The EMBO Journal Vol. 20 No. 19 pp. 5400–5411, 2001

Direct interaction between the *Arabidopsis* disease resistance signaling proteins, EDS1 and PAD4

Bart J.Feys[1], Lisa J.Moisan[1], Mari-Anne Newman[1,2] and Jane E.Parker[1,3,4]

[1]The Sainsbury Laboratory, John Innes Centre, Colney, Norwich NR4 7UH, UK

[2]Present address: Section for Plant Pathology, Department of Plant Biology, Royal Veterinary and Agricultural University, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark
[3]Present address: Max-Planck Institute for Plant Breeding Research, Department of Molecular Plant Pathology, Carl-von-Linne-Weg 10, D-50829 Cologne, Germany

[4]Corresponding author
e-mail: parker@mpiz-koeln.mpg.de

The *Arabidopsis EDS1* and *PAD4* genes encode lipase-like proteins that function in resistance (*R*) gene-mediated and basal plant disease resistance. Phenotypic analysis of *eds1* and *pad4* null mutants shows that *EDS1* and *PAD4* are required for resistance conditioned by the same spectrum of *R* genes but fulfil distinct roles within the defence pathway. *EDS1* is essential for elaboration of the plant hypersensitive response, whereas *EDS1* and *PAD4* are both required for accumulation of the plant defence-potentiating molecule, salicylic acid. *EDS1* is necessary for pathogen-induced *PAD4* mRNA accumulation, whereas mutations in *PAD4* or depletion of salicylic acid only partially compromise *EDS1* expression. Yeast two-hybrid analysis reveals that EDS1 can dimerize and interact with PAD4. However, EDS1 dimerization is mediated by different domains to those involved in EDS1–PAD4 association. Co-immunoprecipitation experiments show that EDS1 and PAD4 proteins interact in healthy and pathogen-challenged plant cells. We propose two functions for EDS1. The first is required early in plant defence, independently of PAD4. The second recruits PAD4 in the amplification of defences, possibly by direct EDS1–PAD4 association.

Keywords: *Arabidopsis*/dimerization/EDS1/PAD4/ salicylic acid

Introduction

Plants have evolved complex recognition and response mechanisms to counter attack by pathogens. Disease occurs only when the pathogen is able to avoid early detection by the plant (Feys and Parker, 2000). One of the most strongly expressed forms of plant disease resistance is conferred by resistance (*R*) genes whose products confer recognition of pathogen avirulence (Avr) proteins (Martin, 1999). Their highly specific interaction occurs within or on the surface of plant cells and leads to the rapid induction of plant defences (Kjemtrup *et al*., 2000). *R* gene-mediated resistance is usually, although not invariably, associated with localized plant cell necrosis, known as the hypersensitive response (HR). Accompanying the HR are a number of early cellular changes within the plant, such as an oxidative burst producing reactive oxygen intermediates (ROI), accumulation of the signaling molecules, nitric oxide (NO) and salicylic acid (SA), and the transcriptional activation of defence-related genes (McDowell and Dangl, 2000). Data suggest that cooperation between NO, ROI and SA molecules contributes to establishment of the HR and to the potentiation of defence signals in surrounding plant tissues (Shirasu *et al*., 1997; Delledonne *et al*., 1998; Klessig *et al*., 2000). However, the precise nature of events determining plant-pathogen recognition and downstream signaling is not known. It is also unclear how localized plant resistance induces systemic immunity (systemic acquired resistance, SAR), a broad spectrum and long lasting resistance that occurs in uninoculated parts of the plant (McDowell and Dangl, 2000).

Mutational analyses in the model plant, *Arabidopsis*, has led to the identification of genes required for *R* gene-mediated resistance or for SAR (Feys and Parker, 2000). The *eds1* (enhanced disease susceptibility) mutation suppresses *R* gene-mediated resistance to the oomycete pathogen, *Peronospora parasitica*, conferred by *RPP1* in accession Wassilewskija (Ws-0), *RPP5* in accession Landsberg-*erecta* (Ler) (Parker *et al*., 1996), and by *RPP2* and *RPP4* in accession Columbia (Col-0) (Aarts *et al*., 1998). Mutations in *EDS1* also abolish *RPS4*-mediated resistance present in all three accessions to the bacterial pathogen, *Pseudomonas syringae* expressing *avrRps4* (Aarts *et al*., 1998). All of these *R* genes belong to a major *R* gene structural class encoding 'TIR-NB-LRR' proteins that have N-terminal (TIR) similarity to the intracellular domains of human and *Drosophila* Toll receptors, a central nucleotide binding (NB) domain and C-terminal leucine-rich repeats (LRRs) (Parker *et al*., 1997; Botella *et al*., 1998; Gassmann *et al*., 1999). *EDS1* is not required for resistance conferred by *RPM1*, *RPS2* or *RPS5*, NB-LRR *R* genes that possess an N-terminal coiled coil (CC) motif and not a TIR domain (Aarts *et al*., 1998), pointing to the possibility that distinct resistance pathways are directed, at least in part, by particular R protein structural types. Analysis of *RPS4*-specified responses in wild-type and *eds1* plants revealed that *EDS1* operates upstream of SA-dependent defences (Falk *et al*., 1999). Moreover, *eds1* plants are hypersusceptible to normally virulent strains of *P.syringae* and *P.parasitica* (a phenotype referred to as 'enhanced disease susceptibility', eds) (Parker *et al*., 1996; Aarts *et al*., 1998), suggesting defects in a basal resistance mechanism against virulent pathogens.

The screen for suppressors of *RPP5* resistance in accession Ler led to the isolation of one defective allele

© European Molecular Biology Organization

of *PAD4* (phytoalexin deficient) (*pad4-2*; Jirage *et al.*, 1999). *PAD4* was first identified in a mutational screen for enhanced disease susceptibility to a virulent isolate of *P.syringae* pv. *maculicola* (Glazebrook *et al.*, 1996) and was found to be required for resistance conferred by *RPP2* and *RPP4* to *P.parasitica* in Col-0 cotyledons (Glazebrook *et al.*, 1997). The eds phenotype of *pad4* was associated with reduced accumulation of the indole phytoalexin, camalexin and the signaling molecule, SA (Glazebrook *et al.*, 1997; Zhou *et al.*, 1998). Neither of these responses was affected in *pad4* plants responding to *P.syringae* expressing *avrRpt2*, indicating that *RPS2*-specified resistance does not require *PAD4* (Zhou *et al.*, 1998). PAD4 was therefore placed as an important regulator of SA accumulation in the plant response to virulent *P.syringae*. However, its position in *R* gene-mediated resistance responses remained unclear.

The isolation of *pad4-2* as a suppressor of *RPP5*-mediated resistance in Ler as well as the requirement for *PAD4* in *RPP2* and *RPP4* resistance in Col-0 shows that *PAD4* participates in several *EDS1*-dependent responses. Both *EDS1* and *PAD4* encode lipase-like proteins (Falk *et al.*, 1999; Jirage *et al.*, 1999). Furthermore, the abundance of *EDS1* and *PAD4* mRNAs is upregulated by applications of SA, suggesting the operation of a positive feedback loop in the expression of both of these genes (Falk *et al.*, 1999; Jirage *et al.*, 1999). This raised the question of whether EDS1 and PAD4 functions are connected in plant defence.

Here we show that EDS1 and PAD4 proteins interact specifically, both in a yeast two-hybrid assay and in plant cells, suggesting that physical association between these two proteins may contribute to their activities in disease resistance. By examining, for the first time, the phenotypes of null *eds1* and *pad4* mutants in the same genetic background we also establish that *EDS1* and *PAD4* are required for resistance conditioned by an identical spectrum of *R* genes. We demonstrate that both *EDS1* and *PAD4* positively regulate SA accumulation in an *EDS1/PAD4*-dependent *R* gene-mediated response and that *EDS1* is additionally required for generation of the plant HR. Furthermore, we establish that *EDS1* is necessary for the upregulation of *PAD4* mRNA, whereas mutations in *PAD4* or depletion of SA only partially compromise enhanced *EDS1* expression. Our results are consistent with placement of *EDS1* and *PAD4* within a defence pathway that is engaged by TIR-NB-LRR-type *R* genes. In this signaling mechanism, we propose two functions for EDS1. One is positioned upstream of PAD4 and triggers early plant defences. The other recruits PAD4 to potentiate plant defences through the accumulation of SA and possibly other molecules.

Results

Suppression of RPP5-mediated resistance in eds1 and pad4

We examined the *RPP5*-mediated resistance phenotypes of wild-type Ler, null *eds1-2* and *pad4-2* mutant plants and Ler-*NahG* plants (expressing the SA-depleting enzyme, salicylate hydroxylase; Bowling *et al.*, 1997) after inoculation with *P.parasitica* isolate Noco2. Two-week-old seedlings were sprayed with Noco2 conidia and infected

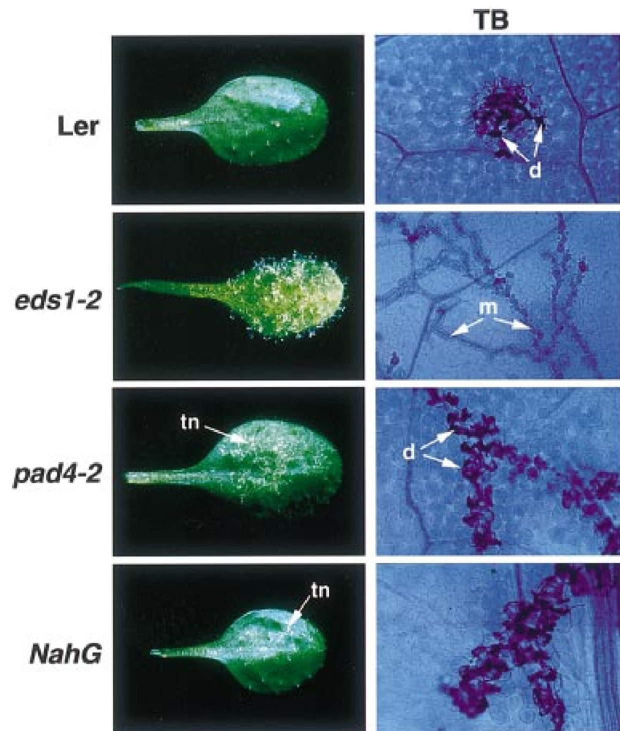

Fig. 1. *RPP5* resistance phenotypes of wild-type Ler, *eds1-2*, *pad4-2* and *NahG* leaves inoculated with *P.parasitica* isolate Noco2. Two-week-old seedlings were spray-inoculated with a suspension of *P.parasitica* conidia (5×10^4/ml) and incubated as described in Materials and methods. Whole leaves were photographed 6 days after inoculation. Trailing necrosis (tn) in *pad4-2* and *NahG* is indicated by an arrow. Leaf tissue was stained with lactophenol Trypan Blue (TB) 5 days after inoculation to visualize pathogen mycelium (m) and necrotic plant cells (d). The TB-stained material, viewed under a light microscope, is shown at ×400 magnification.

leaves assessed up to 7 days after inoculation. As shown in Figure 1, Ler elaborated an HR at points of pathogen penetration that was visible microscopically after staining leaves with lactophenol Trypan Blue (TB) (Parker *et al.*, 1993). Mycelium did not grow beyond these discrete patches of necrotic plant cells. In contrast, Noco2 colonization of *eds1-2* plants was unrestricted and the mycelium rapidly ramified throughout the plant to produce abundant asexual spores on the leaf surface after 6 days (Figure 1). The phenotype of *pad4-2* was strikingly different to that of *eds1-2*. Leaves exhibited trails of necrotic plant cells and permitted the emergence of occasional sporophores after 6–7 days. Lactophenol Trypan Blue staining revealed that *pad4-2* produced an HR but the pathogen was able to grow beyond the initial infection site, giving rise to trails of dead plant cells at the plant-pathogen interface (Figure 1). Ler-*NahG* plants exhibited a similar trailing necrotic phenotype to *pad4-2* in response to Noco2, although mycelial ingress was less extensive in leaves of *NahG* plants than in *pad4-2* (Figure 1).

We concluded from these analyses that *EDS1* and *PAD4* have different functions in *RPP5*-mediated resistance. Whereas *EDS1* is an indispensable component of the HR and is associated with early plant defences, *PAD4* appears to exert a resistance strengthening or potentiating activity that is downstream or independent of HR development.

Similarity between the phenotypes of *pad4-2* and *NahG* leaves suggests that a major role of *PAD4* is to promote SA accumulation in the *RPP5*-conditioned response.

EDS1 and PAD4 interact specifically in a yeast two-hybrid assay

In order to identify potential protein interactors with EDS1, the full-length *EDS1* cDNA was fused to the LexA DNA-binding domain in the bait vector pLexA (pLex-EDS1). A Landsberg-*erecta* two-hybrid cDNA library derived from pathogen-challenged plant material was constructed in the pJG4-5 activation domain (AD) vector (see Materials and methods). The pLex-EDS1 bait was verified not to auto-activate the *LEU2* and *LacZ* reporter genes and to move to the nucleus. After transformation of the two-hybrid library in yeast strain EGY191(p8op-LacZ/pLex-EDS1), 6 000 000 primary transformants were obtained and 60 000 000 yeast clones were screened for potential EDS1 interactors. The dominant class of interactor (in 11 isolates) was identified as PAD4. Only full-length PAD4 inserts were recovered from the screen. EDS1–PAD4 interaction was also tested in the reciprocal combination with PAD4 fused to the LexA domain and was found to be stronger than the original interaction (data not shown). EDS1 did not interact with the control bait protein Bicoid (Figure 2B), making it unlikely that EDS1 is a sticky protein associating non-specifically with PAD4. In addition, a separate two-hybrid screen using full-length PAD4 yielded 50 positive interactors, 37 of which encoded full-length EDS1 (data not shown).

Comparison of the EDS1 and PAD4 protein sequences revealed a novel conserved domain in the C-terminus that we have named the EP domain (for EDS1 and PAD4-defined; Figure 2A), which is not present in other known proteins outside the plant kingdom. The only other *Arabidopsis* gene containing the EP domain is *SAG101*, of unknown function, which is expressed during plant senescence (He *et al.*, 2001). Figure 3 shows a sequence alignment of the EP domain in EDS1, PAD4 and SAG101.

In order to define regions of EDS1 and PAD4 that are required for interaction, we tested combinations of different EDS1 and PAD4 subdomains in the yeast two-hybrid assay. A schematic diagram of the designated EDS1 and PAD4 domains is shown in Figure 2A. Western blot analysis was performed on all combinations to confirm stable expression of the fusion proteins (data not shown). We found that PAD4 interacted with full-length EDS1 but not with any EDS1 subdomain tested, as shown in Figure 2B. PAD4 association with EDS1 was through its N-terminal region, comprising the predicted lipase domain. In particular, the first 15 amino acids of PAD4 were indispensable for interaction with EDS1. We next tested whether EDS1 and PAD4 were each capable of dimerization. EDS1, but not PAD4, strongly interacted with itself, suggesting that EDS1 may function in both homomeric associations as well as in heteromeric complexes with PAD4. The C-terminus of EDS1 was sufficient for interaction with full-length EDS1 (Figure 2B), suggesting that EDS1 dimerization occurs through the C-terminal end. No interaction between full-length EDS1 and its N-terminal domain (amino acids 1–350) was observed (data not shown). The absence of PAD4 dimerization in the two-hybrid system indicates a degree of specificity in interactions between members of this class of lipase-like proteins.

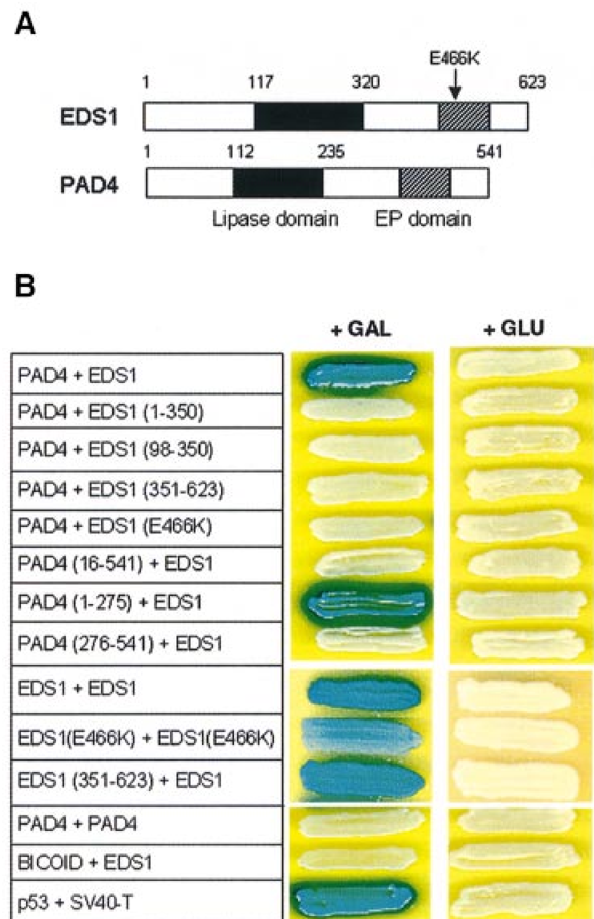

Fig. 2. Interaction between EDS1 and PAD4 in a yeast two-hybrid assay. (**A**) Schematic representation of the domain structure of the *Arabidopsis* EDS1 and PAD4 proteins. The lipase domain (filled box) and EP (EDS1 and PAD4-defined) domain (hatched box) are indicated. The position of the *eds1-1* (E466K) mutation is shown with an arrow. The EP domain lies between residues 405 and 554 (EDS1) and residues 332 and 457 (PAD4). (**B**) Two-hybrid interactions between EDS1 and PAD4. Full-length proteins or defined subdomains of EDS1 and PAD4 were tested for specific interactions under inducing (+GAL) or repressing conditions (+GLU). Combinations are shown with the first protein fused to the LexA domain and the second partner fused to the AD domain. Numbers refer to amino acid positions in the full-length protein. Positive interactions are defined by activation of the LacZ (shown) and LEU2 (same pattern as LacZ; data not shown) reporter genes. The interaction between p53 and SV40-T serves as a positive control for the assay.

Previously, we isolated the *eds1-1* mutant carrying a single amino acid substitution that changes a highly conserved glutamate at position 466 within the EP domain to an oppositely charged lysine residue (E466K in Figure 3). The *eds1-1* mutant has a complete loss of function phenotype (Parker *et al.*, 1996; Falk *et al.*, 1999). Western blot analysis showed that fusions of EDS1 (E466K) to both the LexA and AD domain were stably expressed in yeast cells during two-hybrid interaction assays (data not shown). We assessed the effect of the E466K mutation on two-hybrid interactions and found that EDS1–PAD4 association was abolished, whereas it reduced, but did not abolish EDS1 dimerization (Figure 2B). Quantification of β-galactosidase activity

```
EDS1    387 ...KLAWIED.EYKPKCQAHK..NGYYDSFK..VSNEENDFKANVK...RAELAGVFDEVLGLLKKCQLPDEFEGDIDWI
SAG101  260 MKISMAYIE..WYKKKCKEVK..IGYYDRFKTQLAFPSKEFDINIKNHHKSELNRFWKSVVEEVERRPQSDASILKRRFL
PAD4    309 .SVLPARLEIQWYKDRCDASEEQLGYYDFFKR.YSLK.RDFKVNMS...RIRLAKFWDIVIKMVETNELPFDFHLGKKWI

                     E466K in eds1-1
                          ↓
EDS1    451 KLATRYRRLVEPLDIANYHRHLKNEDTGPYMKRGRPTRYIYAQRGYEHHILKPNGMIAEDVFWNKVNGLNLGLQLEEIQE
SAG101  336 FSGNNYRRMIEPLDIAEYY..LEGRKE..YRTMGRSHHYVMLEKWF........GM..ESILIEKER.....CKKRDLSD
PAD4    378 YASQFYQLLAEPLDIANFYKNRDIKTGGHYLEGNRPKRYEVIDKWQ............KGVKVPEEC.....VRSRYAST

EDS1    521 TLKNSGSECGSCFWAEVEE.......LK...GKPYEEVEVRVKTL...EGMLREWITAGEVDEKEIFLEGSIFRKW...W
SAG101  138 LLT.....FDSCFWAEVEDSLIVINQLNTTVGMRDDEREVLTRKLVEFEGYVWEIITKREVSP.EIFLEESSFMKW...W
PAD4    441 TQ.......DTCFWAKLEQAKEWLDEARKE.SSDPQRRSLLREKIVPFESYANTLVTKKEVSL.DVKAKNSSYSVWEANL

EDS1    579 ITLPKNHKSHSP.LRDYMMDEITDT.....
SAG101  468 KEYKKIKGFNSSYLTEEMNTRKYESYGKSQ
PAD4    494 KEFKCKMGYENE..IEMVVDES.DAMET..
```

Fig. 3. Sequence alignment of the EP domain in EDS1, PAD4 and SAG101. Sequences of the *Arabidopsis* EDS1 and PAD4 proteins were aligned with SAG101, a senescence-associated gene of unknown function (He *et al*., 2001) across the EP domain. The position of the *eds1-1* (E466K) mutation is indicated with an arrow. The alignment was generated using Clustal_W and shaded using BoxShade (see Materials and methods). Positions are relative to the full-length protein. The DDBJ/EMBL/GenBank accession No. for SAG101 is AAF78583.

(see Materials and methods) gave the following values for the interactions: EDS1 + EDS1, 107 ± 16 units; EDS1-(E466K) + EDS1(E466K), 17 ± 4 units. The contrasting effect of the E466K mutation on the EDS1–PAD4 and EDS1–EDS1 interactions provides further evidence that the mode of EDS1 association with PAD4 is different to that of EDS1 dimerization.

In planta interaction between EDS1 and PAD4

In order to study protein interactions in plant cells, a null mutant *pad4* line (*pad4-5*; see below) was stably transformed with wild-type *PAD4* containing an N-terminal c-Myc epitope tag expressed from the native *PAD4* promoter (Myc::PAD4). The c-Myc epitope did not interfere with PAD4 function, as several independent transgenic lines exhibited full resistance to *P.parasitica* isolate Noco2 (data not shown). In addition, we generated anti-EDS1 polyclonal antiserum (see Materials and methods). Both the anti-c-Myc antibody and the anti-EDS1 antiserum detected single protein bands of the expected size (69.6 kDa for Myc::PAD4, 71.5 kDa for EDS1) in total soluble extracts of the *Myc::PAD4* transgenic line, but not in protein extracts from the *pad4-5* or *eds1-1* mutant, respectively (Figure 4A and B).

Immunoprecipitation of EDS1 protein from extracts of the *Myc::PAD4* line using the anti-EDS1 antiserum, followed by detection on western blots using the anti-c-Myc antibody, showed that the EDS1 and PAD4 proteins interact in healthy plant tissue (Figure 4A). Pre-immune serum was not able to co-immunoprecipitate the EDS1–PAD4 complex, indicating that immunoprecipitation was specific for EDS1. In addition, no signal was detected when co-immunoprecipitation was performed with protein extracts from the *pad4-5* mutant line, showing that the Myc::PAD4 protein was specifically detected after immunoprecipitation (Figure 4A). Co-immunoprecipitation experiments, performed by first immunoprecipitating Myc::PAD4 with anti-c-Myc antibodies, followed by western blot detection of EDS1, also showed specific interaction between EDS1 and PAD4 (data not shown). Co-immunoprecipitation experiments using independent *Myc::PAD4* transgenic lines gave identical results (data not shown). These data demonstrate that interaction between EDS1 and PAD4 in the yeast two-hybrid assay reflects their ability to associate in the plant.

We tested, by co-immunoprecipitation, whether the extent of EDS1–PAD4 association changed after pathogen inoculation of plants. EDS1 and PAD4 protein levels in total leaf extracts did not alter substantially after infection with a virulent *P.parasitica* isolate, Emwa1, except at day 6 when increases in the abundance of both proteins were detectable (Figure 4B). At all stages of *P.parasitica* infection, PAD4 co-immunoprecipitated with EDS1. At day 6, enhanced levels of both proteins correlated with increased detection of co-immunoprecipitable PAD4 (Figure 4B), suggesting that EDS1 and PAD4 expression and physical association respond to pathogen colonization. Similar results were obtained after inoculation of plants with avirulent *P.parasitica* isolate, Noco2, although in this interaction no increases in total or co-immunoprecipitable EDS1 and PAD4 were observed by day 6 (data not shown). Leaves of mature plants were infiltrated with the virulent *P.syringae* pv. *tomato* strain, DC3000, or with avirulent DC3000 expressing *avrRps4* (DC3000/*avrRps4*), allowing synchronous infection of a larger area of tissue than could be achieved with *P.parasitica*. Enhanced levels of EDS1 and PAD4 protein were observed in tissues responding to DC3000/*avrRps4* 12 h after infiltration and these persisted for up to 48 h (Figure 4B). Increased EDS1 and PAD4 expression correlated with enhanced detection of co-immunoprecipitable PAD4 (Figure 4B). A slight increase in EDS1 and PAD4 expression was also observed after inoculation with virulent DC3000, although this occurred later (48 h) than in tissues inoculated with DC3000/*avrRps4* (Figure 4B). In addition, leaves were sprayed with the bioactive SA analogue, benzothiadiazole (BTH). Here, elevated EDS1 and PAD4 expression corresponded with higher levels of co-immunoprecipitable PAD4 24 h after treatment (Figure 4C).

Analysis of the spectrum of *Arabidopsis R* genes requiring EDS1 and PAD4

Our findings that EDS1 and PAD4 interact in a two-hybrid assay and in plant cells prompted us to assess the effects of *eds1* and *pad4* mutants on a broader range of *R* gene-mediated responses within the same plant genetic

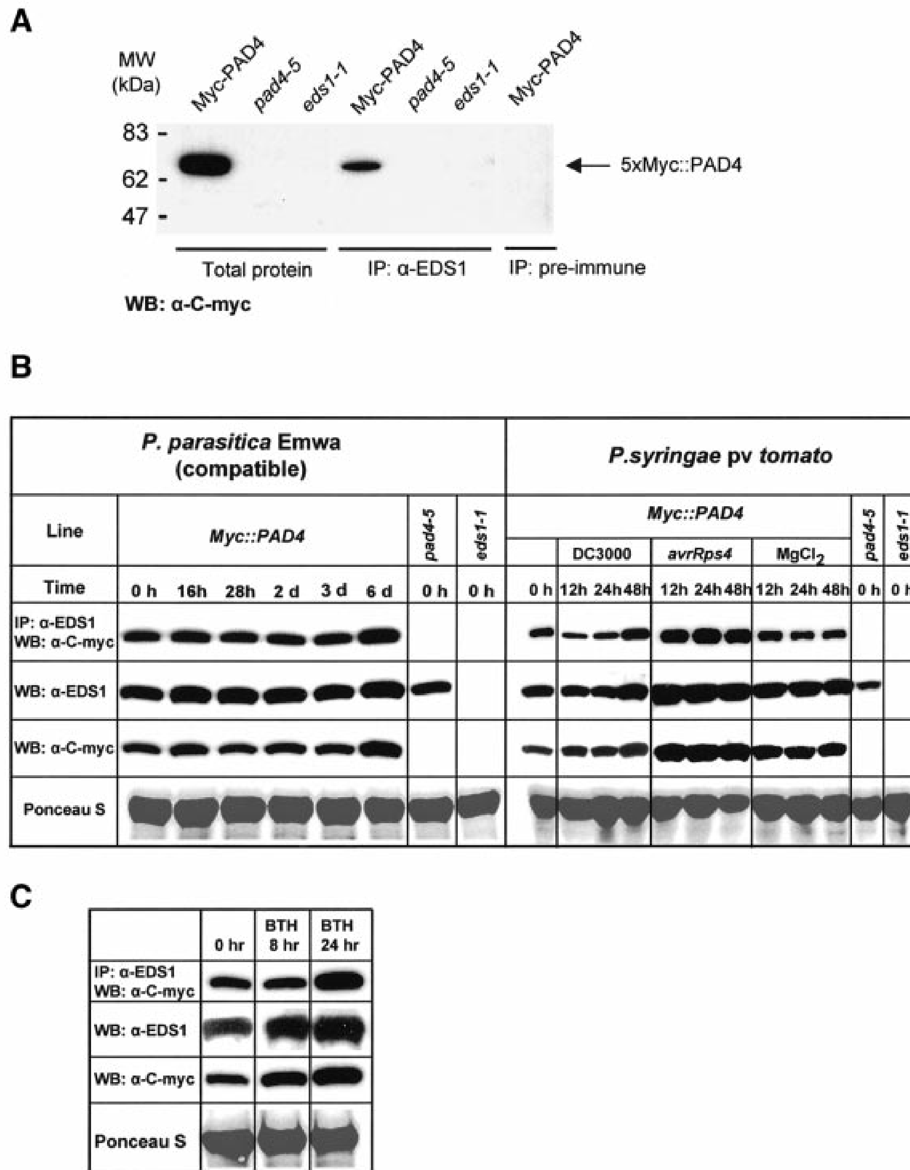

Fig. 4. *In planta* protein interaction between *EDS1* and *PAD4*. (**A**) Co-immunoprecipitation of EDS1 and PAD4 in total plant protein extracts. Protein extracts were prepared from the transgenic *pad4-5* (5× Myc::PAD4) line, indicated as Myc-PAD4, or from the *pad4-5* and *eds1-1* mutants. For immunoprecipitation reactions pre-immune (as control) or EDS1 antiserum was used, followed by western blotting detection with anti-c-Myc antibody. Total protein extracts were analyzed on the same western blot to show the specificity of the anti-c-Myc antibody. (**B**) Analysis of EDS1 and PAD4 protein expression and co-immunoprecipitation in healthy and pathogen-challenged plants. Leaves of the 5× Myc::PAD4 epitope-tagged transgenic line were spray inoculated with *P.parasitica* spores (1×10^5/ml in dH_20) or infiltrated with suspensions (5×10^6/ml colony forming units in 10 mM $MgCl_2$) of DC3000, DC3000 expressing *avrRps4* or 10 mM $MgCl_2$ alone, and tissues harvested at the time points indicated. Levels of EDS1 and PAD4 protein were measured on western blots of total soluble extracts probed with anti-EDS1 and anti-c-Myc antibody, respectively. Co-immunoprecipitations were performed on the same tissue extracts, as described in (A). Equal loading of blots is indicated by Ponceau S staining of total protein. An independent experiment gave similar results. (**C**) Analysis of EDS1 and PAD4 protein expression and co-immunoprecipitation in leaves treated with BTH. Tissues were harvested and analyzed as described in (B). Similar results were obtained in an independent experiment.

background. This would establish whether *EDS1* and *PAD4* functions are tightly associated genetically or can be partially separated into different pathways.

First, the effects of *eds1-2* and *pad4-2* on *RPP* genes recognizing distinct *P.parasitica* isolates were measured in accession Ler. As shown in Table I, resistance responses mediated by *RPP5* and *RPP21* that are fully *EDS1* dependent (Aarts *et al*., 1998) were partially compromised by *pad4*, producing the characteristic trailing necrotic phenotype (see also Figure 1). In contrast, *RPP7*- and *RPP8*-mediated resistance operated independently of both *EDS1* and *PAD4* (Table I). Analysis was extended to *R* genes expressed in accession Ws-0 by isolating a line containing a T-DNA insertion in *PAD4* (*pad4-5*; see Materials and methods). The *pad4-5* mutant is an mRNA null mutant, since transcripts could not be detected using sensitive TaqMan analysis (see below). Here, we found that the *EDS1*-dependent *RPP1A*, *1B* and *1C* genes (Aarts *et al*., 1998) conferred partial resistance in *pad4-5* with a similar phenotype to that observed for *RPP5* and *RPP21* in *pad4-2* (Table I). We then measured the effects of *eds1-2*, *pad4-2* and the *NahG* transgene on *RPM1*-, *RPS2*- and

Table I. Suppression of *RPP* gene-mediated resistance to *P.parasitica* in leaves of *eds1* and *pad4* in accessions Ler and Ws-0

	Plant *R* gene (*P.parasitica* isolate)					
Plant Line	*RPP5* (Noco2)	*RPP8* (Emco5)	*RPP4/8* (Emwa1)	*RPP21* (Maks9)	RPP7[a] (Hiks1)	– (Cala2)
Ler	R	R	R	R	R	S
eds1-2	S*	R	R	S*	R	S*
pad4-2	(S)	R	R	(S)	R	S*
	RPP1A,B,C (Noco2)	*RPP1A,B* (Emoy2)	*RPP1A* (Cala2)	– (Emwa1)		
Ws-0	R	R	R	S		
eds1-1	S*	S*	S*	S-S*		
pad4-5	(S)	(S)	(S)	S-S*		

Two-week-old seedlings were scored 5 and 7 days after inoculation with *P.parasitica*. Ler is genetically susceptible to *P.parasitica* isolate Cala2 and Ws-0 is susceptible to isolate Emwa1. Phenotypes were assigned as R (fully resistant, wild-type HR), S (susceptibility of genetically compatible lines), S* (hypersusceptible, permitting more abundant sporulation than the genetically susceptible line), (S) (partially susceptible, mycelium development accompanied by trailing plant cell necrosis and occasional sporophores). Similar results were obtained in two independent experiments. While *eds1-2* and *pad4-2* reproducibly exhibited hypersusceptibility to Cala2, *eds1-1* and *pad4-5* gave variable results between experiments, as indicated.
[a]*RPP7* in Ler is defined as an *R* locus cosegregating with Col-0 *RPP7* in >4000 Col-0 × Ler F2 seedlings (E.Holub, personal communication).

RPS4-mediated resistance in Ler to *P.syringae* pv. *tomato* DC3000 expressing, respectively, *avrRpm1*, *avrRpt2* and *avrRps4*. Leaves were dipped into bacterial suspensions and bacterial growth and disease symptoms monitored over 5 days. Wild-type plants restricted growth of all the avirulent strains compared with growth of virulent DC3000 (Figure 5A). The *eds1-2* mutation fully suppressed *RPS4* resistance but did not affect resistance mediated by *RPM1* and weakly compromised *RPS2* resistance (Figure 5A). In leaves of *pad4-2* and Ler-*NahG*, growth of DC3000/*avrRps4* was intermediate between that observed in wild-type Ler and *eds1-2* (Figure 5A). This correlated with a slow and sporadic appearance of chlorotic symptoms in *pad4-2* and Ler-*NahG*, compared with *eds1-2* (Figure 5B). Like *eds1-2*, *pad4-2* caused a slight relaxation of *RPS2*-mediated resistance but had no effect on resistance conditioned by *RPM1* (Figure 5A). Interestingly, Ler-*NahG* permitted significant growth of both DC3000/*avrRpt2* and DC3000/*avrRpm1* (Figure 5A), suggesting that SA is furnished in an *EDS1*- and *PAD4*-independent manner in these responses.

The *P.parasitica* and *P.syringae* infection studies reveal that all strongly *EDS1*-dependent *R* gene responses examined have a partial requirement for *PAD4*, and that *EDS1*-independent interactions are also independent of *PAD4*. These data suggest that PAD4 and EDS1 function within the same defence signaling pathway.

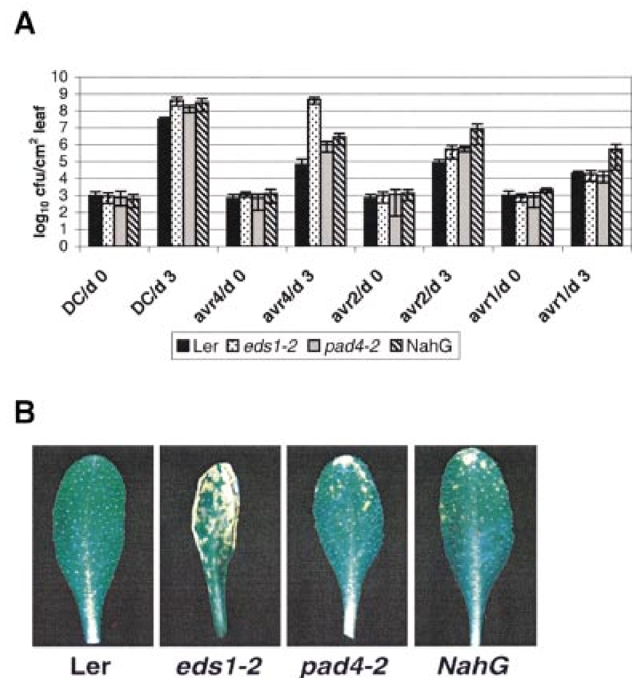

Fig. 5. Growth and symptom development of different *P.syringae* strains in leaves of wild-type Ler, *eds1-2*, *pad4-2* and *NahG* plants. (**A**) Leaves of 4-week-old short day grown plants were infiltrated with a suspension (1×10^5 colony forming units/ml) of *P.syringae* pv. *tomato* strain DC3000 containing an empty vector (DC3000) or DC3000 expressing *avrRps4*, *avrRpt2* or *avrRpm1*. Bacterial titres were measured at 0 and 3 days after inoculation. The measurements and standard errors are derived from four replicates per treatment. An independent experiment gave similar results. (**B**) Leaves were dipped in a suspension (1×10^7 c.f.u./ml) of DC3000 expressing *avrRps4* and disease symptoms observed over 6 days. As shown at day 5, Ler plants appear healthy, *eds1-2* plants develop severe leaf spotting symptoms, while *pad4-2* and *NahG* plants exhibit mild leaf spotting.

Pathogen-induced SA accumulation in eds1 and pad4

In independent studies, *PAD4* and *EDS1* have been implicated as regulators of SA-dependent defences (Zhou *et al*., 1998; Falk *et al*., 1999). We therefore wished to examine the relative contributions of *EDS1* and *PAD4* to SA accumulation in the same *R* gene response. Salicylic acid accumulation profiles of wild-type Ler, *eds1-2* and *pad4-2* leaves were analyzed after triggering an *EDS1*/*PAD4*-dependent *R* gene response (*RPS4*-mediated resistance), an *EDS1*/*PAD4*-independent response (*RPM1*-mediated resistance) or in a compatible interaction with *P.syringae* DC3000. We found that *eds1-2* and *pad4-2* severely depleted SA accumulation after infection with virulent DC3000 (Figure 6). Salicylic acid

accumulation was also abolished in *eds1-2* plants and was strongly reduced in *pad4-2* after inoculation with DC3000/*avrRps4* (Figure 6). In contrast, *eds1-2* and *pad4-2* did not compromise SA accumulation in plants responding to DC3000/*avrRpm1* (Figure 6). The importance of EDS1 and PAD4 as regulators of SA levels therefore correlates with a genetic requirement for their functions in *RPS4*-mediated resistance. In *RPM1*-mediated resistance SA accumulation bypasses both *EDS1* and *PAD4*, consistent with the bacterial growth data in *NahG* plants showing a requirement of *RPM1* resistance for SA but not for *EDS1* or *PAD4* (Figure 5A).

Analysis of EDS1 and PAD4 transcripts

EDS1 (Falk *et al.*, 1999) and *PAD4* (Jirage *et al.*, 1999) mRNAs are induced in response to pathogen inoculation or SA treatment. Here, we examined whether their mode of expression is affected by a mutation in either gene or by the presence of *NahG*. Leaves were infiltrated with a suspension of DC3000/*avrRps4* in 10 mM $MgCl_2$ or with 10 mM $MgCl_2$ alone, or were sprayed with BTH. Total RNA was extracted at various time points up to 48 h after treatment, reverse transcribed into cDNA, and *EDS1* and *PAD4* mRNA levels measured using real-time quantitative PCR and TaqMan chemistry (Holland *et al.*, 1991). This procedure (Wang and Brown, 1999) is particularly suitable for measuring expression levels of rare transcripts, such as *EDS1* (Falk *et al.*, 1999; Clarke *et al.*, 2001). The *Arabidopsis* actin gene *ACT2* was chosen as a normalization standard because of its constitutive expression in nearly all vegetative tissues in juvenile and mature plants (An *et al.*, 1996; see Materials and methods).

The fold induction of *EDS1* and *PAD4* mRNA levels in Ler wild-type and mutant lines after bacterial inoculation or BTH treatment is shown in Figure 7A. *EDS1* expression was induced after inoculation with DC3000 alone or DC3000 expressing *avrRps4*. The presence of *pad4-2* or depletion of SA by *NahG* partially compromised pathogen-induced *EDS1* expression. Expression of *PAD4* mRNA was more strongly induced than *EDS1* in pathogen-treated Ler plants. This induction was severely reduced in *eds1-2* plants and partially compromised by *NahG*. Neither mutant transcript responded strongly to pathogen inoculation, suggesting a requirement for functional protein in optimal upregulation and/or stability of their respective transcripts. As shown previously, applications of BTH induced expression of *EDS1* and *PAD4* mRNAs (Falk *et al.*, 1999; Jirage *et al.*, 1999). Mutations in either gene had little or no effect on the induction of the other gene, suggesting that BTH may lead to increased expression of *EDS1* and *PAD4* independently of each other.

The Ler data show that loss of *EDS1* function has a much stronger negative effect on *PAD4* expression than defective *PAD4* does on *EDS1* expression. The results were reinforced by a similar analysis of Ws-0 wild type and corresponding *eds1-1* and *pad4-5* mutant lines (Figure 7B). Inoculation of Ws-0 with DC3000 or DC3000/*avrRps4* induced levels of both *EDS1* and *PAD4* mRNAs, although the overall fold induction of *PAD4* mRNA was considerably higher than that observed in Ler. Pathogen induction of *PAD4* expression was almost completely abolished in *eds1-1* plants. In contrast, *pad4-5* did not suppress induction of *EDS1* mRNA in the plant response to DC3000/*avrRps4* and had a partial effect on *EDS1* mRNA induction in response to DC3000.

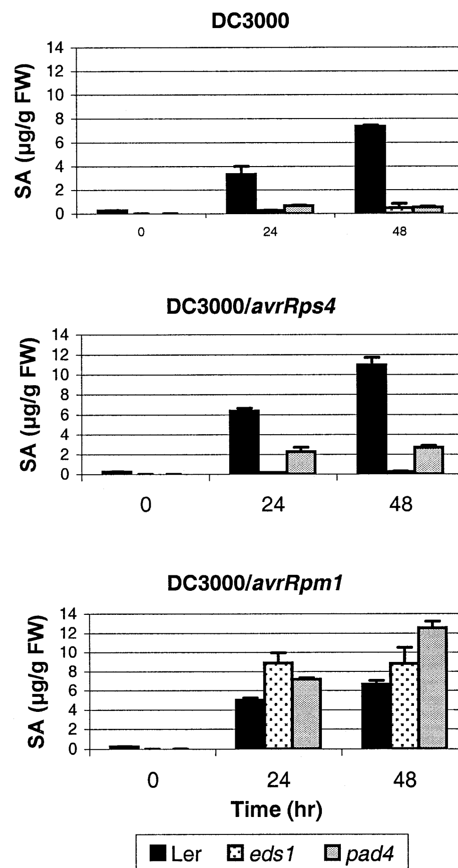

Fig. 6. Accumulation of total salicylic acid in Ler, *eds1-2* and *pad4-2* plants after inoculation with virulent and avirulent *P.syringae* pv. *tomato* DC3000 strains Leaves of 4-week-old short day grown plants were dipped in a suspension (1×10^7 c.f.u./ml) of DC3000 (top panel), DC3000 expressing *avrRps4* (middle panel) or *avrRpm1* (bottom panel). Total salicylic acid (SA) was extracted and quantified after 0, 24 and 48 h by HPLC as described in Materials and methods. Salicylic acid measurements and standard errors are derived from three replicate samples per treatment. Salicylic acid was present in trace amounts in Ler-*NahG* plants at all stages of infection (data not shown).

TaqMan analysis of mRNA abundance was shown previously to accurately reflect *EDS1* expression in wild-type and mutant plants (Clarke *et al.*, 2001). We found that our estimations of *PAD4* mRNA abundance by TaqMan analysis also correlated well with *PAD4* mRNA levels measured on an RNA gel blot (compare Figure 7B bottom panel with Figure 7C). RNA gel blot analysis of selected samples was further used to determine the consequences of the *eds1* and *pad4* mutations on downstream plant defences by measuring expression levels of the SA-responsive marker gene *PR1*. As shown in Figure 7C, pathogen-induced expression of *PR1* in wild-type Ws-0 plants was abolished by *eds1-1* and strongly suppressed by *pad4-5*. In these tests, *PR1* expression was fully rescued in *eds1-2* and *pad4-2* in response to BTH treatment (Figure 7C), consistent with the placement of *EDS1* and *PAD4* upstream of SA accumulation.

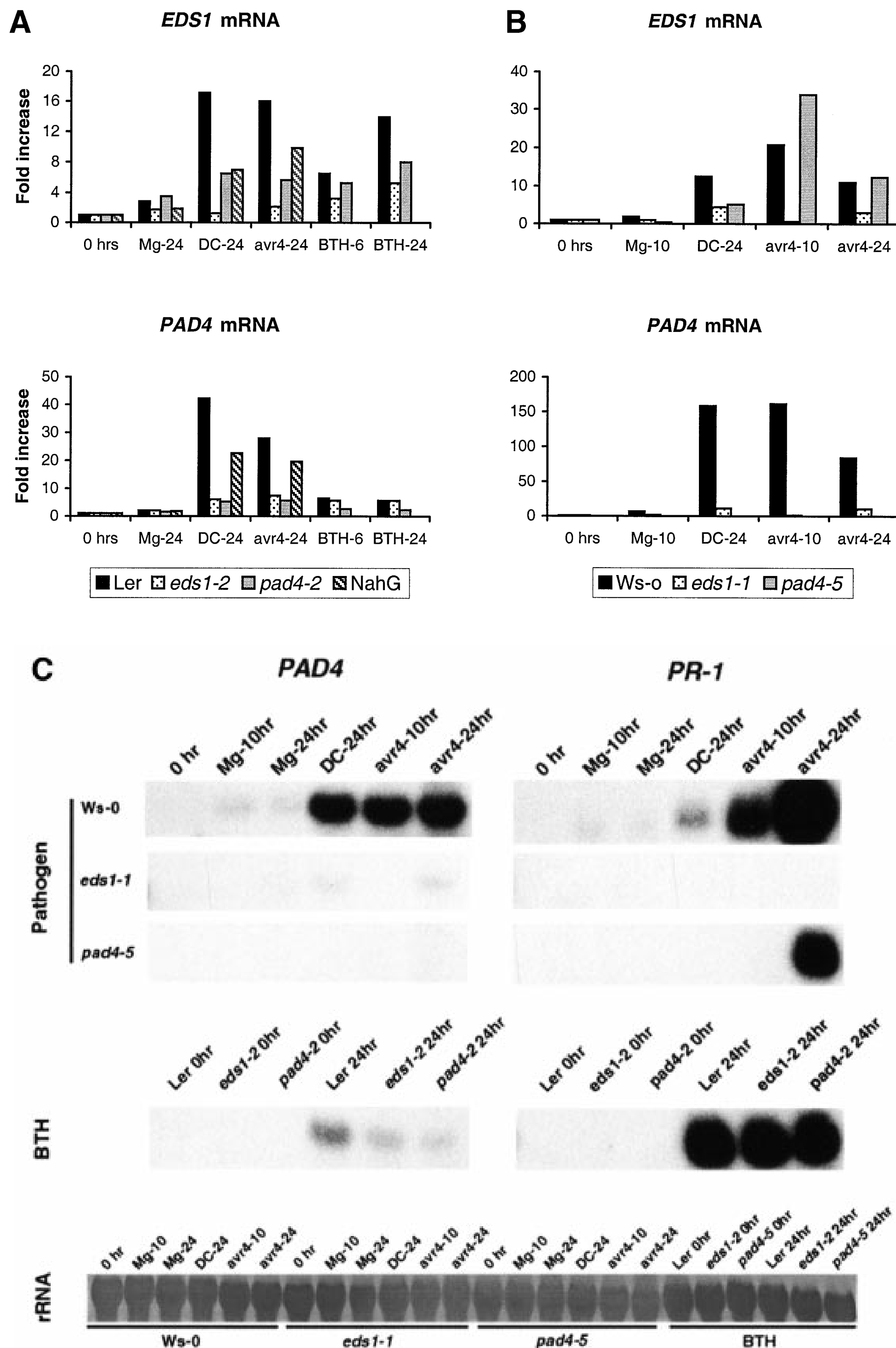

Fig. 7. Abundance of *EDS1*, *PAD4* and *PR-1* mRNAs in pathogen-inoculated and BTH-treated plants. (**A**) Leaves of 5-week-old Ler, *eds1-2*, *pad4-2* and Ler-*NahG* plants were hand-infiltrated with 10 mM $MgCl_2$ (Mg), 1×10^7 c.f.u./ml *P.syringae* DC3000 (DC) or *P.syringae* DC3000 expressing *avrRps4* (avr4), or sprayed with 300 μM benzothiadiazole (BTH). Material was harvested after 24 h for the bacterial inoculations and 6 and 24 h for the BTH treatment. Messenger RNA abundance was determined using TaqMan chemistry (see Materials and methods). *EDS1* and *PAD4* mRNA levels are normalized relative to the internal control *ACT2*, and are calculated relative to expression at 0 h. (**B**) Leaves of 5-week-old Ws-0, *eds1-1* and *pad4-5* plants were pathogen challenged as in (A). Material was harvested 10 and 24 h after challenge. *PAD4* mRNA is undetectable in the *pad4-5* mutant. Relative quantification using TaqMan chemistry is as described in (A). (**C**) RNA gel blot analysis of *PAD4* and *PR-1* mRNA expression. Samples from (B) plus BTH-treated samples from (A) were analysed to verify TaqMan results for *PAD4* and examine the expression of *PR-1*. Results for *PAD4* (left) and *PR-1* (right) are shown after pathogen challenge (top panel) and BTH treatment (middle panel). Control for equal loading is shown in the bottom panel (rRNA).

Discussion

We present genetic and molecular evidence that EDS1 and PAD4, two plant disease resistance signaling proteins, function within the same defence pathway. They are required for an identical spectrum of *R* genes recognizing avirulent *P.parasitica* and *P.syringae* isolates, as well as for restriction of growth of virulent isolates of these pathogens. EDS1 and PAD4 interact specifically in a yeast two-hybrid assay and co-immunoprecipitate in total plant

protein extracts, suggesting that direct association may be important for their cellular roles. Also, *EDS1* and *PAD4* are necessary for accumulation of the plant defence signaling molecule, SA, in response to virulent *P.syringae* or in resistance triggered by an *EDS1/PAD4*-dependent *R* gene, *RPS4*, but not in resistance conferred by an *EDS1/PAD4*-independent *R* gene, *RPM1*. Furthermore, upregulation of *PAD4* expression after pathogen attack depends on *EDS1* function.

In *R* gene-mediated responses that exhibited a requirement for *EDS1* and *PAD4*, the null *eds1* and *pad4* mutant phenotypes were quite distinct. While *eds1* plants failed to elaborate a hypersensitive response and were hypersusceptible to *P.parasitica* infection, *pad4* plants retained the HR and exhibited intermediate susceptibility. Thus, the HR in *pad4* is not sufficient to restrict pathogen colonization fully. We conclude from these data that wild-type EDS1 and PAD4 have intrinsically different functions within the defence pathway. *EDS1* appears to exert a critical, early role during race-specific resistance, whereas *PAD4* serves to reinforce the initial resistance response. This idea is supported by the results of another study showing that *EDS1* but not *PAD4* is required for an HR-associated oxidative burst triggered by either *RPP1*- or *RPS4*-mediated pathogen recognition (Rustérucci *et al.*, 2001).

Zhou *et al.* (1998) demonstrated that *PAD4* is a regulatory component of SA accumulation in plants after inoculation with a virulent *P.syringae* pv. *maculicola* isolate. Salicylic acid is also a necessary component of systemic immunity (Dempsey *et al.*, 1999) and can function as a resistance-potentiating signal in cooperation with ROI (Shirasu *et al.*, 1997; Delledonne *et al.*, 1998; Klessig *et al.*, 2000). Here we demonstrate that *PAD4* is required to furnish SA in *EDS1*-dependent *R* gene-triggered resistance (Figure 6). A major role of *PAD4* in this pathway is therefore likely to positively regulate SA accumulation. This is supported by the similar levels of intermediate *RPS4* resistance observed in *pad4-2* and *NahG* plants after challenge with *P.syringae* carrying *avrRps4* (Figure 5). Partial loss of *RPP5*-mediated resistance to *P.parasitica* Noco2 was also exhibited by *NahG* plants (Figure 1). However, the extent of *P.parasitica* growth and trailing plant cell necrosis was less in *NahG* than in *pad4-2*, suggesting that PAD4 wild-type protein has an additional defence role besides regulating SA accumulation, at least in *RPP5*-mediated resistance.

The *eds1-2* mutation almost completely abolished SA accumulation in *RPS4*-mediated resistance and this correlated with the absence of detectable *PR1* gene expression compared with wild-type plants (Figures 6 and 7C). In contrast, *pad4-2* depleted but did not totally remove SA, tallying with a partial suppression of *PR1* induction. Thus, an *EDS1*-dependent but *PAD4*-independent mechanism exists to generate residual SA, invoking a function for EDS1 that is separable from processes requiring both EDS1 and PAD4. It is possible that the low level of SA accumulation in *pad4* plants is derived from an early, EDS1-regulated mechanism associated with the HR and that subsequent, enhanced SA generation depends on EDS1 and PAD4 activities.

Our data point to a requirement for *EDS1* upstream of *PAD4* in the *R* gene-mediated defence pathway leading to the HR, but together with *PAD4* in driving maximal SA accumulation during defence potentiation, as shown in the model in Figure 8. In this model, PAD4 activity is contingent on the presence of EDS1. This is further supported by the dependence of *PAD4* mRNA upregulation on *EDS1* during pathogen challenge (Figure 7). Thus, there appear to be two distinct EDS1 activities (Figure 8). The fact that *eds1* and *pad4* exhibit an eds phenotype when challenged with a number of virulent pathogens (Glazebrook *et al.*, 1996; Parker *et al.*, 1996; Aarts *et al.*, 1998; see also Figure 5A) reveals functions of both proteins in basal plant resistance. This low level resistance is likely to be at least partially dependent on SA accumulation since *pad4-2* and *eds1-2* were strongly depleted in SA after challenge with virulent *P.syringae* DC3000 (Figure 6). It may be that basal resistance is exerted by the combined EDS1–PAD4 'potentiating' activities, consistent with the presence of a pre-existing EDS1–PAD4 protein complex in unchallenged plant tissues (Figure 4). A different attribute of EDS1 would be engaged to transduce early R-Avr protein-triggered signals leading to the HR. Other recent genetic analyses support our model. First, *EDS1* and *PAD4* operate at a similar position in defence pathways induced by the *cpr1* and *cpr6* (constitutive expressor of *PR* genes) mutations (Clarke *et al.*, 2001; Jirage *et al.*, 2001). Secondly, *EDS1* and *PAD4* are both necessary signaling components of runaway cell death triggered by the *lsd1* mutation, in a mechanism that is separable from events associated with the plant HR (Rustérucci *et al.*, 2001).

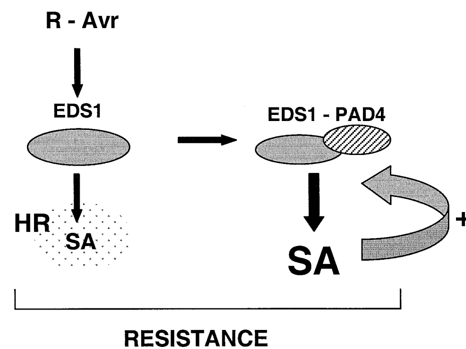

Fig. 8. A model for the roles of EDS1 and PAD4 in *R* gene-mediated resistance. Two functions are proposed for EDS1 in R-Avr protein-triggered resistance at pathogen infection foci. One lies upstream of the plant HR (indicated by the stipled area) and is required for a low level of SA accumulation. The second function recruits PAD4, possibly through direct EDS1–PAD4 interaction, and drives amplification of local defences through enhanced accumulation of SA and other molecules (indicated by the curved arrow). Complete containment of the pathogen requires both the HR and defence signal potentiation.

The yeast two-hybrid data show that EDS1 interacts with itself through the C-terminal half of at least one partner (Figure 2B). EDS1 also associates with PAD4 through the PAD4 N-terminal portion. The differential effect of the *eds1* (E466K) mutation on EDS1 dimerization and EDS1–PAD4 interaction, coupled with the fact that PAD4 interacts with EDS1 through its N-terminus, whereas EDS1 homodimerization requires at least one C-terminus, suggests that the two complexes are arranged in a different way. This could result in the two associations fulfilling quite different functions during a resistance response. Western blotting analysis of the *eds1-1* mutant line shows a lack of detectable mutant EDS1 protein in

plant extracts (Figure 4B), in contrast with the stable expression of EDS1 (E466K) protein in yeast. The absence of detectable mutant EDS1 protein *in planta* may reflect a signaling failure or misfolding of the protein, leading to targeted degradation.

Our co-immunoprecipitation data show that EDS1 associates with PAD4 in healthy and pathogen-challenged plant tissues, suggesting that specific interaction may be important for their roles in defence potentiation, as depicted in Figure 8. Increased abundance of EDS1 and PAD4 proteins after pathogen inoculation or BTH treatment correlated with elevated levels of co-immunoprecipitable protein (Figure 4), indicating that at least a proportion of the increased EDS1 and PAD4 is incorporated into a complex. It is conceivable that pre-existing EDS1–PAD4 complexes play a role in basal resistance against virulent pathogens (discussed above). This raises the question of how an early, PAD4-independent function of EDS1 in TIR-NB-LRR protein-mediated pathogen recognition is exerted. Triggering of the *R* gene pathway does not appear to cause a dramatic alteration in EDS1–PAD4 protein association. However, we cannot discount the possibility that a sub-population of EDS1 molecules or EDS1 homodimers (as implicated by the yeast two-hybrid analysis; Figure 2) performs a critical early signaling role in *R* gene-mediated defence. It is interesting in this context that EDS1 protein stability does not depend on the presence of PAD4 (Figure 4). Alternatively, EDS1 may perform an additional activity within an EDS1–PAD4 complex that does not require PAD4 function.

EDS1 and PAD4 share predicted lipase catalytic motifs (Falk *et al*., 1999; Jirage *et al*., 1999), suggesting that hydrolytic activities may contribute to their signal transduction roles. Indeed, the different enzymatic specificity and/or kinetic properties of an EDS1 homodimer versus an EDS1–PAD4 heterodimer could dictate placement of the respective complexes within the resistance pathway. However, it remains to be established whether these lipase domains are enzymatically functional. There are precedents for dimerization of lipases/esterases in various systems. In mammals, hormone-sensitive lipase exists as a functional dimer with a 40-fold greater activity than the monomer (Shen *et al*., 2000), whereas lipoprotein lipase (LPL) exists as both an inactive monomer and an active dimer *in vivo* (Bergö *et al*., 1996). Interestingly, LPL also has a binding capacity rather than catalytic function, involved in linking triglyceride-rich lipoproteins and cholesterol-rich lipoproteins to the cell surface (Pentikäinen *et al*., 2000). Similarly, EDS1 and/or PAD4 could be involved in binding particular substrates, rather than enzymatically processing them. In bacteria, crystallographic analysis of the *Pseudomonas fluorescens* carboxylesterase, which belongs to the α/β hydrolase family of proteins that also contains lipases, reveals that this protein exists as a homodimer with the two subunits facing each other in a head-to-head fashion, thereby bringing the active sites together (Kim *et al*., 1997). The active LPL homodimer, on the other hand, seems to be arranged in a head-to-tail fashion (Wong *et al*., 1997).

Quantitation of *EDS1* and *PAD4* mRNA levels in wild-type, mutant, and *NahG* plants shows that *EDS1* is essential for the upregulation of *PAD4* mRNA levels in the plant–pathogen interaction, but that *PAD4* contributes in a minor way to the enhanced expression of *EDS1* mRNA (Figure 7A and B). Salicylic acid also appears to be a contributory factor in the expression of both genes (Figure 7A), consistent with participation of SA in a positive feedback loop (Falk *et al*., 1999; Jirage *et al*., 1999). However, other factors are likely to be involved either in the initial induction of *EDS1* and *PAD4* mRNAs or in the amplification of expression. For example, ROI produced during the HR (Rustérucci *et al*., 2001) may influence *EDS1* and *PAD4* expression either directly or indirectly (Levine *et al*., 1994; Orozco-Cárdenas *et al*., 2001). Indeed, the perpetuated HR and associated oxidative burst of pathogen-inoculated *pad4* and *NahG* plants may account for some of the residual *EDS1* expression observed in these backgrounds. Upregulation of *EDS1* and *PAD4* mRNA appears to require protein activity since the mutant *eds1* and *pad4* mRNAs do not respond strongly to pathogen inoculation (Figure 7), again indicative of positive feedback on expression either by the protein itself or by downstream molecules. A fuller examination of the mode of EDS1 and PAD4 protein expression, their biochemical activities and molecular associations in wild-type and mutant plants after pathogen challenge should provide important insights into their signaling roles in plant disease resistance.

Materials and methods

Plant material and pathogen strains

The isolation of *eds1-1* and *eds1-2* (Parker *et al*., 1996; Falk *et al*., 1999) and *pad4-2* (Jirage *et al*., 1999) mutants has been described before. The *pad4-5* mutant was identified by a reverse-genetic screen of the Feldmann T-DNA lines (Ws-0 background; Feldmann *et al*., 1991) for insertions into the *PAD4* gene. A homozygous insertion line was identified in a subpool of 10 single insertion lines (stock CS12182 at the Arabidopsis Biological Resource Center, Columbus, OH). Sequencing of the T-DNA border–*PAD4* junction revealed that the T-DNA had inserted 35 bp 5′ to the end of the unique intron in the *PAD4* gene. *Pseudomonas syringae* strains and *P.parasitica* isolates used in this study have been described before (Aarts *et al*., 1998; McDowell *et al*., 2000).

Construction of c-Myc tagged PAD4 transgenic line

The *PAD4* coding sequence was PCR amplified as a *Nco*I–*Bam*HI fragment cloned into SLJ4C3 upstream of the NOS terminator. The 35S promoter was removed and replaced with 1 kb of *PAD4* promoter amplified as an *Eco*RI–*Nco*I fragment. A *Nco*I cassette containing five copies of the c-Myc epitope (derived from pJR1265, R.Hampton and J.Rine, personal communication) was inserted between the *PAD4* promoter and the *PAD4* coding sequence to give an N-terminal fusion. The construct was transferred to the binary Basta-resistant plasmid SLJ755I5, conjugated to *Agrobacterium tumefaciens* GV3101, and *pad4-5* mutant plants were transformed using the flower-dip method (Clough and Bent, 1998). Several independent homozygous lines with single locus T-DNA inserts were identified and confirmed to confer full resistance to *P.parasitica* isolate Noco2.

Antibody production, immunoprecipitation and western blot analysis

A mutant form of EDS1 (G125R) was highly expressed in *Escherichia coli* M15 (pREP4) as a fusion to a His_6 tag (Qiagen), was purified on TALON metal affinity resin (Clontech) and further purified by electro-elution from a preparative SDS–PAGE gel. New Zealand white male rabbits were immunized and sera were collected.

Total protein extracts were prepared from 5-week-old leaf material after grinding in liquid nitrogen and extracting in 50 mM Tris pH 8.0, 150 mM NaCl and 1 mM EDTA, containing 1× Protease Inhibitor Cocktail (Sigma). Samples were spun at 16 000 *g* for 20 min and protein concentration of the soluble fraction was determined using Bradford reagent (Bio-Rad) and bovine serum albumin as a standard. For

immunoprecipitation (IP) reactions, 1 mg of total protein was incubated with 5 µl of EDS1 antiserum (or pre-immune serum) in a total volume of 1 ml of IP buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.01% Triton X-100) and rotated end-over-end at 4°C for 90 min. Protein A/G Plus beads (35 µl, equilibrated in IP buffer; Santa Cruz Biotechnology) were added and the reactions were incubated for a further 60 min. IP reactions were washed three times with 1 ml of ice-cold IP buffer, resuspended in 40 µl of SDS–PAGE sample buffer, boiled for 5 min and 10 µl run on 7.5% SDS–PAGE gels (Bio-Rad). For standard western blotting analysis, 50 µg of total protein were loaded. Proteins were electroblotted to PVDF membranes (Amersham), blocked for 1 h at room temperature in phosphate-buffered saline (PBS)-Tween containing 5% (wt/vol) non-fat dried milk. Incubation with primary antibodies was in PBS-Tween containing 5% (wt/vol) non-fat dried milk using the following dilutions: EDS1 antiserum (1:7500); anti c-Myc 9E10 (1:5 000; Santa Cruz Biotechnology). Blots were developed using the SuperSignal West Pico Chemiluminescent kit (Pierce).

Pathogen and BTH treatments

Inoculations with *P.parasitica* and *P.syringae*, as well as calculation of growth curves were performed as described by Aarts *et al*. (1998). BTH wettable powder (Novartis, Basel; Lawton *et al*., 1996) was resuspended in water at a concentration of 300 µM active ingredient and plants were sprayed to imminent run off.

Yeast two-hybrid analysis

We used the LexA two-hybrid system (kindly provided by Roger Brent, Massachusetts General Hospital, Boston, MA; Gyuris *et al*., 1993). Details of the construction of the two-hybrid cDNA library are in van der Biezen *et al*. (2000). Two-hybrid analyses were carried out according to Golemis *et al*. (1998). The various *EDS1* and *PAD4* domains were generated by PCR, verified by sequencing on a Perkin-Elmer ABI377 sequencing machine, and subcloned into pLexA and/or pJG4-5. All pLexA constructs were tested for auto-activation of the LacZ and LEU2 reporters. Repression assays with JK101 confirmed protein fusion synthesis and nuclear localization of the LexA fusion protein. pLex-EDS1 showed slight auto-activation of the LEU2 reporter, and was therefore transformed in the less sensitive yeast strain EGY191. All other two-hybrid combinations were performed in the standard yeast strain EGY48. Western blotting analysis was performed on total yeast protein extracts, derived from galactose-induced cultures, using anti-LexA (Santa Cruz Biotechnology) and anti-HA (Roche) antibodies. The *eds1-1* (E466K) mutation was introduced using site-directed mutagenesis with the QuickChange kit (Stratagene). Quantitative β-galactosidase assays were performed on a minimum of six independent yeast transformants for each combination of interactors, and the average for each interaction was calculated as described in Ausubel *et al*. (1996). The experiment was performed twice with similar results.

Determination of SA levels

The procedure for SA extraction and determination is described in Newman *et al*. (2001).

Transcript analysis

Total RNA was isolated using Tri Reagent (Sigma). For TaqMan analysis, 1–2 µg of total RNA were reverse transcribed using Expand Reverse Transcriptase (Roche) and random hexamers (Pharmacia). TaqMan reactions were carried out on an ABI 7700 Sequence Detection System (Perkin-Elmer) according to the manufacturer's instructions. All reactions were done in triplicate.

The *Arabidopsis ACT2* gene was chosen as a normalization standard for TaqMan analysis because of its high, constitutive expression in nearly all vegetative tissues in both juvenile and mature plants (An *et al*., 1996). *ACT2* expression among the various treatments (pathogen challenge and BTH) did not vary more than 2-fold compared with rRNA levels (data not shown), and was therefore considered suitable for normalization. The *ACT2* gene, including part of the 3′ UTR, from accessions Col-0, Ler and Ws-0, was PCR amplified and sequenced in order to design primers and a TaqMan probe that would work in all three accessions. The *ACT2* reverse primer was derived from the 3′ UTR in order to make it specific for *ACT2* mRNA. TaqMan probes for *ACT2*, *EDS1* and *PAD4* were made cDNA specific by designing them across an intron. Primers and probes were designed using Primer Express software (Perkin-Elmer) and are as follows: *ACT2*-F, TCGGTGGTTCCATTCTTGCT; *ACT2*-R, GCTTTT-TAAGCCTTTGATCTTGAGAG; *ACT2*-probe, AGCACATTCCAGCA-GATGTGGATCTCCAA; *EDS1*-F, CAAGAATCTTGAAGCTGTC-ATTGATC; *EDS1*-R, TGTCCTGTGAACACTATCTGTTTTCTACT; *EDS1*-probe, CACAGCCATTTCCACAGAAGCTTGAAATG; *PAD4*-F, TGGTCGACGCTGCCATACT; *PAD4*-R, GGTTGAATGGCCGGTTA-TCA; *PAD4*-probe, AATTCCAATCCTTCCTTGATCTTTAACTGAA-GAAAGAGT. For RNA gel blots, 10 µg of total RNA were separated on a 1.2% formaldehyde agarose gel and blotted to Hybond NX (Amersham) according to the manufacturer's instructions. *PAD4* and *PR-1* ^{32}P-radio-labelled probes were generated with the Oligolabeling kit (Pharmacia).

Sequence alignment

Sequences were aligned using Clustal_W (http://www2.ebi.ac.uk/clustalw) and the alignment was shaded using BoxShade (http://www.ch.embnet.org/software/BOX_form.html).

Acknowledgements

We thank Xinnian Dong (Duke University, Durham, NC) for providing Ler-*nahG* seed and Eric Holub (Horticultural Research International, Wellesbourne, UK) for assessing *pad4-2* cotyledon phenotypes. We also thank Eric Holub, John Rathjen and Paul Schulze-Lefert for helpful discussions, and Susheng Gan (University of Kentucky, Lexington) for communication of results prior to publication. We are grateful to the Arabidopsis Biological Resource Center for seed and DNA stocks. The Sainsbury Laboratory is funded by the Gatsby Charitable Foundation. B.J.F. and L.J.M. are also supported by grants from The Biotechnology and Biological Sciences Research Council (BBSRC).

References

Aarts,N., Metz,M., Holub,E., Staskawicz,B.J., Daniels,M.J. and Parker,J.E. (1998) Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two *R* gene-mediated signaling pathways in *Arabidopsis*. *Proc. Natl Acad. Sci. USA*, **95**, 10306–10311.

An,Y.Q., McDowell,J.M., Huang,S.R., McKinney,E.C., Chambliss,S. and Meagher,R.B. (1996) Strong, constitutive expression of the *Arabidopsis* ACT2/ACT8 actin subclass in vegetative tissues. *Plant J.*, **10**, 107–121.

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (1996) *Current Protocols in Molecular Biology*. John Wiley & Sons, New York, NY.

Bergö,M., Olivecrona,G. and Olivecrona,T. (1996) Forms of lipoprotein lipase in rat tissues: in adipose tissue the proportion of inactive lipase increases on fasting. *Biochem. J.*, **313**, 893–898.

Botella,M.A., Parker,J.E., Frost,L.N., BittnerEddy,P.D., Beynon,J.L., Daniels,M.J., Holub,E.B. and Jones,J.D.G. (1998) Three genes of the *Arabidopsis RPP1* complex resistance locus recognize distinct *Peronospora parasitica* avirulence determinants. *Plant Cell*, **10**, 1847–1860.

Bowling,S.A., Clarke,J.D., Liu,Y.D., Klessig,D.F. and Dong,X.N. (1997) The *cpr5* mutant of *Arabidopsis* expresses both *NPR1*-dependent and *NPR1*-independent resistance. *Plant Cell*, **9**, 1573–1584.

Clarke,J.D., Aarts,N., Feys,B.J., Dong,X. and Parker,J.E. (2001) Constitutive disease resistance requires *EDS1* in the *Arabidopsis* mutants *cpr1* and *cpr6* and is partially *EDS1*-dependent in *cpr5*. *Plant J.*, **26**, 409–420.

Clough,S.J. and Bent,A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.*, **16**, 735–743.

Delledonne,M., Xia,Y.J., Dixon,R.A. and Lamb,C. (1998) Nitric oxide functions as a signal in plant disease resistance. *Nature*, **394**, 585–588.

Dempsey,D.A., Shah,J. and Klessig,D.F. (1999) Salicylic acid and disease resistance in plants. *Crit. Rev. Plant Sci.*, **18**, 547–575.

Falk,A., Feys,B.J., Frost,L.N., Jones,J.D.G., Daniels,M.J. and Parker,J.E. (1999) *EDS*1, an essential component of *R* gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. *Proc. Natl Acad. Sci. USA*, **96**, 3292–3297.

Feldmann,K.A. (1991) T-DNA insertion mutagenesis in *Arabidopsis*: mutational spectrum. *Plant J.*, **1**, 71–82.

Feys,B.J. and Parker,J.E. (2000) Interplay of signalling pathways in plant disease resistance. *Trends Genet.*, **16**, 449–455.

Gassmann,W., Hinsch,M.E. and Staskawicz,B.J. (1999) The *Arabidopsis RPS4* bacterial-resistance gene is a member of the TIR-NBS-LRR family of disease-resistance genes. *Plant J.*, **20**, 265–277.

Glazebrook,J., Rogers,E.E. and Ausubel,F.M. (1996) Isolation of

Arabidopsis mutants with enhanced disease susceptibility by direct screening. *Genetics*, **143**, 973–982.

Glazebrook,J., Zook,M., Mert,F., Kagan,I., Rogers,E.E., Crute,I.R., Holub, E.B., Hammerschmidt,R. and Ausubel,F.M. (1997) Phytoalexin-deficient mutants of *Arabidopsis* reveal that *PAD4* encodes a regulatory factor and that four *PAD* genes contribute to downy mildew resistance. *Genetics*, **146**, 381–392.

Golemis,E.A., Serebriiskii,I., Finley,R.L., Kolonin,M.G., Gyuris,J. and Brent,R. (1998) Interaction trap/two-hybrid system to identify interacting proteins. In Chanda,V.B. (ed.), *Current Protocols in Protein Science*. John Wiley & Sons, New York, NY, pp. 19.2.1–19.2.40.

Gyuris,J., Golemis,E., Chertkov,H. and Brent,R. (1993) Cdi1, a human G_1-phase and S-phase protein phosphatase that associates with Cdk2. *Cell*, **75**, 791–803.

He,Y., Tang,W., Swain,J.D., Green,L., Jack,T.P. and Gan,S. (2001). Networking senescence-regulating pathways by using *Arabidopsis* enhancer trap lines. *Plant Physiol.*, **126**, 707–716.

Holland,P.M., Abramson,R.D., Watson,R. and Gelfand,D.H. (1991) Detection of specific polymerase chain-reaction product by utilizing the 5′-3′ exonuclease activity of *Thermus aquaticus* DNA-polymerase. *Proc. Natl Acad. Sci. USA*, **88**, 7276–7280.

Jirage,D., Tootle,T.L., Reuber,T.L., Frost,L.N., Feys,B.J., Parker,J.E., Ausubel,F.M. and Glazebrook,J. (1999) *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. *Proc. Natl Acad. Sci. USA*, **96**, 13583–13588.

Jirage,D., Zhou,N., Cooper,B., Clarke,J.D., Dong,X. and Glazebrook,J. (2001) Constitutive salicylic acid-dependent signaling in *cpr1* and *cpr6* mutants requires *PAD4*. *Plant J.*, **26**, 395–407.

Kim,K.K., Song,H.K., Shin,D.H., Hwang,K.Y., Choe,S., Yoo,O.J. and Suh,S.W. (1997) Crystal structure of carboxylesterase from *Pseudomonas fluorescens*, an α/β hydrolase with broad substrate specificity. *Structure*, **5**, 1571–1584.

Kjemtrup,S., Nimchuk,Z. and Dangl,J.L. (2000) Effector proteins of phytopathogenic bacteria: bifunctional signals in virulence and host recognition. *Curr. Opin. Microbiol.*, **3**, 73–78.

Klessig,D.F. *et al.* (2000) Nitric oxide and salicylic acid signalling in plant defence. *Proc. Natl Acad. Sci. USA*, **97**, 8849–8855.

Lawton,K.A., Friedrich,L., Hunt,M., Weymann,K., Delaney,T., Kessmann,H., Staub,T. and Ryals,J. (1996) Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *Plant J.*, **10**, 71–82.

Levine,A., Tenhaken,R., Dixon,R. and Lamb,C. (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell*, **79**, 583–593.

Martin,G.B. (1999) Functional analysis of plant disease resistance genes and their downstream effectors. *Curr. Opin. Plant Biol.*, **2**, 273–279.

McDowell,J.M. and Dangl,J.L. (2000) Signal transduction in the plant immune response. *Trends Biochem. Sci.*, **25**, 79–82.

McDowell,J.M., Cuzick,A., Can,C., Beynon,J., Dangl,J.L. and Holub,E.B. (2000) Downy mildew (*Peronospora parasitica*) resistance genes in *Arabidopsis* vary in functional requirements for *NDR1*, *EDS1*, *NPR1* and salicylic acid accumulation. *Plant J.*, **22**, 523–529.

Newman,M.A., von Roepenack-Lahaye,E., Parr,A., Daniels,M.J. and Dow,J.M. (2001) Induction of hydroxycinnamoyl-tyramine conjugates in pepper by *Xanthomonas campestris*: a plant defense response activated by *hrp* gene-dependent and -independent mechanisms. *Mol. Plant Microbe Interact.*, **14**, 785–792.

Orozco-Cardenás,M.L., Narváez-Vásquez,J. and Ryan,C.A. (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. *Plant Cell*, **13**, 179–191.

Parker,J.E., Szabò,V., Staskawicz,B.J., Lister,C., Dean,C., Daniels,M.J. and Jones,J.D.G. (1993) Phenotypic characterization and molecular mapping of the *Arabidopsis thaliana* locus *RPP5*, determining disease resistance to *Peronospora parasitica*. *Plant J.*, **4**, 821–831.

Parker,J.E., Holub,E.B., Frost,L.N., Falk,A., Gunn,N.D. and Daniels, M.J. (1996) Characterization of *eds1*, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different *RPP* genes. *Plant Cell*, **8**, 2033–2046.

Parker,J.E. *et al.* (1997) The *Arabidopsis* downy mildew resistance gene *RPP5* shares similarity to the Toll and Interleukin-1 receptors with *N* and *L6*. *Plant Cell*, **9**, 879–894.

Pentikäinen,M.O., Oorni,K. and Kovanen,P.T. (2000) Lipoprotein lipase (LPL) strongly links native and oxidized low density lipoprotein particles to decorin-coated collagen—roles for both dimeric and monomeric forms of LPL. *J. Biol. Chem.*, **275**, 5694–5701.

Rustérucci,C., Aviv,D.H., Holt,B.,III, Dangl,J.L. and Parker,J.E. (2001). The disease resistance signaling components *EDS1* and *PAD4* are essential regulators of the cell death pathways controlled by *LSD1* in *Arabidopsis*. *Plant Cell*, in press.

Shen,W.J., Patel,S., Hong,R. and Kraemer,F.B. (2000) Hormone-sensitive lipase functions as an oligomer. *Biochemistry*, **39**, 2392–2398.

Shirasu,K., Nakajima,H., Rajasekhar,V.K., Dixon,R.A. and Lamb,C. (1997) Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. *Plant Cell*, **9**, 261–270.

van der Biezen,E.A., Sun,J., Coleman,M.J., Bibb,M.J. and Jones,J.D.G. (2000) *Arabidopsis* RelA/SpoT homologs implicate (p)ppGpp in plant signaling. *Proc. Natl Acad. Sci. USA*, **97**, 3747–3752.

Wang,T. and Brown,M.J. (1999) mRNA quantification by real time TaqMan polymerase chain reaction: validation and comparison with RNase protection. *Anal. Biochem.*, **269**, 198–201.

Wong,H., Yang,D., Hill,J.S., Davis,R.C., Nikazy,J. and Schotz,M.C. (1997) A molecular biology-based approach to resolve the subunit orientation of lipoprotein lipase. *Proc. Natl Acad. Sci. USA*, **94**, 5594–5598.

Zhou,N., Tootle,T.L., Tsui,F., Klessig,D.F. and Glazebrook,J. (1998) *PAD4* functions upstream from salicylic acid to control defense responses in *Arabidopsis*. *Plant Cell*, **10**, 1021–1030.

Received March 13, 2001; revised and accepted August 9, 2001