

# The Arabidopsis *ISR1* Locus Controlling Rhizobacteria-Mediated Induced Systemic Resistance Is Involved in Ethylene Signaling

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In Arabidopsis, the rhizobacterial strain *Pseudomonas fluorescens* WCS417r triggers an induced systemic resistance (ISR) response that is effective against different types of pathogens. The ISR signaling pathway functions independent of salicylic acid, but requires responsiveness to jasmonate (JA) and ethylene. Using the genetic variability of ISR inducibility between Arabidopsis accessions, we recently identified a locus (*ISR1*) on chromosome III that is involved in ISR signaling. Accessions RLD and Wassilewskija (*Ws*) are recessive at the *ISR1* locus and are, therefore, unable to develop ISR. Here we investigated whether the *ISR1* locus is involved in JA or ethylene signaling. Compared with the ISR-inducible accession Columbia (*Col*), accessions RLD and *Ws* were not affected in JA-induced inhibition of root growth and expression of the JA-responsive gene *Atvsp*, suggesting that the *ISR1* locus is not involved in JA signaling. However, RLD and *Ws* showed an affected expression of the triple response and a reduced expression of the ethylene responsive genes *Hel* and *Pdf1.2* after exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylate. Moreover, in contrast to *Col*, RLD and *Ws* did not develop resistance against *P. syringae* pv. *tomato* DC3000 after treatment of the leaves with 1-aminocyclopropane-1-carboxylate. Analysis of the F<sub>2</sub> and F<sub>3</sub> progeny of a cross between *Col* (*ISR1/ISR1*) and RLD (*isr1/isr1*) revealed that reduced sensitivity to ethylene cosegregates with the recessive alleles of the *ISR1* locus. These results suggest that the *ISR1* locus encodes a component of the ethylene response, which is required for the expression of rhizobacteria-mediated ISR.

Localized treatment of plants with specific biotic or abiotic agents can result in the development of enhanced resistance against pathogens in distal plant parts. Resistance induced by such treatments is generally characterized by a restriction of pathogen growth and a reduction of disease severity (Hammer-schmidt, 1999). Induced resistance against pathogens can be subdivided into two categories. The classical way to induce disease resistance is by predisposal infection with a necrotizing pathogen, resulting in a systemic resistance in distal plant parts. This form of induced resistance is generally referred to as systemic acquired resistance (SAR; Ryals et al., 1996; Sticher et al., 1997). SAR is characterized by the endogenous accumulation of salicylic acid (SA; Malamy et al., 1990; Métraux et al., 1990) and a concomitant expression of genes encoding pathogenesis-related proteins (for review, see Van Loon, 1997). The second type of induced resistance develops in response to colonization of plant roots by selected strains of non-pathogenic rhizobacteria, and is often referred to as induced systemic resistance (ISR; Van Loon et al., 1998). Rhizobacteria-mediated ISR has been demonstrated to be effective in a variety of plant species under conditions in which the rhizobacteria remained spatially separated from the challenging

pathogen. Pathogen-induced SAR and rhizobacteria-mediated ISR confer an enhanced defensive capacity that is effective against a broad spectrum of plant pathogens (Ryals et al., 1996; Van Loon et al., 1998). In Arabidopsis the level of induced resistance can be enhanced further when both types of induced resistance are activated simultaneously (Van Wees et al., 2000), indicating that SAR and ISR are additive and constitute two different mechanisms of induced resistance.

Rhizobacteria-mediated ISR has been studied extensively in Arabidopsis using the non-pathogenic rhizobacterial strain *Pseudomonas fluorescens* WCS417r as the inducing agent and *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) as the challenging pathogen (Pieterse et al., 2000). In this combination the ISR signaling pathway clearly differs from the one that controls pathogen-induced SAR. SA-nonaccumulating *NahG* plants expressing the bacterial salicylate hydroxylase gene (*NahG*) fail to express SAR (Gaffney et al., 1993; Lawton et al., 1995), but show normal levels of ISR after treatment of the roots with WCS417r bacteria (Pieterse et al., 1996). This indicates that SA is a necessary signal for the SAR response, but is not required for ISR signaling. Arabidopsis mutants that are impaired in their response to the plant hormones jasmonate (JA) or ethylene, develop normal levels of SAR (Lawton et al., 1995; Pieterse et al., 1998), but are unable to express WCS417r-mediated ISR (Pieterse et al., 1998; Knoester et al., 1999). This demonstrates that, in contrast to SAR, ISR signaling requires components

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of the JA and the ethylene response. Despite these differences, the SAR and the ISR pathway are both controlled by the regulatory protein NPR1/NIM1 (Cao et al., 1994; Delaney et al., 1995; Pieterse et al., 1998). Downstream of NPR1/NIM1 both pathways diverge, indicating that NPR1/NIM1 differentially regulates defense responses depending on the pathway that is activated upstream of it (Pieterse et al., 1998).

We recently identified a novel factor in the ISR signaling pathway by screening 10 Arabidopsis accessions for their ability to express ISR. Two accessions, RLD and Wassilewskija (Ws), failed to develop ISR after treatment of the roots with WCS417r bacteria, whereas they expressed normal levels of pathogen-induced SAR (Ton et al., 1999). This WCS417r-non-responsive phenotype could not be attributed to poor root colonization by the ISR-inducing rhizobacteria, since colonization of the roots of both accessions was unaffected. Furthermore, the WCS417r-non-responsive phenotype of RLD and Ws was associated with a relatively high level of susceptibility to *Pst*. Genetic analysis of progeny of crosses between inducible and noninducible accessions revealed that the potential to express ISR, as well as the relatively high level of basal resistance against *Pst*, are controlled by a single dominant locus (*ISR1*) that maps on chromosome III between cleaved amplified polymorphism sequence (CAPS) markers *B4* and *GL1*. This suggested that the *ISR1* locus encodes a factor that is involved in both ISR and basal resistance against *Pst*.

Not only are the Arabidopsis mutants *etr1-1* and *jar1-1* affected in their ability to express WCS417r-mediated ISR against *Pst*, they also develop more severe disease symptoms upon primary infection with *Pst*, and allow significantly more bacterial growth compared with wild-type Columbia (Col) plants (Pieterse et al., 1998). This strikingly resembles the *isr1* phenotype of accessions RLD and Ws, which are similarly affected in the expression of ISR and basal resistance against *Pst*. The involvement of JA and ethylene in basal defense responses has repeatedly been demonstrated. In many cases, blocking the response to either of these signals can render plants more susceptible to certain pathogens and even insects. For instance, mutants that are affected in JA biosynthesis or signaling are more susceptible to pathogens such as *Pythium mastophorum* (Vijayan et al., 1998) and *P. irregulare* (Staswick et al., 1998), as well as to insect herbivory (McConn et al., 1997; Stout et al., 1999). In a similar manner, ethylene-insensitive tobacco plants transformed with the mutant *etr1-1* gene from Arabidopsis lost their ability to resist the soil-borne pathogen *P. sylvaticum* (Knoester et al., 1998). Furthermore, the ethylene-insensitive Arabidopsis mutant *ein2-1* gained enhanced susceptibility to the necrotrophic fungal pathogen *Botrytis cinerea* (Thomma et al., 1999) and the bacterial leaf pathogen

*Erwinia carotovora* pv. *carotovora* (Norman-Setterblad et al., 2000). Although these examples demonstrate the importance of JA and ethylene in specific basal resistance responses, other pathogens seem to be resisted predominantly through a SA-dependent pathway (Thomma et al., 1998).

The *isr1* phenotype, i.e. inability to express ISR and enhanced susceptibility to *Pst* infection, of accessions RLD and Ws on the one hand and that of mutants *etr1-1* and *jar1-1* on the other hand, prompted us to investigate whether the *ISR1* locus is involved in JA or ethylene signaling. Arabidopsis accessions Col (*ISR1/ISR1*), RLD (*isr1/isr1*), and Ws (*isr1/isr1*) were tested for their ability to respond to JA and ethylene by examining JA- and ethylene-induced inhibition of root growth and by studying JA- and ethylene-responsive gene expression. Here we show that the *isr1* phenotype of accessions RLD and Ws is associated with a reduced sensitivity to ethylene, suggesting that the *ISR1* locus is involved in ethylene signaling.

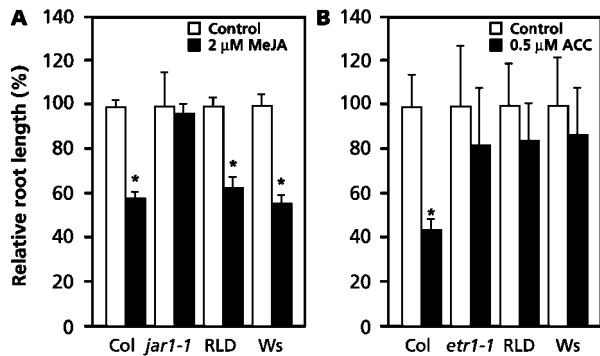
## RESULTS

### The *isr1* Phenotype Is Not Associated with Reduced Sensitivity to Methyl JA (MeJA)

Accessions RLD and Ws resemble the JA-insensitive mutant *jar1-1* in that they are blocked in the ISR signaling pathway and exhibit enhanced susceptibility to *Pst* (Pieterse et al., 1998; Ton et al., 1999). Therefore, we investigated whether the *isr1* phenotype of accessions RLD and Ws is based on reduced sensitivity to JA. It was previously demonstrated that MeJA-induced inhibition of primary root growth and MeJA-induced expression of the *Atvsp* gene is substantially decreased in the *jar1-1* mutant (Staswick et al., 1992, 1998). Both characteristics were examined in Col, *jar1-1*, RLD, and Ws plants. Five days after germination, accession Col showed a 42% inhibition of primary root growth on Murashige and Skoog (MS) agar plates with 2  $\mu$ M MeJA (Fig. 1A). Growth of primary roots of mutant *jar1-1* was not inhibited, whereas RLD and Ws showed a similar response to MeJA as accession Col, resulting in 38% and 45% inhibition of primary root growth, respectively. Moreover, exogenous application of 100  $\mu$ M MeJA to leaves of Col resulted in a strong activation of the JA-responsive gene *Atvsp*, whereas in *jar1-1* plants *Atvsp* transcripts accumulated to a much lower level (Fig. 2). RLD and Ws showed similar responses to MeJA as Col. These results demonstrate that the *isr1* phenotype of RLD and Ws cannot be attributed to reduced responsiveness to JA.

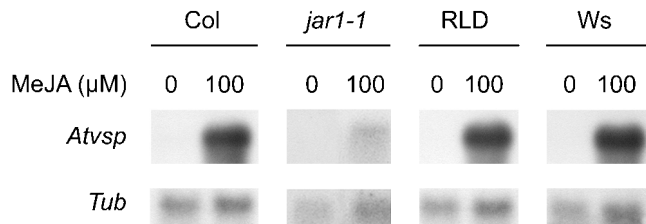
### RLD and Ws Show Reduced Sensitivity to Ethylene

Like *jar1-1*, the ethylene insensitive mutant *etr1-1* is blocked in the expression of ISR and exhibits enhanced disease susceptibility to *Pst* (Pieterse et al., 1998; Knoester et al., 1999). Therefore, we investi-



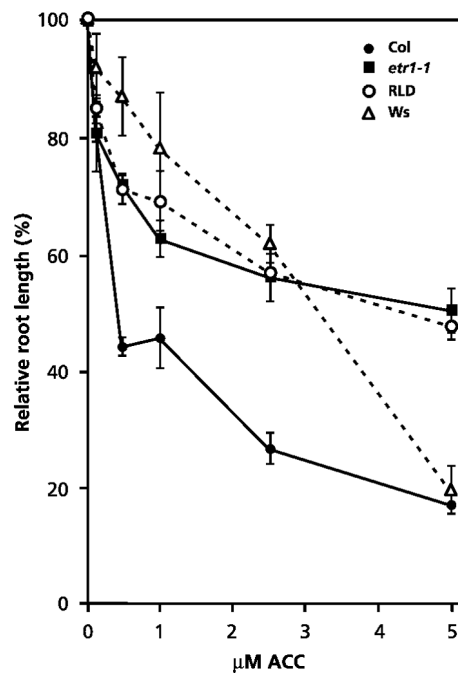
**Figure 1.** MeJA- and ACC-induced inhibition of primary root growth. A, MeJA-induced inhibition of primary root growth in Col, *jar1-1*, RLD, and Ws. Seeds were surface-sterilized, distributed on MS-agar plates containing 0 or 2  $\mu\text{M}$  MeJA, and germinated for 2 d at 4°C in the dark. After an additional growth period of 5 d at 20°C with an 8-h photoperiod, the length of the primary roots was measured. Data are means ( $\pm$  SD;  $n = 15\text{--}25$ ) of the relative root length compared with control plants (0  $\mu\text{M}$  MeJA), which was set at 100%. Asterisks indicate statistically significant differences compared with the control plants (Student's *t* test;  $\alpha = 0.05$ ). B, ACC-induced inhibition of primary root length in Col, *etr1-1*, RLD, and Ws. Seeds were germinated on MS-agar plates containing 0 or 0.5  $\mu\text{M}$  ACC for 2 d at 4°C in the dark. After an additional growth period of 3 to 7 d in the dark at 20°C, the length of the primary root was measured. Data are means ( $\pm$  SD;  $n = 15\text{--}25$ ) of the relative root length compared with control plants (0  $\mu\text{M}$  ACC), which was set at 100%. Asterisks indicate statistically significant differences compared with the control plants (Student's *t* test;  $\alpha = 0.05$ ).

gated whether the *isr1* phenotype of accessions RLD and Ws is based on reduced sensitivity to ethylene. The "triple response" is a reaction of etiolated seedlings to ethylene and has been shown to be a reliable marker for ethylene sensitivity (Guzmán and Ecker, 1990). To assess ethylene sensitivity, we first examined ethylene-induced root length inhibition of etiolating seedlings, which is one of the characteristics of the triple response. Col, *etr1-1*, RLD, and Ws seedlings were grown on MS-agar plates containing 0.5  $\mu\text{M}$  of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC), as this concentration was found to differentiate best for root length inhibition be-

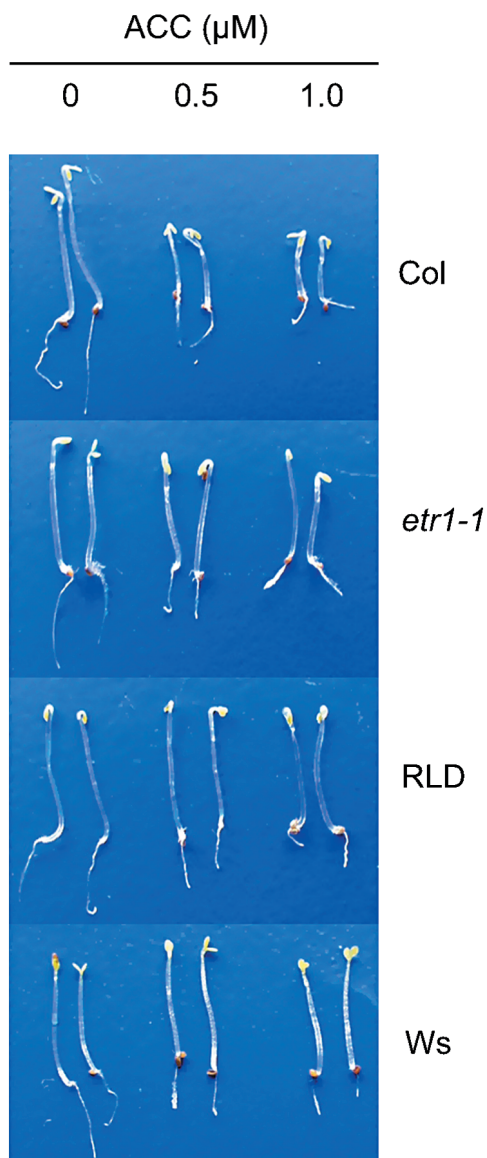


**Figure 2.** RNA gel-blot analysis of MeJA-induced *Atvsp* gene expression in leaves of Col, *jar1-1*, RLD, and Ws. Five-week-old plants were treated by dipping the leaves in a solution containing 0 or 100  $\mu\text{M}$  MeJA. Two days after MeJA-treatment the leaves were harvested. An Arabidopsis *Atvsp* gene-specific probe was used for the RNA gel-blot hybridization. To check for equal loading, the blot was stripped and hybridized with an Arabidopsis gene-specific probe for  $\beta$ -tubulin (*Tub*). The experiment was repeated with similar results.

tween Col and *etr1-1* (data not shown). At 0.5  $\mu\text{M}$  ACC, Col plants showed a statistically significant inhibition of root growth, whereas mutant *etr1-1*, RLD, and Ws responded only weakly (Fig. 1B). In *etr1-1*, RLD, and Ws the weak inhibition of root growth was never statistically significant, indicating that RLD and Ws exhibit a certain degree of insensitivity to ACC. To determine the extent of this insensitivity, the effect of increasing concentrations of ACC on root length inhibition was examined. Figure 3 shows that primary root length of etiolated Col seedlings was reduced by 55% to 80% when grown on 0.5 to 5  $\mu\text{M}$  ACC, respectively. In this concentration range, the inhibition of root elongation of *etr1-1* and RLD was significantly less dramatic (ranging between 25%–45%). Up to 2.5  $\mu\text{M}$  ACC, the effect of ACC on root elongation in accession Ws was even less evident than in RLD and *etr1-1* (ranging between 10%–35%). However, at 5  $\mu\text{M}$  ACC Ws showed the same level of inhibition of root length as Col. Other characteristics of the triple response, i.e. inhibition of hypocotyl elongation and exaggeration of the apical hook, were clearly apparent at 0.5  $\mu\text{M}$  of ACC in Col. In *etr1-1*, RLD, and Ws these characteristics were absent at 0.5  $\mu\text{M}$  and 1  $\mu\text{M}$  (Fig. 4) and only occurred consistently at concentrations above 2.5  $\mu\text{M}$  (data not shown).



**Figure 3.** Dose-response curves of ACC-induced inhibition of primary root growth in Col, *etr1-1*, RLD, and Ws. Seeds were germinated on MS-agar plates containing 0, 0.1, 0.5, 1.0, 2.5, or 5.0  $\mu\text{M}$  ACC for 2 d at 4°C in the dark. After an additional growth period of 3 to 7 d in the dark at 20°C, the length of the primary roots was measured. Data are means ( $\pm$ SD;  $n = 15\text{--}25$ ) of the relative root length compared with that of control-treated plants (0  $\mu\text{M}$  ACC), which was set at 100%.



**Figure 4.** Triple response expression of Col, *etr1-1*, RLD, and Ws. Seeds were germinated on MS-agar plates containing 0, 0.5, or 1.0  $\mu\text{M}$  ACC for 2 d at 4°C in the dark. Photographs were taken after an additional growth period of 4 d in the dark at 20°C.

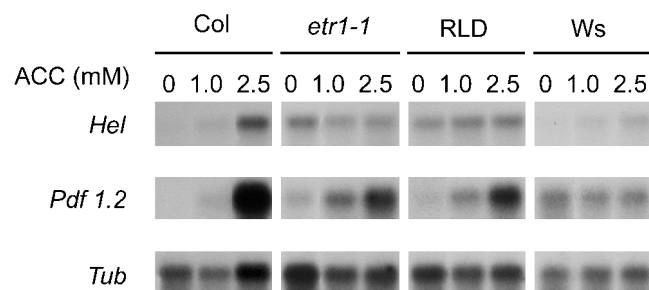
To further investigate the apparent differences in ACC sensitivity, the expression patterns of the ethylene-inducible, defense-related genes *Hel* (Potter et al., 1993) and *Pdf1.2* (Penninckx et al., 1996) were analyzed after application of 0, 1.0, or 2.5 mM ACC to the leaves of Col, *etr1-1*, RLD, and Ws (Fig. 5). In Col, *Hel* and *Pdf1.2* transcripts were evident at 1.0 mM ACC and accumulated to a relatively high level at 2.5 mM ACC. In contrast, at increasing ACC concentrations *etr1-1* showed no increase in the steady-state *Hel* mRNA level. Moreover, only a relatively weak induction of *Pdf1.2* gene expression was apparent in *etr1-1*, which was significantly less pronounced than that observed in Col plants. RLD showed *Hel* and *Pdf1.2* expressions patterns similar to that observed

in *etr1-1*. In Ws, steady-state levels of *Pdf1.2* transcripts did not increase at all after ACC treatment, whereas the *Hel* gene was weakly induced. These results confirm that accessions RLD and Ws show reduced sensitivity to ACC, with RLD resembling *etr1-1* more than Ws.

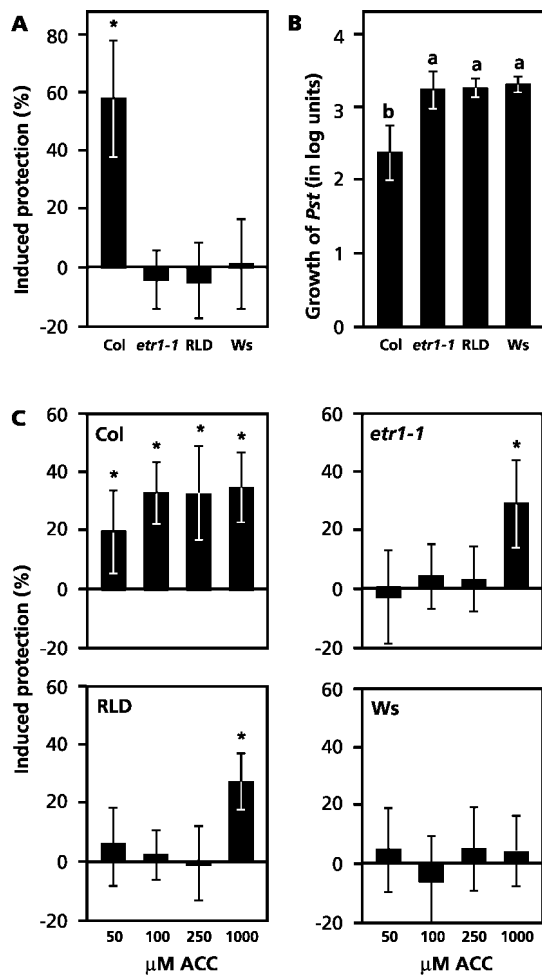
After application of 1 mM ACC, all four genotypes showed similar time kinetics in the emission of ethylene (data not shown), indicating that ACC uptake and ACC converting capacity was similar for all four genotypes. It can thus be concluded that the observed differences in responsiveness to ACC are the result of differences in ethylene sensitivity.

#### RLD and Ws Are Affected in Ethylene-Induced Protection against *Pst*

Comparison of mutant *etr1-1* and accessions RLD and Ws revealed that they are phenotypically similar in that they are non-responsive to WCS417r-mediated induction of ISR (Fig. 6A) and allow statistically significant higher levels of growth of *Pst* compared with Col (Fig. 6B). Both characteristics are likely to be caused by the common reduced sensitivity to ethylene. We previously demonstrated that exogenous application of ACC to the leaves induces protection against *Pst* in Col, but not in *etr1-1* (Pieterse et al., 1998). To investigate whether RLD and Ws are similarly affected in ACC-induced protection, we determined the level of resistance against *Pst* after exogenous application of increasing concentrations of ACC (Fig. 6C). In Col, all ACC concentrations tested induced a statistically significant level of protection against *Pst* compared with water-treated plants. In contrast, *etr1-1* and RLD failed to develop resistance after treatment with the three lowest ACC concentrations (50, 100, and 250  $\mu\text{M}$ ), whereas application of 1 mM ACC induced protection in *etr1-1* and RLD only. In Ws, all ACC concentrations tested failed to induce protection against *Pst*. It can thus be



**Figure 5.** RNA gel-blot analysis of ACC-induced *Hel* and *Pdf1.2* gene expression in the leaves of Col, *etr1-1*, RLD, and Ws. Five-week-old plants were treated by dipping the leaves in a solution containing 0, 1.0, or 2.5 mM ACC. Two days after ACC-treatment the leaves were harvested. Arabidopsis *Hel* and *Pdf1.2* gene-specific probes were used for RNA gel-blot hybridizations. To check for equal loading, the blots were stripped and hybridized with an Arabidopsis gene-specific probe for  $\beta$ -tubulin (*Tub*). The experiment was repeated with similar results.



**Figure 6.** Level of WCS417r-induced protection, basal resistance, and ACC-induced protection against *Pst* in Col, *etr1-1*, RLD, and Ws. A, Quantification of WCS417r-mediated ISR. ISR was triggered by growing plants in soil containing ISR-inducing *P. fluorescens* WCS417r bacteria at  $5 \times 10^7$  cfu  $g^{-1}$ . Five-week-old plants were challenge-inoculated with a bacterial suspension of virulent *Pst* at  $2.5 \times 10^7$  cfu  $mL^{-1}$ . Three days after challenge inoculation the percentage of diseased leaves was assessed and the level of induced protection was calculated. Induced protection is presented as a reduction of disease symptoms relative to challenged control plants. Asterisks indicate statistically significant differences compared with noninduced control plants (Student's *t* test;  $\alpha = 0.05$ ;  $n = 20$ –25). Data presented are means ( $\pm$ SD) from representative experiments that were performed at least twice with similar results. B, Proliferation of *Pst* over a 3-d time interval. Five-week-old plants were infected by pressure infiltrating a suspension of virulent *Pst* at  $5 \times 10^5$  cfu  $mL^{-1}$  into the leaves. Immediately after pressure infiltration and 3 d later, the number of *Pst* bacteria per gram of leaf fresh weight was determined and the proliferation over a 3-d time interval was calculated. Data presented are the means ( $\pm$ SD) of the proliferation values (log cfu  $g^{-1}$ ) of a representative experiment that was repeated twice with similar results. Different letters indicate statistically significant differences between genotypes (Fisher's LSD test;  $\alpha = 0.05$ ;  $n = 6$ ). C, Quantification of ACC-induced protection against *Pst*. Plants were dipped in a solution containing different concentrations of ACC 3 d before challenge inoculation with *Pst*. Three days after challenge, the level of induced protection was assessed as described above.

concluded that, like *etr1-1*, accessions RLD and Ws are impaired in ethylene-induced resistance against *Pst*.

#### Reduced Sensitivity to Ethylene Cosegregates with the *isr1* Phenotype

To investigate whether the reduced sensitivity to ethylene observed in RLD and Ws is associated with the recessive alleles of the *ISR1* locus we tested the  $F_2$  progeny of the RLD  $\times$  Col cross for ethylene sensitivity. The triple response was tested in the  $F_2$  seedlings and in the RLD and Col parents at 0 and  $0.5 \mu M$  ACC. In the  $F_2$  population, responsiveness to  $0.5 \mu M$  ACC segregated in a statistically significant 3:1 ratio ( $X^2 = 0.16$ ,  $P = 0.69$ ; Table I), indicating that the reduced sensitivity to ethylene is caused by a single recessive locus. This experiment was repeated twice, yielding comparable segregation ratios ( $X^2 = 0.04$  and  $0.19$ ;  $P = 0.83$  and  $0.66$ , respectively). Five  $F_3$  families homozygous at the *ISR1* locus (*ISR1/ISR1*), five  $F_3$  families homozygous at the *isr1* locus (*isr1/isr1*), and the corresponding Col and RLD parent were subsequently tested for triple response expression at 0, 0.5, 1, and  $5 \mu M$  ACC. On MS-agar plates without ACC, none of the genotypes showed triple response expression (Table II). On  $0.5 \mu M$  ACC, only the Col parent and the five *ISR1/ISR1*  $F_3$  families exhibited consistent triple response expression, whereas the RLD parent and the *isr1/isr1*  $F_3$  families did not (Table II). At higher concentrations of ACC, RLD and the *isr1/isr1*  $F_3$  families showed triple response expression, although in many cases this was inconsistent (Table II). It can thus be concluded that the reduced ethylene sensitivity observed in RLD is a recessive trait that cosegregates with the *isr1* phenotype.

#### The *ISR1* Locus Is Not Allelic with *Ein3*

The *ISR1* locus was previously mapped at chromosome III between CAPS markers *GL1* and *B4* (Ton et al., 1999). The *Ein3* gene, encoding an activator of the ethylene response pathway (Chao et al., 1997), also maps in this region. Moreover, the *ein3* mutant is unable to express WCS417r-mediated ISR (Knoester et al., 1999). Therefore, we investigated whether *ISR1* and *Ein3* are allelic. For this purpose we designed a CAPS marker based on the nucleotide sequence of the *Ein3* gene (Chao et al., 1997) and performed a CAPS marker analysis on 32 homozygous  $F_3$  families of the RLD  $\times$  Col cross (16 *ISR1/ISR1*  $F_3$  families and 16 *isr1/isr1*  $F_3$  families). Of the 64 chromosomes tested, 13 were recombinant with the *Ein3* CAPS marker, yielding a recombination frequency at 20.3%. Moreover, the  $F_1$  progeny of a complementation cross between RLD and the *ein3* mutant was fully capable of expressing ISR and exhibited a similar level of basal resistance against *Pst* as the Col parent

**Table I.** Genetic segregation of triple response expression at 0.5  $\mu\text{M}$  ACC in Col, RLD, and F<sub>2</sub> plants of the RLD  $\times$  Col cross<sup>a</sup>

Genotype	Treatment ( $\mu\text{M}$ ACC)	Total No. of Seedlings	Triple Response <sup>b</sup>	No Triple Response <sup>c</sup>	Expected Ratio	$\chi^2$ value	<i>P</i>
Col	0	48	0	48	0:1	–	–
	0.5	56	53	3	1:0	–	–
RLD	0	52	3	49	0:1	–	–
	0.5	53	4	49	1:0	–	–
F <sub>2</sub>	0	52	4	48	0:1	–	–
	0.5	74	54	20	3:1	0.16	0.69

<sup>a</sup> Surface-sterilized seeds were plated onto MS-agar plates with or without 0.5  $\mu\text{M}$  ACC. After germination for 2 d at 4°C, seedlings were grown for 5 d at 20°C in the dark and examined for triple-response expression. The experiment was repeated twice, yielding similar results. <sup>b</sup> No. of seedlings exhibiting triple response characteristics. <sup>c</sup> No. of seedlings showing no triple response characteristics.

(data not shown). It can thus be concluded that the *ISR1* locus is not allelic with the *Ein3* gene.

## DISCUSSION

We previously demonstrated that the *ISR1* locus on chromosome III of Arabidopsis controls the expression of WCS417r-mediated ISR and basal resistance against *Pst* (Ton et al., 1999). This study aimed at elucidating the physiological role of the *ISR1* locus in the ISR signaling pathway. In Arabidopsis the ISR pathway requires an intact response to the plant

hormones JA and ethylene (Pieterse et al., 1998). Analysis of MeJA-induced inhibition of primary root growth and *Atvsp* gene expression in the WCS417r-non-responsive accessions RLD (*isr1/isr1*) and *Ws* (*isr1/isr1*), and the ISR-inducible accession Col (*ISR1/ISR1*), revealed that RLD and *Ws* are not affected in their response to MeJA (Figs. 1 and 2). It can thus be concluded that the *ISR1* locus is not involved in JA signaling. In contrast, analysis of the triple response and ethylene-inducible gene expression demonstrated that sensitivity to ethylene is significantly reduced in RLD and *Ws* in comparison with Col (Fig. 3–5). Moreover, RLD and *Ws* were impaired in their ability to express ethylene-induced resistance against *Pst* (Fig. 6). Genetic analysis of the F<sub>2</sub> and F<sub>3</sub> progeny of a RLD  $\times$  Col cross revealed that the reduced sensitivity to ethylene is monogenically inherited as a recessive trait and genetically linked to the recessive alleles of the *ISR1* locus. These results indicate that the *ISR1* locus contains a gene encoding a component involved in ethylene signaling. Using a large set of ethylene response mutants, we previously demonstrated that insensitivity to ethylene causes non-responsiveness to WCS417r (Pieterse et al., 1998; Knoester et al., 1999). Therefore, it is likely that ethylene responsiveness and ISR inducibility are determined by a single gene on the *ISR1* locus. However, the possibility of close linkage between two different genes cannot be eliminated completely.

The *ISR1* locus maps on chromosome III between CAPS markers *B4* and *GL1* (Ton et al., 1999). Two genes from the ethylene signaling pathway, *Ein3* and *Ein4*, map in the vicinity of the *ISR1* locus. The possibility that the *ISR1* locus is allelic with *Ein4* can be ruled out by the observation that accessions RLD and *Ws* exhibit recessive phenotypes (Ton et al., 1999; this study), whereas the *ein4* mutation is dominant (Roman et al., 1995). The considerable recombination between the *ISR1* locus and the *Ein3* gene in F<sub>3</sub> families of the RLD  $\times$  Col cross also rules out the possibility that *ISR1* is allelic with *Ein3*. Moreover, accession RLD and mutant *ein3* showed full complementation of ISR-inducibility and basal resistance

**Table II.** Triple response expression in accessions Col and RLD, and in F<sub>3</sub> families of the RLD  $\times$  Col cross that are homozygous at the *ISR1* locus

Accessions/F <sub>3</sub> Families <sup>a</sup>	Triple Response Assay <sup>b</sup>			
	0 $\mu\text{M}$	0.5 $\mu\text{M}$	1 $\mu\text{M}$	5 $\mu\text{M}$
Col ( <i>ISR1/ISR1</i> )	–	+	+	+
<i>ISR1/ISR1</i> fam 1	–	+	+	+
<i>ISR1/ISR1</i> fam 2	–	+	+	+
<i>ISR1/ISR1</i> fam 3	–	+	+	+
<i>ISR1/ISR1</i> fam 4	–	+	+	+
<i>ISR1/ISR1</i> fam 5	–	+	+	+
RLD ( <i>isr1/isr1</i> )	–	–	–	–
<i>isr1/isr1</i> fam 1	–	–	+/-	+/-
<i>isr1/isr1</i> fam 2	–	–	+	+
<i>isr1/isr1</i> fam 3	–	–	+	+
<i>isr1/isr1</i> fam 4	–	–	+/-	+/-
<i>isr1/isr1</i> fam 5	–	–	–	+/-

<sup>a</sup> The F<sub>3</sub> families homozygous for the dominant *ISR1* alleles of Col are capable of expressing ISR after treatment of the roots with *P. fluorescens* WCS417r and exhibit relatively high basal resistance against *Pst*. The F<sub>3</sub> families homozygous for the recessive *isr1* alleles of RLD fail to express ISR after treatment of the roots with WCS417r and exhibit relatively low levels of basal resistance against *Pst* (Ton et al., 1999). <sup>b</sup> Genotypes were assayed for sensitivity to ACC using the triple response assay. Surface-sterilized seeds (50–100) were plated onto MS-agar plates containing different concentrations of ACC. After germination for 2 d at 4°C, seedlings were grown for 3 to 7 d at 20°C in darkness before being examined for triple response expression. +, Consistent triple response in all plants; +/-, inconsistent triple response; –, none of the plants expressed the triple response.

against *Pst* in their F<sub>1</sub> progeny (data not shown). Therefore, we hypothesize that the Arabidopsis *ISR1* locus encodes a novel component of the ethylene response pathway, which plays an important role in disease resistance signaling.

Although RLD and Ws showed reduced sensitivity to ethylene in comparison to Col, the magnitude of the reduced ethylene response differed between both accessions. For instance, like *etr1-1*, RLD was affected in the inhibition of ethylene-induced primary root growth at all ACC concentrations tested (0.5–5.0  $\mu\text{M}$ ), whereas for Ws this was only apparent at ACC concentrations up to 2.5  $\mu\text{M}$ . At 5  $\mu\text{M}$ , Ws displayed a normal triple response. Analysis of ethylene-responsive gene expression revealed a similar pattern, with RLD resembling *etr1-1* more than Ws. The apparent differences in ethylene responsiveness between RLD and Ws likely result from the considerable genetic diversity between both accessions (Erschadi et al., 2000). Alternatively, RLD and Ws might be affected at different sites in the *ISR1* locus.

In Arabidopsis considerable genetic variation between accessions has been reported for several developmental, physiological, and biochemical traits (Alonso-Blanco and Koornneef, 2000). This naturally occurring variation of Arabidopsis has contributed to the identification of a large number of loci conferring resistance to viral, bacterial, or fungal pathogens (Kunkel, 1996). In this study we provide evidence that reduced sensitivity to ethylene in RLD and Ws is causing the *isr1* phenotype in these accessions. Naturally occurring variation in ethylene sensitivity is known to occur in various plant species. For instance, Voesenek et al. (1996) reported significant differences in ethylene sensitivity between three species of *Rumex*, one of which showed exceptionally high responsiveness to ethylene. This phenotype appeared to be a necessary adaptation for escaping water submergence by mediation of ethylene-induced shoot elongation. Moreover, Emery et al. (1996) reported considerable differences in ethylene responsiveness between ecotypes of *Stellaria longipes*. The enhanced ethylene responsiveness of one ecotype was explained as a critical adaptation to wind stress.

Arabidopsis plants carrying the recessive alleles of the *ISR1* locus show an enhanced susceptibility to *Pst* infection (Ton et al., 1999), suggesting that ethylene signaling is involved in basal resistance against *Pst*. This is supported by the observation that the ethylene response mutant *etr1-1* allows 10-fold higher levels of growth of *Pst* than wild-type plants (Pieterse et al., 1998; this study). Moreover, treatment of Arabidopsis with the ethylene precursor ACC induces resistance against *Pst* (Pieterse et al., 1998; Van Wees et al., 1999). These findings seem to contradict those reported by Bent et al. (1992), who showed that the ethylene response mutant *ein2* allows similar levels of growth of *Pst*, and develops fewer symptoms compared with wild-type plants. However, Bent et al.

(1992) used a 5-fold lower density of *Pst* inoculum. This suggests that the ethylene-dependent basal resistance that we observed is only apparent when the initial inoculum density is above a certain threshold level, although other so far unidentified differences in the experimental setup could also be responsible for the different results. The plant hormones SA and JA are also involved in basal resistance against *Pst*, because genotypes that are impaired in their response to these signaling molecules are more susceptible to *Pst* infection (Delaney et al., 1994; Pieterse et al., 1998). It is apparent that the mechanisms contributing to basal resistance against *Pst* are controlled by a coordinated action of SA-, JA-, and ethylene-dependent signaling pathways.

Knoester et al. (1999) previously tested eight ethylene response mutants on their ability to express WCS417r-mediated ISR. None of these mutants were able to express ISR after application of WCS417r to the roots, demonstrating that an intact ethylene-signaling pathway is required for the expression of ISR. Mutant *eir1-1* that is insensitive to ethylene in the roots only was capable of mounting ISR after application of WCS417r to the leaves, but not after application of WCS417r to the roots, indicating that the ISR signaling pathway requires ethylene sensitivity at the site of WCS417r application. From these results it was hypothesized that ethylene signaling is involved in the generation or translocation of the systemically transported ISR signal. Future research will be focused on cloning of the *ISR1* gene. This will open the way to study the role of the *ISR1* protein in both ISR and ethylene signaling.

## MATERIALS AND METHODS

### Cultivation of Rhizobacteria, Pathogens, and Plants

Non-pathogenic, ISR-inducing *Pseudomonas fluorescens* WCS417r bacteria (Pieterse et al., 1996) were grown on King's medium B (KB) agar plates (King et al., 1954) for 24 h at 28°C. Bacterial cells were collected and resuspended in 10 mM MgSO<sub>4</sub> to a final density of 10<sup>9</sup> colony-forming units (cfu) per mL. The virulent pathogen *P. syringae* pv. *tomato* strain DC3000 (*Pst*; Whalen et al., 1991) used for challenge inoculations was grown overnight at 28°C in liquid KB. After centrifugation, the bacterial cells were resuspended in 10 mM MgSO<sub>4</sub> with 0.015% (v/v) of the surfactant Silwet L-77 (Van Meeuwen Chemicals, Weesp, The Netherlands) to a final density of 2.5 × 10<sup>7</sup> cfu mL<sup>-1</sup>.

Seedlings of wild-type Arabidopsis accessions Col (Col-0), Ws (Ws-0), and RLD (RLD1), and the Col mutants *etr1-1* (Bleecker et al., 1988) and *jar1-1* (Staswick et al., 1992) were grown in quartz sand for 2 weeks. For transfer of the seedlings, a sand/potting soil mixture (5:12, v/v) that had been autoclaved twice for 1 h with a 24-h interval was supplemented with a suspension of ISR-inducing WCS417r bacteria or an equal volume of 10 mM MgSO<sub>4</sub>. Seedlings were then transferred into 60-mL pots containing the sand/potting soil mixture with or without WCS417r. Plants were

cultivated in a growth chamber with an 9-h day (200  $\mu\text{E m}^{-2} \text{s}^{-1}$  at 24°C) and 15-h night (20°C) cycle at 65% relative humidity. For the duration of the experiments, all genotypes remained vegetative and developed at least 10 to 15 mature leaves. Plants were watered on alternate days, and once a week supplied with modified one-half strength Hoagland solution: 2 mM  $\text{KNO}_3$ , 5 mM  $\text{Ca}[\text{NO}_3]_2$ , 1 mM  $\text{KH}_2\text{PO}_4$ , 1 mM  $\text{MgSO}_4$ , and trace elements, pH 7 (Hoagland and Arnon, 1938), containing 10  $\mu\text{M}$  sequestren [Fe-ethylenediamide-di(0-hydroxyphenylacetic acid)]; Novartis, Basel].

### Induction Treatments

For treatment with ISR-inducing rhizobacteria, 2-week-old seedlings were transplanted into soil containing WCS417r bacteria at  $5 \times 10^7$  cfu  $\text{g}^{-1}$ . Induction of ethylene-mediated resistance was performed 2 d before challenge by dipping the leaves of 5-week-old plants in a solution containing the ethylene precursor ACC with 0.015% (v/v) Silwet L-77. For RNA-blot analysis, chemical treatments were performed by dipping the leaves of 5-week-old plants in a solution containing 0.015% (v/v) Silwet L77 and different concentrations of MeJA or ACC.

### Challenge Inoculations and Disease Assessment

For assaying induced resistance, WCS417r- and control-treated plants were challenged when 5 weeks old by dipping the leaves in a suspension of virulent *Pst* bacteria at  $2.5 \times 10^7$  cfu  $\text{mL}^{-1}$  in 10 mM  $\text{MgSO}_4$ , 0.015% (v/v) Silwet L-77. One day before challenge inoculation, the plants had been placed at 100% relative humidity. Three or 4 d after challenge inoculation, the percentage of leaves with symptoms was determined per plant ( $n = 20$ –25). Leaves showing necrotic or water-soaked lesions surrounded by chlorosis were scored as diseased (Pieterse et al., 1996). For assaying basal resistance against *Pst*, leaves of 5-week-old, control-treated plants were inoculated by pressure-infiltration with a suspension of virulent *Pst* at  $5 \times 10^5$  cfu  $\text{mL}^{-1}$  in 10 mM  $\text{MgSO}_4$ . Immediately after pressure infiltration and 3 d later, replicate leaf samples from five plants per genotype were collected, weighed, and homogenized in 10 mM  $\text{MgSO}_4$ . Serial dilutions were plated on selective KB-agar plates supplemented with 100 mg  $\text{L}^{-1}$  cycloheximide and 50 mg  $\text{L}^{-1}$  rifampicin. After incubation at 28°C for 2 d, the number of rifampicin-resistant colony-forming units per gram of infected leaf tissue was determined and bacterial proliferation over the 3-d time interval was calculated.

### Inhibition of Primary Root Length by MeJA and ACC

Seeds of Arabidopsis were surface sterilized for 5 min in 5% (v/v) sodium hypochlorite, washed in 70% (v/v) ethanol, and air dried. Seeds were subsequently distributed evenly on 1.0% (w/v) agar medium containing 0.5% (w/v) MS salts (Duchefa bv, Haarlem, The Netherlands), 0.5% (w/v) Suc, and different concentrations of MeJA or ACC (pH 5.7). MeJA (Serva, Brunschwig Chemie bv, Amsterdam) was added to the autoclaved medium from a filter-

sterilized 1-mm stock (containing 0.96% [v/v] ethanol). ACC (Sigma-Aldrich Chemie bv, Zwijndrecht, The Netherlands) was added from a 10 mM stock in a similar manner. Seeds were germinated in the dark for 2 d at 4°C. The effect of MeJA on primary root growth was determined essentially as described by Staswick et al. (1992). Plates were incubated in a climate chamber at 22°C with an 8-h day (approximately 200  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) and a 16-h night cycle. After 5 d the primary root length was measured under a dissection microscope. In each case, 15 to 25 randomly selected seedlings were measured. The effect of ethylene on primary root length of etiolated seedlings was tested essentially according to Guzmán and Ecker (1990). After germination in the dark for 2 d at 4°C the seedlings were grown for an additional 3 to 7 d at 20°C without light. The primary root length was measured as described above.

### Ethylene Measurements

Leaves of plants pretreated with 1 mM ACC were detached, weighed, and placed in 25-mL gas-tight serum flasks, and subsequently incubated at climate chamber conditions. At different time points over a 28-h interval, cumulative ethylene production was measured by gas chromatography as described by De Laat and Van Loon (1982).

### RNA Gel-Blot Analysis

Total RNA was extracted by homogenizing frozen leaf tissue in extraction buffer (0.35 M Gly, 0.048 N NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% [w/v] SDS, and 1 mL  $\text{g}^{-1}$  of leaf tissue). The homogenates were extracted with phenol and chloroform and the RNA was precipitated using LiCl, as described by Sambrook et al. (1989). For RNA gel-blot analysis, 15  $\mu\text{g}$  of RNA was denatured using glyoxal and dimethyl sulfoxide (Sambrook et al., 1989). Samples were subsequently electrophoretically separated on 1.5% (w/v) agarose gels and blotted onto Hybond-N<sup>+</sup> membranes (Amersham, 's-Hertogenbosch, The Netherlands) by capillary transfer. The electrophoresis buffer and blotting buffer consisted of 10 and 25 mM sodium phosphate (pH 7.0), respectively. RNA gel blots were hybridized and washed as described previously (Pieterse et al., 1994). DNA probes were labeled with  $\alpha$ -<sup>32</sup>P-dCTP by random primer labeling (Feinberg and Vogelstein, 1983). Probes for the detection of *Atvsp*, *Hel*, and *Tub* transcripts were prepared by PCR with primers based on sequences obtained from GenBank accession numbers Z18377, U01880, and M21415, respectively. Probes to detect *Pdfl.2* transcripts were derived from an Arabidopsis *Pdfl.2* cDNA clone (Penninckx et al., 1996).

### Genetic Analysis

The F<sub>1</sub>, F<sub>2</sub>, and F<sub>3</sub> progenies of a cross between Col (*ISR1/ISR1*) and RLD (*isr1/isr1*) were previously tested for ISR inducibility and basal resistance against *Pst* (Ton et al., 1999). From this cross the F<sub>2</sub> progeny, five randomly selected F<sub>3</sub> families homozygous at the *ISR1* locus, and five randomly selected F<sub>3</sub> families homozygous at the *isr1* locus were tested for ethylene sensitivity and compared with that



of the corresponding parents. Ethylene sensitivity was quantified by assaying triple response expression as described by Guzmán and Ecker (1990), using MS-agar plates containing different concentrations of ACC. Recombination between the *ISR1* and the *EIN3* locus was determined on 16 F<sub>3</sub> families homozygous at the *ISR1* locus and 16 F<sub>3</sub> families homozygous at the *isr1* locus, using a CAPS marker (Konieczny and Ausubel, 1993) for the *EIN3* gene (accession no. AF004217; Chao et al., 1997). Amplification of the *EIN3* sequence was performed using the primers 5'-CTCCTTCTTTTCCCATCACCATA-3' (nucleotides 349–372) and 5'-TTCCCATCTCATTAAACATCATTG-3' (nucleotides 975–952). Subsequent digestion with *Bgl*II resulted in a polymorphism between accessions Col (627 bp) and RLD (359 and 268 bp).

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