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Ethylene Regulates the Susceptible Response to Pathogen Infection in Tomato

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Ethylene evolution occurs concomitantly with the progression of disease symptoms in response to many virulent pathogen infections in plants. A tomato mutant impaired in ethylene perception—*Never ripe*—exhibited a significant reduction in disease symptoms in comparison to the wild type after inoculations of both genotypes with virulent bacterial (*Xanthomonas campestris* pv *vesicatoria* and *Pseudomonas syringae* pv *tomato*) and fungal (*Fusarium oxysporum* f sp *lycopersici*) pathogens. Bacterial spot disease symptoms were also reduced in tomato genotypes impaired in ethylene synthesis (1-aminocyclopropane-1-carboxylic acid deaminase) and perception (14893), thereby corroborating a reducing effect for ethylene insensitivity on foliar disease development. The reduction in foliar disease symptoms in *Never ripe* plants was a specific effect of ethylene insensitivity and was not due to reductions in bacterial populations or decreased ethylene synthesis. *PR-1B1* mRNA accumulation in response to *X. c. vesicatoria* infection was not affected by ethylene insensitivity, indicating that ethylene is not required for defense gene induction. Our findings suggest that broad tolerance of diverse vegetative diseases may be achieved via engineering of ethylene insensitivity in tomato.

INTRODUCTION

Susceptible host plants can incur extensive disease damage from infections by virulent pathogens. Although plant pathologists have focused considerable attention on mechanisms of host and non-host resistance to avirulent pathogens in numerous systems (Bent, 1996), our understanding of the physiology underlying disease development in response to infections by virulent pathogens is relatively limited. Understanding how disease development is regulated in compatible plant–microbe interactions is a first step toward engineering plants that are tolerant to virulent pathogen infections. Plants that are disease tolerant have the ability to produce ample yields even when they are infected by pathogens (Agrios, 1997).

The mechanisms underlying the widespread cell death that occurs during susceptible responses are not understood. Numerous studies have focused on the importance of pathogen-derived compounds to compatible interactions and have demonstrated that phytotoxins produced by some pathogens are critical for host recognition and infection; however, there are only a limited number of cases in which phytotoxins have been demonstrated to directly control disease symptom development (DeVay, 1988; Sigee, 1993; Walton, 1996). It is important to note that infections by organisms that produce phytotoxins, such as *Pseudomonas syringae* pv *tomato* (Palmer and Bender, 1995) and *Fusarium oxysporum* f sp *lycopersici* (DeVay, 1988), and those that do not, such as *Xanthomonas campestris* pv *vesicatoria* (R.E. Stall, unpublished data), cause extensive necroses in susceptible tissues. Pathogen-derived ethylene has been suggested to act as a phytotoxin that accelerates the chlorosis, necrosis, and foliar abscission associated with disease development; however, under in vitro conditions, bacterial and fungal pathogens generally do not evolve enough ethylene to be necrogenic in plant tissues (Pegg and Cronshaw, 1976a; Pegg, 1981). Thus, it is most likely that a host-derived signal(s) regulates the widespread cell death during the susceptible response to pathogen infection.

Because ethylene production is known to cause necroses in plant tissues in response to numerous environmental and developmental cues (Abeles et al., 1992), host-derived ethylene has been hypothesized to be an important signal for disease development. Previous studies have shown a correlation between the timing of increased ethylene evolution in response to pathogen infections and the development of chlorotic and wilting symptoms in many plant species (Goto et al., 1980; Pegg, 1981; Stall and Hall, 1984; Ben-David et al., 1986; Boller, 1991), including tomato (Gentile and Matta, 1975; Pegg and Cronshaw, 1976a, 1976b; Elad, 1990). The accumulation and increased activities of the ethylene biosynthetic enzymes 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase have been localized to chlorotic tissue directly surrounding primary lesions in tobacco leaves

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in response to tobacco mosaic virus (de Laat and Van Loon, 1983) and *Phytophthora infestans* (Spanu and Boller, 1989) infections. The localized activities of ethylene biosynthetic enzymes in tissues surrounding primary lesions suggest that host ethylene production may be a response to the cell death that occurs during primary lesion formation.

Controversy remains as to whether the ethylene produced by the host is a signal that acts to amplify cell death during the susceptible response or whether increased ethylene synthesis is simply correlated with advanced disease symptoms and has no biological significance to the process. Bent et al. (1992) were the first to use a genetic approach to determine whether ethylene perception plays a causal role in foliar disease development. By visually scoring for disease phenotypes and assaying for loss of chlorophyll in Arabidopsis, Bent et al. (1992) determined that ethylene-insensitive *etr1-3* mutants had disease phenotypes similar to those of the wild type when each was infected with virulent strains of *X. c. campestris* and *P. s. tomato.* Disease symptoms were suppressed in ethylene-insensitive *ein2-1* mutants. The *ETR1* gene encodes an ethylene receptor, and *EIN*2 encodes a protein of unknown function that acts downstream of ETR1 in the ethylene signal transduction pathway in Arabidopsis (Guzmán and Ecker, 1990; Bleecker and Schaller, 1996). This finding of a critical function for the downstream ethylene signal transduction element EIN2 in the regulation of disease development is not easily reconciled with the relative lack of importance of ETR1, the ethylene receptor. Bent et al. (1992) concluded that either EIN2 is a critical component of additional pathogen response pathways or the *ein2-1* allele is much stronger than is the *etr1-3* allele in reducing ethylene sensitivity. Although the results with Arabidopsis were suggestive of a role for ethylene in disease development during the susceptible response, the importance of ethylene perception to the process remained to be determined. Our objective in the work presented here was to determine whether there is a clear role for ethylene perception in disease development for the economically important solanaceous species tomato.

We hypothesized that if ethylene is an important host-derived signal that promotes disease development in tomato, then mutant lines impaired in ethylene perception should exhibit reduced disease symptoms when infected by some virulent pathogens. An ethylene-insensitive mutant of tomato, *Never ripe* (*Nr*), has been identified in the Pearson cultivar. *Nr* was identified as a spontaneous, naturally occurring mutation and has been backcrossed to an isogenic Pearson parent for several generations, so it is unlikely that additional mutations are closely linked to *Nr.* The ethylene insensitivity conferred by the *Nr* mutation arises from a single base substitution in the N-terminal coding region of the gene *Le-ETR3* (formerly *TXTR-14*; Wilkinson et al., 1995). *Le-ETR3* encodes an ethylene receptor because normal ethylene responses are suppressed or blocked in *Nr* plants (Lanahan et al., 1994), and *Le-ETR3* expressed in yeast has been demonstrated to create high-affinity, saturable binding sites for labeled ethylene gas (A.B. Bleecker, G.E. Schaller, and H.J. Klee, unpublished data). *Le-ETR3* is one member of a gene family consisting of at least five members in tomato (D.M. Tieman and H.J. Klee, unpublished data). During tomato fruit ripening, *Le-ETR3* is developmentally upregulated, and transcript accumulation is correlated with the climacteric burst in ethylene evolution and increased ethylene sensitivity in the fruit. *Le-ETR3* mRNA accumulation is vastly reduced in *Nr* fruit (Wilkinson et al., 1995).

The *Nr* mutation derives its name from the observation that ripening of homozygous (*Nr*/*Nr*) fruit is delayed and incomplete. The fruit exhibits only a yellow color and marginal softening after many months (Rick and Butler, 1956; Hobson, 1967). In comparison to the wild type, *Nr*/*Nr* hypocotyls exhibit a fourfold increase in elongation when germinated on ACC-containing media in the dark. Floral petal senescence and abscission are greatly reduced in *Nr*/*Nr* plants (Lanahan et al., 1994). Ethylene insensitivity conferred by *Nr* is incompletely dominant. Based on triple-response assays and evaluations of fruit coloration and softening, Lanahan et al. (1994) concluded that heterozygous (*Nr*/+) plants are not as ethylene insensitive as *Nr*/*Nr* plants but are much more ethylene insensitive than the wild type. Although the *Nr* mutant has been well characterized developmentally, the potential effects of *Nr* on environmental responses mediated by ethylene had not been previously investigated.

Here, we report on the effect of ethylene insensitivity conferred by *Nr* on the susceptible response to infections by using three pathogens. We show that *Nr* causes reductions in disease development phenotypes in response to compatible infections with one eukaryotic and two prokaryotic pathogens. We provide evidence that the susceptible response to foliar pathogen infection consists of two stages with regard to ethylene action, ethylene-independent primary lesion formation and ethylene-dependent symptom development.

RESULTS

Ethylene Insensitivity Reduces Foliar Disease Development in Tomato

Early symptoms of foliar diseases include the formation of necrotic primary lesions that later expand and are surrounded by regions of chlorotic tissue. Later symptoms consist of widespread necroses and ultimately leaf abscission (Jones, 1991). To test whether ethylene perception regulates the development of foliar disease symptoms, we inoculated wild-type and *Nr*/*Nr* seedlings with *X. c. vesicatoria* and *P. s. tomato*, which cause bacterial spot and bacterial speck diseases, respectively, in tomato. Both diseases are nonsystemic; thus, symptoms only develop on the leaves that initially are inoculated with the bacteria.

Figure 1 shows that 6 days after inoculation (DAI) with *X. c. vesicatoria* (Figures 1A and 1B) or *P. s. tomato* (Figures 1C and 1D), lesions were detected on the abaxial surfaces of both wild-type (Figures 1A and 1C) and *Nr*/*Nr* (Figures 1B and 1D) leaves. There were no visible differences between the two genotypes in the number or size of the lesions that formed in response to infections by either bacterium (data not shown). Comparisons of disease damage at 16 DAI with *X. c. vesicatoria* (Figure 1E) and *P. s. tomato* (Figure 1F), however, reveal that foliar disease symptoms are greatly reduced in *Nr*/*Nr* leaves in comparison with those in wild-type leaves. These data indicate that *Nr* abates the development of bacterial spot and bacterial speck diseases in tomato after primary lesions are detected but before chlorosis and necroses become confluent in the leaves.

We also inoculated a transgenic tomato line, designated 14893, with *X. c. vesicatoria.* This line is homozygous for a constitutively expressed fusion between the N-terminal putative membrane-spanning region of the ETR1-1 mutant from Arabidopsis and the C-terminal putative histidine kinase region of Le-ETR3 (Wilkinson et al., 1997). Based on triple-response assays (Lanahan et al., 1994), 14893 seedlings exhibit an ethylene-insensitive phenotype similar to that of *Nr*/*Nr* seedlings when each is compared with its corresponding wild-type cultivar (J.Q. Wilkinson, S.T. Lund, and H.J. Klee, unpublished data). Reductions in necrotic symptoms of bacterial spot disease after inoculation of 14893 leaves with *X. c. vesicatoria* (Figure 1G) were similar to those in *Nr*/*Nr* leaves (Figure 1E). The 14893 disease phenotype supports the contention that the reduction in disease damage in *Nr*/*Nr* leaves is due specifically to ethylene insensitivity conferred by *Nr* and is not the effect of some closely linked, additional mutation. The reduced disease phenotype of 14893 seedlings supports a role for ethylene perception in regulating foliar disease development in tomato.

Ethylene Synthesis Is Required for Wild-Type Disease Development in Tomato Leaves

To demonstrate further a role for ethylene in the regulation of foliar disease development, we inoculated another tomato line, designated 8338, that contains the ACC deaminase transgene under the control of a constitutive promoter (Klee et al., 1991). ACC deaminase converts ACC, the substrate for ACC oxidase, to α-ketobutyric acid, thereby inhibiting ethylene biosynthesis. There is a $>$95% reduction in ethylene evolution from leaves and fruits of the ACC deaminase line when compared with the wild type (Klee et al., 1991). Reductions in necrotic symptoms of bacterial spot disease after inoculation of the ACC deaminase line with *X. c. vesicatoria* (Figure 1G) were similar to those in *Nr*/*Nr* leaves (Figure 1E). These data, taken together, indicate that both ethylene synthesis and ethylene sensitivity are critical for foliar disease development in tomato.

Ethylene Insensitivity Causes a Quantitative Reduction in Foliar Disease Damage

To test whether ethylene insensitivity causes a quantitative reduction in foliar disease damage, we measured electrolyte leakage in wild-type and *Nr*/*Nr* leaves inoculated with either *X. c. vesicatoria* or *P. s. tomato.* Electrolyte leakage is a quantitative measure of cell injury or death resulting from pathogen infection (Stall and Hall, 1984). Figure 2A shows that mean electrolyte leakage in *Nr*/*Nr* leaves 15 DAI with *X. c. vesicatoria* was reduced fourfold in comparison with the wild type and was only twofold greater than that in mock-inoculated control plants of both genotypes. Mean electrolyte leakage in *Nr*/*Nr* leaves 12 DAI with *P. s. tomato* was reduced fourfold in comparison with the wild type and was only twofold greater than that in mock-inoculated control plants of both genotypes (Figure 2B). The electrolyte leakage data corroborate the phenotypic evidence shown in Figures 1E and 1F and support a reducing effect for ethylene insensitivity on foliar disease development.

Reduced Foliar Disease Development in *Nr* Seedlings Is a Specific Effect of Ethylene Insensitivity

We next examined whether the reduction in disease development in inoculated *Nr*/*Nr* leaves in comparison with that in the wild type is a specific effect of impaired ethylene perception or if it is due to reductions in bacterial populations or ethylene evolution in the leaves. Figures 3A and 3B, respectively, show that both *X. c. vesicatoria* and *P. s. tomato* population sizes at 5, 9, and 13 DAI were equivalent in extracts from wild-type and *Nr*/*Nr* leaves. Thus, a decrease in bacterial populations cannot account for the reduced foliar disease development in *Nr*/*Nr* leaves. The equivalent bacterial population sizes in wild-type and *Nr*/*Nr* leaves infected with either *X. c. vesicatoria* or *P. s. tomato* demonstrate that ethylene insensitivity confers tolerance of these bacterial diseases to *Nr* seedlings.

In comparison with basal levels, which are shown in Figure 4 at 0 DAI, an increase in ethylene evolution was correlated to the onset of expansion and confluence of chlorotic tissue surrounding the primary lesions (observed from 7 to 10 DAI) from leaves of both genotypes in response to inoculation with *X. c. vesicatoria.* In comparison with the wild type, ethylene evolution was not reduced in *Nr*/*Nr* leaves inoculated with *X. c. vesicatoria* (Figure 4). This finding indicates that a decrease in ethylene evolution cannot account for the reduced foliar disease development in *Nr*/*Nr* leaves.

The increased amount of ethylene produced by *Nr*/*Nr* leaves in comparison with the wild type (Figure 4) is consistent with the finding of increased ethylene evolution detected in an ethylene-insensitive petunia line (Wilkinson et al., 1997). Increased ethylene evolution in infected *Nr*/*Nr* leaves in comparison with the wild-type indicates that ethylene synthesis is regulated by an autoinhibition mechanism that is impaired by ethylene insensitivity. An impaired

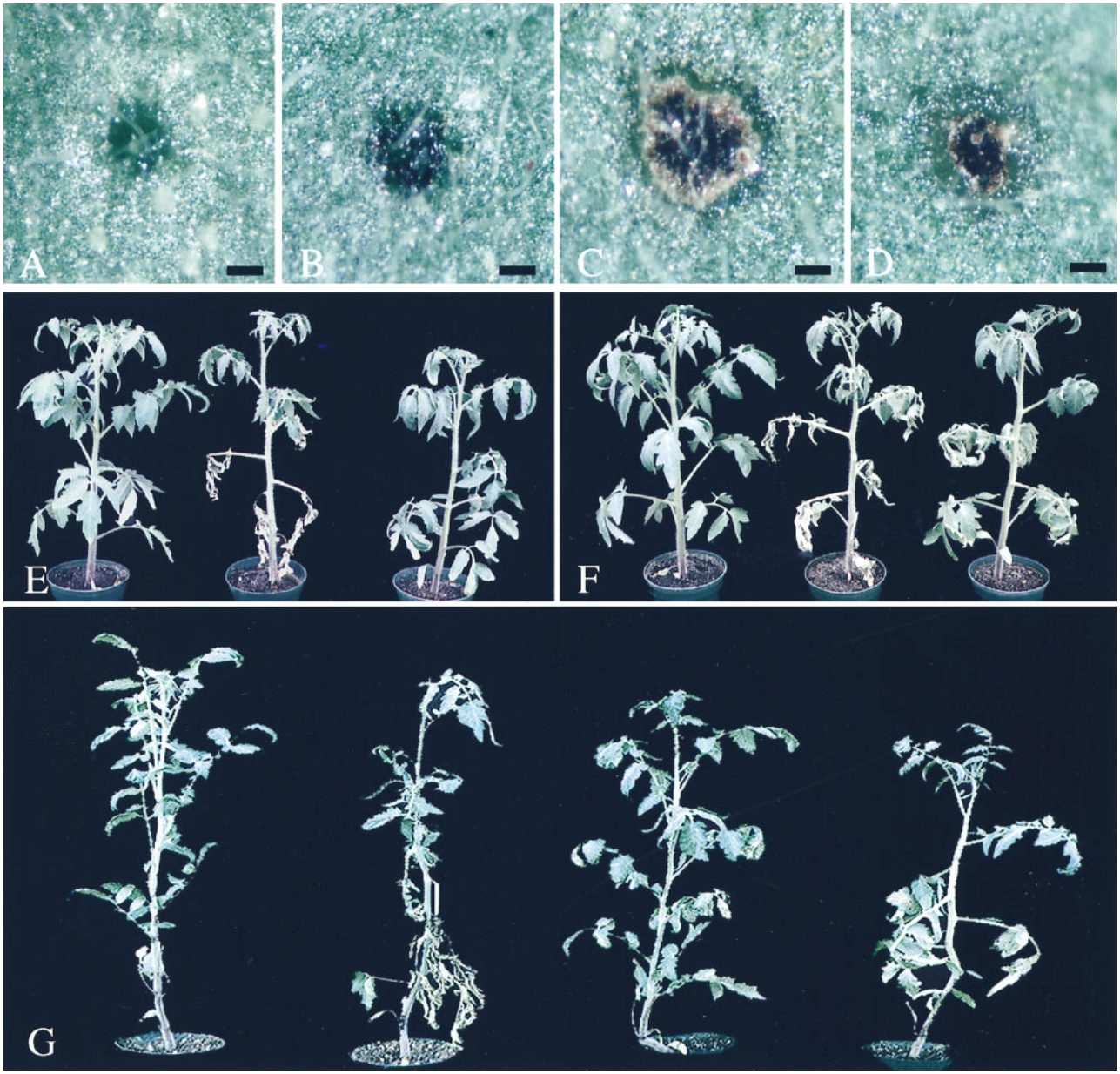

Figure 1. Foliar Disease Phenotypes of Ethylene-Insensitive Genotypes.

(A) A bacterial spot lesion in a wild-type leaf (cv Pearson) 6 DAI with *X. c. vesicatoria.*
(B) A bacterial spot lesion in an *Nr*/*Nr* leaf 6 DAI with *X. c. vesicatoria.*
(C) A bacterial speck lesion in a wild-type leaf (cv Pearson) 6 DAI with *P. s. tomato.*
(D) A bacterial speck lesion in an *Nr*/*Nr* leaf 6 DAI with *P. s. tomato.*
(E) A typical mock-inoculated wild-type plant (cv Pearson, left) and wild-type (cv Pearson, middle) and *Nr*/*Nr* (right) plants 16 DAI with *X. c. vesicatoria.* Note the necrotic, diseased lower leaves in the inoculated wild-type plant versus the relatively healthy appearance of the lower leaves of the inoculated *Nr*/*Nr* plant.
(F) A typical mock-inoculated wild-type plant (cv Pearson, left) and wild-type (cv Pearson, middle) and *Nr*/*Nr* (right) plants 16 DAI with *P. s. tomato.* Note the necrotic, diseased lower leaves in the inoculated wild-type plant versus the relatively healthy appearance of the lower leaves of the inoculated *Nr*/*Nr* plant.
(G) A typical mock-inoculated wild-type plant (cv UC82B, far left) and wild-type (cv UC82B, left), ACC deaminase (right), and 14893 (far right) plants 16 DAI with *X. c. vesicatoria.* Note the necrotic, diseased lower leaves in the inoculated wild-type plant versus the relatively healthy appearance of the lower leaves of the inoculated ACC deaminase and 14893 plants. The UC82B cultivar was used as a control for the 14893 line,

feedback mechanism was also detected in *etr1*/*etr1* leaves in comparison with the wild type when ethylene evolution was measured in each genotype after pretreatment with 10 μL/L ethylene (Bleecker et al., 1988). Inhibition of ethylene synthesis in vegetative tissues is at least partially due to an ethylene-induced reduction in ACC synthase mRNA accumulation (Kim et al., 1997). Taken together, results from the bacterial population size and ethylene evolution experiments indicate that the reduced disease development in *Nr*/*Nr* seedlings is a specific effect of ethylene insensitivity.

Ethylene Insensitivity Confers Increased Survival to Fusarium-Infected *Nr* Seedlings

Many vascular wilt diseases, including Fusarium wilt in tomato, are caused by the gradual, systemic growth of fungal hyphae from the roots into the aerial portion of the seedlings. Fusarium wilt disease symptoms develop over the course of 2 to 4 weeks in young tomato seedlings and consist of foliar chlorosis, wilting, and death of entire seedlings. To test whether ethylene perception is important for the development of Fusarium wilt disease, roots of wild-type and *Nr*/*Nr* seedlings were inoculated with the causal agent, *F. o. lycopersici.* Figure 5A shows that by 21 DAI, inoculated wild-type seedlings were severely stunted in comparison with mock-inoculated wild-type seedlings, and yellowing of some lower leaves was evident. Although inoculated *Nr*/*Nr* seedlings were stunted in comparison with mock-inoculated wild-type and *Nr*/*Nr* seedlings by 21 DAI, they were larger and healthier in appearance than were inoculated wild-type seedlings (Figure 5A). By 35 DAI, all inoculated wild-type seedlings had died, whereas only one *Nr*/*Nr* seedling was necrotic (Figure 5B). We have consistently observed at least 70% survival to maturity in *Nr*/*Nr* seedlings infected with *F. o. lycopersici* in comparison with 0% survival in infected wild-type seedlings. The data shown in Figure 5 indicate that ethylene perception in the host is critical for the development of this systemic fungal disease.

Ethylene Insensitivity Does Not Modify *PR-1* Gene Expression in Response to *X. c. vesicatoria* Infection

In addition to generating visible disease symptoms in plants, pathogen infections have been previously shown to induce the expression of genes that encode defense proteins, including the pathogenesis-related (PR) proteins (Alexander et al., 1994). The local induction of defense gene expression by foliar pathogen infection is likely mediated by endogenous plant signals, including salicylic acid (SA), jasmonic acid, and ethylene (Enyedi et al., 1992). Exogenous ethylene treatments have been shown to induce PR gene expression in leaves of tobacco (Brederode et al., 1991) and tomato (Tornero et al., 1994); however, these experiments were conducted in the absence of pathogen infection. We were interested in determining whether endogenous ethylene is required for *PR-1* gene expression during the susceptible response to pathogen infection in tomato in the absence of exogenous SA, jasmonic acid, or ethylene treatments. If transcript accumulation of the *PR-1* marker were qualitatively or quantitatively regulated by endogenous ethylene in response to virulent pathogen infection, then the levels of marker mRNA accumulation would be absent or reduced, respectively, in *Nr* leaves in comparison with those in the wild type.

We used RNA gel blot analyses of *PR-1B1* mRNA accumulation in wild-type and *Nr*/*Nr* leaves to test whether ethylene insensitivity affects basic *PR-1* gene expression during the susceptible response to *X. c. vesicatoria* infection. Tornero et al. (1993) isolated a cDNA, *PR-1B1*, via differential screening of an ethephon-treated tomato (cv Rutgers) leaf cDNA library and determined that *PR-1B1* encodes a basic member of the tomato *PR-1* family. Tornero et al. (1994) demonstrated that *PR-1B1* mRNA accumulation was inducible by ethephon treatment in tomato leaves. Figure 6 shows that although *PR-1B1* mRNA accumulation was induced by inoculation with *X. c. vesicatoria* in both wild-type and *Nr*/*Nr* leaves, *PR-1B1* mRNA accumulation was not decreased in inoculated *Nr*/*Nr* leaves in comparison with inoculated wild-type leaves. Quantification of hybridization signals corroborated a lack of a reducing effect of ethylene insensitivity on *PR-1B1* mRNA accumulation; maximum *PR-1B1* mRNA accumulation at 10 DAI in inoculated leaves of both genotypes was ~20-fold higher than that in mock-inoculated controls (data not shown). Maximum *PR-1B1* mRNA accumulation in inoculated leaves of both genotypes (Figure 6) was correlated to the timing of increased chlorotic and necrotic symptoms (data not shown). The data shown in Figure 6 indicate that although ethylene may play a role in PR gene induction, wild-type ethylene perception is not required for *PR-1B1* mRNA accumulation in tomato leaves during the susceptible response to *X. c. vesicatoria.*

Figure 1. (continued).

which is in a Floradade background (see Methods). The Floradade cultivar is susceptible to *X. c. vesicatoria* infection, and bacterial spot disease development in Floradade plants is identical to that in the Pearson and UC82B cultivars (data not shown).
Bars in **(A)** to **(D)** = 1 mm.

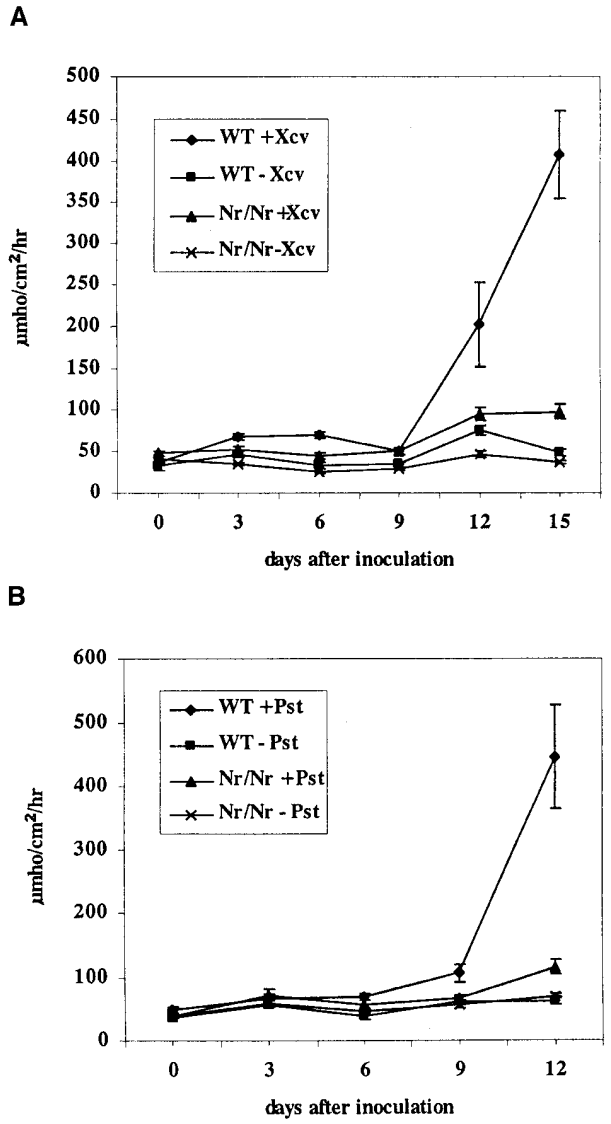

Figure 2. Electrolyte Leakage Response to Foliar Pathogen Infection.

(A) Mean electrolyte leakage response to inoculation with *X. c. vesicatoria.* Vertical bars indicate standard errors. WT, wild type (cv Pearson); + Xcv, inoculated with *X. c. vesicatoria*; − Xcv, mock inoculated.

(B) Mean electrolyte leakage response to inoculation with *P. s. tomato.* Vertical bars indicate standard errors. WT, wild type (cv Pearson); + Pst, inoculated with *P. s. tomato*; − Pst, mock inoculated.

Leaves of 6-week-old wild-type (cv Pearson) and *Nr*/*Nr* seedlings were inoculated with *X. c. vesicatoria* or *P. s. tomato* (10^6 colony-forming units per mL). At the indicated days after inoculation, samples were excised from the leaves and placed in test tubes containing deionized water. Conductivity was measured as described in Methods. μmho, microohms^{-1}.

DISCUSSION

Ethylene Regulates Foliar Disease Development after Primary Lesions Form

The goal of this investigation was to determine whether endogenous ethylene perception regulates susceptible responses in tomato. By comparing foliar disease phenotypes in wild-type and ethylene-insensitive genotypes, we conclude that the regulation of foliar disease development in tomato can be separated into two stages with regard to endogenous ethylene. The early stage occurs independently of ethylene and involves pathogen infection and primary lesion formation, whereas the latter stage requires ethylene and consists of expansion and confluence of the necroses surrounding the primary lesions.

During the early stage of the susceptible response, the host activates diverse defense mechanisms in an attempt to limit infection and spread by the pathogen. These defenses include production of PR proteins, reinforcement of the cell walls, and accumulation of phenolic compounds (e.g., phytoalexins) that are toxic to the pathogen (Dixon and Lamb, 1990; Alexander et al., 1994; Smith, 1996). Evidence for the activation of defense mechanisms during the susceptible response includes the induction of PR gene expression (Van Kan et al., 1992) and hypersusceptiblity of SA-deficient genotypes (Delaney et al., 1994) in response to virulent pathogen infections. If ethylene perception were required for the activation of the defense mechanisms that deter infection and limit pathogen spread, then ethylene-insensitive genotypes would be expected to behave like SA-deficient genotypes (Delaney et al., 1994) and exhibit hypersusceptibility, but this was not evident (Figures 1E to 1G). Furthermore, the lack of an effect of ethylene insensitivity on primary lesion formation and size (Figures 1A to 1D), bacterial population growth (Figures 3A and 3B), and PR gene expression (Figure 6; see also below) in infected leaves indicates that ethylene perception is not important for the regulation of defenses against pathogen infection during the early stage of the susceptible response.

Widespread cell death is symptomatic of the latter stage of the susceptible response. Necrosis of infected tissue presumably occurs because of a failure of host defenses to limit growth and spread of the pathogen. Previously, it was unclear what initiated the widespread cell death that occurs during the susceptible response. Here, we have demonstrated that foliar disease symptoms are greatly reduced in one tomato line deficient in ethylene synthesis (ACC deaminase) and two lines impaired in ethylene perception (*Nr*/*Nr* and 14893) in comparison with the wild-type cultivars. The reductions in widespread cell death in these ethylene-insensitive lines clearly demonstrate that ethylene is a critical inductive signal for disease development in tomato. Although disease symptoms are greatly reduced in the ethylene-insensitive lines, it is important to note that some minor

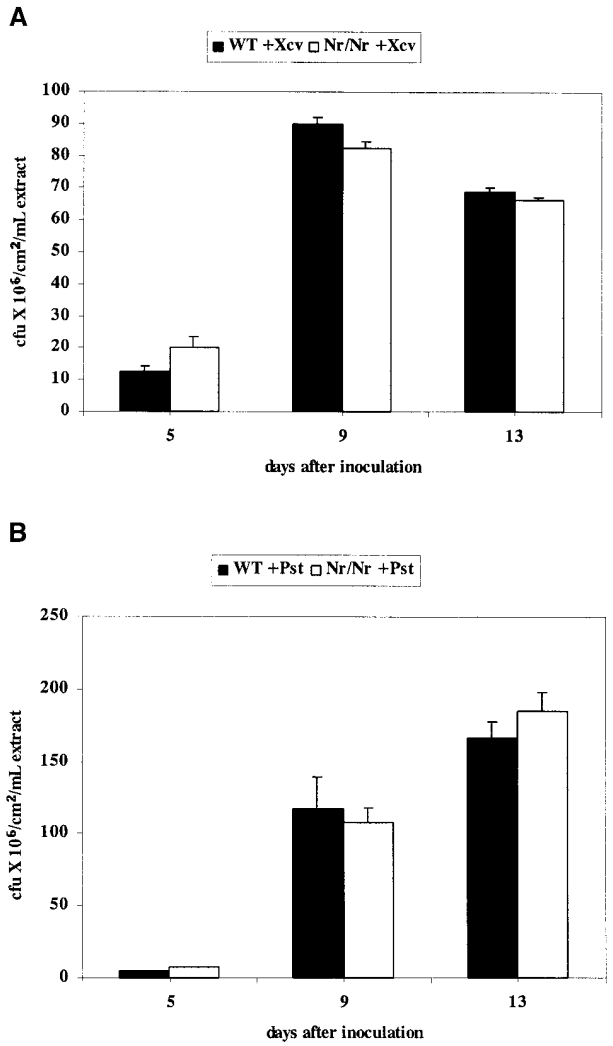

Figure 3. Colony-Forming Units in Extracts from Infected Leaves.

(A) Mean colony-forming units (cfu) in extracts from leaves inoculated with *X. c. vesicatoria.* T-bars indicate standard errors. WT, wild type (cv Pearson); + Xcv, inoculated with *X. c. vesicatoria*.

(B) Mean colony-forming units in extracts from leaves inoculated with *P. s. tomato.* T-bars indicate standard errors. WT, wild type (cv Pearson); + Pst, inoculated with *P. s. tomato*.

Leaves of 6-week-old wild-type (cv Pearson) and *Nr*/*Nr* seedlings were inoculated with either *X. c. vesicatoria* or *P. s. tomato* (10^6 colony-forming units per mL). At the indicated days after inoculation, samples were excised from the leaves and placed in test tubes containing tap water. Samples were ground with a glass rod. Serial dilutions that were made from each of the crude extracts were plated on agar and placed at 30°C for 3 days, at which time colony-forming units were counted as described in Methods.

damage does occur (Figures 1E to 1G and 2). Ethylene by itself cannot induce widespread cell death because an ethylene-overproducing tomato line was shown to be healthy in the absence of pathogen infection (Lanahan et al., 1994). Thus, we conclude that ethylene is a major component of a complex mechanism regulating the spread of necroses that arises during the latter stage of the susceptible response in tomato leaves. To further our understanding of the physiology underlying the susceptible response, we need to determine in future studies how ethylene synthesis is induced and/or augmented after primary lesion formation.

Wild-Type Disease Development in Tomato Requires Ethylene Perception

Our finding of a critical role for a tomato ethylene receptor, Le-ETR3, in the regulation of disease development in tomato is different from that found for ETR1 in Arabidopsis. Like *Le-ETR3*, the *ETR1* gene in Arabidopsis encodes an ethylene receptor. The structure of ETR1 is similar to that of two-component signal transduction regulators in bacteria (Chang and Meyerowitz, 1995). ETR1 is comprised of (1) an N-terminal hydrophobic region, which is membrane localized and most likely involved in sensing the ethylene signal (Schaller et al., 1995); (2) a histidine kinase signaling region; and (3) a C-terminal response regulator region (Bleecker and Schaller, 1996). All characterized mutations in genes encoding ethylene receptors reside within the hydrophobic region and could potentially affect ethylene binding. The function of the response regulator has not been elucidated, but it may

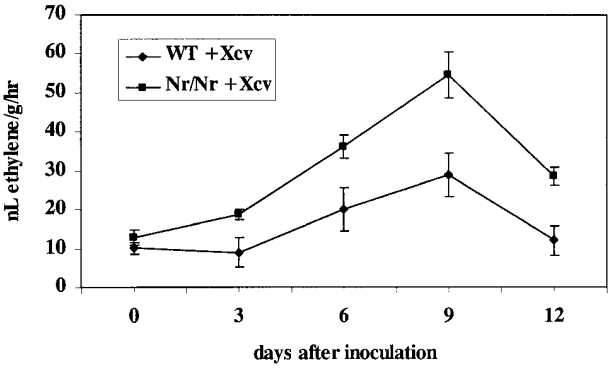

Figure 4. Ethylene Evolution Response in Leaves Infected with *X. c. vesicatoria.*

Leaves of 6-week-old wild-type (cv Pearson) and *Nr*/*Nr* seedlings were inoculated with *X. c. vesicatoria* (10^6 colony-forming units per mL). Leaflets were removed, placed in test tubes for 30 min to allow the escape of wound-responsive ethylene, and then sealed for 2 hr. Gas chromatography was performed as described in Methods. Vertical bars indicate standard errors. WT, wild type (cv Pearson); + Xcv, inoculated with *X. c. vesicatoria.*

Figure 5. Fusarium Wilt Phenotype of *Nr*/*Nr*.

(A) Mock-inoculated wild-type (cv Pearson, far left), inoculated wild-type (cv Pearson, left), inoculated *Nr*/*Nr* (right), and mock-inoculated *Nr*/*Nr* (far right) plants 21 DAI with *F. o. lycopersici.*

(B) A mock-inoculated wild-type plant (cv Pearson, far left), inoculated wild-type (cv Pearson) plants (top row), inoculated *Nr*/*Nr* plants (bottom row), and a mock-inoculated *Nr*/*Nr* plant (far right) 35 DAI with *F. o. lycopersici.*

Roots of 3-week-old wild-type (cv Pearson) and *Nr*/*Nr* seedlings were inoculated with a suspension of *F. o. lycopersici* and transplanted to sterile soil, as described in Methods.

modulate the activity of the histidine kinase. If the histidine kinase domain maintains the ethylene signal transduction pathway "off" in the absence of ethylene binding, as has been hypothesized previously (Bleecker and Schaller, 1996), then mutant ethylene receptors that lack the response regulator may have unrestricted histidine kinase activity and thus a greater ability to maintain ethylene responses off than do mutant ethylene receptors that contain the response regulator. Whereas ETR1 contains the response regulator, Le-ETR3 lacks this domain (Wilkinson et al., 1995). We are currently using several ethylene-insensitive lines in Arabidopsis to test the hypothesis that the basis for the decrease in foliar disease development in *Nr* versus the lack thereof in *etr1-3* is the structural difference between Le-ETR3 and ETR1.

Endogenous Ethylene Is Not Required for PR Gene Expression during the Susceptible Response in Tomato Leaves

The equivalent levels of PR mRNA accumulation detected in wild-type and *Nr*/*Nr* leaves (Figure 6) are consistent with lack of a role for ethylene perception in regulating defense responses in compatible interactions. Previous studies have implicated ethylene in the control of expression of PR genes in response to biotic and abiotic elicitors of defense responses (Chappell et al., 1984; Broglie et al., 1986; Ecker and Davis, 1987; Roby et al., 1991). In these studies, whole plants were usually treated with ethylene gas, even though pathogen-induced ethylene evolution in vivo is likely restricted to specific sites within the leaves (de Laat and Van Loon, 1983; Spanu and Boller, 1989). In other studies, ethylene was applied to individuals via treatment with ethephon, which is converted to ethylene, hydrochloric acid, and phosphorous acid in plants.

Because Brederode et al. (1991) demonstrated in tobacco (cv Samsun) that ethephon treatments primarily induced the accumulation of mRNAs encoding basic PR proteins, we tested whether endogenous ethylene perception is important for the accumulation of mRNAs encoding a basic *PR-1* protein in tomato, a close relative of tobacco. Although *PR-1B1* mRNAs accumulated in tomato leaves in response to infection with a virulent bacterial pathogen, this accumulation was not affected by ethylene insensitivity (Figure 6). This finding indicates that there are multiple mechanisms that regulate defense gene expression in response to diverse environmental cues, including virulent pathogen infections and exogenous ethylene treatments. The induction of PR gene expression by ethephon may be mediated by the catabolites of the compound and by additional stress signals to ethylene, such as SA (Lawton et al., 1994; Tornero et al., 1997). We are currently expanding our investigations to include the role of SA, relative to that of ethylene, in regulating components of the susceptible response to foliar infection by *X. c. vesicatoria.*

Ethylene-Induced Symptom Development Is a Host Response to Virulent Pathogen Infections

X. c. vesicatoria, *P. s. tomato*, and *F. o. lycopersici* are three diverse microorganisms that comprise two genera of

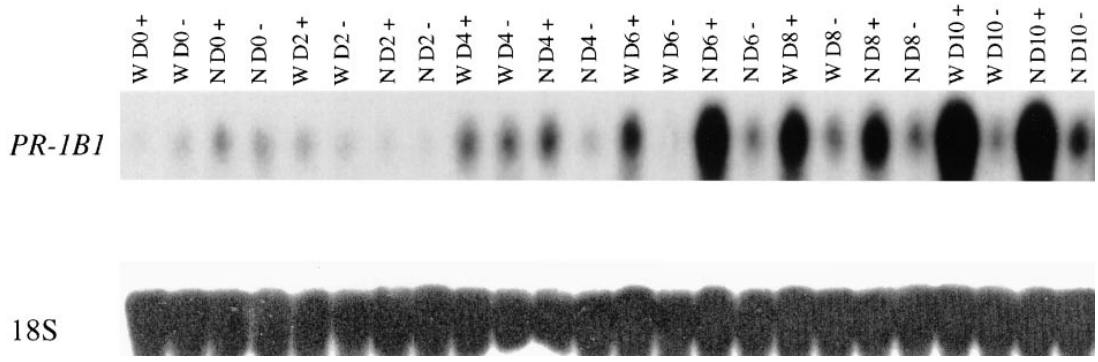

Figure 6. RNA Gel Blot Analysis of *PR-1B1* Expression in Response to Inoculation with *X. c. vesicatoria.*

PR-1B1 (top row) and 18S (bottom row) RNA accumulation in wild-type (cv Pearson) and *Nr*/*Nr* leaves inoculated with *X. c. vesicatoria* is shown. Total RNA was extracted from leaves of 6-week-old wild-type (cv Pearson) and *Nr*/*Nr* leaves inoculated with *X. c. vesicatoria* (10^6 colony-forming units per mL). Ten micrograms of total RNA from each sample was electrophoresed and blotted onto a charged membrane. Blotted RNA samples were probed with either the *PR-1B1* or 18S probes. Replicate RNA gel blot analysis was performed with wild-type (cv Pearson) and *Nr*/*Nr* leaves 12 DAI or mock inoculation with *X. c. vesicatoria*; this was an inoculation separate from that used to generate the data shown in Figure 6. Results from this replicate experiment (data not shown) corroborated a lack of a reducing effect of ethylene insensitivity on *PR-1B1* mRNA accumulation. W, wild type (cv Pearson); N, *Nr*/*Nr*; D0 to D10, 0 to 10 DAI, respectively; +, inoculated; −, mock inoculated.

prokaryotes and one eukaryotic species. Infections by all of these biotrophic pathogens can cause widespread necroses in susceptible tomato cultivars. It seems paradoxical that organisms that must gain nourishment from living tissues would also induce host cell death. In the case of foliar infections by virulent biotrophs, the widespread necroses and foliar abscission may constitute a host strategy in which individual organs are sacrificed to remove the invading pathogen. We postulate, then, that host-derived ethylene is a major component of a drastic response mechanism to foliar infections in susceptible tomato seedlings involving cellular disruption, electrolyte leakage, spread of necroses, and ultimately, foliar abscission.

Host ethylene perception may be an important factor in the development of vascular wilt symptoms that occur in response to systemic infection by *F. o. lycopersici.* VanderMolen et al. (1983) demonstrated that ethylene treatment of intact castor bean plants and excised leaves promoted host gel formation and xylem occlusion, which lead to the wilt phenotype of vascular wilt diseases. Treatment of castor bean leaves with an ethylene synthesis inhibitor drastically reduced the ability of a crude *F. o.* f sp *cubense* enzyme extract to induce xylem occlusion. VanderMolen et al. (1983) concluded that host ethylene signal transduction is required for the xylem occlusion response to infections by wilt pathogens. Considering the findings of VanderMolen et al. (1983), the increased survival of *Nr* seedlings infected with *F. o. lycopersici* is intriguing and will require a more detailed investigation into whether ethylene insensitivity affects xylem occlusion and systemic growth of the fungal hyphae.

From a practical standpoint, we conclude that wild-type ethylene sensitivity is a deleterious agronomic trait in the compatible plant–microbe interactions studied here in tomato. Ethylene insensitivity confers tolerance of bacterial pathogen infections in tomato leaves, causes reductions in vascular wilt symptoms, and does not impart reduced resistance to *Nr* seedlings. In compatible interactions with tolerant plants, pathogen populations are not affected, and selective pressure on the pathogen population for increased virulence is likely minimal or absent. Thus, tolerance is thought to be a more durable means of disease control than is resistance due to the abilities of many avirulent pathogens to regain virulence via natural selection (Crute and Pink, 1996). The results presented here indicate that engineering of ethylene insensitivity in vegetative tissues of tomato can potentially effect durable disease tolerance to a broad spectrum of virulent pathogens.

METHODS

Plant Materials

Seedlings of the homozygous mutant *Nr*/*Nr* (Lanahan et al., 1994), the transgenic line homozygous for the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase construct (8338; Klee et al., 1991), the transgenic line homozygous for the mutant *ETR1–Le-ETR3* fusion (14893; Wilkinson et al., 1997), and the wild-type *Lycopersicon esculentum* cvs Pearson and UC82B were grown in pots under ambient temperature and light conditions in a greenhouse. The 8338 line is in a UC82B background, and the 14893 line is in a Floradade background. Because susceptibility to bacterial spot disease in Floradade is the same as that in UC82B (data not shown), UC82B was used as the wild-type control for the 14893 line.

Inoculum Culture and Inoculations

Bacterial suspensions were prepared by growing *Xanthomonas campestris* pv *vesicatoria* (tomato race 1) or *Pseudomonas syringae* pv *tomato* (race 0, DC3000) overnight at 30°C in nutrient broth (0.8% BBL; Becton Dickinson, Cockeysville, MD) and adjusted to an OD_{600} of 0.4. Ten milliliters of the suspension was diluted 1:100 (v/v) in 0.025% Silwet L-77 (Union Carbide Corp., New York, NY) to a final concentration of 10^6 colony-forming units/mL. The aerial portions of 6-week-old seedlings of the genotypes *Nr*, ACC deaminase, and 14893 and the tomato cultivars Pearson and UC82B were dipped for 15 sec in suspensions containing either *X. c. vesicatoria* or *P. s. tomato.* Mock inoculations were done in an identical manner in 0.025% Silwet L-77. All plants were maintained in a greenhouse under ambient conditions. Subjects for photography were chosen from a population of at least 12 plants per genotype per treatment. All lesions and plants shown for each genotype and treatment are typical. For all experiments with bacterial pathogens described below, samples were taken from only the three most apical leaflets from leaves four to five nodes apical to the cotyledons per genotype per treatment per day.

Fungal suspensions were prepared by streaking stabs of *Fusarium oxysporum* f sp *lycopersici* (race 3) on four 15 × 100 mm plates containing solidified media (1.5% potato dextrose agar) and growing for 3 days at 30°C. Agar containing fungal hyphae and conidia from each of the four plates was combined and ground for 1 min in 200 mL of sterile H_2O in a surface-sterilized blender. The root systems of 3-week-old *Nr*/*Nr* and wild-type seedlings were washed and dipped for 15 sec in sterile H_2O (mock inoculated) or a suspension containing *F. o. lycopersici* (inoculated) before transplanting to sterilized soil. All plants were maintained in a greenhouse under ambient conditions. All experiments were repeated at least once; typical results are shown.

Bacterial Colony Counts

Leaf discs (1 cm^2) were sampled from each of two leaflets from four or five plants per genotype per day. Samples were ground in tap water. Serial dilutions of each sample were plated on solidified nutrient agar (0.8% BBL, 1.5% Difco Bacto-agar; Difco Laboratories, Detroit, MI) and placed at 30°C. *X. c. vesicatoria* colony-forming units were counted on each plate after 3 days, and these counts were used to calculate mean colony-forming units per square centimeter per milliliter of extract.

Electrolyte Leakage and Ethylene Evolution Assays

For electrolyte leakage measurements, 3-cm^2 leaf discs (six discs) were sampled from each of nine to 12 leaflets from three or four plants per genotype per day and placed in tubes containing 3 mL of

deionized H_2O. Conductivity in each sample was assayed immediately after and again at 1 hr after isolation of the leaf discs. After the initial conductivity measurements, vacuum (−25 psi) was applied to the samples for 1 min, and then samples were placed at 37°C with constant shaking (175 rpm). After 1 hr, tubes were removed and vortexed briefly before taking the second conductivity measurements. Change in microohms^{-1} per hr (designated as μmho/hr) was used to calculate mean electrolyte leakage and standard errors.

For ethylene evolution, 15 leaflets from five plants per genotype per day were analyzed. Leaflets were excised and placed in test tubes for 30 min to allow for the escape of wound ethylene, after which time the tubes were sealed for 2 hr. Air samples were then analyzed for ethylene content on a gas chromatograph (model 10A10; Photovac, Inc., Thronhill, Ontario, Canada) equipped with an HP integrator (model 3390A; Hewlett Packard Co., Avondale, PA).

PR Gene Cloning and RNA Gel Blot Analyses

A 348-bp fragment containing *PR-1B1* coding sequence was cloned into pCR-Script (Stratagene, La Jolla, CA) after polymerase chain reactions of tomato (cv Pearson) genomic DNA with forward (5′-TTTCACTCTTGTGAGGCCCA-3′) and reverse (5′-TGCCCGACCACAACCTAGTC-3′) primers. A 2.5-kb 18S rDNA clone from *Zamia floridana* was a gift from R. Ferl (University of Florida, Gainesville).

Total RNA was extracted from 1-g leaflets per genotype per inoculation per day with phenol–SDS, followed by precipitation in 2 M LiCl. Leaflets were sampled from 3 to 5 plants per genotype per inoculation per day. Ten micrograms of total RNA was loaded in all lanes, and electrophoresis was conducted in 1.2% agarose gels containing formaldehyde. Total RNA was blotted overnight onto a charged membrane (Hybond-N$^+$; Amersham Life Science, Inc., Arlington Heights, IL) and then was subjected to ultraviolet cross-linking for 30 sec and air drying for 2 hr.

All prehybridizations were performed at 42°C for 2 to 3 hr (50% formamide, 5 × Denhardt's solution [1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA], 5 × SSPE [1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4], 1% SDS, and 100 μg denatured salmon sperm DNA). ^{32}P-labeled *PR-1B1* and 18S probes were synthesized by random priming (Prime-It II kit; Stratagene). Hybridizations with both probes were done overnight at 42°C in fresh prehybridization solution. Washes for *PR-1B1* analyses were done for 30 min at 65°C (0.3 × SSPE and 0.1% SDS). Washes for 18S analyses were done for 60 min at 80°C (0.1 × SSPE and 0.1% SDS).

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