Response to \textit{Xanthomonas campestris pv. vescatoria} in Tomato Involves Regulation of Ethylene Receptor Gene Expression$^1$

Joseph A. Ciardi, Denise M. Tieman, Steven T. Lund, Jeffrey B. Jones, Robert E. Stall, and Harry J. Klee*

Horticultural Sciences Department, P.O. Box 110690, University of Florida, Gainesville, Florida 32611–0690 (J.A.C., D.M.T., H.J.K.); Genesis Research and Development Corporation, 1 Fox Street, Parnell, Auckland, New Zealand (S.T.L.); and Department of Plant Pathology, University of Florida, Gainesville, Florida 32611–0680 (J.B.J., R.E.S.)

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 \textit{Xanthomonas campestris pv. vescitoria} in tomato (\textit{Lycopersicon esculentum} Mill.). The ethylene-insensitive \textit{Never ripe} (\textit{Nr}) mutant displays increased tolerance to the virulent strain, while maintaining resistance to the avirulent strain. Expression of the ethylene receptor genes \textit{NR} and \textit{LeETR4} was induced by infection with both virulent and avirulent strains; however, the induction of \textit{LeETR4} expression by the avirulent strain was blocked in the \textit{Nr} mutant. To determine whether ethylene receptor levels affect symptom development, transgenic plants overexpressing a wild-type \textit{NR} cDNA were infected with virulent \textit{X. campestris pv. vescitoria}. Like the \textit{Nr} mutant, the \textit{NR} overexpressors displayed greatly reduced necrosis in response to this pathogen. \textit{NR} overexpression also reduced ethylene sensitivity in seedlings and mature plants, indicating that, like \textit{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 (Epplle 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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* Corresponding author; e-mail hjklee@gnv.ifas.ufl.edu; fax 352–846–2063.
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 tabacam) 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. tomoato, 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. tomoato and pv. maculicola as well as X. campestris pv. campestris (Bent et al., 1992). However, ethylene-insensitive transgenic tobacco (Nicotiana tabacam) 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 Phytophthora 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. tomoato (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).

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).
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 inoculated with virulent and avirulent strains of *X. campestris pv. vesicatoria*. Four-week-old plants were inoculated with virulent and avirulent strains of the pathogen. □, Control; □, virulent; ■, avirulent.

![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.](image)

![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.](image)
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). Expression of NR, LeETR4, and LeETR5 in leaves was induced by exogenous ethylene, with LeETR4 and NR showing the greatest induction (Fig. 6). Exogenous 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 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.

**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.”
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...
Pathogen Response Involves Ethylene Receptor Gene Expression

Table 1. Stem elongation in 9-week-old tomato plants

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

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...
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 32P 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′-TCT-TOCCCCATTTCAACTTC and the reverse primer 3′-GTC-CCAAACCTCTTTCAGACACC. 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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