New Phytologist

A core function of EDS1 with PAD4 is to protect the salicylic acid defense sector in Arabidopsis immunity

Haitao Cui, Enrico Gobbato, Barbara Kracher, Jingde Qiu, Jaqueline Bautor and Jane E. Parker

Department of Plant-Microbe Interactions, Max-Planck Institute for Plant Breeding Research, Carl-von-Linné Weg 10, 50829 Cologne, Germany

Author for correspondence:
Jane E. Parker
Tel: +49 2215 062303
Email: parker@mpipz.mpg.de

Received: *8 July 2016*
Accepted: *23 September 2016*

New Phytologist (2017) **213:** 1802–1817
doi: 10.1111/nph.14302

Key words: *Arabidopsis thaliana*, basal immunity, biotic stress network, effector-triggered immunity (ETI), RNA-seq, transcriptional reprogramming.

Summary

- Plant defenses induced by salicylic acid (SA) are vital for resistance against biotrophic pathogens. In basal and receptor-triggered immunity, SA accumulation is promoted by Enhanced Disease Susceptibility1 with its co-regulator Phytoalexin Deficient4 (EDS1/PAD4). Current models position EDS1/PAD4 upstream of SA but their functional relationship remains unclear.
- In a genetic and transcriptomic analysis of Arabidopsis autoimmunity caused by constitutive or conditional EDS1/PAD4 overexpression, intrinsic EDS1/PAD4 signaling properties and their relation to SA were uncovered.
- A core EDS1/PAD4 pathway works in parallel with SA in basal and effector-triggered bacterial immunity. It protects against disabled SA-regulated gene expression and pathogen resistance, and is distinct from a known SA-compensatory route involving MAPK signaling. Results help to explain previously identified EDS1/PAD4 regulated SA-dependent and SA-independent gene expression sectors.
- Plants have evolved an alternative route for preserving SA-regulated defenses against pathogen or genetic perturbations. In a proposed signaling framework, EDS1 with PAD4, besides promoting SA biosynthesis, maintains important SA-related resistance programs, thereby increasing robustness of the innate immune system.

Introduction

In plants, pathogen attack is sensed by innate immune receptors residing at the host cell surface or in the cytoplasm. Binding of conserved microbial molecules (pathogen-associated molecular patterns, PAMPs) by surface receptors induces PAMP-triggered immunity (PTI) which provides early protection against non- or poorly adapted microbes (Dodds & Rathjen, 2010). In the course of host–pathogen coevolution, PTI has been targeted for suppression by pathogen-derived virulence factors (effectors) delivered to host cells to promote infection (Macho & Zipfel, 2015). Disabling of PTI leads to effector-triggered susceptibility associated with a post-infection basal immune response that slows pathogen growth.

Specific pathogen effectors are recognized by intracellular nucleotide-binding/leucine-rich-repeat (NLR) receptors, leading to effector-triggered immunity (ETI). ETI characteristically boosts PTI-associated defense pathways including the production of reactive oxygen species (ROS), mobilization of Ca^{2+}-dependent protein kinase and mitogen-activated protein kinase (MAPK) signaling cascades, generation of the phenolic hormone salicylic acid (SA), and transcriptional reprogramming (Cui *et al.*, 2015; Tsuda & Somssich, 2015). ETI also employs compensatory mechanisms to protect important resistance hubs from pathogen effector interference, making the defense network more resilient (Tsuda *et al.*, 2009, 2013; Kim *et al.*, 2014). Mis-regulated NLRs cause the same programs to be unleashed without a pathogen trigger, producing autoimmunity with negative effects on plant growth and fitness (Zhang *et al.*, 2003; Wirthmueller *et al.*, 2007; Palma *et al.*, 2010; Williams *et al.*, 2011; Wang *et al.*, 2013; Gloggnitzer *et al.*, 2014).

SA contributes to PTI and ETI, and is regulated by transcriptional control of the principal SA biosynthetic enzyme gene *Isochorismate synthase1* (*ICS1*) and SA metabolic genes (Seyfferth & Tsuda, 2014). Responses downstream of SA are mediated by the nucleocytoplasmic regulator Nonexpressor of PR Genes1 (NPR1), which is a transcriptional co-activator of SA-dependent local and systemic immunity pathways (Fu & Dong, 2013). Numerous pathogens use effector molecules to interfere with SA signaling either by targeting SA biosynthesis directly or steering the plant stress response network away from SA accumulation (Brooks *et al.*, 2005; Djamei *et al.*, 2011; Zheng *et al.*, 2012; Caillaud *et al.*, 2013; Gimenez-Ibanez *et al.*, 2014). These interference strategies emphasize the importance of SA-mediated defenses in innate immunity and SA connectivity to other biotic and abiotic stress pathways within the host signaling network (Robert-Seilaniantz *et al.*, 2011).

NLR receptors fall into two major subclasses with different N-terminal domains: CNLs contain a coiled-coil (CC) domain and are present in eudicot and monocot species. TNLs possess a

Toll-Interleukin-1 receptor signaling (TIR) domain and are restricted to eudicot lineages (Maekawa *et al.*, 2011; Jacob *et al.*, 2013). Mutational screens in Arabidopsis revealed that CNLs and TNLs have different genetic requirements in pathogen resistance (Wiermer *et al.*, 2005). Whereas many CNL receptors signal via the plasma membrane-associated protein Non Race-Specific Disease Resistance 1 (NDR1), all tested TNL receptors require the nucleocytoplasmic lipase-like protein, Enhanced Disease Susceptibility 1 (EDS1) for resistance (Wiermer *et al.*, 2005; Day *et al.*, 2006). NDR1 and EDS1 also positively regulate basal immunity against virulent pathogens. Compensatory properties of the ETI network can obscure individual pathway actions (Tsuda *et al.*, 2009; Cui *et al.*, 2015). For example, the Arabidopsis CNL receptor Resistance to *Pseudomonas syringae* 2 (RPS2) specifying resistance to *P. syringae*-secreted effector AvrRpt2, or Hypersensitive Response to TCV (HRT) recognizing turnip crinkle virus, utilize *ICS1*-generated SA and *EDS1* in a genetically redundant manner (Venugopal *et al.*, 2009). Therefore, EDS1 and SA pathways operate in parallel for certain CNL immune responses.

In basal and TNL immunity, EDS1 with its direct partner Phytoalexin Deficient4 (PAD4), promotes *ICS1* expression and SA accumulation, and current models position EDS1/PAD4 upstream of SA signaling (Zhou *et al.*, 1998; Feys *et al.*, 2001; Wiermer *et al.*, 2005; Wang *et al.*, 2009; Rietz *et al.*, 2011; Wagner *et al.*, 2013). A feedback loop in which accumulated SA enhances expression of *EDS1*, *PAD4* and other genes further amplifies resistance outputs (Jirage *et al.*, 1999; Feys *et al.*, 2001; Vlot *et al.*, 2009). Genetic and transcriptomic data also revealed an EDS1/PAD4-regulated branch functioning independently of *ICS1*-generated SA in basal and TNL immunity (Glazebrook *et al.*, 2003; Zhang *et al.*, 2003; Bartsch *et al.*, 2006; Wang *et al.*, 2008; Gloggnitzer *et al.*, 2014).

Here, we show that constitutive or conditional overexpression of Arabidopsis PAD4 together with EDS1 drives plants into an immune response. In a genetic and transcriptomic study, we identify an intrinsic, early function of EDS1/PAD4 signaling which is independent of *ICS1*-generated SA and provides a mechanism for preserving SA-related defense gene expression and pathogen resistance.

Materials and Methods

Plant materials, growth conditions and pathogen strains

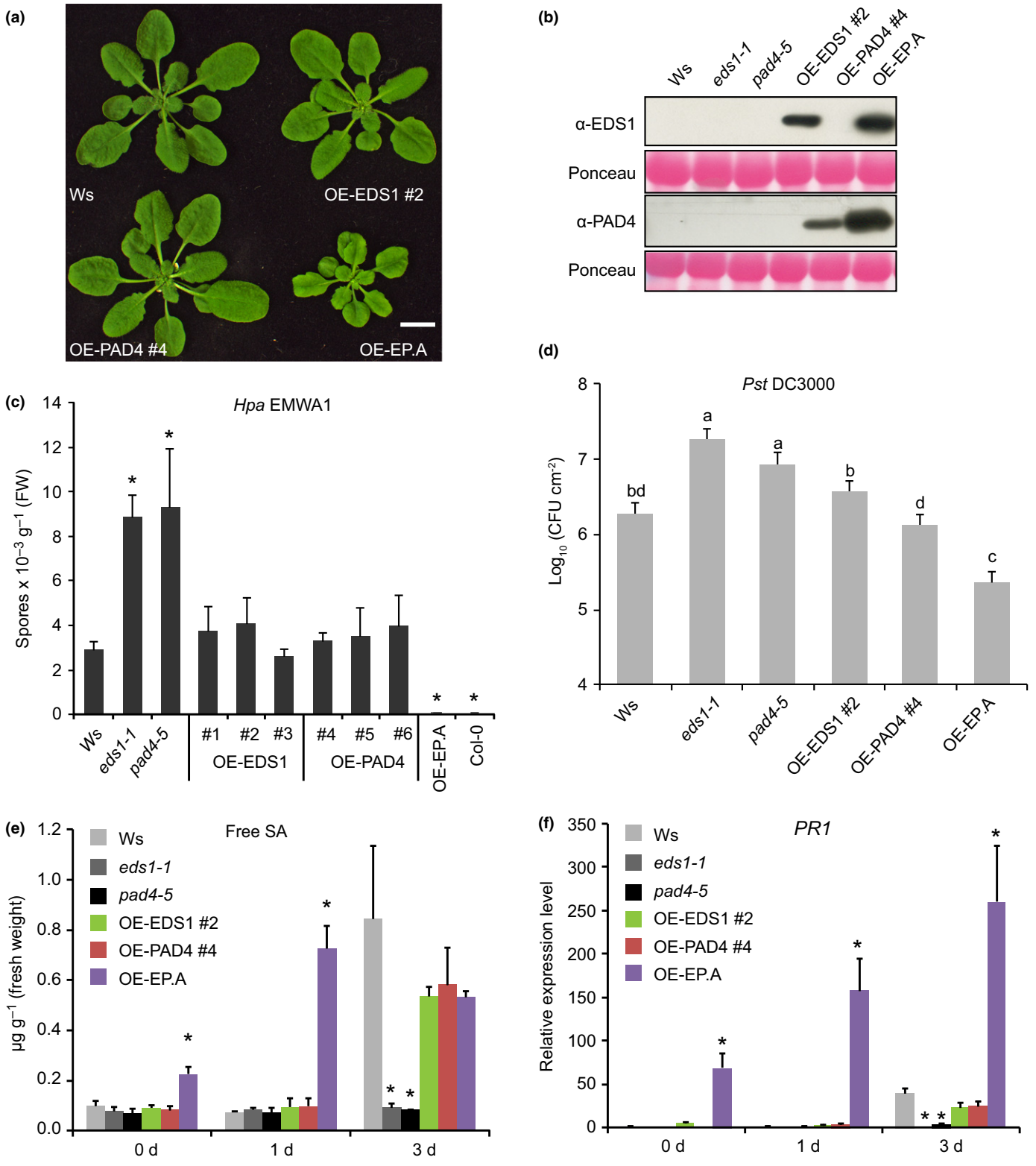
Work and materials are registered under German S1 regulatory code: 01/1/0450/87. *Arabidopsis thaliana* wild-type (WT) accessions used are Wassilewskija-2 (Ws) and Columbia-0 (Col). Ws *eds1-1* and *pad4-5* (Falk *et al.*, 1999; Feys *et al.*, 2001) and Col *eds1-2* (Bartsch *et al.*, 2006), *pad4-1* (Jirage *et al.*, 1999), *eds1-2 pad4-1* (Wagner *et al.*, 2013), *sid2-1* (Wildermuth *et al.*, 2001), *sid2-2, pad4-1 sid2-2*, *rps2rpm1* (Tsuda *et al.*, 2009) and *efr1* (Zipfel *et al.*, 2006) mutants are published. An *eds1-2 sid2-1* double mutant was generated from progeny of a single mutant cross using PCR-based gene-specific markers (available on request). *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (Aarts *et al.*, 1998), *Pst* DC3000 *AvrRpt2* (Bent *et al.*, 1994), *Pst* Δ*cor* (Ma *et al.*, 1991) and *Hyaloperonospora arabidopsidis* (*Hpa*) isolates Noco2 and Emwa1 (McDowell *et al.*, 2000) are described. *Pst* Δ*cor AvrRps4* was made by triparental mating using the helper plasmid pRK2013, as described (Heidrich *et al.*, 2011). Plants were grown on soil in controlled environment chambers with a 10 h photoperiod (200 μmol m^{-2} s^{-1}) at 22°C and 65% relative humidity. For sterile plant analyses, Arabidopsis seeds were surface-sterilized for 5 h with chlorine gas and sown in sealed Magenta pots on solid 0.5× Murashige & Skoog (MS) medium with 0.9% Plant Agar (Duchefa, Haarlem, the Netherlands). After 3 d stratification at 4°C, Magenta pots were moved to growth chambers under the same conditions as soil-grown plants.

Generation of Arabidopsis transgenic plants

EDS1 genomic DNA and *PAD4* cDNA minus stop codons were amplified and cloned into a pENTR/D-TOPO vector by TOPO cloning (Invitrogen). The resulting ENTRY clones were used in a gateway® LR reaction with the binary destination vector pXGCS-strepII (Witte *et al.*, 2004) for overexpression of *EDS1* and *PAD4*, or pER8-strepII-3xHA vector (Zuo *et al.*, 2000) for estradiol-inducible expression of *PAD4*. Expression vectors pXGS-*gEDS1*-strepII, pXGS-*cPAD4*-strepII or pER8-strepII-3xHA-*cPAD4* were mobilized into *A. tumefaciens* strain GV3101RK90 and used to transform Arabidopsis plants. Transformants were selected on soil after spraying with phosphinotricin herbicide (Tissier *et al.*, 1999). Arabidopsis transgenic OE-PAD4 line #4 is the same as 35S:PAD4 (Pegadaraju *et al.*, 2007). Dual *EDS1 PAD4* overexpression (OE) lines OE-EP.A and OE-EP.B were made by crossing OE-EDS1 line #2 with OE-PAD4 #4 to produce OE-EP.A, and OE-EDS1 #1 with OE-PAD4 #5 to produce OE-EP.B. Plants homozygous for both transgenes were identified by immunoblot analysis using α-strepII antibodies (Abcam, ab76949) in F_3 progeny. Homozygosity of *eds1-1* and *pad4-5* alleles was determined using PCR-based markers (Falk *et al.*, 1999; Feys *et al.*, 2001). Estradiol-inducible lines ED-P4E1 and ED-P4 in *eds1-2 pad4-1* were generated by crossing ED-PAD4 *pad4-1* with *35S:EDS1-HA eds1-2* (Wagner *et al.*, 2013). Homozygosity of both transgenes and *eds1-2 pad4-1* was confirmed in F_3 progeny. ED-P4E1 *sid2* was selected from a cross between ED-P4E1 and *eds1-2 sid2-1*.

PAMP elicitation assays

Elongation factor Tu (Ef-Tu)/elf18-mediated growth inhibition assays were performed as described (Navarro *et al.*, 2008). Seedling FW was measured 7–8 d after PAMP treatment. Statistical analysis of $\log_2$-transformed seedling FW was described previously (Tsuda *et al.*, 2009) using the LME4 package in the R programming environment (http://www.r-project.org). The following model was fitted to the data: $\log_2 FW_{gyr} = GY_{gy} + R_r + e_{gyr}$ (GY, genotype : treatment interaction; R, biological replicate; e, residual). For MAPK activation assays, 1 μM elf18 or flg22 peptide was infiltrated into leaves of 4-wk-old



plants. Total protein samples were used for immunoblots with α-p44/42 MAPK antibody (Cell Signaling, Cambridge, UK) to detect activated forms of MPK3, MPK6 and MPK4 (Suarez-Rodriguez *et al.*, 2007; Feng *et al.*, 2012). Two independent assays gave similar results.

Disease resistance assays

Hpa isolates Noco2 and Emwa1 were spray-inoculated onto 2- to 3-wk-old plants and spore numbers determined at 5 d post inoculation (dpi) (Feys *et al.*, 2005). Reacting plant cells and *Hpa* hyphae were detected by trypan blue staining of leaves (Aarts

et al., 1998). Four- to five-week-old plants were hand-infiltrated with *Pst* bacterial suspensions of 2×10^5 colony forming units (CFU) ml^{-1} and bacterial growth measured as described (Feys *et al.*, 2005). Statistical analysis of bacterial growth data was described previously (Tsuda *et al.*, 2009) using the LME4 package in R. Log_{10}-transformed CFU cm^{-2} leaf surface area were calculated and the following model was fitted to the data: $\log_{10} CFU_{gyr} = GY_{gy} + R_r + e_{gyr}$ (GY, genotype : treatment interaction; R, biological replicate; e, residual). All experiments were performed at least two times with similar results.

Protein expression, purification and SA quantification

Total protein extracts (Garcia *et al.*, 2010) were loaded onto 10% SDS-PAGE gels for immunoblot analysis. Equal membrane loading was tested by staining with Ponceau S (Sigma-Aldrich). α-EDS1, α-PAD4 (Rietz *et al.*, 2011), α-HA (Roche), α-p44/42 MAPK (Cell Signaling) antibodies and secondary antibodies coupled to Horseradish Peroxidase (HRP) (Santa Cruz Biotechnology, Dallas, TX, USA) were used. Purification of strepII-tagged PAD4 from Arabidopsis transgenic lines was performed as described (Wagner *et al.*, 2013). Free and total SA was quantified in leaf tissues as described (Straus *et al.*, 2010). Two or three independent assays gave similar results.

qRT-PCR analysis

Total leaf RNA was extracted for quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Rietz *et al.*, 2011) and qRT-PCR was performed using a Bio-Rad iQ5 Real-Time PCR Detection System with Brilliant SYBR Green (Thermo Scientific, Waltham, MA, USA). *Actin1* (*At2g37620*) or *AT4G26410* transcript levels were used as internal reference in all samples (Czechowski *et al.*, 2005). qRT-PCR primers are listed in Supporting Information Table S1. At least two independent experiments, each with three or four technical replicates, gave similar results.

Estradiol treatment, RNA-sequencing and data analysis

Four-week-old ED-P4E1 soil-grown plants were sprayed with 10 μM estradiol in 0.2% DMSO dissolved in water with 0.01% silwet-L77 or 0.2% DMSO in water with 0.01% silwet-L77 (mock). Leaf samples from three independent biological replicates were processed at 6, 12 and 24 h after estradiol treatment. RNA-purification with an RNeasy Plant Mini Kit (Qiagen) was performed according to manufacturer's instructions. RNA-seq libraries were prepared from 1 μg total RNA according to recommendations (TruSeq RNA sample preparation v2 guide; Illumina). Library construction and RNA sequencing were done by the Max-Planck Genome Centre, Cologne, producing 20–50 million 100-base long reads per sample. RNA-seq data are deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database with accession number GSE80585. RNA-seq reads were mapped to the annotated genome of *A. thaliana* (TAIR10) using TOPHAT2 ($a = 10$, $g = 10$) (Kim *et al.*, 2013) and transformed into a read count per gene per sample using htseq-count (s = no, t = exon) (Anders *et al.*, 2015). For statistical analysis, count values for all expressed genes were TMM-normalized and log-transformed using the functions 'CALCNORMFACTORS' (R package EDGER) and 'VOOM' (R package LIMMA) to yield $\log_2$ counts per million ($\log_2$ cpm). Next, a linear model was fitted to each gene using the 'LMFIT' (R package limma) function. Resulting *P*-values for the analysed comparisons were adjusted for false discoveries due to multiple hypotheses testing via the Benjamini–Hochberg procedure (FDR). To extract genes with significant expression differences, a cut-off of FDR < 0.05 and $\log_2$ (Fold Change) ≥ 1 was applied if not specified otherwise. Heatmaps were generated with software CLUSTER using uncentered Pearson correlations and complete linkage clustering, and visualized by TREEVIEW software (Eisen *et al.*, 1998). Transcriptome similarity analysis was performed with the GENEVETIGATOR SIGNATURE tool (https://genevestigator.com/gv/doc/signature.jsp). Gene lists with $\log_2$ fold-change values of estradiol vs mock treatment at 24 h were used as input.

Cell death (HR) and ion leakage assays

Leaves of 4-wk-old plants were infiltrated with *Pst* DC3000 *AvrRpt2* at $OD_{600} = 0.02$ and macroscopic cell death recorded at 24 h. Ion leakage assays on detached leaves were performed as described (Heidrich *et al.*, 2011). Two or more independent assays gave similar results.

Fig. 1 Constitutive EDS1/PAD4 overexpression leads to autoimmunity. (a) Growth phenotypes of Arabidopsis accession Ws and overexpression lines, as indicated. Four-week-old soil-grown plants are shown. Bar, 1 cm. (b) EDS1 or PAD4 protein accumulation in lines from (a) with Ws *eds1-1* and *pad4-5* mutants, monitored on immunoblots probed with α-EDS1 or α-PAD4. Ponceau staining of the blots indicates equal loading. (c) *Hyaloperonospora arabidopsidis* (*Hpa*) isolate Emwa1infection phenotypes of 2-wk-old plant lines, as indicated. Pathogen spores on leaves were counted at 7 d after spray-inoculation with 4×10^4 spores ml^{-1}. Error bars represent + SD of three biological replicates. Significant difference to Ws in a Student's *t*-test: *, $P < 0.05$. (d) Growth of *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 on EDS1 and PAD4 OE lines at 3 d post inoculation (dpi). Leaves of 4-wk-old plants were hand-infiltrated with bacterial suspensions ($OD_{600} = 0.0002$) and CFU (colony-forming units) counted at 3 dpi. Bars represent means + SD calculated from three independent experiments using a mixed linear model. The Benjamini–Hochberg method was used to adjust *P*-values to correct for multiple testing. Statistically significant differences are indicated by different letters (adjusted *P*-value < 0.01). (e) Quantitation of salicylic acid (SA) in 4-wk-old plant lines, as indicated, after spray-inoculation with *Hpa* isolate Emwa1. Leaf samples were collected at 0, 1 and 3 dpi and free SA measured. Error bars represent + SD of three biological replicates. Significant difference to Ws in a Student's *t*-test in each group (0 dpi, 1 dpi, or 3 dpi): *, $P < 0.05$. (f) *PR1* (*Pathogenesis-related gene 1*) expression in the same samples as (e) measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Gene expression was normalized to *Actin1* (*At2g37620*). Error bars represent +SD of three technical replicates. Significant difference to Ws in a Student's *t*-test in each group (0, 1 or 3 dpi): *, $P < 0.05$. EDS1, Enhanced Disease Susceptibility 1; PAD4, Phytoalexin Deficient 4.

Results

Combined EDS1/PAD4 overexpression leads to autoimmunity

We generated multiple transgenic *EDS1-StrepII* and *PAD4-StrepII* OE lines, respectively, in the *eds1-1* and *pad4-5* null mutants of Arabidopsis accession Wassilewskija-2 (Ws). Three independent lines with a single transgene insertion were taken to homozygosity for *p35S:EDS1-StrepII* (OE-EDS1 #1, OE-EDS1 #2 and OE-EDS1 #3) and *p35S:PAD4-StrepII* (OE-PAD4 #4, OE-PAD4 #5 and OE-PAD4 #6). Immunoblotting of leaf extracts with α-EDS1 or α-PAD4 antibodies showed high EDS1 or PAD4 accumulation in the lines compared to corresponding native proteins in uninfected Ws or Ws infected with the virulent *Hyaloperonospora arabidopsidis* (*Hpa*) isolate Emwa1 (Fig. S1a). These lines grew normally in soil over a 4- to 6-wk period, as shown for OE-EDS1 #2 and OE-PAD4 #4 (Figs 1a, S1b). Therefore, EDS1 or PAD4 OE alone does not produce symptoms of autoimmunity, as found previously for EDS1 OE in accession Col-0 (Col) (Wagner *et al.*, 2013). All lines exhibited TNL (*RPP1b*) immunity against *Hpa* isolate Noco2 (Botella *et al.*, 1998), indicated by a hypersensitive response (HR) in OE-EDS1 #2 and OE-PAD4 #4 leaves (Fig. S1c).

We then crossed OE-EDS1 #2 with OE-PAD4 #4 and selected a dual EDS1/PAD4 OE line (denoted OE-EP.A) that was homozygous for both transgenes in an *eds1-1 pad4-5* background. Four-week-old OE-EP.A plants were stunted compared to OE-EDS1 #2 or OE-PAD4 #4 (Figs 1a, S1b). OE-EP.A accumulated more EDS1 and PAD4 protein (Fig. 1b), consistent with mutual stabilizing effects of each partner in an EDS1-PAD4 heteromeric complex (Feys *et al.*, 2005; Wagner *et al.*, 2013).

OE-EP.A plants conferred TNL (*RPP1b*) ETI to *Hpa* Noco2 and a tendency to produce smaller HR lesions than the parental lines (Fig. S1c). To measure basal immunity, OE-EP.A plants were inoculated with virulent *Hpa* isolate Emwa1 and pathogen sporulation counted on leaves. Whereas the single OE-EDS1 and OE-PAD4 lines were susceptible to *Hpa* Emwa1, OE-EP.A plants restricted *Hpa* Emwa1 sporulation to the same degree as the genetically resistant accession Col (Fig. 1c). As expected, *eds1-1* and *pad4-5* mutants had enhanced susceptibility to *Hpa* Emwa1 compared to Ws in these assays (Fig. 1c). Notably, resistance to *Hpa* Emwa1 in OE-EP.A manifested as HR-like lesions (Fig. S1d). In bacterial infection assays, OE-EDS1 #2 and OE-PAD4 #4 displayed WT basal resistance to virulent *Pst* DC3000, which grew even less on OE-EP.A leaves (Fig. 1d).

Crossing two different OE-EDS1 and OE-PAD4 lines (OE-EDS1 #1 with OE-PAD4 #5) produced stunted plants (OE-EP.B) already in the F_1 generation (Fig. S1e). OE-EP.B plants also displayed ETI-like resistance whereas the parental OE-EDS1 #1 and OE-PAD4 #5 lines remained susceptible to virulent *Hpa* Emwa1 infection (Fig. S1f).

Concentrations of free and total SA, and expression of the SA defense marker gene *Pathogenesis-related gene 1* (*PR1*), were determined before and after infection of the different lines with *Hpa* Emwa1. OE-EDS1 #2 and OE-PAD4 #4 plants behaved similarly to WT Ws with low pre-inoculation SA and increased SA accumulation at 3 dpi (Figs 1e, S1g). There was a similar trend in *PR1* expression (Fig. 1f). As anticipated, *eds1-1* and *pad4-5* mutants failed to accumulate SA or induce *PR1* over the 3 d *Hpa* infection time-course (Fig. 1e,f). By contrast, OE-EP.A plants displayed high SA and *PR1* expression before *Hpa* inoculation (Fig. 1e,f). SA amounts and *PR1* expression in OE-EP.A leaves increased further at 1 and 3 dpi (Fig. 1e,f). Together, these results show that combined EDS1/PAD4 OE, but not OE of EDS1 or PAD4 alone, leads to Arabidopsis autoimmunity.

EDS1/PAD4 autoimmunity involves intrinsic defense pathway activation

Because the above assays were performed on soil-grown plants we tested whether OE-EP.A autoimmunity is an intrinsic property or derives from hyper-responsiveness to microbes or PAMPs in the environment. For this, we grew plants on sterile 0.5 × MS media in Magenta boxes. Under these conditions, the OE-EP.A plants had impaired growth (Fig. 2a) and constitutive *PR1* expression phenotypes compared to OE-EDS1 #2, OE-PAD4 #4 or Ws (Fig. 2b). Sterile propagation also led to enhanced growth of *eds1-1* or *pad4-5* mutants relative to WT Ws or OE lines (Fig. 2a). These data suggest that autoimmunity caused by EDS1/PAD4 OE is due to an intrinsic deregulation of resistance pathways and trade-off with growth. In a growth inhibition assay, OE-EP.A seedlings displayed similar responsiveness as WT Ws to 0.1 μM and 1 μM elf18, a bacterial PAMP recognized by EFR (EF-Tu receptor) (Zipfel *et al.*, 2006) (Fig. 2c), suggesting that EDS1/PAD4 overexpression does not strongly affect this PAMP-triggered output.

Estradiol-inducible PAD4 with OE EDS1 reprograms cells for resistance

In order to capture early EDS1/PAD4-conditioned transcriptional changes we generated a transgenic line in Col *pad4-1* expressing PAD4 with an N-terminal StrepII-Hemagglutinin (SIIHA) tag under control of an estradiol-inducible promoter. This line was then crossed with Col *eds1-2* expressing EDS1-HA driven by a 35S promoter (*35S:EDS1-HA eds1-2*) and conferring full basal resistance to *Pst* DC3000 (Fig. S2a) or *Hpa* Noco2 (Fig. S2b) (Wagner *et al.*, 2013). An *eds1-2 pad4-1* line (denoted ED-P4E1) was selected which expressed high levels of EDS1-HA and estradiol-inducible SIIHA-PAD4 (Fig. 3a). A further line (ED-P4) with estradiol-inducible SIIHA-PAD4 in *pad4-1 eds1-2* (thus lacking EDS1), was also selected. In 4-wk-old ED-P4 and ED-P4E1 plants grown on soil, *SIIHA-PAD4* transcripts accumulated to high levels 24 h after a single application of 10 μM estradiol but not mock treatment (Fig. S2c). In the same tissues, SIIHA-PAD4 protein accumulation was much lower in ED-P4 than ED-P4E1 plants (Fig. S2d), consistent with EDS1 stabilizing PAD4 in a complex (Feys *et al.*, 2005). Accordingly, EDS1-HA co-purified with estradiol-induced SIIHA-PAD4 after purification via strepII tag binding to a Strep-Tactin matrix (Fig. 3a).

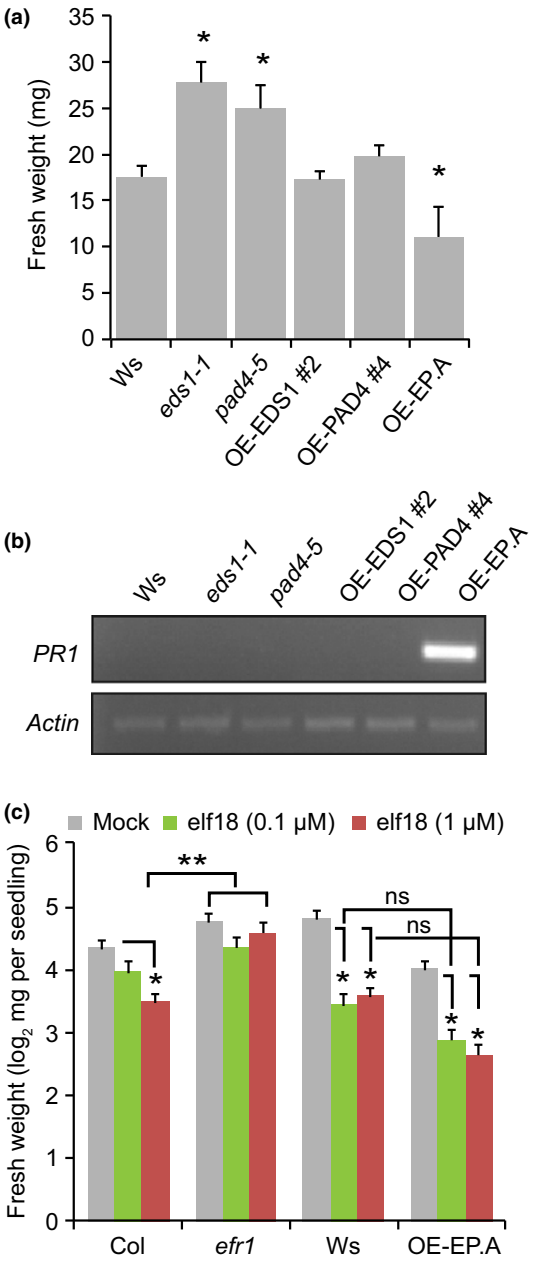


Fig. 2 Autoimmunity is an intrinsic signaling property of EDS1/PAD4 OE. (a) Fresh weight of 5-wk-old Arabidopsis plants, as indicated, growing under sterile conditions in Magenta boxes. Error bars represent + SD of three biological replicates. Significant difference to Ws in a Student's *t*-test: *, $P < 0.05$. (b) *PR1* (*Pathogenesis-related gene 1*) expression in leaf tissues from material in (a) detected by semi-quantitative RT-PCR. *Actin1* (*At2g37620*) was used as a control. (c) Responses of 1-wk-old seedlings of Arabidopsis accession Col, *efr1* (Col background) mutant, Ws and OE-EP.A to 0.1 μM or 1 μM elf18 peptide, as measured by growth inhibition. FW of elf18-treated seedlings (16 per sample) was measured at 7 d. Bars represent $\log_2$ transformed fresh weight + SD calculated from six biological replicates using a mixed linear model. The Benjamini–Hochberg method was used to adjust *P*-values for multiple testing. Significant differences to mock in genotypes: *, adjusted *P*-value < 0.01. Statistically significant difference-of-differences of mock- and elf18- treated samples between genotypes: **, $P < 0.01$; ns, not significant. EDS1, Enhanced Disease Susceptibility 1; PAD4, Phytoalexin Deficient 4.

Estradiol-treated ED-P4E1, but not ED-P4 leaf samples, showed induced expression of several *EDS1*/*PAD4*-dependent defense genes: *CBP60g* (*Calmodulin-Binding Protein 60-Like.g*), *PBS3* (*AvrPphb Susceptible3*), *ICS1* and *FMO1* (*Flavin-Dependent Monooxygenase 1*) at 24 h (Fig. 3b). Therefore, conditionally expressed SIIHA-PAD4 with OE EDS1-HA causes defense gene expression.

We tested whether ED-P4E1 plants express basal resistance to virulent *Pst* DC3000 bacteria inoculated onto leaves 24 h after estradiol treatment. *Pst* DC3000 titers at 3 dpi (4 d after estradiol application) were lower in response to estradiol vs mock-treated ED-P4E1 or estradiol-treated ED-P4, WT Col and *eds1-2 pad4-1* mutant plants (Fig. 3c). Similarly, estradiol pre-treatment of ED-P4E1, but not ED-P4, produced increased resistance to virulent *Hpa* Noco2 (Fig. 3d). These data show that conditional expression of SIIHA-PAD4 in the presence of OE EDS1-HA leads to increased basal immunity.

Inducible PAD4 with OE EDS1 transcriptionally activates SA-responsive genes

We performed an RNA-sequencing (RNA-seq) experiment to identify differentially regulated genes between mock and estradiol treatments at 6, 12 and 24 h in 4-wk-old ED-P4E1 leaves. Only one gene, *PAD4* itself, was induced at 6 h (moderated *t*-test, FDR-adjusted *P*-value < 0.05 and fold change > 2; Table S2) and SIIHA-PAD4 protein was detectable at 12 h after estradiol treatment (Fig. 4a). Totals of 240 and 386 genes were induced at 12 and 24 h, respectively, in estradiol-treated compared to mock samples, but no genes were significantly repressed (Table S2). We speculated that these estradiol-induced genes represent early targets of EDS1/PAD4 signaling. We selected 155 genes that were differentially expressed at both 12 and 24 h as a 'core' set of EDS1/PAD4-induced genes (Fig. 4b; Table S2) and evaluated how many of these were induced in an autoimmunity expression microarray dataset of plants over-expressing the TNL receptor RPS4 (OE-RPS4) (Heidrich *et al.*, 2013; GSE50019; http://www.ncbi.nlm.nih.gov/geo/). Shifting OE-RPS4 plants from a repressive (28°C) to an inductive (19°C) temperature leads to *EDS1*-dependent gene expression changes over 24 h that resemble TNL ETI (Bartsch *et al.*, 2006; Wirthmueller *et al.*, 2007; Heidrich *et al.*, 2013). 93% of the ED-P4E1 core gene set was induced in an *EDS1*-dependent manner at 8 h in the OE-RPS4 system (Fig. 4c). Therefore, in terms of induced defense genes, the conditional ED-P4E1 system represents a small subset of the TNL-ETI transcriptome.

In a fuller transcriptome analysis, we used Genevestigator Signature tool (https://genevestigator.com/gv/doc/signature.jsp) to identify conditions in which expression signatures (genes with its $\log_2$ fold change value) of the 155 core genes in ED-P4E1 (135 of which are present on the Affymetrix ATH1 GeneChip) are most similar. In this analysis, published Arabidopsis gene expression microarray datasets encompassing 2951 perturbations (biotic, chemical, elicitor, hormone, nutrient, stress, temperature and genetic background) were screened. Expression changes

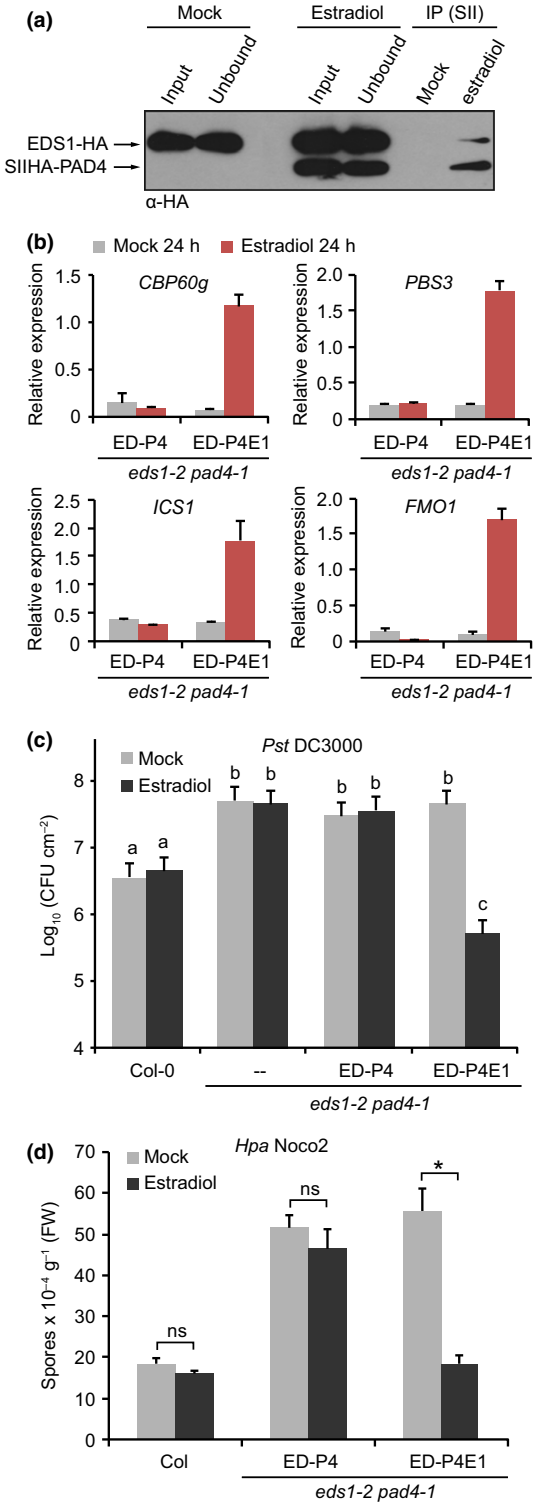


Fig. 3 Estradiol-inducible PAD4 in an EDS1 over expression background increases basal resistance. (a) SIIHA-PAD4 was induced (Input) by estradiol treatment for 24 h and purified (IP) via strepII tag binding to a Strep-Tactin matrix from total protein extracts of Arabidopsis ED-P4E1 transgenic plant leaves. Four-week-old plants were treated with 0.2% DMSO in water (mock) or 10 μM estradiol (in 0.2% DMSO). The immunoblot was probed with α-HA antisera to detect both PAD4 and EDS1. Unbound, protein sample after IP. (b) Estradiol-induced expression of EDS1/PAD4-dependent marker genes *Calmodulin-Binding Protein 60-Like.g* (*CBP60g*), *AvrPphb Susceptible 3* (*PBS3*), *Isochorismate synthase 1* (*ICS1*) and *Flavin-Dependent Monooxygenase 1* (*FMO1*) measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in leaves of 4-wk-old ED-P4 or ED-P4E1 plants at 24 h after 10 μM estradiol or mock (DMSO) treatments. Gene expression was normalized to *AT4G26410*. Error bars represent + SD of three technical replicates. (c) *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 growth on Arabidopsis Col, *eds1-2 pad4-1*, ED-P4 and ED-P4E1 leaves at 3 d post-inoculation (dpi). Four-week-old plants were 10 μM estradiol- or mock- (DMSO) treated 24 h before pathogen inoculation. Bacterial suspensions (OD_{600} = 0.0002) were hand-infiltrated into leaves. Bars represent means + SE calculated from four independent experiments using a mixed linear model. The Benjamini–Hochberg method was used to adjust *P*-values to correct for multiple testing. Statistically significant differences are indicated by different letters (adjusted *P*-value < 0.01). CFU, colony-forming units. (d) *Hyaloperonospora arabidopsidis* (*Hpa*) Noco2 sporulation on Col, ED-P4 and ED-P4E1 leaves. 3-wk-old plants were treated with 10 μM estradiol or DMSO (mock) as in (c), and then inoculated with *Hpa* Noco2 (4×10^4 spores ml^{-1}). Pathogen spores on leaves were counted at 5 d. Error bars represent + SD of three biological replicates. Statistical differences between mock and estradiol treatments (student's *t*-test) *, $P < 0.05$; ns, no significant difference. EDS1, Enhanced Disease Susceptibility 1; PAD4, Phytoalexin Deficient 4.

associated with basal resistance (e.g. *Hpa* Emwa1 on a susceptible Col *rpp4* mutant or powdery mildew (*Golovinomyces orontii*) infection of Col) showed strongest overall similarity to the ED-P4E1 data (Table S3; Fig. 4d). The second most enriched class related more broadly to SA-dependent or SA-induced responses (Table S3; Fig. 4d). Notably, 91% of the ED-P4E1 core genes was induced by SA treatment in a microarray dataset (GSE34047) (Fig. 4d), indicating that these genes respond to SA. By contrast, pathogen-triggered expression changes in *eds1,* SA-biosynthetic mutants, or plants treated with SA-antagonizing metabolites such as methyl jasmonic acid (MeJA), were most different to ED-P4E1 (Fig. 4d; Table S4). These data underscore the role of EDS1 with PAD4 in the transcriptional induction of SA-related defense pathways (Wiermer *et al.*, 2005; Cui *et al.*, 2015).

EDS1/PAD4 autoimmunity involves a significant SA-independent component

Because SA-dependent and SA-independent expression sectors were found in EDS1-dependent TNL ETI (Bartsch *et al.*, 2006; Straus *et al.*, 2010), we investigated whether this is also a property of the estradiol-inducible EDS1/PAD4 system. First, we examined whether EDS1/PAD4-conditioned transcriptional changes require SA accumulation. For this, ED-P4E1 was crossed with *sid2-1* (mutated in the SA-biosynthesis gene *ICS1*) and a homozygous *eds1-2 pad4-1 sid2-1* (ED-P4E1 *sid2-1*) line selected. SIIHA-PAD4 protein accumulation upon estradiol treatment was unaffected by *sid2-1* (Fig. 5a). Of eight tested genes from the ED-P4E1 155 core set (Table S2), induction of five (*ICS1*, *PBS3*, *ARD1-L2*, *MC2* and *AtRLP34-Receptor-Like Protein34*) was independent of *ICS1*-generated SA at 12 and 24 h after estradiol treatment,

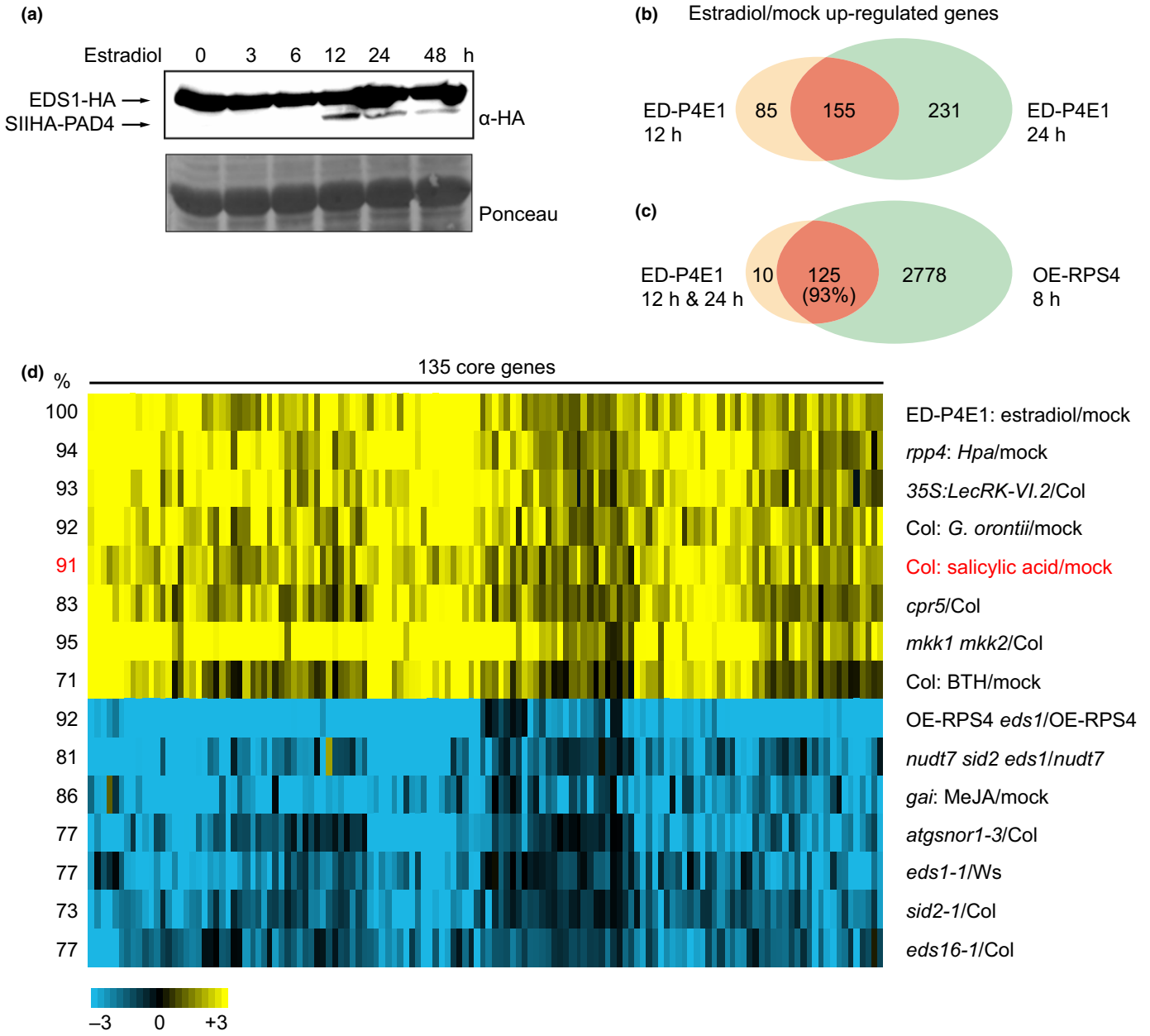


Fig. 4 Induced PAD4 with overexpressed EDS1 transcriptionally activates SA-responsive genes. (a) Accumulation of EDS1-HA and SIIHA-PAD4 protein in Arabidopsis ED-P4E1 plants at the indicated time points after treatment with 10 μM estradiol. Proteins were detected by immunoblotting with α-HA antibodies. Ponceau staining of the blot shows equal sample loading. (b) Overlap of induced genes at 12 and 24 h after estradiol vs mock treatment in ED-P4E1 plants (see the Materials and Methods section and Supporting Information Table S2). Lists of upregulated genes at 12 h were generated by combining genes with $\log_2$ (fold change_12 h) > 1 and False discovery (FDR) *P*-value < 0.05, or genes with $\log_2$ (fold change_12 h) > 0.7, $\log_2$ (fold change_24 h) > 1, and FDR < 0.05. Lists of upregulated genes at 24 h were generated using $\log_2$ (fold change_24 h) > 1, and FDR < 0.05. (c) 135 genes of the 155 'core' genes in (b) have corresponding probes on an Affymetrix ATH1 microarray. 93% (125 of135) of these genes overlap with *EDS1*-dependent induced genes in a microarray experiment of OE-RPS4 at 8 h after shifting plants from 28°C (permissive) to 19°C (inductive) temperature (GSE50019, Heidrich *et al.*, 2013). (d) A heatmap representing fold-changes of 135 core genes (columns) from (c) in microarray experiments (rows) showing strongest similarity or highest differences in expression patterns. Microarray experiments from 2951 perturbations were ranked using the Genevestigator Signature tool (see the Materials and Methods section). %, percent of 135 genes upregulated (yellow, $\log_2$ (fold change) > 1 and FDR < 0.05) or downregulated (blue, $\log_2$ (fold change) < −1 and FDR < 0.05) in corresponding microarray datasets. EDS1, Enhanced Disease Susceptibility 1; PAD4, Phytoalexin Deficient 4.

measured by qRT-PCR (Fig. 5b). Induction of *AT5G41750* and *WRKY54* was *ICS1*-dependent at 12 h but not 24 h (Fig. 5b). Expression of the SA marker gene *PR1* was *ICS1*-dependent at both time points (Fig. 5b). These data suggest that genes induced in the ED-P4E1 system also fall into SA-dependent and SA-independent sectors.

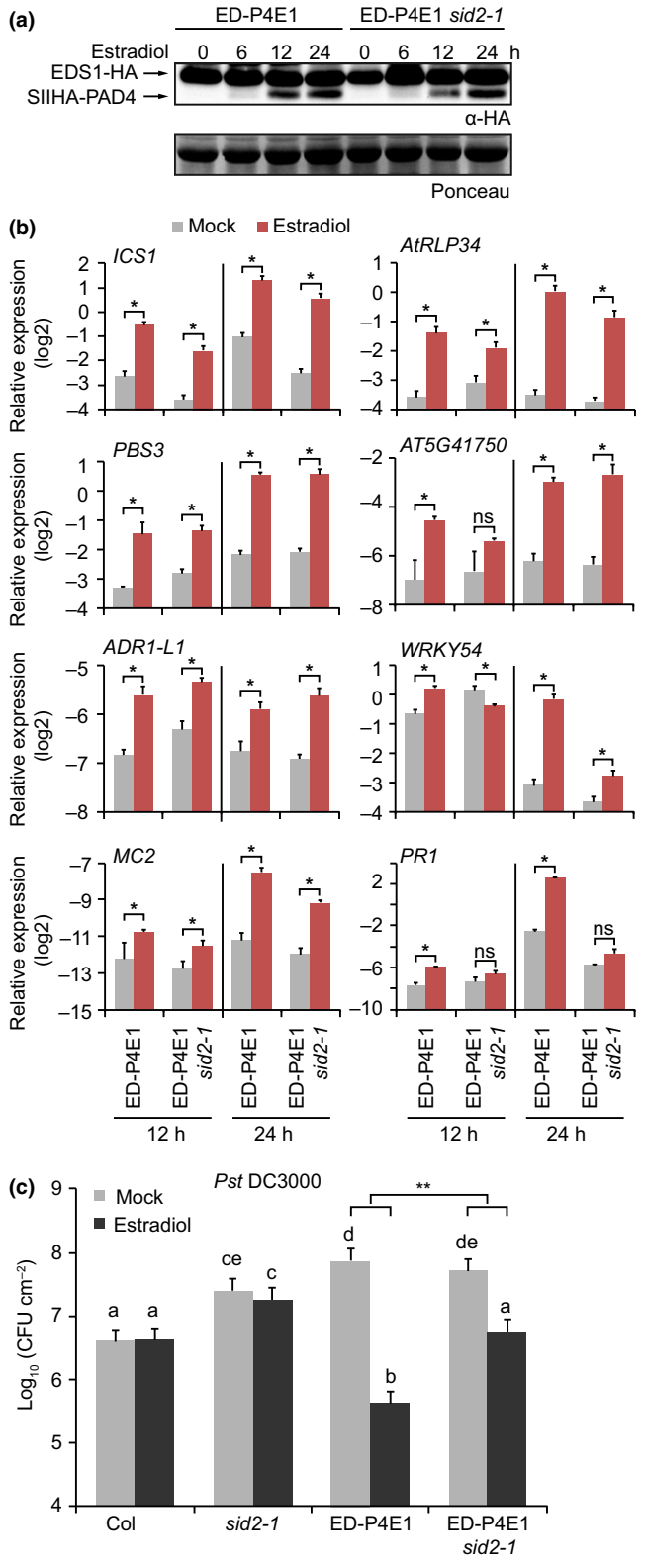


Fig. 5 EDS1/PAD4 signaling involves a major salicylic acid (SA)-independent component. (a) EDS1-HA and SIIHA-PAD4 protein accumulation in Arabidopsis ED-P4E1 and ED-P4E1 *sid2-1* plants at the indicated time points detected on an immunoblot probed with α-HA antibodies. Ponceau staining shows equal sample loading. (b) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of EDS1/PAD4-responsive genes in 4-wk-old ED-P4E1 and ED-P4E1 *sid2-1* plants at 12 h and 24 h after 10 μM estradiol or mock treatment. Log_2 gene expression was normalized to *AT4G26410*. Error bars represent +SD of four technical replicates. *Statistical differences between mock and estradiol treatment, and 'ns' indicates no significant difference (student's *t*-test, $P < 0.01$). These experiments were performed twice with similar results. (c) *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 growth at 3 d post-inoculation (dpi) on lines, as indicated. Leaves of 4-wk-old plants were treated as in Fig. 3(c). Bars represent means and + SE calculated from two independent experiments using a mixed linear model. The Benjamini–Hochberg method was used to adjust *P*-values to correct for multiple testing. Statistically significant differences are indicated by different letters (adjusted *P*-value < 0.01). Statistically significant difference-of-differences of estradiol- and mock- treated samples between EP-P4E1 and ED-P4E1 *sid2-1*: **, $P < 0.01$. CFU, colony-forming units; EDS1, Enhanced Disease Susceptibility 1; PAD4, Phytoalexin Deficient 4.

Second, we tested whether estradiol-induced EDS1/PAD4 basal resistance (observed in Fig. 3c) requires SA by inoculating *Pst* DC3000 onto leaves of ED-P4E1 or ED-P4E1 *sid2-1* plants 24 h after estradiol treatment and measuring bacterial titers at 3 dpi. The *sid2-1* mutation caused a partial loss of estradiol-conditioned resistance, indicating that enhanced basal immunity in ED-P4E1 is composed of SA-dependent and SA-independent sectors (Fig. 5c). We concluded that SA and non-SA expression branches are an intrinsic feature of EDS1/PAD4 basal defense reprogramming.

We then measured the relative contributions of SA-dependent and SA-independent processes to EDS1/PAD4 transcriptional reprogramming in TNL immunity by re-examining a gene expression microarray study (E-MEXP-2405) of *EDS1*-dependent autoimmunity in a Col loss-of-function *Nudix Hydrolase7* (*nudt7-1*) mutant (Straus *et al.*, 2010). Autoimmunity in *nudt7-1* is caused by deregulation of TNL genes including *SNC1* (*Suppressor of Npr1-1, Constitutive 1*) (Wang *et al.*, 2013) and thus represents a TNL immune response. Phenotyping and expression profiling of *nudt7*, *nudt7 eds1-2*, *nudt7 sid2-1* and *nudt7 eds1-2 sid2-1* plants identified SA-promoted and SA-antagonized sectors in *nudt7* autoimmunity (Straus *et al.*, 2010). In our analysis, *EDS1*-dependent genes (378 induced and 43 repressed) were selected by comparing expression changes of *nudt7-1* vs *nudt7-1 eds1-2*. *EDS1*-dependent but *SID2*-independent genes (724 induced and 190 repressed) were selected by comparing *nudt7-1 sid2-1* vs *nudt7-1 sid2-1 eds1-2*. Strikingly, 83% (314 of 378) of the *EDS1*-dependent induced and 51% (22 of 43) repressed genes were unaffected by *sid2-1* (Fig. S3a; Table S5). Pearson correlation and complete linkage clustering of the *EDS1*-dependent genes separated *nudt7* and *nudt7 sid2* expression changes from those of Col, *nudt7 eds1-2* and *nudt7 eds1-2 sid2-1*, as represented in a heat map (Fig. S3b). In a different microarray experiment (GSE34047), 71% (223 of 314) of the *EDS1*-dependent SA-independent induced genes were upregulated by SA treatment (Fig. S3c), indicating that these are SA-responsive genes. Our analysis suggests that a significant portion of EDS1 and SA signaling operates in parallel to regulate a

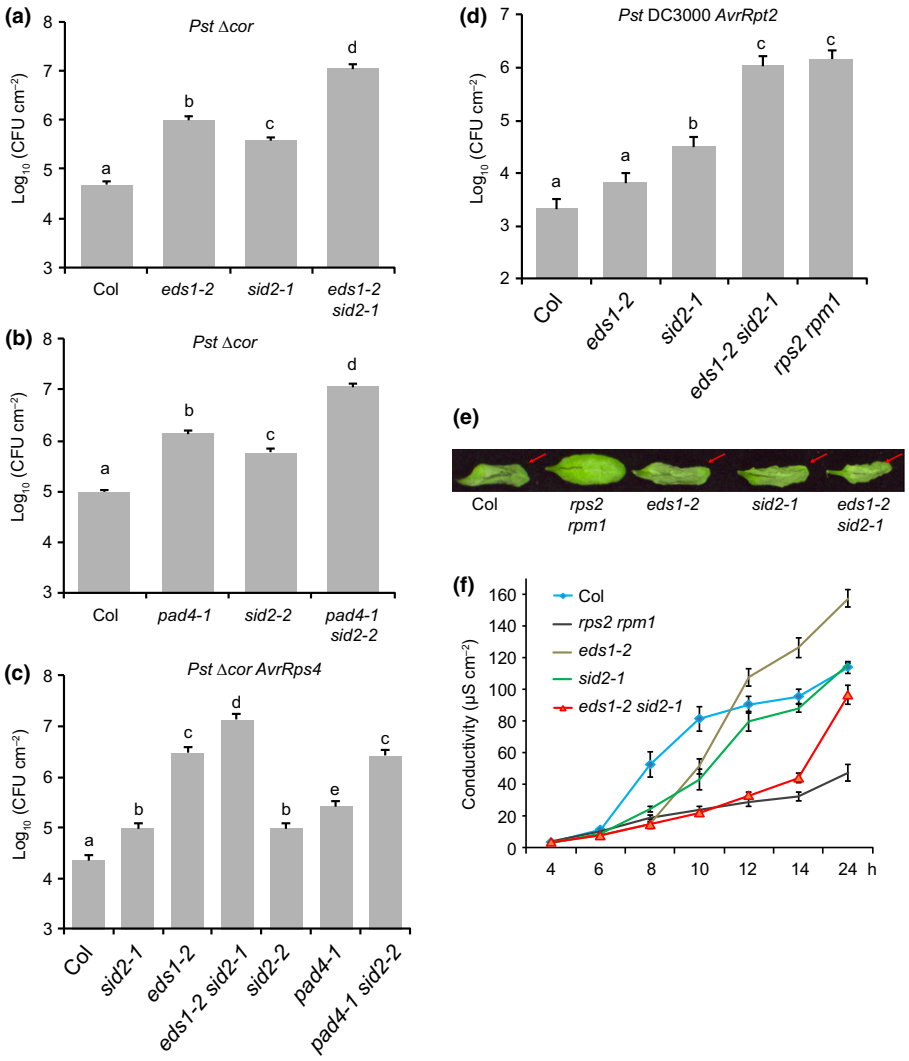


Fig. 6 Separate EDS1/PAD4 and salicylic acid (SA) pathways contribute to basal resistance. (a, b) Growth of a *Pseudomonas syringae* pv. *tomato* (*Pst*) strain lacking coronatine (*Pst* Δ*cor*) at 3 d post-inoculation (dpi) in leaves of the indicated 4-wk-old Arabidopsis plant genotypes. Leaves were hand-infiltrated with bacterial suspensions (OD_{600} = 0.0002). CFU, colony-forming units. (c, d) Bacterial growth assays performed as in (a, b), respectively, with *Pst* Δ*cor AvrRps4* and *Pst* DC3000 *AvrRpt2* in the indicated genotypes. Bars represent means + SE calculated from two independent experiments using a mixed linear model. The Benjamini–Hochberg method was used to adjust *P*-values to correct for multiple testing. Statistically significant differences are indicated by different letters (adjusted *P*-value < 0.01). (e) Macroscopic cell death on leaves (from 10 to 12 tested per line) of the indicated genotypes at 24 h after infiltration of 4-wk-old plants with *Pst* DC3000 *AvrRpt2* at OD_{600} = 0.02. Red arrows indicate leaves showing cell death. (f) Quantitative ion leakage assays over 24 h in leaf discs of 4-wk-old Col genotypes, as indicated, after infiltration with *Pst* DC3000 *AvrRpt2* at OD_{600} = 0.02. Error bars represent + SD of four samples per genotype. EDS1, Enhanced Disease Susceptibility 1; PAD4, Phytoalexin Deficient 4.

common set of defense genes in TNL immunity. It further suggests that EDS1 is able to preserve induction of many SA-responsive genes when SA signaling is disabled.

EDS1/PAD4 and SA work in parallel in bacterial resistance

The above results point to parallel actions of EDS1/PAD4 and SA in basal and TNL immunity. However, *eds1-2 sid2-1* double mutant plants are as susceptible as *eds1-1* or *sid2-1* single mutants to *Pst* DC3000 infection (Fig. S4) (Venugopal *et al.*, 2009), which fits more to EDS1/PAD4 promoting SA in the same pathway, as depicted in models. We therefore tested whether separate EDS1/PAD4 and *ICS1*-generated SA pathways might be obscured by the virulence factor coronatine (COR) which is delivered by *Pst* DC3000 and is a potent JA-Ile mimic that antagonizes host SA signaling to promote infection (Geng *et al.*, 2012; Zheng *et al.*, 2012). In growth assays of a weakly virulent *Pst* strain lacking COR (*Pst* Δ*cor*) (Ma *et al.*, 1991) on WT Col, *eds1-2*, *sid2-1* and *eds1-2 sid2-1* leaves, the *eds1-2* and *sid2-1* single mutants displayed intermediate susceptibility compared to resistant Col and the highly susceptible *eds1-2 sid2-1* double mutant (Fig. 6a). A similar *Pst* Δ*cor* infection trend was observed on *pad4-1* and *sid2-2* single mutants compared to *pad4-1 sid2-2* (Fig. 6b). Genetically additive contributions of *EDS1/PAD4* and *ICS1*-generated SA in resistance to *Pst* Δ*cor* are consistent with parallel actions in basal resistance.

We next tested whether there is genetic additivity between *EDS1/PAD4* and SA pathways in TNL immunity by infecting the above WT and mutant plants with *Pst* Δ*cor* expressing the TNL (*RRS1/RPS4*)-recognized effector AvrRps4 (*Pst* Δ*cor AvrRps4*). Here, *eds1-2 sid2-1* and *pad4-1 sid2-2* plants supported higher amounts of bacterial growth than *eds1-2* or *pad4-1* single mutants (Fig. 6c). From these data, we concluded that parallel EDS1/PAD4 and SA pathways underlie a major portion of basal and TNL-mediated immunity.

EDS1 and *ICS1* contribute additively to CNL RPS2 resistance but not cell death

The above basal and TNL immunity phenotypes against *Pst* Δ*cor* strains reminded us of genetically additive contributions of *EDS1* and *ICS1*-generated SA in ETI reported for Arabidopsis CNL

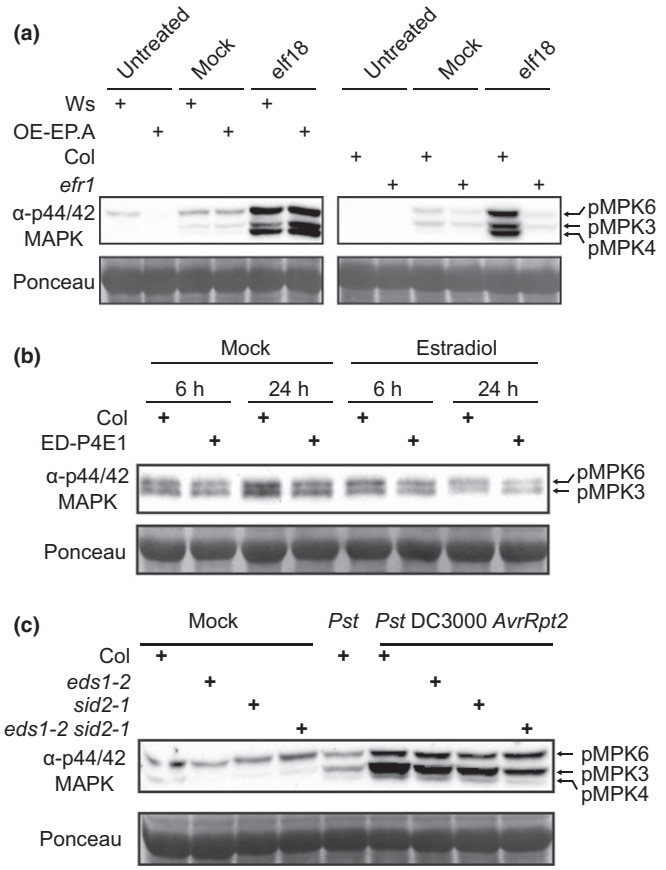


Fig. 7 EDS1/PAD4 core signaling does not involve mitogen-activated protein kinase (MAPK) activation. (a) Total protein extracts from leaves of 4-wk-old Arabidopsis Ws, OE-EP.A, Col and *efr1* plants 15 min after no treatment (untreated), water (mock) or 1 μM elf18. Activated MAPKs were detected on an immunoblot probed with α-p44/42 MAPK antibodies, as indicated. Ponceau staining of the blot shows equal sample loading. (b) Total protein extracts from leaves of 4-wk-old Col and ED-P4E1 plants 6 h and 24 h after spraying with 10 μM estradiol or DMSO (mock). MAPK activation was monitored on immunoblots as in (a). (c) Total protein extracts collected at 4 hpi from leaves of 4-wk-old Col genotypes, as indicated, after infiltration with water (mock), *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) or *Pst* DC3000 *AvrRpt2* (OD600 = 0.01). MAPK activation was monitored on immunoblots as in (a). EDS1, Enhanced Disease Susceptibility 1; PAD4, Phytoalexin Deficient 4.

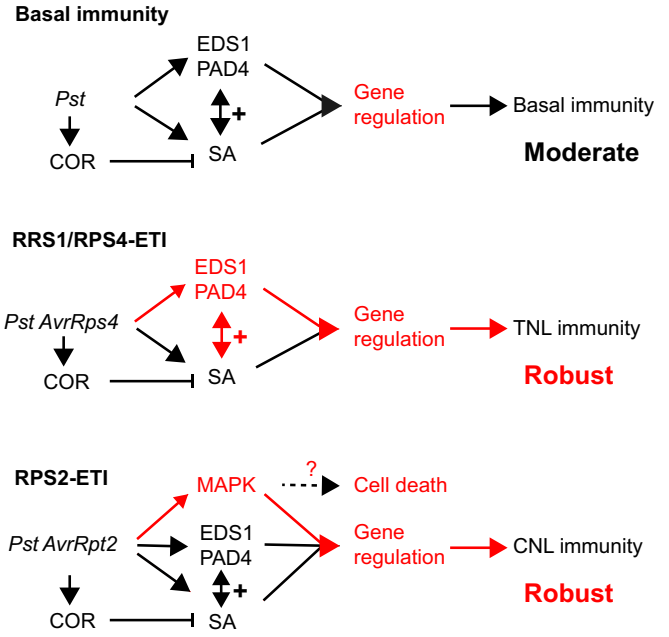


Fig. 8 A model for parallel pathways in basal, TNL and CNL immunity. In the model, separate actions of the EDS1/PAD4, salicylic acid (SA) and mitogen-activated protein kinase (MAPK) pathways enable the plant to regulate a common set of defense genes against biotrophic pathogens. A feedback loop between EDS1/PAD4 and SA (+) mutually reinforces these two immune sectors. Coronatine (COR) produced by *Pseudomonas syringae* (*Pst*) bacteria represents one means of disabling SA signaling which is protected against by a TNL (e.g. *RRS1*/*RPS4*) boosted EDS1/PAD4 pathway or a CNL (*RPS2*) boosted MAPK pathway, as indicated by the thick red arrows, increasing network robustness in Effector-triggered Immunity (ETI). TNL, nucleotide-binding/leucine-rich-repeat (NLR) receptors with a N-terminal Toll-interleukin-1 domain; CNL, NLR receptors with a N-terminal coiled-coiled domain. EDS1, Enhanced Disease Susceptibility 1; PAD4, Phytoalexin Deficient 4.

receptor *RPS2* (Venugopal *et al.*, 2009), which we confirmed (Fig. 6d). Surprisingly, although *eds1-1 sid2-1* leaves were as susceptible to *Pst AvrRpt2* as an *rps2 rpm1* CNL receptor mutant (Fig. 6d) (Venugopal *et al.*, 2009), they produced equivalent macroscopic cell death to that of WT Col or the *eds1-2* or *sid2-1* single mutants at 24 h (Fig. 6e). In a quantitative ion leakage assay, cell death was delayed in *eds1-2 sid2-1* leaves compared to Col but reached the same level at 24 h (Fig. 6f). The delayed death of *eds1-2 sid2-1* leaves was not due to infection-induced necrosis because *Pst* DC3000 *AvrRpt2* titers were equivalent in *rps2 rpm1* leaves which did not produce cell death at 24 h (Fig. 6e,f). These data suggest that parallel EDS1 and SA-driven processes in RPS2 (CNL) resistance are unrelated to host cell death propagation.

Altogether, the bacterial infection data support contributions of separate EDS1/PAD4 and SA signaling pathways in basal, TNL and CNL (RPS2) immunity.

EDS1/PAD4-transcriptional reprogramming does not involve sustained MAPK signaling

In *RPS2* ETI, sustained activation of MAP kinase (MAPK) pathways involving MPK3 and MPK6 confers SA-independent regulation of many SA-responsive genes which partially protects plants against SA pathway perturbations (Tsuda *et al.*, 2013). Having established that EDS1/PAD4 also confers SA-independent regulation of many SA-responsive genes (Figs 5, S3) and partially compensates for SA depletion in biological resistance (Fig. 6), we tested whether elevated or prolonged MAPK signaling contributes to EDS1/PAD4 actions. We first monitored the presence of active, phosphorylated MPK3 and MPK6 in leaf tissues of 4-wk-old OE-EP.A autoimmune plants on an immunoblot probed with α-p44/42 MAPK antibodies (Tsuda *et al.*, 2013) and found no increase in MPK3 and MPK6 phosphorylation compared to WT Ws (Fig. 7a). In both genotypes, MPK3 and MPK6 phosphorylated forms

were induced 15 min after treatment with the PAMP elicitor, elf18 (Fig. 7a). Therefore, OE-EP.A autoimmunity is not associated with increased MAPK activities and does not affect early PAMP-triggered MAPK phosphorylation. There was also no detectable increase in MPK3 and MPK6 phosphorylation status in ED-P4E1 plants at 6 and 24 h after estradiol treatment (Fig. 7b). In the same tissues, estradiol-induced PAD4 accumulation (Fig. S5a) and expression of the EDS1/PAD4-regulated genes *PAD4*, *FMO1, CBP60g* and *ICS1* occurred at 6 and 24 h (Fig. S5b), indicating that plants had responded to estradiol. ED-P4E1 and Col plants produced equivalent MAPK phosphorylation signatures over a 60 min time-course in response to the PAMP flg22 (Fig. S5c). Also, *eds1-2*, *sid2-1* and *eds1-2 sid2-1* mutants exhibited similarly enhanced MPK3 and MPK6 phosphorylation as Col in *RPS2* ETI against *Pst AvrRpt2* bacteria compared to *Pst* or mock treatments (Fig. 7c), indicating that early RPS2-triggered boosting of MAPK signaling (Tsuda *et al.*, 2013) is independent of *EDS1* and *ICS1*-generated SA. Together, the results suggest that EDS1/PAD4 constitutive or induced transcriptional reprogramming does not involve elevated MAPK signaling.

Discussion

Importance of the salicylic acid (SA) defense node in plant host resistance against biotrophic pathogens is well established (Vlot *et al.*, 2009; Fu & Dong, 2013). Here we show that EDS1/PAD4, besides bolstering SA signaling, work in parallel with *ICS1*-generated SA and protect against perturbations to SA in Arabidopsis basal, TNL and CNL receptor immunity. We present evidence that this EDS1/PAD4 protective role does not involve a boost in MAPK signaling and is therefore likely to be a distinct mechanism which plants have evolved for preserving SA-regulated defenses against pathogens, as depicted in a model (Fig. 8). In this model, we propose a signaling framework for basal, TNL and CNL (RPS2) resistance in which EDS1/PAD4 provide an alternative route for conserving SA-related resistance.

Intrinsic properties of EDS1/PAD4 signaling in innate immunity

Previous studies showed that EDS1 and PAD4 are necessary for promoting *ICS1* gene expression and SA accumulation as part of an amplifying loop in Arabidopsis basal and TNL immunity (Jirage *et al.*, 1999; van Wees & Glazebrook, 2003; Wiermer *et al.*, 2005; Vlot *et al.*, 2009). Evidence also emerged for a second EDS1/PAD4-controlled resistance branch operating independently of SA (Glazebrook *et al.*, 2003; Zhang *et al.*, 2003; Bartsch *et al.*, 2006; Wang *et al.*, 2008; Straus *et al.*, 2010; Gloggnitzer *et al.*, 2014). Here, our aim was to identify a basic EDS1/PAD4 signaling function and determine its relationship to SA in immunity. For this, we characterized a transgenic Arabidopsis line (OE-EP.A in accession Ws) that constitutively overexpresses EDS1/PAD4, leading to autoimmunity (Fig. 1), and another Arabidopsis line (ED-P4E1 in accession Col) in which EDS1/PAD4 immune signaling is conditional on estradiol treatment (Fig. 3). In both systems, only combined overexpression of PAD4 with EDS1 led to induction of defense genes and increased basal immunity (Figs 1c,d,f, 3).

In the estradiol-induced ED-P4E1 system, we find that promotion of SA-dependent and SA-independent resistance sectors is an intrinsic property of EDS1/PAD4 signaling (Figs 3, 5). Nevertheless, estradiol-induced *EDS1*/*PAD4*-dependent genes at 12 h and 24 h represent a small subset of expression changes observed in TNL effector-triggered and autoimmune responses (Fig. 4c). It is therefore likely that activated TNL receptors confer additional properties on the EDS1/PAD4 pathway for defense gene reprogramming in ETI. Recently, we reported on Arabidopsis autoimmunity caused by a TNL (*Dangerous Mix2*, *DM2*) gene cluster in accession Landsberg-*erecta* when combined with overexpressed nuclear-enriched EDS1-YFP (Stuttmann *et al.*, 2016). Although OE-EP.A has similar autoimmune characteristics (Fig. 1, S1), it is in accession Ws-2 which lacks the $DM2^{Ler}$ cluster. We speculate that OE-EP.A autoimmunity engages other TNL genes or, alternatively, is due to increased EDS1/PAD4 activity independently of TNLs.

EDS1/PAD4 protect the SA-responsive disease resistance sector

RNA-seq analysis of ED-P4E1 plants (Fig. 4) and a re-evaluation of *EDS1*- and *ICS1*-regulated genes in TNL autoimmunity (Figs 4, 5b, S3) show that a major EDS1/PAD4 activity is independent of *ICS1*-generated SA, allowing EDS1/PAD4 to mitigate defects in SA resistance. Hence, identified EDS1/PAD4-induced core genes in ED-P4E1 overlap extensively with SA-responsive genes in numerous Arabidopsis transcriptomic datasets (Fig. 4).

Estradiol-induced resistance in an ED-P4E1*sid2-1* line against virulent *Pst* DC3000 provides genetic support for EDS1/PAD4 actions independently of SA in basal immunity (Figs 3c, 5c). Reinforcing a parallel pathway model, *eds1-2 sid2-1* and *pad4-1 sid2-2* double mutants showed increased disease susceptibility compared to the respective single mutants against a weakly virulent *Pst* Δ*cor* bacterial strain (Fig. 6a,b). The same genetic relationship was not observed in basal resistance against virulent *Pst* DC3000 which delivers COR (Venugopal *et al.*, 2009) (Fig. S4). We interpret this difference to be the consequence of *Pst* DC3000-derived COR dampening SA defenses in *eds1-2* or *pad4-1* single mutant plants (Brooks *et al.*, 2005; Geng *et al.*, 2012; Zheng *et al.*, 2012). In TNL ETI conferred by *RRS1*/*RPS4* to *Pst* Δ*cor AvrRsp4*, bacterial growth was strongly restricted in the *sid2-1* or *sid2-2* single mutants, indicative of *EDS1/PAD4* mediating TNL resistance independently of *ICS1*-generated SA (Fig. 6c). A major conclusion from our data is that genetically distinct and mutually reinforcing EDS1 and SA pathways reported for CNL RPS2 ETI (Venugopal *et al.*, 2009) (Fig. 6d), in principal, also operate in basal and TNL immunity against *Pst* bacteria (Fig. 8).

EDS1/PAD4 work in parallel with SA and MAPK defense branches

Sustained activation of MAPKs MPK3 or MPK6 was reported to induce many SA-responsive genes and partially compensate for loss of SA signaling in CNL (*RPS2*) ETI against *Pst* bacteria (Tsuda *et al.*, 2013). We did not observe increased or prolonged activation of MPK3/6 in OE-EP.A autoimmune plants or after estradiol-induction of EDS1/PAD4 resistance in ED-P4E1 plants (Fig. 7a,b). These data are consistent with previous findings that prolonged activation of MPK3/6 is not a feature of *EDS1*-dependent *RRS1*/*RPS4* ETI (Tsuda *et al.*, 2013). Moreover, sustained MPK3/6 signaling was detected in *eds1-2 sid2-1* plants responding to *Pst AvrRpt2* (Fig. 7c), indicating that RPS2-triggered activation of MPK3/6 pathways does not require *EDS1* or *ICS1*-dependent SA signaling. Interestingly, RPS2-boosted MPK3/6 activation in *eds1-2 sid2-1* mutant leaves (Fig. 7c) did not limit bacterial growth (Fig. 6d). MAPK signaling might be responsible for the delayed RPS2-triggered cell death response to *Pst AvrRpt2* bacteria in *eds1-2 sid2-1* plants (Fig. 6e,f). Whatever their role, activated MAPKs are insufficient to fully protect against disabled EDS1/PAD4 and SA signaling in RPS2 ETI (Fig. 6d).

The above results suggest that MPK3/6 signaling is not part of an EDS1/PAD4 mechanism for preserving SA defense outputs. Thus, EDS1/PAD4 might represent a separate resistance branch working in parallel with SA and MAPK pathways (Fig. 8). This idea is supported by studies of MAPK pathway mutants. Inhibition of MPKs 3, 4 and 6 by *P. syringae* effector HopAI1 suppresses early PTI responses (Zhang *et al.*, 2007, 2012) and disabled MPK4 causes activation of autoimmunity via the CNL receptor SUMM2 (Suppressor of *mkk1 mkk2*) (Zhang *et al.*, 2012), which depends on *EDS1*/*PAD4* and SA signaling (Petersen *et al.*, 2000; Brodersen *et al.*, 2006; Qiu *et al.*, 2008). Therefore, both EDS1/PAD4 and SA pathways are operational in CNL (SUMM2) resistance when MAPK signaling is disrupted.

As depicted in our model (Fig. 8), we speculate that the MAPK, SA and EDS1/PAD4 nodes function in different ways to maintain certain defense sectors and increase robustness of the immunity network. With this model in mind, a parallel relationship between EDS1/PAD4 and SA signaling becomes more obvious. For example, *EDS1* and *PAD4* are essential for many instances of TNL autoimmunity which, although associated with SA overproduction, show weak *ICS1* dependence (Li *et al.*, 2001; Shirano *et al.*, 2002; Zbierzak *et al.*, 2013). Conversely, *eds1* disease susceptibility was suppressed by high SA accumulation caused by mutations in *DMR6* (*Downy Mildew Resistance6*) (van Damme *et al.*, 2008; Zeilmaker *et al.*, 2015) or *CPR5* (*Constitutive Expression of PRgenes5*) (Clarke *et al.*, 2000, 2001). Thus, SA can also cover for loss of the EDS1/PAD4 sector in immunity.

Evolution of parallel defense pathways in immunity

The signaling model (Fig. 8) might be rationalized in the context of resistance pathway innovations over host–pathogen coevolution. *MPK* orthologs are present in ancient red algal species (Wang *et al.*, 2015). SA signaling genes appear to have evolved later because core SA components are present in the basal land plant *Marchantia* but not algae (Wang *et al.*, 2015). *EDS1* and *PAD4* orthologs are detected in flowering plants but not, for example, the more basal moss *Physcomitrella patens* (Wagner *et al.*, 2013). In one possible scenario, host MAPK signaling becomes targeted and suppressed by pathogen effectors (Feng & Zhou, 2012) and SA signaling has evolved in part to compensate for disabled MAPK pathways. Pathogen targeting of SA-mediated defenses might have rendered necessary an independent EDS1/PAD4 signaling mechanism, co-opted by TNL and certain CNL receptors to protect this important resistance node (Venugopal *et al.*, 2009; Wagner *et al.*, 2013). EDS1 resides in complexes with several nuclear TNL receptors and is required for all measured TNL outputs (Cui *et al.*, 2015). It is therefore likely that initial EDS1/PAD4 signaling in TNL immunity does not involve SA (Feys *et al.*, 2005; Rietz *et al.*, 2011). EDS1 association with the CNL HRT was also reported (Zhu *et al.*, 2011). Involvement of EDS1/PAD4 in ETI governed by CNL receptors such as RPS2 and HRT (Fig. 6) (Venugopal *et al.*, 2009) as well as functional links between EDS1/PAD4 and the Activated Disease Resistance1 (ADR1) family of conserved CNL proteins (Bonardi *et al.*, 2011; Roberts *et al.*, 2013), might explain presence of *EDS1* and *PAD4* orthologs in monocot lineages which have lost TNLs (Pan *et al.*, 2000; Wagner *et al.*, 2013).

Acknowledgements

We thank Kenichi Tsuda and Takaki Maekawa for helpful discussion and Kenichi Tsuda for advice on statistical analysis using R. This work was supported by The Max-Planck Society and an Alexander von Humboldt Foundation postdoctoral fellowship (H.C.), an International Max-Planck Research School (IMPRS) doctoral fellowship (E.G.), a PhD fellowship from China Scholarship Council (J.Q.) and a grant within Deutsche Forschungsgemeinschaft SFB 680 (Molecular Basis of Evolutionary Innovations) (J.E.P., J.B.).

Author contributions

H.C., E.G. and J.E.P. designed the research; H.C., E.G., J.Q. and J.B. performed experiments; H.C. and B.K. analyzed microarray data and RNA-seq data; H.C., E.G. and J.E.P. wrote the manuscript.

References

Aarts N, Metz M, Holub E, Staskawicz BJ, Daniels MJ, Parker JE. 1998. Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two *R* gene-mediated signaling pathways in *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **95**: 10306–10311.

Anders S, Pyl PT, Huber W. 2015. HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**: 166–169.

Bartsch M, Gobbato E, Bednarek P, Debey S, Schultze JL, Bautor J, Parker JE. 2006. Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in *Arabidopsis* immunity and cell death is regulated by the monooxygenase *FMO1* and the Nudix hydrolase *NUDT7*. *Plant Cell* **18**: 1038–1051.

Bent AF, Kunkel BN, Dahlbeck D, Brown KL, Schmidt R, Giraudat J, Leung J, Staskawicz BJ. 1994. *RPS2* of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. *Science* **265**: 1856–1860.

Bonardi V, Tang S, Stallmann A, Roberts M, Cherkis K, Dangl JL. 2011. Expanded functions for a family of plant intracellular immune receptors beyond specific recognition of pathogen effectors. *Proceedings of the National Academy of Sciences, USA* **108**: 16463–16468.

Botella MA, Parker JE, Frost LN, Bittner-Eddy PD, Beynon JL, Daniels MJ, Holub EB, Jones JD. 1998. Three genes of the Arabidopsis *RPP1* complex resistance locus recognize distinct *Peronospora parasitica* avirulence determinants. *Plant Cell* **10**: 1847–1860.

Brodersen P, Petersen M, Bjorn Nielsen H, Zhu S, Newman MA, Shokat KM, Rietz S, Parker J, Mundy J. 2006. Arabidopsis MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. *Plant Journal* **47**: 532–546.

Brooks DM, Bender CL, Kunkel BN. 2005. The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*. *Molecular Plant Pathology* **6**: 629–639.

Caillaud MC, Asai S, Rallapalli G, Piquerez S, Fabro G, Jones JD. 2013. A downy mildew effector attenuates salicylic acid-triggered immunity in Arabidopsis by interacting with the host mediator complex. *PLoS Biology* **11**: e1001732.

Clarke JD, Aarts N, Feys BJ, Dong XN, Parker JE. 2001. Constitutive disease resistance requires *EDS1* in the *Arabidopsis* mutants *cpr1* and *cpr6* and is partially *EDS1*-dependent in *cpr5*. *Plant Journal* **26**: 409–420.

Clarke JD, Volko SM, Ledford H, Ausubel FM, Dong X. 2000. Roles of salicylic acid, jasmonic acid, and ethylene in *cpr*-induced resistance in arabidopsis. *Plant Cell* **12**: 2175–2190.

Cui H, Tsuda K, Parker JE. 2015. Effector-triggered immunity: from pathogen perception to robust defense. *Annual Review of Plant Biology* **66**: 487–511.

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiology* **139**: 5–17.

van Damme M, Huibers RP, Elberse J, Van den Ackerveken G. 2008. *Arabidopsis DMR6* encodes a putative 2OG-Fe(II) oxygenase that is defense-associated but required for susceptibility to downy mildew. *Plant Journal* **54**: 785–793.

Day B, Dahlbeck D, Staskawicz BJ. 2006. NDR1 interaction with RIN4 mediates the differential activation of multiple disease resistance pathways in Arabidopsis. *Plant Cell* **18**: 2782–2791.

Djamei A, Schipper K, Rabe F, Ghosh A, Vincon V, Kahnt J, Osorio S, Tohge T, Fernie AR, Feussner I *et al.* 2011. Metabolic priming by a secreted fungal effector. *Nature* **478**: 395–398.

Dodds PN, Rathjen JP. 2010. Plant immunity: towards an integrated view of plant-pathogen interactions. *Nature Reviews Genetics* **11**: 539–548.

Eisen MB, Spellman PT, Brown PO, Botstein D. 1998. Cluster analysis and display of genome-wide expression patterns. *Proceedings of the National Academy of Sciences, USA* **95**: 14863–14868.

Falk A, Feys BJ, Frost LN, Jones JD, Daniels MJ, Parker JE. 1999. *EDS1*, an essential component of *R* gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. *Proceedings of the National Academy of Sciences, USA* **96**: 3292–3297.

Feng F, Yang F, Rong W, Wu X, Zhang J, Chen S, He C, Zhou JM. 2012. A *Xanthomonas* uridine 5′-monophosphate transferase inhibits plant immune kinases. *Nature* **485**: 114–118.

Feng F, Zhou JM. 2012. Plant-bacterial pathogen interactions mediated by type III effectors. *Current Opinion in Plant Biology* **15**: 469–476.

Feys BJ, Moisan LJ, Newman MA, Parker JE. 2001. Direct interaction between the *Arabidopsis* disease resistance signaling proteins, EDS1 and PAD4. *Embo Journal* **20**: 5400–5411.

Feys BJ, Wiermer M, Bhat RA, Moisan LJ, Medina-Escobar N, Neu C, Cabral A, Parker JE. 2005. *Arabidopsis* SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. *Plant Cell* **17**: 2601–2613.

Fu ZQ, Dong X. 2013. Systemic acquired resistance: turning local infection into global defense. *Annual Review of Plant Biology* **64**: 839–863.

Garcia AV, Blanvillain-Baufume S, Huibers RP, Wiermer M, Li G, Gobbato E, Rietz S, Parker JE. 2010. Balanced nuclear and cytoplasmic activities of EDS1 are required for a complete plant innate immune response. *PLoS Pathogens* **6**: e1000970.

Geng X, Cheng J, Gangadharan A, Mackey D. 2012. The coronatine toxin of *Pseudomonas syringae* is a multifunctional suppressor of *Arabidopsis* defense. *Plant Cell* **24**: 4763–4774.

Gimenez-Ibanez S, Boter M, Fernandez-Barbero G, Chini A, Rathjen JP, Solano R. 2014. The bacterial effector HopX1 targets JAZ transcriptional repressors to activate jasmonate signaling and promote infection in *Arabidopsis*. *PLoS Biology* **12**: e1001792.

Glazebrook J, Chen W, Estes B, Chang HS, Nawrath C, Metraux JP, Zhu T, Katagiri F. 2003. Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. *Plant Journal* **34**: 217–228.

Gloggnitzer J, Akimcheva S, Srinivasan A, Kusenda B, Riehs N, Stampfl H, Bautor J, Dekrout B, Jonak C, Jimenez-Gomez JM *et al.* 2014. Nonsense-mediated mRNA decay modulates immune receptor levels to regulate plant antibacterial defense. *Cell Host & Microbe* **16**: 376–390.

Heidrich K, Tsuda K, Blanvillain-Baufume S, Wirthmueller L, Bautor J, Parker JE. 2013. *Arabidopsis* TNL-WRKY domain receptor RRS1 contributes to temperature-conditioned RPS4 auto-immunity. *Frontiers in Plant Science* **4**: 403.

Heidrich K, Wirthmueller L, Tasset C, Pouzet C, Deslandes L, Parker JE. 2011. *Arabidopsis* EDS1 connects pathogen effector recognition to cell compartment-specific immune responses. *Science* **334**: 1401–1404.

Jacob F, Vernaldi S, Maekawa T. 2013. Evolution and conservation of plant NLR functions. *Frontiers in Immunology* **4**: 297.

Jirage D, Tootle TL, Reuber TL, Frost LN, Feys BJ, Parker JE, Ausubel FM, Glazebrook J. 1999. *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. *Proceedings of the National Academy of Sciences, USA* **96**: 13583–13588.

Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology* **14**: R36.

Kim Y, Tsuda K, Igarashi D, Hillmer RA, Sakakibara H, Myers CL, Katagiri F. 2014. Mechanisms underlying robustness and tunability in a plant immune signaling network. *Cell Host & Microbe* **15**: 84–94.

Li X, Clarke JD, Zhang Y, Dong X. 2001. Activation of an EDS1-mediated *R*-gene pathway in the *snc1* mutant leads to constitutive, NPR1-independent pathogen resistance. *Molecular Plant–Microbe Interactions* **14**: 1131–1139.

Ma SW, Morris VL, Cuppels DA. 1991. Characterization of a DNA region required for production of the phytotoxin coronatine by *Pseudomonas syringae pv. tomato*. *Molecular Plant-Microbe Interactions* **4**: 69–74.

Macho AP, Zipfel C. 2015. Targeting of plant pattern recognition receptor-triggered immunity by bacterial type-III secretion system effectors. *Current Opinion in Microbiology* **23**: 14–22.

Maekawa T, Kufer TA, Schulze-Lefert P. 2011. NLR functions in plant and animal immune systems: so far and yet so close. *Nature Immunology* **12**: 817–826.

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

Navarro L, Bari R, Achard P, Lison P, Nemri A, Harberd NP, Jones JD. 2008. DELLAs control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. *Current Biology* **18**: 650–655.

Palma K, Thorgrimsen S, Malinovsky FG, Fiil BK, Nielsen HB, Brodersen P, Hofius D, Petersen M, Mundy J. 2010. Autoimmunity in *Arabidopsis acd11* is mediated by epigenetic regulation of an immune receptor. *PLoS Pathogens* **6**: e1001137.

Pan Q, Wendel J, Fluhr R. 2000. Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. *Journal of Molecular Evolution* **50**: 203–213.

Pegadaraju V, Louis J, Singh V, Reese JC, Bautor J, Feys BJ, Cook G, Parker JE, Shah J. 2007. Phloem-based resistance to green peach aphid is controlled by

Arabidopsis PHYTOALEXIN DEFICIENT4 without its signaling partner *ENHANCED DISEASE SUSCEPTIBILITY1*. *Plant Journal* **52**: 332–341.

Petersen M, Brodersen P, Naested H, Andreasson E, Lindhart U, Johansen B, Nielsen HB, Lacy M, Austin MJ, Parker JE *et al.* 2000. *Arabidopsis* map kinase 4 negatively regulates systemic acquired resistance. *Cell* **103**: 1111–1120.

Qiu J-L, Zhou L, Yun B-W, Nielsen HB, Fiil BK, Petersen K, MacKinlay J, Loake GJ, Mundy J, Morris PC. 2008. *Arabidopsis* mitogen-activated protein kinase kinases MKK1 and MKK2 have overlapping functions in defense signaling mediated by MEKK1, MPK4, and MKS1. *Plant Physiology* **148**: 212–222.

Rietz S, Stamm A, Malonek S, Wagner S, Becker D, Medina-Escobar N, Vlot AC, Feys BJ, Niefind K, Parker JE. 2011. Different roles of Enhanced Disease Susceptibility1 (EDS1) bound to and dissociated from Phytoalexin Deficient4 (PAD4) in *Arabidopsis* immunity. *New Phytologist* **191**: 107–119.

Roberts M, Tang S, Stallmann A, Dangl JL, Bonardi V. 2013. Genetic requirements for signaling from an autoactive plant NB-LRR intracellular innate immune receptor. *PLoS Genetics* **9**: e1003465.

Robert-Seilaniantz A, Grant M, Jones JD. 2011. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annual Review of Phytopathology* **49**: 317–343.

Seyfferth C, Tsuda K. 2014. Salicylic acid signal transduction: the initiation of biosynthesis, perception and transcriptional reprogramming. *Front Plant Science* **5**: 697.

Shirano Y, Kachroo P, Shah J, Klessig DF. 2002. A gain-of-function mutation in an *Arabidopsis* Toll Interleukin-1 Receptor-Nucleotide Binding Site-Leucine-Rich Repeat type *R* gene triggers defense responses and results in enhanced disease resistance. *Plant Cell* **14**: 3149–3162.

Straus MR, Rietz S, Loren Ver, van Themaat E, Bartsch M, Parker JE. 2010. Salicylic acid antagonism of EDS1-driven cell death is important for immune and oxidative stress responses in *Arabidopsis*. *Plant Journal* **62**: 628–640.

Stuttmann J, Peine N, Garcia AV, Wagner C, Choudhury SR, Wang Y, James GV, Griebel T, Alcazar R, Tsuda K *et al.* 2016. *Arabidopsis thaliana DM2h* (*R8*) within the Landsberg *RPP1-like Resistance* locus underlies three different cases of *EDS1*-conditioned autoimmunity. *PLoS Genetics* **12**: e1005990.

Suarez-Rodriguez MC, Adams-Phillips L, Liu Y, Wang H, Su SH, Jester PJ, Zhang S, Bent AF, Krysan PJ. 2007. MEKK1 is required for flg22-induced MPK4 activation in *Arabidopsis* plants. *Plant Physiology* **143**: 661–669.

Tissier AF, Marillonnet S, Klimyuk V, Patel K, Torres MA, Murphy G, Jones JD. 1999. Multiple independent defective suppressor-mutator transposon insertions in Arabidopsis: a tool for functional genomics. *Plant Cell* **11**: 1841–1852.

Tsuda K, Mine A, Bethke G, Igarashi D, Botanga CJ, Tsuda Y, Glazebrook J, Sato M, Katagiri F. 2013. Dual regulation of gene expression mediated by extended MAPK activation and salicylic acid contributes to robust innate immunity in *Arabidopsis thaliana*. *PLoS Genetics* **9**: e1004015.

Tsuda K, Sato M, Stoddard T, Glazebrook J, Katagiri F. 2009. Network properties of robust immunity in plants. *PLoS Genetics* **5**: e1000772.

Tsuda K, Somssich IE. 2015. Transcriptional networks in plant immunity. *New Phytologist* **206**: 932–947.

Venugopal SC, Jeong RD, Mandal MK, Zhu S, Chandra-Shekara AC, Xia Y, Hersh M, Stromberg AJ, Navarre D, Kachroo A *et al.* 2009. Enhanced disease susceptibility 1 and salicylic acid act redundantly to regulate resistance gene-mediated signaling. *PLoS Genetics* **5**: e1000545.

Vlot AC, Dempsey DA, Klessig DF. 2009. Salicylic Acid, a multifaceted hormone to combat disease. *Annual Review of Phytopathology* **47**: 177–206.

Wagner S, Stuttmann J, Rietz S, Guerois R, Brunstein E, Bautor J, Niefind K, Parker JE. 2013. Structural basis for signaling by exclusive EDS1 heteromeric complexes with SAG101 or PAD4 in plant innate immunity. *Cell Host & Microbe* **14**: 619–630.

Wang Y, Bao Z, Zhu Y, Hua J. 2009. Analysis of temperature modulation of plant defense against biotrophic microbes. *Molecular Plant-Microbe Interactions* **22**: 498–506.

Wang C, Liu Y, Li SS, Han GZ. 2015. Insights into the origin and evolution of the plant hormone signaling machinery. *Plant Physiology* **167**: 872–886.

Wang H, Lu Y, Liu P, Wen W, Zhang J, Ge X, Xia Y. 2013. The ammonium/nitrate ratio is an input signal in the temperature-modulated, *SNC1*-mediated and *EDS1*-dependent autoimmunity of *nudt6-2 nudt7*. *Plant Journal* **73**: 262–275.

Wang L, Mitra RM, Hasselmann KD, Sato M, Lenarz-Wyatt L, Cohen JD, Katagiri F, Glazebrook J. 2008. The genetic network controlling the *Arabidopsis* transcriptional response to *Pseudomonas syringae pv. maculicola*: roles of major regulators and the phytotoxin coronatine. *Molecular Plant–Microbe Interactions* **21**: 1408–1420.

van Wees SC, Glazebrook J. 2003. Loss of non-host resistance of *Arabidopsis NahG* to *Pseudomonas syringae pv. phaseolicola* is due to degradation products of salicylic acid. *Plant Journal* **33**: 733–742.

Wiermer M, Feys BJ, Parker JE. 2005. Plant immunity: the EDS1 regulatory node. *Current Opinion in Plant Biology* **8**: 383–389.

Wildermuth MC, Dewdney J, Wu G, Ausubel FM. 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* **414**: 562–565.

Williams SJ, Sornaraj P, deCourcy-Ireland E, Menz RI, Kobe B, Ellis JG, Dodds PN, Anderson PA. 2011. An autoactive mutant of the M flax rust resistance protein has a preference for binding ATP, whereas wild-type M protein binds ADP. *Molecular Plant–Microbe Interactions* **24**: 897–906.

Wirthmueller L, Zhang Y, Jones JD, Parker JE. 2007. Nuclear accumulation of the *Arabidopsis* immune receptor RPS4 is necessary for triggering EDS1-dependent defense. *Current Biology* **17**: 2023–2029.

Witte CP, Noel LD, Gielbert J, Parker JE, Romeis T. 2004. Rapid one-step protein purification from plant material using the eight-amino acid StrepII epitope. *Plant Molecular Biology* **55**: 135–147.

Zbierzak AM, Porfirova S, Griebel T, Melzer M, Parker JE, Dormann P. 2013. A TIR-NBS protein encoded by *Arabidopsis Chilling Sensitive 1* (*CHS1*) limits chloroplast damage and cell death at low temperature. *Plant Journal* **75**: 539–552.

Zeilmaker T, Ludwig NR, Elberse J, Seidl MF, Berke L, Van Doorn A, Schuurink RC, Snel B, Van den Ackerveken G. 2015. DOWNY MILDEW RESISTANT 6 and DMR6-LIKE OXYGENASE 1 are partially redundant but distinct suppressors of immunity in Arabidopsis. *Plant Journal* **81**: 210–222.

Zhang YL, Goritschnig S, Dong XN, Li X. 2003. A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in *suppressor of npr1-1, constitutive 1*. *Plant Cell* **15**: 2636–2646.

Zhang J, Shao F, Li Y, Cui H, Chen L, Li H, Zou Y, Long C, Lan L, Chai J *et al.* 2007. A *Pseudomonas syringae* effector inactivates MAPKs to suppress PAMP-induced immunity in plants. *Cell Host & Microbe* **1**: 175–185.

Zhang Z, Wu Y, Gao M, Zhang J, Kong Q, Liu Y, Ba H, Zhou J, Zhang Y. 2012. Disruption of PAMP-induced MAP kinase cascade by a *Pseudomonas syringae* effector activates plant immunity mediated by the NB-LRR protein SUMM2. *Cell Host & Microbe* **11**: 253–263.

Zheng XY, Spivey NW, Zeng W, Liu PP, Fu ZQ, Klessig DF, He SY, Dong X. 2012. Coronatine promotes *Pseudomonas syringae* virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. *Cell Host & Microbe* **11**: 587–596.

Zhou N, Tootle TL, Tsui F, Klessig DF, Glazebrook J. 1998. *PAD4* functions upstream from salicylic acid to control defense responses in *Arabidopsis*. *Plant Cell* **10**: 1021–1030.

Zhu S, Jeong RD, Venugopal SC, Lapchyk L, Navarre D, Kachroo A, Kachroo P. 2011. SAG101 forms a ternary complex with EDS1 and PAD4 and is required for resistance signaling against turnip crinkle virus. *PLoS Pathogens* **7**: e1002318.

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JD, Boller T, Felix G. 2006. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* **125**: 749–760.

Zuo J, Niu QW, Chua NH. 2000. Technical advance: an estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *Plant Journal* **24**: 265–273.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Resistance phenotypes of Arabidopsis transgenic lines over expressing EDS1 and/or PAD4.

Fig. S2 Analysis of Arabidopsis transgenic lines expressing estradiol-inducible PAD4 with 35S:EDS1.

Fig. S3 *EDS1*-driven transcriptional reprogramming in an Arabidopsis *nudt7* TNL autoimmune background independently of *ICS1*-generated SA**.**

Fig. S4 Similar growth of *Pst* DC3000 in Arabidopsis *eds1 sid2* double mutant and *eds1* or *sid2* single mutant plants.

Fig. S5 PAMP-induced MAPK activation is intact in Arabidopsis ED-P4E1 plants.

Table S1 Primers used for qPCR.

Table S2 Upregulated genes from analysis of estradiol vs mock treatments at 6, 12, or 24 h in Arabidopsis ED-P4E1 plants.

Table S3 Arabidopsis microarray experiments identified by GENEVESTIGATOR which have highest similarity to 135 core genes from ED-P4E1 RNA-seq.

Table S4 Arabidopsis microarray experiments identified by GENEVESTIGATOR which are most different to 135 core genes from ED-P4E1 RNA-seq.

Table S5 *EDS1*-dependent genes from analysis of *nudt7* vs *nudt7 eds1* gene expression changes in a microarray dataset (E-MEXP-2405).

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.