Enhanced disease susceptibility 1 and salicylic acid act redundantly to regulate resistance gene-mediated signaling

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Enhanced Disease Susceptibility 1 and Salicylic Acid Act Redundantly to Regulate Resistance Gene-Mediated Signaling

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Abstract

Resistance (R) protein–associated pathways are well known to participate in defense against a variety of microbial pathogens. Salicylic acid (SA) and its associated proteinaceous signaling components, including enhanced disease susceptibility 1 (EDS1), non–race-specific disease resistance 1 (NDR1), phytoalexin deficient 4 (PAD4), senescence associated gene 101 (SAG101), and ED5, have been identified as components of resistance derived from many R proteins. Here, we show that EDS1 and SA fulfill redundant functions in defense signaling mediated by R proteins, which were thought to function independent of EDS1 and/or SA. Simultaneous mutations in EDS1 and the SA–synthesizing enzyme SID2 compromised hypersensitive response and/or resistance mediated by R proteins that contain coiled coil domains at their N-terminal ends. Furthermore, the expression of R genes and the associated defense signaling induced in response to a reduction in the level of oleic acid were also suppressed by compromising SA biosynthesis in the eds1 mutant background. The functional redundancy with SA was specific to EDS1. Results presented here redefine our understanding of the roles of EDS1 and SA in plant defense.

Introduction

Plants have evolved highly specific mechanisms to resist pathogens. One of the common ways to counter pathogen growth involves the deployment of resistant (R) proteins, which confer protection against specific races of pathogens carrying corresponding avirulence (Avr) genes [1]. Following recognition of the pathogen, one or more signal transduction pathways are induced in the host plant and these lead to the prevention of colonization by the pathogen. Induction of defense reactions is often accompanied by localized cell death at the site of pathogen entry. This phenomenon, termed the hypersensitive response (HR), is one of the earliest visible manifestations of induced defense reactions and resembles programmed cell death in animals [1–6]. Concurrent with HR development, defense reactions are triggered in both local and distant parts of the plant and accompanied by a local and systemic increase in endogenous salicylic acid (SA) levels and the upregulation of a large set of defense genes, including those encoding pathogenesis-related (PR) proteins [7–9].

The SA signal transduction pathway plays a key role in plant defense signaling (see reviews in [10–12]). Arabidopsis mutants that are impaired in SA responsiveness, such as npr1 (Nonexpressor of PR; [13–15]), or are defective in pathogen-induced SA accumulation, such as eds1 (Enhanced Disease Susceptibility 1; [16]), eds5 (Enhanced Disease Susceptibility 5; [17]), sid2 (isochorismate synthase; [18]) and pad4 (Phytoalexin Deficient 4; [19]), exhibit enhanced susceptibility to pathogen infection and show impaired PR gene expression. The EDS1, EDS5, PAD4, NPR1 and SID2 proteins participate in both basal disease resistance to virulent pathogens as well as R protein-mediated resistance to avirulent pathogens [20]. Defense signaling mediated via a majority of R proteins, which contain Toll-interleukin1-like (TIR) domains at their N-terminal ends, is dependent on EDS1 [21]. Conversely, the NDR1 (Non-race-specific Disease Resistance) protein is required for many R proteins that contain coiled-coil (CC) domains at their N-terminal ends. However, several CC-nucleotide binding site (NBS)-leucine rich repeat (LRR) type of R proteins, including RPP8, RPP13-Nd, HRT, and RPP7, signal resistance via a pathway(s) that is independent of NDR1 [21,22–24]. Strikingly, the CC-NBS-LRR gene HRT, which confers resistance to Turnip Crinkle Virus (TCV), is dependent on EDS1 [23]. Besides HRT, the only other CC domain-containing R protein that utilizes an EDS1-dependent pathway is RPW8, which confers broad-spectrum resistance to powdery mildew [25].
However, RPW8 is not a typical NBS-LRR type of R protein; it contains an N-terminal transmembrane domain in addition to the molecular signaling pathways places salicylic acid and enhanced disease susceptibility 1 downstream of resistant protein activation. In addition, enhanced disease susceptibility 1 is primarily thought to function in the signaling initiated via Toll-interleukin 1-receptor type of resistance proteins. Here, we show that salicylic acid and enhanced disease susceptibility 1 serve redundant functions in defense signaling mediated by coiled-coil-domain containing resistance proteins that were thought to function independent of enhanced disease susceptibility 1. Furthermore, resistance signaling induced under low oleic acid conditions also requires enhanced disease susceptibility 1 and salicylic acid in a redundant manner, but these components are required upstream of resistance gene expression. Together, these results show that the functional redundancy between salicylic acid and enhanced disease susceptibility 1 has precluded their detection as required components of many resistance protein–signaling pathways.

Author Summary

Salicylic acid and enhanced disease susceptibility 1 are important components of resistance gene-mediated defense signaling against diverse pathogens in a variety of plants. Present understanding of plant defense signaling pathways places salicylic acid and enhanced disease susceptibility 1 downstream of resistant protein activation. In addition, enhanced disease susceptibility 1 is primarily thought to function in the signaling initiated via Toll-interleukin 1-receptor type of resistance proteins. Here, we show that salicylic acid and enhanced disease susceptibility 1 serve redundant functions in defense signaling mediated by coiled-coil-domain containing resistance proteins that were thought to function independent of enhanced disease susceptibility 1. Furthermore, resistance signaling induced under low oleic acid conditions also requires enhanced disease susceptibility 1 and salicylic acid in a redundant manner, but these components are required upstream of resistance gene expression. Together, these results show that the functional redundancy between salicylic acid and enhanced disease susceptibility 1 has precluded their detection as required components of many resistance protein–signaling pathways.

Redundancy in Plant Defense

However, RPW8 is not a typical NBS-LRR type of R protein; it contains an N-terminal transmembrane domain in addition to the CC domain. Although several components contributing to resistance against pathogens have been identified, the molecular signaling underlying R gene-mediated resistance still remains obscure. Furthermore, potential relationship(s) among different downstream components and how they relay information leading to resistance remains unknown.

The EDS1 and PAD4 proteins are structurally related to lipase/esterase-like proteins although their lipase-like biochemical functions have not been demonstrated [16,19]. EDS1 interacts with PAD4 and SAG (senescence associated gene) 101 and the combined activities of these proteins are required for HR formation and to restrict the growth of virulent bacterial strains [26]. PAD4 and SAG101 also restrict the post-invasive growth of non-pathogenic fungi in Arabidopsis [27].

In addition to the major phytohormone-mediated defense pathways, fatty acid (FA)-derived signals have emerged as important mediators of defense signaling [28–35]. The Arabidopsis *SSI2/FAB2*-encoded stearoyl-acyl carrier protein-desaturase (SACPD) converts stearic acid (18:0) to oleic acid (18:1). A mutation in *SSI2* results in the accumulation of 18:0 and a reduction in 18:1 levels. The mutant plants show stunting, spontaneous lesion formation, constitutive PR gene expression, and enhanced resistance to bacterial and oomycete pathogens [29,36]. Characterization of *ssi2* suppressor mutants has shown that the altered defense-related phenotypes are the result of the reduction in the levels of the unsaturated FA, 18:1 [30,31,35,37–40]. The altered defense-related phenotypes in *ssi2* plants can be rescued by restoring the 18:1 levels via second site mutations in genes encoding a glycerol-3-phosphate (G3P) acyltransferase [ACT1, 30], a G3P dehydrogenase [GLT1, 31], and an acyl carrier protein [ACP4, 35]. A mutation in *act1* disrupts the acylation of G3P with 18:1 resulting in the increased accumulation of 18:1, thereby restoring wild-type (wt) phenotypes in *ssi2* plants. ACT1 preferentially utilizes 18:1 conjugated to the ACP4 isoform in Arabidopsis [35]. Thus, a mutation in *act1* produces similar phenotypes as the *act1* mutant and suppresses *ssi2*-mediated signaling by increasing 18:1 levels [35]. A mutation in *GLT1* also restores 18:1 levels in *ssi2* *gly1* plants because it disrupts the formation of G3P from dihydroxyacetone phosphate [31]. Reduced availability of G3P in turn impairs the ACT1-catalyzed reaction resulting in accumulation of 18:1 in *ssi2* *gly1* plants. Concurrently, increasing the endogenous G3P levels via exogenous application of glycerol reduces 18:1 levels and induces *ssi2*-like phenotypes in wt plants [31,40]. This effect of glycerol is highly specific because *ssi2*-associated phenotypes are not induced upon glycerol treatment of *act1* (defective in the acylation of G3P with 18:1) or *gli1* (defective in the phosphorylation of glycerol to G3P) mutants [40].

Recently, we showed that a reduction in 18:1 levels upregulates the expression of several R genes in an SA-independent manner [37]. Furthermore, we showed that pathogen resistance induced via this mode bypasses the requirement for components that are normally required for signaling downstream of R protein activation. For example, resistance to TCV mediated by the R gene *HRT* (HR to TCV), requires the recessive locus *rrt* (regulates resistance to TCV), SA, *EDS1* and *PAD4* [23]. Exogenous application of SA induces the expression of *HRT* and overcomes the requirement for *rrt*. However, exogenous SA is unable to induce *HRT* or confer resistance in *pad4* background [23]. Interestingly, even though a reduction in 18:1 levels also upregulates *HRT* expression to confer resistance to TCV, this mode of resistance is independent of *PAD4*, SA, *EDS1* and *EDS5*, which are required for *HRT*-mediated resistance to TCV [37]. Remarkably, induction of R genes in response to reduced 18:1 is conserved in plants as diverse as Arabidopsis and soybean [41]. Furthermore, this low 18:1-mediated induction of defense responses was also demonstrated in rice recently [42]. Together, these studies strengthen the conserved role of 18:1 in plant defense signaling.

Here, we show that R gene expression induced in response to a reduction in 18:1 levels and the associated defense signaling can be suppressed by simultaneous mutations in *EDS1* and the genes governing synthesis of SA. We also show that EDS1 and SA function redundantly in R gene-mediated resistance against bacterial, viral and oomycete pathogens and that EDS1 also regulates signaling mediated by CC domain containing R proteins.

Results

EDS1 and SA are essential but redundant components required for R gene expression induced in response to a reduction in 18:1 levels

Signaling mediated by many R genes is known to require EDS1 and/or NDR1. Previously, we have shown that *ssi2* *eds1* plants continue to express R genes at high levels, including those that are dependent on *EDS1* for their signaling [37]. To determine if *NDR1* played a role in *ssi2*-triggered phenotypes, we generated *ssi2* *ndr1* plants. The double-recessive plants segregated in a Mendelian fashion and all *ssi2* *ndr1* plants showed *ssi2*-like morphology in the F2, F3 and F4 generations (Figure 1A; Table S1). Although the *ssi2* *ndr1* plants accumulated significantly less SA/SAG (Figure 1C), *ssi2* *eds1* *sid2* plants (data not shown). Together, these results suggest that R gene expression induced by low 18:1 levels does not require *EDS1* or *NDR1*.

The SA/SAG levels in *ssi2* *eds1* and *ssi2* *ndr1* plants were significantly higher compared to those in wt plants (Figure 1C). To determine whether high SA in these genotypes was responsible for increased R gene expression, we generated *ssi2* *eds1* *sid2* and *ssi2*
Figure 1. Morphological, molecular, and defense phenotypes of ssi2 ndr1-1 sid2-1 and ssi2 eds1-1 sid2-1 plants. (A) Comparison of the morphological phenotypes displayed by 3-week-old soil-grown plants (scale, 0.5 cm). (B) Microscopy of trypan blue-stained leaves from wt (SSI2, Col-0 ecotype), ssi2, ssi2 eds1-1, ssi2 sid2-1 and ssi2 eds1-1 sid2-1 plants (scale bars, 270 microns). (C) SA and SAG levels in indicated genotypes. The error bars indicate SD. Asterisks indicate data statistically significant from wt No¨ ecotype (SSI2) ( P < 0.05, n = 4). (D) Expression of PR-1 and PR-2 genes in indicated genotypes. Total RNA was extracted from 4-week-old plants and used for RNA gel-blot analysis. Ethidium bromide staining of rRNA was used as the loading control. The PR-1 transcript levels in EDS1 SID2 F2 plants were similar to those of wt plants (data not shown). (E) RT-PCR analysis of various R genes in indicated genotypes. The level of β-tubulin was used as an internal control to normalize the amount of cDNA template. The expression of R genes in EDS1 SID2 F2 plants was similar to that of wt plants (data not shown). (G) Levels of Myc-tagged RPM1 protein in indicated genotypes. Levels of Rubisco were used as the loading control.
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However, the *sii2* mutation overcomes the requirement for *rtr* in *HRT*-containing plants [23,37]. Furthermore, the *sii2* mutation only confers resistance to TCV when *HRT* is present (Figure 3A). The *sii2* mutation also overrides a requirement for EDs1 and SA and consequently *sii2 HRT edb1* as well as *sii2 HRT sid2* plants exhibit resistance to TCV [37] (Figure 3A). Unlike *sii2, HRT ssi2 edb1* or *HRT ssi2 sid2* plants, the *HRT sii2 edb1 sid2* plants showed susceptibility to TCV; ~85% *HRT sii2 edb1 sid2* plants were susceptible to TCV as against ~2–4% of *HRT sii2 sid2* or *HRT sii2 edb1* plants (Figure 3A). TCV-induced expression of *PR-1* is also independent of EDs1 and SA. However, TCV inoculation failed to induce *PR-1* expression in *HRT sii2 edb1 sid2* plants, unlike in *HRT sii2 sid2* plants (Figure 3B). These results showed that both EDs1 and SA have redundant functions in *sii2*-mediated resistance to TCV in *HRT* plants.

**EDS1 and SA function redundantly in signaling mediated by HRT, RPS2, and RPP8 genes that encode CC-NBS-LRR proteins**

To determine the redundancy of EDs1 and SA in signaling mediated by CC-NBS-LRR R proteins, we tested the effects of mutations in EDs1- and/or SID2 on HR to TCV. Earlier, we showed that *HRT*-mediated HR to TCV and *PR-1* gene expression is not affected by mutations in the *EDS1* or *SID2* genes [23]. Consistent with previous results, *Di-17* (*HRT*-containing resistant ecotype), *HRT sid2* and *HRT edb1* plants revealed discrete and similar-sized HR lesions on TCV-inoculated leaves (Figure 3C and 3D). In comparison, *HR* in *HRT edb1 sid2* plants was diffused and formed larger lesions (Figure 3C and 3D). Increased lesion size in *HRT edb1 sid2* plants correlated with increased accumulation of the TCV coat protein (CP) and TCV CP transcript (Figure 3E and 3F). Analysis of *PR-1* and *PR-2* gene expression indicated that TCV-inoculated *HRT edb1 sid2* plants accumulated lower levels of *PR-1* and *PR-2* transcripts, unlike *Di-17, HRT edb1* or *HRT sid2* plants (Figure 3G and 3H). In contrast to *PR, HRT* expression remained unaltered in *HRT edb1 sid2* plants (Figure 3H). Together, these results suggested that EDs1 and SA function redundantly in *HRT*-mediated signaling leading to HR formation and expression of *PR-1*. The functional redundancy with SA was specific to EDs1 and did not extend to PAD4; *HRT pad4 sid2* plants showed normal replication of the virus and wt-like HR and *PR-1* gene expression (Figure 3C–3G).

A majority of CC-domain containing R proteins, including *RPS2*, have been reported as not requiring EDs1 for resistance signaling [21]. To determine the effect of simultaneous mutations in *EDS1* and *SID2* on *RPS2*-mediated resistance, we compared defense phenotypes produced in single or double mutant plants with that of plants lacking a functional *RPS2* gene. Since different alleles of *RPS2* confer varying levels of resistance to *Pseudomonas syringae* (containing *AvrRPT2*) [44], we screened and isolated an *EDS1* knockout (KO) mutant (designated *eds1-22*) in the Col-0 background and crossed it into the *sid2* background (Col-0 ecotype). Inoculation with *P. syringae* expressing *AvrRPT2* induced severe chlorosis on *eds1-22 sid2* leaves (Figure 4A). Similar results were obtained when *P. syringae* expressing *AvrRPT2* was inoculated into *eds1-1 sid2* double mutant plants (Figure S2A). Interestingly, these phenotypes were very similar to those produced on plants lacking a functional *RPS2* gene (*sps2-101e*), while *eds1* and *sid2* showed no or very mild symptoms, respectively (Figure 4A, Figure S2A).

The appearance of symptoms correlated with bacterial growth; *eds1-22 sid2* plants and the *sp2* mutant supported maximum growth of the pathogen, followed by *sid2* plants (Figure 4B). Similarly, the *eds1-1 sid2* double mutant plants supported more pathogen growth compared to *eds1* or *sid2* plants (data not shown). Together, these data suggest that the simultaneous loss of
Figure 2. Restoration of ssi2 phenotypes in ssi2 eds1-1 sid2-1 plants and glycerol responsiveness of eds1-1 sid2-1 plants. (A) Visual phenotypes of water- or BTH–treated wt (SSI2; Col-0 ecotype) and ssi2 eds1-1 sid2-1 plants. The plants were photographed at 2 days post treatment (dpt). (B) Microscopy of trypan blue-stained leaves from BTH–treated wt (SSI2; Col-0 ecotype), sid2, eds1-1 and ssi2 eds1-1 sid2-1 plants. The plants were treated with BTH and stained at 2 dpt (scale bars, 270 microns). (C) RT–PCR analysis of R genes in water- or BTH-treated ssi2 eds1-1 sid2-1 plants. Untreated wt (SSI2; Col-0 ecotype) and ssi2 plants were used as controls. The expression of R genes in EDS1 SID2 F2 plants was similar to that of wt plants (data not shown). The level of β-tubulin was used as an internal control to normalize the amount of cDNA template. (D) RT–PCR analysis of various R genes in water- or glycerol-treated sid2-1 and eds1-1 sid2-1 plants. The glycerol-treated wt (SSI2; Col-0 ecotype) and eds1-1 were included as additional controls. The expression of R genes in water- or glycerol-treated EDS1 SID2 F2 plants was similar to that of water- or glycerol-treated wt plants, respectively (data not shown). The expression of R genes in wt and eds1-1 plants was similar to that seen in sid2-1 or eds1-1 sid2-1 plants. The plants were treated with water or glycerol for three days and analyzed for 18:1 levels and R gene expression. The level of β-tubulin was used as an internal control to normalize the amount of cDNA template. The 18:1 content of each genotype is shown as mol%±SD.

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EDS1- and SA-dependent signals is required to mimic a phenotype produced by the loss of the cognate R gene, RPS2.

To determine if the loss of both EDS1- and SA-dependent signaling impaired resistance by affecting the RPS2 protein, we analyzed R protein levels in eds1-1 and sid2 single and eds1-1 sid2 double mutant plants. Analysis of RPS2 tagged with HA epitope at various times did not detect any significant changes in RPS2 levels in response to inoculation with *P. syringae* expressing AvrRPT2 (Figure 4C). Therefore, RPS2 levels in mutant plants were analyzed at only 12 and 24 h post-pathogen inoculation. The RPS2-HA levels in eds1-1, sid2 or eds1-1 sid2 plants were similar to that in wt plants (Figure 4D). These results suggested that abrogation of resistance in eds1 sid2 double mutants was not due to a defect in the accumulation of the R protein.

We next evaluated the effects of mutations in EDS1 and SID2 on RPP8-mediated resistance to *Hyaloperonospora arabidopsidis* biotype Emco5 encoding Avr8. RPP8 (encodes a CNC-NBS-LRR type R protein)-mediated resistance signaling was previously reported to be independent of both EDS1 and SA [21, 24]. As expected, RPP8 plants (ecotype Ler) inoculated with the Emco5 isolate showed localized HR and did not support growth of the pathogen (Figure 5A). Consistent with earlier reports [21, 24], RPP8 eds1-2 plants also did not support the growth of Emco5, although they did develop trailing necrosis (Figure 5A and 5B). The presence of the nahG transgene did not alter HR formation or pathogen response in the RPP8 nahG plants (Ler ecotype). In contrast, eds1-2 nahG plants were affected in both HR as well as resistance; eds1-2 nahG plants not only showed extensive trailing necrosis but also supported growth and sporulation of the pathogen (Figure 5A-5C). Although RPP8 EDS1 nahG and RPP8 eds1-2 nahG plants showed contrasting phenotypes (Figure 5A-5C), we still wanted to rule out the possibility that susceptibility of eds1-1 nahG plants was not due to the accumulation of catechol, which is formed upon degradation of SA by NAHG. Estimation of SA levels in Emco5 inoculated RPP8 (Ler) plants showed marginal increase in SA and no significant increase in SAG levels compared to mock-inoculated plants (data not shown). This suggests that Emco5 inoculated nahG plants are unlikely to show a significant increase in catechol levels. In addition to this, we tested two independent lines of RPP8 eds1-2 sid2 (in the sid2 background) plants and both showed increased susceptibility to Emco5 (Figure 5D). In comparison, RPP8 eds1-2 or RPP8 sid2 genotypes did not support any growth or sporulation of the pathogen (Figure 5D). Taken together, these results show that EDS1 and SA have redundant functions in RPP8-mediated resistance to *H. arabidopsidis* Emco5.

Exogenous SA and overexpression of EDS1 have additive effects on pathogen resistance in wild-type plants

To determine the relation between EDS1- and SA-derived signaling, we compared PR-1 gene expression and resistance in plants that were either overexpressing EDS1 or were pretreated with SA. EDS1 overexpression was achieved by expressing EDS1 (At3g40090 from the Col-0 ecotype) under control of the CaMV 35S promoter in Col-0 plants (Figure 6A). The 35S-EDS1 plants analyzed in the T2 and T3 generations showed wt-like morphology (data not shown), wt-like expression of the PR-1 gene (Figure 6A) and accumulated wt-levels of SA/SAG (data not shown). In comparison, exogenous application of SA induced PR-1 and EDS1 gene expression [data not shown; 16].

Analysis of RPS4 (encodes a TIR-NBS-LRR type R protein)-mediated resistance showed that exogenous application of SA enhanced resistance to *P. syringae* (expressing AvrRPS4) in wt as well as eds1-22 plants, although wt plants were more resistant to AvrRPS4 bacteria than the eds1-22 plants (Figure 6B). Overexpression of EDS1, on the other hand, did not alter the response to AvrRPS4 bacteria. Strikingly, exogenous application of SA on 35S-EDS1 plants enhanced resistance even more than in the SA-treated wt or eds1-22 plants. Together, these results suggest that EDS1- and SA-derived signaling contribute additively towards pathogen resistance.

Simultaneous defects in EDS1 and SA biosynthesis do not additively lower basal defense

We next evaluated the effect of the eds1 sid2 mutations on basal resistance to virulent *P. syringae*, since both EDS1 and SID2 are known to contribute to basal defense as well. The eds1-1, eds1-22, sid2 and eds1 sid2 plants all showed enhanced susceptibility to virulent bacteria as compared to the respective wt ecotypes (Figure 7A). Interestingly, unlike in the case of the avirulent bacteria, growth of virulent bacteria was similar in eds1 sid2 double mutant plants as compared to that in eds1 or sid2 single mutant plants. These results suggested that loss-of-function mutations in EDS1 and SID2 do not additively reduce basal resistance to virulent *P. syringae*. Similar to the results obtained with the bacterial pathogen, the loss of both EDS1- and SA-dependent signals did not additively lower basal resistance to TCV either (Figure 7B). This further suggested that the redundant functions of EDS1 and SA might be relevant only for *R* gene-mediated signaling.

Mutations in FAD7 FAD8 and EDS5 restore altered defense signaling in sid2 eds1 plants

Besides SID2, mutations in FAD7 FAD8, which catalyze desaturation of 18:2 to 18:3 on membrane glycerolipids, also lower the SA levels in sid2 plants [40]. To test if *fad7* or *fad8* mutations produced a similar effect as *sid2*, these mutations were mobilized into the sid2 eds1 background. The sid2 *eds1* *fad7* and sid2 *eds1* *fad8* plants were bigger in size compared to *sid2* *fad7* or *sid2* *fad7* *fad8* plants (Figure S3A). The *sid2* *eds1* *fad7* *fad8* were wt-
Figure 4. Interaction phenotypes of virulent or AvrRPT2-expressing *P. syringae* with eds1 sid2 plants. (A) Photograph showing phenotypes produced upon infiltration of 10^7 CFU/ml bacteria (AvrRPT2). All genotypes were in the Col-0 background. The leaves were photographed at 3 days post inoculation (dpi). The pathogen-inoculated EDS1 SID2 F2 plants showed absence of any visible symptoms in response to bacterial inoculations, similar to Col-0 plants (data not shown). (B) Growth of virulent or avirulent (expressing AvrRPT2) *P. syringae* on indicated genotypes. The error bars indicate SD. Asterisks and omega symbols indicate data statistically significant from wt (Col-0) or sid2 (P<0.05, n = 4), respectively. All genotypes are in the Col-0 background. (C) Levels of HA-tagged RPS2 protein at 0, 2, 4, 8, and 24 h post inoculation with *P. syringae* expressing AvrRPT2. Levels of Rubisco were used as the loading control. (D) Levels of HA-tagged RPS2 protein in indicated genotypes. Levels of Rubisco were used as the loading control.

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**Figure 5. Interaction phenotypes of H. arabidopsidis biotype Emco5 expressing Atr8 with RPP8 eds1-2 nahG or RPP8 eds1-2 sid2-1 plants.** (A) Whole leaf pictures showing growth of Emco5 on the cotyledons from indicated genotypes. All genotypes were in the Ler background. Cotyledons were photographed 10 days after inoculation. (B) Trypan blue stained leaf showing microscopic HR on Ler and Ler nahG leaves, and trailing necrosis on eds1-2 and eds1-2 nahG leaves (scale bars, 270 microns). Both high (100×) and low magnification (100×) images of eds1-2 nahG leaf are shown. Pathogen inoculations were carried out in F2, F3, and F4 generations with consistent results. The F2 plants showing wt genotype at the mutant locus were resistant to pathogen infection (data not shown). (C) Quantification of pathogen growth on RPP8 EDS1, RPP8 eds1-2 and RPP8 eds1-2 nahG plants. Approximately, 40–60 cotyledons were assayed for each genotype. Asterisks indicate absence of spores. All genotypes were in the Ler background. (D) Quantification of pathogen growth on RPP8 sid2, RPP8 eds1-2, and RPP8 eds1-2 sid2-1 plants. All genotypes were in the ssi2 background. Approximately, 40–60 cotyledons were assayed for each genotype. Asterisks indicate absence of spores.

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Mutations in EDS5 and PAD4 also lower SA/SAG levels in ssi2 plants [40]. To determine if mutations in these can substitute for ssi2 triple mutants containing ssi2 eds1 pad4 and ssi2 eds1 eds5 were generated. The ssi2 eds1 pad4 plants were morphologically similar to ssi2 eds1 or ssi2 pad4 plants and showed spontaneous cell death and increased expression of PR-1 gene (Figure 8A–8C). In comparison, ssi2 eds1 eds5 showed wt-like morphology, greatly reduced cell death and basal expression of PR-1 gene (Figure 8A–8C). Quantification of endogenous SA levels showed that both ssi2 eds1 eds5 and ssi2 eds1 pad4 accumulated lower SA/SAG levels compared to ssi2 eds3 and ssi2 pad4, respectively (Figure 8D and 8E). However, while ssi2 eds1 eds5 plants accumulated basal levels of SA/SAG, the ssi2 eds1 pad4 accumulated significantly higher levels of SA/SAG compared to wt, ssi2 sid2 and ssi2 eds1 eds5 plants (Figure 8D and 8E). Analysis of R gene expression showed greatly reduced levels in ssi2 eds1 eds5 plants but the ssi2 eds1 pad4 expressed ssi2-like levels of R genes (Figure 8F, Figure S1D). Taken together, these results suggest that the suppression of SA levels was required for the normalization of defense phenotypes in the ssi2 eds1 background.

**Discussion**

SA is long known as an essential modulator of R gene-derived signaling in pathogen defense. Several molecular components, including EDS1, have been identified as essential effectors of SA-derived signaling [23,26,45]. Since SA upregulates expression of EDS1, both SA and EDS1 are thought to function in a positive

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**Figure 6. Effect of SA pretreatment and EDS1 overexpression on pathogen resistance.** (A) Expression of EDS1 and PR-1 in EDS1 (Col-0) and 35S-EDS1 (Col-0) plants. Total RNA was extracted from 4-week-old plants and ethidium bromide staining of rRNA was used as the loading control. (B) Growth of P. syringae AvrRPS4 on indicated genotypes (all in Col-0 background). Single asterisks indicate data statistically significant from results for water-treated wt (Col-0) (P < 0.05, n = 4). Two asterisks indicate data statistically significant from results for water-treated wt (Col-0) (P < 0.05, n = 4). The error bars indicate SD. doi:10.1371/journal.pgen.1000545.g006
feedback loop and EDS1 is widely considered an upstream effector of SA [16,19,23,45]. Recent data has shown that EDS1 signals resistance via both SA-dependent as well as SA-independent pathways [46]. Strikingly, EDS1-dependent but SA-independent branch of EDS1 pathway still requires SA pathway for full expression of resistance [46]. In this study, we have characterized the relationship between EDS1 and SA. We show that the two components act in a redundant, and not necessarily sequential manner to regulate R gene expression induced in response to a reduction in the levels of the FA 18:1. Furthermore, EDS1 and SA also function redundantly in R gene-mediated defense against viral, bacterial and oomycete pathogens. It appears that the redundant functions of EDS1 and SA may have prevented their identification as required components for signaling mediated by CC-NBS-LRR R proteins. Indeed, RPS2-mediated signaling is fully compromised only in eds1 sid2 and not in the single mutant

Figure 7. Basal resistance in eds1 sid2 plants. (A) Growth of virulent P. syringae on indicated genotypes. The error bars indicate SD. Asterisks indicate data statistically significant from wt (Col-0 or Ws) (P<0.05, n = 4). The eds1-1 and eds1-22 are in Ws and Col-0 ecotypic backgrounds, respectively. (B) ELISA showing levels of TCV CP in the inoculated leaves of indicated genotypes at 3 dpi. The error bars indicate SD (n = 4).

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Figure 8. Morphology, cell death, SA/SAG levels. PR-1 and R gene expression ssi2 eds1-2 pad4-1 and ssi2 eds1-2 eds5-1 plants. (A) Comparison of the morphological phenotypes displayed by 4-week-old soil-grown wt (SSI2), ssi2, ssi2 eds1, ssi2 pad4, ssi2 eds5, ssi2 eds1 pad4, and ssi2 eds1 eds5 plants. (B) Microscopy of trypan blue-stained leaves from indicated genotypes. (C) Expression of PR-1 gene in indicated genotypes. Total RNA was extracted from 4-week-old plants and used for RNA gel-blot analysis. Ethidium bromide staining of rRNA was used as the loading control. (D) Endogenous SA levels in the leaves of 4-week-old soil-grown plants. Values are presented as mean of three replicates and the error bars represent SD. Statistical significance was determined using Students t-test. Asterisks indicate data statistically significant compared to SSI2 (Col-0) plants (P<0.05, n = 5). (E) Endogenous SAG levels in the leaves of 4-week-old soil-grown plants. Values are presented as mean of three replicates and the error bars represent SD. Asterisks indicate data statistically significant compared to SSI2 (Col-0) plants (P<0.05, n = 5). (F) RT–PCR analysis of R genes in indicated genotypes. The level of β-tubulin was used as an internal control to normalize the amount of cDNA template. The SSI2 ED1, SSI2 PAD4, SSI2 ED1 PAD4, and SSI2 ED1 ED5 F2 plants showed wt–like morphology, accumulated basal levels of SA and showed basal level expression of PR-1 and R genes (data not shown). doi:10.1371/journal.pgen.1000545.g008
plants. Similarly, HRT-mediated signaling leading to HR formation and PR-1 gene expression is only affected in eds1 sid2 plants, while eds1 or sid2 plants behave similar to wt plants. Furthermore, RPP9-mediated resistance, which was previously reported not to require EDS1 or SA [21,24], is compromised in plants lacking both EDS1 and SA. In contrast to their effect on R gene-mediated resistance, loss of both EDS1- and SA-dependent signals did not additively lower basal resistance to P. syringae or TCV. Together, these data suggests that the redundant functions of EDS1 and SA might be relevant only for R gene-mediated signaling.

In contrast to SA application, overexpression of EDS1 was unable to confer increased resistance to the avirulent pathogen P. syringae. Furthermore, unlike SA, overexpression of EDS1 was not associated with the induction of PR-1 gene expression. These findings, together with the observation that SA was able to induce EDS1 expression and that SA application on wt plants resulted in higher resistance than that in eds1, suggests that SA feedback regulates EDS1-derived signaling in a unidirectional manner (Figure 9B). Thus, SA application induces both SA- and EDS1-derived signaling, the additive effects of which enhance resistance in wt plants much more than in eds1-22 plants. Furthermore, the combined effects of SA pretreatment and EDS1 overexpression induced much better resistance than the individual effects of each. This is consistent with a previous report that 35S-EDS1 plants induce rapid and stronger expression of PR-1 in response to pathogen inoculation [47]. The additive effects of EDS1 and SA was also supported by the observation that eds1 sid2 plants showed pronounced chlorosis upon inoculation with AvrRPS4 expressing pathogen, which is recognized by a TIR-NBS-LRR protein RPS4 (Figure S2B). Since mutations in SA-independent branch of EDS1 pathway and sid2 have additive effects on R gene-mediated resistance [46], it is possible that overexpression of EDS1 triggers signaling via both SA-dependent and/or -independent branches of EDS1 pathway.

Although the Col-0 ecotype is thought to contain two functional alleles of EDS1 [26], a KO mutation in At3g48090 was sufficient to compromise both basal and R gene (RPS4)-mediated resistance. However, the Col-0 eds1-22 mutant consistently supported less growth of virulent or avirulent pathogens compared to eds1-1 or eds1-2 plants. This suggests that the second EDS1 allele in the Col-0 ecotype might also contribute towards the resistance response. This is consistent with another study where constitutive defense phenotypes due to the overexpression of the SNC1 gene, encoding a TIR-NBS-LRR R protein, are not completely suppressed by a mutation in eds1 in the Col-0 background but restored by the eds1 mutation in theWs background [48].

The inability to accumulate SA together with a mutation in EDS1 was also required to suppress constitutive defense signaling resulting from the overexpression of R genes induced in response to reduced 18:1 levels. Although eds1 or sid2 plants were entirely competent in inducing R gene expression in response to a reduction in 18:1, eds1 sid2 plants were not. Thus, ss2 eds1 sid2 as well as glycerol-treated eds1 sid2 plants showed wt-like expression of R genes while ss2 eds1, ss2 sid2 and glycerol-treated eds1 or sid2 plants showed increased expression of R genes. Moreover, treatment of ss2 eds1 sid2 plants with exogenous SA restored R transcript induction and cell death in these plants. The fact that glycerol treatment is unable to induce R gene expression in eds1 sid2 plants supports the possibility that EDS1 and SA function upstream of, and not merely serve as a feedback loop in, R gene induction. Signaling induced by low 18:1 levels continues to function in the absence of SA, suggesting a novel SA-independent role for EDS1 in defense signaling.

Since ss2 eds1 sid2 plants contain a mixed ecotypic background (No, Ws/Ler, Col-0, ecotypes), it is possible that ecotypic variations in various genetic backgrounds resulted in the restoration of ss2-triggered defense phenotypes. Indeed, phenotypic variations amongst different Arabidopsis ecotypes have been associated with many physiological processes [48–51]. Moreover, certain alleles can express themselves only in specific ecotypic backgrounds [48,51]. However, since ss2 EDS1 SID2, ss2 EDS1 sid2 or ss2 eds1 SID2 plants (F2 population) always exhibited ss2-like phenotypes, it is highly unlikely that ecotypic variations resulted in the restoration of phenotypes in ss2 eds1 sid2 plants. The effect of ecotypic variations on the observed phenotypes can be further ruled out for the following reasons. First, the effects of different mutations were assessed in multiple backgrounds. For example, we used both eds1-1 (Ws-0 ecotype) and eds1-2 (Ler ecotype) alleles in ss2 sid2 (No, Col-0 ecotypes) and ss2 nahG (No ecotype) backgrounds and all combinations of ss2 with eds1-1/eds1-2 and sid2/nahG produced similar phenotypes (Table S1). Second, all defense phenotypes were assessed over three generations using multiple progeny. Third, similar results were obtained when different ecotypic backgrounds were evaluated for their response to different pathogens. For example, eds1 nahG or eds1 sid2 backgrounds conferred increased susceptibility to H. arabidopsidis.
factor(s) that function downstream of expression. This idea is supported by the fact that in the absence of a pathogen [48,52], it is possible that the induced R expression induced by low 18:1 levels. Since the overexpression of R genes can initiate defense signaling in the absence of a pathogen [48,52], it is possible that the induced defense responses in ssi2 plants are the result of increased R gene expression. This idea is supported by the fact that ssi2-related phenotypes can be normalized by restoring R gene expression to wt-like levels, irrespective of their 18:1 levels. Thus, wt-like defense phenotypes are restored in suppressors containing high 18:1 levels, such as ssi2 ael1 ssi2 gyl1 or ssi2 aep4 [30,31,35], as well as in suppressor containing low 18:1 levels, such as ssi2 ed1 sid2 (this work) and restored in defective crosstalk (rdc) mutants (unpublished data) (Figure 9A). We have also characterized additional ssi2 suppressors that show wt-like phenotypes even though they contain low 18:1 levels and express R genes constitutively (rdc3, rdc4). Together, these results suggest that the ssi2-associated phenotypes can be restored by normalizing R gene expression to wt-like levels either by increasing 18:1 levels, impairing factors downstream of signaling induced by low 18:1 levels, or impairing events downstream of R gene expression induced by low 18:1 levels.

In addition to 18:1 levels or R gene expression, ssi2-related defense signaling could also be normalized by altering some factor(s) that function downstream of R gene induction. Indeed, our preliminary characterizations have identified additional ssi2 suppressors that yield wt-like phenotypes with regards to defense signaling but continue to express R genes at high levels. Reduced 18:1 levels may induce defense signaling by directly regulating the transcription of activators or suppressors of defense gene expression. This is supported by the fact that 18:1-mediated activation of a transcription factor induces the expression of genes required for neuronal differentiation [53]. Similarly, in Sacharomyces cerevisiae as well as mammalian cells, binding of 18:1 to specific transcription factors induces the transcription of genes carrying 18:1 responsive elements in their promoters [54,55]. On the other hand, expression of the oncogene HER2 is inhibited via the 18:1-unregulated expression of its transcriptional repressor [56]. Reduced 18:1 might also directly activate/inhibit/alter protein activities. For example, 18:1 is known to activate the Arabidopsis phospholipase D [57] and inhibit glucose-6-phosphate transporter activity in Brassica embryos [38]. Indeed, we have also identified several Arabidopsis proteins for which enzymatic activities are inhibited upon binding to 18:1 (unpublished data).

In conclusion, results presented here redefine the currently accepted pathway for SA-mediated signaling by showing that EDS1 and SA play a redundant role in plant defense mediated by R proteins and in signaling induced by low 18:1 fatty acid levels. Further biochemical characterization should help determine if 18:1 binds to EDS1 and if cellular levels of 18:1 modulate the as yet undetected lipase activity of EDS1.

Materials and Methods

Plant growth conditions and genetic analysis

Plants were grown in MTPS 144 Conviron (Winnipeg, MB, Canada) walk-in-chambers at 22°C, 65% relative humidity and 14 hour photoperiod. The photon density of the day period was 106.9 μmol m⁻² s⁻¹ and was measured using a digital light meter (Phytotronic Inc, Earth city, MO). All crosses were performed by emasculating the flowers of the recipient genotype and pollinating with the pollen from the donor. All the genotypes and crosses analyzed in this work, their genetic background and number of single, double, or triple mutant plants studied are listed in Table S1.

In most cases, single, double, or triple mutant plants were obtained from more than one combination of crosses and showed similar morphological, molecular, and biochemical phenotypes. F2 plants showing the wt genotype at the mutant locus were used as controls in all experiments. The wt and mutant alleles were identified by PCR, CAPS, or dCAPS analysis and/or based on the FA profile [30,31,38,40]. The EDS1 KO mutant in At3g48090 was, isolated by screening SALK_071051 insertion line, obtained from ABRC. The EDS1 KO was designated eds1-22, based on the previous designation assigned to SALK_071051 T-DNA KO line [48]. The At3g48090 gene showed 98.8% identity at amino acid level to EDS1 allele from Let ecotype. The homozygous insertion lines were verified by sequencing PCR products obtained with primers specific for the T-DNA left border in combination with an EDS1-specific primer. The eds1-22 lines did not show any detectable expression of EDS1.

RNA extraction and northern analyses

Small-scale extraction of RNA from one or two leaves was performed with the TRIzol reagent (Invitrogen, CA), following the manufacturer’s instructions. Northern blot analysis and synthesis of random-primed probes for PR-1 and PR-2 were carried out as described previously [29].

Reverse Transcription–PCR

RNA quality and concentration were determined by gel electrophoresis and determination of A260. Reverse transcription (RT) and first strand cDNA synthesis were carried out using Superscript II (Invitrogen, CA). Two-to-three independent RNA preparations were used for RT-PCR and each of these were analyzed at least twice by RT-PCR. The RT–PCR was carried out for 35 cycles in order to determine absolute levels of transcripts. The number of amplification cycles was reduced to 21–25 in order to evaluate and quantify differences among transcript levels before they reached saturation. The amplified products were quantified using ImageQuant TL image analysis software (GE, USA). Gene-specific primers used for RT–PCR analysis are described in Table S5.

Trypan-blue staining

The leaves were vacuum-infiltrated with trypan-blue stain prepared in 10 mL acidic phenol, 10 mL glycerol, and 20 mL sterile water with 10 mg of trypan blue. The samples were placed in a heated water bath (90°C) for 2 min and incubated at room temperature for 2–12 h. The samples were destained using chloral hydrate (25 g/10 mL sterile water, Sigma), mounted on slides and observed for cell death with a compound microscope. The samples were photographed using an AxioCam camera (Zeiss, Germany) and images were analyzed using Openlab 3.5.2 (Improvement) software.

Pathogen infections

The asexual conidiospores of H. arabidopsidis Emeo5 expressing Atr8 were maintained on the susceptible host Nössen (No) or No
NahG. The spores were removed by agitating the infected leaves in water and suspended to a final concentration of 10^5 spores/mL. Two-week-old seedlings were sprayed with spore suspension and transferred to a MTR30 reach-in chamber (Conviron, Canada) maintained at 17°C, 90% relative humidity and 8 h photoperiod. Plants were scored at ~10–14 dpi and the conidiophores were counted under a dissecting microscope.

The bacterial strain DC3000 derivatives containing pVSP61 (empty vector), AevRpt2 or AevRps4 were grown overnight in King’s B medium containing rifampicin (Sigma, MO). The bacterial cells were harvested, washed and suspended in 10 mM MgCl2. The cells were diluted to a final density of 10^3 to 10^7 CFU/mL (A600) and used for infiltration. The bacterial suspension was injected into the abaxial surface of the leaf using a needle-less syringe. Three leaf discs from the inoculated leaves were collected at 0 and 3 dpi. The leaf discs were homogenized in 10 mM MgCl2, diluted 10^3 or 10^4 fold and plated on King’s B medium.

Transcripts synthesized in vitro from a cloned cDNA of TCV using T7 RNA polymerase were used for viral infections [59,60]. For inoculations, the viral transcript was suspended at a concentration of 0.05 μg/μL in inoculation buffer, and the inoculation was performed as described earlier [56]. After viral inoculations, the plants were transferred to a Conviron MTR30 reach-in chamber maintained at 22°C, 65% relative humidity and 14 h photoperiod. HR was determined visually three-to-four days post-inoculation (dpi). Resistance and susceptibility was scored at 14 to 21 dpi and confirmed by northern gel blot analysis. Susceptible plants showed stunted growth, crinkling of leaves and drooping of the bolt.

Transcriptional profiling
Total RNA isolated from four-week-old plants using TRIZOL as outlined above. The experiment was carried out in triplicate and a separate group of plants was used for each set. RNA was processed and hybridized to the Affymetrix Arabidopsis ATH1 genome array GeneChip following the manufacturers instructions (http://www.affymetrix.com/Auth/support/downloads/manuals/expression_analysis_technical_manual.pdf). All probe sets on the Genechips were assigned hybridization signal above background using Affymetrix Expression Console Software v1.0 (http://www.affymetrix.com/Auth/support/downloads/manuals/expression_console_userguide.pdf). Data was analyzed by one-way Anova followed by post hoc two sample t-tests. The P values were calculated individually and in pair-wise combination for each probe set. The identities of 162 NBS-LRR genes were obtained from the Arabidopsis information resource (TAIR; http://www.arabidopsis.org) and disease resistance gene homolog databases (http://mblhrs.ucdavis.edu/).

Fatty acid profiling
FA analysis was carried out as described previously [61]. For FA profiling, one or few leaves of four-week-old plants were placed in 2 mL of 3% H2SO4 in methanol containing 0.001% butylated hydroxytoluene (BHT). After 30 minutes incubation at 80°C, 1 mL of hexane with 0.001% BHT was added. The hexane phase was then transferred to vials for gas chromatography (GC). One-microliter samples were analyzed by GC on a Varian FAME 0.25 mm × 50 m column and quantified with flame ionization detection. The identities of the peaks were determined by comparing the retention times with known FA standards. Mole values were calculated by dividing peak area by molecular weight of the FA.

SA and SAG quantification
SA and SAG quantifications were carried out from ~300 mg of leaf tissue as described before [23].

Chemical treatment of plants
SA treatments were carried out by spraying or subirrigating 3-week-old plants with 500 μM SA or 100 μM BTH. For glycerol treatment, plants were sprayed with 50 mM solution prepared in sterile water.

Enzyme linked immuno-sorbent assay and western analysis
Total protein was extracted in buffer containing 50 mM Tris pH 8.0, 1 mM EDTA, 12 mM β-mercaptoethanol and 10 μg ml−1 phenylmethylsulfonyl fluoride. Proteins were fractionated on a 10–12% SDS-PAGE to confirm the quality. An antigen-coated enzyme-linked immunoassay was used to determine levels of TCV CP in the infected plants as described before [62].

For protein gel blot analysis, leaf tissue from 4-week-old plants was extracted with a buffer containing 50 mM Tris-HCl, pH 7.5, 10% glycerol, 150 mM NaCl, 10 mM MgCl2, 5 mM EDTA, 5 mM DTT, and 1× proteinase inhibitor (Sigma). Protein concentrations were determined by the Bradford assay (Bio-Rad, CA). For immunodetection, 10–50 μg protein samples were electrophoresed on 10–15% polyacrylamide gels and run in the presence of 0.38 M Tris and 0.1% SDS. Proteins were transferred from the gels to polyvinylidene difluoride membranes by electroblotting, incubated with primary anti-HA antibody (Sigma) and alkaline phosphatase-conjugated secondary antibody (Sigma). Immunoblots were developed using color detection.

Supporting Information
Figure S1 Relative expression levels of R genes in indicated genotypes. One representative quantification is shown for each Figure (noted above the graph) showing RT-PCR results. The R gene transcript levels were normalized for β-tubulin and relative differences in expression levels were quantified using ImageQuant TL image analysis software (GE, USA). Two-to-three independent RNA preparations were used for RT-PCR and each of these were analyzed at least twice by RT-PCR. The fold differences in expression levels were consistent between experiments and between repeats within an experiment. Found at: doi:10.1371/journal.pgen.1000545.s001 (0.16 MB TIF)

Figure S2 Interaction phenotypes of AevRPT2 or AevRPS4 expressing P. syringae with eds1 sid2 plants. (A) Photograph showing phenotypes produced upon infiltration of 10^5 CFU/mL bacteria (AevRPT2). The leaves were photographed at 3 days post inoculation (dpi). The mock- or pathogen-inoculated EDS1 SID2 F2 plants showed absence of any visible symptoms in response to bacterial inoculations, similar to Col-0 or Ws-0 plants (data not shown). (B) Photograph showing phenotypes produced upon infiltration of 10^7 CFU/mL bacteria. The leaves were photographed at 3 dpi. The phenotypes seen on pathogen inoculated eds1-1 sid2-1 leaves were comparable to those seen on RLD (ecotype) plants, which lack a functional RPS4 gene (data not shown). The mock- or pathogen-inoculated EDS1 SID2 F2 plants showed absence of any visible symptoms in response to bacterial inoculations, similar to Col-0 or Ws-0 plants (data not shown). Found at: doi:10.1371/journal.pgen.1000545.s002 (1.09 MB TIF)

Figure S3 Morphology, cell death, PR-1, and R gene expression and SA/SAG levels in sid2 eds1-2 fad7-1 and sid2 eds1-2 fad7-1 fad8-1 plants. (A) Comparison of the morphological phenotypes displayed by 4-week-old soil-grown wt (SSS2), sid2, sid2 eds1, sid2 fad7, sid2 fad7 fad8, sid2 eds1 fad7, and sid2 eds1 fad7 fad8 plants. (B) Microscopy of trypan blue-stained leaves from indicated genotypes. (C) Expression of PR-1 indicated genotypes. Total RNA was...
extracted from 4-week-old plants and used for RNA gel-blot analysis. Ethidium bromide staining of rRNA was used as loading control. (D) Endogenous SA levels in the leaves of 4-week-old plants. Values are presented as average of four replicates and the error bars represent SD. Statistical significance was determined using Student’s t-test. Asterisks indicate data statistically significant between ssii2 fad7 and ssii2 eds1 fad7 or ssii2 fad7 fad8 and ssii2 eds1 fad7 fad8 (P<0.05, n = 5). (E) Endogenous SAG levels in the leaves of 4-week-old plants. Values are presented as mean of three replicates and the error bars represent SD. Asterisks indicate data statistically significant between ssii2 fad7 and ssii2 eds1 fad7 or ssii2 fad7 fad8 and ssii2 eds1 fad7 fad8 (P<0.05, n = 5). (F) RT-PCR analysis of R genes in indicated genotypes. The level of $\beta$-tubulin was used as an internal control to normalize the amount of cDNA template. The SSII EDS1 FAD7 and SSII EDS1 FAD7 FAD8 F2 plants showed wt-like morphology and basal levels expression of PR-1 and R genes (data not shown).

Found at: doi:10.1371/journal.pgen.1000545.s003 (0.96 MB TIF)

Figure S4 Morphology, cell death, PR-1, and R gene expression and SA/SAG levels in ssii2 sag101-1, ssii2 sag101-1 eds1-2 and ssii2 sag101-1 sid2-1 plants. (A) Comparison of the morphological phenotypes displayed by 4-week-old soil-grown wt (SSII; Col-0 ecotype), sag101, ssii2, ssii2 sag101, ssii2 eds1, ssii2 sag101 sid2, ssii2 eds1 and ssii2 sag101 eds1 plants (scale, 0.5 cm). (B) Microscopy of trypan blue-stained leaves from indicated genotypes (scale bars, 270 microns). (C) Expression of PR-1 in indicated genotypes. Total RNA was extracted from 3-week-old plants and used for RNA gel-blot analysis. Ethidium bromide staining of rRNA was used as the loading control. (D) RT-PCR analysis of R genes in indicated genotypes. The level of $\beta$-tubulin was used as an internal control to normalize the amount of cDNA template. (E) Endogenous SA levels in the leaves of 4-week-old soil-grown plants. Values are presented as averages of four replicates and the error bars represent SD. (F) Endogenous SAG levels in the leaves of 4-week-old soil-grown plants. Error bars represent SD. The SSII EDS1 FAD7 and SSII EDS1 FAD7 FAD8 F2 plants showed wt-like morphology, accumulated wt-like levels of SA and showed wt-like expression of PR-1 and R genes (data not shown). Statistical significance was determined using Student’s t-test. Asterisks indicate data statistically significant compared to results from SSII (Col-0) plants. Error bars represent SD. The four replicates and the error bars represent SD. Asterisks indicate data statistically significant between ssii2 fad7 and ssii2 eds1 fad7 or ssii2 fad7 fad8 and ssii2 eds1 fad7 fad8 (P<0.05, n = 5). Statistical significance in (E) and (F) were determined using Student’s t-test. Asterisks indicate data statistically significant compared to results from SSII (Col-0) plants (P<0.05, n = 4).

Found at: doi:10.1371/journal.pgen.1000545.s004 (1.17 MB TIF)

Figure S5 Morphology, cell death, PR-1, SA/SAG levels, and R gene expression in ssii2 pad4-1 sid2-1 and ssii2 eds5-1 sid2-1 plants. (A) Comparison of the morphological phenotypes displayed by 4-week-old soil-grown wt (SSII; Col-0 ecotype), ssii2, ssii2 sid2, ssii2 pad4, ssii2eds5, ssii2 pad4 sid2 and ssii2 eds5 sid2 plants (scale, 0.5 cm). (B) Microscopy of trypan blue-stained leaves shown in (A) (scale bars, 270 microns). (C) Expression of PR-1 gene in indicated genotypes. Total RNA was extracted from 4-week-old plants and used for RNA gel-blot analysis. Ethidium bromide staining of rRNA was used as the loading control. (D) RT-PCR analysis of R genes in indicated genotypes. The level of $\beta$-tubulin was used as an internal control to normalize the amount of cDNA template. The SSII PAD4 SID2 and SSII EDS5 SID2 F2 plants showed wt-like morphology and showed wt-like expression of PR-1 and R genes (data not shown). (E) Endogenous SA levels in the leaves of 4-week-old soil-grown plants. Values are presented as averages of four replicates and the error bars represent SD. (F) Endogenous SAG levels in the leaves of 4-week-old soil-grown plants. Error bars represent SD. The SSII EDS1 FAD7 and SSII EDS5 FAD8 plants showed wt-like morphology, accumulated wt-like levels of SA and showed wt-like expression of PR-1 and R genes (data not shown). Statistical significances in E and F were determined using Student’s t-test. Asterisks indicate data statistically significant compared to results from SSII (Col-0) plants (P<0.05, n = 4).

Found at: doi:10.1371/journal.pgen.1000545.s005 (1.09 MB TIF)

Table S1 A list of genetic crosses analyzed in this study.

Found at: doi:10.1371/journal.pgen.1000545.s006 (0.08 MB DOC)

Table S2 Fold change in transcript levels of R and PR genes in ssii2 sid2 and ssii2 eds1 sid2 plants compared to results from Col-0 (wt) plants. R genes showing 2–2.5, 2.5–3, and >3-fold activation are marked yellow, orange, or red, respectively. Transcriptional profiling was performed using Affymetrix arrays.

Found at: doi:10.1371/journal.pgen.1000545.s007 (0.09 MB DOC)

Table S4 FA composition from leaf tissues of SSII (Col-0), sid2, ssii2, ssii2 eds1, ssii2 sid2, and ssii2 eds1 sid2 plants. All measurements were made on 4-week-old plants grown at 22°C and data are described as mol%±SD calculated for a sample size of six.

Found at: doi:10.1371/journal.pgen.1000545.s008 (0.06 MB DOC)

Table S5 Primer sequences used to amplify various genes.

Found at: doi:10.1371/journal.pgen.1000545.s010 (0.04 MB DOC)

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Author Contributions

Conceived and designed the experiments: SCV RDJ MKM SZ ACCS YX MH AJS DN AK. Performed the experiments: SCV RDJ MKM SZ ACCS YX DN AK. Analyzed the data: SCV RDJ MKM SZ ACCS YX MH AJN DN AK. Contributed reagents/materials/analysis tools: SCV RDJ MKM SZ ACCS YX MH AJN AK. Wrote the paper: AK PK.

References

Redundancy in Plant Defense


