Delayed Symptom Development in *ein2-1*, an *Arabidopsis* Ethylene-Insensitive Mutant, in Response to Bacterial Wilt Caused by *Ralstonia solanacearum*

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ABSTRACT

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Wilt disease caused by the phytopathogenic bacterium *Ralstonia solanacearum* is poorly understood at the molecular level. The possible roles of salicylic acid, jasmonic acid, and ethylene, compounds commonly associated with the plant response to pathogens, in wilt symptom development were investigated using various *Arabidopsis thaliana* mutants in a Col-0 background, an ecotype that develops wilt symptoms in response to the virulent GMI1000 strain. Following root inoculation, wilt symptoms were delayed in *ein2-1*, an ethylene-insensitive mutant, in

response to several virulent strains of the pathogen. In *ein2-1*, bacteria invade the plant and multiply, reaching concentrations slightly lower than those detected in susceptible plants but 1 to 2 logs higher than in Nd-1, an *A. thaliana* ecotype resistant to strain GMI1000. This delay in disease symptom development of *ein2-1* plants suggests that ethylene signaling plays a critical role in wilt disease development. Furthermore, a strong accumulation of transcripts corresponding to *PR-3* and *PR-4*, two ethylene-responsive genes, was observed in susceptible Col-0 plants, but not in *ein2-1* and Nd-1 plants, providing additional evidence for a role of ethylene in wilt symptom production. However, this hormone is probably not involved in the establishment of resistance to *R. solanacearum*, because homozygous *ein2-1* plants in a resistant background remain fully resistant to strain GMI1000.

The plant hormone ethylene regulates a number of developmental processes and stress responses in plants. This gaseous compound is involved in physiological processes as diverse as senescence, germination, fruit ripening, wounding, and sex determination (1). Participation of ethylene in plant responses to pathogens is well documented although its effect varies greatly according to the nature of the pathogen. The role of ethylene in plant pathogenesis and disease resistance was first hypothesized based on physiological studies: ethylene levels have been shown to increase upon infection by pathogens (6,15,46). Furthermore, ethylene biosynthesis genes are activated in response to pathogen attack (31), and the expression of defense genes is induced by ethylene treatment (10,20,38).

Although the role of ethylene in the plant response to biotic stresses has long remained controversial because of apparently conflicting results, there is now compelling genetic evidence that ethylene signaling can contribute either to disease resistance or disease susceptibility, depending on the plant-pathogen interaction. The use of mutant or transgenic plants impaired in their response to ethylene has been instrumental in establishing the role of the ethylene signal transduction pathway in the plant response to microbial infection. Although ethylene-insensitive plants of different species such as *Arabidopsis thaliana* (4,51), tobacco (29), soybean (27), and tomato (14), have all shown normal resistance to a range of different avirulent pathogens, ethylene insensitivity results in reduced resistance to the soft rot fungus *Botrytis cinerea* (51) and to the potato soft rot agent *Erwinia carotovora* subsp. *carotovora* (35) in the *A. thaliana ein2-1* mutant (2).

Similarly, soybean ethylene-insensitive mutants display disease symptoms in response to an avirulent strain of the fungal pathogen *Phytophthora sojae*, and increased susceptibility to virulent strains of *Septoria glycines* and *Rhizoctonia solani* (27). Likewise, tobacco plants transformed with the mutant *etr1-1* gene from *A. thaliana* that confers dominant ethylene-insensitivity are susceptible to the normally nonpathogenic soilborne fungus *Pythium sylvaticum* (29). In tomato, a reduction of the expression of *LeTR4*, one of the five tomato ethylene receptor genes identified so far, which results in increased sensitivity to ethylene, leads to an enhanced hypersensitive response and reduced growth of an avirulent strain of *Xanthomonas campestris* pv. *vesicatoria* compared with wild-type plants (13). Finally, the *A. thaliana cev1* mutation, which stimulates both the jasmonic acid (JA) and ethylene pathways, also causes a higher level of resistance to powdery mildew diseases (21).

Conversely, ethylene insensitivity greatly reduces susceptibility of *A. thaliana* to virulent strains of *Pseudomonas syringae* pv. *tomato*, *Pseudomonas syringae* pv. *maculicola*, or *X. campestris* pv. *campestris* (4), and to the sugar beet cyst nematode *Heterodera schachtii* (56). Soybean ethylene-insensitive mutants display reduced symptoms in response to virulent *Phytophthora sojae* and *Pseudomonas syringae* pv. *glycineae* (27). In tomato, *Nr* (Never-ripe), a dominant ethylene-insensitive mutant affected in an ethylene receptor (30), exhibits reduced necrosis in response to the virulent pathogen *X. campestris* pv. *vesicatoria*, suggesting that the hormone may be involved in pathogen-induced cell death pathways in wild-type plants (33). Thus, ethylene signaling both contributes to disease resistance to particular classes of pathogens, and is involved in symptom development in response to infection by other classes of disease-causing agents.

Other signaling molecules such as salicylic acid (SA) and JA, which accumulate in plants during pathogen infection, play an im-

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portant role in resistance establishment (18,22,41), whereas their involvement in disease symptom production still remains poorly documented.

Ralstonia solanacearum is a gram-negative, soilborne pathogen that induces wilt disease development on more than 200 plant species (26). Paradoxically, little is known about the molecular mechanisms underlying resistance and susceptibility to this important pathogen. In tomato, resistance to *R. solanacearum* is complex and polygenic (52,53). The demonstration that this bacterium, and in particular strain GMI1000, a well characterized tomato strain (7), is capable of wilting many *A. thaliana* ecotypes in a manner comparable to that described in tomato, as well as the existence of resistant ecotypes, led to the recent isolation of the *RRS1-R* and *RRS1-S* genes, the alleles present in the resistant Nd-1 ecotype and the susceptible Col-0 ecotype, respectively. The *RRS1* genes encode a novel family of R proteins (16,17).

The objectives of this study were the identification of signaling pathways involved in wilt disease development. Several *A. thaliana* mutant or transgenic lines altered in signaling pathways commonly associated with resistance or susceptibility to pathogens were tested for their response to the virulent GMI1000 strain of *R. solanacearum*. Whereas the development of wilt symptoms appeared to be independent of SA and JA, *ein2-1* ethylene-insensitive plants in a susceptible Col-0 genetic background presented a delayed symptom production in response to several virulent *R. solanacearum* strains, indicating that ethylene signaling plays an important role in the susceptible response of *A. thaliana* to *R. solanacearum*. Our data are corroborated by the preferential accumulation of the ethylene-inducible *PR-3* and *PR-4* transcripts in infected Col-0 plants, whereas the steady-state mRNA levels of these two genes are much weaker in *ein2-1* as well as Nd-1 plants.

MATERIALS AND METHODS

Plant materials and inoculations. *A. thaliana* plants were grown and root-inoculated with the various *R. solanacearum* strains as previously described (17). Leaf inoculations were performed by puncturing leaves with a needle dipped in a bacterial colony according to the protocol of Yang and Ho (57). Wilting of the susceptible Col-0 plants was observed 4 to 8 days after inoculation. Pathogenicity tests were conducted until resistant Nd-1 plants presented a yellowing of the older leaves (usually 12 to 16 days after inoculation). The ethylene signaling mutants as well as the *jar1-1* mutant were obtained from the Arabidopsis Stock

Center (Nottingham Arabidopsis Stock Center, UK). The *nahG A. thaliana* plants were provided by J. Ryals.

Bacterial strains. All the *R. solanacearum* strains used in this study were supplied by C. Boucher and A. Trigalet. They were grown at 28°C in bacto-agar glucose triphenyltetrazolium chloride (BGT) medium (7). The GMI1000 strain is a wild-type isolate which is virulent on tomato and induces the hypersensitive response on tobacco. The Δhrp strain is a derivative of strain GMI1000 (C. A. Boucher, unpublished data) whose *hrp* cluster was replaced by an Ω cassette (40).

Disease scoring. Disease symptoms were scored regularly, up to 16 days after inoculation, on each individual inoculated plant according to the percentage of wilted leaves using the following 0-to-4 scale: 0 = no wilting, 1 = 25%, 2 = 50%, 3 = 75%, and 4 = 100% of wilted leaves.

Bacterial internal growth curves. Internal bacterial populations in the aerial parts of plants after root-inoculation in a suspension of 10⁸ bacteria per ml were estimated in triplicate for each time point according to the protocol of Deslandes et al. (17). Serial dilutions of bacteria present in the ground extracts of aerial leaves were spread on solid BGT medium containing the appropriate antibiotic (rifampicin 50 µg ml⁻¹).

Generation of homozygous *ein2-1* plants in an *RRS1-R* homozygous background. Nd-1 plants were crossed with *ein2-1* plants. Lines homozygous for the *ein2-1* mutation were selected in the F₂ generation by identifying plants that displayed an ethylene-insensitive phenotype in the triple response assay. For triple response experiments, seedlings were grown in the dark on agar medium in sterile, gas-tight jars in which ethylene (final concentration 50 µl liter⁻¹) was applied by injecting the appropriate volume of ethylene gas with a syringe through a rubber septum. The *EIN2* gene was then amplified by polymerase chain reaction (PCR) and sequenced in order to check that the ethylene-insensitive plants were indeed homozygous for the *ein2-1* mutation. Plants homozygous for *RRS1-R*, the allele that confers resistance to *R. solanacearum* strain GMI1000, were selected by a PCR-based marker that distinguishes between the *RRS1-R* and *RRS1-S* alleles present in ecotypes Nd-1 and Col-0, respectively. The primers that were used have the following sequences: 5'-ATGT-TATATCGACGTTGG-3' and 5'-AACTCCTCCATGTCCGTC-3'. Genomic DNA for PCR analysis was isolated from leaves according to the protocol of Saghai-Marouf et al. (43). PCR reactions were performed in a total volume of 50 µl containing 10 ng of genomic DNA, 0.2 µM each primer, 0.2 mM each dNTP, 1.5 mM MgCl₂, and 1.25 units of *Taq* polymerase. The PCR program used

TABLE 1. Response to strain GMI1000 of various *Arabidopsis thaliana* mutants altered in their response to pathogens

Mutants	Comments	Ref.	Response to strain GMI1000 ^a
<i>nahG</i>	Depletion of endogenous salicylic acid	(32)	S
<i>cpr1</i>	Constitutively elevated levels of salicylic acid and PR gene expression	(8)	S
<i>cpr5</i>	Constitutively elevated levels of salicylic acid and PR gene expression	(9)	S
<i>jar1-1</i>	Jasmonic acid-insensitive mutant	(47)	S
<i>etr1-3</i>	Ethylene-insensitive mutant	(25)	S
<i>ein4-1</i>	Ethylene-insensitive mutant	(28)	S
<i>ein2-1</i>	Ethylene-insensitive mutant	(25)	DSP (7/63)
<i>ein3-1</i>	Ethylene-insensitive mutant	(12)	S
<i>eto2</i>	Ethylene-overproducing mutant	(54)	S
<i>eto3</i>	Ethylene-overproducing mutant	(55)	DSP (14/63)

^a S = susceptible (disease symptom production identical to that of wild-type Col-0 plants). DSP = delayed symptom production compared with Col-0 plants. Ratio indicates the percentage of mutant plants wilted 10 days after inoculation versus the percentage of Col-0 plants wilted at this stage. These experiments were performed twice using 30 plants and similar results were obtained.

Fig. 1. Symptom development in *ein2-1* and Col-0 plants. Fifty-six mutant and wild-type plants were root-inoculated with strain GMI1000 at a concentration of 10⁸ bacteria per milliliter. Plants were considered completely wilted when 100% of the rosette leaves were wilted. This experiment was repeated three times and reproducible results were obtained.

included an initial denaturation step (3 min at 94°C), 7 amplification cycles (1 min at 94°C, 2 min at 50°C, and 2 min at 72°C), an additional 31 amplification cycles (1 min at 94°C, 1 min 30 s at 52°C, and 1 min 30 s at 72°C), and a final termination step (5 min at 72°C).

RNA gel blot analysis. Total RNA was isolated from frozen leaf material with the Extract-All solution according to the recommendations of the manufacturer (Eurobio, Les Ulis, France). For northern blots, total RNA (20 µg) was separated on 1% agarose gels according to the protocol of McMaster and Carmichael (34), transferred to Nylon membranes (Hybond N+, Amersham Pharmacia Biotech, Buckinghamshire, England) in a 20× SSC blotting solution (3 M NaCl plus 0.3 M sodium citrate, pH 7.0), and cross-linked to the filters with a UV Stratalinker 2400 (Stratagene, Ozyme, France).

Expressed sequence tag stocks 92G1T7 (*PR-3*) and 245P5T7 (*PR-4*), used for DNA probe synthesis, were ordered from the Arabidopsis Biological Resource Center, Ohio State University, Columbus. Plasmid DNAs were extracted with the Concert Rapid Plasmid Miniprep System (Invitrogen Life Technologies, San Diego, CA) according to the protocol provided by the manufacturer. After *Sall*/*NotI* digestion of plasmid vectors, and prior to labeling, DNA inserts were gel-purified with a gel extraction kit (JETSORB; GENOMED, Bad Oeynhausen, Germany). Labeling of the probes was performed by random priming (Ready-To-Go Kit; Amersham Pharmacia Biotech) with (α -³²P)dCTP according to the manufacturers' instructions. Membranes were prehybridized for 2 h and hybridized overnight at 65°C in 0.5 M sodium phosphate buffer (pH 7.2), 25 mM EDTA (pH 8), 0.1% bovine serum albumin, 7% sodium dodecyl sulfate (SDS), and 0.1 mg ml⁻¹ of heat-denatured salmon sperm DNA. The membranes were then washed twice at 65°C in 2× SSC, 0.1% SDS for 10 min, and once at the same temperature in 0.1× SSC, 0.1% SDS for 5 min. Blots

were exposed to autoradiogram film (Fuji Medical X-ray films, Dusseldorf, Germany).

To check for equal loading of RNA, the blots were stripped and hybridized with a ³²P-labeled radish 18S rDNA probe (obtained from T. Tremousaygue).

RESULTS

Responses to strain GMI1000 of various *A. thaliana* mutants altered in signaling pathways associated to plant resistance.

In order to identify which signaling pathways are important for symptom development caused by *R. solanacearum*, several mutant or transgenic lines in a susceptible Col-0 background (Table 1) were root-inoculated with strain GMI1000. *NahG* transgenic plants (32) affected in SA accumulation and *cpr1* (9) and *cpr5* (8) plants overexpressing a number of pathogenesis-related (PR) proteins did not present detectable differences in wilt symptom production compared with the wild-type Col-0 plants. Responses similar to that of wild-type susceptible plants were also observed in various mutants affected in the ethylene signaling pathway such as *etr1-3* (25), *ein4-1* (42), and *ein3-1* (12), as well as in *jar1-1* (47), a JA-insensitive mutant present in a Col-0 susceptible genetic background. For all of these mutants, no differences were observed either in the timing of symptom appearance or in the intensity of wilt symptoms compared with wild-type plants. In contrast, *ein2-1* (25) and *eto3* (55) plants exhibited delayed and weaker wilt symptoms after inoculation with strain GMI1000. Although disease symptom production was strongly delayed in *eto3* plants, the study of their response to the bacterial pathogen was hindered because these plants are severely altered in the development of their root system. It was therefore difficult to assess the reasons of the observed delay in symptom appearance. As shown in Figure 1, the development of wilt symptoms was strongly delayed in *ein2-1* plants compared with Col-0 plants. Seven days after inoculation, 46 of the 56 Col-0 plants tested versus 3 of the 56 *ein2-1* plants were wilted. All Col-0 plants were wilted 8 days after inoculation, whereas complete wilting of *ein2-1* was observed only after 12 days. Figure 2A illustrates the differences of symptom development on *ein2-1*, Col-0, and Nd-1 plants 7 days after root inoculation. Needle inoculation of leaves with the pathogen led to spreading chlorosis in susceptible Col-0 plants infected with strain GMI1000, whereas resistant Nd-1 plants remained symptomless. As shown in Figure 2B, *ein2-1*

Fig. 2. Phenotypic responses of *ein2-1*, Col-0, and Nd-1 plants **A**, 7 days after root-inoculation with strain GMI1000 (10^8 bacteria per milliliter) of *Ralstonia solanacearum* and **B**, 5 days after leaf inoculation with strain GMI1000. Leaf inoculation was performed by dipping a needle in a bacterial colony grown on bacto-agar glucose triphenyltetrazolium chloride medium on a petri dish according to the protocol described by Yang and Ho (57).

Fig. 3. Bacterial multiplication of strain GMI1000 in Col-0, *ein2-1*, and Nd-1 plants. For each time point, triplicate assays were performed on three plants for each *Arabidopsis thaliana* accession and the *ein2-1* mutant. Values are means $\pm$ standard deviation.

plants reacted similarly to Nd-1 plants, confirming that this mutation affects disease development regardless of the mode of inoculation.

Interestingly, ethylene insensitivity does not affect *RRS1-R*-mediated resistance because *ein2-1/ein2-1* plants crossed in an *RRS1-R* homozygous background remained fully resistant to strain GMI1000 (data not shown).

Bacterial multiplication in *ein2-1* plants. In order to determine the mechanisms by which the mutation affects symptom development, bacterial populations were estimated in *ein2-1* and wild-type plants after inoculation with strain GMI1000. The pathogen multiplied extensively in susceptible plants reaching densities of approximately 10¹⁰ CFU per gram of fresh weight, 3 to 4 orders of magnitude higher than those found in resistant plants (Fig. 3). Bacterial levels in *ein2-1* plants were only slightly lower than in susceptible plants but 1 to 2 orders of magnitude higher than in resistant plants.

The *ein2-1* mutation affects symptom development induced by several strains of *R. solanacearum*. We checked whether the delay in symptom development observed in *ein2-1* inoculated plants was specific to the GMI1000 strain. These plants were root-inoculated with various strains of the pathogen (Table 2) which induce different responses in the Col-0 and Nd-1 ecotypes (17). The *ein2-1* mutation led to delayed symptom development upon inoculation with virulent strains isolated from different parts of the world (Table 2). Interestingly, the appearance of symptoms induced by the Rd15 strain, a strain isolated from radish, which is highly virulent on Nd-1, an *A. thaliana* ecotype that is resistant to strain GMI1000, was also strongly delayed in the *ein2-1* mutant (Fig. 4). Additionally, this mutation had no effect on the resistance developed by Col-0 plants in response to an avirulent tomato strain, 14.25 (L. Godiard, unpublished data).

Strong accumulation of transcripts corresponding to *PR-3* and *PR-4*, two ethylene-responsive genes, detected in susceptible Col-0 plants but not in *ein2-1* and Nd-1 plants. In order to confirm a possible role of ethylene in the production of wilt symptoms, the accumulation of transcripts corresponding to two ethylene-responsive genes, *PR-3* (or *ChiB*), encoding a basic chitinase (44), and *PR-4* (or *HEL*), encoding a hevein-like protein (39), was assessed by RNA blot analysis. Four-week-old Col-0, *ein2-1*, and Nd-1 plants were root-inoculated either with strain GMI1000 or with the Δhrp strain, a nonpathogenic derivative of the GMI1000 strain deleted of the *hrp* cluster. As shown in Figure 5, a sustained induction of *PR-3* gene expression was detected in susceptible Col-0 plants inoculated with the GMI1000 strain. *PR-3* mRNAs started to accumulate in Col-0 plants 2 days after inoculation. This early, weak induction, was followed by a very strong (over 100-fold) accumulation of *PR-3* transcripts 6 days after inoculation, which coincided with the onset of wilt symptoms in Col-0 plants. *PR-3* levels continued to rise 8 and 10 days after inoculation, as wilt symptoms spread. No such induction was detected in Δhrp -inoculated plants, because *PR-3* transcripts remained weak or undetectable in these plants. *PR-3* induction was abolished in *ein2-1*

plants, indicating that pathogen-induced expression of this gene requires a functional ethylene response pathway. Surprisingly, no *PR-3* transcripts were detected in Nd-1 plants infected with strain GMI1000.

A similar expression pattern in response to *R. solanacearum* infection was observed for *PR-4*. However, a strong (20-fold) induction of *PR-4* expression preceded the appearance of disease symptoms in Col-0 plants, and *PR-4* expression was also induced in *ein2-1* and Nd-1 plants, but to a much lesser extent. The signal observed in this particular experiment in Col-0 plants 8 days after inoculation with the Δhrp strain was not reproducibly detected.

DISCUSSION

In several plant species, inoculation of virulent strains of *R. solanacearum* leads to the development of wilt symptoms, a plant response poorly characterized at the molecular level. *A. thaliana* mutants affected in their response to signaling compounds related to the plant response to pathogens and/or perception of pathogens constitute powerful tools for deciphering the transduction pathways activated during resistance establishment or disease development. Our results indicate that SA is probably not involved in symptom production in response to strain GMI1000. No modification in the timing and intensity of wilt symptoms was observed between *nahG* plants (unable to accumulate SA), *cpr* lines altered in the expression of pathogenesis-related genes, and wild-type susceptible Col-0 plants inoculated with strain GMI1000. Previous studies in tomatoes challenged with a bacterial pathogen, *X. campestris* pv. *vesicatoria*, demonstrated that SA is an important component of the susceptible disease response (36). Failure to

Fig. 4. Symptom development in *ein2-1* plants root-inoculated with strain Rd-15 compared with Col-0 and Nd-1 plants. Thirty plants were tested for each accession and mutant line. Plants were considered completely wilted when 100% of the rosette leaves were wilted. This experiment was repeated three times and reproducible results were obtained.

TABLE 2. Responses of the *ein2-1* mutant to the inoculation by various strains of *Ralstonia solanacearum*^a

<i>R. solanacearum</i> strains	Col-0 phenotype	<i>ein2-1</i> phenotype ^b	Host plant	Geographical origin
GMI1000	S	DSP (3/67)	Tomato	French Guyana
GA4	S	DSP (7/60)	Eggplant	French West Indies
8084	S	DSP (17/67)	<i>Solanum melongena</i>	Australia
8085	S	DSP (13/93)	<i>Xanthium pungens</i>	Australia
Rd-15	S	DSP (10/93)	Radish	Taiwan
14.25	R	R	Tomato	France

^a All the strains listed are virulent on Col-0 plants, except strain 14.25 which does not induce apparent symptoms on this *Arabidopsis thaliana* ecotype. Strain Rd-15 is virulent both on ecotypes Nd-1 and Col-0. The plants from which these strains were originally isolated as well as their geographical origin are indicated. S = susceptible, R = resistant.

^b DSP = delayed symptom production compared with Col-0. Ratio indicates the percentage of mutant plants wilted 10 days after inoculation versus the percentage of Col-0 plants wilted at this stage (30 *ein2-1* and Col-0 plants were tested for each *R. solanacearum* strain). These experiments were performed at least twice for each strain and similar results were obtained.

accumulate SA was associated with reduced disease symptoms. The results of this study suggested that coordinated phytohormone signals are involved in the plant susceptible response. Our data suggest that the implication of SA differs according to the nature of the pathogen and the type of symptoms it induces in the host plant. Interestingly, SA does play a role in the resistance response of *A. thaliana* to *R. solanacearum* because Nd-1 plants homozygous for the *nahG* transgene are susceptible to strain GMI1000 (16). The wilting is, however, delayed in comparison to Col-5 susceptible plants, indicating the implication of other factors necessary for resistance establishment.

Our data also indicate that JA, whose role in disease development is still poorly documented, is probably not involved in wilt symptom production.

Most of the ethylene response mutant lines tested (*etr1-3* and *ein4-1*, two ethylene insensitive mutants that are mutated in ethylene receptors [11,28], *ein3-1*, an ethylene insensitive mutant affected in a gene encoding a transcription factor that acts downstream of EIN2 [12], and *eto2*, an ethylene-overproducing mutant [54]) are not affected in symptom development, whereas the *ein2-1* mutation has a clear effect on this response. Similar results were obtained with ethylene-insensitive *etr1-3* plants, which responded to three different virulent pathogens in a manner comparable to wild-type plants, whereas the susceptibility response of *ein2-1* plants was strongly affected (4). A possible explanation for these apparently contradictory data might be that an alternative pathway independent of the ETR1 and EIN4 ethylene receptors may activate EIN2. However, the fact that a number of ethylene responses have been shown to be only partially blocked in the *etr1-3* mutant (38) may also explain our results. The *ein3* mutant displays an intermediate ethylene-insensitive phenotype (19), probably due to overlapping functions of other members of the EIN3-LIKE multi-gene family (12), which may explain its unaltered phenotype in response to pathogen attack. If ethylene promotes the development of wilt symptoms, ethylene-overproducing mutants such as *eto2* would be expected to exhibit accelerated wilt symptoms compared with wild-type plants, which is not the case. This result does not, however, rule out a possible role of ethylene in wilt symptom expression, because although the *eto2* mutation strongly increases ethylene biosynthesis in etiolated seedlings, ethylene production in unchallenged adult tissues of the *eto2* mutant is only slightly higher than in wild-type tissues (54).

EIN2, an ethylene signal transduction component that acts downstream of the ethylene receptors, is a structurally novel protein with an N-terminal integral membrane domain that shows similarity to the Nrmp family of metal-ion transporters (2). This protein is a central component in a number of signaling pathways, including the cytokinin and ABA signaling pathways (3,24,49), as well as pathways involved in the response to a number of

microbes and pests (4,35,48,51,56). Our results clearly implicate EIN2 in the susceptibility of *A. thaliana* to *R. solanacearum* strain GMI1000. However, whether ethylene itself plays a role in the expression of wilt symptoms remains unclear. A first step toward addressing this question is to determine whether ethylene production correlates with the appearance of wilt symptoms in infected plants. A correlation between the timing of increased ethylene production in plants challenged with pathogens and the development of symptoms was reported in several studies (15,33). Additionally, enzymes involved in the biosynthesis of this compound were localized in tissues surrounding primary lesions in tobacco leaves infected by two different pathogens (15,45), which also suggests that the ethylene production may somehow be associated with cell death. In the case of the interaction between *A. thaliana* and *R. solanacearum*, attempts to detect increases in ethylene production during disease development were unsuccessful, due to very low ethylene production in leaves of control and root-inoculated plants (data not shown). However, highly localized and subtle changes in ethylene production may be involved. Additionally, previous studies have shown that *R. solanacearum* is capable of synthesizing ethylene (23) although the biological significance of this observation is not understood.

The possibility that ethylene is implicated in wilt symptom production is strengthened by the observation that the ethylene-responsive genes *PR-3* and *PR-4* are strongly expressed in Col-0 plants inoculated with strain GMI1000, whereas the corresponding transcripts are either not detected or present at a low level in *ein2-1* and in Nd-1 plants. The *PR-3* and *PR-4* genes have been previously shown to be inducible by exogenous application of ethylene (39,44). Pathogen-induced expression of these genes, considered as defense markers, has been observed in *A. thaliana* in response to a number of viral (39), fungal (5,50), and bacterial (35,37) pathogens. The specific activation of these genes in susceptible Col-0 plants, but not in resistant Nd-1 plants, infected with strain GMI1000 of *R. solanacearum* is puzzling. However, *PR-3* and *PR-4* also constitute markers of the ethylene response pathway, and as such, their preferential activation during disease development provides further support for a role of ethylene in wilt symptom production.

Resistance to *R. solanacearum*, even though it requires SA (16), appears to be independent of ethylene signaling. Homozygous *ein2-1/ein2-1* plants in a homozygous *RRS1-R* background are indeed resistant to strain GMI1000. Additionally, *ein2-1* exhibits an unaltered (wild-type) response to the 14.25 strain, a tomato strain that induces no apparent symptoms on Col-0 plants. These results are in agreement with previous observations showing that the *ein2-1* mutation does not affect the interactions of *A. thaliana* with avirulent strains of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (4).

Fig. 5. Expression of ethylene-inducible genes in leaves of *Arabidopsis thaliana* infected with *Ralstonia solanacearum* strain GMI1000. 32 P-labeled *PR-3* and *PR-4* probes were used for northern blot analysis of *ein2-1*, wild-type Col-0, and Nd-1 plants after root inoculation with a 10^8 CFU per milliliter bacterial suspension of the GMI1000 strain or Δhrp strain. Equal RNA loading was controlled by rehybridization of the blot with a 32 P-labeled 18S rDNA probe. These results were reproducibly obtained. Number of days after inoculation (dai) is indicated on top of the wells. Abbreviations are as follows: e = *ein2-1*; C = Col-0; and N = Nd-1.

This study underlines the important role of ethylene signaling in the development of disease symptoms induced by the bacterial wilt pathogen *R. solanacearum*. Transcriptome analysis of susceptible wild-type and mutant plants altered in their response to a virulent strain of *R. solanacearum* was initiated and should lead to a better understanding of the molecular mechanisms underlying disease development. This approach should, in particular, provide some information on the expression of other ethylene-associated genes in the plant response to virulent strains of the pathogen. *R. solanacearum* is a pathogen of more than 200 plant species. It would therefore be interesting to determine whether plants of agronomic value affected in ethylene production or perception, such as *Nr* tomatoes for example, also display a delayed symptom production in response to this bacterium. In this case, modulating the ethylene transduction pathway in these plants may constitute an alternative strategy to control bacterial wilt.

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