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# Uncoupling Salicylic Acid-Dependent Cell Death and Defense-Related Responses From Disease Resistance in the Arabidopsis Mutant *acd5*

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Manuscript received February 10, 2000
Accepted for publication May 22, 2000

ABSTRACT

Salicylic acid (SA) is required for resistance to many diseases in higher plants. SA-dependent cell death and defense-related responses have been correlated with disease resistance. The ***a**ccelerated **c**ell **d**eath 5* mutant of Arabidopsis provides additional genetic evidence that SA regulates cell death and defense-related responses. However, in *acd5*, these events are uncoupled from disease resistance. *acd5* plants are more susceptible to *Pseudomonas syringae* early in development and show spontaneous SA accumulation, cell death, and defense-related markers later in development. In *acd5* plants, cell death and defense-related responses are SA dependent but they do not confer disease resistance. Double mutants with *acd5* and *nonexpressor of PR1*, in which SA signaling is partially blocked, show greatly attenuated cell death, indicating a role for *NPR1* in controlling cell death. The hormone ethylene potentiates the effects of SA and is important for disease symptom development in Arabidopsis. Double mutants of *acd5* and *ethylene insensitive 2*, in which ethylene signaling is blocked, show decreased cell death, supporting a role for ethylene in cell death control. We propose that *acd5* plants mimic *P. syringae*-infected wild-type plants and that both SA and ethylene are normally involved in regulating cell death during some susceptible pathogen infections.

ROBUST disease resistance in plants is often mediated by host recognition of pathogen-derived proteins, called Avirulence (Avr) proteins, and/or metabolites. In many cases, recognition leads to the coordinated activation of host responses such as hypersensitive cell death (HR), cell wall crosslinking, and defense-related gene induction (Greenberg 1997). Collectively, these responses are referred to as resistance responses. Recently it has become clear that resistance responses are not mediated by a single genetic program. Rather, different genes and signaling molecules are used by plants in response to different pathogens (Century *et al.* 1997; Aarts *et al.* 1998). For example, in Arabidopsis infected with the bacterial pathogen *Pseudomonas syringae*, the HR requires the defense signal molecule salicylic acid (SA) only in response to some strains carrying avirulence genes (Rate *et al.* 1999), indicating that the HR can occur by multiple mechanisms. Resistance responses that occur locally can induce long-term resistance at the whole plant level in a process that requires SA and is called systemic acquired resistance (SAR; Ryals *et al.* 1996). SAR, but not the HR, requires the ***N**onexpressor of **PR**1/**N**o **Im**munity 1* (*NPR1/NIM1*) gene (Cao *et al.* 1994; Delaney *et al.* 1995). *npr1/nim1* mutants of Arabidopsis have extra susceptibility to multiple pathogens (Cao *et al.* 1994; Delaney *et al.* 1995).

Attempts to tease apart the role of cell death in the resistance response have been difficult due to the lack of mutations or pharmacological agents that specifically block cell death. It has been suggested that cell death can be uncoupled from the resistance response because in ***d**efense **n**o **d**eath 1* (*dnd1*) Arabidopsis mutants, resistance to *P. syringae* occurs without the HR (Yu *et al.* 1998). However, since the *dnd1* mutant shows constitutively active defenses, it is difficult to infer the normal role of cell death in plants in which defenses are not already active. In barley, one functional allele of the *Mlg* resistance gene is sufficient to confer resistance to 80% of invading *Blumeria graminis* without extensive cell death (Gorg *et al.* 1993). However, two copies of the functional *Mlg* allele provided quantitative resistance to the fungus possibly due to the more extensive HR that occurred (Gorg *et al.* 1993).

Cell death is also associated with host-pathogen interactions that do not involve a resistance response. For example, *P. syringae* induces water-soaked cell death patches and loss of chlorophyll in a susceptible interaction on Arabidopsis. Concomitant with the onset of these symptoms is the activation of some defense-related responses that require SA for their full induction. These include the low-molecular-weight antimicrobial compound camalexin and transcripts of the ***P**athogenesis **Re**lated-1* (*PR-1*) gene (Glazebrook and Ausubel 1994; Greenberg *et al.* 1994). Camalexin induction by *P. syrin-*

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*gae* is compromised in *nahG* plants, in which SA accumulation is blocked by the salicylate hydroxylase activity encoded by *nahG* (Zhou *et al.* 1998). Its induction by *P. syringae* is also compromised in ***p****hyto****a****lexin* ***d****eficient 4* (*pad4*) mutant plants unless the plants are treated with SA (Zhou *et al.* 1998). An indication that cell death associated with susceptible interactions in Arabidopsis is genetically conditioned comes from the observation that ***e****thylene* ***in****sensitive 2 (ein2)* plants, in which ethylene signaling is blocked, show attenuated cell death but do not show disease resistance (Bent *et al.* 1992). *ein2* plants are also compromised for the induction of camalexin by *Alternaria brassicicola* (Thomma *et al.* 1999) and *P. syringae* (J. T. Greenberg, unpublished observations), indicating a possible link between the regulation of cell death and other susceptible responses.

To understand more about what regulates pathogen susceptibility and disease symptom development, we isolated and characterized a mutant called ***a****ccelerated* ***c****ell* ***d****eath 5* (*acd5*). We report here that *acd5* plants are modestly more susceptible to *P. syringae* early in development and show SA-dependent cell death and defense-related responses spontaneously late in development. However, although *acd5* accumulates SA, it does not induce SAR, suggesting that SA can control cell death without inducing disease resistance. We also show that the *NPR1* and *EIN2* genes play key roles in controlling cell death in *acd5*, and we propose that these genes may play a role in controlling disease symptoms in pathogen-infected wild-type plants.

## MATERIALS AND METHODS

**Plant growth, pathogenicity assays, chemical treatments and metabolite measurements:** *Arabidopsis thaliana* plants were grown in a 12-hr light/12-hr dark cycle for all experiments except ethylene measurements, which were made with plants grown in a 16-hr light/8-hr dark cycle. All experiments done in the 12-hr day showed similar results in the 16-hr day (data not shown). Plants were grown on Pro Mix BX supplemented twice weekly with Peter's 15-16-17. Infections with *P. syringae* were done using 1-ml blunt syringes to hand-inoculate leaves as described (Rate *et al.* 1999). Unless otherwise indicated, plants were grown in 50% relative humidity conditions. *P. syringae pv. maculicola* strain *PsmES4326* and *P. syringae pv. tomato* strain *PstDC3000* were obtained from F. M. Ausubel (Harvard University and Massachusetts General Hospital, Boston). A *hrcU* mutant of *PstDC3000* was obtained from B. Staskawicz (University of California, Berkeley, CA). *Xanthomonas campestris pv. campestris* strain *BP109* was obtained from Spencer Benson (The University of Maryland, College Park, MD). Leaf discs for pathogen growth assays were 6 mm in diameter. SA in the form of sodium salicylate was used at concentrations of 0.5 and 5 mm. Treatments with SA or benzo(1,2,3) thiadiazole-7-carbothioic acid (BTH) were performed as described (Rate *et al.* 1999). BTH was a gift from Novartis, Inc. (Research Triangle Park, NC). Camalexin determinations were performed according to Glazebrook and Ausubel (1994). SA levels were determined from 0.5 g of tissue as described (Seskar *et al.* 1998). Yields, determined from samples spiked with SA, were 52%. Values were adjusted for yield losses. Ethylene measurements were made on whole plants by placing plants in their pots in a sealed quart mason jar. Plants were kept at 22° in the light for 18 hr and the ethylene composition of the head space was determined by sampling using a syringe and injecting into an HP 5890 GC with a packed Hayesep T column coupled to a flame ionization detector. The ethylene content of samples was determined by comparison with known standards.

Seeds of *npr1-1, ein2-1,* Landsberg *erecta*, Nossen (Nos), Wassilewskija (Ws), Columbia (Col-0), Col-0 with a *BGL2-uidA* transgene, and $M_2$ seeds of Col-0 mutagenized with ethyl methanesulfonate were from F. M. Ausubel. Seeds of Cape Verdi Island (Cvi) were obtained from Daphne Preuss (The University of Chicago, Chicago). Seeds of *nahG* line "B15" in the Col-0 background was from Novartis, Inc. *acd5* was backcrossed to its parent (Col-0) four times and used for the studies described here. *acd5npr1* plants were constructed by using *npr1* as a female recipient for *acd5* pollen. *acd5* homozygous plants from the $F_2$ population were self-fertilized and screened in the $F_3$ generation for the *npr1* mutation by polymerase chain reaction (PCR) as described (Cao *et al.* 1997). *acd5-nahG* plants were constructed by pollinating *nahG* plants with *acd5* pollen. $F_2$ plants from the cross were sprayed with 100 μm BTH to identify potential *nahG-acd5* plants. $F_2$ plants showing cell death after 4 days were tested for the presence of *nahG* by PCR of a linked kanamycin resistance marker (*nptII*). $F_3$ progeny of individuals showing BTH-induced cell death were retested for spontaneous and BTH-induced cell death separately. Plants in the $F_3$ that showed no spontaneous cell death but showed BTH-induced cell death and scored positive for the *nahG* transgene were test-crossed with wild-type and *acd5* plants, respectively, and followed for two generations to confirm that the original plant was homozygous for *acd5*. To construct *acd5ein2* plants, *ein2-1* plants were pollinated with *acd5* pollen. $F_2$ progeny that were homozygous *acd5* were examined for large curled leaves typically seen in *ein2* homozygous plants. *acd5ein2* double mutants were confirmed for the presence of *ein2* by outcrossing to *ein2* plants and testing the progeny for lack of a triple response after growth in the dark for 3 days on MS medium (Mirashige and Skoog basal salts with B5 vitamins and 0.7% bactoagar plates, pH 5.8) with 50 μm 1-aminocyclopropane-1-carboxylic acid.

To map *acd5* initially, Landsberg *erecta* was crossed with pollen from *acd5*. $F_2$ progeny showing the *acd5*-conferred cell death phenotype were used for recombination analysis. To score recombinant plants, we used the technique of cleaved amplified polymorphic sequences using 34 published markers on all five chromosomes (Konieczny and Ausubel 1993). We detected linkage to *LFY* on chromosome 5 (see text). Additional mapping was performed on the same $F_2$ population that was treated with 100 μm BTH to induce the *acd5*-conferred cell death phenotype and with untreated plants from the $F_2$ progeny of crosses of *acd5* to Ws. These additional experiments indicated linkage of *acd5* to *DFR* (11%), *LTI78* (2.5%), and *LFY* (15.5%) on chromosome 5.

**RNA gel blot analysis:** RNA was isolated and gels were run as described (Rate *et al.* 1999). The probe used for detecting *PR-1* was described previously (Greenberg *et al.* 1994). A PhosphorImager (Molecular Dynamics, Sunnyvale, CA) was used to record the *PR-1* expression data.

**Visualization of dead cells and histochemical analysis:** Fresh tissue was boiled in lactophenol (10 ml of lactic acid, 10 ml of glycerol, 10 ml of liquid phenol, and 10 ml of distilled $H_2O$) containing 10 mg trypan blue and cleared as described previously (Rate *et al.* 1999). Stained tissue was visualized on a petri dish with a Wild M3Z binocular microscope (Leica, Inc., Rockleigh, NJ) with a magnification of ×40. Photographs were taken with Kodak 100 Elite Chrome slide film, scanned

with a Microtek ScanMaker 4 using Adobe PhotoShop 4.0LE software (Adobe Systems Inc.), assembled into a composite, and annotated using Canvas 5 (Deneba Software).

Transgenic plants harboring a *BGL2-uidA* fusion were stained with x-gluconase (Rose Scientific, Toronto, Canada) for 48 hr as described (Bowling *et al.* 1994), cleared in an ethanol series, and photographed.

**Statistical analysis:** All analyses presented were done with a statistical software package from StatView (SAS Institute, Inc., Cary, NC).

## RESULTS

**Identification of novel mutant with increased pathogen susceptibility and spontaneous cell death:** To identify new genes important for disease resistance and/or disease symptom development, we screened 5000 $M_2$ ethyl methanesulfonate-mutagenized Arabidopsis ecotype Columbia for mutants with altered disease symptoms after *P. syringae pv. maculicola* strain *PsmES4326* infection as described previously (Rate *et al.* 1999). One mutant (named *acd5*, see below), infected at week 3, showed enhanced disease symptoms after *PsmES4326* attack relative to that seen in wild type (Figure 1, A and B). Similar results were obtained with *P. syringae pv. tomato* strain *PstDC3000* (data not shown). Mock inoculation or infection with a nonpathogenic version of *PstDC3000* (due to a mutation in *hrcU*) did not elicit any symptoms (data not shown), indicating that the mutant was not generally more sensitive to inoculation stress or the presence of bacteria. The enhanced symptom development after pathogen attack was accompanied by a moderate increase in pathogen growth of strain *PsmES4326* or *PstDC3000* (Figure 2, A and B). This increase was manifest as a small but reproducible increase in the highest bacterial titers achieved in *acd5 vs.* wild-type plants. Infection of *acd5* and wild-type plants with *X. campestris pv. campestris* strain *BP109* caused a mild increase in symptom development in *acd5* (data not shown); however, the growth of *BP109* in *acd5* was not different from that seen in wild type in most experiments (Figure 2C). Inoculation with *PsmES4326* carrying either of two *avr* genes (*avrRpt2* and *avrRpm1*) elicited the normal hypersensitive response, indicating

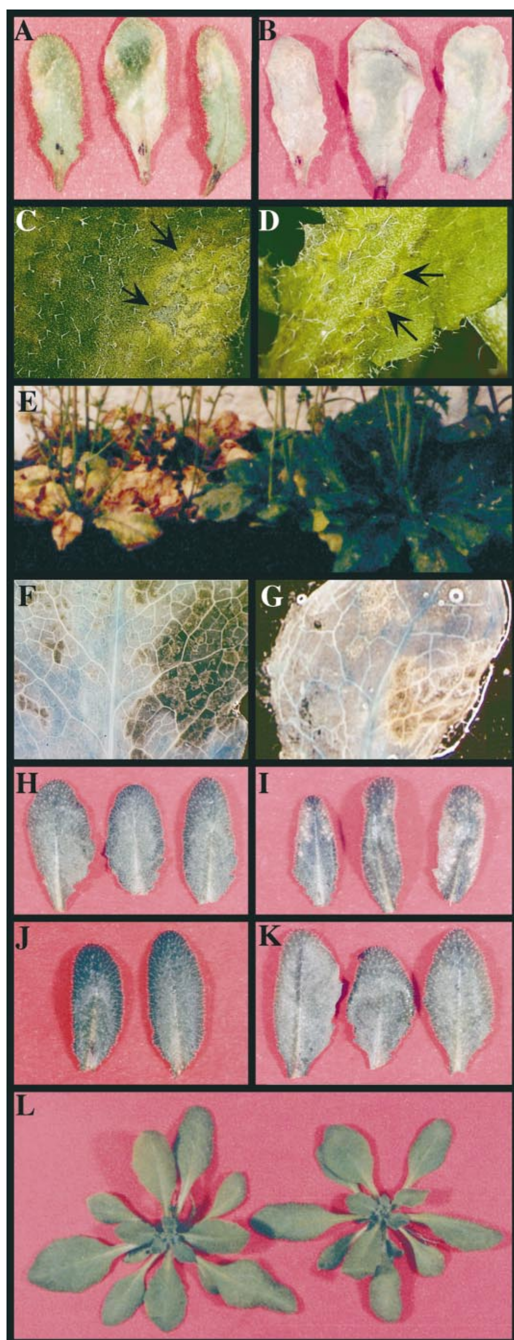


Figure 1.—Cell death and defense phenotypes of *acd5* and *acd5npr1* plants. (A and B) Young leaves from 24-day-old wild-type (A) and *acd5* (B) plants infected with *P. syringae* strain *PsmES4326* at a dose of $OD_{600} = 0.002$ photographed 3 days after the infection. Similar symptom enhancement was seen with infections using lower doses of *P. syringae.* (C and D) Five-week-old *ACD5 BGL2-uidA* (C) and *acd5 BGL2-uidA* (D) leaves grown in 95% relative humidity. Leaf in C was infected with *PsmES4326* at a dose of $OD_{600} = 0.0002$ and photographed 4 days later. Arrows indicate representative disease lesions. Leaf in D shows spontaneous cell death in *acd5* that resembles leaf spot disease shown in C. Arrows indicate representative spontaneous lesions. *acd5* plants without *BGL2-uidA* looked indistinguishable from plants with the transgene (data not shown). (E) *acd5* (left side) and *acd5npr1* (right side) plants photographed at 9 wk. (F and G) Five-week-old *ACD5 BGL2-uidA* (F) and *acd5 BGL2-uidA* (G) leaves grown in 95% relative humidity. Leaf in F was infected with *PsmES4326* at a dose of $OD_{600} = 0.0002$ and photographed 4 days later. Leaves were stained for β-glucuronidase activity. Mock-treated wild-type and *acd5* leaves without lesions showed no staining (data not shown). (H–K) Three-week-old *acd5* (H and I) or wild-type (J and K) leaves treated with water (H and J) or 100 μm BTH (I and K) and photographed 4 days later. Note lesions induced in leaves in I only. (L) Twenty-nine-day-old *npr1* (left) and *acd5npr1* (right) plants photographed 8 days after treatment with 100 μm BTH. Water-treated controls were indistinguishable from BTH-treated plants (data not shown). These experiments were repeated twice under short-day and twice under long-day conditions with similar results.

that this type of cell death response was not altered in *acd5* plants (data not shown).

At week 5 after planting, the *acd5* mutant showed spontaneous disease-like lesions, which resembled leaf spot symptoms caused by *P. syringae* (Figure 1, C and D), on the youngest leaves of the rosette (sink leaves). These spontaneous lesions typically showed modest spreading. Similar disease-like lesions could also be precociously induced by treatment of *acd5* plants with the SA agonist BTH (see below). Trypan blue staining showed that the spontaneous lesions were composed of dead cells (Figure 3, A and B). Over time, additional leaves showed spontaneous cell death as did stems (Figure 1E) and siliques (data not shown). *acd5* is named for its spontaneous *accelerated cell death* phenotype and falls into the category of "disease lesion mimic" mutants found in numerous plant species (Greenberg 1997). Prior to the onset of cell death, *acd5* plants had normal sized rosettes (data not shown), but mature plants were shorter than wild type (Figure 4) due to premature death of *acd5* floral inflorescences, not altered internode length (data not shown). *acd5* had reduced fitness as evidenced by the lower seed yield (Table 1), probably as a result of the cell death. In *acd5* plants, spontaneous cell death cosegregated with enhanced disease symptoms after pathogen attack. The spontaneous cell death phenotype was not due to *P. syringae* spreading, as *P. syringae* was not recovered from the spontaneous lesions. In addition, aseptically grown mutant plants developed spontaneous lesions independent of infection (data not shown). Based on these phenotypes, and the observation that *acd5* is recessive (see below), the *ACD5* gene appears to negatively regulate cell death and pathogen susceptibility.

**Genetic analysis of *acd5*:** *acd5*-conferred cell death segregated as a single recessive trait in a backcross to its wild-type Col parent (Table 2). Using cleaved amplified polymorphic sequences (see materials and methods) for mapping, *acd5* was tightly linked to *LFY* on chromosome 5 (2 out of 66 chromosomes or 3% ± 2.2% recombination). Although *acd5* segregated as a single Mendelian locus in backcrosses to Col, in the $F_2$ progeny from crosses to the Landsberg ecotype, plants with a mutant phenotype were underrepresented (segregating at a 16:1 ratio), suggesting reduced penetrance of the mutant phenotype, reduced viability of *acd5* seedlings, or the presence of a modifying gene (Table 2). Despite a survey of the entire Arabidopsis genome (see materials

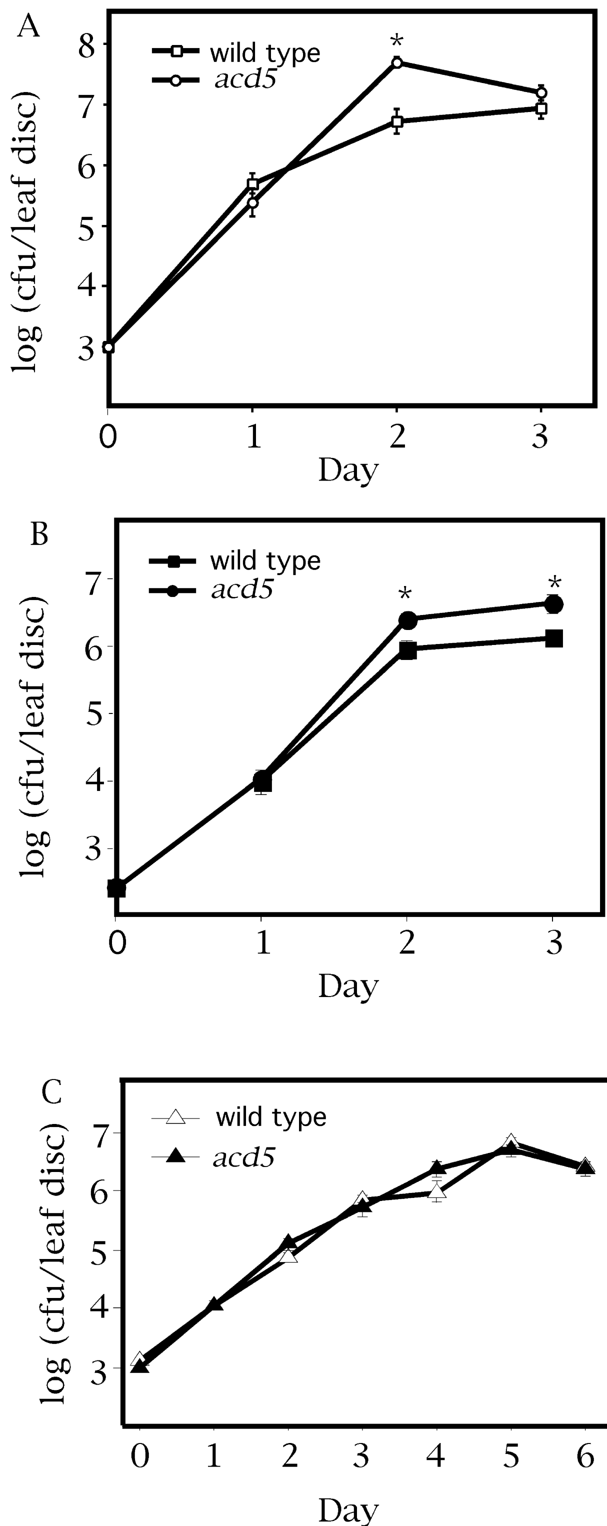


Figure 2.—Analysis of the growth of *P. syringae* strains *PsmES4326* and *PstDC3000* and *X. campestris* stain *BP109* in *acd5* plants. Lesion-free plants were inoculated with bacteria at 3 wk of age. The mean value of the growth of bacteria in six leaves is indicated in each case. Bars indicate standard error. (A) Plants were inoculated with *PsmES4326* ($OD_{600}$ = 0.002). *Growth of *PsmES4324* in *acd5* was significantly different from the growth in wild type ($P < 0.002$, unpaired *t*-test day 2). (B) Plants were inoculated with *PstDC3000* ($OD_{600}$ = 0.002). *Growth of *PstDC3000* in *acd5* was significantly different from the growth in wild type ($P = 0.03$, unpaired *t*-tests days 2 and 3). (C) Plants were inoculated with *X. campestris BP109* ($OD_{600}$ = 0.002). There was no significant difference in the growth of *BP109* in *acd5* and wild type. This experiment was repeated once under short-day and three times under long-day conditions with similar results.

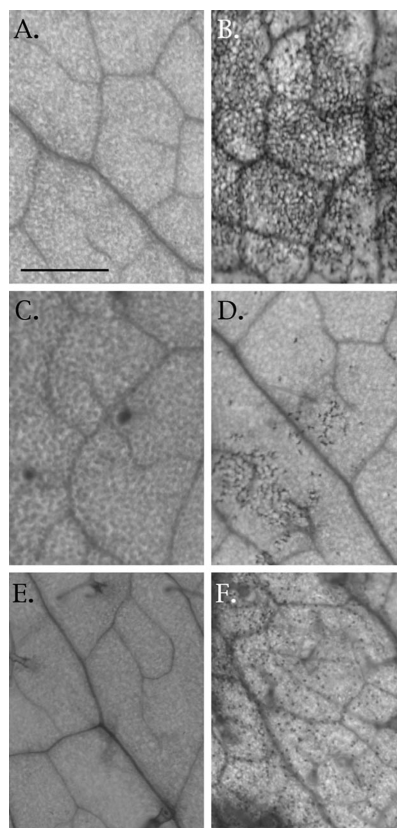


Figure 3.—Microscopic cell death in *acd5*, *acd5npr1*, and *acd5ein2* leaves. Representative leaves from 7-wk-old plants stained with trypan blue are shown. Dark spots are condensed, dead cells. Bar, 1 mm. (A) Wild-type; (B) *acd5*; (C) *npr1*; (D) *acd5npr1*; (E) *ein2*; and (F) *acd5ein2*. This experiment was repeated once under short-day and once under long-day conditions with similar results.

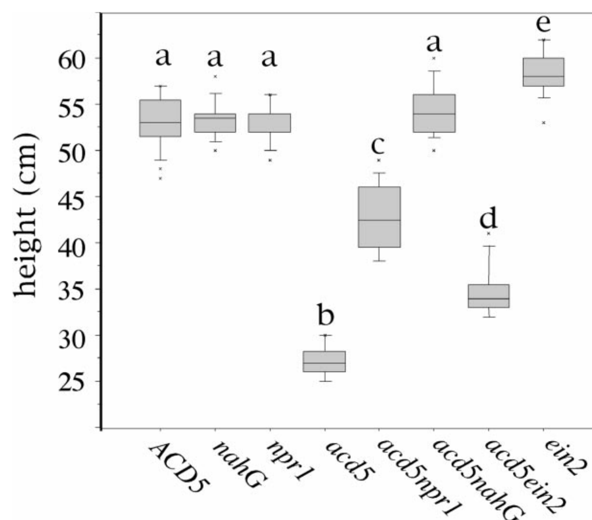


Figure 4.—Height analysis of *acd5* and other genotypes of plants affecting SA and ethylene signaling. Three-month-old plants of the indicated genotypes were used for height measurements. At least 12 plants were used for each determination. Box plots show the mean (center line of the box) and the second and third quartiles, which indicate the dispersion of 50% of the data points (shaded boxes) and the range (vertical lines above and below the boxes). Statistical outliers are indicated with x's. The distribution of heights for all possible combinations were compared using Fisher's least-squares test. Each letter represents a height class that is different from other height classes with a different letter designation ($P < 0.002$). This experiment was repeated once under short-day conditions and once under long-day conditions with similar results.

and methods), we were unable to identify a modifying locus; only the previously identified *LFY* region cosegregated with the *acd5* phenotype. In addition, to test whether *acd5* homozygotes might have reduced viability, we monitored the segregation of the *LFY* marker in the Ler $F_2$ population. This marker segregated 1:2:1 (45 $LFY_{Col}$: 91 $LFY_{Col/Ler}$: 44 $LFY_{Ler}$), suggesting that the underrepresentation of *acd5* homozygous phenotypic plants was due to the lack of penetrance of the cell death phenotype in the mapping cross. Interestingly, the lack of *acd5* penetrance was relieved when $F_2$ individuals from the same cross were treated with 100 μm BTH; *acd5* phenotypic plants (scored as plants with cell death patches) were found in a quarter of the plants, as expected for a single recessive trait (Table 2). In the BTH-treated $F_2$ population, the *acd5*-conferred cell death phenotype showed 15.5% recombination (39 out of 252 chromosomes) with *LFY*, 2.5% recombination (7 out of 252 chromosomes) with *LTI178a*, and 11% recombination (26 out of 242 chromosomes) with *DFR*. The larger sample size of the second mapping experiment is likely to be largely responsible for the different recombination frequency of *acd5* with *LFY* seen in the BTH-treated *vs.* the untreated plants.

Similar reductions in *acd5* penetrance were seen in crosses of *acd5* to the ecotypes Ws, Cvi, and Nos with recovery of *acd5* phenotypic plants in 21, 20, and 2.4% of the $F_2$ progeny, respectively. In agreement with the

**TABLE 1**

**Seed yield in *acd5* plants**

| Genotype | g seed per 50 siliques[a] ± SE | *P* value[b] |
|---|---|---|
| *ACD5* | 0.059 ± 0.001 | |
| *nahG* | 0.057 ± 0.001 | 0.3846[c] |
| *npr1* | 0.053 ± 0.001 | 0.0466[c] |
| *ein2* | 0.065 ± 0.003 | 0.0298[c] |
| *acd5* | 0.024 ± 0.002 | <0.0001[c] |
| *acd5nahG* | 0.057 ± 0.003 | <0.0001[d] |
| *acd5npr1* | 0.055 ± 0.001 | <0.0001[d] |
| *acd5ein2* | 0.039 ± 0.001 | <0.0001[d] |

SE, standard error of the mean. This experiment was repeated once under long-day conditions.

[a] Four samples were used for each genotype.

[b] Fisher's least-squares difference test was used.

[c] Indicated genotype was compared to *ACD5*.

[d] Indicated genotype was compared to *acd5*.

**TABLE 2**

**Genetic analysis of the *acd5* mutant**

| Cross | Type | Total | Mutant[a] | Wild type | $\chi^2$ |
|---|---|---|---|---|---|
| *ACD5/ACD5* × *acd5/acd5* | $F_1$ | 15 | 0 | 15 | |
| | $F_2$ | 132 | 32 | 100 | $0.040^b$ ($P > 0.90$) |
| *nahG/nahG ACD5/ACD5* × *acd5/acd5* | $F_1$ | 21 | 0 | 21 | |
| | $F_2$ | 94 | 5 | 89 | $0.18^c$ ($P > 0.50$) |
| | $F_2$+ BTH[d] | 99 | 26 | 73 | $0.05^b$ ($P > 0.80$) |
| *ACD5/ACD5*(Ler) × *acd5/acd5* | $F_1$ | 12 | 0 | 12 | |
| | $F_2$ | 1212 | 83 | 1129 | $212.9^b$ ($P < 0.01$)[e] |
| | $F_2$+ BTH[d] | 522 | 128 | 394 | $0.092^b$ ($P > 0.70$) |

[a] Mutant phenotype scored is spontaneous cell death on leaves or, in the case of BTH treatment, induced cell death.

[b] The $\chi^2$ is given for the ratio of 3:1 (wild type/mutant).

[c] The $\chi^2$ is given for the ratio of 15:1 (wild type/mutant).

[d] BTH was used at 100 μM and plants were scored 4–7 days later.

[e] $P < 0.05$ indicates that the segregation does not conform to the predicted 3:1 pattern.

linkage data from the BTH-treated Ler × *acd5* $F_2$ progeny, we detected 18% linkage of *acd5* to *LFY* (19 out of 104 chromosomes) in the $F_2$ progeny of the cross of *acd5* to Ws. Taken together, these data suggest that *acd5* maps 2.5 cM north of the *LTI78* marker on chromosome 5. The variable penetrance of the *acd5* phenotype in the different crosses may indicate that there are many modifiers that collectively behave as quantitative traits, as no distinct loci have yet been detected that are responsible for modifying the *acd5*-conferred cell death phenotype. No other mutants affecting spontaneous cell death that we know of have been reported to map to this region. Thus, *acd5* represents a new cell death mutant.

**TABLE 3**

**Camalexin levels in *acd5* plants**

| Genotype[a] | Treatment[b] | μg/cm²[c] ± SE |
|---|---|---|
| *ACD5* | None | <0.001 |
| *ACD5* | *PsmES4326* | 0.46 ± 0.04 |
| *ACD5* | 10 mM $MgSO_4$ | <0.001 |
| *ACD5* | 100 μM BTH | <0.001 |
| *ACD5* | Water | <0.001 |
| *acd5*[d] | None | <0.001 |
| *acd5*[e] | 100 μM BTH | 0.26 ± 0.05 |
| *acd5*[d] | Water | <0.001 |
| *acd5-nahG*[d] | Water | <0.001 |
| *acd5-nahG*[e] | 100 μM BTH | 0.34 ± 0.05 |
| *nahG* | Water | <0.001 |
| *nahG* | 100 μM BTH | <0.001 |
| *ACD5*[f] | None | <0.001 |
| *acd5*[e,f] | None | 0.56 ± 0.09 |
| *ein2*[f] | None | <0.001 |
| *acd5ein2*[e,f] | None | 0.16 ± 0.03 |

SE is standard error of the mean. This experiment was repeated once under short-day and once under long-day conditions.

[a] Unless indicated, 3-wk-old plants were used to determine camalexin levels.

[b] For BTH and water treatment, measurements were made 4 days after treatment. For *P. syringae* and 10 mM $MgSO_4$ (mock inoculation) treatments, measurements were made 2 days after treatment. *P. syringae* was used at a dose of $OD_{600} = 0.01$.

[c] Four to six samples were used for each measurement.

[d] Plants were asymptomatic.

[e] Plants had lesions.

[f] Plants were 7 wk old.

**Activation of multiple defense-related responses without disease resistance in *acd5*:** Cell death activation often requires the accumulation of and/or sensitivity to the defense signal SA. To test whether other defense-related markers that require SA were activated in *acd5*, we analyzed camalexin levels and steady-state *PR-1* transcript levels. Additionally, we analyzed β-glucuronidase activity in *acd5* plants harboring a SA-inducible *BGL2-uidA* gene fusion (Bowling *et al.* 1994). Camalexin levels in *acd5* were elevated to levels similar to those seen in *PsmES4326*-infected wild-type plants (Table 3). The steady-state gene transcript level of *PR-1* was strongly

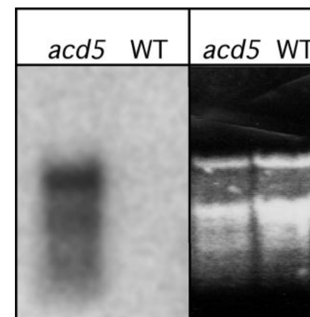


Figure 5.—Steady-state levels of *PR-1* gene transcripts in *acd5* and wild type. Leaves from 5-wk-old plants were used for RNA gel blot analysis of *PR-1* transcript levels. (Left) A PhosphorImager image; (right) ethidium staining of the rRNA from the same blot. Sample in the *acd5* lane was from leaves with spontaneous lesions. This experiment was repeated once under short-day and once under long-day conditions with similar results.

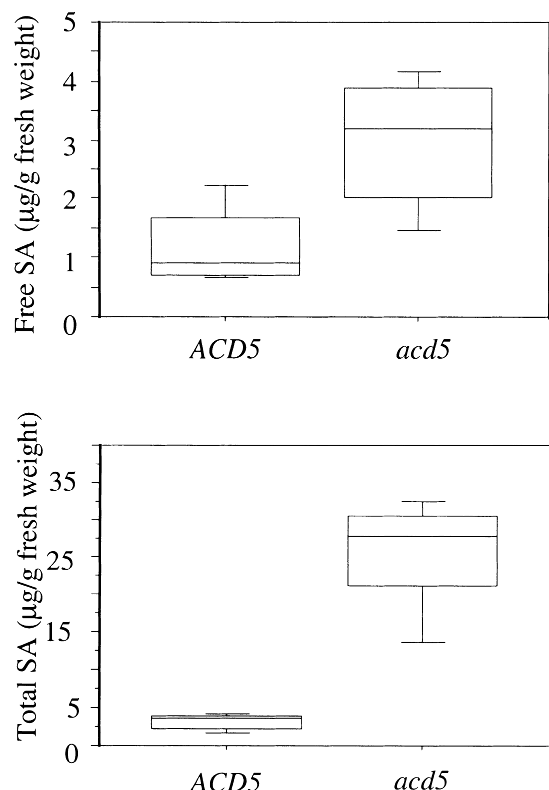


FIGURE 6.—Free and total salicylic acid levels in *acd5* plants. Leaves from 5-wk-old plants were used for SA extractions. Five replicates were used for each sample. Box plot parameters are the same as described in the legend to Figure 4. SA levels in *ACD5* and *acd5* were statistically different ($P = 0.016$ for free SA, $P = 0.002$ for total SA). This experiment was repeated once under long-day conditions with similar results.

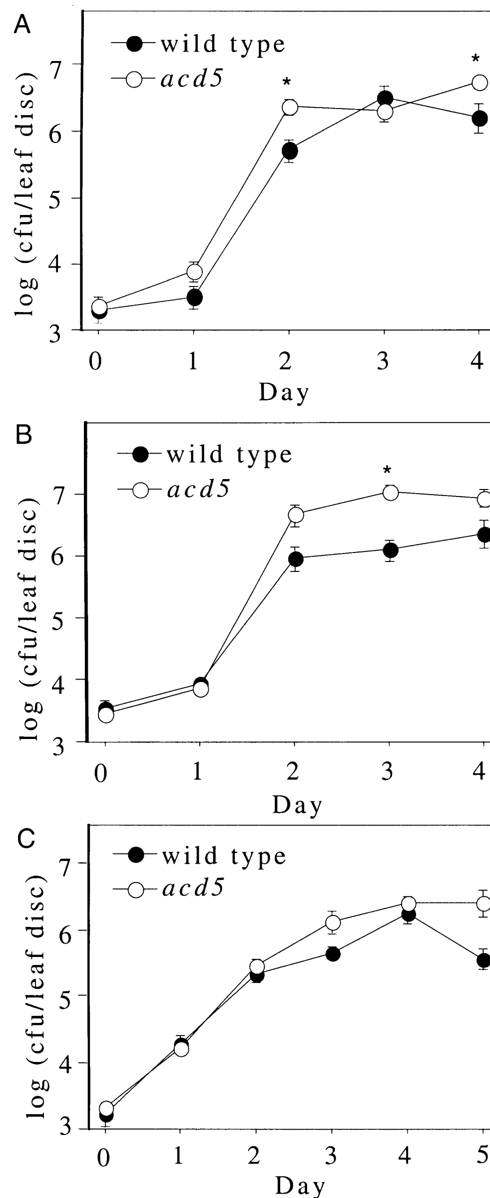


FIGURE 7.—Analysis of the growth of *P. syringae* and *X. campestris* in *acd5* plants with lesions. Plants were inoculated with bacteria as described in the legend to Figure 2. Bars indicate standard errors. (A) Plants were inoculated with *PsmES4326*. *Significant differences between bacterial growth in *acd5* and wild type ($P = 0.03$). (B) Plants were inoculated with *PstDC3000*. *Significant differences between bacterial growth in *acd5* and wild type ($P = 0.003$). (C) Plants were inoculated with *X. campestris* strain *BP109*. There was no significant difference in the bacterial growth in *acd5* and wild type. This experiment was repeated once under short-day and twice under long-day conditions with similar results.

elevated in *acd5* plants showing lesions relative to the wild-type control (Figure 5). Leaves taken from plants prior to lesion formation showed no *PR-1* mRNA accumulation (data not shown). The defense-related gene fusion *BGL2-uidA* was also induced in *acd5* around the spontaneous lesions in a similar fashion to what was found in *P. syringae*-infected wild-type plants (Figure 1, F and G). Consistent with these defense-related phenotypes, *acd5* accumulated high levels of free and total SA relative to wild-type plants (Figure 6).

To determine if SA, cell death, and/or defense-related markers were associated with SAR in *acd5*, we monitored the growth of two strains of *P. syringae* and one strain of *X. campestris pv. campestris* on *acd5* plants with preformed spontaneous lesions. The growth of both strains of *P. syringae* grew slightly better in *acd5* than wild type (Figure 7, A and B). Additionally the growth of *X. campestris* was equivalent in *acd5* and wild-type plants (Figure 7C). Thus, *acd5* was not more resistant than wild type to *P. syringae* or *X. campestris*, indicating that SA, cell death, and defense-related responses of *acd5* were uncoupled from disease resistance.

**A role for SA signaling and the *NPR1* gene for the *acd5*-conferred phenotypes:** The cell death, defense-related phenotypes and elevated SA levels of *acd5* raised the possibility that SA, an important defense signal, might be required for one or more of the *acd5*-conferred phenotypes. To determine this, we crossed *acd5* with a well-characterized transgenic plant harboring the *nahG* gene, whose product metabolizes SA to the inactive catechol. The *acd5*-conferred cell death phenotype segre-

gated 15 to 1 in the $F_2$ progeny (Table 2). The 1 out of 16 plants showing the *acd5*-conferred cell death phenotype lacked the *nahG* transgene, while the other homozygous *acd5* plants, lacking a cell death phenotype, had 1 or 2 copies of the *nahG* transgene. This result suggests that *nahG* dominantly suppressed the cell death of *acd5* plants. Treating the $F_2$ progeny with 100 μm BTH, an SA agonist, reversed the suppression and yielded the expected 3 to 1 segregation of plants with the *acd5*-conferred cell death phenotype (Table 2). Plants homozygous for *acd5* and *nahG* were indistinguishable in size from wild type or *nahG* (Figure 4) and had wild-type seed yield (Table 1), indicating that removal of SA also suppressed the growth defect and reduced fitness of *acd5*. Application of 100 μm BTH to *acd5-nahG* plants induced camalexin synthesis to a similar level as that seen in *acd5* plants (Table 3). Interestingly, spraying *acd5* with BTH (or SA, data not shown) 1–2 wk prior to the onset of the spontaneous visible phenotype induced cell death (Figure 1, H–K) and camalexin synthesis (Table 3) in the young leaves in the same pattern that occurred spontaneously in older plants.

The *NPR1* gene is required for some aspects of SA signaling and *npr1* mutants show highly increased susceptibility to *P. syringae* (Cao *et al.* 1994; Delaney *et al.* 1995; Shah *et al.* 1997). Susceptibility of *acd5npr1* mutants to *P. syringae* was indistinguishable from that seen in *npr1* alone (data not shown), indicating that the increased susceptibility of *acd5* was not additive with *npr1*. However, in *acd5npr1* double mutants, the spontaneous cell death was highly attenuated. In some *acd5npr1* plants, no spontaneous cell death was seen associated with leaves, while stems showed a modest amount of cell death. When cell death did occur on *acd5npr1* leaves, it was typically very mild (Figure 1E, and Figure 3, compare B and D) and started 2–3 wk after the initiation of cell death of the *acd5* single mutant. The attenuated phenotype resulted in plants that were much taller than the *acd5* plants, indicating that *npr1* partially suppressed the reduced stature of *acd5* (Figure 4). *npr1* also suppressed the reduced fitness of *acd5* and restored the seed yield to that seen in *npr1* (Table 1). *NPR1* was strictly required for the BTH- or SA-induced cell death of young *acd5* plants, as no visible (Figure 1L) or microscopic (not shown) cell death was seen after treatment of the *acd5npr1* double mutant. Thus *NPR1* was required for mediating BTH- or SA-induced early cell death and was largely required for spontaneous cell death in *acd5* plants, while SA was necessary and sufficient to induce cell death.

**A partial requirement for ethylene signaling for *acd5*-conferred phenotypes:** Ethylene signaling has been shown to be important for some bacterial pathogen-induced symptoms, but not resistance, in susceptible interactions in Arabidopsis (Bent *et al.* 1992). As *acd5* showed lesions that appeared to mimic *P. syringae*-induced symptoms, we tested the involvement of ethylene signaling in the *acd5* phenotypes. First we measured the amount of ethylene evolved from whole *acd5* plants with lesions and found that they evolved significantly more ethylene than the wild-type plants (Table 4). *acd5* plants without lesions showed a more modest increase (twofold) in ethylene levels (data not shown). To further test the involvement of ethylene in the *acd5*-conferred phenotypes we crossed *acd5* with the ethylene-insensitive *ein2* mutant in which ethylene signaling is blocked (Guzman and Ecker 1990). Spontaneous cell death of the *acd5ein2* plants lagged 1 wk or more behind those seen in *acd5* plants alone. In addition, the severity of the BTH- or SA-induced cell death of young plants (data not shown) and the spontaneous cell death of older plants was reduced relative to the *acd5* single mutant (Figure 3, compare B and F). The attenuated phenotype resulted in slightly taller *acd5ein2* double mutant plants relative to the *acd5* single mutant (Figure 4). In addition, *ein2* partially suppressed the fitness defect of *acd5*, as *acd5ein2* seed yield was intermediate between *acd5* and *ein2* (Table 1). Interestingly, *ein2* plants were taller and produced more seed than wild type, suggesting that *ein2* conferred improved fitness under these growth conditions (Table 1). However, the magnitude of these effects (comparing *ein2* with wild type) was much smaller than that seen when comparing *acd5ein2* with *acd5* (Table 1 and Figure 4). The *ein2* mutation also caused a reduction in camalexin production in *acd5* (Table 3). Despite the attenuated spontaneous symptoms of *acd5ein2* plants, *P. syringae* growth in *acd5ein2* was not decreased relative to the *acd5* single mutant (data not shown). This is consistent with the previous report that *ein2* shows attenuated symptoms with *P. syringae* but does not increase disease resistance (Bent *et al.* 1992).

**TABLE 4**

**Ethylene evolution from *acd5* plants**

| Genotype | Number sampled | nl/g/hr ± SE |
|---|---|---|
| *ACD5* | 8 | 0.84[a] ± 0.09 |
| *acd5*[b] | 8 | 4.69[a] ± 0.62 |

SE is standard error of the mean. This experiment was repeated twice with similar results. Four-week-old plants were used for ethylene determinations.

[a] $P < 0.0001$, unpaired *t*-test.

[b] Plants had spontaneous lesions.

## DISCUSSION

We identified a new mutant, *acd5*, with altered susceptibility to the bacterial pathogen *P. syringae*. *acd5* shows both modestly increased growth of *P. syringae* and increased symptom development similar to other previously described ***e****nhanced* ***d****isease* ***s****usceptibility* (*eds*) mutants of Arabidopsis. However, unlike the *eds* mutants,

*acd5* also has a spontaneous cell death phenotype that is correlated with decreased fitness of the plants. This cell death phenotype of *acd5* is strictly dependent on the defense signal molecule SA, as inferred from the phenotype of the *acd5-nahG* plants and the reversibility of the suppressed phenotype by application of the synthetic SA analogue BTH. *acd5* also accumulated high levels of SA relative to wild-type plants. Several other mutants of Arabidopsis such as *lsd6*, *lsd7*, *ssi1*, *acd6*, and *cpr20cpr21* also show SA-dependent cell death (Weymann *et al.* 1995; Rate *et al.* 1999; Shah *et al.* 1999; Silva *et al.* 1999). However, unlike these other disease-resistant cell death mutants, *acd5* does not show increased resistance to *P. syringae*, *X. campestris*, or *P. parasitica* (J. T. Greenberg, unpublished data) under any conditions tested despite the activation of several defense-related markers. Thus, *acd5* is unique in that it uncouples SA-dependent cell death and defense-related markers from disease resistance. The observation that *acd5* mutation is recessive suggests that the *ACD5* gene acts to repress some SA-dependent responses.

In addition to requiring SA for developmentally induced cell death and camalexin synthesis, *acd5* shows precocious stimulation of these events when the SA pathway is activated. This phenotype of *acd5* is similar to what was reported for the Arabidopsis cell death mutant *lsd1*. Under short-day conditions, the repressed cell death phenotype of *lsd1* is induced by SA (Dietrich *et al.* 1994). However, *lsd1*, unlike *acd5*, shows disease resistance and uncontrolled spreading of cell death. One possibility is that SA is required for the induction of distinct cell death pathways, one that is associated with disease resistance and one that is associated with disease susceptibility. Alternatively, *acd5* and *lsd1* might induce the same SA-dependent cell death, but *acd5* might only induce a subset of defense-related responses (not enough to confer disease resistance). Since it is not known which SA-dependent defense(s) is required for resistance to *P. syringae*, it is difficult to determine whether the appropriate antibacterial defense(s) is activated in *acd5*. It is also possible that *acd5* activates a disease susceptibility factor that overrides the defense response.

*NPR1* is required for some aspects of SA signal transduction and for disease resistance (Cao *et al.* 1994; Delaney *et al.* 1995; Shah *et al.* 1997). We showed previously that *NPR1* influences the induction of cell death in the *acd6* mutant of Arabidopsis (Rate *et al.* 1999). *acd6npr1* plants show modestly delayed and reduced cell death that is coupled to a cell growth response (Rate *et al.* 1999). The characterization of *acd5* plants further suggests that *NPR1* plays a key role controlling cell death. *npr1* suppressed much of the spontaneous cell death of *acd5* and completely blocked cell death induced by early activation of the SA signaling pathway. This effect of the *npr1* mutation raises the possibility that *NPR1* functions to control cell death in wild-type plants during pathogen infection. If this is the case, it is unclear why *npr1* mutants show earlier and more severe symptom development after attack by pathogens such as *P. syringae* than wild-type plants. It is possible that when NPR1 is removed, cells under pathogen attack die by a mechanism distinct from that which occurs in cells that contain functional NPR1. An examination of the mechanism of cell death in *NPR1* plants and *npr1* mutants will be necessary to resolve this question. Interestingly, plants lacking SA due to the presence of the *nahG* gene die by a different mechanism after ozone treatment than wild-type plants (Rao and Davis 1999). It has been suggested that ozone induces cell death by activating an HR (Sharma and Davis 1997; Sandermann *et al.* 1998). We found recently that Arabidopsis *nahG* plants are compromised for inducing the HR after infection with a subset of normally HR-inducing *P. syringae* strains (Rate *et al.* 1999). Thus, it is clear that there are both SA-dependent and -independent modes of the HR in plants. Cell death during plant-pathogen interactions that do not involve the HR could similarly show SA-dependent and -independent mechanisms.

Unlike SA, which plays an essential role in *acd5*-conferred cell death, the hormone ethylene plays a more minor role in the expression of this phenotype. *acd5* plants produce more ethylene than wild-type plants. The *ein2* mutation, which blocks ethylene signal transduction, partially suppresses the *acd5*-conferred phenotypes. Thus, the *acd5ein2* double mutants were delayed for the developmental induction of cell death, showed reduced camalexin synthesis, showed less intense cell death after BTH or SA treatment, and had higher fitness than *acd5* single mutants. *ein2* plants were shown previously to have reduced pathogenic symptoms without decreasing the growth of *P. syringae* (Bent *et al.* 1992). Recently it was also shown that *ein2* plants were compromised for camalexin induction by the fungal pathogen *A. brassicicola* (Thomma *et al.* 1999). We have found a similar result with *P. syringae* (J. T. Greenberg, unpublished observations). Since *ein2* partially suppresses both pathogen-induced and *acd5*-conferred cell death and camalexin synthesis, it is possible that these two incidences of cell death and defense induction share a common mechanism of activation and/or execution. If this were true, it would imply that *acd5* plants truly mimic a pathogenic infection and would furthermore suggest that pathogenic symptoms caused by *P. syringae* are caused largely by host-encoded functions when the ethylene and SA signaling pathways are intact.

We thank D. Guttman, J. Mach, D. Rate, H. Vanacker, J. H. Lu, D. Preuss, and J. Malamy for helpful discussions. We thank one of the anonymous reviewers for helpful comments on the mapping experiments. We thank Michael Spaly for help with the mapping experiment and James Cuenca for excellent technical assistance. J.T.G. is a Pew Scholar and an American Cancer Society Research Fellow (no. JFRA-623). This research was supported by a grant to J.T.G. from the National Institutes of Health (no. 1R29GM54292-01) and by an award to the University of Chicago's Division of Biological Sciences under

the Research Resources Program for Medical Schools of the Howard Hughes Medical Institute.

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Communicating editor: V. L. Chandler