Response to *Xanthomonas campestris* pv. *vesicatoria* in Tomato Involves Regulation of Ethylene Receptor Gene Expression[1]

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Although ethylene regulates a wide range of defense-related genes, its role in plant defense varies greatly among different plant-microbe interactions. We compared ethylene's role in plant response to virulent and avirulent strains of *Xanthomonas campestris* pv. *vesicatoria* in tomato (*Lycopersicon esculentum* Mill.). The ethylene-insensitive *Never ripe* (*Nr*) mutant displays increased tolerance to the virulent strain, while maintaining resistance to the avirulent strain. Expression of the ethylene receptor genes *NR* and *LeETR4* was induced by infection with both virulent and avirulent strains; however, the induction of *LeETR4* expression by the avirulent strain was blocked in the *Nr* mutant. To determine whether ethylene receptor levels affect symptom development, transgenic plants overexpressing a wild-type *NR* cDNA were infected with virulent *X. campestris* pv. *vesicatoria*. Like the *Nr* mutant, the *NR* overexpressors displayed greatly reduced necrosis in response to this pathogen. *NR* overexpression also reduced ethylene sensitivity in seedlings and mature plants, indicating that, like LeETR4, this receptor is a negative regulator of ethylene response. Therefore, pathogen-induced increases in ethylene receptors may limit the spread of necrosis by reducing ethylene sensitivity.

Plant response to pathogen infection can determine both the extent of pathogen growth and the amount of damage caused by it. During a compatible interaction, a virulent pathogen spreads from the point of entry and causes cell damage far beyond the site of infection. During an incompatible interaction, cell death is limited to the site of infection and colonization of the plant by the avirulent pathogen is greatly reduced. An incompatible interaction often results in a hypersensitive response in which damage is limited to the rapid death of a small number of cells (Goodman and Novacky, 1994).

Several differences between compatible and incompatible interactions may explain how the plant limits both pathogen growth and cell death. One of the first differences is the greater increase in reactive oxygen intermediates observed during an incompatible interaction (Keppler et al., 1989; Orlandi et al., 1992). This oxidative burst may kill the pathogen directly (Keppler et al., 1989; Wu et al., 1995) or limit its spread by killing infected plant cells (Greenberg et al., 1994) and inducing cross-linkage of cell wall proteins (Bradley et al., 1992; Brisson et al., 1994). During an incompatible interaction, cell walls are also strengthened through increased deposition of hydroxy-Pro-rich glycoproteins (Showalter et al., 1985), callose (Parker et al., 1993), and lignin (Moerschbacher et al., 1990). Infection with an avirulent pathogen often causes a stronger and more rapid increase in pathogenesis-related (PR) proteins (Linthorst, 1991), which may enhance resistance to fungi (Broglie et al., 1991; Zhu et al., 1994). Increased synthesis of other antimicrobial compounds such as phytoalexins (Hain et al., 1993), thionins (Epple et al., 1995), and defensins (Penninckx et al., 1996) observed during incompatible interactions may also limit pathogen growth. Many of these resistance responses are also a component of compatible interactions, but occur much later in the progression of the disease (Staskawicz et al., 1995). Therefore, whether infection results in a compatible or an incompatible interaction may be determined more by the speed of the response than by qualitative differences between these interactions.

Synthesis of the plant hormones salicylic acid (SA), ethylene, and jasmonic acid increases greatly during many incompatible interactions (Malamy et al., 1990; Boller, 1991; Penninckx et al., 1996). These hormones regulate a wide range of defense-related genes, making them likely candidates as signals that coordinate plant response to pathogens (Penninckx et al., 1998; Thomma et al., 1998). SA in particular is essential in

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mounting the resistance response in many plant-pathogen interactions (Delaney et al., 1994). Ethylene regulates several genes involved in defense responses, including those encoding PR proteins such as chitinases, β-1,3-glucanases, and PR1 (Deikman, 1997), phytoalexin synthesis enzymes (Ecker and Davis, 1987), defensin (Penninckx et al., 1996), and hydroxy-Pro-rich glycoproteins (Toppan et al., 1982). Since ethylene induces the expression of many defense-related genes, increasing ethylene synthesis during infection would be one way of initiating a defense response. Increased ethylene synthesis in infected tissue has been reported for a wide range of pathogens (Boller, 1991). For some, such as tobacco mosaic virus in tobacco (*Nicotiana tabacum*) and *Uromyces phaseoli* in bean (*Phaseolus vulgaris*), the increase in ethylene levels during an incompatible interaction is greater and more rapid than during a compatible interaction (Montalbini and Elstner, 1977; De Laat and van Loon, 1983). This increase in ethylene synthesis may be one way that the plant activates a more rapid defense response after infection with an avirulent pathogen.

Ethylene responses can also be regulated by changes in ethylene perception. Several genes encoding ethylene receptors have been isolated from Arabidopsis (Chang et al., 1993; Hua et al., 1995, 1998) and tomato (*Lycopersicon esculentum* Mill.) (Wilkinson et al., 1995; Lashbrook et al., 1998; Tieman and Klee, 1999). In Arabidopsis, loss-of-function mutations in four of these receptor genes, *ETR1*, *ETR2*, *EIN4*, and *ERS2*, have been identified. Plants containing all four of these mutations showed strong constitutive ethylene responses, demonstrating that these receptors are negative regulators of the ethylene response (Hua and Meyerowitz, 1998). In tomato, five different members of an ethylene receptor gene family, *LeETR1*, *LeETR2*, *NR*, *LeETR4*, and *LeETR5*, have been isolated. In transgenic tomato plants, reduced expression of one of these receptor genes, *LeETR4*, also resulted in constitutive ethylene responses such as leaf epinasty and flower senescence, indicating that a reduction in receptor level causes an increase in ethylene sensitivity (Tieman et al., 2000). Although the effect of increasing ethylene receptor levels has not been reported previously, the evidence cited above suggests that an increase in receptors would reduce sensitivity. Therefore, plants may be capable of reducing sensitivity of specific tissues through the induction of receptor gene expression. To determine whether greater ethylene receptor gene expression does in fact reduce ethylene sensitivity, we analyzed transgenic tomato plants overexpressing the wild-type *NR* gene.

In contrast to the loss-of-function mutants, Arabidopsis plants containing dominant mutations in the ethylene receptor genes are insensitive to ethylene. For one of these mutants, *etr1-1*, this insensitivity is due to the inability of the mutant ETR1 protein to bind ethylene (Schaller and Bleecker, 1995). In wild-type plants, binding of ethylene by the receptor is thought to inactivate its function as a negative regulator, allowing the ethylene response to occur. Since the mutant receptors are unable to bind ethylene, they cannot be inactivated and remain constitutive suppressors of the ethylene response (Hua and Meyerowitz, 1998). The tomato *NR* gene is homologous to *ETR1* and other Arabidopsis ethylene receptor genes and, like ETR1, the wild-type NR protein is able to bind ethylene (G.E. Schaller, F. Rodriguez, and A.B. Bleecker, personal communication). The *Never ripe* (*Nr*) mutant displays ethylene insensitivity in several developmental processes, including hypocotyl elongation, flower senescence, and fruit ripening (Lanahan et al., 1994).

Analysis of ethylene-insensitive plants in several different species has demonstrated a role for ethylene in both compatible and incompatible interactions, yet the effect of ethylene insensitivity on pathogenesis varies greatly among pathogens. In tomato, the ethylene-insensitive *Nr* mutant showed increased tolerance to virulent strains of *Fusarium oxysporum*, *Pseudomonas syringae* pv. *tomato*, and *Xanthomonas campestris* pv. *vesicatoria* (Lund et al., 1998). In Arabidopsis, the ethylene-insensitive *ein2* mutant displayed increased tolerance to virulent strains of the bacterial pathogens *P. syringae* pv. *tomato* and pv. *maculicola* as well as *X. campestris* pv. *campestris* (Bent et al., 1992). However, ethylene-insensitive transgenic tobacco (*Nicotiana tabacum*) plants expressing a mutant form of the Arabidopsis ETR1 ethylene receptor gene were more susceptible to the soil-borne fungal pathogen *Pythium sylvaticum* (Knoester et al., 1998). Soybean (*Glycine max*) mutants with reduced ethylene sensitivity displayed less severe symptoms in response to virulent *P. syringae* pv. *glycinea* and *Phytopthora sojae*, but more severe symptoms to *Septoria glycines* and *Rhizoctonia solani* (Hoffman et al., 1999).

The role of ethylene in incompatible interactions also appears to vary from one pathogen to another. Ethylene-insensitive Arabidopsis mutants maintained their resistance to *P. syringae* pv. *tomato* (Bent et al., 1992), *Peronospora parasitica*, and *Alternaria brassicicola* (Thomma et al., 1999). Likewise, ethylene-insensitive transgenic tobacco plants were resistant to an incompatible strain of tobacco mosaic virus (Knoester et al., 1998), and soybean mutants with reduced ethylene sensitivity maintained resistance to avirulent *P. syringae* pv. *glycinea* (Hoffman et al., 1999). However, resistance to the avirulent fungal pathogen *P. sojae* was compromised in these soybean mutants (Hoffman et al., 1999), and an ethylene-insensitive Arabidopsis mutant was more susceptible to a normally avirulent strain of the fungus *Botrytis cinerea* (Thomma et al., 1999). Therefore, ethylene is

involved in the resistance response for some plant-pathogen interactions.

We compared the role of ethylene in compatible and incompatible interactions with *X. campestris* pv. *vesicatoria*, the causal agent of bacterial spot in tomato and pepper (*Capsicum annuum*). Wild-type and ethylene-insensitive *Nr* mutant plants were infected with virulent (Xv 93-1) and avirulent (Xv 87-7) strains of this pathogen. Transgenic tomato plants overexpressing the wild-type NR protein were also infected to determine the effect of increased NR expression on disease development.

RESULTS

Response to *X. campestris* pv. *vesicatoria* Infection in Wild-Type and *Nr* Mutant Plants

Both wild-type and *Nr* plants infected with the compatible strain of *X. campestris* pv. *vesicatoria* first developed water-soaked lesions on leaves 5 to 6 d after inoculation (DAI). Chlorosis began in the wild-type plants 10 to 12 DAI. In the wild-type plants, this area of chlorosis enlarged and was followed by complete necrosis of entire leaflets 13 to 14 DAI. As reported previously (Lund et al., 1998), in the *Nr* mutant chlorosis and the spread of necrosis were greatly reduced (Fig. 1A). Wild-type and *Nr* plants inoculated with the incompatible *X. campestris* pv. *vesicatoria* strain Xv 87-7 developed small, light-brown lesions on the abaxial surface of the leaf 3 to 4 DAI. These lesions darkened and increased in quantity up to 5 DAI, but did not increase in size or quantity after this time. There was no further symptom development except for small areas of necrosis that developed along the margins of a few leaflets (Fig. 1A).

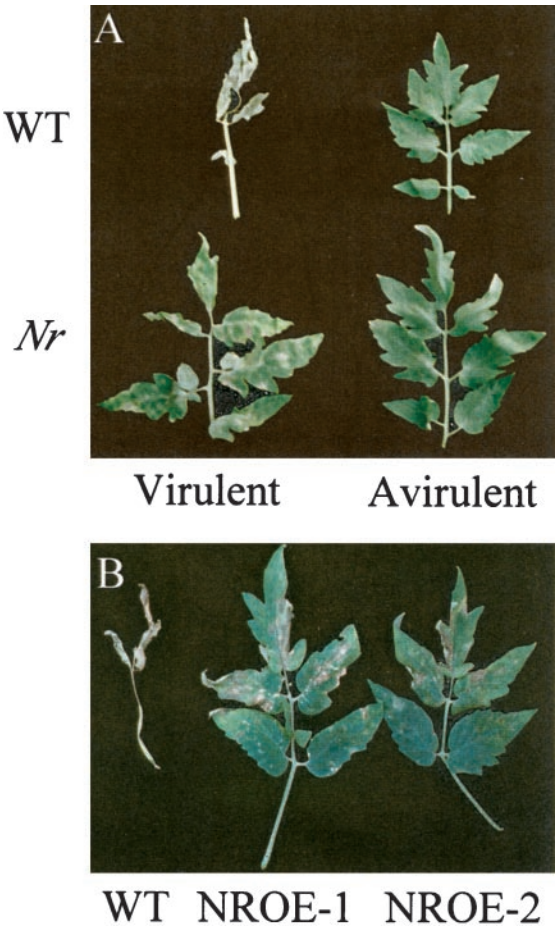

Figure 1. Disease severity in tomato leaves 14 DAI with *X. campestris* pv. *vesicatoria*. A, Four-week-old wild-type (WT) and ethylene-insensitive *Nr* mutant plants were inoculated with virulent and avirulent strains of the pathogen. B, Four-week-old wild-type and NROE-1 and NROE-2 were infected with a virulent strain of the pathogen.

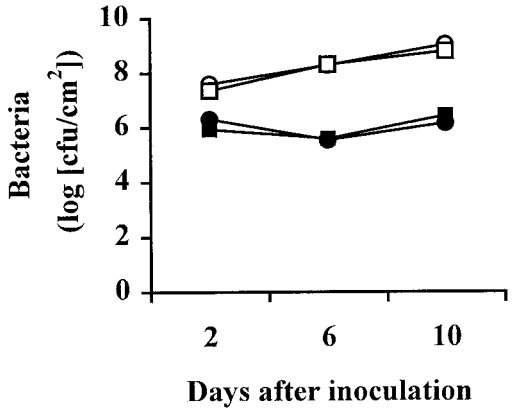

Figure 2. Growth of *X. campestris* pv. *vesicatoria* in leaves of wild-type and *Nr* tomato plants. Four-week-old plants were inoculated with virulent and avirulent strains of the pathogen. SE bars are smaller than the symbols. ○, Wild-type virulent; ●, wild-type avirulent; □, *Nr* virulent; ■, *Nr* avirulent.

Levels of virulent *X. campestris* pv. *vesicatoria* increased approximately 50-fold in both wild-type and *Nr* plants in the period from 2 to 10 DAI, and there was no difference in bacterial populations between the wild-type and the mutant (Fig. 2). Populations of the avirulent strain Xv 87-7 were approximately 100-fold lower than those of the virulent strain in both the wild-type and the mutant during this same time period, indicating that there was no change in resistance of the *Nr* plants to this strain.

An earlier and greater increase in ethylene levels occurred in plants infected with an avirulent strain of *X. campestris* pv. *vesicatoria* compared with plants infected with a virulent strain. Ethylene began to increase 8 to 24 h after inoculation with the avirulent strain and peaked 4 to 5 DAI, with ethylene levels eight times higher than in mock-inoculated plants (Fig. 3). In plants infected with the virulent strain, ethylene levels did not begin to increase until 4 DAI, and did not increase more than 4-fold by 12 DAI. Similar levels of ethylene were observed in infected wild-type and *Nr* mutant plants (Fig. 3).

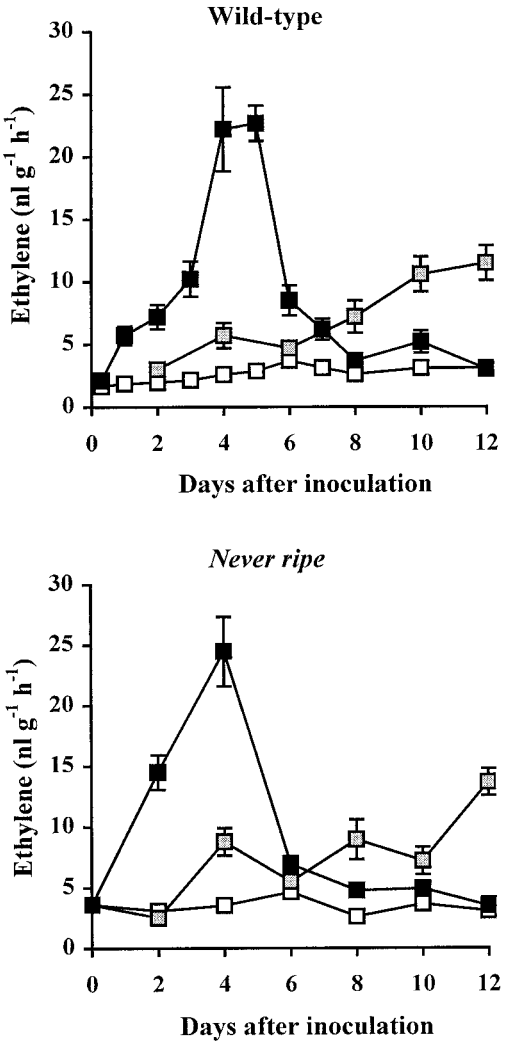

Figure 3. Ethylene synthesis in leaves of wild-type and *Nr* mutant plants inoculated with *X. campestris* pv. *vesicatoria*. Four-week-old plants were inoculated with virulent and avirulent strains of the pathogen. □, Control; ▨, virulent; ■, avirulent.

PR Gene and Ethylene Biosynthesis Gene Expression

The expression of PR genes and an ethylene biosynthetic gene was measured after inoculation with virulent and avirulent strains of *X. campestris* pv. *vesicatoria*. RNA levels of three basic, intracellular PR genes, *PR1b1*, chitinase, and β-1,3-glucanase, began to increase 1 to 2 DAI (data for 1 DAI not shown) after inoculation with the avirulent strain (Fig. 4). In contrast, *PR1b1* gene expression did not increase until 8 DAI in response to the virulent strain, and there was little or no induction of chitinase and β-1,3-glucanase. The expression pattern of a wound-inducible 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase gene (*ACO1*) was similar to that of *PR1b1* except that there was an increase in expression 12 DAI in response to the avirulent strain. Induction of β-1,3-glucanase by the avirulent strain was almost completely inhibited in the *Nr* mutant, and induction of chitinase and *ACO1* gene expression by the avirulent strain was reduced in the *Nr* mutant compared with wild type. *PR1b1* mRNA levels were the same in wild-type and mutant plants (Fig. 4).

LeETR Gene Expression

Expression of five members of the tomato ethylene receptor gene family, *LeETR1*, *LeETR2*, *NR*, *LeETR4*, and *LeETR5*, was measured in leaves of plants infected with virulent and avirulent strains of *X.*

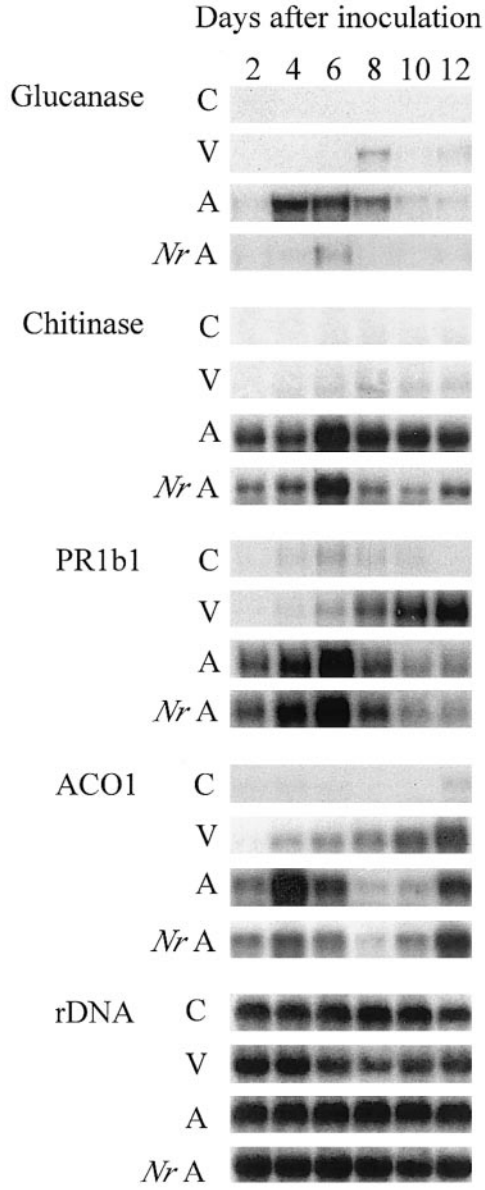

Figure 4. Pathogenesis-related and ethylene biosynthesis gene expression in leaves of wild-type and *Nr* mutant tomato plants inoculated with *X. campestris* pv. *vesicatoria*. Four-week-old plants were inoculated with virulent and avirulent strains of the pathogen. RNA levels were determined by RNA gel-blot analysis. Plants are wild type unless otherwise indicated. C, Control; V, virulent; A, avirulent.

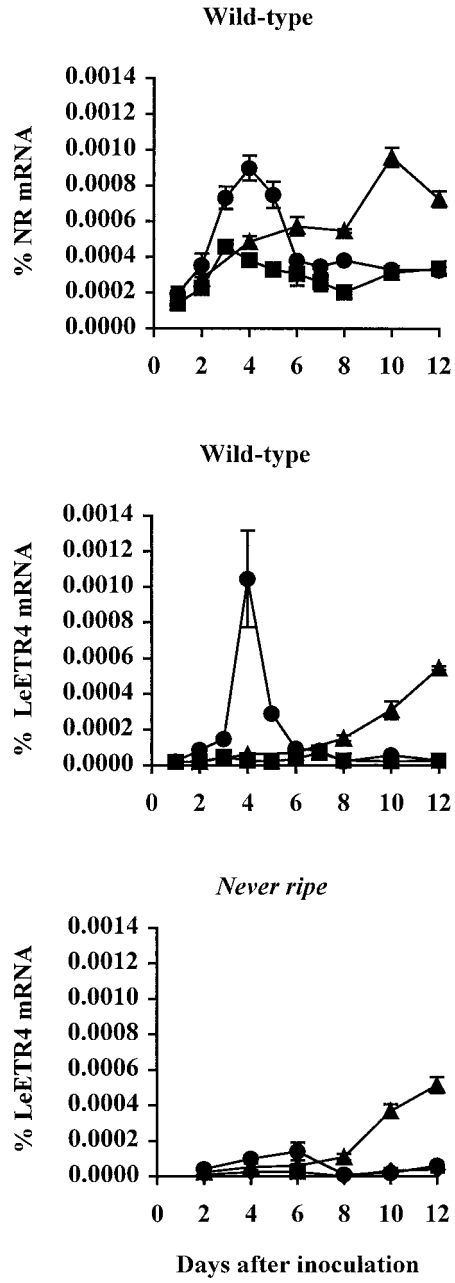

Figure 5. Expression of the tomato ethylene receptor genes *LeETR4* and *NR* in leaves following inoculation with *X. campestris* pv. *vesicatoria*. Four-week-old plants were inoculated with virulent and avirulent strains of the pathogen. Percent mRNA was quantified by RNase protection assays as described in "Materials and Methods." ■, Control; ▲, virulent; ●, avirulent.

campestris pv. *vesicatoria. NR* and *LeETR4* mRNA levels began to increase 2 DAI in response to the avirulent strain and peaked 4 DAI, when there was a 3-fold increase in *NR* mRNA and an approximately 30-fold increase in *LeETR4* (Fig. 5). A similar level of induction of *NR* and *LeETR4* expression was observed in response to the virulent strain, but did not begin until 8 DAI. The induction of *LeETR4* expression by the avirulent strain was greatly reduced in the *Nr* mutant, but there was no difference in induction by the virulent strain (Fig. 5). *NR* gene expression was similar in *Nr* mutant and wild-type plants (data not shown). There were no significant changes in mRNA levels of *LeETR1*, *LeETR2*, and *LeETR5* during disease progression (data not shown). Expression of *NR*, *LeETR4*, and *LeETR5* in leaves was induced by exogenous ethylene, with *LeETR4* and *NR* showing the greatest induction (Fig. 6). Exogenous

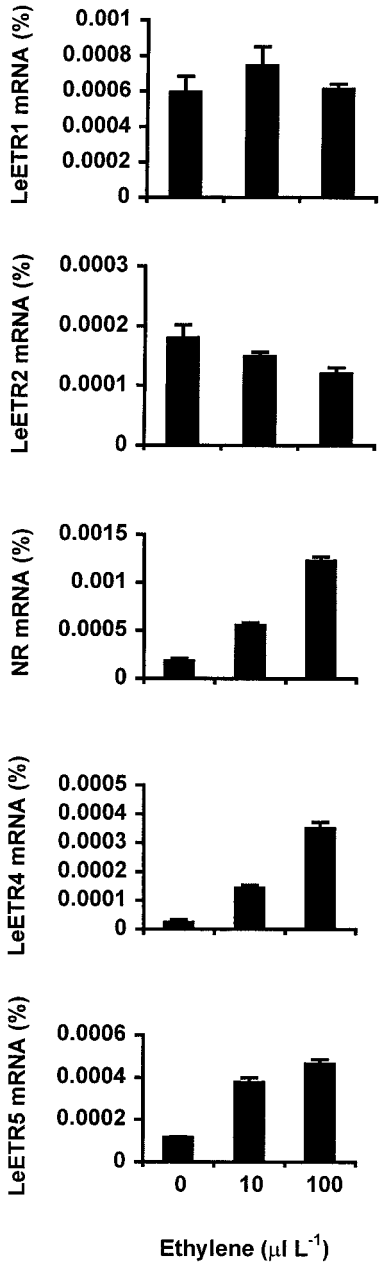

Figure 6. Ethylene receptor gene expression in tomato leaves in response to exogenous ethylene. Four-week-old wild-type (cv Pearson) plants were treated with ethylene for 1 h. Percent mRNA of ethylene receptor genes was quantified by RNase protection assays as described in "Materials and Methods."

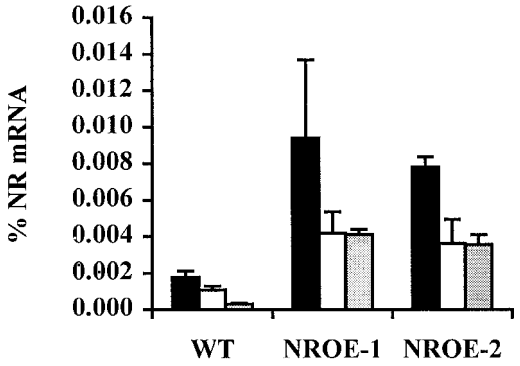

Figure 7. Expression of the ethylene receptor gene *NR* in stems (black bars), etiolated seedlings (white bars), and leaves (gray bars) of wild-type (WT) and NROE-1 and NROE-2 tomato plants. Percent mRNA was quantified by RNase protection assays as described in "Materials and Methods."

ethylene did not affect *LeETR1* and *LeETR2* mRNA levels.

Ethylene Sensitivity of NR-Overexpressing Lines

Transgenic tomato plants overexpressing a cDNA of the wild-type *NR* gene were analyzed to determine the effect of *NR* expression on ethylene sensitivity. *NR* mRNA levels were 4- to 10-fold higher than the wild type in seedlings, stems, and leaves of two independent transgenic lines overexpressing the tomato ethylene receptor gene *NR* (NROE-1 and NROE-2) (Fig. 7). However, *NR* expression was lower in fruit of the transgenic lines, apparently due to co-suppression of the native gene (data not shown). To determine the effect of increased NR levels on ethylene sensitivity, seedlings were grown in the dark on medium containing varying amounts of the ethylene precursor ACC. ACC is converted to ethylene by the plant and reduces hypocotyl and root elongation in wild-type tomato seedlings (Lanahan et al., 1994). Etiolated seedlings of both *NR*-overexpressing lines were taller and had longer roots than the wild type, even in the absence of exogenous ethylene (Fig. 8).

Growing seedlings in the presence of the ethylene action inhibitor 1-methylcyclopropene (Sisler and Serek, 1997) reduced this difference in hypocotyl and root length (data not shown), indicating that it was dependent on ethylene sensitivity. At levels of ACC below 0.1 μM, ACC treatment did not reduce seedling length, and *NR*-overexpressing lines remained longer than the wild type. Concentrations of ACC above 0.1 μM reduced seedling elongation in all three lines, and at 1 μM there was no difference in seedling length between the transgenic lines and the wild type (Fig. 8). *Nr* mutant seedlings were longer than wild type, even at ACC concentrations above 10 μM, indicating that they were less sensitive to ethylene than the *NR*-overexpressing lines (Fig. 8).

Like hypocotyl elongation, stem elongation is regulated by endogenous ethylene and can be inhibited by exogenous ethylene (Abeles et al., 1992). Ethylene insensitivity results in increased stem elongation, as illustrated by the greater internode length and plant height of the *Nr* mutant (Table I). To further analyze the ethylene sensitivity of the NR overexpressors, stem elongation was measured in 9-week-old greenhouse-grown plants. At this age the plants had approximately 12 internodes and had begun to flower. Like the *Nr* mutant, the *NR*-overexpressing lines are taller and have longer internodes than wild-type plants, indicating that they have reduced sensitivity to ethylene (Table I).

Pathogen Response of *NR*-Overexpressing Lines

Two independent *NR*-overexpressing lines were infected with virulent and avirulent strains of *X. campestris* pv. *vesicatoria*. In general, symptom development in the *NR* overexpressors was similar to that in the *Nr* mutant. There was no visible difference in symptoms between wild-type and *NR*-overexpressing lines in response to infection with the avirulent

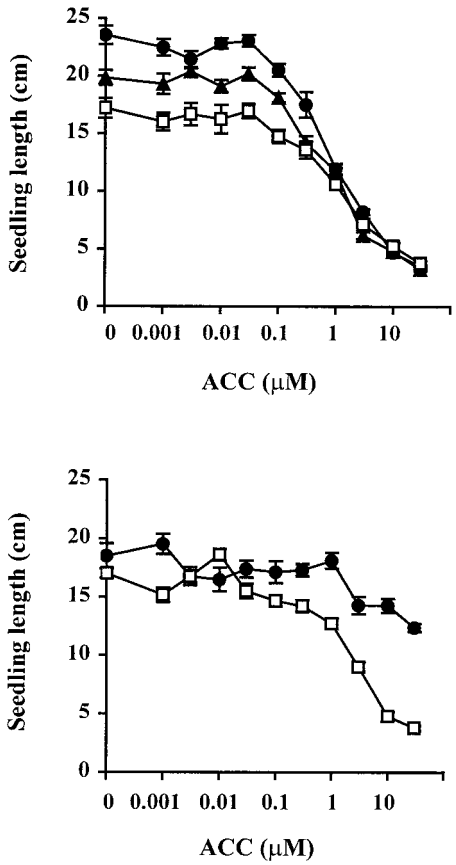

Figure 8. Triple response assay of NROE-1 and NROE-2, *Nr* mutant, and wild-type tomato seedlings. Seedling length is the sum of hypocotyl and root length. Seedlings were grown in the dark for 2 weeks on 1% (w/v) agar containing varying concentrations of ACC. Seedlings in the top panel are cv Floradade (●, NROE-1; ▲, NROE-2; □, wild type); seedlings in the bottom panel are cv Pearson (●, *Nr*; □, wild type).

Table I. *Stem elongation in 9-week-old tomato plants*

NROE-1, NROE-2, Independent transgenic lines overexpressing the ethylene receptor gene *NR;* WT, wild type. Transgenic lines are cv Floradade, *Nr* mutant is cv Pearson. *, Significantly different from wild type ($P \leq 0.05$); **, significantly different from wild type ($P \leq 0.01$).

Plant	Internode No.	Average Internode Length	Plant Height
		cm	
WT (cv Floradade)	12.6 ± 0.4	5.7 ± 0.3	84 ± 2
NROE-1	12.4 ± 0.2	6.8 ± 0.4**	94 ± 3**
NROE-2	12.7 ± 0.4	6.9 ± 0.4*	99 ± 4**
WT (cv Pearson)	20.4 ± 0.2	8.5 ± 0.4	167 ± 4
Nr	18.0 ± 0.3**	11.0 ± 0.4**	176 ± 3*

strain (data not shown). However, *NR*-overexpressing plants infected with the virulent strain displayed greatly reduced necrosis 14 DAI relative to wild type (Fig. 1B). As with the *Nr* mutant, reduced necrosis in the *NR* overexpressors was a result of tolerance (rather than resistance) to the pathogen, since there was no difference in bacterial growth between wild-type and transgenic plants (data not shown).

Electrolyte leakage from leaf tissue of inoculated plants was also assayed to quantify the extent of disease damage. At 12 and 13 DAI, electrolyte leakage was significantly higher in wild-type plants, and this increased membrane permeability was accompanied by chlorosis and the spread of necrosis (Fig. 9). The *NR* overexpressors showed only limited chlorosis at this stage. By 14 DAI, the wild-type leaves were completely necrotic, while the *NR* overexpressors showed only small areas of necrosis. By 16 DAI, leaves of the *NR*-overexpressing lines contained large areas of necrosis as well, illustrating that overexpression of *NR* delayed necrosis but did not completely prevent it. Therefore, tolerance to *X. campestris* pv. *vesicatoria* was not as strong in the NR overexpressors as it was in the *Nr* mutant, which showed little necrosis even 16 DAI (data not shown). There were no significant differences in PR gene expression between wild-type and NR-overexpressing lines (data not shown).

DISCUSSION

In tomato, symptom development in response to avirulent *X. campestris* pv. *vesicatoria* strain Xv 87-7 is a hypersensitive response involving the formation of small, distinct lesions that do not spread. Since response to this pathogen has not been characterized at the molecular level, we measured PR gene expression in plants infected with virulent and avirulent strains. Expression of PR1b1, β-1,3-glucanase, and chitinase increased more quickly during the incompatible interaction than during the compatible interaction, indicating a faster response to the avirulent strain (Fig. 4). A faster induction of PR genes also occurs during a hypersensitive response to other tomato pathogens such as *Cladosporium fulvum* (van Kan et al., 1992) and *Pseudomonas syringae* (Jia and Martin, 1999), as well as during several other incompatible plant-pathogen interactions (Linthorst, 1991). Therefore, response to *X. campestris* pv. *vesicatoria* strain Xv 87-7 at the molecular level appears typical of hypersensitive responses to other pathogens. Since all *L. esculentum* genotypes tested are resistant to this strain, it could serve as a useful tool for studying the hypersensitive response in the wide range of transgenic and mutant tomato lines that lack resistance genes to other pathogens.

Increases in ethylene synthesis (Fig. 3) and *ACO1* expression (Fig. 4) also indicated a more rapid response to the avirulent strain than to the virulent strain. Earlier increases in ethylene synthesis have been observed for several other incompatible interactions (Montalbini and Elstner, 1977; De Laat and van Loon, 1983; Boller, 1991), suggesting that ethylene may be one of the signals that initiates the faster defense response. However, no increase in ethylene synthesis occurred during the first 8 h after inoculation with the avirulent strain; therefore, ethylene does not appear to play a role in the earliest resistance responses to *X. campestris* pv. *vesicatoria*. Furthermore, the peak in ethylene synthesis occurred relatively late in the disease progression, at the time of lesion formation and spread, suggesting that ethylene could be involved in regulating the spread of cell death during infection. Since *ACO1* expression

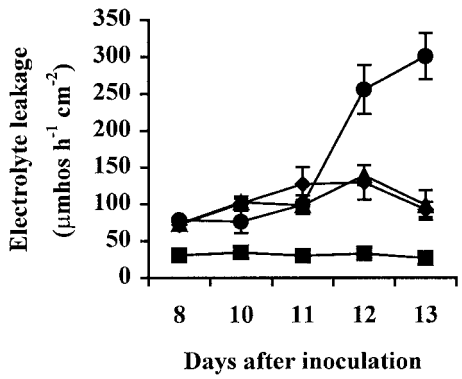

Figure 9. Electrolyte leakage from tomato leaves infected with a virulent strain of *X. campestris* pv. *vesicatoria*. Plants were 4 weeks old at the time of inoculation. ■, Wild-type control; ●, wild-type virulent; ▲, NROE-1 virulent; ♦, NROE-2 virulent.

was reduced in the *Nr* mutant but ethylene levels were not, other enzymes appear to be involved in regulating ethylene synthesis in response to this pathogen.

Chitinase and β-1,3-glucanase expression was reduced in the ethylene-insensitive *Nr* mutant (Fig. 4) and was correlated with endogenous ethylene levels during infection (Fig. 3 and Fig. 4), indicating that the induction of these genes is ethylene regulated. However, there were no differences in disease symptoms (Fig. 1) or bacterial populations (Fig. 2) between the wild-type and mutant plants infected with the avirulent strain. Therefore, these enzymes do not appear to be critical for resistance to this pathogen. A similar result was observed in the ethylene-insensitive *ein2* mutant of Arabidopsis in response to the fungal pathogen *Alternaria brassicicola*. Pathogen-induced expression of three PR genes was eliminated or greatly reduced in the mutant plants, but resistance to this pathogen was maintained (Thomma et al., 1999). Ethylene-insensitive Arabidopsis (Bent et al., 1992; Thomma et al., 1999), tobacco (Knoester et al., 1998), and soybean (Hoffman et al., 1999) have all shown normal resistance to a range of different avirulent pathogens, while ethylene insensitivity increased susceptibility to *B. cinerea* in Arabidopsis (Thomma et al., 1999) and to *P. sojae* in soybean (Hoffman et al., 1999). Therefore, while ethylene does regulate specific components of the defense response, the importance of those components in plant resistance varies greatly from one pathogen to another.

Expression of two tomato ethylene receptor genes, *NR* and *LeETR4*, was induced during infection, suggesting that these genes may play a role in pathogen response (Fig. 5). Induction of *LeETR4* gene expression by the avirulent pathogen was reduced in the *Nr* mutant (Fig. 5), indicating that this induction during the incompatible interaction is ethylene regulated. *LeETR4* expression was induced by exogenous ethylene and was closely correlated with endogenous ethylene synthesis during infection (Figs. 3 and 6), further indicating that *LeETR4* mRNA levels are regulated by ethylene. It is interesting that *LeETR4* mRNA levels were not reduced in *Nr* mutant plants infected with virulent *X. campestris* pv. *vesicatoria*, demonstrating that additional signals control *LeETR4* expression during the compatible interaction. A similar result was observed in tomato in response to *P. syringae* pv. *tomato*, in which the ethylene action inhibitor norbornadiene blocked induction of glucanase and osmotin expression during an incompatible interaction, but had no effect on expression during the compatible interaction (Thara et al., 1999).

NR gene expression was also induced by exogenous ethylene and was correlated with endogenous ethylene during infection (Figs. 3 and 6). However, induction of *NR* expression by *X. campestris* pv. *vesicatoria* was similar in mutant and wild-type plants, indicating that this induction is not ethylene dependent. Although the level of ethylene induction was similar for *LeETR4* and *NR*, the induction of *LeETR4* during infection was much greater. Furthermore, *LeETR5* mRNA levels also increased in response to exogenous ethylene, but not during infection. These data indicate that the ethylene inducibility of these genes is only one component of pathogen induction.

The effect of pathogen infection on LeETR protein levels has not been determined. The abundance of NR protein is correlated with transcript levels in both wild-type and NR antisense plants (D.M. Tieman, unpublished data); antibodies to the other LeETR proteins are not yet available. Although increased expression of *LeETR4* and *NR* mRNA during infection indicates that protein levels may also increase, it is not yet known what role post-transcriptional regulation plays in determining ethylene receptor abundance.

In Arabidopsis, loss-of-function mutations in four ethylene receptor genes greatly increased sensitivity to ethylene, identifying these genes as negative regulators of the ethylene response (Hua and Meyerowitz, 1998). Similarly, decreased *LeETR4* expression in antisense tomato lines caused constitutive ethylene responses such as leaf epinasty and accelerated flower senescence (Tieman et al., 2000). Therefore, a reduction in ethylene receptor levels increases sensitivity to ethylene. According to this model, an increase in receptor levels would be expected to decrease sensitivity. In fact, *NR*-overexpressing lines are less sensitive to ethylene, as indicated by increased stem elongation in mature plants (Table I) and increased hypocotyl elongation in etiolated seedlings (Fig. 8).

Based on seedling response to ACC, the *NR*-overexpressing lines are not as ethylene insensitive as the *Nr* mutant. It has been suggested that the mutant receptors cannot be inactivated due to their inability to bind ethylene (Schaller and Bleecker, 1995). This model would explain why the *NR*-overexpressing seedlings show no difference in length at higher ACC concentrations, while the *Nr* mutant is longer than wild type even at the highest concentrations tested (Fig. 8). At high ethylene levels, the additional wild-type NR protein in the overexpressors would be inactivated, while the mutant protein would continue to suppress the ethylene response. The reduced ethylene sensitivity of the *NR* overexpressors also indicates that NR, like LeETR4, is a negative regulator of ethylene response. Therefore, the induction of *LeETR4* and *NR* gene expression observed in response to *X. campestris* pv. *vesicatoria* infection would decrease the ethylene sensitivity of infected tissue.

Given the function of *LeETR4* and *NR* as negative regulators of ethylene response, it is intriguing that these genes are ethylene inducible. Treatment with 10 ppm ethylene for 1 h induced expression of both genes approximately 10-fold (Fig. 6), indicating that

one of the plant's responses to increased ethylene levels is a fairly rapid reduction in ethylene sensitivity. Ethylene induction of these genes may serve to regulate the magnitude and duration of ethylene responses. Regulation of ethylene action at the level of both synthesis and perception would allow for an initial response to increased ethylene levels to be quickly dampened by greater *LeETR* expression. As receptor levels increase, more ethylene would be required to inactivate these suppressors and continue the response.

A strong induction in *NR* expression also occurs during tomato fruit ripening and is highly correlated with a large increase in ethylene synthesis (Lashbrook et al., 1998). In tissues with autocatalytic ethylene synthesis, such as ripening fruit, an additional level of regulation may be necessary to control the ethylene response. Similar dampening mechanisms exist for other hormones such as auxin, in which increases in endogenous indole-3-acetic acid levels are accompanied by conjugation to inactive forms (Cohen and Bandurski, 1982), and SA, in which pathogen-induced increases are accompanied by conjugation to SA glucosides (Malamy et al., 1992). These mechanisms provide a means of inducing a rapid but brief hormone response, allowing a large initial increase in hormone synthesis while preventing a prolonged activation of these responses.

Like the *Nr* mutant (Fig. 1A), transgenic plants overexpressing wild-type *NR* displayed tolerance to virulent *X. campestris* pv. *vesicatoria*, as evidenced by reduced necrosis (Fig. 1B) and greater membrane integrity (Fig. 9) in infected *NR*-overexpressing lines. Therefore, an increase in the expression of the wild-type *NR* gene is sufficient to confer this tolerance, indicating that the plant may be able to control its response to pathogens through the regulation of this and other ethylene receptor genes. Specifically, an increase in *LeETR* expression may help to limit the spread of necrosis in response to infection, as it did in the *NR* overexpressing lines. Induction of the *LeETR* genes during an incompatible interaction may play a similar role, limiting cell death to the site of infection by decreasing the ethylene sensitivity of the surrounding tissue. Although the induction of *LeETR4* expression was blocked in the *Nr* mutant and the spread of cell death was still limited, this tissue is already insensitive to ethylene and would not require the induction of *LeETR4* expression to limit necrosis. Analysis of disease progression in *LeETR4* and *NR* antisense lines will be necessary to determine whether blocking the induction of these genes alters the extent of cell death.

MATERIALS AND METHODS

Plant Material

The homozygous *Nr* tomato (*Lycopersicon esculentum* Mill.) mutant and wild-type cv Pearson lines are isogenic (Rick and Butler, 1956). The *NR*-overexpressing transgenic lines were produced through *Agrobacterium tumefaciens*-mediated transformation of cv Floradade (McCormick et al., 1986). A *NR* cDNA under transcriptional control of the figwort mosaic virus promoter (Richins et al., 1987) was inserted into the tomato genome, along with a glyphosate-resistance gene as a selectable marker. Insertion of the transgene was confirmed by PCR amplification of the glyphosate resistance gene. Primary transformants were self-pollinated to produce lines that were homozygous for the transgene.

Inoculations and Disease Development

Four-week-old tomato plants were inoculated with *Xanthomonas campestris* pv. *vesicatoria* strains Xv 93-1 (virulent) and Xv 87-7 (avirulent). *X. campestris* pv. *vesicatoria* strain Xv 87-7 is avirulent on all *L. esculentum* genotypes tested, but virulent on pepper cv Early Calwonder (Canteros, et al., 1991). Xv 87-7 contains the avirulence gene *avrBs3-2*; conjugation of a virulent strain (Xv 75-3) with this gene converts the strain to avirulent in tomato (Bonas et al., 1993). Inoculations were performed by dipping plants for 15 s into an inoculum containing 1×10^8 colony forming units (cfu)/mL and 0.025% (v/v) Silwet 77 (Lehle Seeds, Round Rock, TX) in sterile tap water. Control plants were dipped in sterile tap water containing 0.025% (v/v) Silwet 77. Plants were grown under standard greenhouse conditions. Electrolyte leakage and bacterial growth were measured as described previously (Lund et al., 1998). All experiments were repeated at least twice.

Ethylene samples were collected by placing single leaflets from the third or fourth leaf from the base of the plant into 5-mL containers and incubating at room temperature for 1 h. Ethylene concentration from a 1-mL sample was determined by gas chromatograph (model 5890, Hewlett-Packard, Palo Alto, CA).

RNA Isolation and Quantification

RNA was isolated from leaflets of the third and fourth leaf from the base of the plant; these were the two youngest fully expanded leaves at the time of inoculation. For the *NR*-overexpressing lines, RNA was also isolated from 2-week-old etiolated seedlings grown on 1% (w/v) agar and the 10th internode (from the base of the plant) of 9-week-old greenhouse-grown plants. RNA was extracted in SDS-phenol and purified by LiCl precipitation. Northern-blot analysis was performed as described using 10 μg of total RNA (Kneissl and Deikman, 1996). All DNA probes were labeled with ^{32}P by random primer labeling, as described by Sambrook et al. (1989). The template for PR1b1 was a 348-bp PCR fragment (Lund et al., 1998). A 655-bp PCR fragment from a basic intracellular β-1,3-glucanase (GenBank accession no. M80608; van Kan et al., 1992) was amplified using the forward primer 5′-TCTTGCCCCATTTCAACTTC and the reverse primer 3′-GTCCCAAACTCTTTCAGACACC. The template for the basic intracellular chitinase (GenBank accession no. Z15140;

Danhash et al., 1993) was isolated from a Lambda Zap II cDNA library prepared from phosphate-stressed tomato roots. The template for *ACO1* (ethylene biosynthesis gene *ACC Oxidase 1*) was a full-length cDNA (GenBank accession no. X04792; Holdsworth et al., 1987). Blots were probed with labeled 18S rDNA from *Zamia floridana* to ensure equal levels of total RNA. RNase protection assays were performed with 20 μg of total RNA using gene-specific probes as described previously (Lashbrook et al., 1998; Tieman and Klee, 1999).

Ethylene and ACC Treatment

Ethylene treatments were conducted by sealing 4-week-old wild-type tomato plants (cv Pearson) in glass containers and adding ethylene to 10 or 100 μL L^{-1}. Control plants were sealed in glass containers containing potassium permanganate, an ethylene absorbant. Triple response assays were performed by germinating surface-sterilized seed on 1% (w/v) agar containing varying concentrations of ACC. Seedlings were grown in the dark for 2 weeks at room temperature.

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LITERATURE CITED

Abeles FB, Morgan PW, Saltveit ME (1992) Ethylene in Plant Biology, Ed 2. Academic Press, San Diego, pp 264–296

Bent AF, Innes RW, Ecker JR, Staskawicz BJ (1992) Disease development in ethylene-insensitive *Arabidopsis thaliana* infected with virulent and avirulent *Pseudomonas* and *Xanthomonas* pathogens. Mol Plant-Microbe Interact **5:** 372–378

Boller T (1991) Ethylene in pathogenesis and disease resistance. *In* AK Matoo, JC Suttle, eds, The Plant Hormone Ethylene. CRC Press, Boca Raton, FL, pp 293–314

Bonas U, Conrads-Strauch J, Balbo I (1993) Resistance in tomato to *Xanthomonas campestris* pv *vesicatoria* is determined by alleles of the pepper-specific avirulence gene *avrBs3*. Mol Gen Genet **238:** 261–269

Bradley DJ, Kjellbom P, Lamb CJ (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. Cell **70:** 21–30

Brisson LF, Tenhaken R, Lamb CJ (1994) Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. Plant Cell **6:** 1703–1712

Broglie K, Chet I, Jolliday M, Cressman R, Biddle P, Knowlton S, Mauvais CJ, Broglie R (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science **254:** 1194–1197

Canteros B, Minsavage G, Bonas U, Pring D, Stall R (1991) A gene from *Xanthomonas campestris* pv. *vesicatoria* that determines avirulence in tomato is related to *avrBs3*. Mol Plant-Microbe Interact **4:** 628–632

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) *Arabidopsis* ethylene response gene *ETR1*-similarity of product to two-component regulators. Science **262:** 539–544

Cohen JD, Bandurski RS (1982) Chemistry and physiology of the bound auxins. Annu Rev Plant Physiol **33:** 403–430

Danhash N, Wagemakers CAM, van Kan JAL, de Wit PJGM (1993) Molecular characterization of four chitinase cDNAs obtained from *Cladosporium*-infected tomato. Plant Mol Biol **22:** 1017–1029

Deikman J (1997) Molecular mechanisms of ethylene regulation of gene transcription. Physiol Plant **100:** 561–566

De Laat AMM, van Loon LC (1983) The relationship between stimulated ethylene production and symptom expression in virus-infected tobacco leaves. Physiol Plant Pathol **22:** 261–273

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

Ecker JR, Davis RW (1987) Plant defense genes are regulated by ethylene. Proc Natl Acad Sci USA **84:** 5202–5206

Epple P, Apel K, Bohlmann H (1995) An *Arabidopsis thaliana* thionin gene is inducible via a signal transduction pathway different from that for pathogenesis-related proteins. Plant Physiol **109:** 813–820

Goodman RN, Novacky AJ (1994) The Hypersensitive Reaction in Plants to Pathogens: A Resistance Response. APS Press, St. Paul, pp 117–119

Greenberg JT, Guo A, Klessig DF, Ausubel FM (1994) Programmed cell death in plants: a pathogen-triggered response activated coordinately with multiple defense functions. Cell **77:** 551–563

Hain R, Reif H, Krause E, Langebartels R, Kindl H, Vonram B, Wiese W, Schmelzer E, Schreier PH, Stocker RH, Stenzel K (1993) Disease resistance results from foreign phytoalexin expression in a novel plant. Nature **361:** 153–156

Hoffman T, Schmidt JS, Zheng X, Bent AF (1999) Isolation of ethylene-insensitive soybean mutants that are altered in pathogen susceptibility and gene-for-gene disease resistance. Plant Physiol **119:** 935–949

Holdsworth MJ, Bird CR, Ray J, Schuch W, Grierson D (1987) Structure and expression of an ethylene-related mRNA from tomato. Nucleic Acids Res **15:** 731–739

Hua J, Chang C, Sun Q, Meyerowitz EM (1995) Ethylene insensitivity conferred by Arabidopsis *ERS* gene. Science **269:** 1712–1714

Hua J, Meyerowitz EM (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell **94:** 261–271

Hua J, Sakai H, Nourizadeh S, Chen QG, Bleecker AB, Ecker JR, Meyerowitz EM (1998) *EIN4* and *ERS2* are members of the putative ethylene receptor gene family in *Arabidopsis*. Plant Cell **10:** 1321–1332

Jia YL, Martin GB (1999) Rapid transcript accumulation of pathogenesis-related genes during an incompatible interaction in bacterial speck disease-resistant tomato plants. Plant Mol Biol **40:** 455–465

Keppler LD, Baker CJ, Atkinson MM (1989) Activated oxygen production during a bacteria induced hypersensitive reaction in tobacco suspension cells. Phytopathology **79:** 974–978

Kneissl ML, Deikman J (1996) The tomato *E8* gene influences ethylene biosynthesis in fruit but not in flowers. Plant Physiol **112:** 537–547

Knoester M, van Loon LC, van den Heuvel J, Hennig J, Bol JF, Linthorst HJM (1998) Ethylene-insensitive tobacco lacks non-host resistance against soil-borne fungi. Proc Natl Acad Sci USA **95:** 1933–1937

Lanahan MB, Yen H-C, Giovannoni JJ, Klee HJ (1994) The *Never ripe* mutation blocks ethylene perception in tomato. Plant Cell **6:** 521–530

Lashbrook CC, Tieman DM, Klee HJ (1998) Differential regulation of the tomato ETR gene family throughout plant development. Plant J **15:** 243–252

Linthorst HJM (1991) Pathogenesis-related proteins in plants. Crit Rev Plant Sci **10:** 123–150

Lund ST, Stall RE, Klee HJ (1998) Ethylene regulates the susceptible response to pathogen infection in tomato. Plant Cell **10:** 371–382

Malamy J, Carr JP, Klessig DF, Raskin I (1990) Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science **250:** 1002–1004

Malamy J, Hennig J, Klessig DF (1992) Temperature-dependent induction of salicylic acid and its conjugates during the resistance response to tobacco mosaic virus infection. Plant Cell **4:** 359–366

McCormick S, Neidermeyer J, Fry J, Barnason A, Horsch R, Fraley R (1986) Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Rep **5:** 81–84

Moerschbacher BM, Noll U, Gorrichon L, Reisener HJ (1990) Specific inhibition of lignification breaks hypersensitive resistance of wheat to stem rust. Plant Physiol **93:** 465–470

Montalbini P, Elstner EF (1977) Ethylene evolution by rust-infected, detached bean (*Phaseolus vulgaris* L.) leaves susceptible and hypersensitive to *Uromyces phaseoli* (Pers.) Wint. Planta **135:** 301–306

Orlandi EW, Hutcheson SW, Baker CJ (1992) Early physiological responses associate with race-specific recognition in soybean leaf tissue and cell suspensions treated with *Pseudomonas syringae* pv. *glycinea*. Physiol Mol Plant Pathol **40:** 173–180

Parker JE, Szabo V, Staskawicz B, Lister C, Dean C, Daniels MJ, Jones J (1993) Phenotypic characterization and molecular mapping of the *Arabidopsis thaliana* locus RPP5, determining disease resistance to *Peronospora parasitica*. Plant J **4:** 821–831

Penninckx IAMA, Eggermont K, Terras FRG, Thomma BPHJ, De Samlanx GW, Buchala A, Metraux J-P, Manners JM, Broekaert WF (1996) Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. Plant Cell **8:** 2309–2323

Penninckx IAMA, Thomma BPHJ, Buchala A, Metraux J-P, Broekaert WF (1998) Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. Plant Cell **10:** 2103–2113

Richins RD, Scholthof HB, Shepard RJ (1987) Sequence of the figwort mosaic virus DNA (caulimovirus group). Nucleic Acid Res **15:** 8451–8466

Rick C, Butler L (1956) Phytogenetics of the tomato. Adv Gen **8:** 267–382

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schaller GE, Bleecker AB (1995) Ethylene binding sites generated in yeast expressing the *Arabidopsis ETR1* gene. Science **270:** 1809–1811

Showalter AM, Bell JN, Cramer CL, Bailey JA, Varner JE, Lamb CJ (1985) Accumulation of hydroxyproline-rich glycoprotein mRNAs in response to fungal elicitor and infection. Proc Natl Acad Sci USA **82:** 6551–6555

Sisler EC, Serek M (1997) Inhibitors of ethylene responses in plants at the receptor level: recent developments. Physiol Plant **100:** 577–582

Staskawicz BJ, Ausubel FM, Baker BJ, Ellis JG, Jones JDG (1995) Molecular genetics of plant disease resistance. Science **268:** 661–667

Thara VK, Tang X, Gu YQ, Martin GB, Zhou JM (1999) *Pseudomonas syringae* pv *tomato* induces the expression of tomato *EREBP*-like genes *Pti4* and *Pti5* independent of ethylene, salicylate and jasmonate. Plant J **20:** 475–483

Thomma BPHJ, Eggermont K, Penninckx IAMA, Mauch-Mani B, Vogelsang R, Cammue BPA, Broekaert WF (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc Natl Acad Sci USA **95:** 15107–15111

Thomma BPHJ, Eggermont K, Tierens KFMJ, Broekaert WF (1999) Requirement of functional *Ethylene-Insensitive 2* gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea*. Plant Physiol **121:** 1093–1101

Tieman DM, Klee HJ (1999) Differential expression of two novel members of the tomato ethylene receptor family. Plant Physiol **120:** 165–172

Tieman DM, Taylor MG, Ciardi JA, Klee HJ (2000) The tomato ethylene receptors NR and LeETR4 are negative regulators of ethylene response and exhibit functional compensation within a multigene family. Proc Natl Acad Sci USA (in press)

Toppan A, Roby D, Esquerre-Tugaye M-T (1982) Cell surfaces in plant-microorganism interactions: III. In vivo

effect of ethylene on hydroxyproline-rich glycoprotein accumulation in the cell wall of diseased plants. Plant Physiol **70:** 82–86

van Kan JAL, Joosten MHAJ, Wagemakers CAM, van den Berg-Velthius GCM, de Wit PJGM (1992) Differential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum*. Plant Mol Biol **20:** 513–527

Wilkinson JQ, Lanahan MB, Yen H-C, Giovannoni JJ, Klee HJ (1995) An ethylene-inducible component of signal transduction encoded by *Never-ripe*. Science **270:** 1807–1809

Wu GS, Short BJ, Lawrence EB, Levine EB, Fitzsimmons KC, Shah DM (1995) Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. Plant Cell **7:** 1357–1368

Zhu Q, Maher EA, Maoud S, Dixon RA, Lamb CJ (1994) Enhanced protection against fungal attack by constitutive coexpression of chitinase and glucanase genes in transgenic tobacco. Biotechnology **12:** 807–812