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Disease Development in Ethylene-Insensitive *Arabidopsis thaliana* Infected with Virulent and Avirulent *Pseudomonas* and *Xanthomonas* Pathogens

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The plant hormone ethylene has been hypothesized to play roles both in disease resistance and in disease susceptibility. These processes were examined by using isogenic virulent and avirulent bacterial pathogens and mutants of *Arabidopsis thaliana* that were altered in ethylene physiology. Ethylene-insensitive *ein1* and *ein2* mutants of *Arabidopsis* were resistant to *Pseudomonas syringae* pv. *tomato* made avirulent by the addition of the cloned avirulence genes *avrRpt2*, *avrRpm1*, or *avrB*; this suggests that ethylene is not required for active resistance against avirulent bacteria. In a second set of experiments, susceptibility was monitored with virulent *P. s.* pv. *tomato*, *P. s.* pv. *maculicola*, or *Xanthomonas campestris* pv. *campestris* strains. Wild-type *Arabidopsis* and *ein1* mutants were susceptible to these strains, but *ein2* mutants developed only minimal disease symptoms. Despite these reduced symptoms, virulent *P. s.* pv. *tomato* grew extensively within *ein2* leaves. The *Pseudomonas* phytotoxin coronatine induces ethylene biosynthesis and diseaselike symptoms on many plant species, but the reduced symptomology of *ein2* mutants could not be attributed to insensitivity to coronatine. The enhanced disease tolerance of *ein2* plants suggests that ethylene may mediate pathogen-induced damage, but the absence of tolerance in *ein1* mutants has yet to be explained.

Additional keywords: *eto1*, *etr1*.

The plant hormone ethylene induces plant responses associated with disease resistance, but also induces responses associated with disease susceptibility (Ben-David *et al.* 1986; Boller 1991; Pegg 1976; Stall and Hall 1984; Yang and Hoffman 1984). Plant ethylene synthesis is often significantly increased during infection by pathogens and can also be induced by treatment with pathogen-derived elicitors (Boller 1991; Pegg 1976; Yang and Hoffman 1984). Accordingly, it has been proposed that ethylene acts as a second messenger during plant-microbe interactions.

Support for the involvement of ethylene in resistance comes primarily from its ability to induce synthesis of "defense proteins" such as β-1,3-glucanase, chitinase, L-phenylalanine ammonia lyase, and hydoxyproline-rich glycoproteins (Broglie *et al.* 1986; Chappell *et al.* 1984; Ecker and Davis 1987; Mauch *et al.* 1984; Toppan *et al.* 1982). Synthesis of these proteins is observed after pathogen infection or treatment with pathogen-derived elicitors, and roles for these proteins in defense have been postulated (Dixon and Harrison 1990; Dixon and Lamb 1990; Lamb *et al.* 1989). There is little direct evidence, however, that ethylene plays a causal role in resistance. Many defense proteins are also inducible by ethylene-independent pathways (Dixon and Harrison 1990; Dixon and Lamb 1990; Lamb *et al.* 1989), and treatment of plants with inhibitors of ethylene biosynthesis does not necessarily alter the macroscopic responses of plants to pathogens or block synthesis of phytoalexins (Nemestothy and Guest 1990; Paradies *et al.* 1980). Thus, ethylene production correlates with the induction of resistance responses but does not necessarily cause resistance.

Ethylene may play a greater role in symptom development (an aspect of disease susceptibility) than in disease resistance. Chlorosis, senescence, and abscission are well-known responses of plants to ethylene, and there is a clear correlation between ethylene production and pathogen-induced tissue damage (Ben-David *et al.* 1986; Boller 1991; Pegg 1976; Stall and Hall 1984; Yang and Hoffman 1984). Ethylene production can also be stimulated by application of pathogen compounds associated with virulence, such as the *Pseudomonas syringae* van Hall phytotoxin, coronatine (Ferguson and Mitchell 1985).

We sought evidence that could causally link ethylene to either disease resistance or disease susceptibility. We utilized plant mutants defective in their responsiveness to ethylene as an alternative to previous approaches based on physiological inhibitors of ethylene biosynthesis. Several mutants of *Arabidopsis thaliana* (L.) Heynh. altered in ethylene biosynthesis or sensitivity have been isolated (Bleeker *et al.* 1988; Guzman and Ecker 1990; Harpham *et al.* 1991). Among these are "ethylene-insensitive" (*ein*) or "ethylene-resistant" (*etr*) mutants that do not display classical ethylene responses (such as hypocotyl swelling, inhibition of seedling elongation, tightening of the apical hook, induction of peroxidase activity, and increased chlorosis) even when treated with high concentrations of exogenous ethylene. Two unlinked *EIN* loci, *EIN1* and *EIN2*, have been reported (Guzman and Ecker 1990); *EIN1*

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is apparently the same locus as *ETR1* (Bleeker *et al.* 1988; Guzman and Ecker 1990). If ethylene plays a causal role in resistance, then we would expect ethylene-insensitive mutants of *Arabidopsis* to show susceptibility to pathogens against which the wild type is resistant. Conversely, if ethylene plays a role in symptom development, we would predict that symptoms induced by virulent pathogens would be attenuated in ethylene-insensitive *Arabidopsis*. Ethylene could conceivably affect both processes; the two possibilities are not mutually exclusive.

The resistant or susceptible outcome of a plant-pathogen interaction is frequently determined by the presence of race-specific plant resistance genes and pathogen avirulence genes (Crute 1985; Keen 1990). A given resistance gene is only effective against pathogens that carry a particular avirulence gene. If the plant and pathogen carry resistance and avirulence genes with matched specificity, disease spread is curtailed and a hypersensitive response (HR) involving localized cell death and physical isolation of the pathogen typically occurs (Klement 1982). In the absence of matched resistance and avirulence genes, colonization and tissue damage proceed past the site of initial infection, and disease is observed.

To test the above hypotheses about ethylene, resistance, and susceptibility, we used a pathogen system that we and others have developed for use with *Arabidopsis* (Debener *et al.* 1991; Dong *et al.* 1991; Whalen *et al.* 1991). Strain DC3000 of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Okabe) Young *et al.* is virulent on *Arabidopsis* ecotype Col-0 (Whalen *et al.* 1991). A *P. s.* pv. *tomato* avirulence gene (*avrRpt2*) that can convert strain DC3000 from virulent to avirulent on *Arabidopsis* ecotype Col-0, and does not cause observable reduction in virulence on susceptible plant lines, has been isolated (Dong *et al.* 1991; Whalen *et al.* 1991). It is, therefore, possible to study the resistant and susceptible responses of *Arabidopsis* by using isogenic *P. s.* pv. *tomato* strains that differ by only a single gene.

We infected the ethylene-insensitive *Arabidopsis* mutants with virulent and avirulent *P. s.* pv. *tomato* strains and compared the ensuing responses to those of wild-type plants. We also investigated responses to other bacterial pathogens and to strains carrying other avirulence genes, and examined a related *Arabidopsis* mutant (*eto1-1*) that constitutively produces ethylene at higher levels than the wild type (Guzman and Ecker 1990). The *ein1*, *ein2*, and *eto1* mutations had no observable effect on resistance to avirulent bacterial strains. Induction of disease symptoms by virulent bacteria, however, was markedly reduced in *ein2* mutants of *Arabidopsis*.

MATERIALS AND METHODS

The materials and methods used were those of Whalen *et al.* (1991), except as noted below.

Bacterial strains, plasmids, and plant lines. For *P. s.* pv. *tomato*, strain DC3000 derivatives were used in all cases. The plasmids carrying cloned avirulence genes were pABL18 or pLH12 for *avrRpt2* (Whalen *et al.* 1991), pPSG0002 for *avrB* (Staskawicz *et al.* 1987), K48 for *avrRpm1* (Debener *et al.* 1991). Virulent *P. s.* pv. *tomato* strains were DC3000, or DC3000 carrying pLAFR3 (vector with no insert), or DC3000 carrying pLH12Ω (insertionally inactivated *avrRpt2* on plasmid pLAFR3 [Whalen *et al.* 1991]). *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson strain 2669 from R. Stall (grown on NYGA medium [Daniels *et al.* 1984]) and *P. s.* pv. *maculicola* (McCulloch) Young *et al.* strain 4326 (Whalen *et al.* 1991) were used. Homozygous *ein1*, *ein2*, and *eto1* *Arabidopsis* lines are described by Guzman and Ecker (1990); homozygous *etr1* *Arabidopsis* plants are from Bleeker *et al.* (1988). The ethylene mutants were derived from the same *Arabidopsis* ecotype (Col-0) as was used to study resistance to *P. s.* pv. *tomato* (Whalen *et al.* 1991), allowing integration of the two experimental systems. Plants were grown in growth chambers at 24° C for 8 hr per day.

Inoculation and disease scoring methods. For pipet infiltration, freshly grown bacteria were resuspended and diluted in 10 mM $MgCl_2$ to an OD_{600} of 0.001 (approximately 1×10^6 cfu/ml) for *P. s.* pv. *tomato* and *P. s.* pv. *maculicola* or OD_{600} of 0.02 (approximately 2×10^7 cfu/ml) for *X. c.* pv. *campestris*; a plastic Pasteur pipet was used to introduce approximately 10 μl into the mesophyll of intact leaves. Leaves were scored 5 days after inoculation unless otherwise noted. For each leaf, severity of visible disease symptoms was rated on a scale of 1 (no symptoms) to 5 (confluent necrosis) (Whalen *et al.* 1991). To assay for the hypersensitive response, *P. s.* pv. *tomato* strains were resuspended at OD_{600} of 0.02, applied by pipet infiltration, and leaves were scored for tissue collapse 1 day after inoculation. For surfactant inoculation, freshly grown bacteria were resuspended in 10 mM $MgCl_2$ at an OD_{600} of between 0.2 and 0.3, and Silwet L-77 (Union Carbide, Danbury, CT) was added to a final concentration of 0.02%. Entire rosettes (grown under light of approximately 150 $mmol \cdot s^{-1} \cdot m^{-2}$) were dipped briefly in this solution, maintained at high relative humidity for 1 day and then in normal growth chamber conditions, and lesions were scored on the fourth day after inoculation unless otherwise noted. For vacuum infiltration, freshly grown bacteria were resuspended and diluted in 10 mM $MgCl_2$ to approximately 1×10^5 cfu/ml. Entire rosettes were infiltrated, and plants were returned to growth chambers. For all of the above methods, no disease lesions were observed in control inoculations that did not contain bacteria. For monitoring growth of bacteria in *Arabidopsis* leaves, two leaf disk samples (0.125 cm^2 each) were removed from each vacuum-infiltrated plant at the designated times after infection, samples from two plants were pooled and homogenized in 10 mM $MgCl_2$, and the number of bacterial colony-forming units per square centimeter of leaf area (cfu/cm^2) was determined by dilution plating on King's B agar containing rifampicin (100 mg/L) and cyclohexamide (50 mg/L) (Whalen *et al.* 1991). Bacteria were also enumerated by using individual 0.125-cm^2 disks taken from leaves inoculated by pipet infiltration. Leaf chlorophyll content was determined by the method of Lichtenthaler and Wellburn (1983).

Coronatine. For coronatine preparation, *P. s.* pv. *tomato* strains DC3000 and DC3661 were grown at 16° C in Wooley's medium (Wooley *et al.* 1952). A volume (1/15) of concentrated HCl was added to culture supernatant,

which was then extracted with ethyl acetate. The organic phase was flash-evaporated at 45° C and resuspended either in methanol for storage at −20° C or in H_2O for use in inoculations. Plants were inoculated by placing a 10- or 15-μl droplet on the leaf surface and then piercing the leaf with a syringe needle. Quantities of crude toxin preparation are presented as colony-forming unit equivalents (cfu equivalents), relating toxin concentration (unknown) to the known quantity of original bacterial culture represented in a given toxin aliquot. Pure coronatine was prepared as in Bender *et al.* (1987) and stored in methanol; dried samples were resuspended in H_2O immediately before being used.

RESULTS

The response of *Arabidopsis* ethylene-insensitive and ethylene-overproducing mutants to infection by virulent and avirulent *P. s.* pv. *tomato* strains was first characterized by visual scoring of infected leaves. It had previously been shown that the leaves of resistant plants inoculated with avirulent *P. s.* pv. *tomato* strains at lower bacterial concentrations develop few or no symptoms, whereas susceptible hosts or hosts infected with virulent *P. s.* pv. *tomato* strains display necrotic lesions with chlorotic margins 3–5 days after infection (Whalen *et al.* 1991). When plants were inoculated by pipet infiltration with 1×10^6 cfu/ml of *P. s.* pv. *tomato* carrying *avrRpt2*, we observed that the leaves of *ein1-1*, *ein2-1*, and *eto1-1* mutants resembled wild-type Col-0 and developed only minimal disease symptoms (Table 1). Similar results were obtained with *Arabidopsis* lines carrying different alleles of the ethylene-insensitivity loci, *etr1*, *ein2-3*, *ein2-4*, and *ein2-5* (data not shown).

Disease lesions closely resembling those formed in moderate to severe natural infestations involving *P. s.* pv. *tomato* can be produced by dipping leaves in a bacterial suspension containing the surfactant L-77 or by vacuum infiltration (Whalen *et al.* 1991). Using these methods, we inoculated mutant *ein1-1*, *ein2-1*, and *eto1-1* plants with *P. s.* pv. *tomato* carrying *avrRpt2*. All plant genotypes tested resembled the wild type in displaying only occasional and minor disease symptoms after inoculation with this avirulent strain.

Resistant *Arabidopsis* infected with avirulent bacteria induce a hypersensitive response (HR) within 24 hr (Whalen *et al.* 1991). Pipet inoculation of leaves with a high concentration of bacteria ($>2 \times 10^7$ cfu/ml) provides a convenient assay for active resistance, because the HR is readily detectable 24 hr after inoculation as a large region of collapsed tissue (Whalen *et al.* 1991). This collapse is not observed if the plant lacks resistance or the pathogen lacks avirulence. Wild-type Col-0 plants and those carrying *ein1-1*, *etr1*, *ein2-1*, *ein2-4*, and *eto1-1* mutations were inoculated by this method with isogenic *P. s.* pv. *tomato* DC3000(pABL18) and DC3000(pLAFR3). Mutants for each of the three plant loci reproducibly elaborated a strong HR after inoculation with *P. s.* pv. *tomato* carrying *avrRpt2* and gave no HR in response to virulent controls.

Additional pathogen avirulence genes were sought to test if the above results could be generalized beyond resistance to pathogens carrying *avrRpt2*. Previously cloned avirulence genes were tested for function during infection of *Arabidopsis*, and the well-characterized *avrB* locus from *P. s.* pv. *glycinea* (Coerper) Young *et al.* (Huynh *et al.* 1989; Keen and Buzzel 1990; Staskawicz *et al.* 1987; Tamaki *et al.* 1988) converted virulent *P. s.* pv. *tomato* strains to avirulent on *Arabidopsis* ecotype Col-0 (R. W. Innes, A. F. Bent, and B. J. Staskawicz, unpublished results). In addition, Dangl and colleagues recently isolated a gene (*avrRpm1*) from *P. s.* pv. *maculicola* that causes avirulence in infections of *Arabidopsis* Col-0 (Debener *et al.* 1991). When wild-type, *ein1-1*, *ein2-1*, and *eto1-1* lines were inoculated with *P. s.* pv. *tomato* carrying *avrB* or *avrRpm1*, resistance was observed in all cases. Disease development was minimal in inoculations at the lower bacterial concentration (Table 1). Furthermore, a clear and obvious HR was observed on wild-type, *ein1-1*, *ein2-1*, and *eto1-1* plants after pipet inoculation at 2×10^7 cfu/ml with *P. s.* pv. *tomato* carrying *avrB* or *avrRpm1*, whereas no tissue collapse was seen on separate leaves (on the same plants) inoculated with virulent *P. s.* pv. *tomato* controls.

In contrast to the results with *P. s.* pv. *tomato* strains carrying *avrRpt2*, *avrB*, or *avrRpm1*, the development of disease symptoms following inoculation with virulent *P. s.* pv. *tomato* strains was clearly different for one class of mutants. The disease severity scores for pipet inoculations

Table 1. Disease phenotypes[a] of *Arabidopsis* plants inoculated with avirulent and virulent bacterial pathogens

	***Arabidopsis* line**			
Bacterial strain	**Col-0**	***ein1-1***	***ein2-1***	***eto1-1***
Pseudomonas syringae pv. *tomato* + *avrRpt2*	1.8 ± 0.2 n = 49	2.3 ± 0.2 n = 41	1.2 ± 0.1 e n = 39	2.5 ± 0.3 n = 27
P. s. pv. *tomato* + *avrB*	1.5 ± 0.3 n = 10	1.7 ± 0.3 n = 9	1.0 ± 0.1 n = 8	1.7 ± 0.4 n = 9
P. s. pv. *tomato* + *avrRpm1*	1.3 ± 0.2 n = 15	1.7 ± 0.3 n = 18	1.0 ± 0 n = 9	1.5 ± 0.2 n = 17
Virulent *P. s.* pv. *tomato*	4.1 ± 0.2 n = 42	4.2 ± 0.2 n = 35	2.1 ± 0.2 e n = 35	4.0 ± 0.2 n = 28
Virulent *P. s.* pv. *maculicola*	4.3 ± 0.2 n = 26	4.0 ± 0.4 n = 13	2.2 ± 0.3 e n = 14	4.5 ± 0.2 n = 15
Virulent *Xanthomonas campestris* pv. *campestris*	3.6 ± 0.3 n = 16	3.2 ± 0.2 n = 16	1.9 ± 0.2 e n = 16	4.2 ± 0.2 n = 22

[a]Disease severity scored on a scale of 1 (no symptoms) to 5 (confluent necrosis). Values are the mean ± one standard error of the mean. n is the number of replications. Ratings followed by the letter e are significantly different from the score for Col-0 infected with the same bacterial strain ($P < 0.05$, Wilcoxon two-sample test). Leaves inoculated by pipet infiltration of bacteria at OD_{600} of 0.001 (*P. s.* pv. *tomato* and *P. s.* pv. *maculicola* strains) or OD_{600} of 0.02 (*X. c.* pv. *campestris*); lesions scored 5 days after inoculation (see text).

are presented in Table 1. Col-0, *ein1-1*, and *eto1-1* leaves developed pale necrotic or watersoaked lesions surrounded by chlorotic tissue, but *ein2-1* plants showed almost no chlorosis and less frequent water-soaking. When plants were inoculated with virulent *P. s.* pv. *tomato* by the L-77 surfactant or vacuum infiltration methods, disease symptoms were again observed on wild-type, *ein1-1*, and *eto1-1* genotypes but were largely absent from *ein2-1* plants. Figure 1 shows Col-0 and *ein2* leaves taken from vacuum-infiltrated plants 4 days after inoculation. Col-0 leaves had developed individual and coalesced necrotic lesions and showed extensive chlorosis (*ein1* and *eto1* leaves were extremely similar to Col-0 in appearance). Leaves on *ein2-1* mutants, on the other hand, were for the most part unaffected (Fig. 1). By the seventh day after inoculation, many of the leaves on wild-type Col-0 plants had become completely chlorotic and dead, whereas the *ein2-1* mutants showed only minimal symptoms (not shown). By any of the three inoculation methods, *ein2* leaves that were relatively free of symptoms 4 or 5 days after inoculation remained similarly free of symptoms when observed 2 wk after inoculation.

We also inoculated plants with virulent strains of two other *Arabidopsis* pathogens, *P. s.* pv. *maculicola* (Debener *et al.* 1991; Dong *et al.* 1991; Whalen *et al.* 1991) and *X. c.* pv. *campestris* (Simpson and Johnson 1990; Tsuji *et al.* 1991), to test whether the *ein2* result was in some way unique to the *Arabidopsis-P. s.* pv. *tomato* interaction. After inoculation with *P. s.* pv. *maculicola* or *X. c.* pv. *campestris*, the *ein2* plants again showed significantly less disease than wild type, *ein1-1*, or *eto1-1* plants (Table 1).

In a subset of the experiments summarized in Table 1, we also scored leaves 3 days after inoculation to assay for exceptionally early symptom appearance. The only statistically significant difference from wild-type Col-0 observed was for *eto1-1* leaves inoculated with *X. c.* pv. *campestris* (day 3 disease scores: Col-0-*X. c.* pv. *campestris*, 1.8 ± 0.3; *eto1-1-X. c.* pv. *campestris*, 3.4 ± 0.3). Prematurely strong symptoms were also observed in some *eto1-1* leaves inoculated with virulent *P. s.* pv. *tomato*, but this difference was not reproducible (day 3 disease scores: Col-0-*P. s.* pv. *tomato* 2.9 ± 0.3; *eto1-1-P. s.* pv. *tomato* 3.3 ± 0.2).

Differences in the extent of leaf chlorosis between infected *ein2-1* and wild-type *Arabidopsis* were quantified by chlorophyll assays. By the fourth day after inoculation with virulent *P. s.* pv. *tomato*, total leaf chlorophyll (chlorophyll a + b) in wild-type Col-0 had dropped to 47% of the original level, whereas total chlorophyll only fell to 89% of the original level in *ein2-1* plants (Fig. 2).

To confirm that decreased susceptibility to virulent *P. s.* pv. *tomato* was caused by alterations at *EIN2* and not by other coincidentally present mutations, we examined plants carrying independently derived *ein2* alleles. Leaves were inoculated by pipet infiltration with a 1- × 10^6-cfu/ml suspension of virulent *P. s.* pv. *tomato*. Whereas wild-type Col-0 plants developed extensive lesions, individuals homozygous for *ein2-3*, *ein2-4*, or *ein2-5* resembled *ein2-1* and showed few if any disease symptoms. Mean disease severity scores plus or minus one standard error of the mean for *ein2-3*, *ein2-4*, *ein2-5* plants were 2.3 ± 0.3, 2.2 ± 0.4, and 2.0 ± 0.2, respectively (compare with Table 1). *Arabidopsis* carrying *etr1*, a stronger allele of the *EIN1* locus (Bleeker *et al.* 1988; Guzman and Ecker 1990), were similar to *ein1-1* plants; they developed extensive disease symptoms after inoculation with virulent *P. s.* pv. *tomato* by both pipet and vacuum infiltration methods.

A common characteristic of plant resistance to bacterial infection is the relative restriction of pathogen multiplication within the plant. Populations of virulent *P. s.* pv. *tomato* strains have been shown to increase more than 10^5-fold in *Arabidopsis* Col-0 leaves, whereas isogenic strains carrying the cloned avirulence gene *avrRpt2* on RP4-derived plasmids grow to 50-fold lower levels (Whalen *et*

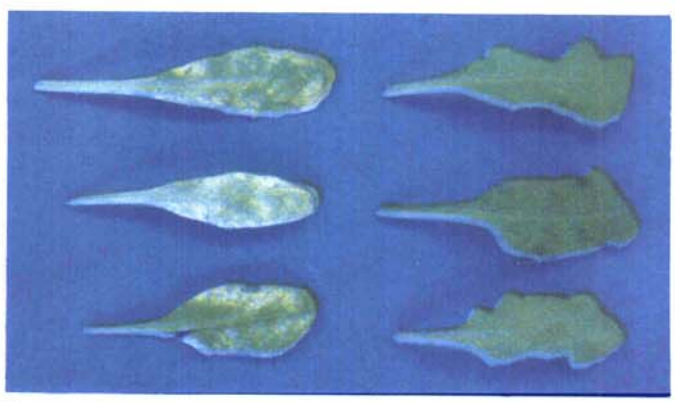

Fig. 1. Leaves of *Arabidopsis* Col-0 and *ein2-1* plants 4 days after inoculation with the virulent *Pseudomonas syringae* pv. *tomato* strain DC3000 (pLH12Ω). Leaves of intact plants were inoculated by vacuum infiltration of a 1- × 10^5-cfu/ml suspension of bacteria in 10 mM $MgCl_2$ (see text). Three representative leaves from each genotype were removed immediately before photography. Left, Col-0; right, *ein2-1*.

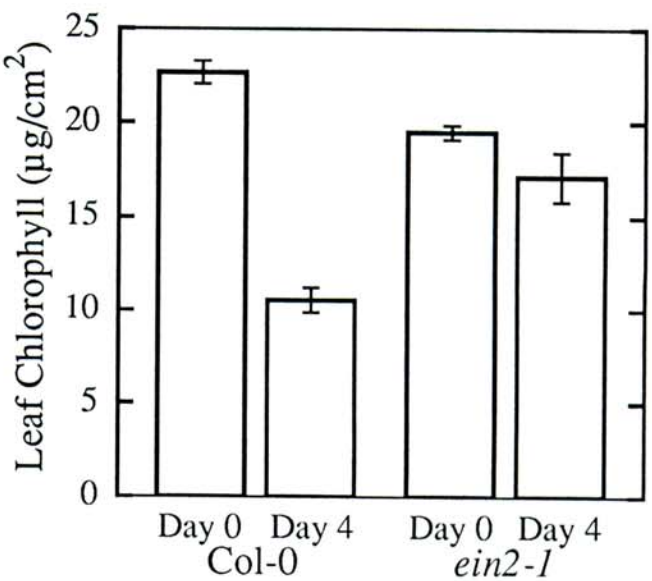

Fig. 2. Loss of chlorophyll in *Arabidopsis* Col-0 or *ein2-1* leaves inoculated with the virulent *Pseudomonas syringae* pv. *tomato* strain DC3000. Plants were inoculated by vacuum infiltration of a 1- × 10^5-cfu/ml bacterial suspension (see text). Leaf samples were taken 1 hr or 4 days after inoculation and assayed for chlorophyll content (Lichtenthaler and Wellburn 1983). Values presented are the mean plus or minus one standard error of the mean; n = 12.

al. 1991). Bacterial population sizes in *ein2* and wild-type Col-0 leaves were examined after inoculation by vacuum infiltration of a solution containing 1×10^5 cfu/ml of bacteria (Fig. 3). As expected from visual scoring of infected leaves, growth of avirulent *P. s.* pv. *tomato* was restricted by wild-type and *ein2-5* plants. Suprisingly, however, the isogenic virulent *P. s.* pv. *tomato* strain (without *avrRpt2*) grew to similar population levels in wild-type and *ein2* plants. Visible damage was minimal on *ein2* plants infected with virulent *P. s.* pv. *tomato* (discussed above and shown in Fig. 1) even though bacteria were present in large numbers. Similar results were obtained with plants carrying the *ein2-1* allele. Five days after inoculation by vacuum infiltration, *P. s.* pv. *tomato* had grown to levels (mean [log(cfu/cm^2)] $\pm$ standard error) of 7.3 ± 0.2 in Col-0 and 7.3 ± 0.1 in *ein2-1*, and 6 days after pipet infiltration leaf *P. s.* pv. *tomato* population sizes of 7.5 ± 0.1 were observed for both Col-0 and *ein2-1*. Thus, symptom development differed between *ein2* and wild-type *Arabidopsis* despite the presence of similar numbers of the virulent *P. s.* pv. *tomato* strain DC3000.

Toxin production plays a significant role in the virulence of bacterial plant pathogens, and *P. s.* pv. *tomato* strains produce a toxin called coronatine that stimulates ethylene production in plants (Gross 1991; Mitchell 1984). We therefore tested if the relative absence of symptoms on *ein2* plants infected with virulent *P. s.* pv. *tomato* was due to a diminished response to coronatine. In the first set of experiments, crude toxin preparations were made from *P. s.* pv. *tomato* DC3000. Tomato (Peto76) plants inoculated with the DC3000 toxin preparations showed the chlorotic response that is characteristic of coronatine treatment (Bender *et al.* 1987; Moore *et al.* 1989). In contrast, *Arabidopsis* Col-0 leaves inoculated with the same preparations developed a strong purple hue in the region surrounding the inoculation site. This coloration is attributable to increased anthocyanin production (J. Greenberg, unpublished results), a commonly observed stress response.

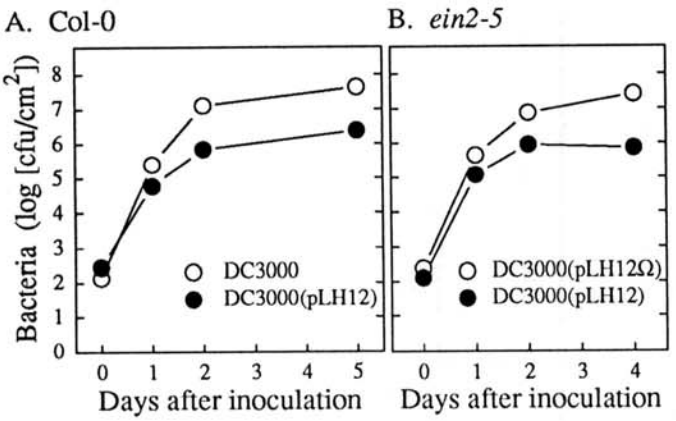

Fig. 3. Growth of virulent (○) or avirulent (●) *Pseudomonas syringae* pv. *tomato* in *Arabidopsis* **A,** Col-0 and in **B,** Col-0 *ein2-5* mutants. DC3000(pLH12) carries *avrRpt2* as a 1.4-kb fragment cloned into pLAFR3; pLH12Ω carries an insertionally inactivated allele of *avrRpt2* (Whalen *et al.* 1991). Leaves of intact plants were inoculated by vacuum infiltration of a 1×10^5-cfu/ml suspension of bacteria and sampled as in text. Values presented are the mean for six plants; standard error of the mean was less than 0.15 log(cfu/cm^2) for every data point.

The severity of the toxin response was dose-dependent over a 500-fold range of concentrations, with discoloration evident after applications of as little as 1×10^7 cfu equivalents (see Materials and Methods) of crude toxin. Extensive anthocyanin production extending into the petiole could be observed after treatment with 5×10^8 cfu equivalents, and entire leaves frequently collapsed after application of $\geq 10^9$ cfu equivalents. Significantly, *ein1-1*, *ein2-1*, and *eto1-1* plants exhibited responses to toxin treatment that were qualitatively very similar to those seen in wild-type Col-0. As a negative control in these experiments, preparations were also made from *P. s.* pv. *tomato* DC3661. This strain is a tox$^-$ derivative of DC3000 carrying a Tn*5* insertion in a locus required for coronatine production (Moore *et al.* 1989). No response was observed in *Arabidopsis* or tomato leaves inoculated with DC3661 preparations.

A second group of experiments was performed with highly purified coronatine (gift of Carol Bender, Oklahoma State University, Stillwater, OK). Fifty nanograms of pure coronatine produced clearly observable responses of similar strength to those observed with approximately 5×10^7 cfu equivalents of crude preparation. Plant reactions to pure coronatine were very similar to those obtained with DC3000 toxin preparations: tomato leaves turned yellow, *Arabidopsis* leaves turned purple, symptoms were dose-dependent, and the responses of *ein1-1*, *ein2-1*, and *eto1-1* plants were qualitatively indistinguishable from those of Col-0.

DISCUSSION

Ethylene has been shown to induce elevated levels of defense-associated mRNAs and proteins, but definitive evidence that ethylene or ethylene-mediated responses are involved in disease resistance is lacking (Dixon and Harrison 1990; Dixon and Lamb 1990; Lamb *et al.* 1989). Our findings with ethylene-insensitive mutants of *Arabidopsis* provide no evidence for a role of ethylene in plant resistance elicited by avirulent pathogens. Despite the inability to respond to ethylene, resistance to *P. s.* pv. *tomato* carrying *avrRpt2* was functional in *ein1* and *ein2* mutants. These plants retained the ability to initiate a HR and to restrict bacterial growth within leaves, and the leaves remained essentially healthy in appearance after inoculation by three different methods. To study resistance specified by different triggering mechanisms, we also examined reactions to bacteria carrying avirulence genes *avrB* and *avrRpm1*. The ethylene mutants examined all expressed active resistance to these strains as well. Although every ethylene response previously examined in *ein1* (including *etr1*) and *ein2* mutants was found to be disrupted (Bleeker *et al.* 1988; Guzman and Ecker 1990), it is possible that these mutations do not knock out all ethylene responses. The functions that are disrupted, however, were clearly not required for macroscopic, whole-plant level resistance in the *A. thaliana-P. s.* pv. *tomato* interaction.

Resistance was also observed when *P. s.* pv. *tomato* carrying *avrRpt2*, *avrB*, or *avrRpm1* was inoculated on *Arabidopsis eto1-1* mutants. The question here was if overproduction of ethylene would make *eto1-1* plants more

resistant. The data in Table 1 give no indication of reduced disease severity relative to the wild type. These mutants are constitutive ethylene overproducers, however, and mature rosettes only make two to three times as much ethylene as wild-type Col-0 (Guzman and Ecker 1990).

Ethylene is produced during susceptible interactions with bacterial, viral, and fungal pathogens (Boller 1991; Pegg 1976; Yang and Hoffman 1984). The utility of this ethylene synthesis is unclear, however, with both beneficial and detrimental roles proposed (Ben-David *et al.* 1986; Boller 1991; Pegg 1976; Stall and Hall 1984; Yang and Hoffman 1984). Mutant *ein2* plants infected with virulent *P. s.* pv. *tomato*, *P. s.* pv. *maculicola*, or *X. c.* pv. *campestris* developed much less severe and/or less frequent disease lesions than wild-type plants. Leaf chlorophyll content provided a quantitative index that differentiated *ein2* from the wild type (Fig. 2), but visual assessment was even more striking (Fig. 1). In many experiments, infected areas of wild-type leaves died, whereas *ein2* leaves almost all survived. Similar phenotypes were observed in plants carrying any of four different *ein2* mutant alleles. The term "resistant" is generally applied to plants that limit the growth of pathogens, whereas plants that survive extensive infection with minimal injury or crop loss are termed "tolerant" (Agrios 1988; Mussel 1980). Similar numbers of bacteria grew in *ein2* and wild-type individuals, indicating that disruption of wild-type *EIN2* function conferred tolerance. Our findings with *ein2* plants suggest that when *Arabidopsis* is infected by virulent bacteria, ethylene-mediated responses are largely detrimental.

If ethylene promotes the development of disease symptoms, then more extensive and/or earlier appearance of bacterial disease lesions on ethylene-overproducing *eto1-1* mutants would be expected. The experiments summarized in Table 1 did not reveal significantly greater lesion severity in *eto1-1* plants (relative to the wild type) for any of the six bacterial strains employed. Because of the small (two- to threefold) and constitutive nature of increased ethylene production in *eto1-1* plants (Guzman and Ecker 1990), however, any effects may have been subtle. When leaves were scored for earlier onset of disease, *eto1-1* leaves did exhibit more severe symptoms than the wild type for one of the pathogens tested (*X. c.* pv. *campestris*). Work with *eto1*, thus, lent only modest additional support to the hypothesis that ethylene promotes disease severity.

It is of interest that *ein1* mutants responded to virulent pathogens like wild-type plants and not like *ein2* plants. All other ethylene-related processes that have been examined are disrupted in both *ein1* and *ein2* mutants, and these mutants had previously been phenotypically differentiated only by the dominance-recessiveness and strength of a given allele (Guzman and Ecker 1990; J. R. Ecker, unpublished data). Taken independently, the results with *ein1* plants suggest that ethylene is not involved in susceptibility, whereas the *ein2* results suggest the opposite. One explanation for the distinct disease phenotypes could be that the *EIN1* gene product is active after a branch point in the ethylene response pathway and is not involved in the generation of pathogen-induced symptoms. Differences in disease phenotype may also be attributable to quantitative differences in ethylene responsiveness among the alleles tested. Alternatively, the disease tolerance observed in *ein2* plants may be due to additional, unknown effects of mutations at the *EIN2* locus that are essentially unrelated to ethylene. Additional research will clearly be necessary to resolve this question.

The disease tolerance of *ein2* mutants could not be attributed to a decrease in sensitivity to coronatine. However, our experiments with this toxin also addressed the open question as to whether coronatine action is mediated by auxin or ethylene (Ferguson and Mitchell 1985; Gross 1991; Mitchell 1984; Sakai 1980). The responsiveness of ethylene-insensitive *Arabidopsis* to coronatine suggests that ethylene is not a required component of the plant response. Our data also support the finding (Gnanamanickam *et al.* 1982) that factors other than coronatine can play the central role in inducing lesion formation after infection by pseudomonads. The difference between the responses observed in *Arabidopsis* and tomato after coronatine treatment was unexpected and may merit additional investigation.

The *Arabidopsis RXC1* locus reduces chlorosis in plants infected by virulent *X. c.* pv. *campestris* strains, without affecting bacterial growth (Tsuji *et al.* 1991). Similarities between these findings and our work with *ein2* plants highlight the possibility that *RXC1* may encode functions related to ethylene metabolism.

Our findings seemingly conflict with the theory that ethylene can induce resistance responses (Boller 1991; Pegg 1976; Yang and Hoffman 1984). However, the infection responses mediated by ethylene could exacerbate the destruction caused by some pathogens yet limit the spread and damage caused by others. It is worth re-emphasizing that our study examined race-specific resistance to necrogenic bacterial pathogens.

Control of ethylene responses is being pursued for the improvement of the quality and longevity of products such as market flowers and fresh tomatoes. Transgenic tomato lines that synthesize less ethylene have recently been constructed by a number of groups (Hamilton *et al.* 1990; Klee *et al.* 1991; Oeller *et al.* 1991); an alternative approach would be to isolate plants that show decreased sensitivity to ethylene. Concern has been voiced, however, that decreases in ethylene production or sensitivity might adversely affect disease resistance. From our data, one might predict that resistance elicited by avirulent pathogens will not be disrupted in these plants. Furthermore, if the tolerance of *ein2* plants is in fact due to disruption of ethylene-mediated responses, this would suggest the possibility of enhancing disease tolerance through engineered reduction of ethylene-mediated responses.

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