The Plant Cell, Vol. 11, 1695–1708, September 1999, www.plantcell.org © 1999 American Society of Plant Physiologists

The Gain-of-Function Arabidopsis *acd6* Mutant Reveals Novel Regulation and Function of the Salicylic Acid Signaling Pathway in Controlling Cell Death, Defenses, and Cell Growth

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We isolated a dominant gain-of-function Arabidopsis mutant, *accelerated cell death 6* (*acd6*), with elevated defenses, patches of dead and enlarged cells, reduced stature, and increased resistance to *Pseudomonas syringae*. The *acd6*-conferred phenotypes are suppressed by removing a key signaling molecule, salicylic acid (SA), by using the *nahG* transgene, which encodes SA hydroxylase. This suppression includes phenotypes that are not induced by application of SA to wild-type plants, indicating that SA acts with a second signal to cause many *acd6*-conferred phenotypes. *acd6–nahG* plants show hyperactivation of all *acd6*-conferred phenotypes after treatment with a synthetic inducer of the SA pathway, benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH), suggesting that SA acts with and also modulates the levels and/or activity of the second defense signal. *acd6* acts partially through a *NONEXPRESSOR OF PR 1* (*NPR1*) gene–independent pathway that activates defenses and confers resistance to *P. syringae*. Surprisingly, BTH-treated *acd6–nahG* plants develop many tumor-like abnormal growths, indicating a possible role for SA in modulating cell growth.

INTRODUCTION

Plants have evolved a number of mechanisms to defend themselves against environmental stresses, such as pathogen invasion. One defense mechanism, a resistance response, involves the specific recognition of secreted pathogen-derived factors and the rapid induction of localized host cell death (the hypersensitive response [HR]), cross-linking of components of the cell wall, and the coordinate activation of many defense-related genes (Greenberg, 1997). Recognition occurs through a gene-for-gene interaction between a plant resistance (*R*) gene product and a pathogen avirulence (*avr*) gene product that elicits the plant defense response (Baker et al., 1997). A secondary consequence of the resistance response is termed systemic acquired resistance (SAR). SAR involves the production of salicylic acid (SA) both locally and systemically and may involve translocation of SA and/or additional signals (Vernooij et al., 1994; Shulaev et al., 1995) that lead to the induction of a battery of *PATHOGENESIS-RELATED* (*PR*) genes. The end result of SAR induction is resistance against future assaults from a broad range of pathogens (e.g., see Uknes et al., 1992; Ryals et al., 1996).

A combination of approaches has implicated SA in a number of different aspects of defense regulation in Arabidopsis. Treatment of Arabidopsis with exogenous SA induces some defenses such as *PR* gene expression and resistance to diverse pathogens (Uknes et al., 1992). Conversely, removing SA by using the bacterial *nahG* transgene, the product of which metabolizes SA to the inactive catechol, has been used to demonstrate a role for SA in the activation of SAR and in the full expression of the resistance response to some pathogens (Delaney et al., 1994). Plants unable to transduce the SA signal due to a mutation in the *NONEXPRESSOR OF PR 1* (*NPR1*)/*NO IMMUNITY 1* (*NIM1*) gene are hypersusceptible to pathogens and exhibit no induction of SAR (Cao et al., 1994; Delaney et al., 1995).

Whereas SA is sufficient on its own to activate some defenses, to modulate others, SA must act with additional signals. In this role, SA can act as an amplifier of defenses that are only induced to low levels in the absence of SA. For example, camalexin, the major low molecular weight antimicrobial metabolite (phytoalexin) in Arabidopsis, is not induced by SA, yet removal of SA impairs the induction of camalexin by amino acid starvation and by *Pseudomonas syringae* (Zhao and Last, 1996; Zhao et al., 1998; Zhou et al., 1998). This role for SA as an amplifier of defense induction was reported in Arabidopsis *phytoaxelin deficient 4* (*pad4*) mutant plants (Zhou et al., 1998) and has been documented for other plants as well (Rao et al., 1997; Shirasu et

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al., 1997; Thulke and Conrath, 1998). SA can also function as a coactivator, where it is strictly required with a second signal to obtain any activation of defense. For example, SA on its own does not induce the HR, yet it is needed for the induction of cell death in the Arabidopsis *lesion simulating disease* mutants *lsd6* and *lsd7* (Weymann et al., 1995; Lawton et al., 1996) and the *suppressor of SA insensitivity1* (*ssi1*; Shah et al., 1999) mutant, and it can be required for the induction of the HR in response to bacterial pathogens (Tenhaken and Rubel, 1997). Thus, SA can act as a sole activator of pathogenesis-related genes, an amplifier of camalexin synthesis, and a coactivator of cell death.

To better understand how Arabidopsis resists the bacterial pathogen *P. syringae*, we isolated a pathogen-resistant mutant with a gain-of-function allele of a locus we have designated as *ACCELERATED CELL DEATH 6* (*acd6*). These plants show small spontaneous patches of cell death and high levels of resistance to *P. syringae*. In addition, *acd6* plants have high levels of defenses that require SA as a sole activator as well as defenses that require SA as a coactivator. All of the *acd6*-conferred phenotypes are dependent on SA. Our analysis suggests that SA not only acts with a second signal to regulate defenses but also modulates the level or the activity of this signal. Finally, we have uncovered an *NPR1*-independent but SA-dependent pathway conferring partial resistance to *P. syringae*, and we show an unexpected role for SA in modulating cell growth.

RESULTS

Isolation of a Novel Gain-of-Function Arabidopsis Mutant with Reduced Disease Symptoms and an Accelerated Cell Death Phenotype

To identify genes that are involved in regulating pathogen defenses, we screened 5000 individual M_2 plants, grown from ethyl methanesulfonate–mutagenized seed, for reduced symptoms after infection with a high dose of the virulent pathogen *P. s.* pv *maculicola* ES4326 (10^7 colony-forming units [cfu]/mL). We identified one plant that showed very little of the expected disease symptoms (chlorosis and water soaking) after infection and a few punctate cell death patches on the uninfected leaves and characterized it further. We named this plant *acd6* because of the cell death phenotype. Other *acd* mutants (*acd3* to *acd5*) identified in this screen will be described elsewhere.

Table 1 shows that after four backcrosses, reciprocal crosses, and self-fertilization of the original mutant, the *acd6*-conferred cell death phenotype segregated as a semidominant trait. This phenotype cosegregated with enhanced resistance to *P. syringae* (data not shown). All other *acd6*-conferred defense phenotypes also behaved as dominant traits (see below). To determine whether the dominance of *acd6* resulted from a haploinsufficient or a gain-of-function

Table 1. Genetic Analysis of *acd6* Plants

Cross (Recipient × Pollen Donor)	Type	Phenotype: Healthy	Mild Cell Death[a]	Cell Death[b]	χ^2	Hypothesis[c]	Fit?
acd6/ACD6(M_2)[d]	Self	22	43	19	0.262[e]	1:2:1	Yes
ACD6 × *acd6/ACD6*(M_2)	F_1	39	37	0	0.053[e]	1:1:0	Yes
ACD6 × *acd6/ACD6*(M_2)	F_2[f]	28	63	30	0.149[e]	1:2:1	Yes
ACD6 × *acd6/ACD6*(M_2)	F_2[g]	79	0	0		1:0:0	
acd6/ACD6 × *ACD6*	F_1	16	18	0	0.118[e]	1:1:0	Yes
acd6/ACD6	Self	36	65	30	0.561[e]	1:2:1	Yes
acd6 × *ACD6*	F_1	0	29	0		0:1:0	
ACD6 × *acd6*	F_1	0	17	0		0:1:0	
ACD6 × *acd6/ACD6*	F_1	37	40	0	0.117[e]	1:1:0	Yes
CS3432[h] × *ACD6*	F_1	46	0	0		1:0:0	
ACD6 × CS3432	F_1	59	0	0		1:0:0	
CS3432 × *acd6*	F_1	0	53	0		0:1:0	
acd6 × CS3432	F_1	0	67	0		0:1:0	

[a] Plants in this phenotypic class had slightly yellowed leaves and microscopic cell death and were of medium stature.
[b] Plants in this phenotypic class had many visible punctate cell death patches and were of reduced stature.
[c] The hypothesis is that *acd6* segregates as a semidominant trait with respect to cell death.
[d] M_2 designates the original mutant plant.
[e] Not significantly different from the expected value ($P > 0.7$ to 0.9 in each of these cases).
[f] This is the progeny of a mild cell death and medium stature F_1 plant.
[g] This is the progeny of a healthy, normal stature F_1 plant.
[h] CS3432 is a *gi-2 co-1* tetraploid Arabidopsis in the Col-0 background (*acd6* is from the same background).

mutation, we created triploid plants with two wild-type copies of *ACD6* and one copy of the *acd6* mutation. If the mutation caused haploinsufficiency, then we would expect the triploid plants to have a wild-type phenotype. If, however, the *acd6* mutation was a gain-of-function allele, then the triploids should have a phenotype reminiscent of the *acd6/ACD6* heterozygotes. Triploid plants harboring *acd6* showed a cell death phenotype (Table 1), indicating that *acd6* is likely a gain-of-function mutant. Moreover, the *acd6/ACD6/ACD6* triploid plants also showed all of the defense phenotypes seen in the *acd6* homozygous and heterozygous diploids (see below).

We used cleaved amplified polymorphic sequence (CAPS) mapping (see Methods) to localize the *acd6* locus to chromosome IV. The *acd6* locus maps between SC5 and mi128 at a distance of 1.8 ± 0.52 centimorgans (cM) from SC5 and 0.43 ± 0.21 cM from mi128. No other previously published mutant(s) with all of the *acd6*-conferred phenotypes has been mapped to this particular region of chromosome IV.

To further characterize the cell death phenotype of *acd6*, we observed plants at various ages for macroscopic and microscopic cell death symptoms. Figure 1A shows an example of the early cell death phenotype, characterized by yellowing leaves with cell death patches, of *acd6* homozygous and heterozygous plants, visible on day 13 after planting. By day 21, *acd6* homozygous plants were considerably smaller in stature than wild-type plants (data not shown), whereas plants that were heterozygous for *acd6* were intermediate in size (data not shown). Microscopic cell death was visualized by trypan blue staining of *acd6* homozygous and heterozygous plants, which revealed single dead cells as well as small clusters of dead cells (Figures 2B, 2C, and 2K). Interestingly, we also saw clusters of dead cells interspersed with enlarged cells, which appeared whitish in *acd6*, *acd6/ACD6*, and *acd6/ACD6/ACD6* plants (Figures 2B, 2C, and 2K). Occasionally, we saw enlarged cells protruding above the abaxial leaf surface of *acd6/ACD6* leaves (Figure 2D). Cell death was also visible in aseptically grown *acd6* plants (Figure 1B), indicating that the *acd6*-conferred cell death phenotype did not depend on the presence of an infectious agent.

Multiple Defenses Activated in *acd6* Plants in the Absence of Pathogens

The presence of cell death patches on *acd6* plants and the lack of symptoms after *P. syringae* infection suggested that defenses had been constitutively activated. To learn whether this was true, we measured the levels of camalexin and the steady state mRNA levels of a number of different defense-related genes. As shown in Table 2, camalexin levels were modestly elevated above wild-type levels in the *acd6*, *acd6/ACD6*, and *acd6/ACD6/ACD6* plants. The RNA gel blot analyses shown in Figures 3 and 4 revealed that the steady state level of the SA-responsive *PR-1* gene transcript was elevated in *acd6*, *acd6/ACD6*, and *acd6/ACD6/ACD6* plants as compared with the levels in *ACD6* diploid and triploid plants, respectively. In addition, the steady state mRNA levels of the defense-related gene transcripts encoded by the *GLUTATHIONE S-TRANSFERASE 1* (*GST1*) and *AVR-INDUCED GENE 1* (*AIG1*) genes were modestly elevated in *acd6* and *acd6/ACD6* plants relative to the control plants (Figure 3). *GST1* and *AIG1* are induced in wild-type plants undergoing a resistant (hypersensitive) response after infection with *P. syringae* carrying *avrRpt2* (Greenberg et al., 1994; Reuber and Ausubel, 1996).

acd6 Plants Show High-Level Resistance to Virulent and Avirulent *P. syringae*

The reduction of pathogenic symptoms and the constitutive activation of defenses in *acd6* leaves suggested that pathogen growth might be compromised in the *acd6* plants. We tested virulent and avirulent *P. syringae* strains for their growth in *acd6* plants. Figures 5A and 5B show that the growth of virulent *P. s. maculicola* ES4326 was reduced in *acd6*, *acd6/ACD6*, and *acd6/ACD6/ACD6* plants as compared with *ACD6* diploid and triploid plants, respectively. Similar results were obtained with a second virulent *P. syringae* strain (*P. s.* pv *tomato* DC3000, data not shown). The growth reduction of virulent *P. s. maculicola* ES4326 in *acd6* plants was similar to that seen in wild-type or *nahG* plants treated with an inducer of the SA signaling pathway, benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH) (Lawton et al., 1996).

We also examined the growth of avirulent strains of *P. syringae* (*P. s. maculicola* ES4326 carrying *avrRpt2* or *avrRpm1*) that should be recognized in the strain background of *acd6*. In three independent experiments, we saw no difference between the growth of avirulent (*P. s. maculicola* ES4326 carrying *avrRpt2*) and that of congenic virulent bacteria. Similarly, we observed a slight or no difference between the growth of bacteria carrying a second *avr* gene (*avrRpm1*) and that of the virulent control bacteria on *acd6* or *acd6/ACD6* plants (Figure 5C). Thus, resistance mediated by an *R* gene–*avr* strain interaction does not significantly contribute to resistance in the *acd6* mutant.

acd6 Antagonizes the Further Induction of Defense Responses during Infection

The lack of a clear growth difference between virulent and avirulent *P. syringae* in *acd6* plants suggested that other aspects of the plant responses to infection, such as the HR and defense induction, might be altered in the *acd6* mutant. After infection with two different avirulent *P. syringae* strains (*P. s. maculicola* ES4326 carrying *avrRpt2* and *avrRpm1*, respectively), the wild type gave an HR at doses as low as 2×10^7 cfu/mL, whereas *acd6* rarely gave an HR, even at a 20-fold higher dose (Figure 1C). *acd6/ACD6* plants showed an

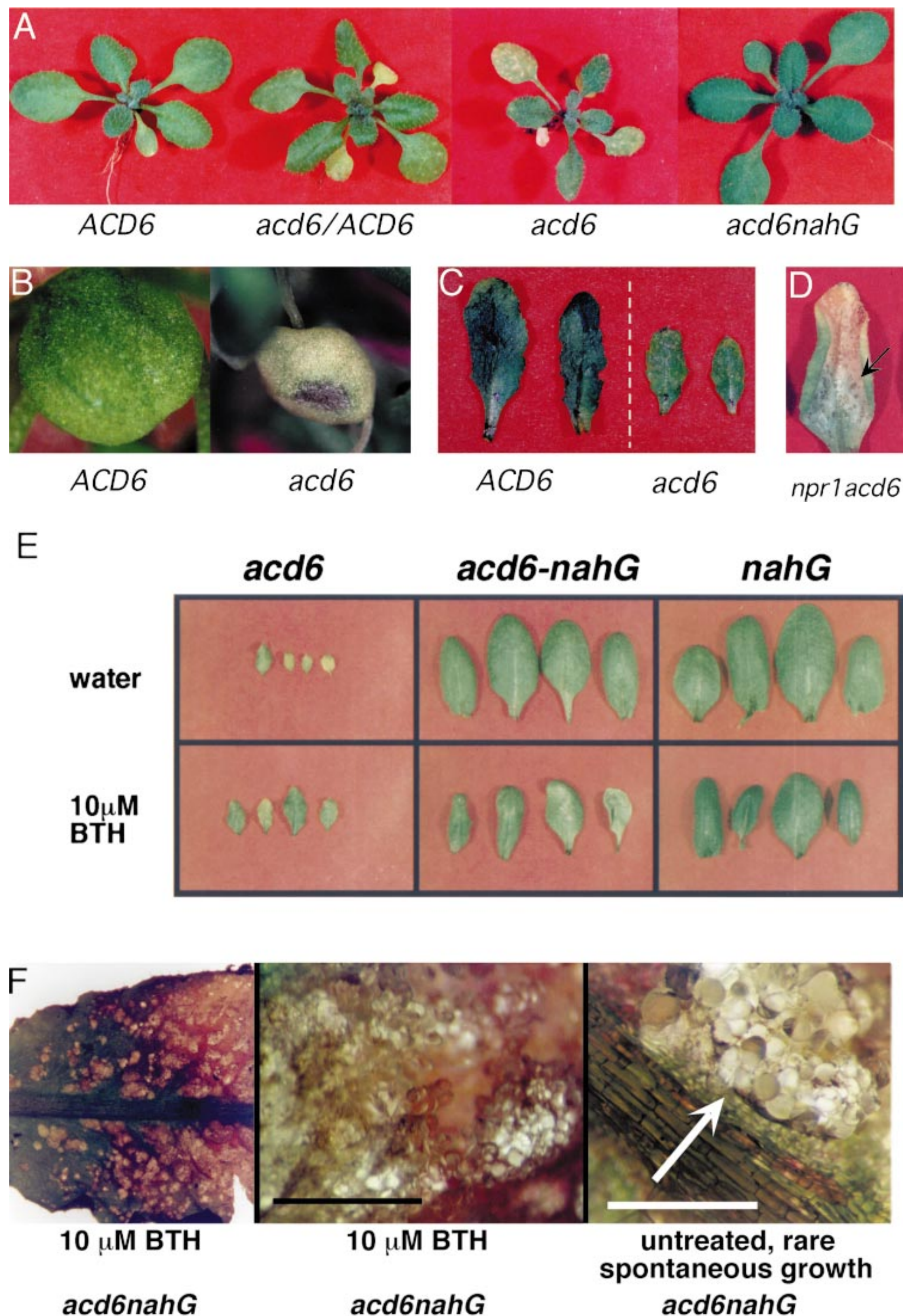

Figure 1. Visible and Defense Phenotypes of *acd6*, *npr1 acd6*, and *acd6–nahG* Plants.

(A) Thirteen-day-old plants photographed at the same distance. *nahG* control plants were indistinguishable in appearance from *acd6–nahG* plants (not shown).

(B) First true leaf of 8-week-old aseptically grown plants of the indicated genotypes, showing enhanced chlorophyll loss and cell death in *acd6*.

(C) Two representative leaves of the indicated genotypes photographed 24 hr after infection with 4×10^8 cfu/mL *P. s.* pv *maculicola* ES4326/*avrRpt2*, showing induced hypersensitive cell death of *ACD6* leaves and no cell death on *acd6* leaves beyond that which was already present on the leaves prior to inoculation. *ACD6* leaves treated with *P. s.* pv *maculicola* ES4326/vector control alone or mock-treated showed no visible symptoms at this time point (24 hr). *acd6* leaves treated with *P. s.* pv *maculicola* ES4326 or mock treated were indistinguishable from those shown here.

intermediate response: at low doses of bacteria, no HR was apparent; however, at higher doses, the HR was obvious (data not shown). The strong HR$^-$ phenotype of *acd6* homozygous plants cosegregated with the small stature/punctate cell death phenotype in two separate experiments.

acd6 plants also failed to induce camalexin synthesis upon infection with avirulent and virulent *P. syringae*. Strain *P. s. tomato* DC3000 carrying *avrRpt2* induces camalexin 24 hr after infection, whereas the congenic virulent strain does not induce camalexin at that time (Glazebrook and Ausubel, 1994). Figure 6A shows that camalexin was constitutively elevated in the *acd6* plants but could not be further induced after infection with *P. s. tomato* DC3000 carrying *avrRpt2*. Infection by a strong camalexin-inducing virulent strain (*P. s. maculicola* ES4326) also failed to induce camalexin in the *acd6* homozygous or heterozygous plants but did induce camalexin in the *ACD6* plants (Figure 6A).

The lack of response to bacterial infection could result from a failure of the bacterial elicitor proteins encoded by *avr* genes to gain access to the plant cells, an instability of the elicitor(s) in the plant cells, a block in the perception of the elicitor(s), or a block in the downstream signal transduction pathway. To examine these possibilities, we bypassed the pathogen delivery system of elicitors by directly expressing one *avr*-encoded elicitor in the *acd6* or *acd6*/*ACD6* leaves. If the elicitor still functioned when directly expressed in plants, this would indicate that the signal transduction pathway was in principle still functional. We introduced the gene encoding the AvrRpm1 elicitor under the control of a strong promoter into plant cells by using biolistic transformation (see Methods). To assay for AvrRpm1 function, we coexpressed the *avrRpm1* gene with *uidA*, which encodes β-glucuronidase. When AvrRpm1 is recognized by an *RPM1*$^+$ plant, the expression of the *uidA* gene should be blocked (Mindrinos et al., 1994; Leister et al., 1996). Figure 7 shows that cotransformation of *avrRpm1* and *uidA* in *acd6* or *acd6*/*ACD6* leaves prevented the expression of the *uidA* gene to the same extent as that seen in the wild type. Thus, the *acd6* plants may not be receiving the *avrRpm1*-encoded elicitor.

SA Is Required for the *acd6*-Conferred Phenotypes

Because of the central role of SA in regulating defense responses, we tested its involvement in the *acd6*-conferred phenotypes. We removed SA from *acd6* by crossing *acd6* with a plant harboring a well-characterized insertion of a *nahG* transgene whose product metabolizes SA. *acd6*–*nahG* homozygous plants were normal in appearance and stature and lacked macroscopic (Figures 1A and 1E) and microscopic cell death patches as well as the enlarged cells (Figure 2G). The *nahG* transgene also suppressed the resistance of *acd6* plants to *P. syringae* (Figure 5A), the elevation of *PR-1* and *AIG1* transcripts (Figure 8A), and the elevation of camalexin levels (Table 2 and Figure 6B). *nahG* also suppressed all of the *acd6*/*ACD6* phenotypes (Table 2 and data not shown).

To determine whether the *nahG* transgene also suppressed the HR$^-$ phenotype of *acd6*, we infected *acd6*–*nahG* plants with avirulent bacteria (*P. s. maculicola* ES4326 carrying *avrRpt2* or *avrRpm1*) and scored cell death after 14 to 20 hr. Upon infection with *P. s. maculicola* ES4326/*avrRpm1*, the HR in *acd6*–*nahG* plants was identical to that seen in the *ACD6* and *nahG* control plants (data not shown). In contrast, *acd6*–*nahG* plants infected with *P. s. maculicola* ES4326/*avrRpt2* showed no HR after 20 hr, even with very high doses of bacteria (data not shown). However, *nahG* control plants also failed to show an HR response specifically with *P. s. maculicola* ES4326/*avrRpt2*, indicating a role for SA in mediating the HR in this interaction (data not shown). We concluded that SA was required for all of the phenotypes conferred by the *acd6* mutation and was also required for *P. s. maculicola* ES4326/*avrRpt2*–mediated cell death in wild-type plants.

The Presence of *acd6* Sensitizes *acd6*–*nahG* Plants to an Inducer of the SA Pathway

To determine whether the effects of the *nahG* transgene resulted from blocking the SA signaling pathway specifically, we treated the *acd6*–*nahG* plants with different levels of the synthetic SA analog BTH (1, 10, 100, and 300 μM) and examined cell death, cell enlargement, and defense activation. The lowest dose of BTH (1 μM) had no effect on the plants. However, even a low level of BTH (10 μM) reversed and hyperactivated all of the suppressed phenotypes of *acd6*–*nahG* plants. Application of 10, 100, or 300 μM BTH induced cell death in small patches on the young leaves within 24 hr of the treatment and caused hyperactivation of cell death in the oldest leaves by 48 hr (Figure 1E; note the completely

Figure 1. (continued).

(D) Four-week-old *npr1 acd6* leaves. Arrow points to an abnormal growth. *npr1*, wild-type, and *acd6* control plants showed no obvious abnormal growths.

(E) Three-week-old plants were treated as indicated and photographed 2 days later.

(F) Four-week-old plants were treated as indicated and photographed 4 days later. The middle panel shows a close-up view of BTH-induced abnormal growths that have coalesced to reveal large, disorganized unpigmented cells along the bottom surface of the leaf. Arrow points to an abnormal growth. This experiment was repeated four times with similar results. Bars = 1 mm of leaf tissue.

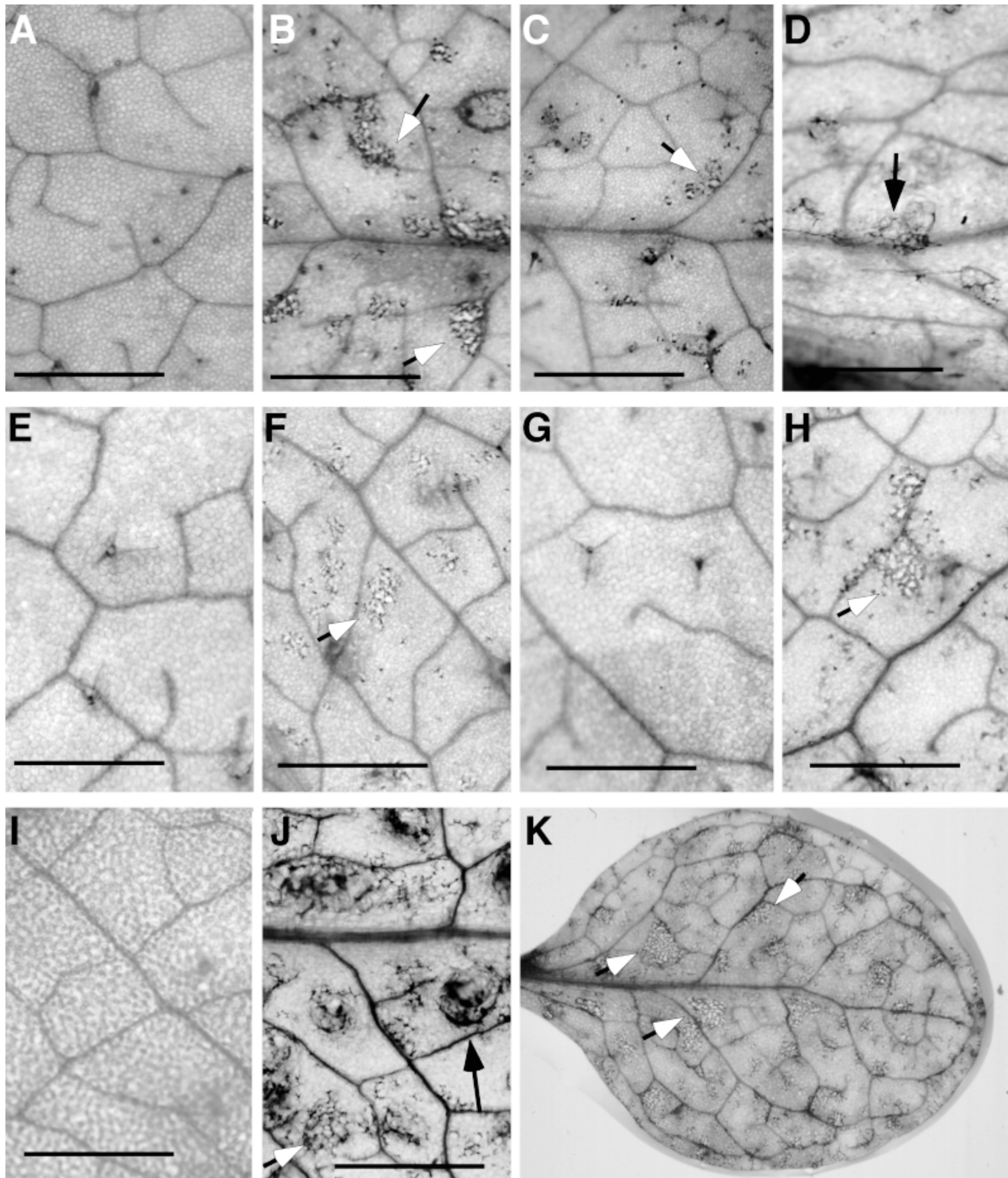

Figure 2. Microscopic Cell Death and Cell Growth Phenotypes of *acd6* Plants.

Representative leaves stained with trypan blue are shown.
(A) to **(C)** The first true leaves from 11-day-old plants. **(A)** *ACD6* leaf; **(B)** *acd6* leaf; and **(C)** *acd6*/*ACD6* leaf.
(D) Thirty-day-old *acd6*/*ACD6* leaf with a small growth.
(E) and **(F)** Twenty-four-day-old triploid leaves. **(E)** *ACD6* triploid leaf; **(F)** *acd6*/*ACD6*/*ACD6* triploid leaf.
(G) and **(H)** Thirty-day-old *acd6–nahG* leaves. **(G)** shows a leaf 4 days after water treatment. **(H)** shows a leaf 4 days after treatment with 100 μM BTH. BTH did not cause any microscopic cell death or cell enlargement in the wild-type or *nahG* control plants (data not shown).
(I) and **(J)** Twenty-four-day-old leaves. **(I)** *npr1* leaf; **(J)** *npr1 acd6* leaf.
(K) First true leaf of an *acd6* plant. Leaf was 6 mm long.
White arrows point to clusters of enlarged and dead (stained) cells. Black arrows point to small growths. These experiments were repeated twice with similar results. Bars in **(A)** to **(J)** = 1 mm.

collapsed leaf at the right end of the panel). On a microscopic level, the BTH-treated *acd6–nahG* leaves showed individual cell death as well as clusters of cell death and enlarged cells reminiscent of those seen in the *acd6* leaves (Figure 2H). BTH did not induce cell death or cell enlargement in *ACD6* or *nahG* leaves (data not shown).

Camalexin levels in the BTH-treated *acd6–nahG* plants exceeded the levels seen in *acd6* plants by at least fivefold, even when only 10 μM BTH was applied (Figure 6B). *AIG1* gene transcript accumulation was induced in the *acd6–nahG* plants at 10, 100, and 300 μM BTH to higher levels than were seen in *acd6* plants (Figure 8A). BTH did not induce camalexin accumulation or *AIG1* gene expression in control *ACD6* or *nahG* plants (Figures 6B and 8A). Finally, accumulation of the *PR-1* gene transcript was induced by 10 and 100 μM BTH to significantly higher levels and for longer times in the *acd6–nahG* plants compared with wild-type or *nahG* plants (Figures 8A and 8B). Suppression of the *acd6/ACD6* heterozygous phenotypes by *nahG* was also reversed and hyperactivated by BTH application (data not shown). The reversibility of all of the *acd6–nahG* phenotypes by even low levels of BTH treatment indicates that SA or a component of the SA signaling pathway is required for all of the *acd6*-conferred phenotypes (see Discussion).

acd6 Acts Partially through *NPR1*

The suppression of the *acd6*-conferred phenotypes by the *nahG* transgene prompted us to examine the possible role of *NPR1*, a gene required for some aspects of SA signal transduction (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996), in the *acd6* plants. We crossed *acd6* with *npr1-1* and identified *npr1 acd6* homozygous plants (see Methods). A comparison of the *acd6* and *npr1 acd6* double mutants revealed that the onset of visible and microscopic cell death was delayed by 4 to 10 days when *npr1* was present, and the extent of cell death was less than that seen in *acd6* plants (data not shown). By days 21 to 25, the *npr1 acd6* plants showed a novel cellular phenotype: in addition to patches of enlarged and dead cells, they also had numerous visible clusters of large cells (abnormal growths; see below) that protruded above the abaxial leaf surface (Figures 1D and 2J). *npr1 acd6* plants also appeared white in the center of the rosettes and had bleached stems, a phenotype that has been reported for *npr1* plants treated with SA (Cao et al., 1994) or crossed with plants with high levels of SA, such as the *constitutive expressor of PR 5* (*cpr5*) mutant (Bowling et al., 1997). The *npr1 acd6* double mutants had rosettes intermediate in size between those seen in *npr1* and *acd6*, respectively, indicating that *npr1* partially suppressed the reduced stature of *acd6* plants (data not shown).

To determine whether *npr1* suppressed resistance or other defense phenotypes in the *acd6* plants, we first examined the growth of *P. syringae* in the *npr1 acd6/ACD6* and *npr1 acd6* plants. *npr1 acd6/ACD6* plants were nearly as susceptible to *P. syringae* as were the *npr1* plants, indicating that resistance of *acd6/ACD6* plants was *NPR1* dependent (Figure 5A). However, the *P. syringae* susceptibility of *npr1 acd6* plants was intermediate between that observed in *npr1* and *acd6* (Figure 5A). *npr1 acd6* plants were clearly more resistant than were *acd6–nahG* plants (Figure 5A), confirming a role for SA in the resistance mediated by *acd6* that is independent of *NPR1*. Despite the lack of clearly enhanced resistance in *npr1 acd6/ACD6* plants, Figure 4 shows that *npr1 acd6/ACD6* and *npr1 acd6* plants did show modest increases in the steady state levels of the *PR-1* gene transcript. Individual progeny of an *npr1 acd6/ACD6* plant showing the cell death phenotype had camalexin levels comparable to those seen in *acd6* and *acd6/ACD6* plants (Table 2). *npr1 acd6* plants also had levels of *AIG1* gene

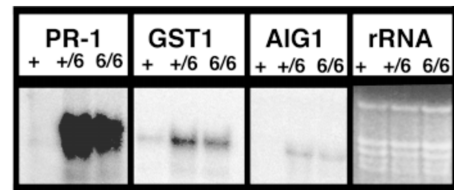

Figure 3. Steady State Transcript Levels of Defense-Related Genes in *acd6* Plants.

RNA gel blot analysis of total RNA extracted from leaves of 20-day-old plants is shown. The blot was probed with the defense-related genes *PR-1*, *GST1*, and *AIG1*. +, *ACD6*; +/6, *acd6/ACD6*; 6/6, *acd6*. This experiment was repeated once with similar results.

Table 2. Accumulation of Camalexin in Various Backgrounds Harboring the *acd6-1* Mutation

Genotype/Phenotype[a]	μg Camalexin cm^{-2} (SD)
ACD6	<0.001
acd6	0.086 (0.040)
acd6/ACD6	0.089 (0.040)
acd6/nahG F_1	<0.001
acd6-nahG	<0.001
nahG	<0.001
npr1	0.001
npr1 acd6 F_3[b]	0.207
npr1 acd6 F_3[b]	0.207
npr1 acd6 F_3[b]	0.107
npr1 acd6 F_3[b]	0.089
ACD6/ACD6/ACD6	0.003 (0.001)
acd6/ACD6/ACD6	0.157 (0.049)

[a] Four to six replicates were assayed for each genotype.
[b] An *npr1 acd6/ACD6* F_2 individual was self-fertilized. Individual F_3 progeny that showed slightly yellowed leaves were assayed. These plants may have been homozygous or heterozygous for *acd6* (see text). This experiment was repeated twice with similar results.

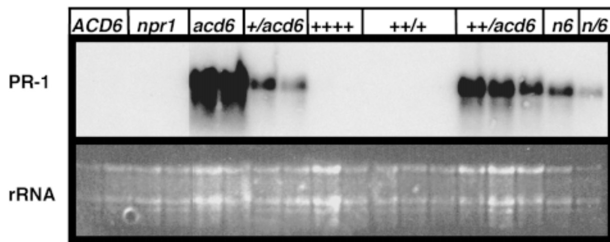

Figure 4. Steady State Levels of *PR-1* Gene Transcripts in *npr1 acd6* Plants and *acd6/ACD6/ACD6* Triploids.

RNA gel blot analysis of *PR-1* transcript levels determined from leaves from the indicated genotypes at 24 days after planting. +/*acd6*, *acd6/ACD6*; ++++, CS3432 (tetraploid Arabidopsis); ++/+, CS3432 × *ACD6* F_1; ++/*acd6*, CS3432 × *acd6* F_1; *n6*, *npr1 acd6*; *n/6*, *npr1 acd6/ACD6*. This experiment was repeated once with similar results.

transcripts comparable to those seen in *acd6* plants alone (data not shown). Thus, only some of the *acd6*-conferred phenotypes were suppressed by the *npr1-1* mutant, which suggests that a second *NPR1*-independent pathway acts to mediate some SA signal transduction events in the *acd6* plants.

The SA Pathway Can Influence Cellular Growth

As mentioned above, treatment of *acd6–nahG* plants with low levels of the SA analog BTH restored many of the *acd6* phenotypes. However, we observed a novel cellular phenotype in the BTH-treated plants 3 to 6 days after the initial treatment. Plants treated with 10, 100, or 300 μM BTH developed abnormal growths on the undersides of the rosette leaves. Figure 1F shows that BTH-induced abnormal growths on *acd6–nahG* leaves formed in large numbers and were composed of clusters of greatly enlarged unpigmented cells that erupted through the epidermal layer of the leaves. All BTH-treated *acd6–nahG* plants developed these abnormal growths on most of the expanded and young leaves, whereas the oldest leaves died (Figure 1E). Plants treated with 10 μM BTH had more abnormal growths relative to cell death area than did plants treated with higher levels of BTH. Five-week-old *acd6–nahG* plants also showed rare spontaneous abnormal growths on the older leaves (Figure 1F). Induction of these abnormal growths by BTH was dependent on the developmental stage of the plants. Before bolting or at the transition to flowering, plants were competent to initiate growths in response to BTH. However, at 1 week past flowering, plants treated with BTH showed the induction of cell death and enlarged cells (Figure 2H) but formed few visible abnormal growths. We were unable to initiate these abnormal growths on wild-type or *nahG* plants inoculated with an extract from the leaves showing this phenomenon, indicating that the abnormal growths were likely not the result of an infectious agent.

DISCUSSION

We identified a novel mutant, *acd6*, that shows high levels of resistance to *P. syringae* and constitutive activation of several defense-related markers. The phenotype of triploid Arabidopsis with one copy of *acd6* and two copies of the wild-type locus indicates that *acd6* is a dominant gain-of-function mutation resulting in the activation of defenses.

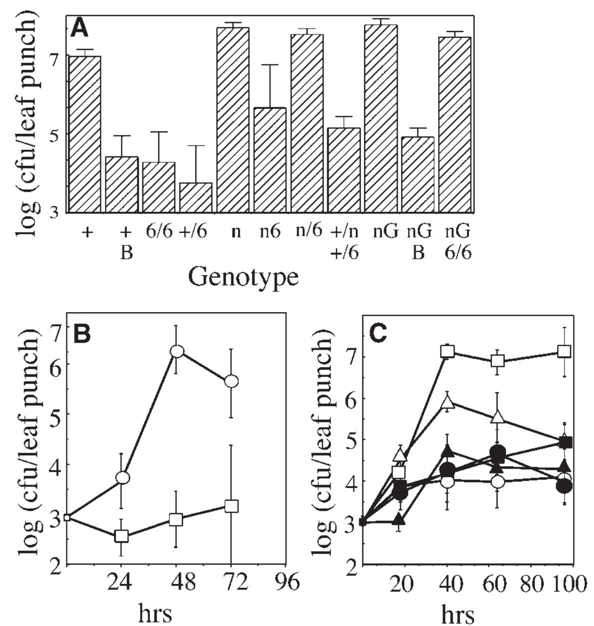

Figure 5. Analysis of Growth of *P. syringae* Strains in *acd6* Plants.

For all graphs, error bars indicate standard deviations.

(A) Growth of *P. s. maculicola* ES4326 in *ACD6* and *nahG* plants after treatment with 100 μM BTH compared with *acd6* double mutants with *npr1* and *nahG*, respectively. Infections were done to give 3.5 × 10^3 cfu/cm^2 leaf area 3 days after BTH treatment, and bacterial growth determinations were done 3 days after the infection. +, *ACD6*; +B, *ACD6* with 100 μM BTH; 6/6, *acd6*; +/6, *acd6/ACD6*; n, *npr1*; n6, *npr1 acd6*; n/6, *npr1 acd6/ACD6*; +/n +/6, *npr1/NPR1 acd6/ACD6*; nG, *nahG*; nG B, *nahG* with 100 μM BTH; nG 6/6, *acd6–nahG*.

(B) Growth of *P. s. maculicola* ES4326 in triploid *ACD6* (circles) and *acd6/ACD6/ACD6* plants (squares). Each experiment was repeated at least once with similar results.

(C) Growth of *P. s. maculicola* ES4326/vector, *P. s. maculicola* ES4326/*avrRpt2*, and *P. s. maculicola* ES4326/*avrRpm1* in *ACD6* (open symbols) and *acd6* (closed symbols) plants. Squares, *P. s. maculicola* ES4326/vector control; triangles, *P. s. maculicola* ES4326/*avrRpm1*; circles, *P. s. maculicola* ES4326/*avrRpt2.*

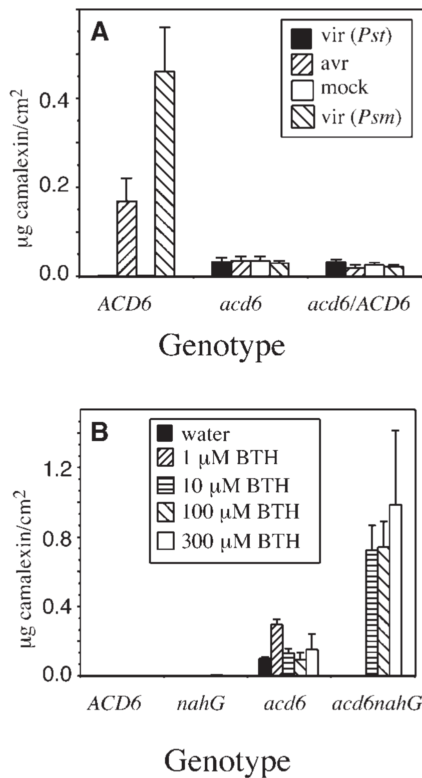

Figure 6. Camalexin Levels in *P. syringae*–Infected *acd6* Plants and BTH-Treated Plants.

(A) Plants were treated with *P. s. tomato* DC3000 (vir [*Pst*]) or *P. s. tomato*/*avrRpt2* (avr) for 1 day or with 10 mM $MgSO_4$ (mock) or *P. s. maculicola* ES4326 (vir [*Psm*]) for 2 days. Six samples per treatment were used. Error bars show standard deviations. This experiment was repeated once with similar results.

(B) Plants were treated with the indicated doses of BTH, and camalexin was extracted and quantitated after 2 days. Four samples were used for each treatment. Error bars show standard deviations. This experiment was repeated twice with similar results.

Because *acd6* is a gain-of-function mutant, ACD6 may be a positive regulator of defenses if the *acd6* mutation is hypermorphic, or ACD6 may play no role in defense if the mutation is neomorphic. The isolation of a loss-of-function *acd6* allele and the cloning of the *ACD6* gene will clarify this issue. Nevertheless, our study has implicated both SA and at least one other unknown signal in regulating defenses, cell death, and, unexpectedly, cell growth. Furthermore, SA may act to modulate the activity and/or levels of the second signal, fine-tuning the defense response to pathogens.

acd6 Plants Are Resistant and Insensitive to *P. syringae*

acd6 plants are highly resistant to several virulent and avirulent strains of *P. syringae* and fail to induce the HR and camalexin synthesis upon infection. In addition, avirulent *P. syringae* and the congenic virulent strain show the same growth potential in *acd6* plants. The HR$^-$ phenotype of *acd6* plants is reminiscent of that seen in the *dnd1* (*defense no death*) mutant (Yu et al., 1998), with an important difference. *dnd1* plants fail to show an HR with several avirulent *P. syringae* strains, but they can still respond to avirulent signals:

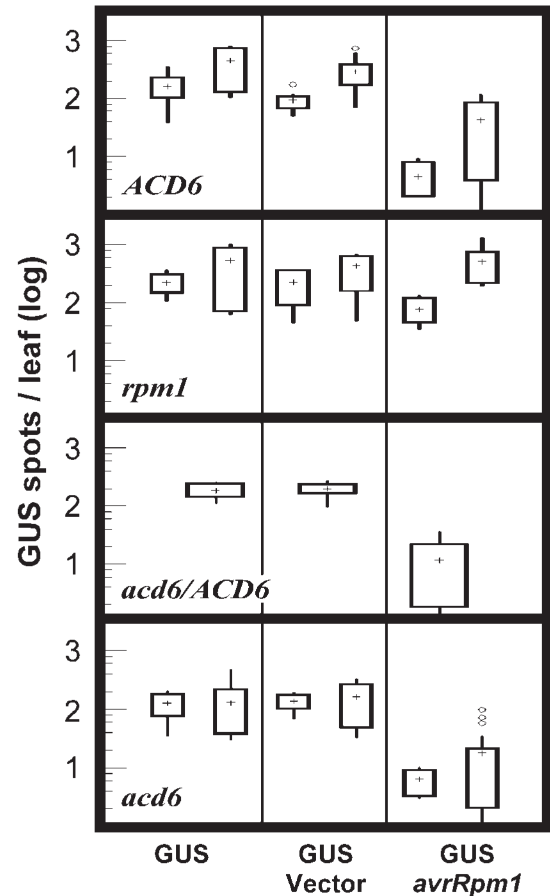

Figure 7. Transient Assay of *avrRpm1* and β-Glucuronidase (GUS) Expression in Four Different Plant Genotypes.

Three sets of DNA constructs (pKEx4tr-GUS [carries 35S-GUS]; pKEx4tr-GUS plus pKEx4tr [vector control]; and pKEx4tr-GUS plus pKEx4tr-*avrRpm1*) were biolistically transformed or cotransformed into plant leaves, and GUS activity was assayed by counting the number of leaf cells staining blue. Two replicate experiments were performed except in the case of transformation into the *acd6*/*ACD6* heterozygote. In each case, the box at left is from experiment 1 and the box at right is from experiment 2. In the case of *acd6*/*ACD6*, data were obtained in experiment 1. The box plots show the mean (+ in the center of the box) and the second and third quartiles, which indicate the dispersion of 50% of the data points (open boxes) and the range (vertical lines above and below the boxes). Statistical outliers are indicated by open circles.

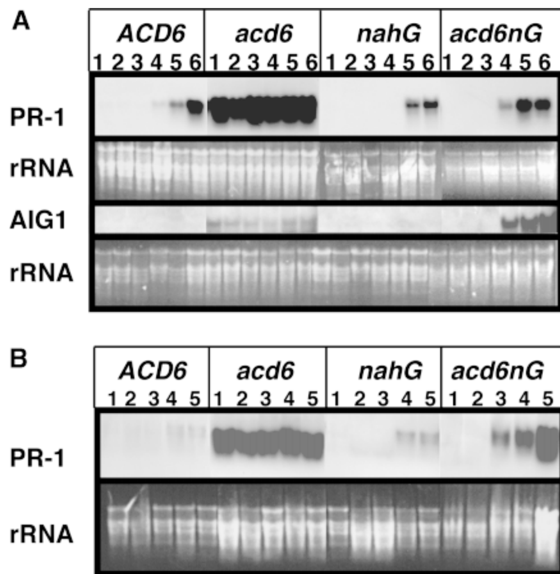

Figure 8. Time Course of the Steady State Levels of Defense-Related Transcripts in BTH-Treated Plants.

(A) Three-week-old plants were untreated (lane 1) or treated with water (lane 2), 1 μM BTH (lane 3), 10 μM BTH (lane 4), 100 μM BTH (lane 5), or 300 μM BTH (lane 6) for 24 hr and then harvested. *acd6nG*, *acd6–nahG*.

(B) Three-week-old plants were treated with water (lane 1), 1 μM BTH (lane 2), 10 μM BTH (lane 3), 100 μM BTH (lane 4), or 300 μM BTH (lane 5) for 48 hr and then harvested. This experiment was repeated three times with similar results.

virulent *P. syringae* grow more than avirulent *P. syringae*, and *PR-1* is induced to a greater extent in response to avirulent strains of *P. syringae* than in response to virulent strains. *dnd1* shows constitutive *PR-1* expression and approximately the same level of resistance to virulent *P. syringae* as does *acd6*. The differences between *dnd1* and *acd6* suggest that the basis for their HR$^-$ phenotypes may be different. *P. syringae* exerts many of its effects as a pathogen through the secretion of toxins (Mo and Gross, 1991; Mittal and Davis, 1995; Lavermicocca et al., 1997; Bender et al., 1998) as well as the secretion of proteins (including *avr* proteins) that are thought to act inside plant cells (Gopalan et al., 1996; Leister et al., 1996). Results of our experiments with the *acd6* plants suggest that they are blocked at a step before the internalization of the Avr proteins into the *acd6* cells.

acd6 Uncovers an *NPR1*-Independent but SA-Dependent Pathway for *P. syringae* Resistance and *AIG1* Regulation

Many phenotypes of *acd6* plants are *NPR1* independent but require SA. For example, the elevated camalexin levels and *AIG1* expression in *acd6* strictly require SA, as inferred from the *acd6–nahG*—conferred phenotypes, but are entirely *NPR1* independent (Table 2; J.V. Cuenca and J.T. Greenberg, unpublished observations). Previously, it was shown that SA is an *NPR1*-independent amplifier of camalexin synthesis during pathogen attack (Glazebrook et al., 1996; Zhao and Last, 1996; Zhao et al., 1998; Zhou et al., 1998). Our results suggest that there are also conditions under which SA is strictly required with another signal to coactivate camalexin synthesis. Steady state *PR-1* gene transcript levels were somewhat reduced in the *npr1 acd6* double mutants as compared with those seen in *acd6* homozygous plants, indicating that both an *NPR1*-dependent pathway and an *NPR1*-independent pathway for regulating *PR-1* expression are operating in *acd6* plants. In the pathogen-resistant Arabidopsis *cpr6* mutant, removal of *NPR1* function abolishes resistance to *P. syringae* but does not abolish *PR-1* activation (Clarke et al., 1998). In contrast, *acd6* plants partially retain both *PR-1* activation and *P. syringae* resistance in the absence of *NPR1* function. The recently described *ssi1* mutant shows high *PR-1* activation and *P. syringae* resistance, but unlike *acd6*, these *ssi1* phenotypes are entirely *NPR1* independent (Shah et al., 1999). *acd6* plants require SA for both *PR-1* activation and *P. syringae* resistance, suggesting that there is a *NPR1*-independent pathway that is SA dependent and can confer resistance to *P. syringae*. Alternatively, the *npr1* mutant may retain some functional *NPR1* product, although this seems unlikely, because *npr1* plants show no *PR-1* induction by SA or BTH treatment (Cao et al., 1994, 1997; J.V. Cuenca and J.T. Greenberg, unpublished observations).

acd6 Acts through SA and at Least One Additional Signal

SA is required for all the *acd6*-conferred phenotypes, as inferred from the suppression of defenses, cell death, and cell growth phenotypes in *acd6–nahG* plants. The *acd6*-conferred phenotypes could result from a heightened sensitivity to SA and/or elevated levels of SA. Whereas SA is required for the *acd6*-conferred phenotypes, it is not sufficient on its own to cause many of the *acd6*-conferred phenotypes in wild-type plants. This observation implicates a second signal in addition to SA that is strictly required for the activation of many defenses, cell death, and cell growth in *acd6* plants.

The fact that all of the phenotypes of *acd6* require SA and that *NPR1*-dependent and *NPR1*-independent pathways are activated suggests a model for how defenses are regulated in *acd6* (Figure 9). We hypothesize that the mutation in *acd6* causes plants to activate and/or become sensitized to two signals, SA and a second signal that acts as an obligatory coactivator with SA. The elevated sensitivity to and/or the elevated levels of SA in *acd6* plants lead to an upregulation of the *NPR1*-dependent pathway 1 and pathway 2 defenses. Camalexin, *AIG1*, cell death, and cell growth are part of pathway 2, because these events require SA, but SA is not

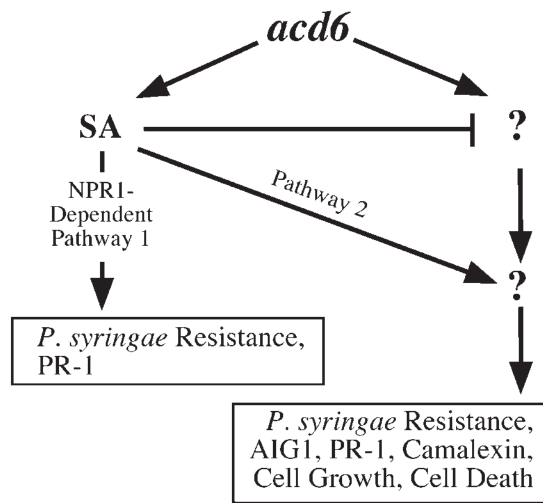

Figure 9. A Model for How SA Acts to Activate Cell Death, Cell Growth, and Defenses in *acd6* Plants.

The suppression of all the *acd6*-conferred phenotypes by *nahG* suggests that SA is required for all the *acd6*-conferred phenotypes. Only some phenotypes of *acd6* are suppressed by *npr1*; therefore, *acd6* acts through the *NPR1*-dependent pathway 1 and the *NPR1*-independent pathway 2. Because all *acd6*-conferred phenotypes require SA, although SA is not sufficient to induce these phenotypes when applied to wild-type plants, we hypothesize that SA acts together with a second unknown signal (?) to induce the pathway 2 phenotypes. The second signal thus requires activation by *acd6* and the presence of SA to activate pathway 2. Furthermore, because *acd6–nahG* plants treated with BTH showed hyperactivation of the *acd6*-conferred phenotypes, we hypothesize that the second signal is negatively regulated by SA in addition to acting with SA. The unknown second signal might be two signals.

sufficient to induce them in wild-type plants on its own. An alternative model also formally consistent with some of our data is that pathway 2 is induced by SA but suppressed by a second signal. The *acd6* mutation in this scenario would inactivate the second repressive signal, which in turn would lead to the activation of pathway 2 defenses by SA.

SA is not simply permissive for the second signal required for pathway 2 (Figure 9), but it may modulate the level and/or activity of the second signal. Indeed, plants from which SA has been removed are hypersensitive to low levels of exogenous BTH, indicating that SA or an SA-controlled factor negatively regulates the level and/or activity of the second signal. Removal of SA from *acd6* plants by using the *nahG* transgene may allow the second signal to build up to high levels (Figure 9). When the SA pathway is reactivated, the second signal is now so high that camalexin production, *AIG1* gene transcript accumulation, cell death, and cell growth are hyperactivated. A critical test of this model awaits the identification of the second signal(s) activated in *acd6* plants. It has previously been reported that the induction of *PR-1* can require only very low levels of SA if plants are pretreated with low levels of ethylene (Lawton et al., 1994). Thus, the highly sensitized state of *acd6–nahG* plants to BTH might result from elevated ethylene levels, but this remains to be determined.

Does SA Regulate Coupled Cell Growth and Cell Death?

Our results on the influence of SA on the occurrence of cell death and cell growth in *acd6* plants suggest that these events can be coupled in plants, a phenomenon that is well established in animals. For example, in animals, deregulation of cell growth and cell death can be caused by misregulation of a single gene such as c-*myc*, *Rb*, or *E1A* (Evan and Littlewood, 1998). Cell growth and cell death are coupled in animals, and loss of control over growth regulation can lead to programmed cell death (Evan and Littlewood, 1998). In some situations, blocking programmed cell death can also suppress proliferation (Evan and Littlewood, 1998).

Both cell death and cell enlargement in *acd6* plants may be a consequence of the unique physiology of this mutant. However, it is possible that coupled cell death and enlargement are part of a normal response to some pathogens. For example, during the interaction of tomato with an elicitor of the resistance response, tomato cells enlarged before dying (Hammond-Kosack et al., 1996). We have observed persistent cell enlargement coupled with cell death in *P. syringae*–infected wild-type Arabidopsis (J.T. Greenberg, unpublished observation). The cell death and enlarged cell phenotypes of *acd6* were suppressed by *nahG*, implying an involvement of SA in these phenotypes. Low-level BTH or SA (H. Vanacker and J.T. Greenberg, unpublished observations) treatment of *acd6–nahG* plants resulted in cell death and clusters of very enlarged, unpigmented cells. These abnormal growths may represent a hyperactivation of the cell enlargement seen in *acd6* plants; however, we do not know whether these two phenotypes represent a continuum of the same phenomenon.

Physiological levels of *O*-acetylsalicylic acid were previously shown to promote growth in maize protoplasts (Carswell et al., 1989). The sporadic abnormal growths formed on the oldest leaves of *acd6–nahG* plants, even in the absence of BTH, suggests that (1) *acd6–nahG* plants are not entirely lacking SA, (2) a signal other than SA can weakly activate cell growth, or (3) SA amplifies a growth-promoting signal present in *acd6–nahG* plants. Abnormal growths on *npr1 acd6* plants may be caused by an imbalance of these activation signals. Genetic tumors resembling the abnormal growths described here have been reported in certain intraspecific and interspecific hybrid tobacco lines (Ahuja, 1998). It is not known whether the SA pathway influences the production of tumors in these plants, although some are influenced by environmental conditions (Fujita et al., 1994; Ahuja, 1998).

A possible link between the cell growth, cell death, and defense phenotypes of *acd6* might be elevated levels of second messengers such as hydrogen peroxide and/or superoxide. SA can amplify the induction of a hydrogen peroxide burst, which influences *GST1* gene induction and cell death after pathogen attack (Shirasu et al., 1997). Superoxide can also cause cell death, for example, in *lsd1* plants grown under long days (Jabs et al., 1996). Interestingly, tumor production in Agrobacterium-infected *Kalanchoe* plants is greatly influenced by the levels of superoxide (Jia et al., 1996). Thus, the same or similar signals can influence cell death, defense activation, and cell growth in different contexts.

In summary, through the study of a gain-of-function *acd6* mutant, we have shown that SA can act in an *NPR1*-independent manner to enhance resistance to *P. syringae* and regulate multiple defense responses. We have also shown that SA must act with at least one additional signal whose activity and/or levels are influenced by SA or an SA-controlled factor to control plant defenses, cell death, and, unexpectedly, cell growth. These observations suggest that to regulate defense responses, cell death, and cell growth, plants integrate two or more signals that act together to promote the induction of novel pathways that can be further fine-tuned by antagonistic interactions between the signals themselves.

METHODS

Plant Growth, Pathogenicity Assays, Chemical Treatments, and Metabolite Measurements

Plants (*Arabidopsis thaliana*) were grown in growth rooms or aseptically, as described elsewhere (Greenberg et al., 1994; Butt et al., 1998), in a 16-hr-light and 8-hr-dark cycle. *Pseudomonas syringae* pv *maculicola* ES4326 and *P. s.* pv *tomato* DC3000 and its derivatives were obtained from F.M. Ausubel (Massachusetts General Hospital and Harvard University, Boston, MA), except for *P. s. maculicola* ES4326/*avrRpm1*, which was obtained from J.L. Dangl (University of North Carolina, Chapel Hill). Strains harboring *avrRpt2* or *avrRpm1* carried these genes on derivatives of pLAFR (plasmid pLH12 and pK48, respectively). Infections and growth determinations were done as described previously (Greenberg et al., 1994), except that plants were covered with a plastic dome for the duration of the experiment to enhance the local humidity, and only young leaves were used. Leaf discs were 6 mm in diameter. Benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH) was a gift from Novartis, Inc. (Research Triangle Park, NC). Plants treated with BTH were sprayed until liquid dripped off the leaves. Camalexin determinations were performed with four to six replicates, as described previously (Glazebrook and Ausubel, 1994).

The M_2 population of ethyl methanesulfonate–mutagenized Columbia (Col-0) seeds used for this study, *nonexpressor of PR 1* (*npr1-1*), and Landsberg *erecta* seeds were from F.M. Ausubel. All experiments reported were performed with a single allele of *accelerated cell death 6* (*acd6*), designated *acd6-1* and referred to in the text as *acd6*. CS3432 (tetraploid *gi-2 co-1* Col-0 Arabidopsis) was obtained from G. Copenhaver and D. Preuss (University of Chicago, IL). *nahG* "B15" was obtained from Novartis, Inc. *RPM1*-deficient ecotype HS12 was obtained from R. Mauricio and J. Bergelson (University of Chicago). The genotype of *npr1-1* was confirmed as described (Cao et al., 1997). Isolation of the *npr1 acd6* double mutant was done by selecting plants in the F_2 generation of a *npr1* × *acd6* cross that were homozygous *npr1* (determined by molecular characterization) and showed slightly yellowed leaves and/or a bleached stem late in development. The presence of *acd6* was confirmed in test crosses to wild-type, *acd6*/*ACD6*, and *acd6* plants. Isolation of the *acd6* homozygous plants that were also homozygous for the *nahG* transgene (*acd6–nahG*) was done by spraying an F_2 population of progeny from a *acd6* × *nahG* cross with 100 μM BTH. Plants that showed the induction of small cell death patches after 1 day were test crossed to *acd6* and followed for two generations to confirm that the original plant was homozygous for *acd6*. The presence of the *nahG* transgene was confirmed by polymerase chain reaction (PCR) amplification of the *nptII* gene.

For mapping, a cross between *acd6* (Col-0) and Landsberg *erecta* was performed. F_2 plants displaying the *acd6* homozygous cell death phenotype were used for recombination analysis by using the PCR-based technique of cleaved amplified polymorphic sequences (CAPS). Published CAPS markers for all five chromosomes were scored on 34 of the recombinant progeny to link the *acd6* locus to chromosome IV. New markers were identified on chromosome IV by sequencing mi markers obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus) and searching for CAPS or using published sequences to create CAPS as the *acd6* locus was approached. The two markers used in this study were based on SC5 and DR1 (derived from mi128). For SC5, the primers 5′-TCGACGACTCTCAAGAACCC-3′ and 5′-CACAAGCTATACGATGCTCACC-3′ were used for PCR amplification, and the product was cleaved with AccI. For DR1, the primers 5′-CGCTTGTACTACTGAGGTTG-3′ and 5′-CGCTATTCGGTTGCTGTGA-3′ were used for PCR amplification, and the product was cleaved with BfaI. Details regarding these markers are available upon request.

RNA Gel Blot Analysis

Total RNA was isolated as described (Nagy et al., 1988). The total RNA (~10 μg) was separated by electrophoresis on a 1% agarose gel, as described previously (Greenberg et al., 1994); however, the gel and running buffer contained 0.22% formaldehyde. Probes were made using primer extension from PCR products (Greenberg et al., 1994). The probes used for detecting *PATHOGENESIS RELATED 1* (*PR-1*) and *GLUTATHIONE S-TRANSFERASE* (*GST1*) gene transcript accumulation were described previously (Greenberg et al., 1994). The probe for *AVR INDUCED GENE 1* (*AIG1*) was made by using PCR to amplify a portion of this gene from genomic DNA and performing a primer extension reaction. The primers used were 5′-GCCACGGGGAACAGCATC-3′ (sense primer) and 5′-CTTCCATTTCAGCACGCATACG-3′ (antisense primer).

Visualization of Dead Cells, Enlarged Cells, and Abnormal Growths

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) contain-

ing 10 mg of trypan blue for 1 min. Tissue was cleared in alcoholic lactophenol (2:1 95% ethanol:lactophenol) for 2 min, washed in 50% ethanol at room temperature, and stored in water. For analysis, tissue was spread on a plastic Petri dish and visualized using a Leica Wild M3Z binocular microscope (Leica, Inc., Rockleigh, NJ) with a magnification of ×10 to ×40.

Transient Expression of *avrRpm1* and Histochemical Analysis

Transient expression assays of the *P. syringae avr* gene *avrRpm1* were performed in wild-type, *acd6*/*ACD6*, *acd6*, or *rpm1* (ecotype HS12) plants with the aid of a Biolistic PDS-1000/HE particle delivery system (Bio-Rad). The *avrRpm1* gene cloned into pKEX-4tr behind the 35S promoter was provided by F. Katagiri (Novartis Agricultural Discovery Institute, San Diego, CA) and F.M. Ausubel. In addition, a construct with *uidA* cloned behind the 35S promoter (pKE4tr-G) was used for the expression of β-glucuronidase in leaves. Biolistic transformation and histochemical staining were performed as described previously (Mindrinos et al., 1994), except that leaves were soaked for 1 min in 90% acetone and blotted dry before staining. Four replicate bombardments were performed for each combination of DNA constructs. Twenty to 40 samples were analyzed for each genotype.

ACKNOWLEDGMENTS

We thank Adam Driks, Laurence Mets, Hélène Vanacker, Daphne Preuss, and members of Daphne Preuss's laboratory for helpful discussions. We thank Jennifer Mach, Manfred Ruddat, the anonymous reviewers, and the coeditor for useful comments on the manuscript. We thank Kenneth Yliniemi, John Zdenek, and Sandra Suwanski for excellent assistance in growing Arabidopsis at the University of Chicago greenhouse. We thank Michael Spaly for performing crosses. We thank the Arabidopsis Biological Resource Center for clones. D.N.R. was supported by STAR Fellowship No. U914960-01 from the Environmental Protection Agency. D.S.G. was supported by Fellowship No. 1 F32 GM20024-01 from the National Institutes of Health. J.T.G. is a Pew Scholar and an American Cancer Society Research Fellow (No. JFRA-623). Research in the J.T.G. laboratory was supported by Grant No. 1R29GM54292-01 from the National Institutes of Health 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.

Received May 5, 1999; accepted June 25, 1999.

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