 1989; Mauch et al., 1992). However, ethylene application has not been demonstrated to lead to heightened disease resistance, so the role of ethylene as a signal involved in disease resistance is unclear.

We examined the effects of ethephon (2-chloroethylphosphonic acid), a chemical that releases ethylene, hydrochloric acid (HCl), and phosphonic acid (H_3PO_3) as breakdown products when applied to plants (Yang, 1969), as well as ethylene and the chemical SAR activators SA and 2,6-dichloroisonicotinic acid (INA) on the SAR response using Arabidopsis mutants inhibited in the triple response to ethylene (Bleecker et al., 1988; Guzmán and Ecker, 1990; Chang et al., 1993). These mutants were altered in their ability to perceive and react to the action of ethylene. We showed that ethephon induction of PR gene expression is an SA-dependent process that is not the result of ethylene release during the breakdown of the chemical but rather is an effect of the acids. This effect was enhanced by ethylene. Moreover, we found that activation of SAR gene expression and resistance by SA and INA was unaffected in the ethylene response mutants, demonstrating that ethylene is not required for SAR in Arabidopsis.

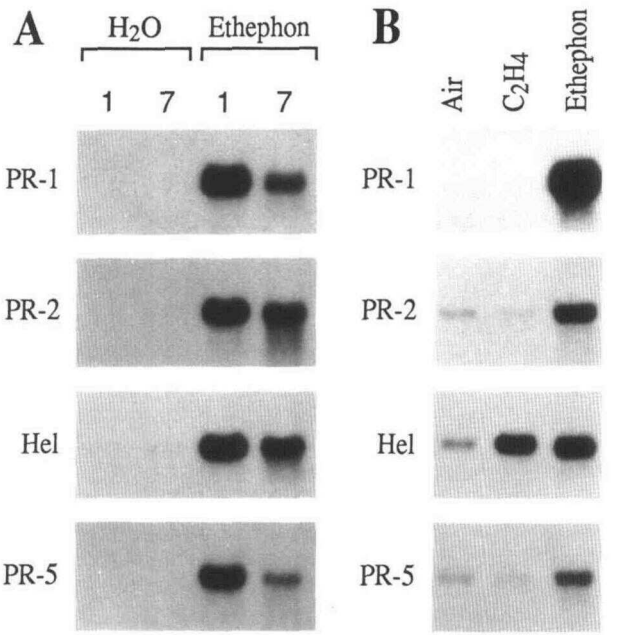

Figure 1. Accumulation of SAR-Related mRNAs in Response to Ethephon and Ethylene.

(A) SAR transcript abundance at 1 and 7 days after the application of 7 mM ethephon.

(B) SAR transcript abundance following 24 hr of exposure to 100 μL/L ethylene or 7 mM ethephon.

Tissue was harvested concurrent with the day 1 sample shown in **(A).** Control samples were enclosed in a chamber without ethylene. The probes were derived from ^{32}P-labeled cDNAs encoding the Arabidopsis PR-1, PR-2, PR-5 (Uknes et al., 1992), and HEL (Potter et al., 1993) transcripts. The apparent slight suppression of PR-2 and PR-5 transcripts by ethylene was not consistent and was indicative of the variable basal levels of expression of these transcripts as has been observed previously (Uknes et al., 1992).

RESULTS

Effects of Ethylene, Ethephon, and Ethephon Components on Gene Induction in Arabidopsis

To clarify the role of ethylene in the induction of SAR, ethephon was applied to leaves of Arabidopsis plants. Leaves were harvested at 1 and 7 days after treatment, and the accumulation of PR-1, PR-2, hevein-like (HEL), and PR-5 mRNA was determined by RNA gel blot hybridization. We have previously shown that the HEL gene is expressed at high levels in response to ethylene and pathogen infection (Potter et al., 1993). In this study, HEL mRNA was measured as an indicator of ethylene response. Expression of PR-1, PR-2, and PR-5 mRNA has been shown to be strongly induced by SA, INA, and pathogen infection (Uknes et al., 1992). Thus, expression of these genes is indicative of the SAR response. As shown in Figure 1A, all four gene families were strongly induced within 1 day of ethephon treatment, and the mRNA levels remained elevated for at least 7 days after treatment.

To further study this effect, plants were treated with gaseous ethylene. Figure 1B shows the results of gel blot analysis of RNA extracted from tissue 24 hr after either enclosure in 100 μL/L ethylene or treatment with 7 mM ethephon. The HEL transcript was induced by both ethylene and ethephon, whereas the transcript levels of the other gene families increased in response to ethephon, but not in response to ethylene. This result suggested that ethephon induction of gene expression was not due to the release of ethylene gas during the breakdown of ethephon, but rather to some other effect of ethephon treatment.

To determine in a more direct fashion if the induction of the genes by ethephon was an effect of ethylene, we used Arabidopsis plants that were altered in ethylene responsiveness (Bleecker et al., 1988; Guzmán and Ecker, 1990). Both wild-type and ethylene-insensitive mutants of Arabidopsis were

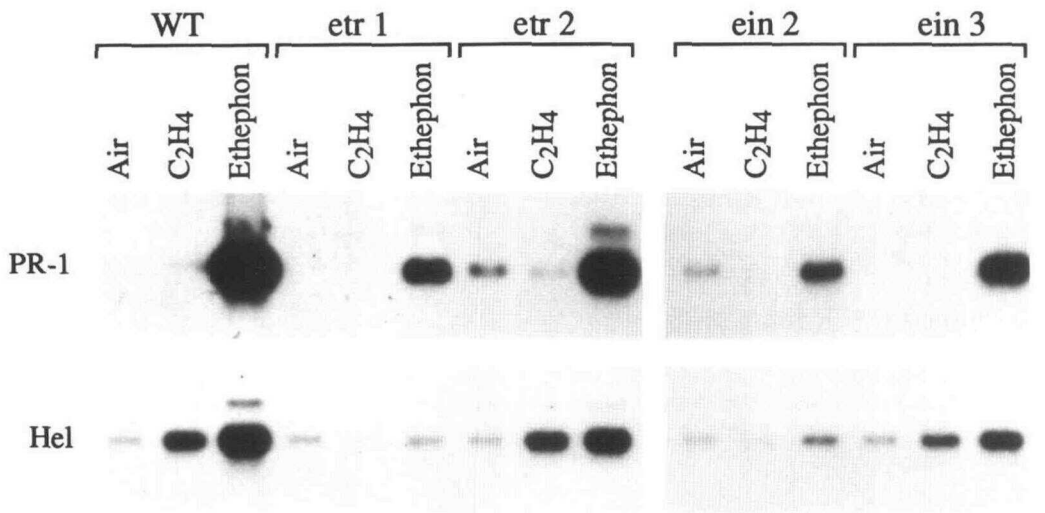

Figure 2. Ethylene and Ethephon Induction of PR-1 and HEL mRNA in the Wild Type and Ethylene Response Mutants.

PR-1 and HEL transcript abundance was determined in tissue harvested 24 hr after the application of gaseous ethylene (100 μL/L) or ethephon (7 mM). Control plants were enclosed in a chamber without ethylene. PR-1 and HEL probes are described in Figure 1. *WT, wild type.*

treated with either ethephon or gaseous ethylene. Figure 2 shows the accumulation of mRNA for PR-1 and HEL in response to either ethylene or ethephon treatment in Columbia wild type and the ethylene-response mutants *etr1*, *etr2*, *ein2*, and *ein3*. The HEL message was induced by ethylene and ethephon in the wild type, *etr2*, and *ein3*. In *etr1* and *ein2*, the HEL message was not induced by either ethephon or ethylene, indicating that these mutants are the least sensitive to ethylene. In contrast, ethephon treatment strongly induced PR-1 transcript accumulation in the wild type as well as in all four of the ethylene-response mutants, including the *etr1* and *ein2* plants. However, ethephon induction of PR-1 mRNA accumulation in *etr1* and *ein2* was less than observed in wild-type plants. From these experiments, it was clear that the action of ethylene alone could account for the accumulation of HEL mRNA following ethephon treatment. Furthermore, consistent with previous results in the wild-type plants, these results suggested that ethephon was not inducing PR-1 mRNA accumulation through the action of ethylene. The *etr1* and *ein2* mutants were chosen for further experiments because the mature plants exhibited the least sensitivity to ethylene.

Ethephon spontaneously degrades and releases ethylene gas, HCl, and H_3PO_3 when applied to plants. To determine if the gene-inducing effects of ethephon might be due to the acids, we treated Arabidopsis plants with ethylene, ethephon, HCl, and H_3PO_3, or combinations thereof. The results in Figure 3 demonstrate that both HCl and H_3PO_3 significantly induced PR-1 mRNA accumulation, and this effect was increased substantially in the presence of ethylene. As in previous experiments, ethylene induced HEL mRNA accumulation but not the accumulation of PR-1 mRNA. These results showed that although ethylene alone was not capable of inducing PR-1 mRNA, the combination of acid and ethylene could induce PR-1 mRNA to approximately the level observed with ethephon treatment.

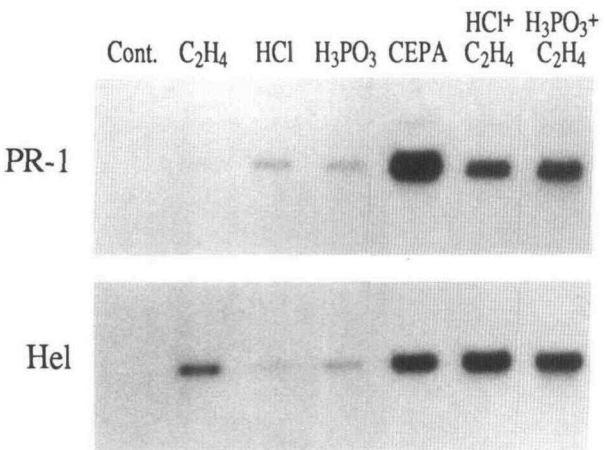

Figure 3. Accumulation of PR-1 and HEL mRNA in Acid- and Ethylene-Treated Plants.

Responses to treatment with ethylene (C_2H_4), ethephon (2-chloroethylphosphonic acid; CEPA), hydrochloric acid (HCl), and phosphonic acid (H_3PO_3), or combinations thereof were determined. Transcript abundance was analyzed in tissues harvested 24 hr after treatment. Probes for PR-1 and HEL are as described in Figure 1. Cont., control.

Ethephon Induction of PR-1 mRNA Accumulation Is Dependent on SA

We considered the possibility that treatment with HCl or H_3PO_3 somehow activates the production of SA in Arabidopsis and that SA then leads to the induction of PR-1 mRNA. To test this hypothesis, wild-type and transgenic plants expressing SAH were treated with either ethephon or ethylene. Like the tobacco plants that express SAH (Gaffney et al., 1993), the transgenic Arabidopsis plants expressing SAH are unable to accumulate SA or mount the SAR response (K. Weymann, B. Vernooij, T. Delaney, E. Ward, S. Uknes, and J. Ryals, unpublished data). Figure 4 shows that ethephon could induce high levels of PR-1 mRNA in wild-type but not in transgenic Arabidopsis plants expressing SAH, suggesting that ethephon induction is dependent on the accumulation of SA. Both ethylene and ethephon induced the accumulation of HEL in plants expressing SAH to levels comparable to those observed in wild-type plants, indicating that the ethylene induction of HEL is not dependent on SA.

Independence of PR-1 mRNA Induction and SAR from Ethylene Sensitivity

To investigate the role of ethylene in acquired resistance, the *etr1* and *ein2* mutants were tested for their ability to induce resistance to the downy mildew *P. parasitica* and PR-1 mRNA in response to SA and INA. As shown in Figure 5 and Table 1, water-treated control plants exhibited heavy downy mildew infection 7 days after inoculation, but pretreatment with SA or INA strongly inhibited the disease in both wild-type and ethylene-insensitive plants. Furthermore, the results shown in Figure 6 demonstrated that both SA and INA treatment could induce PR-1 mRNA accumulation to high levels in both wild-type and ethylene-response mutant plants. Taken together, these results suggested that SAR is not dependent on ethylene.

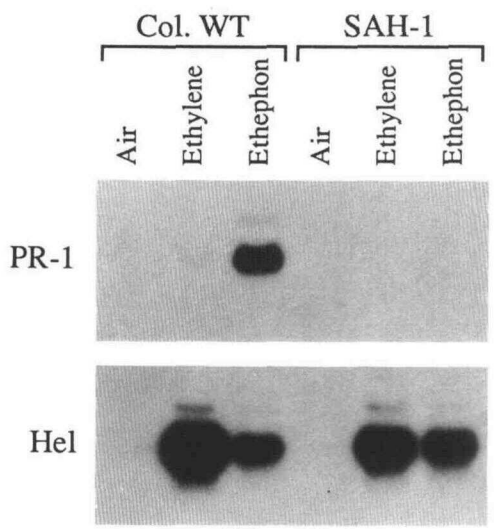

Figure 4. Accumulation of PR-1 and HEL mRNA in Wild-Type and Transgenic SAH-Expressing Plants.

Transcript abundance in response to ethylene and ethephon was determined by RNA gel blot hybridization. Tissue was harvested for RNA analysis following 24 hr of treatment with 100 μL/L ethylene or 7 mM ethephon. Control plants were enclosed in a chamber without ethylene. Probes for PR-1 and HEL are as described in Figure 1. Col. WT, Columbia wild type.

Interaction of Ethylene with SA

In the previous experiments, we observed that H_3PO_3 or HCl treatment could induce SAR gene expression and that this induction was enhanced by ethylene treatment. Furthermore, whereas ethephon strongly induced PR-1 mRNA expression in *etr1* and *ein2*, the induction was reduced relative to the wild type. These observations are consistent with the idea that ethylene may potentiate the effects of SA. To examine this possibility, we carried out an SA dose–response experiment in the presence or absence of ethylene. As shown in Figure 7, exposure to ethylene immediately following SA application resulted in an increase in PR-1 mRNA at lower SA concentrations than observed with SA alone. These results indicated that ethylene can enhance the sensitivity of plants to low concentrations of SA.

DISCUSSION

Contrary to what has been previously reported for tobacco (Raz and Fluhr, 1992), acquired resistance in Arabidopsis does not require ethylene sensitivity; therefore, it is unlikely that ethylene mediates this signal transduction pathway. This conclusion is based on the following lines of evidence. Ethylene application did not induce expression of the Arabidopsis SAR genes. Moreover, the application of SA or INA to the ethylene response mutants *etr1* and *ein2* prior to challenge with the pathogen *P. parasitica* resulted in resistance comparable to that observed in wild-type plants; SAR gene induction by SA and INA was also uncompromised in these mutants. Thus, it follows that ethylene is neither sufficient nor required for SAR signal transduction in Arabidopsis. This is consistent with reports that ethylene exposure does not induce resistance to *Botrytis cinerea* in carrots (Hoffman and Heale, 1987). In addition, our results support and extend the findings of Bent et al. (1992); they showed that ethylene sensitivity is not required for resistance to an avirulent bacterial pathogen.

Whereas SAR is ethylene independent, ethephon induction of SAR gene expression is dependent on the accumulation of SA. In transgenic plants that are unable to accumulate SA, ethephon application did not result in PR-1 mRNA accumulation (Figure 4); these plants responded normally to ethylene. This result supports the idea that the SA and ethylene signal

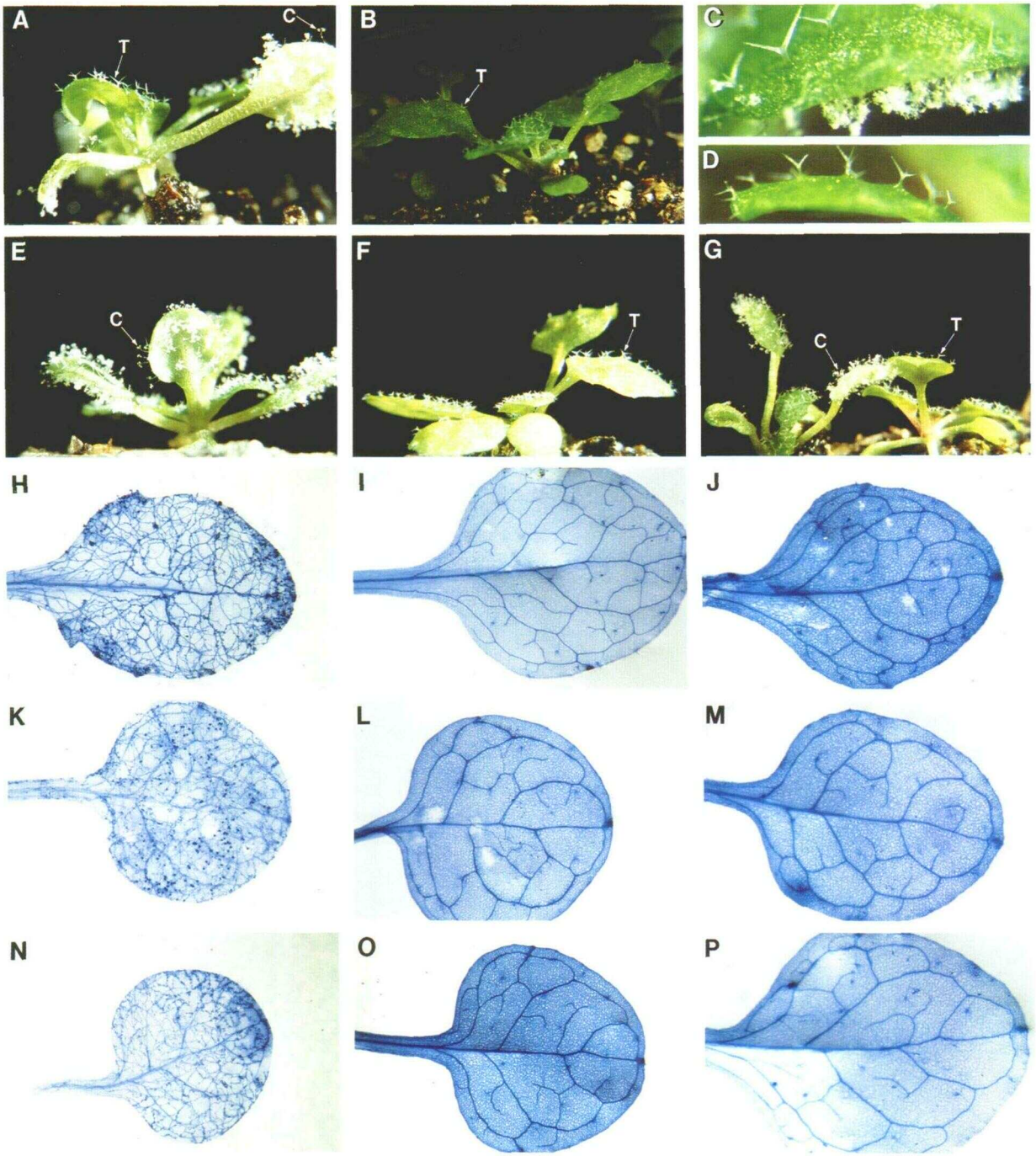

Figure 5. Effects of SA and INA Treatment on *P. parasitica* Infection of the Wild Type and Ethylene Response Mutants.

(A), **(C)**, and **(E)** show control plants infected with *P. parasitica*. **(B)**, **(D)**, and **(F)** show plants pretreated with INA. **(C)** and **(D)** are close-up views of leaves inoculated with *P. parasitica*; trichomes are shown on the upper leaf surface in contrast to conidiophores on the lower leaf surface of the control plant shown in **(C)** or trichomes only on a leaf from an INA-treated plant shown in **(D)**. Plants were photographed 7 days postinoculation.

(A) to **(D)** Columbia wild-type plants.

(E) and **(F)** Columbia *ein2* plants.

(G) Columbia *etr1* plants. At left is the control plant; at right is an INA-treated plant.

(H) to **(P)** Trypan blue-stained leaves. In **(H)** to **(J)** are Columbia wild-type plants; **(K)** to **(M)**, *etr1* plants; **(N)** to **(P)**, *ein2* plants. Plants were treated with water in **(H)**, **(K)**, and **(N)**, SA in **(I)**, **(L)**, and **(O)**, or INA in **(J)**, **(M)**, and **(P)** prior to inoculation with *P. parasitica*. Extensive fungal sporulation and hyphal growth are observed only in leaves of control plants.

C, conidiophore; T, trichome.

Table 1. *P. parasitica* Infection Ratings

Plant	Treatment	Average Rating[a]	No. of Plants
Columbia Wild Type	Control	3.16	31
	SA	0.73	33
	INA	0.08	37
etr1	Control	2.40	53
	SA	0.91	43
	INA	0.35	67
ein2	Control	2.8	33
	SA	1.2	45
	INA	0	52

[a] The ratings scale used the following parameters to define infection severity: 0, no conidiophores on plant; 1, at least one leaf with 1 to 5 conidiophores; 2, at least one leaf with 5 to 20 conidiophores; 3, many leaves with 5 to 20 conidiophores; 4, all inoculated leaves with greater than five conidiophores; 5, all inoculated leaves with >20 conidiophores. Plants were scored for infection 7 days after inoculation.

transduction pathways are distinct. However, ethylene does exert some influence on the SA signaling pathway because ethylene enhanced the sensitivity of the tissue to the action of SA (Figure 7). Although the magnitude of enhancement varied from experiment to experiment, the effect on PR-1 mRNA expression was most pronounced at low SA levels and diminished as SA levels increased. This observation is similar to results of experiments with beans, in which ethylene alone did not induce PAL in resistant cultivars but caused an increase in PAL activity following induction by low concentrations of a fungal elicitor (Hughes and Dickerson, 1989).

Ethylene is released during the chemical degradation of ethephon, and the effects of ethephon application are considered indicative of an ethylene response. However, our results indicated that some ethephon effects were not due to ethylene. Whereas HEL mRNA accumulation in response to ethephon was apparently due to ethylene action, SAR gene expression was not. Therefore, it appeared that ethephon stimulated two independent signal transduction pathways by different mechanisms. Because of these bifunctional effects, previous experiments in which ethephon has been used as an ethylene source should be reevaluated.

METHODS

Plant Material

Arabidopsis thaliana ecotype Columbia (Lehle Seeds, Tucson, AZ) seeds were surface sterilized, sown in growing media that was autoclaved, and grown in a phytotron as described previously (Uknes et al., 1992). Ethylene response mutants *etr1*, *etr2*, *ein2*, and *ein3* in the Columbia background were grown under conditions that were identical to those for the Columbia wild-type plants.

Chemical Application

For gene induction experiments, chemicals were applied as a spray to 3- to 4-week-old plants. Salicylic acid (SA) was applied at concentrations indicated in the legends to Figures 6 and 7. 2,6-Dichloroisonicotinic acid (INA) was applied as a spray at concentrations indicated for the gene induction experiments in Figure 6. For the *Peronospora parasitica* protection experiments, 5 mM SA or 0.25 mg/mL (25% active ingredient in a wettable powder) INA was applied as a combination spray/soil drench to seedlings 2 to 3 days after germination. Control plants were sprayed with water. Ethephon was applied at the rate of 7 mM. Acids (HCl and H_3PO_3) were applied as 7-mM aqueous solutions. For ethylene experiments, plants were enclosed in a chamber equilibrated to 100 μL/L ethylene with gaseous ethylene for the times indicated. Ethylene concentration was confirmed by gas chromatography. Control plants were enclosed in an identical chamber without ethylene. Each experiment was repeated at least three times with similar results.

RNA Analysis

RNA was isolated from frozen tissue samples by phenol–chloroform extraction followed by lithium chloride precipitation (Lagrimini et al., 1987). Total RNA samples (10 μg) were electrophoretically separated through formaldehyde–agarose gels and blotted to hybridization membrane (GeneScreen Plus; Du Pont–New England Nuclear), as described by Ausubel et al. (1987). Equal loading of samples was confirmed by including 40 μg/mL ethidium bromide in the sample loading buffer, allowing visualization of RNA by photography under UV light. ^{32}P-labeled cDNA probes were synthesized by random priming of isolated insert DNA using the random primers DNA labeling system (Gibco BRL). The cDNAs to PR-1, PR-2, and PR-5 were described by Uknes et al. (1992). The HEL cDNA was described by Potter et al. (1993). Hybridization and washing conditions were as described by Church and Gilbert (1984). Relative amounts of transcript were determined by detecting β decay of phosphorus-32 with a Betascope analyzer (Betagen, Waltham, MA).

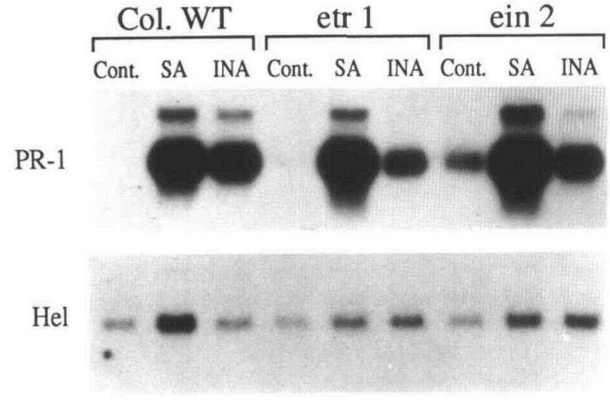

Figure 6. Accumulation of PR-1 and HEL mRNA in Wild-Type and Ethylene-Insensitive Arabidopsis in Response to SA and INA.

Gel blot analysis of RNA extracted from plants 24 hr after application of 2.5 mM SA or 7 mM INA. Probes for PR-1 and HEL are described in Figure 1. Col. WT, Columbia wild type; Cont., control.

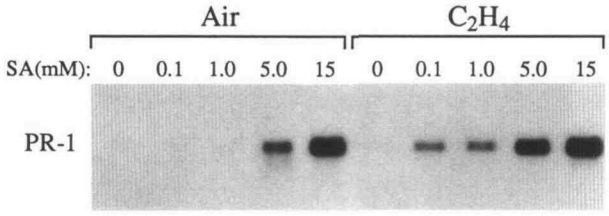

Figure 7. Effects of Ethylene on PR-1 mRNA Accumulation in Response to SA.

Columbia plants were sprayed with increasing concentrations of SA, then held in air or enclosed in 100 μL/L ethylene for 24 hr. Tissue was harvested for RNA analysis immediately following ethylene treatment, which was ~25 hr after SA application. The PR-1 probe is described in Figure 1.

P. parasitica Inoculation

Columbia wild type and ethylene-insensitive mutants were surface sterilized and sown in sterile growing media as described above. Pots were held at 4°C for 2 to 3 days following sowing for vernalization and to enhance uniformity of germination. At 2 to 3 days after germination, seedlings were sprayed to runoff with 5 mM SA or 25 μg/mL INA (25% active ingredient, wettable powder), effectively providing a spray and soil drench application to the plants. Control plants were sprayed with sterile water. At 4 days after SA and INA application, the plants were sprayed with a conidial suspension containing 4 to 5 × 10^4 spores per mL. Following inoculation, plants were placed in a covered flat to maintain high humidity and grown at 17°C with a 14-hr light/10-hr dark cycle in a moist growth chamber to encourage fungal sporulation. Plants were scored for fungal growth at 5 and 7 days after inoculation by viewing with a dissecting microscope. In addition, several plants were randomly chosen from each treatment and stained with lacto-phenol trypan blue (Keogh et al., 1980) for microscopic examination. Inoculations were repeated several times with essentially the same results.

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