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Constitutive disease resistance requires *EDS1* in the *Arabidopsis* mutants *cpr1* and *cpr6* and is partially *EDS1*-dependent in *cpr5*

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Summary

The systemic acquired resistance (SAR) response in *Arabidopsis* is characterized by the accumulation of salicylic acid (SA), expression of the pathogenesis-related (*PR*) genes, and enhanced resistance to virulent bacterial and oomycete pathogens. The *cpr* (constitutive expressor of *PR* genes) mutants express all three SAR phenotypes. In addition, *cpr5* and *cpr6* induce expression of *PDF1.2*, a defense-related gene associated with activation of the jasmonate/ethylene-mediated resistance pathways. *cpr5* also forms spontaneous lesions. In contrast, the *eds1* (enhanced disease susceptibility) mutation abolishes race-specific resistance conferred by a major subclass of resistance (*R*) gene products in response to avirulent pathogens. *eds1* plants also exhibit increased susceptibility to virulent pathogens. Epistasis experiments were designed to explore the relationship between the *cpr- and EDS1*-mediated resistance pathways. We found that a null *eds1* mutation suppresses the disease resistance phenotypes of both *cpr1* and *cpr6*. In contrast, *eds1* only partially suppresses resistance in *cpr5*, leading us to conclude that *cpr5* expresses both *EDS1*-dependent and *EDS1*-independent components of plant disease resistance. Although *eds1* does not prevent lesion formation on *cpr5* leaves, it alters their appearance and reduces their spread. This phenotypic difference is associated with increased pathogen colonization of *cpr5 eds1* plants compared to *cpr5*. The data allow us to place *EDS1* as a necessary downstream component of *cpr1*- and *cpr6*-mediated responses, but suggest a more complex relationship between *EDS1* and *cpr5* in plant defense.

Keywords: *cpr*, *eds1*, SAR, disease resistance, signaling, epistasis.

Introduction

In plants, effective defense against disease often relies on the ability of the plant to recognize an invading pathogen. Specific recognition between a plant resistance (*R*) gene product and a pathogen avirulence (*avr*) gene product elicits a rapid response at the site of pathogen invasion (Martin, 1999; Staskawicz *et al.*, 1995). Interaction between these proteins is commonly, although not exclusively, associated with localized plant-cell necrosis, a form of programmed cell death known as the hypersensitive response (HR) (McDowell and Dangl, 2000; Parker, 2000). The HR is accompanied by a battery of defense-related processes, including rapid ion-flux changes, an oxidative burst giving rise to reactive oxygen intermediates, the generation of potent signaling molecules such as nitric oxide and salicylic acid (SA), and the local and systemic activation of defense-related genes (Grant and Mansfield, 1999; McDowell and Dangl, 2000).

Although the precise nature of events leading to gene-for-gene resistance are not understood, studies suggest that the co-operation and balance of reactive oxygen intermediates, nitric oxide and SA molecules generated early in the plant defense response are crucial for the timely elaboration of an HR (Delledonne *et al.*, 1998; Durner *et al.*, 1998; Shirasu *et al.*, 1997). Depletion of SA

in transgenic plants expressing the SA-degrading enzyme salicylate hydroxylase (NahG) compromises certain *R* gene-mediated resistances (Delaney *et al.*, 1994). Salicylic acid is also an important component of systemic plant resistance (systemic acquired resistance; SAR), an immune response that is induced in uninfected parts of the plant after local pathogen attack (Gaffney *et al.*, 1993). SAR is characterized by an increase in endogenous SA levels and expression of a subset of pathogenesis-related (*PR*) genes, as well as enhanced resistance to a broad spectrum of virulent pathogens (Dempsey *et al.*, 1999). Furthermore, exogenous application of SA or its chemical analogues INA (2,6-dichloroisonicotinic acid; Métraux *et al.*, 1991) or BTH (benzothiodiazole; Görlach *et al.*, 1996) activates *PR* gene expression and induces SAR. While induced *PR* gene expression correlates with increased pathogen resistance and SAR, the molecular determinants of plant immunity are not known.

More recently, the plant growth regulators jasmonic acid (JA) and ethylene (ET) have been implicated as important modulators of local and systemic disease-resistance pathways that operate independently of SA (Pieterse and van Loon, 1999). For example, induced systemic resistance is activated by non-pathogenic, root-colonizing bacteria and requires JA and ET signaling (Pieterse *et al.*, 1996, Pieterse *et al.*, 1998). Systemic resistance responses to several necrotrophic pathogens also require JA and ET, and are associated with induced expression of the plant defensin *PDF1.2* (Penninckx *et al.*, 1996, Penninckx *et al.*, 1998) and thionin, *Thi2.1* (Epple *et al.*, 1995) genes. In addition, these genes are responsive to exogenous applications of JA but not SA. Thus the plant possesses discriminatory mechanisms that efficiently channel response pathways according to a particular pathogen stimulus (McDowell and Dangl, 2000; Parker, 2000).

The identification and characterization of mutants that alter both local and systemic resistance pathways has dramatically increased our understanding of signaling in plant disease resistance (Feys and Parker, 2000; Glazebrook, 1999). Mutant screens designed to isolate genes required for *R* gene-mediated resistance have revealed both *R* gene-specific and more generally recruited components. Results show that *R* genes engage partially overlapping sets of genes to elicit resistance, leading ultimately to the HR and SAR (Feys and Parker, 2000; Glazebrook, 1999). A similar trend is observed in systemic plant defenses, and the analysis of mutations affecting SA, JA and ET accumulation or perception suggests a complex interplay of signaling molecules determining the plant response (Reymond and Farmer, 1998).

The *Arabidopsis* mutation *eds1* (enhanced disease susceptibility) suppresses resistance conditioned by a subset of *R* genes that share a common structural motif (Aarts *et al.*, 1998; Parker *et al.*, 1996). These encode nucleotide-binding site (NB), leucine-rich repeat (LRR) proteins that have sequence similarities in their amino termini to the cytoplasmic effector domains of the *Drosophila* and mammalian Toll receptor family which is involved in innate immune responses (Medzhitov and Janeway, 1998; Whitham *et al.*, 1994). *EDS1* encodes a lipase-like protein that functions upstream of SA-dependent *PR1* gene expression in response to pathogen infection (Falk *et al.*, 1999). Applications of SA also amplify *EDS1* mRNA accumulation, pointing to the existence of an SA-generated positive feedback loop in the defense circuit (Falk *et al.*, 1999). Other *Arabidopsis* mutations affecting defense signaling upstream of SA accumulation include *pad4* (Glazebrook *et al.*, 1997; Zhou *et al.*, 1998), *eds5/sid1* and *sid2* (Nawrath and Métraux, 1999; Rogers and Ausubel, 1997). Like *EDS1*, the wild-type *PAD4* gene encodes a lipase-like protein whose expression is amplified by applications of SA (Jirage *et al.*, 1999). Both *eds1* (Aarts *et al.*, 1998; Parker *et al.*, 1996) and *pad4* (Glazebrook *et al.*, 1997) plants, as well as a number of other plant defense mutants (Glazebrook *et al.*, 1996; Reuber *et al.*, 1998), exhibit enhanced susceptibility to virulent pathogens, suggesting that *R* gene-mediated resistance to avirulent pathogens and general restriction of growth of virulent pathogens may have features in common.

Mutational screens in *Arabidopsis* have also targeted components of SAR. One mutation, *npr1* (non-expressor of *PR* genes), has been identified that compromises the plant response downstream of SA accumulation, severely impairing induction of SAR and associated expression of SA-responsive *PR* genes (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997). In contrast to *npr1*, the recessive *cpr1* and *cpr5* (constitutive expressor of *PR* genes) and dominant *cpr6* mutants activate SAR responses, enhancing resistance to virulent isolates of the bacterial pathogen *Pseudomonas syringae* ES4326 and the oomycete pathogen *Peronospora parasitica* Noco2 (Bowling *et al.*, 1994, Bowling *et al.*, 1997; Clarke *et al.*, 1998). *cpr5* also forms spontaneous lesions in the absence of pathogens, and has abnormal trichomes (Boch *et al.*, 1998; Bowling *et al.*, 1997). The *cpr* mutants interfere with plant defense signaling at different points along the SAR cascade. Thus *cpr5* and *cpr6*, but not *cpr1*, also constitutively activate the JA/ET-mediated pathways represented by enhanced expression of *PDF1.2* (Boch *et al.*, 1998; Bowling *et al.*, 1997; Clarke *et al.*, 1998), suggesting points of connection between these distinct signaling processes. In *cpr5*, mutations in *NPR1* were found to suppress *PR1* gene expression without diminishing *PDF1.2* mRNA levels or heightened pathogen resistance (Bowling *et al.*, 1997). In contrast, *cpr6* uncouples *PR* gene expression from the *npr1* mutation while maintaining *NPR1*-independent pathogen resistance (Clarke *et al.*, 1998). These studies

reveal a divergence between *NPR1*-dependent and *NPR1*-independent processes that may resemble systemic and local resistances, respectively. Analysis of *NPR1*-independent resistance demonstrates that it requires components from both the SA and JA/ET-mediated signaling pathways (Clarke *et al.*, 2000).

In order to explore the relationship between components of *R* gene-mediated resistance and SAR, we designed an epistasis experiment between *eds1* and the *cpr* mutants *cpr1*, *cpr5* and *cpr6*. Our aim was to place *EDS1* on the SA-dependent signal cascade defined by these *cpr* mutants. We also wished to examine the effect of *eds1* on expression of the JA/ET-dependent pathways in *cpr5* and *cpr6*. We present results showing that a null *eds1* mutation suppresses the disease resistance phenotypes of both *cpr1* and *cpr6*. In contrast, *eds1* only partially suppresses resistance in *cpr5*, leading us to conclude that *cpr5* expresses both *EDS1*-dependent and *EDS1*-independent components of plant resistance. Jirage *et al.* (2001) show in an accompanying paper that the *cpr1*-, *cpr5*- and *cpr6*-conditioned phenotypes have similar requirements for the SA regulatory gene *PAD4* as for *EDS1*. We conclude that EDS1 and PAD4 have similar positions in the *cpr*-induced signaling cascades.

Results

Characterization of the cpr eds1 *double mutants*

In this analysis we used the Landsberg *erecta* (L*er*) null mutation *eds1-2* (Falk *et al.*, 1999) and the Col-0 mutant lines *cpr1-1* (Bowling *et al.*, 1994), *cpr5-1* (Bowling *et al.*, 1997) and *cpr6-1* (Clarke *et al.*, 1998) for epistasis studies. *cpr1 eds1*, *cpr5 eds1* and *cpr6 eds1* double mutant lines were generated as described in Experimental procedures. Due to the combination of L*er* (*eds1*) and Col-0 (*cpr1*, *cpr5* and *cpr6*) accessions, multiple F_3 *cpr eds1* double mutant families were selected alongside corresponding single mutant control families (*cpr1 EDS1*, *CPR1 eds1*, *cpr5 EDS1*, *CPR5 eds1*, *cpr6 EDS1 and CPR6 eds1*). This allowed us to assess the effects of the mixed genetic background on penetrance of the *cpr* mutant phenotypes. Previous L*er*–*eds1-2* × Col-0 segregation data had established that penetrance of the *eds1-2* disease-susceptibility phenotype is not significantly altered in Col-0 (Aarts *et al.*, 1998; Falk *et al.*, 1999). It was also known from previous studies that the *cpr5* and *cpr6* morphological and SAR-related phenotypes penetrate well into the L*er* accession (Bowling *et al.*, 1997; Clarke *et al.*, 1998). Multiple *cpr5 eds1* and *cpr6 eds1* F_3 families and respective *cpr5 EDS1*, *cpr6 EDS1* or *CPR5 eds1* and *CPR6 eds1* control families were therefore used only during initial double mutant characterizations. These experiments confirmed that the presence of a mixed Col-0 and L*er* background did not significantly alter the mutant phenotypes (data not shown). We chose two F_3 double mutant families for more extensive analysis, of which one representative line per genotype is presented in the figures.

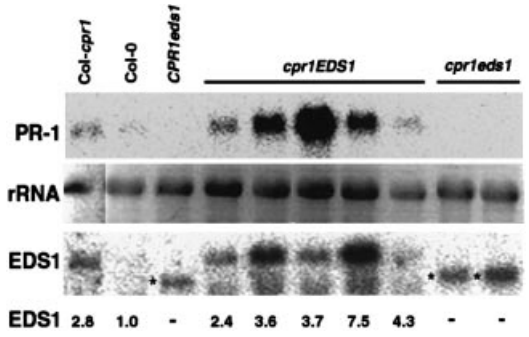

Figure 1. Expression of *PR1* and *EDS1* mRNAs in different *cpr1 EDS1* families.
Total RNA was extracted from 2-week-old soil-grown plants of various genotypes, as indicated. *PR1 and EDS1* expression levels were analyzed by hybridizing an RNA gel blot with respective gene-specific probes (PR-1 and EDS1). Equal loading was established using an 18S ribosomal RNA (rRNA) probe after stripping the blot. The wild-type *EDS1* transcript is 1.4 kb. A shorter (500 bp) transcript expressed in *eds1-2* mutant plants is indicated by an asterisk. *EDS1* mRNA levels (lower panel) were also measured by real-time quantitative PCR on a TaqMan machine. The numbers shown represent the -fold induction of *EDS1* expression over wild type (Col-0) after normalization in all samples for expression of the *ACT2* gene (see Experimental procedures). Each TaqMan reaction was done in triplicate.

In contrast, penetrance of the Col-*cpr1* SAR phenotype was suspected to be weaker in L*er* (L. Anderson and X. Dong, unpublished results). A preliminary analysis was therefore conducted on five independent *cpr1 EDS1* families to establish expression levels of the SAR-related marker gene *PR1*. In young (2-week-old) plants, we observed differences in the extent of constitutive *PR1* expression in these lines (Figure 1). However, all lines exhibited elevated *PR1* mRNA, to levels similar to or higher than those found in Col-*cpr1*. Older (4-week-old) plants of the *cpr1 EDS1* families and Col-*cpr1* had overall higher *PR1* expression levels than younger plants, and the differences among plant lines were less extreme (data not shown). As shown in Figure 1, the *eds1* mutation abolished *PR1* expression in *cpr1*. Due to the genetic variation, experiments were conducted on three or more *cpr1 eds1* F_3 families and control *cpr1 EDS1* families. Despite variable penetrance of *cpr1* in Col-0 × L*er* mixed populations, the effect of the *eds1* mutation on *cpr1*-mediated SAR phenotypes was consistent in different experiments, and a representative data set is shown.

The *cpr1*, *cpr5* and *cpr6* mutations cause plant stunting (Boch *et al.*, 1998; Bowling *et al.*, 1994, Bowling *et al.*, 1997; Clarke *et al.*, 1998). We were unable to conclude whether *eds1* has a significant influence on *cpr1*, *cpr5* or *cpr6* plant stature, due to the considerable variation in leaf size

between different mutant and wild-type families. However, we observed that *cpr5*-induced lesion formation was delayed by 3–4 days, and was qualitatively different in *cpr5 eds1* families. As shown in Figure 2(a), lesions in *cpr5 eds1* are less diffuse than in *cpr5*. Furthermore, the *cpr5* lesions appear whiter and more necrotic in *cpr5 eds1* plants. To demonstrate that the difference in lesion formation between *cpr5* and *cpr5 eds1* is not caused by the L*er* ecotype, a *cpr5 EDS1* control leaf is also shown. A more detailed microscopic analysis was conducted in both non-lesioning and lesioning *cpr5 EDS1* and *cpr5 eds1* plants after staining for plant cell death with lactophenol trypan blue (LPTB). These studies revealed that areas of necrotic plant cells were more discrete in *cpr5 eds1* compared with *cpr5* (Figure 2B) or *cpr5 EDS1* plants. They also showed that in both *cpr5 eds1* and *cpr5 EDS1* leaves, formation of micro-lesions that were not visible to the naked eye preceded macro-lesion development (Figure 2b). Thus, while *EDS1* is not required for lesion initiation in *cpr5* plants, it appears to contribute to lesion propagation.

Effect of eds1 *on defense-related gene expression in the* cpr *mutants*

cpr1, *cpr5* and *cpr6* plants constitutively express the *PR* genes *PR1*, *PR2* (*BGL-2*) and *PR5*, which are markers for SAR (Boch *et al.*, 1998; Bowling *et al.*, 1994, Bowling *et al.*, 1997; Clarke *et al.*, 1998). Also, *cpr5 and cpr6* induce expression of *PDF1.2* mRNA, a marker of JA/ET-dependent signaling. In contrast, *eds1* suppresses the strong induction of *PR1* gene expression that follows infection with the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 or avirulent *P.s. tomato* DC3000 expressing *avrRPS4* (Falk *et al.*, 1999). We therefore examined the effect of *eds1* on the expression of these defense-related genes in the *cpr* mutant backgrounds.

The results of RNA gel-blot analyzes using *PR1* and *PDF1.2* probes are shown in Figure 3(a). Elevated *PR-1* gene expression observed in *cpr1 EDS1* (see also Figure 1) or *cpr6 EDS1* plants was strongly suppressed in the *cpr1 eds1* and *cpr6 eds1* lines. *PR1* mRNA was undetectable in *cpr1 eds1* plants, but was detected at a low level in *cpr6 eds1* families. These were consistent phenotypes in different experiments using 2-week-old and 4-week-old plants. Analysis of *PR2* (*BGL2*) and *PR5* gene expression in these lines showed similar trends to *PR1* in *cpr1 eds1* plants, but their expression was not affected in *cpr6 eds1* (data not shown). Several *cpr1* or *CPR1* plant genotypes were selected for the presence of the *BGL2-Glucuronidase* (*BGL2::GUS*) reporter gene that had been used in the initial screen for *cpr*-type mutants (Bowling *et al.*, 1994). In this analysis, although penetrance of the *cpr1* phenotype was weaker in two *cpr1 EDS1* families examined than in Col-*cpr1*, expression of *BLG2::GUS* was strongly suppressed in the presence of the *eds1* mutation (Figure 3b).

In contrast to the *PR1* gene expression profiles of *cpr1 eds1* and *cpr6 eds1* plants, *eds1* altered *PR1* mRNA levels in *cpr5* in a lesion-dependent manner (Figure 3a). A detailed analysis of *cpr5 EDS1* and *cpr5 eds1* plants at different developmental stages showed that in young (2-week-old) plants with no macroscopic lesions (Figure 2b), *eds1* caused a partial downregulation of *PR1* mRNA levels. In older (4–5-week-old) plants exhibiting lesions, *eds1* had little or no effect on *cpr5*-induced *PR1* gene expression. Applications of SA restored *PR-1* gene expression in all the *cpr eds1* double mutants to levels attained in similarly treated wild-type plants, thus fully rescuing the *eds1* defect in *PR1* expression, as shown for *cpr6 eds1* plants in Figure 3(c).

The *cpr1* mutant did not cause elevated expression of *PDF1.2* mRNA, in contrast to *cpr5* and *cpr6* plants which showed constitutive upregulation of *PDF1.2* (Figure 3a), as observed previously (Bowling *et al.*, 1997; Clarke *et al.*, 1998; Penninckx *et al.*, 1996). The combination of *eds1* and *cpr1* also did not result in detectable increases in *PDF1.2* expression (Figure 3a). In contrast, *cpr6 eds1* lines exhibited enhanced *PDF1.2* expression to levels significantly higher than those of *cpr6 EDS1* lines or *cpr6* (Figure 3a). The increase in *PDF1.2* expression in *cpr6 eds1* is reminiscent of observations with other mutants disrupting SA-mediated responses that become sensitized for activation of the JA/ET pathway (Clarke *et al.*, 1998; Clarke *et al.*, 2000; Gupta *et al.*, 2000). We examined whether hyperinduction of JA/ET signaling in *cpr6 eds1* plants was a direct consequence of depletion of SA by the *eds1* mutation. We found that *eds1* caused a similar upward shift in *PDF1.2* mRNA expression in *cpr6 eds1* compared to *cpr6 EDS1* plants, irrespective of whether they were untreated or treated with SA (Figure 3c). However, absolute *PDF1.2* expression levels were lower in both *cpr6 eds1* and *cpr6 EDS1* after SA application (Figure 3c). This result suggests that *eds1* interferes with *cpr6* signaling at another level than by simply removing SA.

Analysis of *cpr5 eds1* plants showed that *eds1* affected *cpr5*-induced *PDF1.2* expression in a way that is different from the *cpr1*- or *cpr6*-conditioned responses. In young (non-lesioning) plants, *PDF1.2* expression was variable in *cpr5*, *cpr5 EDS1* or *cpr5 eds1* genotypes. Older (lesioning) *cpr5* or *cpr5 EDS1* plants expressed *PDF1.2* mRNA constitutively and there was no detectable effect of *eds1*, mirroring the expression patterns of *PR1* in these plants (Figure 3a).

Expression of EDS1 *mRNA in the* cpr *mutants*

Previously, expression of the *EDS1* gene itself was shown to be upregulated in response to pathogen infection or

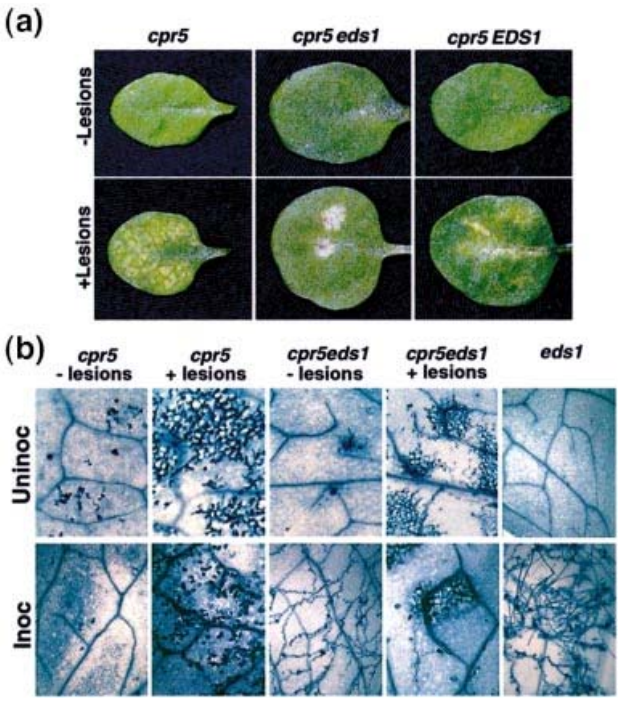

Figure 2. Lesion morphology and *P. parasitica* development in *cpr5* and *cpr5 eds1* plants.
(a) The *eds1* mutation alters the appearance of *cpr5*-induced macroscopic lesions. Leaves shown are a representative sample from a population of approximately ten 4-week-old plants per genotype.
(b) Leaves of different *cpr5* plant lines without macroscopic lesions (– lesions) or with extensive lesions (+ lesions), that were either unchallenged (Uninoc) or had been inoculated 7 days previously with *P. parasitica* isolate Noco2 (Inoc), were stained with lactophenol trypan blue and visualized under a light microscope. In all *cpr5* genotypes, micro-lesions precede the formation of macroscopic lesions. However, patches of dead plant cells are more discrete in *cpr5 eds1* plants than in *cpr5*. Presence of the *eds1* mutation permits some pathogen colonization of *cpr5* leaves that is most extensive in leaves without visible necrosis. Pictures are representative from three replicate samples.

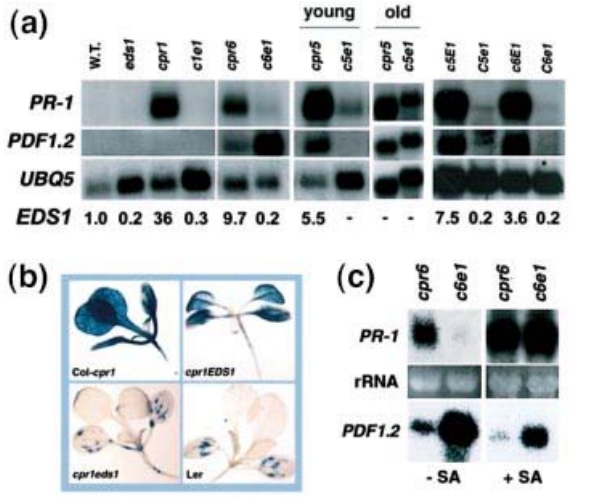

Figure 3. Effect of *eds1* on defense-related gene expression in the *cpr* mutants.
(a) *PR-1* and *PDF1.2* gene-specific probes were used for RNA gel-blot analysis of different plant genotypes, as indicated. The *UBQ5* transcript was used as a loading standard. Total RNA was extracted from 3.5-week-old soil-grown plants, except the *cpr5* samples which contain RNA from 2-week-old (young) and 4-week-old (old) plants. WT is a *BGL2-GUS* transgenic Col-0 line. Other plant genotypes are L*er-eds1-2* (*eds1*); Col-*cpr1* (*cpr1*); *cpr1 eds1* (*c1e1*); Col-*cpr6* (*cpr6*); *cpr6 eds1* (*c6e1*); *Col-cpr5* (*cpr5*); *cpr5 eds1* (*c5e1*); *cpr5 EDS1* (*c5E1*); *CPR5 eds1* (*C5e1*); *cpr6 EDS1* (*c6E1*); *CPR6 eds1* (*C6e1*). The -fold induction of *EDS1* transcript levels shown in the lower panel (*EDS1*) was measured as described for Figure 1.
(b) Expression of BGL2::GUS (β-glucuronidase under the control of the *β1,3-glucanase2* gene promoter; Bowling *et al.*, 1994) was examined in different *cpr1* mutant lines. Constitutive expression of *BGL2-GUS* was observed in *Col-cpr1* and in two independent *cpr1 EDS1* families that were segregating for the transgene reporter. The presence of *eds1* in the *cpr1* mutant background suppresses *BGL2-GUS* expression to levels observed in wild-type L*er* plants expressing the same *BGL2-GUS* transgene.
(c) *PR1* and *PDF1.2* expression was examined in *cpr6* and *cpr6 eds1* (*c6e1*) soil-grown plants that were untreated (–SA) or 20 h after spraying with 0.5 mM SA in water (+SA). Application of SA fully rescues *PR1* gene expression in *cpr6 eds1* to levels observed in SA-treated wild-type plants (not shown). The *eds1* mutation enhances *PDF1.2* expression in a *cpr6* mutant background to a similar degree in untreated and SA-treated plants. Equal loading was established by ethidium bromide staining of ribosomal RNA (rRNA). Similar results were obtained in an independent experiment.

applications of SA (Falk *et al.*, 1999). We therefore examined whether any of the *cpr* mutations altered expression of the wild-type *EDS1* gene in the Col-*cpr* mutant lines and corresponding *cpr1 EDS1*, *cpr5 EDS1* and *cpr6 EDS1* families. Quantification of *EDS1* mRNA levels was performed using real-time quantitative RT–PCR, as overall *EDS1* transcript levels are low and difficult to quantify accurately on RNA gel blots (Figure 1). This analysis revealed that *EDS1* mRNA expression was enhanced in all the *cpr* mutants (Figures 1 and 3a). However, there was no strict correlation between the extent of *EDS1* expression and the level of *PR1* expression in the *cpr* mutant lines. In combinations of *cpr* mutations with the *eds1-2* null mutant allele, *eds1* mRNA was detectable at low levels as a shorter transcript (Figures 1 and 3a), consistent with earlier observations that a 500 bp internal deletion in *eds1-2* either compromises transcription or destabilizes the mRNA (Falk *et al.*, 1999).

Analysis of pathogen development in the cpr eds1 *mutants*

We determined the effect of *eds1* on the enhanced resistance phenotypes of *cpr1*, *cpr5* and *cpr6* plants. Growth of the bacterial pathogen *P.s. maculicola* ES4326 was examined in *cpr eds1* double and *cpr EDS1* single-mutant families or wild-type controls. As shown in Figure 4(a), growth of *P.s. maculicola* ES4326 was reduced in *cpr1*, *cpr5* and *cpr6* plants compared to wild-type Col-0 or L*er*. In contrast, *cpr1 eds1* and *cpr6 eds1* plants were highly susceptible to *P.s. maculicola* ES4326, permitting bacterial growth to levels attained in *eds1* single mutant lines. Thus the combination of *eds1* with *cpr1* or *cpr6* caused an

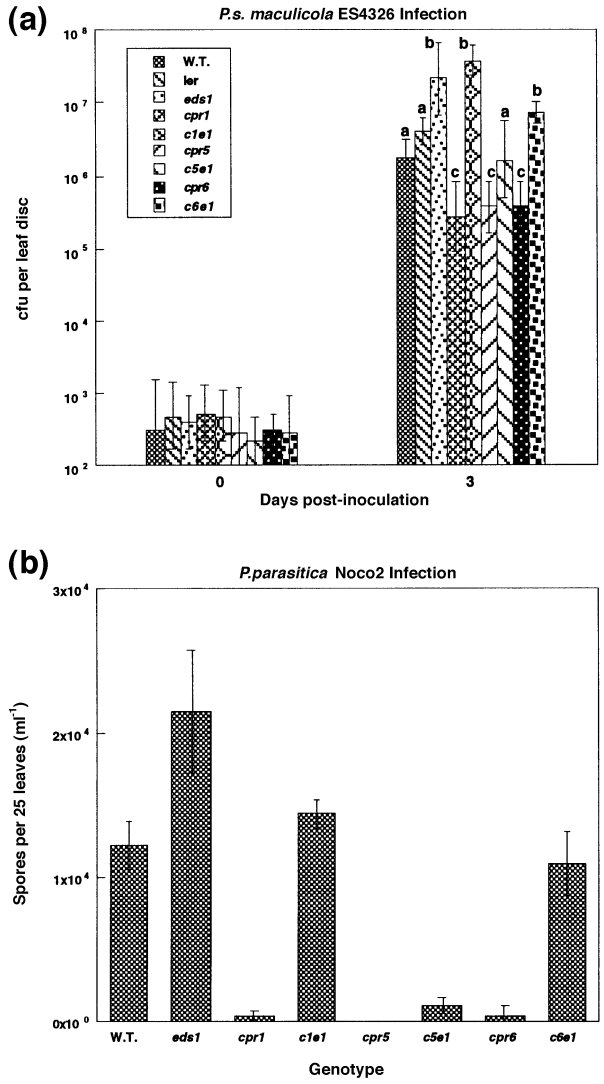

Figure 4. Effect of *eds1* on disease resistance in the *cpr* mutants.
(a) Growth of *P.s. maculicola* ES4326. Different *cpr* plant genotypes were infected by hand infiltrating a 10 mM $MgCl_2$ suspension of *P.s. maculicola ES4326* corresponding to an OD_{600} of 0.001. Leaf disc samples were collected immediately following inoculation (day 0) and at 3 days after infection (day 3), and bacterial growth measured as described in Experimental procedures. Error bars represent 95% confidence limits of log_{10}-transformed data. Letters above bars indicate significant differences between values. In several independent experiments, growth of bacteria in mixed-background *cpr EDS1* families was found to be similar to their corresponding *Col-cpr* mutant lines (not shown). Plant genotype designations are as indicated in Figure 3(a). Cfu, colony-forming units.
(B) Growth of *P. parasitica Noco2*. Plants were inoculated with *P. parasitica* Noco2 by spraying a suspension of conidiospores (3×10^4 spores ml^{-1}) onto 2-week-old plants and assaying for pathogen growth 7 days later. Infection was quantified by counting conidiospores on leaves, as described in Experimental procedures. Error bars represent standard deviation of three replicate samples. Plant genotype designations are as indicated in Figure 3(a).

'enhanced disease susceptibility' (eds) phenotype, reflected by a five- to tenfold increase in bacterial growth over titers measured in either Col-0 or L*er* wild-type plants (Figure 4a). The results indicated that *eds1* is epistatic to *cpr1* and *cpr6* with respect to infection with *P.s. maculicola* ES4326. In contrast to the above results, *cpr5 eds1* was found to be partially resistant to *P.s. maculicola* ES4326, but was not as resistant as *cpr5* plants (Figure 4a). Although growth of *P.s. maculicola* ES4326 was not statistically enhanced in *cpr5 eds1* compared to *cpr5 EDS1* plants or Col-*cpr5*, a consistent trend towards increased susceptibility was observed in three independent *cpr5 eds1* F_3 families, and was not detected in *cpr5 EDS1* control families (data not shown). We concluded that *eds1* partially compromises the *cpr5*-induced enhanced resistance to *P.s. maculicola* ES4326. The *eds1* mutation had previously been shown to confer an eds phenotype to plants infected with *P.s. tomato* DC3000 (Parker *et al.*, 1996). This strain behaved similarly to *P.s. maculicola* ES4326 in the various *cpr eds1* mutant combinations, although *cpr5 eds1* plants did not exhibit significantly different levels of resistance to DC3000 compared with *cpr5* (data not shown).

Plants were also inoculated with isolate Noco2 of the oomycete pathogen *Peronospora parasitica*. The isolate *P. parasitica* Noco2 is virulent on accession Col-0, but avirulent on accession L*er*, being specifically recognized by the *RPP5* gene on the lower arm of chromosome 4 (Parker *et al.*, 1997). As *eds1* fully suppresses *RPP5*-mediated resistance to Noco2 (Aarts *et al.*, 1998; Parker *et al.*, 1996), segregation of the L*er-RPP5* allele was not monitored in homozygous *CPR eds1* or *cpr eds1* families. However, in *cpr EDS1* families expressing the functional Col-*EDS1* allele, it was important to select the Col-*rpp5* susceptibility allele so that resistance to *P. parasticia* Noco2 could be measured without interference from the L*er RPP5* resistance gene. This was easily achieved as *cpr1* is genetically linked (approximately 4 cM) to Col-*rpp5*. When *cpr1 eds1*, *cpr5 eds1* and *cpr6 eds1* were tested for resistance against *P. parasitica* Noco2, a similar trend to that observed in bacterial inoculations was observed. As shown in Figure 4(b), *cpr1 eds1* and *cpr6 eds1* were susceptible to the oomycete pathogen, while *cpr5 eds1* was resistant. The disease susceptibility of *cpr1 eds1* resembled *eds1* plants, whereas that of *cpr6 eds1* was similar to wild-type plants. We concluded from this result that the enhanced susceptibility phenotype of *eds1* plants to Noco2 is not expressed in *cpr6 eds1*. Addition of SA restored resistance to both bacterial and oomycete pathogens in all *cpr eds1* double mutants (data not shown).

We examined more closely the resistance phenotype exhibited by *cpr5 eds1* double mutants by staining *P. parasitica* Noco2-infected leaves with LPTB. Infection analyses were routinely performed on young (2-week-old), non-lesioning plants. *Peronospora parasitica* Noco2 was able to colonize approximately 50% of *cpr5 eds1* leaves examined. Mycelium development in *cpr5 eds1* was

more extensive in leaves without macroscopic lesions, but was altogether poor compared to *CPR5 eds1* or L*er-eds1* leaves (Figure 2b). In contrast, *P. parasitica* Noco2 mycelium was never observed in *cpr5* or *cpr5 EDS1* leaves with or without macroscopic lesions. These data suggest that the *eds1* mutation significantly antagonizes the effects of *cpr5*, leading to an intermediate resistance phenotype in the *cpr5 eds1* double mutant.

Salicylic acid accumulation in cpr eds1 *double mutants*

Accumulation of SA was measured in the *cpr eds1* double mutants to see if the differences in resistance between *cpr1 eds1*, *cpr6 eds1* and *cpr5 eds1* are reflected in the levels of endogenous SA. Because *eds1* does not strongly affect the function of *RPS2* (Aarts *et al.*, 1998), *eds1* infected with *P.s. maculicola* ES4326 expressing *avrRpt2* was used as a control to show that *eds1* is capable of accumulating SA. As shown in Figure 5, *cpr1 eds1* and *cpr6 eds1* exhibited significantly reduced levels of SA compared with *cpr1* or *cpr6* plants. The depletion of SA was severe in *cpr1 eds1* plants, whereas it was partial in *cpr6 eds1*. In contrast, *cpr5 eds1* accumulated approximately the same level of SA as observed in *cpr5*.

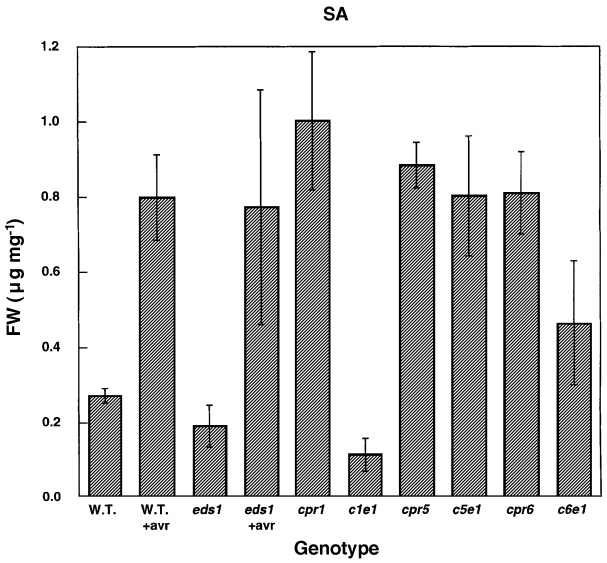

Figure 5. Quantification of SA levels in various *cpr* mutant lines.
Free SA in the *cpr* single mutants compared to the *cpr eds1* double mutant lines was measured as described in Experimental procedures. Data are from leaves of 4-week-old soil-grown plants. Values are average of three replicates ±SD. WT, wild-type *BGL2-GUS* transgenic Col-0; +avr, plants infected with *P.s. maculicola* ES4326/*avrRpt2* 3 days prior to tissue harvest. Other plant genotype designations are as indicated in Figure 3(a).

Discussion

Genetic epistasis analysis was performed between the *cpr1*, *cpr5* and *cpr6* mutations that constitutively activate SAR pathways (Bowling *et al.*, 1994, Bowling *et al.*, 1997; Clarke *et al.*, 1998), and *eds1* that, in contrast, abolishes resistance conditioned by a subset of *R* genes and causes enhanced disease susceptibility to a number of virulent pathogens (Aarts *et al.*, 1998; Parker *et al.*, 1996). Although the nature of the *cpr* mutant alleles is not yet known, it has been shown that they activate biologically relevant resistance pathways (Clarke *et al.*, 2000). *cpr1* and *cpr5* are recessive alleles, and it is therefore likely that their corresponding wild-type proteins repress plant defense signaling. Loss of CPR5 activity may cause deregulation of plant cell death, and this in turn may activate SAR-type defenses. The *CPR5* gene was recently cloned and encodes a novel membrane-associated protein (L. Anderson and X.D., unpublished results). The function of wild-type CPR6 is not clear, as *cpr6* is a dominant mutation and could be interfering with plant resistance pathways in a dominant negative or gain-of-function manner. Any effects of *eds1* on the *cpr*-induced phenotypes could be attributed to a complete loss of EDS1 function due to the use of a null allele, *eds1-2* (Falk *et al.*, 1999). The aim of these studies was to address whether EDS1 is a required component of *cpr*-induced resistance.

eds1 *is epistatic to cpr1 and cpr6*

We established that *EDS1* is required for the SAR-related resistance phenotypes induced by *cpr1* and *cpr6*. *PR-1* gene expression is abolished in *cpr1 eds1* and is substantially reduced in *cpr6 eds1* plants (Figure 3a). Furthermore, enhanced resistance to *P.s. maculicola* ES4326 and *P. parasitica* Noco2 conferred by *cpr1* and *cpr6* is suppressed by the *eds1* mutation (Figure 4). Interference with the *cpr1-and cpr6*-mediated responses by *eds1* is probably due to blocking the signal cascade upstream of SA accumulation, as signified by the reduced levels of SA in *cpr1 eds1* and *cpr6 eds1* plants (Figure 5). Accumulation of SA was strongly depleted in *cpr1 eds1*, but only partially reduced in *cpr6 eds1*. This residual flux did not confer measurable pathogen resistance to *cpr6 eds1*, but it may contribute to the different levels of virulent pathogen growth between the *cpr1 eds1* and *cpr6 eds1* backgrounds (Figure 4b). Whereas *cpr1 eds1* lines routinely exhibited an eds phenotype after inoculation with either *P.s. maculicola* ES4326 or *P. parasitica* Noco2, resembling the *eds1* single mutants (Aarts *et al.*, 1998; Parker *et al.*, 1996), *cpr6 eds1* plants exhibited levels of susceptibility that varied between those measured in wild-type and *eds1* lines (Figure 4). It is possible that a low level of pathogen resistance in *cpr6 eds1* results from residual signaling through an *EDS1*-independent pathway which involves SA. *EDS1*-independent signaling may also contribute to

the maintenance of constitutive *PR2* and *PR5* expression in *cpr6 eds1* plants. Uncoupling *PR1* expression from regulation of *PR2* and *PR5* mRNAs has been observed in other SA-signaling mutants (Nawrath and Métraux, 1999; Rogers and Ausubel, 1997; Zhou *et al.*, 1998). Alternatively, residual resistance in *cpr6 eds1* may be a consequence of the extremely high *PDF1.2* expression observed (Figure 3a). However, studies have shown that PDF1.2 does not affect resistance to *P.s. maculicola* ES4326 or *P. parasitica* Noco2, even at such exaggerated levels (Clarke *et al.*, 2000; Falk *et al.*, 1999; Thomma *et al.*, 1998).

Salicylic acid signaling antagonizes PDF1.2 *expression in* cpr6

Our data complement other studies which show that blocking the SA pathway in *cpr6* results in a dramatic increase in *PDF1.2* expression (Clarke *et al.*, 1998; Clarke *et al.*, 2000), reinforcing evidence of antagonism between SA- and JA/ET-mediated plant defenses (Doares *et al.*, 1995; Felton *et al.*, 1999). We found that *eds1* causes a hyper-induction of *PDF1.2* expression in *cpr6*, irrespective of exogenously added or endogenously elevated SA (Figures 3c and 5). These data suggest that antagonism by *eds1* with JA/ET signaling in *cpr6* occurs at other levels than simply through depletion of SA. It is possible that defects exist in *cpr6 eds1* plants in transduction of the SA signal. This idea is supported by other studies showing that *PDF1.2* is hyper-induced in the *cpr6 npr1* double mutant, even though endogenous SA levels in the double mutant are several times higher than those found in *cpr6* alone (Clarke *et al.*, 2000).

eds1 *partially suppresses* cpr5*-induced resistance*

The pleiotropic nature of *cpr5* suggests a complex interplay of signals that undoubtedly complicates genetic epistasis studies (Boch *et al.*, 1998; Bowling *et al.*, 1997). We found that initiation of lesions (Figure 2a), pathogen resistance (Figure 4) and SA accumulation (Figure 5) in *cpr5 eds1* plants resembles that in *cpr5* or *cpr5 EDS1* lines. We therefore concluded that *cpr5* induces plant defenses essentially independently of *EDS1*. It is likely that the enhanced resistance of *cpr5* depends on SA accumulation as *eds5*, a mutation suppressing SA biosynthesis, was found to be epistatic to *cpr5* in pathogen resistance and SA accumulation (Clarke *et al.*, 2000).

A more detailed examination of *cpr5 eds1* revealed a partial effect of *eds1* on *cpr5*-conditioned responses that was most evident in plants without macroscopic lesions. Microscopic examination of *cpr5 eds1* plants after *P. parasitica* Noco2 infection showed that the presence of the *eds1* mutation allowed colonization of the leaf tissue in both lesioning and non-lesioning (micro-lesioning) leaves. However, pathogen development was more extensive in younger, micro-lesioning tissue (Figure 2b). In contrast, *P. parasitica* Noco2 mycelium was not observed at any stage in *cpr5* plants or *cpr5 EDS1* lines. Additionally, *cpr5*-induced *PR1* and *PDF1.2* gene expression was suppressed by *eds1* in younger tissue without macroscopic lesions, but was unaffected in older, macroscopically lesioning tissue (Figure 3a). This reinforces other mutant analyzes showing that defense-related gene expression, in particular *PDF1.2*, is lesion-dependent (Clarke *et al.*, 2000; Penninckx *et al.*, 1996; Pieterse and Van Loon, 1999; Shah *et al.*, 1999).

Thus there appears to be an *EDS1*-dependent component to the *cpr5* resistance phenotype that becomes redundant as macroscopic lesions form. It is notable in this context that a number of *Arabidopsis R* genes do not require *EDS1* to elicit a local HR (Aarts *et al.*, 1998; McDowell *et al.*, 2000). It is therefore possible that the major *EDS1*-independent component of *cpr5*-induced resistance resembles such local *R* gene-mediated responses. Evidence has also been presented that *cpr5* can activate an *NPR1*-independent resistance response that resembles local *R* gene-mediated resistance (Bowling *et al.*, 1997; Clarke *et al.*, 2000). However, the relationship between EDS1 and NPR1 function in plant defense is not clear, and our recent analyzes show that several strongly *EDS1*-dependent *R* gene-mediated responses operate independently of *NPR1* (E. van der Biezen, J.E.P. and J.D.G. Jones, unpublished results). Placement of *EDS1* relative to *NPR1* in the *cpr*-driven SAR responses would require further genetic analyzes of *cpr*, *npr1* and *eds1* mutant combinations. It is known that the addition of SA can rescue the *eds1*, but not the *npr1* susceptibility phenotype. This, together with studies that suggest *NPR1* is primarily involved in signaling for 'systemic' resistance (Clarke *et al.*, 2000; McDowell *et al.*, 2000), would position *EDS1* upstream of *NPR1* or in a separate 'local' signaling pathway.

Placement of EDS1 on the cpr*-conditioned resistance signaling pathways*

EDS1 encodes a lipase-like protein that is crucial for local, early resistance responses conditioned by the TIR-NB-LRR type of R proteins (Aarts *et al.*, 1998; Falk *et al.*, 1999). Wild-type EDS1 also contributes to restriction of pathogen growth in several compatible plant–pathogen interactions (Aarts *et al.*, 1998; Parker *et al.*, 1996). It is not known whether these two phenotypes reflect one or more biochemical attributes of EDS1. We found that all the *cpr* mutations enhance *EDS1* expression (Figures 1 and 3a). This phenotype is reminiscent of pathogen-inoculated plants or plants treated with exogenous SA that exhibit increased *EDS1* mRNA (Falk *et al.*, 1999). Thus the *cpr*

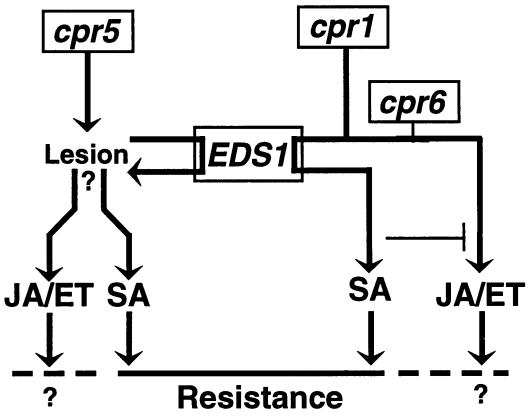

Figure 6. Genetic relationship of *EDS1* to *cpr*-induced plant resistance responses.
A model places *EDS1* in two different signal cascades. *EDS1* is positioned downstream of *cpr1*- and *cpr6*-induced resistance in one pathway leading to SA-mediated plant defenses. *EDS1* is also placed downstream of *cpr5* in a lesion-propagation loop of another pathway. All SAR-related phenotypes in *cpr1* are channelled through *EDS1*. Enhanced pathogen resistance in *cpr6* is strongly dependent on *EDS1*. In contrast, a minor effect of the *eds1* mutation on *cpr5*-induced responses is observed in young, non-lesioning plants, suggesting that resistance in *cpr5* lesioning tissues operates independently of *EDS1*. The *eds1* mutation further enhances *cpr6*-conditioned *PDF1.2* expression, indicating that interference with JA/ET–SA pathway cross-talk occurs when *EDS1*-dependent signaling is disrupted. The contribution of JA/ET signaling in resistance to *P. syringae* or *P. parasitica* is not known (see Discussion).

mutations positively influence *EDS1* expression. This may be through increased accumulation of SA (Figure 5), as well as other factors involved in signal potentiation during plant defense (Delledonne *et al.*, 1998; Shirasu *et al.*, 1997).

The results draw an important connection between *EDS1* and processes associated with resistance to virulent pathogens, raising questions about the precise position of *EDS1* and the *cpr* mutants on the defense signaling cascade. In the model shown in Figure 6, we place EDS1 in two distinct signaling pathways. EDS1 is essential for SA-mediated defenses in *cpr1*, and is a major component of SA signaling and associated defenses in *cpr6*. In *cpr6 eds1* there remains some SA accumulation that occurs in an EDS1-independent manner and may contribute to the low level of resistance retained in *cpr6 eds1* plants to *P. parasitica*. In a different resistance pathway initiated by *cpr5*, *EDS1* has only a minor role that is most apparent in non-lesioning plants. Significantly, *EDS1* is not required for lesion formation in *cpr5*, but contributes to lesion propagation. It is probable that suppression of lesion spread by the *eds1* mutation directly or indirectly permits some pathogen growth in *cpr5* plants. While SA accumulation clearly has an important role in resistance to *P.s. maculicola* ES4326 and *P. parasitica* Noco2 induced by *cpr1*, *cpr5* and *cpr6* (Bowling *et al.*, 1997; Clarke *et al.*, 1998; Clarke *et al.*, 2000), the contribution of JA/ET signaling to resistance against these pathogens is unclear.

In a parallel study, Jirage *et al.* (2001) performed genetic epistasis analysis between the SA regulatory mutant *pad4* and *cpr1*, *cpr5* and *cpr6*. PAD4 was found to be required for the same *cpr*-induced resistance phenotypes as *eds1*, suggesting that EDS1 and PAD4 function at a similar position in the *cpr*-driven signaling network. Significantly, *pad4* suppressed *cpr1- and cpr6*-induced resistance to *P.s. maculicola* ES4326 and *P. parasitica* Noco2, and SA-dependent *PR* gene expression (Jirage *et al.*, 2001). Also, *pad4* partially suppressed *cpr5*-induced pathogen resistance, but had no significant effect on SA accumulation or *PR* gene induction. Strikingly, *cpr6 pad4* plants exhibited a partial reduction in SA accumulation and displayed hyper-accumulation of the JA/ET-responsive gene *PDF1.2*, as observed in the *cpr6 eds1* combinations. However, unlike *cpr6 eds1*, residual SA in *cpr6 pad4* did not appear to contribute to pathogen resistance as the plants exhibited full expression of *pad4*-conditioned enhanced susceptibility. This distinction may point to differences in EDS1 and PAD4 function. However, we cannot rule out the possibility that differences in plant genotypes or the experimental conditions used contribute to different pathogen growth levels attained in the two studies. It is notable, however, that *pad4* did not suppress the stunted growth habit of *cpr6* plants, whereas *cpr1 pad4* plants resembled *pad4*, indicating that certain *cpr6*-conditioned processes are triggered independently of PAD4, as suggested for EDS1 in our analysis. *PAD4* encodes a lipase-like protein with the same catalytic motifs as EDS1 (Jirage *et al.*, 1999), suggesting further mechanistic similarities between these two regulatory proteins. However, they are not redundant as mutations in either gene confer an enhanced susceptibility phenotype to virulent pathogens (Aarts *et al.*, 1998; Glazebrook *et al.*, 1997; Parker *et al.*, 1996). Further insights into the role of EDS1 and PAD4 within the complex interplay of plant defense signaling networks should be gained from examination of their biochemical functions and molecular associations in wild-type and mutant backgrounds, as well as through expression analysis of a much wider array of plant genes.

Experimental procedures

Plant growth conditions

Arabidopsis thaliana plants were grown on soil (Metro-Mix 200; Grace-Sierra, Malpitas, CA, USA) in a growth room under a 14 h photoperiod and a light intensity of 100–200 μE m^{-2} sec^{-1}. All seeds were kept at 4°C for at least 2 days prior to placement in the growth environment to aid uniform germination.

Double mutant isolation

The *cpr1 eds1*, *cpr5 eds1* and *cpr6 eds1* double mutants were generated using the pollen from *cpr* plants in accession Columbia

(Col) to fertilize *eds1* plants in accession Landsberg *erecta* (L*er*). Loss of the recessive *erecta* (*er*) morphology was used in the F_1 generation to score for a successful cross between the *cpr* mutants and *eds1*. F_1 plants were allowed to self-pollinate and F_2 seeds were collected. As it was already known that penetrance of the *cpr5* and *cpr6* morphological phenotypes into the L*er* ecotype is high (Bowling *et al.*, 1997; Clarke *et al.*, 1998), the *cpr5 eds1* and *cpr6 eds1* double mutants were first isolated by screening F_2 plants for the homozygous *cpr* morphology. The *cpr* positives were then screened by PCR to distinguish the null *eds1-2* deletion mutant (Falk *et al.*, 1999) from wild-type *EDS1* DNA using a diagnostic triple DNA primer set (details available on request from J.E.P.). F_3 seeds were collected from plants that passed both screens. These were planted on soil to confirm presence of the *cpr* mutation and the *eds1* mutation. Penetrance of the *cpr1* phenotype was known from previous analyzes to be variable in the L*er* ecotype (L. Anderson and X.D., unpublished results). We first genotyped the F_2 population for the likely presence of *cpr1* using the linked co-dominant amplified polymorphic marker, *AG* (http://www.Arabidopsis.org:80/aboutcaps.html). Selected plants were back-crossed to Col-*cpr1* to verify presence of the *cpr1* mutation. These lines were genotypes for *eds1-2* as described above. Plant lines were then scored for resistance or susceptibility to *P. parasitica* Noco2 and for the presence or absence of *BGL2-GUS* reporter gene expression by X-gluc staining (Bowling *et al.*, 1997). Due to the mixing of the L*er* (*eds1*) and Col (*cpr*) accessions, several control lines (*cpr1EDS1*, *CPR1eds1*, *cpr5EDS1*, *CPR5eds1*, *cpr6EDS1* and *CPR6eds1*) were established, along with multiple double mutant lines, as described above. Genotypes of selected lines were checked in the F_3 generation.

RNA analysis

Tissue samples for RNA gel-blot analysis were collected from 2- or 4-week-old *Arabidopsis* seedlings grown on soil. RNA was extracted as described previously (Bowling *et al.*, 1997; Clarke *et al.*, 1998). 10 μg samples were separated by electrophoresis through formaldehyde–agarose gels and transferred to a hybridization membrane (GeneScreen; DuPont–New England Nuclear, Boston, MA, USA) as described by Ausubel *et al.* (1994). ^{32}P-labeled DNA probes for *PR-1*, *PR-2*, *PR-5*, *PDF1.2* and *UBQ5* were generated using a strand-biased PCR in a protocol modified from Schowalter and Sommer (1989). The template for *PDF1.2* was generated by PCR using primers described by Penninckx *et al.* (1996). The templates for *PR-1*, *PR-2*, *PR-5* rRNA and *UBQ5* were derived from PCR fragments generated from genomic DNA using primers described by Rogers and Ausubel (1997). PCR amplification of the templates is described by Bowling *et al.* (1997). Hybridization and washing conditions were as previously described (Cao *et al.*, 1994).

Real-time quantitative RT–PCR was performed on a PE Applied Biosystems 7700 Sequence Detection System (Foster City, CA, USA) using TaqMan chemistry. The *Arabidopsis ACT2* gene was used as an internal control for relative quantification because of its strong constitutive expression in vegetative tissues (An *et al.*, 1996). The *ACT2* gene from *Arabidopsis* ecotypes Col-0, L*er* and Wassilewskija was sequenced, and primers were designed that would amplify all three alleles. By placing the *ACT2* reverse primer in the 3′ UTR region, only *ACT2* sequences were amplified. Briefly, 1 μg total RNA was reverse transcribed using random hexamers, diluted to 500 μl with water, and 5 μl per reaction was used. Reactions for *ACT2* and *EDS1* were performed individually, and were done in triplicate using the TaqMan Universal 2× Mastermix (PE Applied Biosystems). TaqMan probes for both *ACT2* and *EDS1* carry a 5′ FAM reporter and were designed across introns to give cDNA-specific signals. Primer and probe sequences are as follows: ACT2-forward: TCGGTGGTTCCATTCTTGCT; ACT2-reverse: GCTTTTTAAGCCTTTGATCTTGAGAG; ACT2-probe: AGCACATTCCAGCAGATGTGGATCTCCAA; EDS1-forward: CAAGAATCTTGAAGCTGTCATTGATC; EDS1-reverse: TGTCCTGTGAACACTATCTGTTTTCTACT; EDS1-probe: CACAGCCATTTCCACAGAAGCTTGAAATG.

Salicylic acid analysis

Tissue for SA extraction was harvested from 4-week-old, soil-grown plants. The procedure used to extract SA is described by Li *et al.* (1999). This procedure had an approximately 25% recovery rate, determined by extracting known amounts of SA.

Pathogen infections

Infection of plants with *Pseudomonas syringae* pv. *maculicola* ES4326 (*P.s. maculicola* ES4326) or *Peronospora parasitica* Noco2 (*P. parasitica* Noco2) were performed as described previously (Clarke *et al.*, 1998), with minor modifications. Plants used for *P.s. maculicola* ES4326 infection were grown on soil for 4 weeks and injected with a bacterial suspension of OD_{600} 0.001 (1×10^6 ml^{-1} colony-forming units) with a blunt syringe on the abaxial side of the leaf. At 0 and 3 days post-inoculation, four to six infected leaves were harvested per plant genotype. An 8 mm disc from each harvested leaf was ground in 500 μl 10 mM $MgCl_2$ and a succession of 20-fold dilutions were made in 10 mM $MgCl_2$. 50 μl from a predetermined range of dilutions from each sample was plated on King's B medium (King *et al.*, 1954) and incubated at 30°C for 2 days. Statistical analyzes were performed by Student's *t*-test of the differences between two means of log-transformed data (Sokal and Rohlf, 1981). Plants to be inoculated with *P. parasitica* Noco2 had grown for 2 weeks on soil when they were sprayed to imminent run-off with a dH_2O suspension of 10^4 spores ml^{-1}. Seven days after inoculation the degree of pathogen infection was determined by harvesting 25 leaves per sample (approximately five plants) in 1 ml H_2O. After vigorous vortexing, the spores in two 10 μl aliquots from one sample were counted in a haemocytometer and averaged. Three replicate samples per genotype were assayed to obtain a standard deviation.

Histochemistry and microscopy

Leaf samples were taken from 2- and 4-week-old soil-grown plants for lactophenol trypan blue (LPTB) staining of dead plant cells, and for *P. parasitica* mycelium. Samples were submerged in a 70°C LPTB solution (2.5 mg ml^{-1} trypan blue, 25% [w/v] lactic acid, 23% water-saturated phenol, 25% glycerol, H_2O) and slow-release vacuum-infiltrated for 5 min, then re-infiltrated for an additional 5 min. Samples were then heated over boiling water for 2 min and cooled before replacement of the LPTB solution with a chloral hydrate solution (25 g in 10 ml H_2O) for de-staining. After multiple exchanges of chloral hydrate solution, samples were equilibrated for several hours in 70% glycerol and mounted on slides. Staining for β-glucuronidase (GUS) activity driven by the β *1,3-glucanase* promoter was performed as described previously (Bowling *et al.*, 1997).

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