

Characterization of *Arabidopsis* enhanced disease susceptibility mutants that are affected in systemically induced resistance

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Summary

In *Arabidopsis*, the rhizobacterial strain *Pseudomonas fluorescens* WCS417r triggers jasmonate (JA)- and ethylene (ET)-dependent induced systemic resistance (ISR) that is effective against different pathogens. *Arabidopsis* genotypes unable to express rhizobacteria-mediated ISR against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) exhibit enhanced disease susceptibility towards this pathogen. To identify novel components controlling induced resistance, we tested 11 *Arabidopsis* mutants with enhanced disease susceptibility (*eds*) to pathogenic *P. syringae* bacteria for WCS417r-mediated ISR and pathogen-induced systemic acquired resistance (SAR). Mutants *eds4-1*, *eds8-1* and *eds10-1* failed to develop WCS417r-mediated ISR, while mutants *eds5-1* and *eds12-1* failed to express pathogen-induced SAR. Whereas *eds5-1* is known to be blocked in salicylic acid (SA) biosynthesis, analysis of *eds12-1* revealed that its impaired SAR response is caused by reduced sensitivity to this molecule. Analysis of the ISR-impaired *eds* mutants revealed that they are non-responsive to induction of resistance by methyl jasmonate (MeJA) (*eds4-1*, *eds8-1* and *eds10-1*), or the ET precursor 1-aminocyclopropane-1-carboxylate (ACC) (*eds4-1* and *eds10-1*). Moreover, *eds4-1* and *eds8-1* showed reduced expression of the plant defensin gene *PDF1.2* after MeJA and ACC treatment, which was associated with reduced sensitivity to either ET (*eds4-1*) or MeJA (*eds8-1*). Although blocked in WCS417r-, MeJA- and ACC-induced ISR, *eds10-1* behaved normally for several other responses to MeJA or ACC. The results indicate that EDS12 is required for SAR and acts downstream of SA, whereas EDS4, EDS8 and EDS10 are required for ISR acting either in JA signalling (EDS8), ET signalling (EDS4), or downstream JA and ET signalling (EDS10) in the ISR pathway.

Keywords: defence signalling, ISR, jasmonic acid, ethylene, salicylic acid, SAR.

Introduction

Plants possess multiple strategies to resist infection by virulent pathogens. The signalling molecules salicylic acid (SA), jasmonate (JA) and ethylene (ET) play important roles in defence signalling, because plant genotypes that are affected in the response to either of these signals show enhanced disease susceptibility to various virulent pathogens or insects (Delaney *et al.*, 1994; Knoester *et al.*, 1998; McConn *et al.*, 1997; Pieterse *et al.*, 1998; Staswick *et al.*, 1998; Thomma *et al.*, 1998; Ton *et al.*, 2001; Vijayan *et al.*,

1998). Previously, responses to virulent pathogens have been subjected to a series of mutant screens using *Arabidopsis thaliana* as a model host plant. From a screen for enhanced disease susceptibility to *Pseudomonas syringae* pv. *maculicola*, 12 unique *eds* mutants have been characterized (*eds2-eds13*; Glazebrook *et al.*, 1996; Volko *et al.*, 1998). All these mutants allow at least tenfold higher levels of growth of *P. syringae* pv. *maculicola* on infection of their leaves, but they vary in their susceptibility

to other pathogens (Glazebrook *et al.*, 1996; Rogers and Ausubel, 1997; Volko *et al.*, 1998). The role of some of these *EDS* genes in basal disease resistance has been elucidated. For instance, mutant *eds5-1* was recently demonstrated to be allelic with the SA induction-deficient mutant *sid1-1* (Nawrath and Métraux, 1999). This mutation affects pathogen-induced accumulation of SA, and renders the plant more susceptible to a broad range of pathogens, including *Peronospora parasitica*, *Erysiphe orantii*, *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000), and *Xanthomonas campestris* pv. *raphani* (Nawrath and Métraux, 1999; Volko *et al.*, 1998). Furthermore, *eds4-1* was recently characterized as a mutant affected in SA-dependent defence responses (Gupta *et al.*, 2000).

In addition to basal resistance that protects the plant to some extent against primary attack by virulent pathogens, plants have the ability to develop an enhanced defensive capacity against a broad spectrum of pathogens after stimulation by specific biological or chemical agents. In *Arabidopsis*, two forms of biologically induced disease resistance have been characterized: systemic acquired resistance (SAR), triggered on infection by a necrotizing pathogen (Ryals *et al.*, 1996; Sticher *et al.*, 1997); and induced systemic resistance (ISR), triggered by selected strains of non-pathogenic rhizobacteria (Pieterse *et al.*, 2001; Van Loon *et al.*, 1998). SAR and rhizobacteria-mediated ISR are both effective against different pathogens, but they are regulated by distinct signalling pathways. Pathogen-induced SAR requires SA, whereas rhizobacteria-mediated ISR functions independently of SA (Gaffney *et al.*, 1993; Pieterse and Van Loon, 1999; Pieterse *et al.*, 1996). In the ISR-signalling pathway, components from the JA and the ET response act in sequence in triggering a defence reaction that, like SAR, depends on the defence regulatory protein NPR1/NIM1 (Cao *et al.*, 1994; Delaney *et al.*, 1995; Pieterse *et al.*, 1998; Pieterse *et al.*, 2000). Simultaneous activation of both types of induced defence results in an enhanced level of induced protection against *Pst* DC3000, demonstrating that the defence responses activated via both pathways are compatible and additive (Van Wees *et al.*, 2000).

Previously we reported that two *Arabidopsis* accessions, RLD1 and Wassilewskija (Ws-0), are impaired in their ability to express ISR after root treatment with ISR-inducing *Pseudomonas fluorescens* WCS417r bacteria, whereas they express normal levels of pathogen-induced SAR (Ton *et al.*, 1999). This ISR-non-inducible phenotype was associated with a remarkably low level of basal resistance against *Pst* DC3000 in comparison to the ISR-inducible accession Columbia (Col-0). By using this naturally occurring variation among *Arabidopsis* accessions, a genetic approach was initiated. Analysis of the progeny from an RLD1 × Col-0 cross revealed that ISR inducibility and basal resistance against *Pst* DC3000 are controlled by

a single dominant locus (*ISR1*) that maps on chromosome III between cleaved amplified polymorphic sequence (CAPS) markers *EIN3* and *GL1* (Ton *et al.*, 1999; Ton *et al.*, 2001). Genotypes carrying the recessive alleles of *ISR1* exhibit reduced sensitivity to ET, indicating that the *ISR1* locus encodes a component of the ET response that plays an important role in disease resistance signalling (Ton *et al.*, 2001).

The observed association between the inability to express WCS417r-mediated ISR on the one hand, and enhanced susceptibility to *Pst* DC3000 on the other hand, prompted us to investigate whether a similar relationship might hold in mutants selected for enhanced disease susceptibility to *P. syringae* pathogens. To this end, mutants *eds3-eds13* were examined for their ability to develop *P. fluorescens* WCS417r-mediated ISR and SAR. Here we show that out of the 11 mutants tested, three mutants are affected in their ability to express ISR, whereas two other mutants are SAR-deficient. The ISR- and SAR-deficient mutants were further characterized with regard to their responsiveness to SA, JA and ET.

Results

Biologically induced ISR and SAR in Arabidopsis mutants eds3-eds13

To investigate whether EDS3 to EDS13 play a role in the ISR-signalling pathway, mutants *eds3-1* to *eds13-1* were tested for their ability to express *P. fluorescens* WCS417r-mediated ISR against *Pst* DC3000. In addition, the 11 mutants were tested for their capacity to express pathogen-induced SAR against this pathogen. Mutant *npr1-1*, which is blocked in both the ISR and the SAR response, was tested as a negative control. ISR was induced by growing the plants in soil containing WCS417r bacteria for 3 weeks. SAR was induced 3 days before challenge inoculation by injecting two lower leaves with avirulent *Pst* DC3000 carrying the avirulence gene *avrRpt2* [*Pst* DC3000(*avrRpt2*); Kunkel *et al.*, 1993]. ISR- and SAR-mediated protection was quantified by assessing the disease incidence 3 days after challenge inoculation with virulent *Pst* DC3000. Except for *eds4-1*, *eds8-1*, *eds10-1* and *npr1-1*, all genotypes showed a statistically significant suppression of disease symptoms after treatment of the roots with WCS417r bacteria, indicating that the corresponding *EDS* genes do not influence WCS417r-mediated ISR (Figure 1a). The non-responsiveness to WCS417r of *eds4-1*, *eds8-1* and *eds10-1* could not be attributed to poor root colonization by the ISR-inducing bacteria, because the extent of root colonization at the end of the bioassays was always above 10^6 cfu g⁻¹ root FW in all genotypes tested (data not shown).

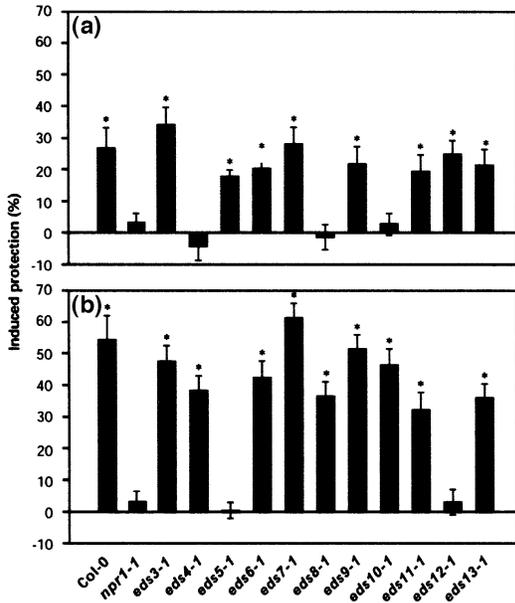


Figure 1. Levels of induced protection against *Pst* DC3000 as a result of *P. fluorescens* WCS417r-mediated ISR (a) and pathogen-induced SAR (b) in wild-type Col-0, *npr1-1* and *eds* mutants of *Arabidopsis*.

(a) Resistance was triggered by growing plants for 3 weeks in soil containing ISR-inducing *P. fluorescens* WCS417r bacteria at 5×10^7 cfu g⁻¹. Five-week-old plants were challenge-inoculated with a bacterial suspension of virulent *Pst* DC3000 at 2.5×10^7 cfu ml⁻¹. Three days after challenge inoculation, the percentage of diseased leaves was assessed and the level of induced protection calculated on the basis of the reduction in disease symptoms relative to challenged control plants.

(b) Induction of SAR was performed 3 days before challenge inoculation by pressure-infiltrating two or three lower leaves with a suspension of *Pst* DC3000(*avrRpt2*) bacteria at 10^7 cfu ml⁻¹. Challenge inoculation and disease assessment were performed as described above.

Asterisks indicate statistically significant differences compared to non-induced 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.

With regard to pathogen-induced SAR, all genotypes except *eds5-1*, *eds12-1* and *npr1-1* showed a statistically significant reduction of disease incidence in *Pst* DC3000(*avrRpt2*)-pretreated plants (Figure 1b). From these results it can be concluded that *eds4-1*, *eds8-1* and *eds10-1* are blocked in the ISR pathway, whereas *eds5-1* and *eds12-1* are blocked in the SAR pathway. For *eds5-1*, this result confirms the finding of Nawrath and Métraux (1999) that mutant *sid1-1*, which is allelic to mutant *eds5-1*, is impaired in its ability to express pathogen-induced SAR.

Chemically induced resistance in ISR- and SAR-deficient eds mutants

In *Arabidopsis*, exogenous application of either methyl jasmonate (MeJA) or 1-aminocyclopropane-1-carboxylate (ACC) has been shown to induce significant levels of protection against *Pst* DC3000 (Pieterse *et al.*, 1998; Van

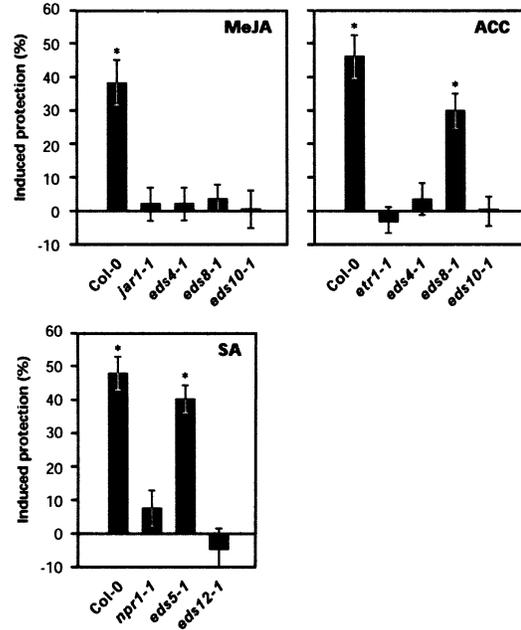


Figure 2. Levels of MeJA-, ACC- and SA-induced protection against *Pst* DC3000 in wild-type Col-0 plants, and in ISR- and SAR-impaired *eds* mutants.

Five-week-old plants were induced by dipping the leaves in a solution containing either 100 μ M MeJA, 0.5 mM ACC or 1 mM SA. For challenge inoculation and disease assessment, see caption to Figure 1.

Wees *et al.*, 1999). In the ISR-signalling pathway leading to induced defence against *Pst* DC3000, components from the JA response act upstream of the ET response, because MeJA-induced protection against *Pst* DC3000 is blocked in the ET-response mutant *etr1-1*, while in the JA-response mutant *jar1-1*, ACC-induced protection is unaffected (Pieterse *et al.*, 1998). To determine the positions of EDS4, EDS8 and EDS10 in the ISR pathway, we tested whether *eds4-1*, *eds8-1* and *eds10-1* are able to mount resistance against *Pst* DC3000 in response to MeJA and ACC. Dipping the leaves of wild-type Col-0 plants in a solution containing 100 μ M MeJA reduced the percentage of leaves with symptoms by almost 40% compared to non-treated control plants (Figure 2). The mutants *eds4-1*, *eds8-1* and *eds10-1* all resembled the JA-response mutant *jar1-1* in that they failed to develop MeJA-induced protection against *Pst* DC3000 (Figure 2). This indicates that EDS4, EDS8 and EDS10 all function downstream of JA perception in the ISR pathway.

Dipping the leaves in a solution containing 0.5 mM ACC resulted in a significant level of protection in Col-0 and *eds8-1* plants. However, *eds4-1* and *eds10-1* plants, like ET-insensitive *etr1-1* plants, failed to develop resistance after treatment with ACC (Figure 2). All genotypes converted ACC to ET with similar kinetics (data not shown), indicating that ACC uptake and ACC-converting capacity did not differ

for all genotypes tested. It can thus be concluded that, in the ISR pathway, EDS8 functions downstream of the perception of JA but upstream of ET signalling, whereas EDS4 and EDS10 both function downstream of the perception of ET.

To determine the locations of the mutations in the SAR-signalling pathway, we dipped the leaves of Col-0, *eds5-1* and *eds12-1* plants in a solution containing 1 mM SA, and quantified the level of protection against *Pst* DC3000. As a control, mutant *npr1-1*, which is blocked downstream of SA in the SAR-signalling pathway (Cao *et al.*, 1994), was also tested. Of the two SAR-impaired mutants, *eds5-1* was fully capable of expressing SAR on treatment with SA (Figure 2). This intact responsiveness of *eds5-1* to SA is consistent with its inability to synthesize this signalling molecule in response to pathogen infection (Nawrath and Métraux, 1999). In contrast, *eds12-1* plants, like *npr1-1*, failed to develop resistance in response to SA treatment (Figure 2), suggesting that *eds12-1* is affected in its sensitivity to SA.

PDF1.2 gene expression in ISR-impaired eds mutants

To further investigate the JA and ET responsiveness of the ISR-impaired mutants *eds4-1*, *eds8-1* and *eds10-1*, we examined the expression pattern of *PDF1.2* after exogenous application of MeJA or ACC. Dipping the leaves of wild-type Col-0 plants in a solution containing either 50 μ M MeJA or 0.5 mM ACC strongly activated *PDF1.2* transcription (Figure 3a). Mutant *eds4-1* showed strongly reduced levels of *PDF1.2* transcript accumulation after treatment with either MeJA or ACC (Figure 3a), consistent with its impaired expression of MeJA- and ACC-induced resistance against *Pst* DC3000 (Figure 2). In mutant *eds8-1*, the level of *PDF1.2* transcripts was also strongly reduced on treatment with MeJA or ACC. However, *eds8-1* was unaffected in ACC-induced resistance (Figure 2). As *PDF1.2* gene expression is synergistically induced by JA and ET (Penninckx *et al.*, 1998), the responsiveness of *eds8-1* to ACC with regard to the induction of resistance suggests that *eds8-1* is affected in JA signalling. Mutant *eds10-1* showed normal levels of *PDF1.2* expression after treatment with either MeJA or ACC (Figure 3a). Thus the inability of *eds10-1* to develop MeJA- and ACC-induced resistance (Figure 2) cannot be explained by impaired sensitivity to JA or ET. Therefore this mutant appears to be affected in a different trait. As neither MeJA nor ACC induced resistance in *eds10-1*, this trait is likely to act downstream of JA and ET signalling in the ISR pathway. When treated with 1 mM SA, *eds4-1*, *eds8-1* and *eds10-1* did not exhibit a reduced accumulation of *PR-1* transcripts compared to wild-type Col-0 plants (data not shown), indicating that they are not impaired in SA responsiveness.

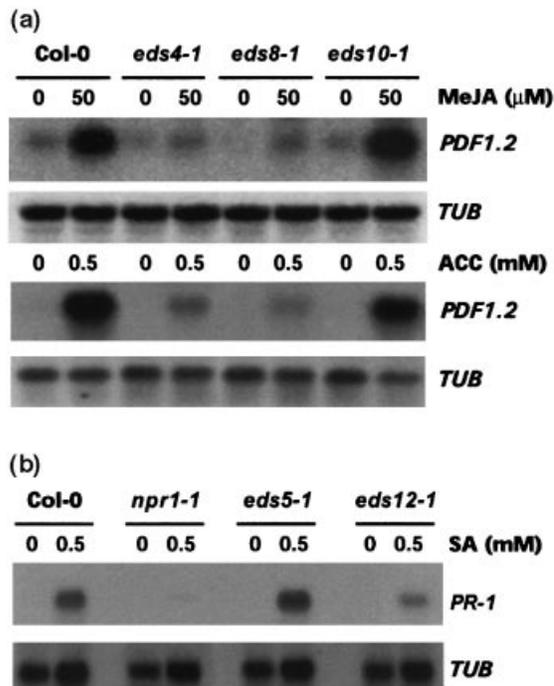


Figure 3. RNA blot analysis of the JA/ET-inducible *PDF1.2* gene in the ISR-impaired mutants *eds4-1*, *eds8-1* and *eds10-1* (a) and of the SA-inducible *PR-1* gene in the SAR-impaired mutants *npr1-1*, *eds5-1* and *eds12-1* (b).

Five-week-old plants were induced by dipping the leaves in a 0.015% (v/v) Silwet solution containing 0.5 mM SA, 0.5 mM ACC or 50 μ M MeJA 3 days before harvesting the leaves. Control-treated plants were dipped in a solution containing only 0.015% (v/v) Silwet L77. To check for equal loading, the blots were stripped and hybridized with a gene-specific probe for β -tubulin (*TUB*). Both experiments were performed twice, yielding similar results.

Pathogen-induced accumulation of SA and SA-induced PR-1 expression in SAR-impaired eds mutants

To further examine the locations of EDS5 and EDS12 in the SAR signalling pathway, we determined the levels of systemically accumulating free SA in the leaves of Col-0, *eds5-1* and *eds12-1* plants after infection of two lower leaves with *Pst* DC3000(*avrRpt2*). Three days after infection, both Col-0 and *eds12-1* plants showed statistically significant enhanced levels of free SA, indicating that *eds12-1* is not affected in SA biosynthesis (Figure 4). By contrast, mutant *eds5-1* failed to enhance SA levels on infection with *Pst* DC3000(*avrRpt2*), confirming the finding of Nawrath and Métraux (1999) that this mutant behaves as an SA-induction mutant. Furthermore, we examined *PR-1* transcript accumulation in the leaves of Col-0, *npr1-1*, *eds5-1* and *eds12-1* plants 3 days after treatment with 0.5 mM SA. In response to SA, *eds5-1* accumulated wild-type levels of *PR-1* transcripts (Figure 3b). In contrast, SA-induced *PR-1* transcription was severely reduced in *eds12-1* plants and nearly abolished in *npr1-1* plants

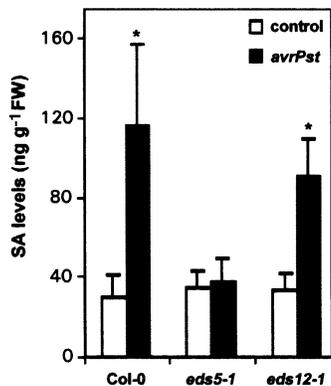


Figure 4. Endogenous levels of free SA in systemic leaf tissue of Col-0, *eds5-1* and *eds12-1* plants 3 days after pressure-infiltrating two lower leaves with water (control) or a suspension of *Pst* DC3000(*avrPst*) bacteria at 10^7 cfu ml⁻¹ (*avrPst*). Values presented are means \pm SD ($n = 5$). Asterisks indicate statistically significant differences compared to control plants (Student's *t*-test: $\alpha = 0.05$).

(Figure 3b). These results indicate that mutant *eds12-1* has reduced sensitivity to SA, suggesting that EDS12 functions downstream of SA in the SAR pathway. Treatment of the SAR-impaired mutants *eds5-1* and *eds12-1* with either 100 μ M MeJA or 0.5 mM ACC resulted in normal levels of expression of the JA- and ET-inducible, defence-related gene *PDF1.2* (data not shown), indicating that they are not affected in JA or ET signalling.

Mutant *eds4-1* is impaired in ET signalling

The 'triple response' is a reaction of etiolated seedlings to ET, and is commonly used as a reliable marker for ET sensitivity (Guzmán and Ecker, 1990). To investigate whether the ISR non-inducibility and impaired *PDF1.2* gene expression of *eds4-1* and *eds8-1* are caused by a reduced sensitivity to ET, we examined ET-induced growth inhibition of the roots and hypocotyls, both characteristics of the triple response. In a comparative analysis, etiolated seedlings of Col-0, *eds4-1*, *eds8-1* and *eds10-1*, as well as the ET response mutant *etr1-1*, were grown on MS agar plates containing different concentrations of ACC. Five days after germination the lengths of hypocotyls and roots were measured. At increasing concentrations of ACC, wild-type Col-0 and mutant *eds8-1* and *eds10-1* plants responded similarly to ACC in a dose-dependent manner, resulting in a 45–50% reduction of hypocotyl length at 5 μ M ACC (Figure 5a). In contrast, mutant *eds4-1* behaved as *etr1-1* in failing to respond to 0.5 μ M ACC and in showing significantly less responsiveness at increasing concentrations of ACC, with no more than 25% reduction of hypocotyl length at 5 μ M ACC (Figure 5a). Similarly, the inhibition of root growth at increasing concentrations of

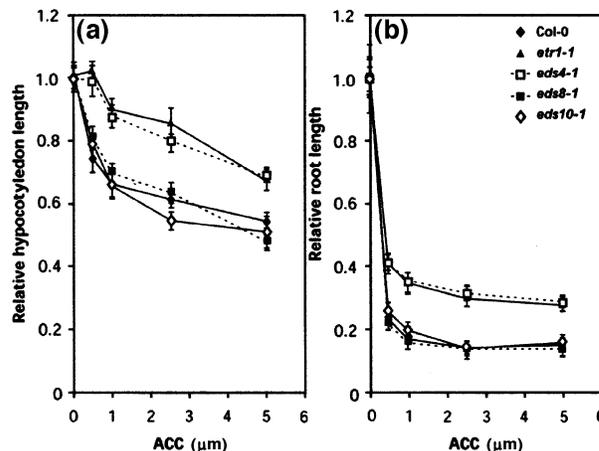


Figure 5. ACC-induced inhibition of growth of hypocotyls (a) and primary roots (b) in wild-type Col-0, *etr1-1* and the ISR-impaired *eds* mutants of *Arabidopsis*.

Seeds were pre-germinated for 2 days at 4°C in darkness on MS agar plates containing increasing concentrations of ACC. After an additional growth period of 5 days in darkness at 20°C, the lengths of etiolated seedlings were measured. Hypocotyl and root lengths were normalized relative to the control treatment (0 μ M ACC) which was set at 1. Values presented are means \pm SEM ($n = 25$). The experiment was repeated several times, yielding similar results.

ACC was significantly less extreme in *etr1-1* and *eds4-1* plants than that in Col-0, *eds8-1* and *eds10-1* (Figure 5b). These findings indicate that the recessive *eds4-1* mutant is as unresponsive to ET as the dominant *etr1-1* mutant.

Mutant *eds8-1* is impaired in JA signalling

Previously it was demonstrated that primary root growth of *Arabidopsis* seedlings is inhibited in response to treatment with MeJA (Staswick *et al.*, 1992). To investigate whether the ISR non-inducibility and impaired *PDF1.2* gene expression of *eds4-1* and *eds8-1* result from a reduced sensitivity to JA, we examined the level of MeJA-induced growth inhibition of the primary roots. Seedlings of Col-0, *eds4-1*, *eds8-1*, *eds10-1* and the JA-response mutant *jar1-1* were grown on MS agar plates containing different concentrations of MeJA. Five days after germination, Col-0, *eds4-1* and *eds10-1* plants showed significant inhibition of root growth at 0.1 μ M MeJA (Figure 6a). In contrast, mutant *jar1-1* exhibited no significant root-growth inhibition at 0.1 and 0.5 μ M MeJA, and only weak inhibition at 1.0 μ M MeJA (Figure 6a). Mutant *eds8-1* exhibited an intermediate phenotype: at the relatively low concentration of 0.1 μ M MeJA it resembled *jar1-1* in not showing a statistically significant response in root growth. However, at concentrations of 0.5 and 1.0 MeJA, *eds8-1* responded intermediately (Figure 6a), indicating that the roots of *eds8-1* are weakly insensitive to JA.

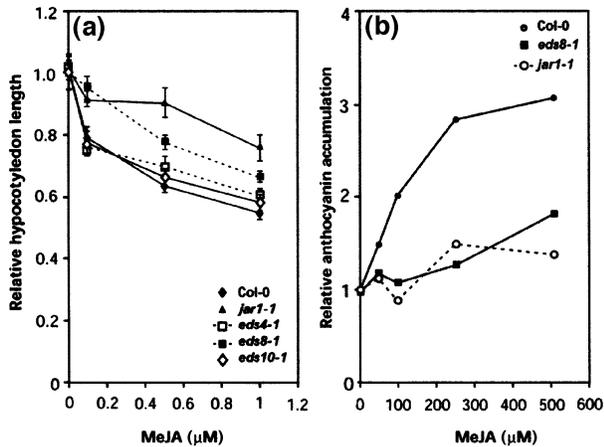


Figure 6. MeJA-dependent inhibition of primary root length (a) and accumulation of anthocyanins in leaves (b).

(a) Seeds of Col-0, *eds4-1*, *eds8-1*, *eds10-1* and *jar1-1* were pre-germinated on MS agar plates containing different concentrations of MeJA for 2 days at 4°C in the dark. After an additional growth period of 5 days at 20°C with an 8 h photoperiod, the length of the primary root was measured. Root lengths were normalized relative to the control treatment (0 μM MeJA) which was set at 1. Values presented are means ± SEM ($n = 25$).

(b) Leaves of 5-week-old Col-0, *eds8-1* and *jar1-1* were dipped in a solution of 0.01% (v/v) Silwet containing different concentrations of MeJA 3 days before harvesting leaves. Values presented are absorbencies (A_{615}) of the anthocyanin-containing extracts normalized with respect to control values.

Application of MeJA has been reported to induce accumulation of anthocyanins in the leaves (Feys *et al.*, 1994). Because this response was severely impaired in the JA-insensitive mutant *coi1-1*, we further quantified the extent of JA insensitivity in *eds8-1* by determining the level of anthocyanin accumulation in response to MeJA. Three days after treatment with increasing concentrations of MeJA, wild-type Col-0 plants had accumulated anthocyanins in a dose-dependent manner, resulting in a threefold increase at 500 μM MeJA compared to water-treated control plants (Figure 6b). In contrast, mutant *eds8-1*, similarly to *jar1-1*, failed to enhance anthocyanin accumulation at concentrations lower than 250 μM MeJA, and showed only weakly increased anthocyanin accumulation at concentrations of 250 and 500 μM MeJA (Figure 6b). These results confirm that *eds8-1* exhibits reduced sensitivity to JA.

The *ISR1* locus is not allelic with *EDS4*

The phenotype of *eds4-1* resembles the phenotype of *isr1* genotypes in its inability to express WCS417r-mediated ISR, enhanced susceptibility to *Pst* DC3000, and reduced sensitivity to ET (Ton *et al.*, 2001; Ton *et al.*, 1999). To investigate whether the *EDS4* gene is allelic with the *ISR1* locus, we performed a complementation cross between

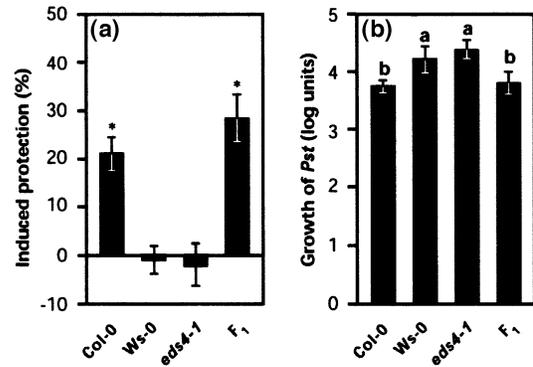


Figure 7. Levels of resistance against *Pst* DC3000 as result of *P. fluorescens* WCS417r-mediated ISR (a) and basal resistance (b) in Col-0, Ws-0, *eds4-1* and F_1 plants of a cross between Ws-0 and *eds4-1*.

(a) For induction of ISR, challenge inoculation and disease assessment, see caption to Figure 1(a).

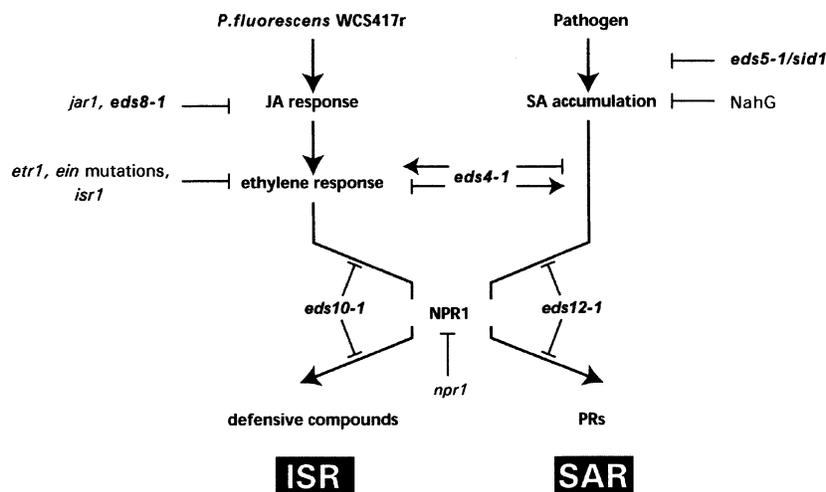
(b) Plants were infected by pressure-infiltrating a suspension of virulent *Pst* DC3000 at 5×10^5 cfu ml⁻¹ into the leaves. Immediately afterwards and 3 days later, the number of *Pst* DC3000 bacteria per g FW was determined and proliferation calculated. Values presented are means (±SD) of the log of the proliferation of *Pst* DC3000. Different letters indicate statistically significant different values between genotypes (LSD test; $\alpha = 0.05$; $n = 20-25$).

accession Ws-0 (*isr1*) and the *eds4-1* mutant. The F_1 progeny of this cross was fully capable of expressing WCS417r-mediated ISR (Figure 7a) and exhibited a similar level of basal resistance against *Pst* DC3000 as Col-0 wild-type plants, as indicated by similar symptom severity (data not shown) and similar rates of proliferation of the pathogen (Figure 7b). It can thus be concluded that the *ISR1* locus is not allelic with the *EDS4* gene.

Discussion

Previously we demonstrated that *Arabidopsis* genotypes that are unable to express *P. fluorescens* WCS417r-mediated ISR against *Pst* DC3000 show enhanced disease susceptibility towards this pathogen (Pieterse *et al.*, 1998; Ton *et al.*, 1999). Independently, two mutant screens for enhanced disease susceptibility to *P. syringae* pathogens yielded a large number of mutants with deficiencies in basal resistance against these pathogens (Glazebrook *et al.*, 1996; Volko *et al.*, 1998). To identify novel components of the ISR pathway, we made use of 11 of these *eds* mutants. Our results demonstrate that *eds4-1*, *eds8-1* and *eds10-1* are impaired in *P. fluorescens* WCS417r-mediated ISR against *Pst* DC3000 (Figure 1a), whereas *eds5-1* and *eds12-1* are affected in pathogen-induced SAR against this pathogen (Figure 1b). The ISR-impaired mutants showed normal levels of pathogen-induced SAR. Conversely, the SAR-impaired mutants showed normal levels of rhizobacteria-mediated ISR. These results corroborate our earlier demonstration that pathogen-

Figure 8. Putative positions of the ISR- and SAR-impaired *eds* mutants in the ISR- and SAR-signalling pathway (adapted from Pieterse *et al.*, 1998).



induced SAR and WCS417r-mediated ISR are regulated by distinct defence pathways that are both effective against *Pst* DC3000.

More detailed analysis of the ISR-impaired *eds* mutants revealed that they are blocked in their ability to develop induced resistance against *Pst* DC3000 in response to exogenous application of MeJA (*eds4-1*, *eds8-1* and *eds10-1*) or ACC (*eds4-1* and *eds10-1*) (Figure 2). Moreover, *eds4-1* showed reduced sensitivity to ET (Figure 3a, Figure 5), whereas mutant *eds8-1* showed reduced sensitivity to JA (Figure 3a, Figure 6). Mutant *eds10-1* showed normal sensitivity to both JA and ET (Figure 3a, Figure 5, Figure 6a). These results indicate that the corresponding gene products act in either the JA response (EDS8), the ET response (EDS4), or downstream of the JA and ET response (EDS10) in the ISR-signalling pathway. Figure 8 presents a model outlining the positions of the *eds* mutations in the ISR- and SAR-signalling pathways.

Genotypes carrying the recessive alleles of the *ISR1* locus are non-responsive to ISR treatment, show enhanced susceptibility to *Pst* DC3000 infection, and exhibit reduced sensitivity to ET (Ton *et al.*, 1999; Ton *et al.*, 2001). These characteristics resemble the phenotype of *eds4-1*. However, F_1 plants of a cross between accession Ws-0 (*isr1/isr1*) and *eds4-1* showed full complementation of ISR inducibility and basal resistance against *Pst* DC3000 (Figure 7), indicating that *ISR1* and *EDS4* are not allelic. Surprisingly, Gupta *et al.* (2000) reported that *eds4-1* failed to develop SAR upon induction treatment with avirulent *P. syringae* pv. *maculicola* carrying *avrRpt2*. Concurrently, *eds4-1* exhibited reduced *PR-1* gene expression after SA treatment, whereas it showed enhanced levels of *PDF1.2* gene expression upon treatment with MeJA. These findings clearly differ from our results. However, Gupta *et al.* (2000) also reported that, at low humidity, the SA

responsiveness in *eds4-1* was restored to wild-type levels. Therefore the behaviour of *eds4-1* plants appears to be strongly influenced by environmental conditions. It may be significant that we cultivated *eds4-1* at a 9 h light/15 h dark cycle at 65% relative humidity, whereas Gupta *et al.* (2000) used a 12/12 h light/dark cycle at 85% relative humidity. Depending on the growth conditions, *eds4-1* may act as a mutant affected in SA-dependent defences (Gupta *et al.*, 2000), or as a mutant affected in ET-dependent defences (this study). This suggests that EDS4 is involved in the control of the balance between SA-dependent and JA/ET-dependent defence pathways, as influenced by the prevailing environmental conditions (Figure 8).

Our results indicate that mutant *eds8-1* is impaired in the response to JA (Figure 2, Figure 3a, Figure 6). So far, two well characterized JA-insensitive mutants have been described in *Arabidopsis*: the jasmonic acid-response mutant *jar1-1* (Staswick *et al.*, 1992), and the coronatin-insensitive mutant *coi1-1* (Feys *et al.*, 1994). Based on the JA-insensitive phenotype of *eds8-1*, this mutant might be allelic with *jar1-1*. However, both mutants differed in other characteristics. First, *eds8-1* showed an intermediate phenotype with regard to MeJA-induced inhibition of root growth (Figure 6a). Second, *jar1-1* and *eds8-1* showed striking differences in leaf morphology. Mutant *eds8-1* developed serrated leaves with relatively short petioles (Glazebrook *et al.*, 1996; data not shown), whereas the leaf morphology of *jar1-1* resembles that of wild-type plants. Therefore it is unlikely that both mutants are allelic. Furthermore, it is highly unlikely that *eds8-1* is allelic with the JA-insensitive mutant *coi1-1*, as the latter mutant, unlike *eds8-1*, is male-sterile (Feys *et al.*, 1994). Moreover, the *coi1-1* mutant exhibited enhanced resistance, rather than enhanced susceptibility to *P. syringae* pv. *atropurpurea*, probably as a result of its insensitivity to coronatin

which functions as a virulence factor of *P. syringae* pathovars (Feys *et al.*, 1994). Hence, we postulate that *EDS8* encodes a novel component in the JA-response pathway that plays an important role in disease resistance.

The only ISR-impaired *eds* mutant that was not affected in sensitivity to either JA or ET was *eds10-1*. However, it failed to develop induced resistance on treatment with MeJA or ACC, indicating that *eds10-1*, like the defence regulatory mutant *npr1-1* (Pieterse *et al.*, 1998), is blocked downstream of the ET response in the ISR pathway (Figure 8). Nevertheless, NPR1 and EDS10 clearly differ, because mutant *eds10-1* was fully capable of expressing pathogen-induced SAR, whereas *npr1-1* is not (Cao *et al.*, 1994; Figure 1b). In this respect, it is tempting to speculate that EDS10 plays a role in the regulation of ISR-specific, NPR1-dependent defence responses.

Further characterization of the SAR-impaired mutants revealed that *eds12-1* is not affected in SA biosynthesis, but is impaired in SA-induced resistance and *PR-1* gene expression. Conversely, *eds5-1* showed full responsiveness to SA, but impaired induction of SA synthesis upon SAR induction. For *eds5-1*, this confirms previous work by Nawrath and Métraux (1999), who showed that EDS5/SID1 regulates the accumulation of SA upon pathogen infection and thus participates in the SAR signalling pathway upstream of SA (Figure 8). The reduced responsiveness of *eds12-1* to SA indicates that EDS12, like the defence regulator NPR1, functions downstream of SA in the SAR signalling pathway (Figure 8). However, it is unlikely that *EDS12* is allelic with *NPR1*, because mutants *eds12-1* and *npr1-4* showed full complementation of basal resistance in their F_1 progeny (Volko *et al.*, 1998). Moreover, mutant *npr1-1* is affected in the expression of WCS417r-mediated ISR (Pieterse *et al.*, 1998; Figure 1a), whereas mutant *eds12-1* is not (Figure 1a). Therefore EDS12 may play a role in the regulation of SAR-specific, NPR1-dependent defence responses.

Our finding that *eds5-1* and *eds12-1* are affected in the SAR response contradicts the results reported by Volko *et al.* (1998), who found that both mutants were capable of expressing SAR. One possible explanation for these differences may be that our induced resistance assays are based on the rating of disease symptoms, whereas the assays described by Volko *et al.* (1998) were based on bacterial replication. However, the bioassay system used in this study has repeatedly been demonstrated to provide similar results for growth data and symptom-based data (Pieterse *et al.*, 1996, Pieterse *et al.*, 1998; Van Wees *et al.*, 2000). Moreover, under conditions similar to those of the induced resistance assays, *eds5-1* failed to enhance SA accumulation upon SAR induction (Figure 4), and *eds12-1* exhibited reduced responsiveness to treatment with SA (Figure 3b). Both characteristics are indicative for an impaired SAR response (Ryals *et al.*, 1996; Sticher *et al.*,

1997). Furthermore, our finding that mutant *eds5-1* is affected in SAR is supported by Nawrath and Métraux (1999), who demonstrated that this mutant is affected in SAR against the oomycete *P. parasitica*. It is likely that the conflicting results are related to differences in growth conditions and SAR induction. We cultivated the plants under a 9 h light/15 h dark cycle. Under these conditions the plants had developed 10–15 leaves by 5 weeks. At this age, we performed SAR induction by infecting two lower leaves. On the other hand, Volko *et al.* (1998) cultivated plants under a 12 h light/12 h dark cycle and triggered SAR by infecting three fully expanded leaves in 4-week-old plants. These plants had fewer leaves at the time of SAR induction, and therefore had relatively more leaves undergoing necrosis on SAR induction treatment. This probably results in a stronger SAR signal, which may have masked the reduced ability of *eds5-1* and *eds12-1* to express SAR.

The *Arabidopsis* genotypes that are impaired in either ISR or SAR all exhibited enhanced susceptibility to *P. syringae* pathovars (Glazebrook *et al.*, 1996; Volko *et al.*, 1998). This association supports the earlier notion that induced resistance is an enhancement of extant basal defence mechanisms (Van Loon, 1997). However, six mutants with enhanced disease susceptibility to *P. syringae* were unaffected in their expression of SAR and ISR against *Pst* DC3000 (Figure 1), indicating that they are affected in basal defence components that do not contribute to induced resistance. In view of the low frequency of allelic pairs in the *eds* mutant screens (Glazebrook *et al.*, 1996; Volko *et al.*, 1998), basal resistance must be controlled by a large number of genes regulating multiple defence mechanisms. Apparently only subsets of these mechanisms are enhanced in plants expressing induced resistance, depending on the type of induced resistance that is activated.

Experimental procedures

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 agar plates (King *et al.*, 1954) for 24 h at 28°C. Bacterial cells were collected by centrifugation and resuspended in 10 mM MgSO₄ to a final density of 10⁹ colony-forming units (cfu) ml⁻¹. An avirulent strain of *Pseudomonas syringae* pv. *tomato* DC3000 carrying the avirulence gene *avrRpt2* (*Pst* DC3000 (*avrRpt2*); Kunkel *et al.*, 1993) was used for SAR induction. *Pst* DC3000(*avrRpt2*) bacteria were grown overnight at 28°C in liquid KB supplemented with 25 mg ml⁻¹ kanamycin to select for the plasmid. The virulent pathogen *Pst* DC3000 (Whalen *et al.*, 1991) used for challenge inoculations was cultivated in a similar manner in liquid KB without kanamycin. After centrifugation, the bacterial cells were resuspended in 10 mM MgSO₄ with 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals, Weesp, the Netherlands) to a final density of 2.5 × 10⁷ cfu ml⁻¹.

Seedlings of wild-type *Arabidopsis thaliana* accession Columbia (Col-0), the Col-0 mutants *etr1-1* (Bleecker *et al.*, 1988), *npr1-1* (Cao *et al.*, 1994), *jar1-1* (Staswick *et al.*, 1992), *eds3-1*, *eds4-1*, *eds5-1*, *eds6-1*, *eds5-1*, *eds6-1*, *eds7-1*, *eds8-1* (Glazebrook *et al.*, 1996), *eds9-1* (Rogers *et al.*, 1997), *eds10-1*, *eds11-1*, *eds12-1* and *eds13-1* (Volko *et al.*, 1998) were grown in quartz sand for 2 weeks, and transferred to a sand/potting soil mixture as described previously (Pieterse *et al.*, 1996). Plants were cultivated in a growth chamber with a 9 h day (200 $\mu\text{E m}^{-2} \text{sec}^{-1}$ at 24°C) and 15 h night (20°C) cycle at 65% relative humidity. Plants were watered on alternate days, and once a week received modified half-strength Hoagland's solution (Hoagland and Arnon, 1938; Pieterse *et al.*, 1996).

Induction treatments

Induced systemic resistance was achieved by transplanting 2-week-old *Arabidopsis* seedlings into the sand/potting soil mixture containing ISR-inducing WCS417r bacteria. Prior to transfer of the *Arabidopsis* seedlings to pots, a suspension of ISR-inducing WCS417r bacteria (10^9 cfu ml^{-1}) was mixed thoroughly through the soil to a final density of 5×10^7 cfu g^{-1} . Control soil was supplemented with an equal volume of 10 mM MgSO_4 . Induction of SAR was performed 3 days before challenge inoculation by pressure-infiltrating two lower leaves with a suspension of *Pst* DC3000(*avrRpt2*) bacteria at 10^7 cfu ml^{-1} . Induction treatments with salicylic acid (SA), methyl jasmonate (MeJA) and 1-aminocyclopropane-1-carboxylate (ACC) were performed 3 days before challenge inoculation by dipping the leaves in a solution containing either SA, MeJA or ACC in 0.015% (v/v) Silwet L77. Control-treated plants were dipped in a solution containing only 0.015% (v/v) Silwet L77.

Challenge inoculations and disease assessment

For assaying induced resistance, plants were challenged when 5 weeks old by dipping the leaves in a suspension of virulent *Pst* DC300 bacteria at 2.5×10^7 cfu ml^{-1} in 10 mM MgSO_4 , 0.015% (v/v) Silwet L-77. One day before challenge inoculation the plants were placed at 100% RH. Three or 4 days 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* DC3000, leaves of 5-week-old, control-treated plants were inoculated by pressure infiltration with a suspension of virulent *Pst* DC3000 at 5×10^5 cfu ml^{-1} in 10 mM MgSO_4 . Immediately, and again 3 days later, replicate leaf samples from five plants per genotype were collected, weighed and homogenized in 10 mM MgSO_4 . Serial dilutions were plated on selective KB agar plates supplemented with 100 mg l^{-1} cycloheximide and 50 mg l^{-1} rifampicin. After incubation at 28°C for 2 days, the number of rifampicin-resistant cfu per g infected leaf tissue was determined, and bacterial proliferation over the 3-day time interval was calculated.

Analysis of MeJA and ACC sensitivity

Seeds of *Arabidopsis* were surface-sterilized for 5 min in 5% sodium hypochlorite, washed in 70% ethanol, and air-dried. Subsequently, seeds were distributed evenly on 1.0% (w/v) agar medium containing 0.5% (w/v) Murashige and Skoog salts (Duchefa BV, Haarlem, the Netherlands), 0.5% (w/v) sucrose,

and different concentrations of either MeJA or ACC (pH 5.7). MeJA (Serva, Brunschwig Chemie BV, Amsterdam, the Netherlands) was added to the autoclaved medium from a filter-sterilized 1 mM stock solution (containing 0.96% ethanol). ACC (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) was added from a 10 mM stock solution in a similar manner.

Seeds were pre-germinated in the dark for 2 days 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 $\mu\text{E m}^{-2} \text{sec}^{-1}$) and a 16 h night cycle. After 5 days the primary root length was measured under a dissection microscope. In each case, 15–25 randomly selected seedlings were measured. The effect of ET on hypocotyl and primary root length in etiolated seedlings was determined essentially according to Guzmán and Ecker (1990). After pre-germination in the dark for 2 days at 4°C, seedlings were grown for an additional 3–7 days at 20°C in darkness and the hypocotyl and primary root length were measured as described above.

ET measurements

Leaves of plants pretreated with 1 mM ACC were detached, weighed, placed in 25 ml gas-tight serum flasks, and incubated under climate chamber conditions. At different intervals over a 28 h period, cumulative ET production was measured by gas chromatography as described by De Laat and Van Loon (1982).

Extraction and quantification of anthocyanins

Three days after treatment with MeJA, leaf material was collected and homogenized in extraction buffer (0.35 M glycine, 48 mM NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% (w/v) SDS; 1 ml g^{-1} leaf tissue). After centrifugation for 2 min at 16 000 g to pellet non-soluble cell fragments, the supernatant was extracted twice with an equal volume of phenol/chloroform/isoamylalcohol (25 : 24 : 1, v/v), and once with chloroform/isoamylalcohol (24 : 1, v/v). Subsequently the relative amount of anthocyanin in the water phase (visible as a purple colour) was assayed spectrophotometrically by determining the absorption spectrum between A_{400} and A_{800} . All samples containing anthocyanins showed an absorption maximum at A_{615} .

Extraction and quantification of SA

Selected leaves were weighed, frozen in liquid nitrogen and pulverized. For analysis of free SA, 200 ng of the internal standard ortho-anisic acid was added per g FW. Subsequently, extraction and quantification of the free SA was carried out as described by Meuwly and Métraux (1993).

RNA gel-blot analysis

Three days after induction treatments, total RNA was extracted as described previously (Ton *et al.*, 2001). For RNA gel-blot analysis, 15 μg RNA was denatured using glyoxal and DMSO (Sambrook *et al.*, 1989), electrophoretically separated on 1.5% agarose gels, and blotted onto Hybond- N^+ membranes (Amersham, 's-Hertogenbosch, the Netherlands) by capillary transfer. The electrophoresis and blotting buffer consisted of 10 and 25 mM sodium phosphate (pH 7.0), respectively. RNA gel blots were hybridized with specific probes and washed, as described previously (Ton *et al.*, 2001). To check for equal loading, the

blots were stripped and hybridized with a probe for constitutively expressed β -tubulin (*TUB*) gene. DNA probes were labelled with α -³²P-dCTP by random primer labelling (Feinberg and Vogelstein, 1983). The probes to detect *PDF1.2* and *PR-1* transcripts were derived from an *Arabidopsis PDF1.2* and a *PR-1* cDNA clone, respectively (Penninckx *et al.*, 1996; Uknes *et al.*, 1992). The probe for detection of *TUB* transcripts was prepared by PCR with primers based on the sequence obtained from GenBank accession number M21415.

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