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 EDS5, 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.

Citation: Venugopal SC, Jeong R-D, Mandal MK, Zhu S, Chandra-Shekara AC, et al. (2009) Enhanced Disease Susceptibility 1 and Salicylic Acid Act Redundantly to Regulate Resistance Gene-Mediated Signaling. PLoS Genet 5(7): e1000545. doi:10.1371/journal.pgen.1000545

Editor: Gregory P. Copenhaver, The University of North Carolina at Chapel Hill, United States of America

Received March 26, 2009; Accepted June 1, 2009; Published July 3, 2009

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Funding: This work was supported by grants from NSF (MCB#0421914), USDA-NRI (2004-03287), and KSEF (419-RDE-004, 555-RDE-005, 622-RDE-006). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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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 responses 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].
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.

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 (SACP) 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 (*GLI1*, 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 isofrom 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 *GLI1* 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 *rt* (regulates resistance to TCV), SA, EDS1 and PAD4 [25]. Exogenous application of SA induces the expression of HRT and overcomes the requirement for *rt*. However, exogenous SA is unable to induce HRT or confer resistance in *pad4* background [25]. 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 edd1* 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), compared to *ssi2* plants, they showed *ssi2*-like *PR-1* and *R* gene expression (Figure 1D and 1E, Figure S1A). Exogenous glycerol application, which reduces 18:1 levels, also induced *R* gene expression in *edd1* and *ndr1* 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 edd1* 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 edd1 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. 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. doi:10.1371/journal.pgen.1000545.g001
Redundancy in Plant Defense

nsb1 sid2 plants. Interestingly, only the ssi2 eds1 sid2 plants showed wt-like morphology and did not develop visible or microscopic cell death (Figure 1A and 1B). In contrast, ssi2 sid2, ssi2 sid1, ssi2 nsb1 sid2 or ssi2 eds1 plants exhibited ssi2-like phenotypes. PR-1 gene expression was restored to wt-like levels in the ssi2 eds1 sid2 and ssi2 nsb1 sid2 plants, due to the sid2-derived reduction in SA levels (Figure 1D). In contrast, expression of the SA-independent PR-2 gene was restored to basal levels only in ssi2 eds1 sid2 [43], but not in ssi2 nsb1 or ssi2 nsb1 sid2 plants (Figure 1D, Table S2). Most importantly, ssi2 eds1 sid2 showed basal expression of R genes, unlike ssi2 nsb1 and ssi2 nsb1 sid2 plants (Figure 1E and 1F; Figure S1A, S1B; Table S1). R gene induction was further confirmed by comparing the transcript profiles of 162 NBS-LRR genes in ssi2 sid2 with that of wt plants using Affymetrix ATH1 GeneChips arrays. Twenty-one NB-LRR genes were specifically expressed at 2-fold or higher levels in ssi2 sid2 plants as compared to wt (Col-0) or eds1 plants (P<0.05) (Table S2). All 21 NB-LRR genes were expressed at low levels in ssi2 eds1 sid2 plants, further confirming the results from the RT-PCR analysis. Transcriptional profiling performed using Affymetrix arrays showed that the induction of several R genes (RPM1, RPS2, RPP5, RPS4) was lower than 2-fold in ssi2 or ssi2 sid2 compared to wt plants (Table S2, data not shown for ssi2). To determine if this low-level induction translated to a significant increase in R protein levels, we analyzed the levels of RPM1 in ssi2 plants. Indeed, ssi2 plants accumulated significantly higher levels of the RPM1-Myc protein (Figure 1G).

To rule out the effects of the varied ecotypes of the ssi2 sid2 eds1 (Nössen, Col-0, Ler) plants we introduced eds1-1 (Ws-0 ecotype) and eds1-2 (Ler ecotype) alleles in ssi2 and ssi2 nsb1G (Nössen ecotype) backgrounds (Table S1). All combinations of ssi2 with eds1-1/eds1-2 and sid2/sid2G produced similar phenotypes (data not shown). FA profiling showed that the ssi2 eds1 sid2 plants contained low 18:1 levels, similar to ssi2 plants (Table S3). We thus concluded that EDS1 and SA function downstream of 18:1 levels, but upstream of R gene expression. Furthermore, ssi2 eds1 sid2 plants were wt-like, even though neither ssi2 eds1 nor ssi2 sid2 were restored for defense signaling. Therefore, EDS1 and SA likely fulfill redundant functions in defense signaling induced in response to a reduction in 18:1 levels.

To further test the redundancy for EDS1 and SA, ssi2 eds1 sid2 plants were treated with SA or its active analog benzo(1,2,3)thiazole-7-carbothioic acid (BTH). Application of SA or BTH induced lesion formation on ssi2 eds1 sid2 plants but not on wt, eds1, sid2, eds1 sid2 or EDS1 SID2 F2 plants (Figure 2A and 2B, data not shown for eds1 sid2 and EDS1 SID2). Also, application of SA or BTH induced R gene expression in ssi2 eds1 sid2 plants (Figure 2C). Thus, application of SA restored ssi2-like phenotypes in ssi2 eds1 sid2 plants. Since glycerol application mimics the effects of the ssi2 mutation, we generated eds1 sid2 plants and evaluated them for their ability to induce R genes in response to glycerol. Exogenous application of glycerol lowered 18:1 levels in all genotypes, but induced the expression of R genes only in wt, eds1, sid2 and EDS1 SID2 F2 plants (Figure 2D, Figure S1C). Only a marginal or no increase in R gene expression was observed in the eds1 sid2 plants (Figure 2D). These results confirmed that EDS1 and SA function redundantly downstream of signaling induced by low 18:1 levels, but upstream of R gene expression.

EFS1 and SA function redundantly in pathogen resistance induced in response to reduction in 18:1 levels

We next evaluated the effect of simultaneous mutations in EDS1- and SA-signaling pathways on resistance to TCV in the ssi2 background. We reported previously that resistance to TCV is dependent on the R gene, HRT, and a recessive locus mt [23]. However, the ssi2 mutation overcomes the requirement for mt in HRT-containing plants [23,37]. Furthermore, the ssi2 mutation only confers resistance to TCV when HRT is present (Figure 3A).

The ssi2 mutation also overrides a requirement for EDS1 and SA and consequently ssi2 HRT eds1 as well as ssi2 HRT sid2 plants exhibit resistance to TCV [37] (Figure 3A). Unlike HRT ssi2, HRT ssi2 eds1 or HRT ssi2 sid2 plants, the HRT ssi2 eds1 sid2 plants showed susceptibility to TCV. ~85% HRT ssi2 eds1 sid2 plants were susceptible to TCV as against ~2–4% of HRT ssi2 sid2 or HRT ssi2 eds1 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 ssi2 eds1 sid2 plants, unlike in HRT ssi2 sid2 plants (Figure 3B). These results showed that both EDS1 and SA have redundant functions in ssi2-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 eds1 plants revealed discrete and similar-sized HR lesions on TCV-inoculated leaves (Figure 3C and 3D). In comparison, HR in HRT eds1 sid2 plants was diffused and formed larger lesions (Figure 3C and 3D). Increased lesion size in HRT eds1 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 eds1 sid2 plants accumulated lower levels of PR-1 and PR-2 transcripts, unlike Di-17, HRT eds1 or HRT sid2 plants (Figure 3G and 3H). In contrast to PR, HRT expression remained unaltered in HRT eds1 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 phenotype 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. 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 rps2 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 eds1-1 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.

doi:10.1371/journal.pgen.1000545.g002
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 double mutant plants. Analysis of RPS2 tagged with HA epitope in indicated genotypes at 3 dpi. Ethidium bromide staining of rRNA was used as the loading control. (G) Expression of PR-1 gene in indicated genotypes. Total RNA was extracted from inoculated leaves at 3 dpi. Ethidium bromide staining of rRNA was used as the loading control. The error bars indicate SD. (F) Transcript levels of TCV CP in the inoculated leaves of indicated genotypes. The plants were inoculated with TCV and leaf samples were harvested 24 h post inoculation. The level of β-tubulin was used as an internal control to normalize the amount of cDNA template.

doi:10.1371/journal.pgen.1000545.g003

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

We next evaluated the effect of mutations in EDS1 and SID2 on RPP8-mediated resistance to Hyaloperonospora arabidopsidis biotype Emco5 encoding Avr8. RPP8 (encodes a CC-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 nahG plants was not due to the accumulation of catechol, which is formed upon degradation of SA by NAHY. 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 ss1 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.
Figure 4. Interaction phenotypes of virulent or AvrRPT2-expressing *P. syringae* with *eds1 sid2* plants. (A) Photograph showing phenotypes produced upon infiltration of 10^5 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. doi:10.1371/journal.pgen.1000545.g004
**Figure 5. Interaction phenotypes of H. arabidopsidis biotype Emc05 expressing Atr8 with RPP8 eds1-2 nahG or RPP8 eds1-2 sid2-1 plants.**

(A) Whole leaf pictures showing growth of Emc05 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.

doi:10.1371/journal.pgen.1000545.g005
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 eds5 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.

**PAD4, SAG101, and EDS5 are not functionally redundant with SA in low 18:1-mediated signaling**

Besides EDS1, the SA signaling pathway is also regulated by PAD4 and EDS5 and via the physical association of EDS1 with SAG101 and PAD4 [17,19,45]. To determine if PAD4, SAG101 or EDS5 also function redundantly with SA, we introduced the pad4, sag101 and eds5 mutations in the ssi2 and ssi2 sid2 backgrounds.

The ssi2 sag101, ssi2 pad4 and ssi2 eds5 plants showed ssi2-like morphology, visible and microscopic cell death and constitutive PR-1 gene expression (Figure S4A, S4B, S4C and Figure S5A, S3E, S5C). Consistent with these phenotypes, the ssi2 sag101, ssi2 pad4, ssi2 eds5 plants showed increased expression of R genes (Figure S4D and Figure S5D) and accumulated elevated levels of SA and SAG (Figure S4E, S4F and Figure S5E, S5F). Notably, the SA levels in ssi2 sag101 plants were ~6-fold lower than in ssi2 plants, suggesting that SAG101 contributed to the accumulation of SA in ssi2 plants. To determine if the reduced SA in the sag101 background could restore wt-like phenotypes in ssi2 eds1 plants, triple mutant ssi2 eds1 sag101 plants were generated. Although the ssi2 eds1 sag101 plants accumulated significantly lower levels of SA/SAG (Figure S4E, S4F), these plants were only slightly bigger than ssi2 eds1 or ssi2 sid2 plants (Figure S4A), showed spontaneous cell death (Figure S4B) and expressed PR-1 (Figure S4C) and R genes constitutively (Figure S4D). We next analyzed the triple mutant ssi2 sag101 sid2, ssi2 pad4 sid2 and ssi2 eds5 sid2 plants. All the triple mutants contained wt-like levels of SA and SAG (Figure S4E, S4F and Figure S5E, S5F). The ssi2 sag101 sid2 plants were morphologically similar to ssi2 plants, showed spontaneous cell death and expressed R genes constitutively (Figure S4A, S4B, S4C, S4D). In comparison, the ssi2 pad4 sid2 and ssi2 eds5 sid2 plants were bigger in morphology. However, plants of both genotypes showed cell death (Figure S5A, S5B) and expressed R genes constitutively (Figure S5D). Together, these data suggest that the functional redundancy with SA was specific only to EDS1 and did not extend to PAD4, SAG101 or EDS5.

**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

**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 SA–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 ed1 sid2 and not in the single mutant

Figure 7. Basal resistance in ed1 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 ed1-1 and ed1-22 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). doi:10.1371/journal.pgen.1000545.g007
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. 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 EDS1, SSI2 PAD4, SSI2 EDS1 PAD4, and SSI2 EDS1 EDS5 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 the Ws 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, sid2 eds1 sid2 as well as glycerc-treatment-treated eds1 sid2 plants showed wt-like expression of R genes while sid2 eds1, ss2 sid2 and glycerc-treatment-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 glycerc 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 sid2 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 sid2-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 sid2-like phenotypes, it is highly unlikely that ecotypic variations resulted in the restoration of phenotypes in sid2 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 sid2 sid2 (No, Col-0 ecotypes) and sid2 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.

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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 flux density of the day period was 106.9 μmol s⁻¹ m⁻² 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 L. 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 AxiosCam camera (Zeiss, Germany) and images were analyzed using Openlab 3.5.2 (Improvision) software.

Pathogen infections

The asexual conidiospores of H. arabidopsidis Emco5 expressing Atβ were maintained on the susceptible host Nossen (No) or No

P. syringae and TCV, even though only the genotypes used for TCV were of mixed ecotypic backgrounds. Fourth, F2 plants containing wild-type alleles behaved like wild-type parents. Finally, the effects of various mutant backgrounds on ssi2 phenotypes were also confirmed by glycerol application on individual mutants.

Although glycerol treatment failed to induce R gene expression in eds1 ssi2 plants, it did induce cell death. This is in contrast to the absence of a cell death phenotype in ssi2 eds1 ssi2 leaves. One possibility is that the glycerol-triggered cell death is not due to a reduction in 18:1 levels. However, significant overlap between ssi2- and exogenous glycerol-triggered signaling pathways lessens such a possibility [40]. An alternate possibility is that, while EDS1 affects a majority of the responses induced by low 18:1 levels, the cell death phenotype is also governed by some additional molecular factor(s). This is supported by the fact that ssi2 pad4 ssi2 plants exhibit improved morphology and reduced cell death even though they are not restored for other defense-related phenotypes.

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 act1, ssi2 gly1 or ssi2 apoA [30,31,35], as well as in suppressor containing low 18:1 levels, such as ssi2 eds1 sid2 (this work) and restored in defective crosstalk (sid2) (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 (sid3, sid4). 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 S. 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-upregulated 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.
NabG. The spores were removed by agitating the infected leaves in water and suspended to a final concentration of 10^6 spores/mL. Two-week-old seedlings were sprayed with spore suspension and transferred to a MSTR30 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), AacRpt2 or AacRps4 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 10^6 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, crumbling 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; 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 x 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 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 AvrRPT2 or AvrRPS4 expressing P. syringae with eds1 sid2 plants. (A) Photograph showing phenotypes produced upon infiltration of 10^6 CFU/ml bacteria (AvrRPT2). 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 (SS2), 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 averages of four replicates and the error bars represent SD. Statistical significance was determined using Student’s t-test. Asterisks indicate data statistically significant between ssi2 fad7 and ssi2 eds1 fad7 or ssi2 fad7 fad8 and ssi2 eds1 fad7 fad8 (P<0.05, n = 5). (E) Endogenous SAG levels in the leaves of 4-week-old plants. Values are presented as means of three replicates and the error bars represent SD. Asterisks indicate data statistically significant between ssi2 fad7 and ssi2 eds1 fad7 or ssi2 fad7 fad8 and ssi2 eds1 fad7 fad8 (P<0.05, n = 5). (F) RT-PCR analysis of PR genes in indicated genotypes. The level of β-tubulin was used as an internal control to normalize the amount of cDNA template. The SSI2 ED5 FAD7 and SSI2 ED5 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 ssi2 sag101-1, ssi2 sag101-1 eds1-2 and ssi2 sag101-1 ssi2-1 plants. (A) Comparison of the morphological phenotypes displayed by 4-week-old soil-grown wt (SSI2; Col-0 ecotype), ssi2, ssi2 sag101, ssi2 sid2, ssi2 sag101 sid2, ssi2 eds1 and ssi2 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 gene 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 PR genes in indicated genotypes. The level of β-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 SSI2 FAD4 and SSI2 ED53 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 SSI2 (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 ssi2 sid2 and ssi2 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 SSI2 (Col-0), eds1, sid2, ssi2, ssi2 eds1, ssi2 sid2, and ssi2 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)

Acknowledgments
We thank David Smith for critical comments; John Johnson for help with gas chromatography; Ludmila Lapchyk, Thomas Muse, and Lev Orlov for help with fatty acid extractions and genotyping; and Amy Crume for maintaining the growth facility. We thank Jeff Dangl for rps2-101c, RPM1-MHC, and RPS2-2HA seeds; Keiko Yoshikawa for Ler NahG, and Jane Parker for sag101 seeds. We thank Walter Grassmann for the Pseudomonas syringae strain containing AvrRPS4 and Barbara Kunke for the AvrRpt2 strain. We thank Jack Morris for providing anti-TCV CP antisera.

Author Contributions
Conceived and designed the experiments: SCV RDJ MKM SZ ACCS YX DN AK PK. Wrote the paper: AK PK. Performed the experiments: SCV RDJ MKM SZ ACCS YX DN AK PK. Helped with fatty acid extractions and genotyping; and Amy Crume for maintaining the growth facility. John Johnson for help with gas chromatography; Ludmila Lapchyk, Thomas Muse, and Lev Orlov for help with fatty acid extractions and genotyping; and Amy Crume for maintaining the growth facility. Jeff Dangl for rps2-101c, RPM1-MHC, and RPS2-2HA seeds; Keiko Yoshikawa for Ler NahG, and Jane Parker for sag101 seeds. Walter Grassmann for the Pseudomonas syringae strain containing AvrRPS4 and Barbara Kunke for the AvrRPT2 strain. Jack Morris for providing anti-TCV CP antisera.

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