Overexpression of *Pto* Induces a Salicylate-Independent Cell Death But Inhibits Necrotic Lesions Caused by Salicylate-Deficiency in Tomato Plants

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Tomato plants overexpressing the disease resistance gene *Pto* (35S::*Pto*) exhibit spontaneous cell death, accumulation of salicylic acid (SA), elevated expression of pathogenesis-related genes, and enhanced resistance to a broad range of pathogens. Because salicylate plays an important role in the cell death and defense activation in many lesion mimic mutants, we investigated the interaction of SA-mediated processes and the 35S::*Pto*-mediated defense pathway by introducing the *nahG* transgene that encodes salicylate hydroxylase. Here, we show that SA is not required for the 35S::*Pto*-activated microscopic cell death and plays a minor role in defense gene activation and general disease resistance in 35S::*Pto* plants. In contrast, temperature greatly affects the spontaneous cell death and general resistance in 35S::*Pto* plants, and high temperature inhibits the cell death. The NahG tomato plants develop spontaneous, unconstrained necrotic lesions on leaves. These lesions also are initiated by the inoculation of a virulent strain of *Pseudomonas syringae* pv. *tomato*. However, the NahG-dependent necrotic lesions are inhibited in the NahG/35S::*Pto* plants. This inhibition is most pronounced under conditions favoring the 35S::*Pto*-mediated spontaneous cell death development. These results indicate that the signaling pathways activated by *Pto* overexpression suppress the cellular damage that is caused by SA depletion. We also found that ethylene is dispensable for the 35S::*Pto*-mediated general defense.

Plants often react to incompatible pathogens with a rapid killing of the infected cells (hypersensitive response, HR) and an array of defense responses, including a burst of reactive oxygen species, ion flux, cell wall modification, accumulation of phytoalexins, and activation of defense-related genes (Hammond-Kosack and Jones 1996). The mechanism of the HR is unclear, but it is thought to be an active suicide program in plants, which restricts pathogens to the infection site. A pathogen-independent, HR-like cell death can be induced by mutations that presumably mimic the signaling process during infection by incompatible pathogens (Dangl et al. 1996). Lesion mimic mutants have been identified from many plant species including *Arabidopsis* (Dietrich et al. 1994; Greenberg et al. 1994; Shah et al. 1999), rice (Takahashi et al. 1999; Yin et al. 2000), maize (Walbot et al. 1983), and barley (Wolter et al. 1993). In addition, the lesion mimic phenotype can also be induced by transgenes that appear

to disrupt the normal metabolic processes (Abad et al. 1997; Mittler et al. 1995; Tadege et al. 1998).

The onset of spontaneous lesions in many lesion mimic mutants is regulated by environmental conditions such as light and temperature (Abad et al. 1997; Dietrich et al. 1994; Johal et al. 1994; Mittler et al. 1995; Tadege et al. 1998). Light intensity and day length was reported to affect lesion formation in a number of lesion mimic mutants of maize (Johal et al. 1994) and *Arabidopsis* (Dietrich et al. 1994). High temperature was shown to suppress lesion formation in tobacco and potato transgenic plants expressing bacterial proton pump and potato transgenic plants expressing pyruvate decarboxylase (Abad et al. 1997; Mittler et al. 1995; Tadege et al. 1998). An environmental effect was also observed in the *Arabidopsis dnd1* mutant that formed spontaneous lesions only under certain environmental conditions (Clough et al. 2000).

The formation of spontaneous lesions is often associated with SA accumulation, activation of multiple defense-related genes, and elevated general disease resistance (Dangl et al. 1996). In many *Arabidopsis* lesion mimic mutants, a high level of SA is required for the induction of spontaneous lesions and the activation of defense-related genes and general disease resistance (Bowling et al. 1997; Dietrich et al. 1994; Greenberg 2000; Greenberg et al. 1994; Rate et al. 1999; Shah et al. 1999; Weymann et al. 1995). However, in the *Arabidopsis lsd2* and *lsd4* mutants, SA is dispensable (Hunt et al. 1997), while in the *ssi2* mutant, SA is partially required for the induction of spontaneous cell death and defense responses (Shah et al. 2001). In tomato plants, the removal of SA induces spontaneous necrotic lesions but does not significantly impact resistance to *Xanthomonas campestris* pv. *vesicatoria* and *Cladosporium fulvum* (Brading et al. 2000; O'Donnell et al. 2001).

The tomato disease resistance (*R*) gene *Pto* encodes a protein kinase that recognizes the bacterial avirulence protein AvrPto and leads to HR and resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Martin et al. 1993; Tang et al. 1996). Tomato plants overexpressing *Pto* (35S::*Pto*) exhibit spontaneous cell death, accumulation of SA, constitutive activation of defense-related genes, and enhanced general resistance (Tang et al. 1999; Xiao et al. 2001). The environmental regulation and the role of SA in the 35S::*Pto*-mediated defense responses have not been examined. Here, we report that the defense responses activated by 35S::*Pto* are severely inhibited by high temperature. In addition, we found that depletion of SA had only a minor effect on 35S::*Pto*-mediated

general resistance, but the necrotic lesions caused by SA depletion were suppressed by 35S::*Pto*.

RESULTS

35S::*Pto*-mediated general resistance is drastically affected by temperature.

Multiple transgenic tomato plants carrying the 35S::*Pto* transgene showed striking differences when grown in different seasons in the greenhouse. The seasonal effect was studied in detail with MM48, a Money Maker (MM) transgenic line homozygous for the 35S::*Pto* locus. MM48 plants exhibited massive microscopic cell death when grown during the November through March season, resulting in severe inhibition of plant growth (Fig. 1A). When infected with virulent *P. syringae* pv. *tomato* T1 strains, these plants often exhibited approximately 100- to 500-fold more resistance than the nontransgenic plants, as measured by leaf bacterial populations. However, the same transgenic plants showed only minor cell death along the veins and almost normal growth during the June through September season (Fig. 1B). In this season, the MM48 plants showed only approximately 10- to 20-fold more resistance than the nontransgenic plants, as measured by leaf bacterial populations.

The major differences in our greenhouse between the two seasons were day length and temperature: approximately 10 h light and 16 to 23°C during the November through March season and approximately 15 h light and 27 to 33°C during the June through September season. As induction of the spontaneous lesions requires light (Tang et al. 1999), we first determined whether day length affected the spontaneous lesions by adding artificial light to the greenhouse during winter. We found that increasing the photoperiod to 16 to 20 h November through March did not reduce the spontaneous cell death in 35S::*Pto* plants. We then tested the influence of temperature on spontaneous lesions by growing the 35S::*Pto* plants in growth chambers with a 12-h photoperiod (approximately 120 μEm^{-2}

s^{-1} of light), 70% humidity, and a constant temperature of 20°C and 30°C, respectively. Plants grown at 20°C showed massive cell death, while plants grown at 30°C were almost free of cell death (Fig. 1C, D, and E).

SA plays a minor role in the 35S::*Pto*-mediated defense.

The 35S::*Pto* plants exhibited SA accumulation, microscopic cell death, pathogenesis-related (PR) gene induction, and broad resistance to pathogens (Tang et al. 1999). In many Arabidopsis lesion mimic mutants, these reactions are mediated by a SA-dependent pathway (Rate et al. 1999; Shah et al. 1999; Weymann et al. 1995). To determine whether SA is required for the induction of the various defense responses in 35S::*Pto* plants, we crossed MM48 with an MM line homozygous for the 35S::*nahG* transgene (Brading et al. 2000). MM48 plants were also crossed with nontransgenic MM to generate control plants. The SA content was measured in the F1 plants. MM48 $\times$ MM F1 plants accumulated a high level of SA (approximately 27 μg of SA per g of fresh leaves). However, the SA level in the MM48 $\times$ NahG F1 plants was below the detection limit (<1 μg of SA per g of fresh leaves) of the high-pressure liquid chromatography (HPLC) analysis, similar to that in the parental NahG plants. These results demonstrated that *nahG* expression effectively blocked the accumulation of SA caused by *Pto* overexpression.

We then examined whether depletion of SA affected the spontaneous, microscopic cell death in 35S::*Pto* plants. The leaves of MM48 $\times$ MM and MM48 $\times$ NahG F1 plants were examined with a fluorescence microscope for autofluorescence under UV light, a reliable indicator of spontaneous cell death in 35S::*Pto* plants. Figure 2 shows cell death in plants grown in March. Both the MM48 $\times$ MM and MM48 $\times$ NahG F1 plants showed a similar level of fluorescence, indicating that depletion of SA did not affect the microscopic cell death caused by *Pto* overexpression. The two F1 lines also showed similar low levels of cell death in the summer (approximately 27 to 33°C; data not shown).

Fig. 1. Low temperature enhances microscopic cell death leading to growth retardation of 35S::*Pto* plants. **A**, MM, MM48 $\times$ NahG F1, and MM48 plants grown in a greenhouse in February (approximately 16 to 20°C). **B**, MM and MM48 plants grown in a greenhouse in June (approximately 27 to 30°C). **C**, Leaves of MM48 plants grown in two growth chambers at 30 and 20°C, respectively. **D**, Microscopic cell death of MM48 plants grown in a growth chamber at 30°C. **E**, Microscopic cell death of MM48 plants grown in a growth chamber at 20°C. Cells with strong fluorescence are dead cells.

To determine how SA depletion affects PR gene expression, we examined the expression of six PR genes in the MM48 × MM F1, MM48 × NahG F1, and wild-type MM plants. Figure 3 shows that all the PR genes were expressed at a higher level in plants expressing the 35S::Pto gene than in the nontransgenic MM plants. The elevated expression of PR genes, except for *Glub*, was partially repressed in the presence of the *nahG* transgene. The repression was more pronounced for *Glua* than it was for *PR1a-1*, *PR1b-1*, *Chia*, and *Osmotin* (*Osm*).

We studied how the removal of SA affected the general disease resistance caused by 35S::Pto. F1 plants of MM48 × NahG and MM48 × MM were compared for resistance to *P. syringae* pv. *tomato* T1 strain, which does not carry the *avrPto* gene. Figure 4A shows bacterial growth in plants grown in June. Four days after inoculation, reduced bacterial growth was detected in the MM48 × NahG and MM48 × MM F1 plants compared with nontransgenic MM plants. However, bacterial numbers in the MM48 × NahG F1 plants were approximately 4- to 5-fold higher than those in the MM48 × MM F1 plants, suggesting that SA is partially required for the general resistance caused by 35S::Pto. A similar experiment was conducted in February. Both the MM48 × MM and MM48 × NahG F1 plants showed strong resistance, and the bacterial numbers in the MM48 × NahG F1 plants were slightly higher than those in the MM48 × MM F1 plants (Fig. 4B).

The NahG plants also were compared with the MM plants for susceptibility to the T1 infection. In several experiments conducted in different seasons, we observed that the NahG plants supported <2-fold more bacterial growth than did the MM plants, again indicating a minimal role of SA in basal resistance to virulent *P. syringae* pv. *tomato*.

The action of 35S::Pto suppresses necrotic lesions caused by SA depletion.

Tomato NahG plants form spontaneous, necrotic lesions on mature leaves and leaf petioles that often result in death of the whole leaf (Fig. 5A and B) (Brading et al. 2000). These lesions are large and morphologically distinct from the 35S::Pto-induced spontaneous cell death that forms bleached, tiny speckles on the adaxial surface of mature leaves. Spontaneous necrotic lesions were also observed in 6- to 7-week-old MM48 × NahG F1 plants grown in summer, albeit fewer than in plants carrying *nahG*

Fig. 2. Spontaneous cell death in 35S::Pto plants was not affected by SA depletion and *Nr* mutation. Plants were grown in a greenhouse at 18 to 23°C. Leaves from MM, MM48 × MM, MM48 × NahG, and MM48 × *Nr* are indicated.

alone (data not shown). Unlike the 35S::Pto-induced microscopic cell death that was enhanced by low temperature, the large necrotic lesions were completely suppressed in the MM48 × NahG F1 plants in the winter (Fig. 5A and B). However, the formation of the large necrotic lesions in NahG plants was not affected by temperature or other seasonal changes.

The NahG-dependent necrotic lesions were also triggered by bacterial infection. When inoculated with the virulent T1 strain of *P. syringae* pv. *tomato*, both nontransgenic MM plants and NahG transgenic plants developed small necrotic “specks” 3 to 4 days after inoculation (Fig. 5C). The disease lesions showed marginal enlargement in the following week in MM plants. However, the lesions in NahG plants showed uncontrolled expansion after 4 days and eventually engulfed the entire leaf (Fig. 5D and E). Uncontrolled expansion of the disease lesions was also observed in the MM48 × NahG F1 plants grown in summer (Fig. 5D). The MM48 × NahG F1 plants grown in winter showed only an average of one to two disease lesions per leaf, which did not expand (Fig. 5E). Interestingly, the uncontrolled lesion expansion in NahG plants and MM48 × NahG F1 plants was not associated with a further increase of bacterial growth 4 days after inoculation (Fig. 4A), indicating that lesion expansion was not caused by increased susceptibility.

The *Nr* mutation does not affect defense responses in 35S::Pto plants.

Ethylene regulates lesion formation in Arabidopsis *acd5* and *cpr5* mutants (Clarke et al. 2000; Greenberg et al. 2000), and recent studies indicated that ethylene and SA regulate

Fig. 3. Salicylic acid depletion partially reduced the pathogenesis-related gene expression in 35S::Pto plants. Total RNA was extracted from fully expanded leaves of MM48 × MM, MM48 × NahG, and MM plants grown in a greenhouse at 18 to 23°C. Total RNA (10 µg) was separated in a denaturing agarose gel. Duplicated RNA blots were hybridized with cDNA probes for *Glua*, *PR-1a1*, *PR-1b1*, *Chia*, *Glub*, and *Osm*. Equal loading was verified prior to blotting by visualizing rRNA in the gel stained with ethidium bromide (bottom).

different sets of defense reactions (Clarke et al., 2000; Penninckx et al. 1996). Because the defense responses in 35S::*Pto* plants were only partially dependent on SA, we examined whether these responses were regulated by ethylene. The MM48 plant was crossed with the *Nr* mutant. *Nr* is a dominant mutation that blocks ethylene signal transduction in tomato (Lanahan et al. 1994). As a control, MM48 was also crossed with the tomato line Pearson (Prsn), the wild-type parent of the *Nr* mutant. The *Nr* mutant shows a number of ethylene-insensitive phenotypes, such as lack of flower senescence and fruit ripening (Lanahan et al. 1994). MM48 × *Nr* F1 plants did not show flower senescence and fruit ripening, indicating disruption of the ethylene signal transduction. MM48 × Prsn F1 plants displayed normal ethylene sensitivity as indicated by normal flower senescence and fruit ripening. The F1 plants of both MM48 × *Nr* and MM48 × Prsn exhibited similar cell death (Fig. 2), suggesting that ethylene is not required for induction of spontaneous lesions in tomato plants overexpressing *Pto*. In addition, PR gene expression was indistinguishable in the MM48 × *Nr* and MM48 × Prsn F1 plants (Fig. 6). The F1 plants of MM48 × *Nr* and MM48 × Prsn were also examined for resistance to *P. syringae* pv. *tomato* T1 strain. Both F1 plants supported similarly reduced bacterial growth compared with that in MM × Prsn F1 (Fig. 7). The MM48 × *Nr* and MM48 × Prsn F1 plants developed similar disease symptoms as measured by disease lesion size and number. These data suggest that ethylene is not required for the induction of defense reactions in 35S::*Pto* plants.

DISCUSSION

We investigated environmental regulation and the roles of SA and ethylene in the general disease resistance activated by 35S::*Pto*. We demonstrated that the general defense responses in the 35S::*Pto* plants were severely inhibited by high temperature. By removing SA and disrupting ethylene signal transduction in 35S::*Pto* plants, we showed that SA played only a minor role in general defense responses activated by 35S::*Pto* while ethylene was dispensable for this general resistance. In addition, we found that the necrotic lesions associated with *nahG* expression were suppressed by *Pto*-overexpression, indicating an interaction between general resistance and cellular damage caused by SA deficiency.

Temperature can modulate both *R*-gene mediated gene-for-gene resistance and general defense reactions. For example, it has been reported that high temperature inhibits the *R*-gene mediated resistance to *Puccinia coronata* in oat, *P. syringae glycinea* in soybean, and *Tobacco mosaic virus* in tobacco (Dinesh-Kumar et al. 1995; Keen et al. 1981; Zimmer and Schaffer 1961). In addition, high temperature also inhibits spontaneous cell death and general disease resistance in transgenic plants expressing the bacterial proton pump and pyruvate decarboxylase genes (Mittler et al. 1995; Abad et al. 1997; Tadege et al. 1998). In these cases, high temperature may inactivate *R* gene products, recognition of the *R* gene products and *avr* gene products, or components of the defense signal transduction pathways. We observed that the 35S::*Pto*-mediated defense responses were severely inhibited at high temperatures. It is yet unclear whether high temperature inactivates *Pto* kinase or the *Pto* pathway components. However, it is interesting to note that high temperature was never reported to suppress the *Pto*-mediated gene-for-gene resistance. If this is indeed the case, it would suggest two possibilities. First, *Pto*-mediated gene-for-gene resistance and 35S::*Pto*-mediated general resistance involve different signal transduction components, and the components in the gene-for-gene pathway are insensitive to temperature, while some components required for the 35S::*Pto*-mediated general resistance are sensitive to high temperature. Second, both *Pto*-mediated gene-for-gene resistance and 35S::*Pto*-mediated general resistance are temperature-sensitive, but *Pto*-mediated gene-for-gene resistance is much stronger than 35S::*Pto*-mediated general resistance (Tang et al. 1999), and therefore, even though a significant portion of the gene-for-gene resistance is lost at high temperature, the remaining resistance is still sufficient to completely suppress the disease.

Accumulation of SA is observed in lesion mimic mutants with enhanced general disease resistance (Dangl et al. 1996). In many Arabidopsis lesion mimic mutants, high levels of SA are required for lesion formation, induction of PR genes, and enhanced general resistance (Rate et al. 1999; Shah et al. 1999; Vanacker et al. 2001; Weymann et al. 1995). Although tomato plants overexpressing *Pto* exhibit similar defense characteristics as many Arabidopsis lesion mimic mutants, depletion of SA by the *nahG* transgene had no detectable effects on microscopic lesions of the 35S::*Pto* plants, indicating that SA is not

Fig. 4. Salicylic acid depletion slightly reduced the 35S::*Pto*-mediated general resistance. MM, MM48 × MM, and MM48 × NahG F1 plants were vacuum-infiltrated with 10^4 CFU per ml *Pseudomonas syringae* pv. *tomato* T1 strain. The growth of the T1 strain on MM, MM48 × MM, and MM48 × NahG plants grown in a greenhouse at 27 to 30°C (June) and 16 to 20°C (February) were measured by grinding two leaf discs in 10 mM MgCl₂, plating diluted suspensions on appropriate KB plates, and counting CFU. **A**, Bacterial growth in June. **B**, Bacterial growth in February.

Fig. 5. 35S::*Pto* suppresses necrotic lesions caused by salicylic acid deficiency. Leaves from the 7-week-old MM, NahG, and MM48 $\times$ NahG F1 plants grown in a greenhouse in January (approximately 16 to 20°C) were photographed to show the spontaneous necrotic lesions on the **A**, leaf petioles and **B**, leaves of the NahG plants but not the MM and MM48 $\times$ NahG F1 plants. The MM48 $\times$ NahG leaves in **A**, and **B**, are magnified three times. One-month-old MM, NahG, MM48 $\times$ MM F1, and MM48 $\times$ NahG F1 plants grown in a greenhouse in **C** and **D**, June (approximately 27 to 30°C) or **E**, February (approximately 16 to 20°C) were inoculated with 10^4 CFU of *Pseudomonas syringae* pv. *tomato* T1 strain per ml. Leaves were photographed at **C**, 4 days and **D** and **E**, 6 days after inoculation to show disease lesion expansion in the NahG plants and the MM48 $\times$ NahG F1 plants grown in summer and the suppression of necrotic lesions in MM48 $\times$ NahG F1 plants grown in winter.

necessary for the microscopic cell death caused by 35S::Pto. In addition, depletion of SA in the 35S::Pto tomato plants resulted in only a small increase in bacterial growth. The relatively small role of SA in inhibiting the *P. syringae* pv. *tomato* growth was consistent with the fact that defense-related genes were expressed at high levels in the MM48 × NahG F1 plants. Ectopic expression of *Pto* in tomato constitutively activates the expression of at least 70 genes (Tang et al. 1999; Xiao et al. 2001). However, only a small fraction of these genes require SA for their elevated expression in 35S::Pto plants (Xiao et al. 2001). The depletion of SA had only a minor effect on expression of *PR-1a1*, *PR-1b1*, *Chia*, and *Osmotin* in 35S::Pto plants. The SA-independent defense gene expression may be responsible for the SA-independent resistance to *P. syringae* pv. *tomato* in 35S::Pto plants.

While depletion of SA in 35S::Pto plants reduced the resistance to *P. syringae* pv. *tomato* by approximately four- to five-fold, the *nahG* transgene in plants lacking 35S::Pto had even less impact on the growth of *P. syringae* pv. *tomato*. In several experiments, we found that the bacterial numbers in NahG plants were less than twofold higher than those in the nontransgenic tomato plants. The insignificant role of SA in resistance to *P. syringae* pv. *tomato* was consistent with previous reports that SA played only a minimal role in resistance to *Xanthomonas campestris* pv. *vesicatoria* (O'Donnell et al. 2001) and *Cladosporium fulvum* in tomato plants (Brading et al. 2000). The lack of an effect of SA in tomato disease resistance is not likely to be caused by pleiotropic effects of *nahG* that might have masked SA function, although we cannot completely rule out this possibility. SA-independent disease resistance is not unique to tomato. Arabidopsis plants, in which SA was found to play a critical role in resistance against various pathogens, including *P. syringae* pv. *tomato* (Delany et al. 1994; Dong 1998), are also able to confer disease resistance in the absence of SA (Hunt et al. 1997; McDowell et al. 2000; Penninckx et al. 1996; Raidan et al. 2001; Roetschi et al. 2001; Shah et al. 2001). The different requirements for SA by tomato and Arabi-

dopsis plants for *P. syringae* pv. *tomato* resistance suggest that different plant species may employ distinct mechanisms for resistance against the same pathogen.

Tomato plants expressing the *nahG* gene form unrestricted necrotic lesions both spontaneously and in response to *P. syringae* pv. *tomato* infection. The mechanism responsible for the NahG-dependent spontaneous lesions and the uncontrolled disease lesion expansion is not clear, but they appear to be related to a lack of control on cellular damage. Uncontrolled expansion of spontaneous necrotic lesions and disease lesions has been also observed in rice plants expressing *nahG* (Yang and Qi 2000). Like tomato, rice plants also contain a high basal level of SA (Brading et al. 2000; Lee et al. 2001). The similar reactions in rice and tomato plants expressing the *nahG* gene suggest a conserved function of SA in protection against cellular damage, probably because of the antioxidant function of SA. However, the uncontrolled expansion of disease lesions was not observed in the NahG tomato plants infected by *X. campestris* pv. *vesicatoria* (O'Donnell et al. 2001). How the *X. campestris* pv. *vesicatoria* infection suppressed the lesion expansion in NahG plants was unclear. Several observations in this study indicate that the uncontained necrotic lesions associated with *nahG* expression can be inhibited by a 35S::Pto-mediated function. For example, the MM48 × NahG F1 plants grown in summer exhibited much less spontaneous necrotic lesion development compared with that on the NahG plants and were completely free of *nahG*-dependent spontaneous lesions in winter. The stronger suppression in winter is correlated with the more pronounced defense responses conferred by 35S::Pto under lower temperatures. Similarly, uncontrolled disease lesion expansion in the MM48 × NahG F1 plants was observed only in summer and not in winter. The effect of temperature on *nahG*-dependent lesions in MM48 × NahG F1 plants was mediated by 35S::Pto, as lesion initiation and expansion in NahG plants were not affected by temperature. These results suggest that one (or more) of the responses activated by 35S::Pto may act to limit cellular damage caused by SA deficiency. In fact, MM48 plants constitutively accumulate high levels of at least three catalase mRNA species (Xiao et al. 2001). It may be that

Fig. 6. *Nr* mutation does not affect pathogenesis-related gene expression in 35S::Pto plants. Total RNA was extracted from fully expanded leaves of MM48 × *Nr*, MM48 × Prsn, and MM × Prsn plants grown in a greenhouse in March (approximately 18 to 23°C). Total RNA (10 µg) was loaded in each lane and separated in a denaturing agarose gel. The blots were hybridized with the cDNA probes for *PR-1a1*, *Glub*, *PR-1b1*, *Osm*, and *Chia*. Equal loading was verified by staining the gel with ethidium bromide (bottom).

Fig. 7. *Nr* mutation does not affect 35S::Pto-conferred general resistance. MM × Prsn, MM48 × Prsn, and MM48 × *Nr* F1 plants grown in a greenhouse in June (approximately 27 to 30°C) were vacuum-infiltrated with 2×10^4 CFU of *Pseudomonas syringae* pv. *tomato* T1 strain per ml. Bacterial growth was measured by grinding two leaf discs in 10 mM MgCl₂, plating diluted suspensions on appropriate King's B medium, and counting CFU. Each data point represents the average of three measurements. Error bars indicate standard error.

increased antioxidant gene expression upon *Pto* overexpression compensated for the antioxidant function of SA.

The *Nr* mutation did not affect spontaneous cell death or the expression of the PR genes in 35S::*Pto* plants. As measured by leaf bacterial population, the *Nr* mutation also had no effect on resistance to *P. syringae* pv. *tomato* in 35S::*Pto* plants. This is consistent with the report that the growth of *P. syringae* pv. *tomato* was not affected by *Nr* mutation in tomato plants lacking the 35S::*Pto* transgene (Lund et al. 1998). These results collectively suggest that ethylene does not play a significant role in disease resistance mediated by 35S::*Pto*. However, the possibility that ethylene plays a role in 35S::*Pto*-mediated resistance can not be completely ruled out, because the *Nr* mutant sometimes shows residual ethylene responses (Lanahan et al. 1994).

MATERIALS AND METHODS

Plant materials.

MM48 line was a transgenic Money Maker line containing a single insertion of the 35S::*Pto* transgene (Tang et al. 1999). The NahG transgenic plants were generated by Brading and associates (2000) and kindly provided by J. Jones. Seeds of the *Nr* mutant and Pearson plants were kindly provided by H. Klee. F1 seeds were generated by reciprocal crosses. Tomato plants were grown in a greenhouse or growth chamber. As tomato plants containing the *nahG* gene developed uncontrolled lesions when they were older than 6 to 7 weeks, assays were conducted on one-month-old plants.

RNA extraction and RNA gel blot analysis.

Tomato plants grown in February were used for RNA blot analysis. Well-expanded leaves from one-month-old plants were collected for gene expression analysis. Leaves were immediately frozen in liquid nitrogen after collection, and RNA was isolated as previously described (Tang et al. 1994). Total RNA (10 µg) from each sample was loaded on a formaldehyde-agarose gel, separated by electrophoresis, transferred to nitrocellulose membrane, and immobilized to the nitrocellulose membrane by UV-crosslinking. cDNA probes for *PR-1a1*, *PR-1b1*, *Chia*, *Gluc*, *Glub*, and *Osm* genes are as previously described (Tang et al. 1999). All probes were radiolabeled with ³²P-dCTP using a random-primed labeling kit (Amersham, Piscataway, NJ.). RNA gel blot analysis was performed as previously described (Tang et al. 1994).

Histochemistry and microscopy.

Methods described by Tang and associates (1999) were used for the detection of autofluorescent materials. Briefly, leaves were cleared by boiling in lactophenol and rinsed first in 50% ethanol and then in water. To visualize autofluorescence, cleared leaves were examined with a UV epifluorescence microscope. Images were obtained using a CCD (charge-coupled device) camera coupled to the microscope.

Measurement of SA.

SA was extracted as previously described (Bowling et al. 1994) and analyzed with an HPLC coupled with a C18 stainless steel column and a photodiode array detector.

Pathogen infections of tomato leaves.

P. syringae pv. *tomato* T1 strain was inoculated in liquid King's B (KB) medium (Martin et al. 1993) containing 50 mg of rifampicin per l and grown overnight. The bacterial cells were washed twice with 10 mM MgCl₂ and resuspended in 10 mM MgCl₂. Bacterial density was determined by absorbance at 600 nm optical density. Bacteria were diluted to the 2 × 10⁴ CFU per ml in 10 mM MgCl₂ plus 0.04% Silwet L-77 (Osi,

Danbury, CT, U.S.A.) for inoculation. One-month-old plants were inoculated by vacuum infiltration, and the inoculated plants were kept in a greenhouse. Leaf bacteria were measured by grinding two leaf discs in 10 mM MgCl₂, plating diluted suspensions on appropriate KB plates, and counting CFU.

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