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Systemic Resistance in Arabidopsis Induced by Rhizobacteria Requires Ethylene-Dependent Signaling at the Site of Application

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Root colonization of *Arabidopsis thaliana* by the nonpathogenic, rhizosphere-colonizing, biocontrol bacterium *Pseudomonas fluorescens* WCS417r has been shown to elicit induced systemic resistance (ISR) against *Pseudomonas syringae* pv. *tomato* (Pst). The ISR response differs from the pathogen-inducible systemic acquired resistance (SAR) response in that ISR is independent of salicylic acid and not associated with pathogenesis-related proteins. Several ethylene-response mutants were tested and showed essentially normal symptoms of Pst infection. ISR was abolished in the ethylene-insensitive mutant *etr1-1*, whereas SAR was unaffected. Similar results were obtained with the ethylene-insensitive mutants *ein2* through *ein7*, indicating that the expression of ISR requires the complete signal-transduction pathway of ethylene known so far. The induction of ISR by WCS417r was not accompanied by increased ethylene production in roots or leaves, nor by increases in the expression of the genes encoding the ethylene biosynthetic enzymes 1-aminocyclopropane-1-carboxylic (ACC) synthase and ACC oxidase. The *eir1* mutant, displaying ethylene insensitivity in the roots only, did not express ISR upon application of WCS417r to the roots, but did exhibit ISR when the inducing bacteria were infiltrated into the leaves. These results demonstrate that, for the induction of ISR, ethylene responsiveness is required at the site of application of inducing rhizobacteria.

Additional keywords: bacterial speck disease, ethylene response mutants.

Induced resistance is the phenomenon by which a plant, upon appropriate stimulation, acquires an enhanced level of resistance against a broad spectrum of pathogens. The classic way of obtaining induced resistance is achieved by infection with a necrotizing pathogen, upon which the plant becomes more resistant to subsequent pathogen attack, both locally and systemically. This type of induced resistance is called systemic acquired resistance (SAR) and was first characterized in tobacco infected with tobacco mosaic virus (TMV) (Ross 1961a, 1961b). Pathogen-induced SAR is accompanied by an early increase in salicylic acid (SA) (Malamy et al. 1990; Métraux et al. 1990). Accumulation of SA is essential for the development of SAR, since transgenic plants that are unable to accumulate SA have lost their ability to express SAR (Gaffney et al. 1993). Infection of a plant by a necrotizing pathogen is also accompanied by a burst of ethylene production (Ross and Williamson 1951; De Laat and Van Loon 1982; Mauch et al. 1984; Spanu and Boller 1989). The role of ethylene in SAR seems to be plant species dependent. In Arabidopsis, Lawton et al. (1995) showed that the ethylene-insensitive *etr1-1* mutant is fully capable of mounting an SAR response. In contrast, in transgenic tobacco plants expressing the Arabidopsis mutant *etr1-1* gene, a substantially reduced SAR response was observed (Knoester 1998). SAR is associated with the expression of the so-called SAR genes (Ward et al. 1991), including genes coding for pathogenesis-related proteins (PRs) (Van Loon 1985; Linthorst 1991), which are often used as markers for the induced state. Exogenous application of the chemicals SA, 2,6-dichloroisonicotinic acid (INA), or benzothiadiazole mimic pathogen-induced SAR by inducing the same set of SAR genes (Ward et al. 1991; Uknes et al. 1992; Friedrich et al. 1996; Görlach et al. 1996; Lawton et al. 1996).

Another type of induced resistance was reported in 1991, when selected strains of nonpathogenic, root-colonizing, plant growth-promoting rhizobacteria (PGPR) were shown to induce systemic resistance in carnation (Van Peer et al. 1991) and cucumber (Wei et al. 1991). To distinguish this enhanced defensive capacity from pathogen-induced SAR, the induced protection was termed rhizobacteria-mediated induced systemic resistance (ISR) (Pieterse et al. 1996; Van Loon 1997; Van Loon et al. 1998). Previous research on PGPR had demonstrated their capability to control soilborne pathogens by competition for nutrients, siderophore-mediated competition for iron, or antibiosis (Bakker et al. 1991; Schippers 1992; Thomashow and Weller 1996). ISR was found to constitute a novel mechanism by which these rhizobacteria can suppress

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plant diseases. Enhanced disease resistance is induced irrespective of whether the rhizobacteria are applied to the roots or the leaves (Hoffland et al. 1995; Pieterse et al. 1996).

Rhizobacteria-mediated ISR differs from pathogen-induced SAR in several ways. Contrary to the situation with SAR, ISR is not accompanied by the accumulation of PRs (Hoffland et al. 1995; Pieterse et al. 1996; Van Wees et al. 1997). Moreover, it was demonstrated by Pieterse et al. (1996) that in Arabidopsis ISR is independent of SA accumulation. When the ethylene response mutant *etr1-1* was used, it was found that this mutant had lost the ability to be induced by rhizobacteria (Pieterse et al. 1998), whereas it was still capable of expressing SAR (Lawton et al. 1994; Pieterse et al. 1998). Thus, responsiveness to ethylene appears to be necessary for the induction of ISR. Conversely, treatment of Arabidopsis with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) induced resistance against *Pseudomonas syringae* pv. *tomato* (Pst), indicative of a role for ethylene in the ISR signal-transduction pathway.

Ethylene signaling has been studied extensively in Arabidopsis with mutants that are altered in the triple response phenotype of dark-grown seedlings in the presence of ethylene. Characteristics of the ethylene-induced triple response include the inhibition of root and hypocotyl elongation, radial swelling of the hypocotyl and root, and exaggeration of the curvature of the apical hook. Several mutants were isolated that either fail to respond to exogenous ethylene, or constitutively display the response in the absence of the hormone (Guzman and Ecker 1990). This resulted in the identification of several genes that constitute a signal-transduction pathway resembling the osmolarity response pathway in yeast (Ota and Varshavsky 1993; Maeda et al. 1994). The earliest steps in the pathway are defined by the ethylene receptor type ETR1, EIN4, ETR2, and ERS (Chang et al. 1993; Hua et al. 1997). Receptor mutants all display dominant ethylene insensitivity, indicating that they have similar or overlapping roles in ethylene perception and signal transduction. Downstream of the receptors is the *ctr1* gene, which is thought to be a negative regulator of the ethylene response, since the *ctr1-1* mutant shows complete activation of all known ethylene-induced phenotypes in the absence of ethylene (Kieber et al. 1993). CTR1 is followed by EIN2, which displays ethylene insensitivity when mutated. CTR1 is thought to negatively regulate EIN2, possibly via a MAP kinase cascade (Ecker 1995). The *ein3* gene has been cloned, and characterized as coding for a positive regulator of ethylene responses, acting downstream of EIN2 (Chao et al. 1997). The positions of EIN5, EIN6, and EIN7 are not yet known. However, they all appear to act downstream of CTR1. Additional mutations known to abolish (some) ethylene responses are less well characterized.

To elucidate which parts of the ethylene signal-transduction pathway are required for rhizobacteria-mediated ISR, we analyzed a set of these ethylene response mutants for their ability to express ISR. For comparison, the mutants were similarly analyzed for their potential to express pathogen-induced SAR.

RESULTS

Disease development in ethylene mutants.

To investigate the involvement of ethylene in induced resistance against Pst, it is important that symptoms of Pst infection in the ethylene mutants do not deviate from those of wild-type plants to any gross extent. Because ethylene plays a role in the regulation of leaf senescence, insensitivity to ethylene could interfere with the pathogen-induced accelerated aging resulting in chlorophyll loss and yellowing symptoms. The ethylene response mutants analyzed are listed in Table 1. Mutant *etr1-1* plants have enhanced levels of chlorophyll compared with wild-type Col-0 (Bleecker et al. 1988), but after the plants were dipped in a solution containing 2.5×10^7 CFU ml^{-1} of virulent Pst, typical symptoms of bacterial speck disease, consisting of extensive chlorosis progressing into spreading necrosis, were observed. For *ein2-1*, Bent et al. (1992) described that these plants showed only minimal symptoms after vacuum infiltration with 10^5 CFU ml^{-1} of virulent Pst. In our experiments, *ein2-1* developed clear symptoms after the plants were dipped into a suspension of 2.5×10^7 CFU ml^{-1} of virulent Pst. Although the appearance of the symptoms was different from those on Col-0 in that leaves lacked the characteristic yellowing, clearly visible water-soaked lesions were apparent. The *ein3-1* plants also showed water-soaked lesions, but less obvious than those on *ein2-1* plants, with more yellowing. The other ethylene response mutants tested, except for *ein6*, *axr1-12*, and *ctr1-1*, showed extensive disease symptoms similar to Col-0 and *etr1-1* plants. Mutants *ein6* and *axr1-12* hardly developed symptoms even after inoculation with 1.25×10^8 CFU ml^{-1} of virulent Pst.

Mutant *ctr1-1* constitutively displays ethylene responses. In this mutant, Pst infection did not result in symptom formation. To analyze whether the lack of symptom formation was caused by an inability of Pst to enter and multiply in *ctr1-1* leaves, the number of bacteria was determined immediately

Table 1. Ethylene mutants used in bioassays

Mutant	Phenotype	Ecotype	Reference
etr1-1	Ethylene insensitive, dominant	Col-0	Bleecker et al. 1988
ein2-1	Ethylene insensitive, recessive	Col-0	Guzman and Ecker 1990
ein3-1	Ethylene insensitive, recessive	Col-0	Kieber et al. 1993
ein4-1	Ethylene insensitive, dominant	Col-0	Roman et al. 1995
ein5-1	Ethylene insensitive, recessive	Col-0	Roman et al. 1995
ein6	Ethylene insensitive, recessive	L*er*	Roman et al. 1995
ein7	Ethylene insensitive, semidominant	Col-0	Roman et al. 1995
eto1-1	Ethylene overproducer, recessive	Col-0	Guzman and Ecker 1990
eir1-1	Ethylene insensitive root, recessive	Col-0	Roman et al. 1995
axr1-12	Ethylene and auxin insensitive root, recessive	Col-0	Estelle and Somerville 1987; Lincoln et al. 1990
ctr1-1	Constitutive triple response, recessive	Col-0	Kieber et al. 1993

after challenge inoculation by dipping and 4 days later. In wild-type Col-0, Pst increased from 1.75×10^5 CFU g^{-1} fresh weight (t = 0) to 1.2×10^7 CFU g^{-1} (t = 4 days). In *ctr1-1*, these values were 4.9×10^5 and 4.7×10^7 CFU g^{-1}, respectively, an increase of the same order of magnitude as in Col-0 plants, indicating that the lack of symptoms on *ctr1-1* plants was not caused by reduced entry or growth of Pst in the leaves. In addition to the lack of symptoms, the extremely small phenotype of *ctr1-1*, due to its constitutive triple response, rendered this mutant unsuitable for further assays.

Analysis of ISR and SAR in ethylene mutants.

To investigate which parts of the ethylene signal-transduction pathway are required for induced resistance, the expression of ISR and SAR was tested in *etr1-1*, the ethylene-insensitive *ein* mutants, and the ethylene overproducer *eto1-1*.

For wild-type Col-0, induction of ISR by WCS417r resulted in between 20 and 30% fewer diseased leaves. Induction of SAR was more effective than that of ISR, resulting in a reduction of the proportion of diseased leaves by 50%. None of the ethylene-insensitive mutants *etr1-1*, *ein2-1*, *ein3-1*, *ein4-1*, *ein5-1*, and *ein7* expressed ISR in response to treatment with WCS417r. In contrast, their SAR response was unimpaired, and as strong as observed in Col-0 plants (Fig. 1). Also, for *ein6*, in the L*er* background, the capacity to express ISR was abolished, whereas SAR was maintained. The ethylene overproducer *eto1-1* developed symptoms to the same extent as wild-type Col-0. In spite of its constitutive ethylene production, it likewise did not show an ISR response, whereas the SAR response was normal.

As the induction of ISR is established by colonization of the rhizosphere, we analyzed whether the absence of ISR in the ethylene response mutants was due to an inability of WCS417r to colonize the roots of these mutants. In each bioassay, root colonization by WCS417r was determined for both wild-type and mutant plants. Root colonization of wild-type plants varied between 3×10^6 and 6×10^7 CFU g^{-1} fresh weight. Root colonization of the ethylene mutants never showed a significant difference from that of wild-type plants (data not shown).

Rhizobacteria-mediated ISR requires ethylene sensitivity at the site of application.

eir1-1 and *axr1-12* are two mutants that are insensitive to ethylene in the roots only. To investigate whether the inability of the *ein* mutants to express ISR results from an inability to react to ethylene in the root or in the shoot, the *eir1-1* and *axr1-12* mutants were tested by applying WCS417r either in the soil, at transplanting, or to the leaves, by pressure infiltration 3 days before challenge inoculation.

When WCS417r was applied to the roots, both mutants behaved like the *ein* mutants, in that no ISR was expressed, whereas SAR could be induced normally in these mutants (Fig. 2A). Upon challenge inoculation, control plants of the *axr1-12* mutant showed a low percentage of diseased leaves, whereas *eir1-1* control plants were as diseased as Col-0 controls. Therefore, further analyses were performed on *eir1-1* mutants only.

To check ethylene responsiveness of the *eir1-1* mutant in the leaves, the expression of the ethylene-responsive gene *hel* was analyzed upon treatment with a concentration range of ACC, and compared with *hel* gene expression in Col-0, *ein2-*

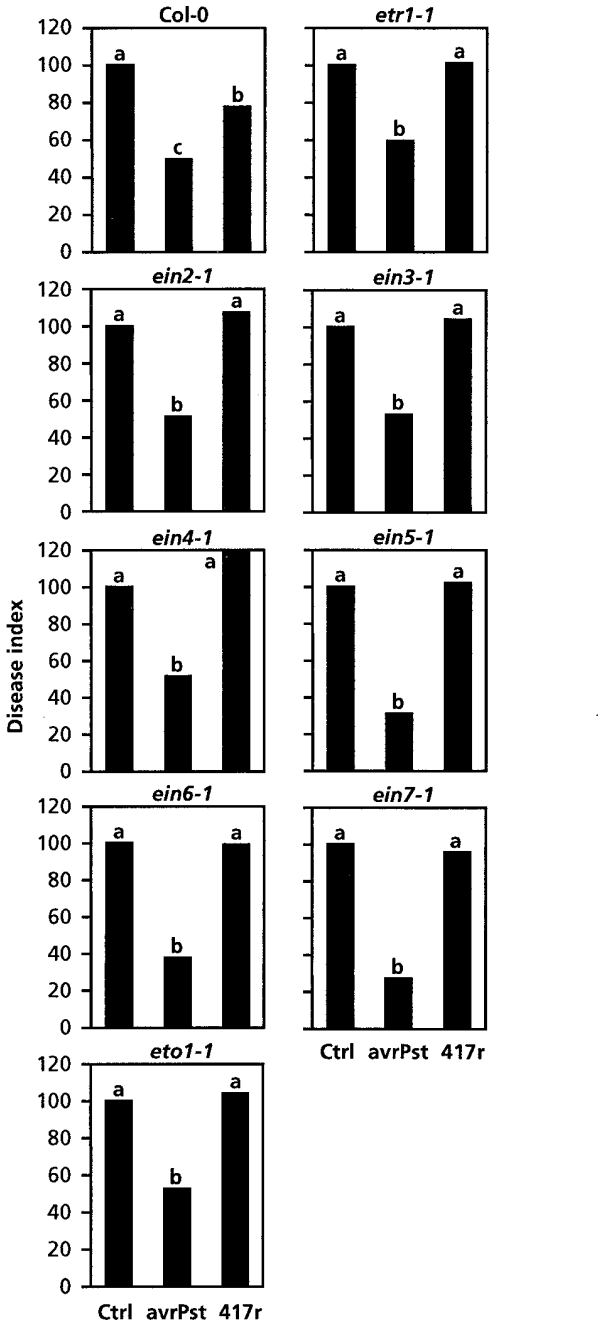

Fig. 1. Quantification of induced systemic resistance against *Pseudomonas syringae* pv. *tomato* infection in Col-0 and ethylene mutants grown in soil amended with 10 mM $MgSO_4$ (Ctrl) or *P. fluorescens* WCS417r (417r), or pressure infiltrated in the leaves with the avirulent *P. syringae* pv. *tomato* (*avr*Pst). Four days after challenge inoculation with virulent *P. syringae* pv. *tomato*, disease severity was scored by determining percent diseased leaves per plant. Disease index is the mean (n = 20) of percent of diseased leaves per plant relative to control treatment. Absolute values for percentages of diseased leaves in controls: Col-0 (52.3), *etr1-1* (64.9), *ein2-1* (62.5), *ein3-1* (55.8), *ein4-1* (58.5), *ein5-1* (71.3), *ein6* (31.1), *ein7* (76.4), and *eto1-1* (53.0). Within each frame, different letters indicate statistically significant differences between treatments by Fisher's least significant difference test (α = 0.05).

1, and *eto1-1* (Fig. 3). As expected, noninduced Col-O and *eir1-1* plants showed a higher basal level of *hel* gene expression than ethylene-insensitive *ein2-1*. Expression was strongly increased upon application of ACC to Col-0 and *eir1-1*, but not in *ein2-1*, providing proof that *eir1-1* leaves are similar to wild-type Col-0 leaves in sensitivity to ethylene. Ethylene-overproducing *eto1-1* plants showed an elevated background level of *hel* gene expression, which was not further increased upon application of ACC.

Figure 2B shows the percentage of diseased leaves in WCS417r-treated plants relative to control plants when ISR was induced by infiltrating the rhizobacteria into the leaf. The level of resistance induced by leaf infiltration was somewhat less than that resulting from application of the bacteria to the roots. Nevertheless, Col-0 treated with WCS417r showed a significant reduction in the percentage of diseased leaves of 20%, compared with noninduced control plants. The *eir1-1* mutant likewise showed a reduction of as much as 25%, whereas no significant ISR was seen in the ethylene-insensitive *etr1-1* plants. While the *eir1-1* mutant did not develop ISR upon application of WCS417r to its roots, it expressed ISR at least as well as wild-type Col-0 when the bacterium was infiltrated in the ethylene-responsive leaves. The ISR response was not induced in ethylene-insensitive leaves of *etr1-1*. These results clearly demonstrate that, for ISR to develop, ethylene-dependent signaling is required at the site of application of the ISR-inducing bacteria.

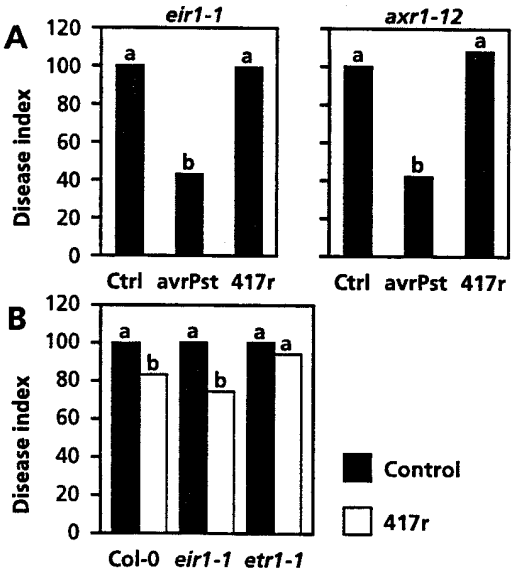

Fig. 2. A, Quantification of induced systemic resistance against *Pseudomonas syringae* pv. *tomato* infection in *eir1-1* and *axr1-12* plants grown in soil amended with 10 mM $MgSO_4$ (Crtl) or *P. fluorescens* WCS417r (417r), or pressure infiltrated in the leaves with the avirulent pathogen *P. syringae* pv. *tomato* (*avr*Rpt2) (avrPst). **B,** Quantification of induced systemic resistance against *P. syringae* pv. *tomato* infection in Col-O, *eir1-1*, and *etr1-1* upon infiltration of three leaves per plant with either 10 mM $MgSO_4$ (Ctrl) or 10^7 CFU ml^{-1} *P. fluorescens* WCS417r (417r) 3 days before challenge inoculation. For bioassays shown in **A** and **B,** 4 days after challenge inoculation with virulent *P. syringae* pv. *tomato*, disease severity was scored by determining percent diseased leaves per plant. Disease index is the mean (n = 20) of percent diseased leaves per plant relative to control treatment. Absolute values for percentages of diseased leaves in controls from *eir1-1* and *axr1-12:* 56.6 and 24.8%, respectively (**A**). In **B,** absolute values for percentages of diseased leaves per plant in controls from Col-O, *eir1-1*, and *etr1-1*: 57.4, 59.9, and 64.4%, respectively. Different letters indicate statistically significant differences between treatments by Fisher's least significant difference test (α = 0.05). Experiments were performed twice with similar results.

P. fluorescens WCS417r does not induce ethylene biosynthesis in Col-0.

For ISR expression, ethylene has to act at the site of application of ISR-inducing bacteria. Moreover, our results with the ethylene-insensitive mutants demonstrate that the ISR response requires all known components of the ethylene-signaling pathway. It was shown recently that ACC induces a resistance against Pst infection similar to WCS417r-mediated ISR (Pieterse et al. 1998). The similarities in ACC-induced resistance and WCS417r-mediated ISR raised the question whether application of WCS417r to the roots results in locally elevated ethylene levels. Such increased ethylene levels could be instrumental in the generation of the systemic signaling compound that renders the plant more resistant to challenging pathogens. Further questions are whether expression of ISR also requires ethylene and whether, upon application of WCS417r to the roots, leaf ethylene levels are also elevated.

Ethylene measurements were performed on roots of Col-0 plants potted in soil with or without WCS417r. Results of a

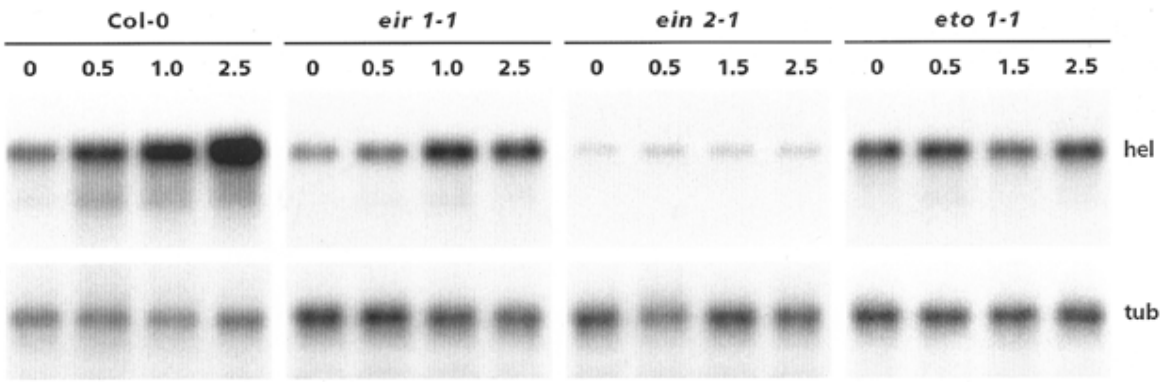

Fig. 3. Analysis of *hel* gene expression in leaves of Col-0, *eir1-1*, *ein2-1*, and *eto1-1* plants. *hel* gene expression was analyzed 2 days after treatment with either 0.01% Silwet L-77 (0), or 0.5, 1, and 2.5 mM ACC (1-aminocyclopropane-1-carboxylic acid) + 0.01% Silwet L-77. To check for equal loadings, blot was stripped and hybridized with a proble for β-tubulin (tub).

typical experiment are shown in Table 2. During the period of 3 weeks between transplant of the seedlings in bacterized soil and challenge inoculation, when bacteria were present on the roots at levels triggering ISR, a statistically significant difference in ethylene levels of roots from control and WCS417r-treated plants was never found. Leaves were analyzed similarly but, likewise, no statistically significant differences in ethylene production were observed between control and WCS417r-treated plants.

To examine the possibility that bacterization of roots is accompanied by an early, transient increase in ethylene production, expression of the ethylene biosynthetic genes ACC-synthase (*acs*) and ACC-oxidase (*aco*) was analyzed in the first week after seedling roots were exposed to the rhizobacteria. The *acs* gene family of Arabidopsis consists of five members, of which *acs3* is a pseudogene of *acs1* (Liang et al. 1992). *acs1, acs4,* and *acs5* were tested but showed no expression in roots at the level of detection. In contrast, *acs2* was clearly expressed (Fig. 4). However, *acs2* revealed similar expression patterns for control and WCS417r-treated roots. For both treatments, expression of *acs2* increased between 1 and 3 days after application of the bacteria and remained elevated, or occasionally declined by 7 days. *aco* gene expression showed a largely similar pattern with no apparent differences between control and WCS417r-treated roots. Thus, no significant alterations in the expression of ethylene biosynthetic genes occurred associated with the induction of ISR.

Table 2. Ethylene production (pmol g^{-1} h^{-1}) for roots and leaves of Col-0 control plants (Ctrl) or WCS417r-treated plants, 1, 2, or 3 weeks after potting[z]

Time (weeks)	Root		Leaf	
	Ctrl	WCS417r	Ctrl	WCS417r
1	138 ± 38 a	129 ± 23 abc	136 ± 41 c	102 ± 39 cd
2	103 ± 29 cd	122 ± 30 abc	175 ± 20 ab	184 ± 15 a
3	75 ± 9 de	66 ± 7 e	144 ± 10 c	155 ± 16 bc

[z] Different letters indicate statistically significant differences between treatments by Fisher's least significant difference test ($\alpha = 0.05$). Experiments were performed three times with similar results.

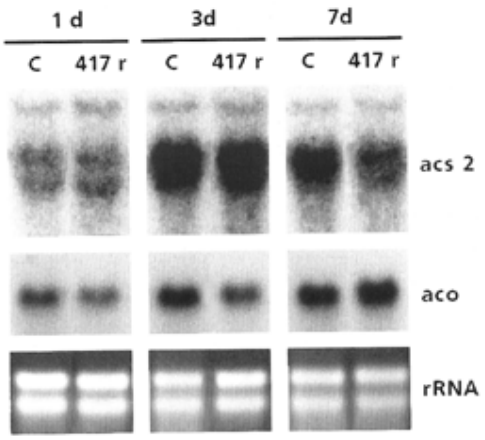

Fig. 4. Analysis of *acs2* and *aco* gene expression in roots of Col-0 plants. On rock-wool cubes, Col-0 roots were treated with either 10 mM $MgSO_4$ mixed with talcum powder as a carrier (C), or with *Pseudomonas fluorescens* WCS417r applied in the same manner (417r). *acs2* and *aco* gene expression were analyzed 1, 3, and 7 days after treatment, compared with patterns of rRNA stained with ethidium bromide.

DISCUSSION

ISR is the mechanism by which *P. fluorescens* WCS417r has been shown to reduce disease caused by Pst in Arabidopsis (Pieterse et al. 1996, 1998; Van Wees et al. 1997). The pathway by which ISR is induced is poorly understood. Clear differences were observed when ISR was compared with the well-established SAR response, in which SA accumulation and induction of *PR* gene expression are associated with the resistance response. Neither occurs during the development of ISR and markers for the ISR response are not yet identified. However, responsiveness to both jasmonic acid (JA) and ethylene is necessary for the expression of ISR, and application of either methyl jasmonate (MeJA) or ACC induces a resistance in Arabidopsis similar to ISR (Pieterse et al. 1998). Application of MeJA to *etr1-1* plants did not result in protection. However, treatment of the JA-nonresponsive mutant *jar1* with ACC did induce resistance, indicating that JA and ethylene responsiveness are successively engaged in signal transduction. Both SAR and ISR are dependent on NPR1, suggesting that at least some of the later steps leading to the resistant state are shared by the two types of induced resistance (Pieterse et al. 1998).

We have attempted to gain further insight into the pathway leading to ISR by performing a more extended analysis of the involvement of the ethylene signal-transduction pathway in the expression of ISR. Several steps in the ethylene signal-transduction pathway have been identified (Ecker 1995). The ethylene response mutants *etr1-1, ein2-1, ein3-1, ein4-1, ein5-1, ein6,* and *ein7* were tested for the WCS417r-mediated ISR response and the pathogen-induced SAR response. WCS417r-mediated ISR was abolished in all mutants tested, whereas all mutants were able to express pathogen-induced SAR. These observations support earlier findings by Lawton et al. (1994, 1995) that in Arabidopsis induction of SAR is ethylene independent. In contrast, ISR requires an intact ethylene response and, although the exact locations of EIN5, EIN6, and EIN7 in the ethylene signal-transduction pathway are not known, this indicates that all known *ein* genes in the pathway are required for the expression of ISR.

Because the ethylene signal-transduction pathway is necessary for full expression of the ISR response, the involvement of ethylene in the induction of ISR was further investigated. Bioassays with the *eir1-1* mutant, displaying ethylene insensitivity in the roots only, shed additional light on the pathway by which ISR is induced. In this mutant, application of WCS417r to the roots did not result in an ISR response in the leaves. In contrast, infiltration of bacteria into the leaves did result in an ISR response. The *eir1-1* leaves are normally sensitive to ethylene, in contrast to *etr1-1* and *ein* mutants, which display complete ethylene insensitivity. This demonstrates that ethylene-dependent signaling is required at the site of application of inducing bacteria, be it roots or leaves, to obtain a full ISR response.

Whether ethylene sensitivity is required also at the site where the resistance is expressed cannot be assessed at present. However, this seems unlikely, because SAR can be expressed in ethylene-insensitive Arabidopsis. Thus, expression

of resistance upon challenge inoculation is not impaired when ethylene cannot be perceived. The ethylene-overproducing mutant *eto1-1* constitutively displays high levels of ethylene, but did not express enhanced resistance. Because *hel* gene transcript accumulation was not increased in response to ACC in the *eto1-1* mutant, it appears that the ethylene signaling pathway was somehow saturated and that application of ISR-inducing bacteria did not provide additional stimuli for the induction of ISR.

Application of ISR-inducing WCS417r did not alter ethylene production or gene expression of ethylene biosynthetic genes in roots or leaves. Thus, there are no indications that WCS417r induced ISR in Arabidopsis by inducing ethylene biosynthetic genes or ethylene production. Some *Pseudomonas* spp. are capable of producing ethylene (Weingart and Völksch 1997), and it might be envisaged that not the plant, but WCS417r itself is producing the ethylene by which the ethylene signal transduction in the plant is activated. No conclusive evidence about ethylene production by WCS417r has been obtained so far (P. A. H. M. Bakker, *personal communication*). However, the ethylene-responsive genes *hel* (Potter et al. 1993) and *pdf1.2* (Penninckx et al. 1996) are not induced in roots bacterized with WCS417r, making it unlikely that WCS417r produces biologically active amounts of ethylene (Pieterse et al. 1998).

Thus, induction of ISR requires all known components of the ethylene signaling pathway in the absence of an increase in ethylene production. This situation resembles the compatible interaction of rice with the rice blast fungus *Magnaporthe grisea* (Hebert) Barr., in which JA-inducible genes are activated without a concomitant increase in endogenous JA (Schweizer et al. 1997). In the JA-promoted senescence of detached rice leaves, ethylene is the hormone responsible for chlorophyll loss. However, ethylene levels decreased rather than increased, but apparently JA increased the sensitivity to ethylene (Tsai et al. 1996). Because induction of ISR by WCS417r is similarly dependent on both JA and ethylene, but no increase in ethylene production is apparent, it may also be envisaged that JA increases sensitivity to ethylene. JA appears to act before ethylene in the ISR signaling pathway (Pieterse et al. 1998). Whether application of ISR-eliciting rhizobacteria increases JA levels in the plant remains to be determined. However, it can be concluded that for expression of ISR in Arabidopsis, ethylene-dependent signaling is required at the site of application of inducing rhizobacteria, and that induction of ISR is not accompanied by elevated ethylene levels in the plant.

MATERIALS AND METHODS

Bacterial cultures.

A rifampicin-resistant mutant of the bacterial biocontrol strain *Pseudomonas fluorescens* WCS417 (WCS417r), effective in inducing systemic resistance in carnation (Van Peer et al. 1991), radish (Hoffland et al. 1995), and Arabidopsis (Pieterse et al. 1996), was used as the inducing rhizobacterium. WCS417r was grown on King's medium B (KB) agar plates (King et al. 1954) for 24 h at 28°C. Bacteria were collected and resuspended in 10 mM $MgSO_4$. The virulent bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 (Whalen et al. 1991) was grown overnight at 28°C in liquid KB. The cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$. Its avirulent derivative DC3000 (*avr*Rpt2), carrying plasmid pLAFR3 containing the avirulence gene *avr*Rpt2 (Whalen et al. 1991), was similarly cultured in liquid KB without $MgSO_4$, supplemented with tetracycline (40 µg/ml).

Cultivation of plants.

Seeds of *Arabidopsis thaliana* ecotypes Columbia (Col-0) and Landsberg *erecta* (L*er*) and their mutants were sown in sand. Seedlings were cultivated in a growth chamber with a 9-h light (200 µE s^{-1} m^{-2} at 24°C) and 15-h dark period (20°C) at 70% relative humidity. Ten- to 14-day-old plants were transplanted individually in 60-ml pots containing a 12:5 (vol/vol) potting soil/sand mixture, autoclaved twice for 1 h, and supplemented with either 10 mM $MgSO_4$ or a suspension of WCS417r in 10 mM $MgSO_4$, as described for the ISR bioassay.

Plants were watered regularly and supplied once a week with half-strength Hoagland solution (2 mM KNO_3, 5 mM $Ca[NO_3]_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, and trace elements, pH 7) (Hoagland and Arnon 1938), containing 10 µM Sequestreen (Fe-ethylenediamine-di[*o*-hydroxyphenylacetic acid]; CIBA-Geigy, Basel, Switzerland).

ISR and SAR induction.

Plants for the ISR and SAR bioassays were randomized. For each treatment, 20 plants were used. For induction of ISR through colonization of the roots, seedlings germinated on sand were transplanted in the potting soil/sand mixture supplemented with either 50 ml of 10 mM $MgSO_4$ containing 10^9 CFU ml^{-1} WCS417r per kg of soil, or an equal amount of 10 mM $MgSO_4$ as control treatment. For induction of ISR through leaf infiltration, 5-week-old plants were used. One day before treatment, plants were placed at 100% relative humidity. Three lower leaves per plant were infiltrated with a solution containing 10^7 CFU ml^{-1} of WCS417r, using a syringe without needle (Swanson et al. 1988). For induction of SAR the procedure used for the induction of ISR was followed, with 10^7 CFU ml^{-1} of avirulent Pst (*avr*Rpt2).

Challenge inoculation and disease assessment.

Plants were subjected to challenge inoculation 3 weeks after transplanting in rhizobacteria-containing soil mixture or 3 days after leaf infiltration with avirulent Pst. One day before challenge, relative humidity was raised to 100%, and kept at 100% during inoculation. Challenge inoculation was performed by dipping the leaves in a suspension of 2.5×10^7 CFU ml^{-1} of virulent Pst in 10 mM $MgSO_4$, supplemented with 0.01% of the surfactant Silwet L-77. Four days later the proportion of diseased leaves per plant was determined (Pieterse et al. 1996, 1998). The number of Pst bacteria in inoculated leaves was determined in three sets of 20 randomly selected leaves per treatment. Leaves were weighed, rinsed thoroughly in sterile water, and homogenized in 10 mM $MgSO_4$. Subsequently, appropriate dilutions were plated onto KB agar supplemented with 50 mg $liter^{-1}$ rifampicin and 100 mg $liter^{-1}$ cycloheximide. After 48 h of incubation at 28°C, the number of rifampicin-resistant CFU g^{-1} infected leaf tissue was determined.

Data were statistically analyzed with analysis of variance followed by Fisher's test for least significant differences at $\alpha = 0.05$.

Rhizosphere colonization.

Roots (0.5 to 1.0 g fresh weight) from three to four plants were harvested in duplicate, and shaken vigorously for 30 s in 10 mM $MgSO_4$ (10 ml per 1 g of roots), containing 0.5 g of glass beads (0.17 mm). Serial dilutions were plated on KB agar supplemented with cycloheximide (100 mg liter^{-1}), ampicillin (50 mg liter^{-1}), chloramphenicol (13 mg liter^{-1}), and rifampicin (150 mg liter^{-1}), which is selective for rifampicin-resistant, fluorescent *Pseudomonas* spp. (Geels and Schippers 1983). After 2 days of incubation at 28°C, the number of rifampicin-resistant CFU g^{-1} root fresh weight was determined.

Ethylene determination.

One, 2, and 3 weeks after potting of the plants, roots and leaves were harvested and enclosed in 30-ml vials ($n = 10$). After incubation for 3 days under growth chamber conditions, the accumulated ethylene was measured by gas chromatography as described previously (De Laat and Van Loon 1983).

RNA analysis.

To study the expression of the ethylene-inducible *hel* gene, 5-week-old plants were dipped in a solution containing various concentrations of ACC, 0.01% Silwet L-77, or 0.01% Silwet L-77 as control. Two days after treatment, leaf material was harvested, frozen in liquid nitrogen, and stored at –80°C until RNA was isolated.

A time course experiment to analyze ISR-related *acs* and *aco* gene expression in seedling roots was performed by placing 2-week-old Col-0 seedlings horizontally on rock wool cubes drenched in half-strength Hoagland solution (Pieterse et al. 1996). A suspension of 5×10^8 CFU ml^{-1} WCS417r in 10 mM $MgSO_4$, or 10 mM $MgSO_4$ alone, was mixed 1:1 (vol/wt) with talcum powder and applied to the roots, which were then covered with rock wool cubes. At 1, 3, and 7 days after treatment, roots were harvested and talcum mix was removed by washing the roots in water. After drying, the roots were frozen in liquid nitrogen and stored at –80°C for RNA isolation.

RNA was isolated as described previously (Linthorst et al. 1993). Total RNA (20 µg) was separated on a 1% agarose gel in 15 mM sodium phosphate pH 6.5, and transferred to Hybond N^+ filters (Amersham, 's-Hertogenbosch, The Netherlands). Hybridization was performed at 65°C in 250 mM sodium phosphate pH 7.0, 1 mM EDTA, 7% sodium dodecyl sulfate, 1% bovine serum albumin, with randomly labeled probes of *acs1*, *-2*, *-4*, and *-5* (Liang et al. 1992), *aco* (Gómez-Lim et al. 1993), and *hel*. The template for the *hel* probe was prepared by polymerase chain reaction with primers based on the published cDNA sequence (Potter et al. 1993).

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