The Role of Salicylic Acid in the Induction of Cell Death in Arabidopsis *acd11*[1]

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Salicylic acid (SA) is implicated in the induction of programmed cell death (PCD) associated with pathogen defense responses because SA levels increase in response to PCD-inducing infections, and PCD development can be inhibited by expression of salicylate hydroxylase encoded by the bacterial *nahG* gene. The *acd11* mutant of Arabidopsis (*Arabidopsis thaliana* L. Heynh.) activates PCD and defense responses that are fully suppressed by *nahG*. To further study the role of SA in PCD induction, we compared phenotypes of *acd11/nahG* with those of *acd11/eds5-1* and *acd11/sid2-2* mutants deficient in a putative transporter and isochorismate synthase required for SA biosynthesis. We show that *sid2-2* fully suppresses SA accumulation and cell death in *acd11*, although growth inhibition and premature leaf chlorosis still occur. In addition, application of exogenous SA to *acd11/sid2-2* is insufficient to restore cell death. This indicates that isochorismate-derived compounds other than SA are required for induction of PCD in *acd11* and that some *acd11* phenotypes require NahG-degradable compounds not synthesized via isochorismate.

Plants possess an immune system to defend themselves against pathogen infection. An intensively studied inducible immune response occurs when a pathogen carrying an avirulence (*avr*) gene is recognized directly or indirectly by a cognate resistance (*R*) gene in the plant. This leads to activation of defenses that restrict pathogen growth in infected tissues and in noninfected tissues by a process referred to as systemic acquired resistance (SAR). These defense responses are typically accompanied by localized programmed cell death (PCD) around the site of infection in the hypersensitive response (HR; Nimchuk et al., 2003). In the absence of an *R-avr* interaction, basal resistance responses are also activated, although they may not successfully restrict pathogen growth, and disease symptoms may develop (Glazebrook et al., 1997).

The importance of salicylic acid (SA) in the induction of such resistance responses is supported by both gain- and loss-of-function evidence. SA levels increase upon many avirulent and some virulent infections (Malamy et al., 1990; Métraux et al., 1990; Heck et al.,

2003), and application of exogenous SA, or generation of high endogenous SA levels by expression of bacterial SA synthesizing enzymes, is sufficient to induce resistance to many normally virulent pathogens (White, 1979; Ward et al., 1991; Verberne et al., 2000; Mauch et al., 2001). Loss-of-function analyses have relied upon SA depletion by transgenic expression of a bacterial SA hydroxylase encoded by *nahG*. NahG abrogates local *R* function elicited by a range of bacterial, oomycete, and viral pathogens (Delaney et al., 1994; Rairdan and Delaney, 2002) as well as SAR (Gaffney et al., 1993) and basal resistance responses to virulent bacteria, fungi, and oomycetes (Delaney et al., 1994; Reuber et al., 1998). These results have been supported by the characterization of two SA-deficient mutants of Arabidopsis (*Arabidopsis thaliana* L. Heynh.), *sid2* and *eds5* (Rogers and Ausubel, 1997; Nawrath and Métraux, 1999; Dewdney et al., 2000). Both *sid2* and *eds5* show enhanced susceptibility to many virulent infections and strongly reduced induction of SAR. However, only some *R*-mediated local resistance responses are affected in *sid2* and *eds5* (Nawrath and Métraux, 1999), and in most cases their hypersusceptibility is not as pronounced as in NahG plants. *SID2* encodes a pathogen induced isochorismate synthase (ICS; Wildermuth et al., 2001), while *EDS5* encodes an orphan multidrug and toxin extrusion transporter that may be involved in a positive feedback loop stimulating SA accumulation as *EDS5* expression is induced by SA (Nawrath et al., 2002). The strong reduction of SA accumulation in *sid2* mutants in response to infection establishes the isochorismate pathway as the major route to defense-associated SA. A second Phe ammonia lyase (PAL)-dependent SA biosynthesis pathway from cinnamic acid via benzoic acid has also been described (Yalpani et al., 1993; Ribnicky et al., 1998),

although the functional relevance of this pathway is unclear.

The involvement of SA in activation of PCD in the HR is supported by similar lines of evidence but remains less clear. SA does not induce HR-like PCD on its own in whole plants, although it may induce PCD in cell culture (Kawai-Yamada et al., 2004). HR induced by two *Peronospora parasitica* isolates avirulent on Arabidopsis appears to depend on SA since *nahG*, *eds5*, and *sid2* blocked the HR in response to infection, although trailing necrosis surrounding growing hyphae was still observed (Nawrath and Métraux, 1999). Similarly, *nahG* delays the HR of tobacco (*Nicotiana tabacum*) in response to tobacco mosaic virus (Mur et al., 1997). Consistent with these observations of PCD attenuation by SA removal, exogenous SA strongly accelerated HR cell death in soybean (*Glycine max*) suspension cells (Shirasu et al., 1997) and induced cell death in Arabidopsis *lsd1* mutants and *RPW8* enhanced transcription lines kept under conditions nonpermissive for spontaneous HR-like cell death development (Dietrich et al., 1994; Xiao et al., 2003). On the other hand, HR is normally induced in *eds5* and *sid2* in response to high doses of *Pseudomonas syringae* pv *tomato* DC3000 expressing the *avr* gene *avrRpm1*, *avrRpt2*, or *avrRps4* (Nawrath and Métraux, 1999; Dewdney et al., 2000). Clearer evidence pointing to a role of SA in PCD comes from the analysis of Arabidopsis *acd* and *lsd* mutants that spontaneously activate PCD and defense responses. In many of these mutants, including *acd6-1*, *acd11*, *ssi1*, and *lsd6*, *nahG* expression completely suppresses PCD development, while this can be restored by application of SA agonists such as 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH; Weymann et al., 1995; Rate et al., 1999; Shah et al., 1999; Brodersen et al., 2002). Similarly, *nahG* inhibits PCD induction by the mycotoxin fumonisin B1 (Asai et al., 2000). Thus, the clearest links of SA to PCD induction are based on analysis of NahG plants.

However, recent reports comparing phenotypes of NahG plants with SA-deficient *eds5* and *sid2* mutants indicate that the effects of *nahG* expression are not as straightforward as previously thought. For example, the loss of nonhost resistance toward *P. syringae* pv *phaseolica* in Arabidopsis expressing *nahG* was not due to reduced SA levels but to the generation of SA breakdown products (van Wees and Glazebrook, 2003), whereas the failure of *nahG*-expressing plants to accumulate camalexin and ethylene in response to *P. syringae* pv *tomato* infections resulted from the action of salicylate hydroxylase on as yet unidentified compounds different from SA (Heck et al., 2003).

We previously characterized the lethal *acd11* mutant in which PCD and defense responses activated at the seedling stage can be completely suppressed by *nahG* (Brodersen et al., 2002). Here, we use the *acd11* mutant in combination with *nahG*, *eds5*, and *sid2* to further examine the role of SA in PCD. To our knowledge,

such a side-by-side comparison of the effect of *nahG*, *eds5*, and *sid2* on PCD and SA accumulation using the same system has not been reported. We show that while SA may play a role in PCD initiation in this system, it is not the only isochorismate-derived compound required for PCD induction. In addition, some *acd11* phenotypes are dependent on NahG degradable compounds not derived from isochorismate.

RESULTS

Penetrance of *acd11* Phenotypes in Columbia and Landsberg *erecta* Accessions

Our previously isolated *acd11-1* deletion mutant allele is in the Landsberg *erecta* (L*er*) accession, while mutant alleles of *EDS5* (*eds5-1* to *eds5-3*) and *SID2* (*sid2-1* and *sid2-2*) were only available in Columbia (Col-0) at the beginning of this study. Since mixed genetic backgrounds arising from crosses of mutants in nonisogenic parental accessions may complicate interpretations of observed double mutant phenotypes, we first made use of a cross of *acd11-1* to wild-type Col-0 to examine phenotypes conferred by *acd11-1* in Col-0. In the F2 population, 19% of the progeny (18/95) showed a typical Acd⁻ phenotype whose onset occurred at the same time as the *acd11-1*^L*er* control. None of the 77 phenotypically wild-type plants were homozygous for *acd11-1*, indicating that the slightly distorted segregation ratio was not due to the presence of a recessive *acd11* suppressor in Col-0. In addition, we recently isolated an *acd11* T-DNA insertion allele in Col-0 (*acd11-2*, SALK_018628) with an Acd⁻ phenotype identical to that of *acd11-1*. We next analyzed total SA levels in *acd11-1*, *acd11-2*, and *acd11-1*^L*er*×Col and found that they accumulated similar high levels of total SA compared with the corresponding ACD11 controls (data not shown). This indicates that the Acd⁻ and SA accumulation phenotypes are not affected in a mixed L*er*/Col-0 genetic background, so that *acd11-1* crossed to *eds5-1* and *sid2-2* may be suitable for examining roles of SA in PCD induction.

Phenotypes of *acd11/eds5-1* and *acd11/sid2-2* Mutants

Families homozygous for *eds5-1* and *sid2-2* and heterozygous for *acd11-1* were selected in the F3 generation of crosses of *eds5-1* and *sid2-2* to *acd11-1*. These lines were grown together with *acd11-1* and *acd11/nahG* (Brodersen et al., 2002) for phenotypic comparison. In three independent F3 families, *acd11/eds5-1* exhibited cell death symptoms 4 to 5 d later than *acd11* and consequently grew much larger than *acd11* (average leaf area over all leaves 32 mm² versus 11 mm² in *acd11-1*; Fig. 1A). However, once leaf yellowing initiated in *acd11/eds5-1*, it developed as quickly as in *acd11* to engulf the rosette. Like *acd11*, *acd11/eds5-1* exhibited strong shoot inhibition, and stem and inflorescence formation was rarely observed, although a few flowers and siliques typically formed directly at
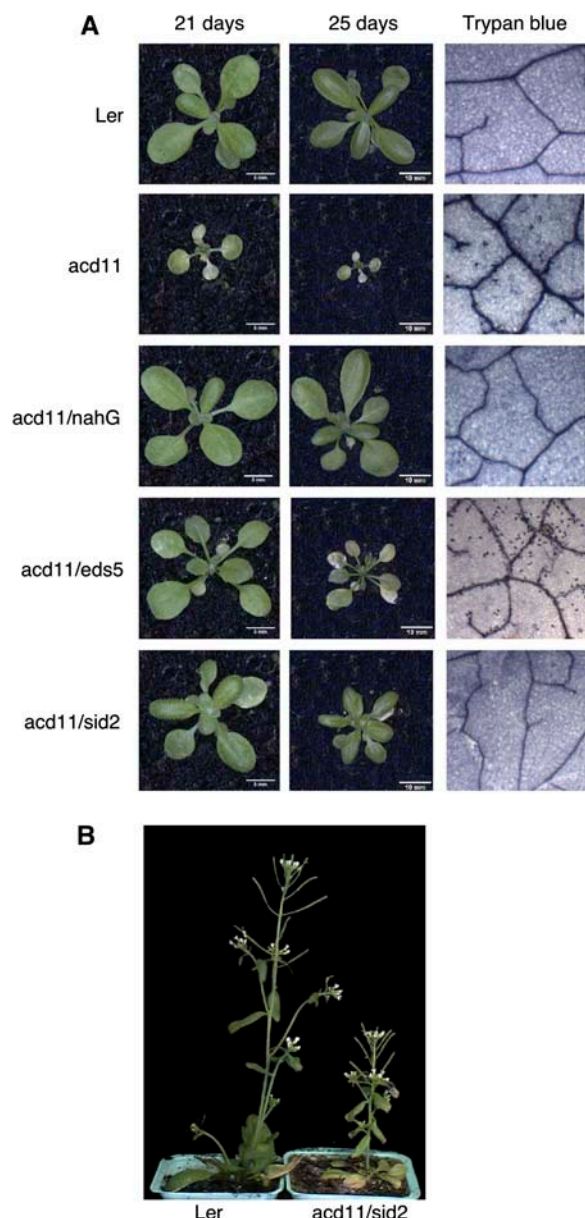
Figure 1. Phenotypes of *acd11/eds5*, *acd11/sid2*, and *acd11/nahG*. A, Phenotypes and trypan blue stainings of leaves at the rosette stage. Trypan blue stainings were performed at day 25. B, Stature of mature *acd11/sid2* relative to wild-type L*er*.

the rosette. Microscopic examination of trypan blue-stained leaves showed that they contained numerous dead cells similar to *acd11* (Fig. 1A). Thus, except for being delayed, *acd11/eds5-1* exhibited the same phenotypes as *acd11*.

The effect of the *sid2-2* mutation was much more pronounced. In four independent F3 families, rosettes of *acd11/sid2* were found to develop normally, although they were reduced in size compared with wild type or *acd11/nahG* (average leaf areas over all leaves 45 mm^2 in *acd11-1/sid2-2*; 76 mm^2 in *acd11/nahG* and 76 mm^2 in L*er*; Fig. 1A). Around the time of bolting, some leaf chlorosis occurred, but flower and silique

bearing bolts were produced. Although smaller than wild type and *acd11/nahG* plants, *acd11/sid2-2* did not show the strong shoot inhibition observed in *acd11* and *acd11/eds5-1* (Fig. 1B). Neither the young green nor the older yellowing *acd11/sid2-2* leaves contained dead cells, as revealed by trypan blue staining. Thus, most but not all *acd11* phenotypes were suppressed by *sid2-2*. As previously described, no differences were observed between the *acd11-1/nahG*^{LerxCol} line and the wild type. We also constructed an *acd11-1/nahG* line in L*er* that showed complete suppression of the Acd$^-$ phenotype (Cui et al., 2002; data not shown). These results demonstrate marked differences between the effects of *nahG* expression and *sid2-2* and *eds5-1* mutations on the *acd11* phenotype in which the strength of suppression decreases in the order *nahG* > *sid2-2* > *eds5-1*. The observation that double mutant phenotypes were the same in independent families of the *acd11-1/sid2-2* and *acd11-1/eds5-1* crosses confirms that the mixed genetic background has little, if any, influence on the double mutant analysis. For this reason, experiments described below were performed with a single double mutant family from each cross.

SA Accumulation in Double Mutants

Due to the proposed key role of SA in PCD and defense induction, the double mutant results may be explained by different effects of *nahG*, *sid2-2*, and *eds5-1* on SA accumulation in *acd11*. To test this, we compared total (the sum of free and Glc conjugated) SA levels in the different genetic backgrounds (Table I). This showed that *acd11/eds5-1* accumulated SA to approximately 10% of the levels observed in *acd11*, whereas only basal SA levels could be detected in *acd11/nahG* or *acd11/sid2-2*. Thus, the weaker suppression of *acd11* by *eds5-1* might be due to incomplete reduction of SA accumulation, whereas this cannot explain the differences observed between *acd11/sid2-2*

Table I. *Accumulation of total SA in single and double mutants*

SA and SA-glucoside levels determined by HPLC in the wild type, in single mutants and *nahG*, and in the *acd11/eds5*, *acd11/sid2*, and *acd11/nahG* double mutants as shown. ''Young'' refers to 18-d-old rosettes (third pair of true leaves emerging; all samples except ''acd11/sid2 old''), and ''old'' refers to yellowing leaves on 34-d-old bolting plants. Each measurement is an average of triplicate samples, and the entire experiment was repeated twice with similar results.

Genotype	Total SA
	μg/g FW
acd11	82 ± 14
L*er*	1.3 ± 0.48
acd11/sid2 young	1.5 ± 0.52
acd11/sid2 old	1.7 ± 0.38
sid2	1.2 ± 0.52
acd11/eds5	8.5 ± 2.9
eds5	1.3 ± 0.35
acd11/nahG	0.87 ± 0.14
nahG	1.01 ± 0.82

and *acd11/nahG*. More specifically, these findings appear to exclude the possibility that SA synthesized via the SID2-independent PAL pathway could cause the phenotypic differences between *acd11/sid2* and *acd11/nahG* because *sid2* mutation alone leads to full suppression of SA accumulation in *acd11*.

Expression of Cell Death and Defense Marker Genes

To characterize the phenotypes of the double mutants at the molecular level, we first monitored the expression of molecular markers for cell death and SA-dependent defense. We chose the SA-dependent defense marker *PR1* (At2g14610) and the cell death-associated genes *SAG13* (At2g29350) and *FMO* (At1g19250), encoding a putative short-chain alcohol dehydrogenase and a putative flavin containing mono-oxygenase, respectively. These genes are all strongly expressed in *acd11* (Brodersen et al., 2002). RNA-blot analysis showed that these three markers were strongly expressed in *acd11/eds5-1*, while their expression was almost completely suppressed in *acd11/sid2-2* and *acd11/nahG* (Fig. 2A; Brodersen et al., 2002). Thus, although these markers do not distinguish between *acd11/nahG* and *acd11/sid2-2*, this molecular analysis supports the whole plant and cellular phenotypes described above.

In contrast to *sid2*, *nahG* has been reported to suppress ethylene accumulation following virulent and avirulent bacterial infections (Heck et al., 2003). This leaves open the possibility that the phenotypic differences between *acd11/sid2* and *acd11/nahG* may be due to differential ethylene accumulation and/or signaling in the two backgrounds. To test this hypothesis, we used real-time reverse transcription (RT)-PCR to monitor the expression of the ethylene-induced genes *PDF1.2* (At5g44420), *PR3* (At3g12500), *ERF1* (At3g23240), and *EBP* (At3g16770), encoding plant defensin, basic chitinase, and two ethylene response element binding transcription factors, respectively (Buttner and Singh, 1997; Solano et al., 1998). No significant induction of *PDF1.2* and *EBP* was observed in *acd11* (data not shown), whereas *PR3* and *ERF1* were clearly induced in *acd11* and *acd11/eds5*. Importantly, expression of both genes was equally strongly suppressed in *acd11/sid2* and *acd11/nahG* (Fig. 2B). Thus, this analysis does not support differences in ethylene accumulation and/or signaling as a cause of the phenotypic differences between *acd11/sid2* and *acd11/nahG*. This result is consistent with the fact that the *ein2* mutation conferring complete insensitivity to ethylene does not appreciably influence the *acd11* phenotype (Brodersen et al., 2002).

nahG Is Epistatic to *sid2-2*

Two models may explain why *nahG* expression results in stronger *acd11* suppression than *sid2-2*. First, catechol produced by NahG-catalyzed decarboxylative hydroxylation of SA may act to fully suppress
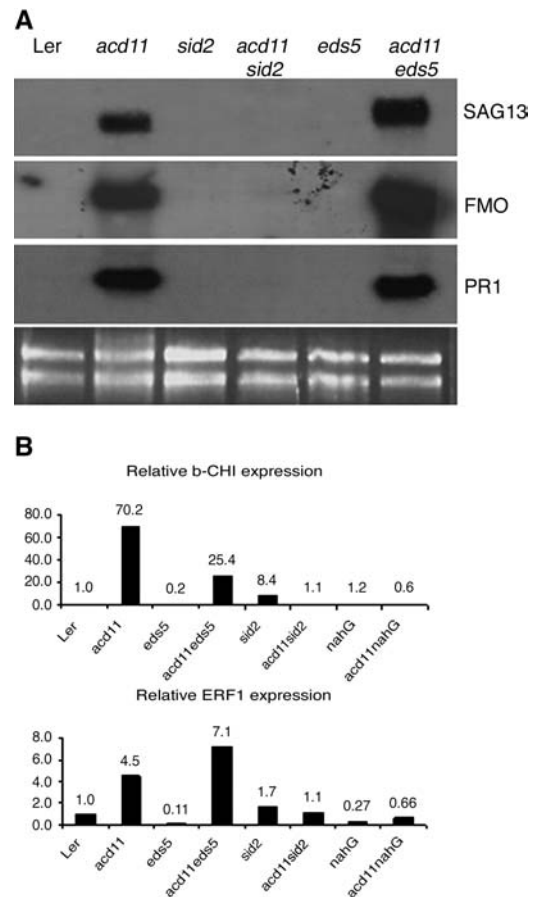

Figure 2. Expression of defense and cell death markers in single and double mutants. A, RNA-blot analysis of steady-state levels of FMO, SAG13, and PR1 mRNA in the wild type, in single mutants, and in the *acd11/eds5* and *acd11/sid2* double mutants. rRNA controls for equal loading are shown at the bottom. In some experiments, weak FMO, SAG13, and PR1 signals were detected in *acd11/sid2-2*. B, Real-time RT-PCR analysis of mRNA accumulation of ethylene response genes PR3 and ERF1 in single and double mutants. Expression of ubiquitin (UBQ10) was used to normalize cDNA input from the different genetic backgrounds.

acd11 phenotypes in concert with SA depletion. Alternatively, NahG acts to modify cellular metabolites other than SA (or other isochorismate-derived metabolites) that are required for development of the residual chlorosis and growth phenotypes observed in *acd11/sid2-2*.

Analysis of the epistatic relationship between *sid2* and *nahG* may distinguish between these possibilities (Heck et al., 2003; van Wees and Glazebrook, 2003). In the first model noted above, *sid2* would be epistatic to *nahG* since catechol cannot be produced in the SA-deficient *sid2* background. In the second model, *nahG* would be epistatic to *sid2* since NahG could still inactivate the additional set of putative, active metabolites in a *sid2* background. To examine this, *acd11/nahG* was crossed to *acd11/sid2-2*. In the segregating F2 generation, none of 10 yellowing plants typical of the *acd11/sid2-2* phenotype contained the *nahG* transgene,

whereas several completely green plants were found by PCR to be *sid2-2* homozygous and to contain the *nahG* transgene. F3 progeny from many of these plants segregated 3:1 for the fully green:yellowing phenotypes, demonstrating that the effects of *nahG* are epistatic to *sid2-2*.

SA Does Not Restore Cell Death in *acd11/sid2-2*

To more directly study the role of SA in cell death induction in *acd11*, we analyzed the effect of application of exogenous SA to *acd11/eds5-1* and *acd11/sid2-2* mutants. Treatment with BTH that completely restores cell death in *acd11/nahG* was used as a control. Both application of 1 and 2 mM SA and 100 μM BTH significantly accelerated cell death in *acd11/eds5-1*, suggesting that the delayed death phenotype of *acd11/eds5-1*, relative to *acd11*, was due to reduced SA levels (Fig. 3C). In contrast, trypan blue staining showed that neither 1 nor 2 mM SA was sufficient to restore leaf cell death in *acd11/sid2-2*, although a dose-dependent growth reduction was observed. However, cell death in *acd11/sid2-2* was fully restored by treatment with 100 μM BTH (Fig. 3A) or 325 μM INA (data not shown). When SA concentrations were elevated to 5 mM, some *acd11/sid2-2* individuals wilted quickly but did not exhibit the phenotype typical of *acd11* after BTH application. Notably, some *sid2-2* single mutant plants also wilted following this treatment with 5 mM SA, indicating that this level of application affects plants lacking the *acd11* mutation.

In addition to growth inhibition (Fig. 3B), SA treatments efficiently induced accumulation of *PR1* mRNA in L*er* and *acd11/sid2*, confirming that gene expression typical of SA application was induced by the treatments (Fig. 3D).

DISCUSSION

NahG Substrates Different from SA May Be Involved in *acd11* Phenotypes

The failure to restore PCD in *acd11/sid2* by exogenous SA could have a trivial cause, such as reduced SA uptake or responsiveness of *acd11/sid2*. Two lines of evidence suggest that this is not the case. First, the SA treatment led to a similar fold induction of *PR1* in L*er* and *acd11/sid2*, demonstrating that responsiveness to SA is intact in *acd11/sid2*. Second, the same treatment was sufficient to accelerate cell death in *acd11/eds5-1*. This indicates that there is no technical problem precluding cell death induction by SA in our treatments.

Assuming that the only function of SID2 is to convert chorismate to isochorismate, two models may explain the lack of cell death induction by SA in *acd11/sid2*. In the first model, ICS mutation leads to accumulation of PCD inhibitory compounds, while in the second model, it impairs accumulation of PCD activating compounds. We do not find accumulation of PCD inhibitory compounds a likely explanation because of the central position of chorismate, the ICS
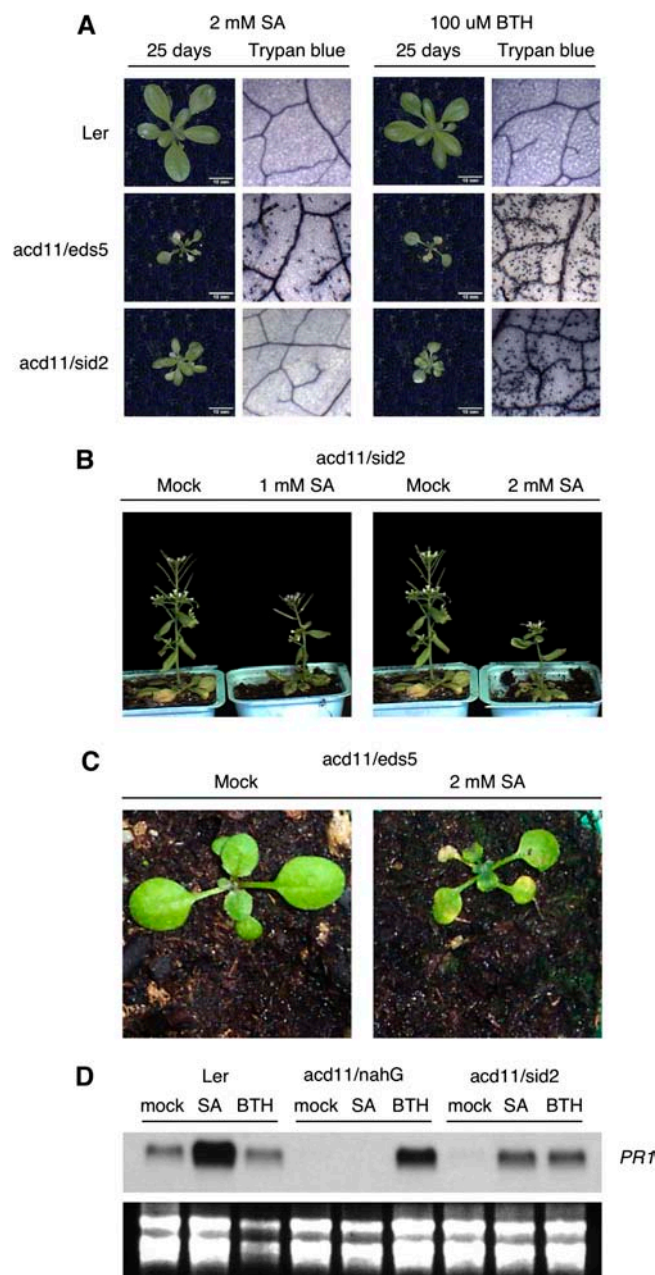
Figure 3. Responses of *acd11/eds5* and *acd11/sid2* to SA and BTH. The compounds were sprayed onto leaves at concentrations of 2 mM (SA) and 100 μM (BTH). Treatments were done at day 18 and pictures taken at day 25. Trypan blue stainings were also performed at day 25. Nontreated control plants are shown in Figure 1. A, Visible phenotypes and microscopic analysis of trypan blue-stained leaves after treatments. Mock-treated controls behaved as plants shown in Figure 1A. B, Dose-dependent growth inhibition of *acd11/sid2* by SA. Plants were allowed to continue growth for 14 d after a single treatment with SA. C, Acceleration of the Acd⁻ phenotype in *acd11/eds5* by exogenous SA. Plants were treated at day 12 and photographed 5 d later. D, Accumulation of PR1 mRNA in L*er*, *acd11/nahG*, and *acd11/sid2* 24 h after treatments with either 2 mM SA or 100 μM BTH. *sid2* and *nahG PR1* inductions were tested on a separate blot and were similar to the double mutants shown.

substrate, in primary metabolic pathways (Fig. 4A). As a precursor to Phe, Tyr, and Trp, chorismate is a branchpoint in aromatic amino acid biosynthesis, and given the high fluxes of chorismate to metabolites other than isochorismate, it is not certain that it would even accumulate in *acd11/sid2-2*. Rather, it is likely that SA is not the only isochorismate-derived compound required for PCD induction. We refer to this other group of PCD-promoting isochorismate-derived compounds as group 1 (Fig. 4, B and C).

Although we cannot exclude leakiness of the *eds5-1* splice acceptor site mutant allele, the accumulation of group 1 compounds appears to be independent of EDS5 since PCD was only delayed in *acd11/eds5-1* and could be accelerated by exogenous SA. Importantly, group 1 compounds or their precursors must be substrates of the NahG salicylate hydroxylase because PCD was completely blocked in *acd11/nahG*. In addition, the action of group 1 compounds must be mimicked by BTH and INA since PCD was fully restored by BTH and INA in both *acd11/sid2-2* and

acd11/nahG. Based on these observations, it is likely that group 1 compounds are structurally related to SA because a test of the substrate specificity of salicylate hydroxylase using 15 differently substituted benzoic and naphtoic acids suggested a requirement for a free carboxyl group and hydroxyl groups in the *ortho*-position (Yamamoto et al., 1965). Nonetheless, since BTH is not particularly closely related to SA structurally (Fig. 4D), we cannot exclude the possibility that NahG acts to deplete an SA-related, inactive precursor for a PCD-promoting compound unrelated to SA.

As shown in Figure 4A, isochorismate is a precursor for a range of plant metabolites, including phylloquinone (vitamin K1; Poulsen and Verpoorte, 1991), other naphthoquinones (Müller and Leistner, 1978), and anthraquinones (Inoue et al., 1984; Sieweke and Leistner, 1992; Stalman et al., 2003). While phylloquinone has no apoptosis-inducing activity in animal cells, such activity has been found for both the related menaquinone (vitamin K2) and several anthraquinones (Miyazawa et al., 2001; Lin et al., 2003; Yeh
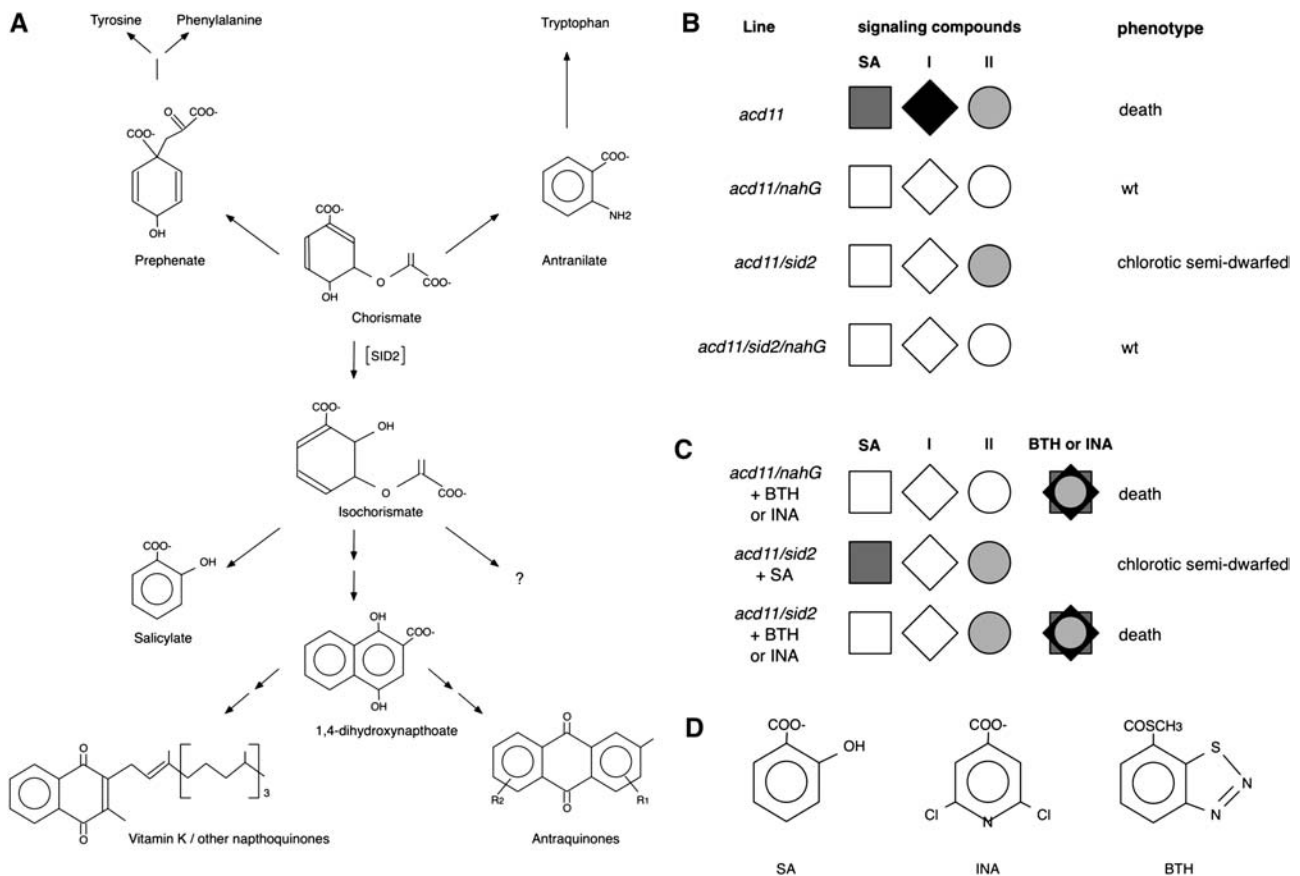

Figure 4. Overview of classes of metabolites required for *acd11* phenotypes. A, Elements of biochemical pathways around isochorismate. The ensemble of isochorismate-derived metabolites different from SA is candidate group 1 compounds. The naphthoquinone/anthraquinone intermediate 1,4-dihydroxynaphtoic acid is highlighted due to its structural similarity with NahG substrates. The question mark indicates that there may be other, as yet unknown, biochemical pathways that use isochorismate as a precursor for synthesis of group 1 compounds. B and C, A black box model illustrating contributions to *acd11* phenotypes of the two classes of compounds discussed in the text. It is assumed that the two classes of compounds are necessary but not sufficient for cell death and chlorosis and that BTH and INA are able to imitate the actions of both classes of compounds. D, Comparison of the chemical structures of SA, BTH, and INA.

et al., 2003). The synthetic, water-soluble menadione (vitamin K3) is also a potent inducer of PCD in tobacco protoplasts (Sun et al., 1999), and menadione bisulphite induces rapid and extensive leaf necrosis when sprayed onto Arabidopsis plants at 1 to 3 mM concentration (P. Brodersen and F.G. Malinovsky, unpublished data). This raises the possibility that isochorismate-derived naphthoquinones and/or anthraquinones may be used as endogenous PCD regulatory signals in plants such that inhibition of their accumulation by ICS mutation leads to defective PCD induction. In this regard, it is interesting to note that the anthraquinone/naphthoquinone biosynthesis intermediate 1,4-dihydroxynaphtoic acid (Fig. 4A) is a likely NahG substrate, as 1-hydroxynapthoic acid is a NahG substrate and 2,5-dihydroxybenzoic acid is hydroxylated more efficiently than SA by NahG (Yamamoto et al., 1965).

The observation that *nahG* acts as a stronger *acd11* suppressor than *sid2-2* may be explained by three scenarios: (1) by protective action of SA degradation products in *acd11/nahG*, (2) by accumulation of isochorismate precursors promoting chlorosis and growth inhibition in *acd11/sid2-2* due to ICS mutation, and (3) by NahG degradation of chlorosis-promoting and growth-inhibiting compounds that accumulate in *acd11/sid2-2* because they are synthesized independently of ICS. The first scenario of a significant contribution of SA degradation products to *acd11* suppression may be excluded because *nahG* was found to be epistatic to SA-deficient *sid2-2*. The second scenario of an accumulation of isochorismate precursors is unlikely because they would have to be NahG substrates whose minimal structural requirements are not met by isochorismate precursors. Thus, the third scenario appears likely. If so, then a second group of compounds (group 2), distinct from the isochorismate-derived group 1 described above, is involved in promoting growth inhibition and chlorosis (Fig. 4, B and C). Like group 1, group 2 may be, or may be derived from, compounds closely related to SA. The PAL pathway is a possible source of such compounds since both benzoic acid and SA can be synthesized via this pathway in several species (Métraux, 2002). However, repeated treatments starting at the seedling stage with the PAL inhibitor 2-aminoindane-2-phosphonic acid (AIP; Zön and Amrhein, 1992) did not reduce or delay leaf chlorosis in *acd11/sid2* rosettes, although these treatments consistently resulted in sterility, indicating that they were effective (data not shown). These preliminary results do not support involvement of the PAL pathway in the generation of group 2 compounds.

We note that while our results suggest that compounds possibly related to SA act to promote cell death, they do not exclude a role for SA itself in promoting this process. On the contrary, the acceleration of PCD by exogenous SA in *acd11/eds5-1* confirms that SA does potentiate PCD and suggests that it can be observed in *acd11/eds5-1* because other PCD-promoting compounds are already present in this

background. If so, comparative metabolite profiling in *acd11/eds5-1* versus *acd11/sid2-2* could identify the group 1 compounds of isochorismate-derived PCD-promoting signals in an unbiased manner.

Pathways of SA Biosynthesis in PCD Induction

The biosynthetic pathway of SA potentiating cell death is unclear at present. PAL-dependent SA biosynthesis has been shown to operate during tobacco mosaic virus-induced resistance in tobacco (Yalpani et al., 1993; Ribnicky et al., 1998). A study using AIP for PAL inhibition concluded that HR and resistance mediated by RPP4 in Arabidopsis also requires PAL-dependent SA synthesis (Mauch-Mani and Slusarenko, 1996). Nonetheless, the characterization of SID2 has established that the isochorismate pathway is the major source of SA in resistance responses, at least in Arabidopsis (Nawrath and Métraux, 1999; Wildermuth et al., 2001). To reconcile these findings, Wildermuth et al. (2001) proposed that while SID2-dependent SA synthesis is required in cells expressing defense responses, the PAL pathway may be important for SA synthesis in cells about to undergo PCD.

Our results provide an example that an SA pool potentiating PCD may be derived entirely from isochorismate synthesized by SID2. However, this does not exclude participation of the PAL pathway in SA synthesis for PCD in other contexts. Indeed, it is reasonable to question the general applicability of our results with a PCD/resistance system based solely upon the *acd11* mutation. Nonetheless, *acd11* cell death and resistance activation share genetic requirements with responses controlled by some *R* genes of the Toll/Interleukin-1 receptor-NBS-LRR class, such as dependence on NPR1 for *PR* gene expression, full dependence on EDS1 and PAD4, as well as complete independence of NDR1, PBS3, RAR1, and components of ethylene and jasmonate signaling (Brodersen et al., 2002; P. Brodersen and N.H.T. Petersen, unpublished data). This suggests that our results may extend to PCD controlled by some Toll/Interleukin-1 receptor-NBS-LRR class *R* genes.

BTH and INA as SA Agonists

BTH and INA have been widely used as SA agonists in the plant defense field because they induce typical SA target genes and provide resistance to the same set of pathogens as SA (Uknes et al., 1992; Lawton et al., 1996). Our study provides an example that BTH and INA may have physiological effects in addition to those that overlap with SA effects, suggesting that results of experiments using these SA analogs should be interpreted with caution with respect to roles of endogenous SA.

Comparison of *eds5* and *sid2*

The SA-deficient mutants *eds5-1* and *sid2-2* behaved differently in two important ways in this study. First,

acd11/sid2-2 exhibited complete suppression of SA accumulation, while approximately 10% of the total SA level in *acd11* remained in *acd11/eds5-1*. This compares well with analyses of total SA levels induced by ozone and *P. syringae* pv *tomato* DC3000(*avrRpt2*) infection in Col-0, *sid2*, and *eds5*, which showed that *sid2* total SA remained at basal levels, while *eds5* accumulated approximately 10% of the wild-type induced level (Nawrath and Métraux, 1999; Heck et al., 2003). Second, *sid2-2*, but not *eds5-1*, appeared to affect other as yet unidentified compounds required for PCD induction. Thus, as has been shown for *nahG*, care should be taken when evaluating the importance of SA solely based on analyses of the *sid2* mutant.

MATERIALS AND METHODS

Construction of Double and Triple Mutants

Crosses of *ACD11/acd11* (pollen donor) to *eds5-1* and *sid2-2* were confirmed by selection of kanamycin-resistant F1 progeny, transferred to soil, and allowed to self-pollinate. Forty F2 plants from each cross were again allowed to self-pollinate, and 25 F3 families segregating for kanamycin resistance were screened for homozygosity of *eds5-1* by DNA sequencing (Nawrath et al., 2002) or for *sid2-2* by PCR with primers detecting the approximately 50-bp deletion in exon IX of the *SID2* gene (Wildermuth et al., 2001). Three *ACD11/acd11;eds5-1/eds5-1* and four *ACD11/acd11;sid2-2/sid2-2* families were identified and used for initial phenotypic analysis, and a single family from each cross was used for subsequent SA measurements, gene expression, and phenotypic analyses. *acd11/sid2-2* was later kept as a double homozygote isolated from the segregating F3 family.

acd11/sid2-2/nahG was constructed by crossing *acd11/nahG* (pollen donor) to *acd11/sid2-2*. F1 progeny were phenotypically wild type and contained nahG detected by PCR. In F2, plants with typical *acd11/sid2-2* phenotypes were checked for *nahG* by PCR and were in all cases shown to lack the *nahG* transgene. Wild type-looking plants were screened for *sid2-2* homozygotes by PCR. Most of these families segregated roughly 3:1 for green versus yellowing plants, but our numbers were not high enough to deduce statistically significant ratios for segregating (*nahG* heterozygous) to nonsegregating (*nahG* homozygous) families.

Sequences of primers used to detect *acd11-1*, *eds5-1*, *sid2-2*, and *nahG* as well as all other novel materials developed in this study are available from the authors upon request.

RNA Analyses

Total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA). Northern blotting and synthesis of radiolabeled probes was done according to standard protocols. cDNA templates for PR1, SAG13, and FMO were amplified by PCR as described (Brodersen et al., 2002). For RT-PCR and quantitative (Q)-PCR analysis, RNA samples were first treated with RQ1 DNase (Promega, Madison, WI). RT reactions were done with 1 μg of RNA and 0.5 μg of (dT)$_{21}$ primer at 42°C with 0.1 unit of RT (Promega) and 2 units of RNasin (Promega) for 1 h in 20-μL reactions. Q-PCR was performed using the SYBR Green protocol (Applied Biosystems, Foster City, CA) with 10 pmol of each primer and 0.5-μL aliquot of RT reaction product in a 25-μL reaction. Q-PCR reactions were in triplicate and averaged for each line individually. Quantification of the threshold cycle (C_T) values obtained by Q-PCR analysis was done by the $2^{-\Delta\Delta C}T$ method (Livak and Schmittgen, 2001) after verifying that C_T(ubiquitin) − C_T(target) remained constant for each of the target genes tested over a 100-fold cDNA dilution series.

SA Measurements

Extraction and quantitation of total SA levels were done on 2- to 3-week-old leaf tissue by the procedure described by Newman et al. (2001).

Trypan Blue Staining

Trypan blue staining was carried out as described by Bowling et al. (1997).

Plant Treatments

Plants were grown in Percival Scientific growth chambers (Perry, IA) at 16 h light/8 h darkness with a day temperature of 21°C and a night temperature of 16°C. A 1 M stock of SA (Sigma-Aldrich, St. Louis) dissolved in ethanol was diluted into aqueous solution at 1 or 2 mM final concentration and sprayed onto the leaves. A 50:50 weight granular formulation (Novartis Crop Protection, Copenhagen) of BTH was dispersed in water (100 μM BTH) and sprayed onto the leaves.

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