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ACD6, a Novel Ankyrin Protein, Is a Regulator and an Effector of Salicylic Acid Signaling in the Arabidopsis Defense Response

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The previously reported Arabidopsis dominant gain-of-function mutant *accelerated cell death6-1* (*acd6-1*) shows spontaneous cell death and increased disease resistance. *acd6-1* also confers increased responsiveness to the major defense signal salicylic acid (SA). To further explore the role of ACD6 in the defense response, we cloned and characterized the gene. *ACD6* encodes a novel protein with putative ankyrin and transmembrane regions. It is a member of one of the largest uncharacterized gene families in higher plants. Steady state basal expression of ACD6 mRNA required light, SA, and an intact SA signaling pathway. Additionally, ACD6 mRNA levels were increased in the systemic, uninfected tissue of *Pseudomonas syringae*–infected plants as well as in plants treated with the SA agonist benzothiazole (BTH). A newly isolated *ACD6* loss-of-function mutant was less responsive to BTH and upon *P. syringae* infection had reduced SA levels and increased susceptibility. Conversely, plants overexpressing ACD6 showed modestly increased SA levels, increased resistance to *P. syringae*, and BTH-inducible and/or a low level of spontaneous cell death. Thus, ACD6 is a necessary and dose-dependent activator of the defense response against virulent bacteria and can activate SA-dependent cell death.

INTRODUCTION

The small phenolic compound salicylic acid (SA) plays a central role in disease resistance in higher plants. Its synthesis is induced in response to many types of pathogens (Ryals et al., 1996). SA is both necessary and sufficient for general resistance to many pathogens. Plants carrying a *nahG* transgene whose product catabolizes SA or plants harboring a mutation in the SA biosynthetic pathway or signaling are more susceptible to many pathogens (Gaffney et al., 1993; Delaney et al., 1994; Wildermuth et al., 2001). Conversely, plants engineered to produce high SA levels constitutively and plants treated exogenously with SA or an SA agonist such as benzothiazole (BTH) have enhanced disease resistance (Friedrich et al., 1996; Verberne et al., 2000).

SA plays multiple roles in the regulation of plant defenses. It is required for the induction of broad-spectrum disease resistance in the systemic tissue of plants previously infected with a necrotizing pathogen (a phenomenon termed systemic acquired resistance) (Gaffney et al., 1993). Some plants also require SA to mount a strong resistance response during so called gene-for-gene resistance. In this response, plants have a resistance (*R*) gene allele that confers the ability to recognize specific pathogen proteins encoded by *avr* genes (Staskawicz, 2001). In some *R-avr*–mediated interactions, SA is required for the *R* gene–dependent host programmed cell death (called the hypersensitive response [HR]) and/or for disease resistance (Delaney et al., 1994; Brading et al., 2000; McDowell et al., 2000; Rate and Greenberg, 2001; Rairdan and Delaney, 2002). However, SA on its own is not sufficient to activate an HR and some defenses when produced at high levels in plants, suggesting that SA acts as a coactivator with another signal(s) to induce these responses (Rate et al., 1999). One such coactivator appears to be light, because the light receptors called phytochromes are important for some SA responses (Genoud et al., 2002).

The molecular basis of SA perception remains unclear, although several SA binding proteins have been identified (Chen et al., 1993; Klessig et al., 2000; Slaymaker et al., 2002). Several genes important for SA accumulation in response to pathogen attack and for its transduction have been found. Positive regulators of SA production during infection by some pathogens include NDR1 (Non-Race-Specific Disease Resistance1), a possible membrane protein (Century et al., 1997; Shapiro and Zhang, 2001), and EDS1 (Enhanced Disease Susceptibility1) and PAD4 (Phytoalexin Deficient4), which are interacting proteins that resemble lipases (Zhou et al., 1998; Falk et al., 1999; Jirage et al., 1999; Feys et al., 2001). Additionally, the Arabidopsis *eds3* (Glazebrook et al., 2003), *eds4* (Gupta et al., 2000), *eds8* (Glazebrook et al., 2003), *pad1* (Glazebrook et al., 1997), and *pad2* (Glazebrook et al., 1997) mutants are impaired for SA production in response to the bacterial pathogen *Pseudomonas syringae*. NPR1/NIM1 (Nonexpressor of PR1/Noninducible Immunity1), an ankyrin repeat–containing protein, is important for transducing the SA signal. The ankyrin repeat is a motif containing ~33 amino acids involved in protein–protein interactions (Sedgwick and Smerdon, 1999). *npr1/nim1* Arabidopsis plants are hypersusceptible to many pathogens and fail to express SA-induced *PR* (pathogenesis-related) genes after treatment with SA or its agonists (Cao et al., 1994, 1997). Arabidopsis harboring a *sni1* mutation (*suppressor of npr1-1, inducible1*) is potentiated for SA-induced *PR* gene expression. Thus, SNI1 is a negative regulator of the SA pathway (Li et al., 1999).

A number of Arabidopsis mutants constitutively accumulate high levels of SA. Generally, these mutants show increased dis-

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ease resistance that requires SA, PAD4, EDS1, and/or NPR1, as well as other phenotypes, such as reduced size, altered morphology, and/or spontaneous cell death. Such mutants include *accelerated cell death* (*acd*) (Rate et al., 1999; Brodersen et al., 2002), *constitutive expressor of PR genes* (Bowling et al., 1997; Clarke et al., 2000), *lesion-simulating disease* (*lsd*) (Weymann et al., 1995; Rusterucci et al., 2001; Aviv et al., 2002), *defense no death* (*dnd*) (Yu et al., 1998), *aberrant growth and death* (*agd*) (Rate and Greenberg, 2001), and *suppressor of salicylic acid insensitivity* (Shirano et al., 2002). Most genes previously identified by this class of mutants are not known to be induced by SA; rather, their functions are influenced by the presence of SA or changes in SA signaling. One example is LSD1, a zinc-finger protein with similarity to GATA-type transcription factors. LSD1 is not controlled by SA signaling, at least with respect to its steady state transcript accumulation (Dietrich et al., 1997). However, *lsd1* plants show uncontrolled cell death that is suppressed by the *eds1* and *pad4* mutations (Rusterucci et al., 2001). Another example is ACD11, a sphingosine transfer protein that may negatively regulate cell death and defenses in vivo by altering sphingolipid metabolism (Brodersen et al., 2002). Although the *acd11* cell death phenotype requires SA, ACD11 transcript accumulation is not SA responsive (this work). These observations suggest that many *ACD/LSD*-type genes function by interacting with SA-controlled activities.

We previously reported the isolation of a dominant gain-of-function mutant in Arabidopsis, *acd6-1*, whose phenotypes all require SA. *acd6-1* has reduced stature, increased SA, spontaneous cell death, high resistance to *P. syringae*, and constitutive defense responses. Treatment of SA-depleted *acd6-1* plants with the SA agonist BTH results in the hyperactivation of defense-related genes (Rate et al., 1999). This finding suggests that *acd6-1* is sensitized to SA signaling and that ACD6 could be involved in amplifying SA responses. We report here the cloning of *ACD6*, which encodes a novel protein containing putative ankyrin and transmembrane regions. Unlike most other *LSD*- and *ACD*-type genes characterized to date, the expression of *ACD6* simultaneously requires both SA and light. Plants lacking *ACD6* are less responsive to the SA agonist BTH, and upon infection they produce less SA and are more susceptible to *P. syringae*. Plants with extra genomic copies of *ACD6* are more resistant to *P. syringae*, have modestly increased SA levels, and show BTH-inducible and/or spontaneous cell death. These facts suggest that ACD6 regulates SA and also is an effector of the SA pathway involved in disease resistance and cell death control.

RESULTS

The *acd6-1* Mutant Has Altered Defense Regulation and Cell Wall Properties

acd6-1 plants have increased levels of SA (Vanacker et al., 2001), whereas SA-depleted *acd6-1* plants are sensitized to the SA agonist BTH (Rate et al., 1999). These results indicate that the levels of SA signaling components may be altered in *acd6-1* plants. To test this possibility, we examined the expression of the positive regulators of SA signaling, EDS1 and PAD4, and the SA-transducing component NPR1. Indeed, the abundance of EDS1, PAD4, and NPR1 mRNAs was increased in *acd6-1* (Figure 1A). As a positive control for SA signaling activation, the level of PR1 mRNA also was increased in *acd6-1*, in agreement with our previous findings (Rate et al., 1999). Interestingly, the steady state mRNA levels of the SA-interacting and cell death–repressing genes *ACD11* and *LSD1* also were increased in *acd6-1* (Figure 1A).

To determine if any of the SA regulatory genes were important for the *acd6-1*–conferred phenotypes, we constructed *acd6-1 pad4* double mutants. The *pad4* mutation partially suppressed the SA-dependent dwarfism of *acd6-1*, as measured by rosette diameter. Additionally, *pad4* partially suppressed the increased SA levels and disease resistance of *acd6-1* to *P. syringae* pv *maculicola* strain DG3 (*Pma* DG3) (Table 1). Thus, the SA regulatory component PAD4 was partially required for the *acd6-1*–conferred dwarfism and disease resistance phenotypes. *acd6-1* plants also inhibit the extracellular bacterial pathogen *P. syringae* from delivering Avr proteins into host cells via the type-III secretion apparatus during infection, resulting in a reduced HR. However, *acd6-1* plants retain the ability to respond to Avr proteins when they are expressed directly in *acd6-1* cells (Rate et al., 1999). Thus, the cell walls of *acd6-1* may have altered properties that prevent the penetration of the type-III apparatus. Indeed, callose, the primary cell wall component induced by wounding and pathogen infection (Adam and Somerville, 1996), was increased in the *acd6-1* leaf cells (Figure 1B). Some *acd6-1* leaf cells also showed strong autofluorescence, possibly resulting from the accumulation and cross-linking of phenolic compounds in and around the cells that had died spontaneously (Figure 1B). These changes in *acd6-1* cell wall properties could reflect cell wall strengthening, a response reported in pathogen-infected plants (Hachler and Hohl, 1982; Kuc, 1990).

ACD6 Is a Novel Ankyrin Protein

We fine-mapped *ACD6* to a 93.1-kb region on chromosome IV. To identify the mutation, most of the coding sequences were

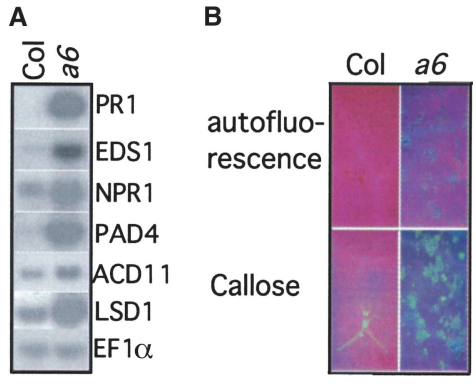

Figure 1. Defense Responses in the *acd6-1* Mutant.

(A) RNA gel blot analysis of defense gene induction. EF1α was used as a loading control. This experiment was repeated three times with similar results.

(B) Staining of cell walls. Fourth and fifth leaves from the wild type (Columbia [Col]) and the *acd6-1* mutant (*a6*) were examined for autofluorescence (top row) and callose (bottom row).

Table 1. Role of PAD4 in *acd6-1* Rosette Growth, Pathogenicity, and Defense Phenotypes

Genotype	Rosette Diameter (mm ± SE)	Growth of *Pma* DG3 (cfu/leaf disc ± SE)	Free SA (μg/g Fresh Weight ± SD)	Total SA (μg/g Fresh Weight ± SD)
acd6-1	6.6 ± 0.3[a] ($n = 30$)	$7.0 \times 10^3 \pm 4.2 \times 10^{3a}$ ($n = 6$)	6.8 ± 0.6[a] ($n = 3$)	89.6 ± 5.6[a] ($n = 3$)
acd6-1 pad4	17.3 ± 0.4[b] ($n = 30$)	$1.1 \times 10^5 \pm 2.4 \times 10^{4b}$ ($n = 6$)	2.6 ± 0.2[b] ($n = 3$)	38.7 ± 13.3[b] ($n = 3$)
pad4	47.9 ± 0.8[c] ($n = 13$)	$1.4 \times 10^8 \pm 1.4 \times 10^{7c}$ ($n = 6$)	0.8 ± 0.08[c] ($n = 3$)	0.9 ± 0.01[c] ($n = 3$)
Wild type	46.4 ± 0.5[c] ($n = 14$)	$4.3 \times 10^6 \pm 1.9 \times 10^{6d}$ ($n = 6$)	0.7 ± 0.06[c] ($n = 3$)	1.1 ± 0.14[c] ($n = 3$)

For rosette diameter measurements, 20-day-old plants were measured. Growth measurements were made 3 days after infection. The starting inoculum was 100 colony-forming units (cfu)/leaf disc. This experiment was repeated twice with similar results. Superscript letters indicate that values are statistically different ($P < 0.0001$ for rosette diameters; $P < 0.008$ for bacterial growth and SA levels).

amplified by PCR from both the wild type and the *acd6-1* mutant and sequenced on both strands. Only one point mutation (a C-to-T change) was found in one of the open reading frames (At4g14400), which was confirmed to be *ACD6*. Three additional lines of evidence confirmed the cloning of *ACD6*. First, a PCR-based derived cleaved amplified polymorphic sequence marker based on this point mutation cosegregated with the *acd6-1*–conferred phenotype in the mapping population and in backcrosses (data not shown). Second, an *ACD6* RNA interference (RNAi) construct introduced into the *acd6-1* mutant (*a6Ri*) suppressed the dwarfism, cell death, and disease resistance phenotypes (Figures 2A and 2B). Finally, the *ACD6-1* genomic clone introduced into the wild type (*a6G*) conferred the dwarfism, cell death, increased PR-1 transcript accumulation, and disease resistance phenotypes seen in the *acd6-1* plants (Figures 2A to 2C).

ACD6 encodes a novel protein containing ankyrin repeats and putative transmembrane regions (Figure 3). Isolation of the cDNA clone confirmed the size of the ACD6 mRNA detected by RNA gel blot analysis (2 kb). *ACD6* is composed of five exons and is predicted to encode a protein with 670 amino acids. The first exon is untranslatable because it contains in-frame stop codons. Although not well described in plants, such untranslated exons are common in animals (for example, see Chen et al., 1996). The N-terminal region (~70 amino acids) does not match any regions with known functions. The middle part contains nine ankyrin repeats, based on the SMART protein domain prediction program. Several transmembrane helices were predicted in the C-terminal region, although the exact number (usually between five and seven) varied depending on the algorithm. The missense mutation in *acd6-1* caused a Leu-to-Phe substitution at position 591 of the protein in a predicted transmembrane helix (Figure 3). This change did not alter the predicted topology of the transmembrane-spanning region.

Many sequences from higher plants in GenBank shared significant similarity with ACD6, both at the amino acid level and in the organization of the predicted functional regions. With an expectation value of $<10^{-4}$, there were 34 genes encoding ACD6-like proteins in the Arabidopsis genome that contained ankyrin and transmembrane regions. The overall similarity between ACD6 and the ACD6-like proteins varied from 9 to 64%.

ACD6 Expression Is SA Dependent

acd6-1 and wild-type plants harboring the ACD6-1 transgene both showed increased steady state ACD6-1 and/or ACD6 mRNA accumulation (Figure 2C). Because SA levels in *acd6-1* are increased (Vanacker et al., 2001), it seemed possible that ACD6 could be regulated by SA. Indeed, ACD6 mRNA abundance increased after BTH treatment in both time-course and dose-response experiments (Figures 4A and 4B). Compared with *ACD6*, the *PR1* gene was much less responsive to BTH (Figures 4A and 4B). Unlike *ACD6*, the *ACD11* and *LSD1* genes associated with defense and cell death were not induced by BTH (Figure 4B).

Consistent with the BTH treatment results, mutants with high SA levels, such as *agd2*, *dnd1*, and *acd5* (Yu et al., 1998; Greenberg et al., 2000; Rate and Greenberg, 2001), had high steady state *ACD6* mRNA levels (Figure 4C). Conversely, plants with low SA levels (*nahG*), impaired SA regulation (*pad4*), or impaired signaling (*npr1*) had greatly reduced accumulation of *ACD6* mRNA. In addition, a mutation in a negative regulator of systemic acquired resistance (SNI1) potentiated the expression of ACD6 mRNA. Consistent with the findings that *acd6-1* acts partially through NPR1 and PAD4 (Rate et al., 1999) (Figure 1A, Table 1), the *acd6-1 npr1* and *acd6-1 pad4* double mutants had reduced *ACD6-1* mRNA levels relative to that seen in *acd6-1* alone. The pattern of *PR1* mRNA accumulation was similar to that of ACD6, with some variations in the level of induction (Figure 4C). These data suggest that a threshold level of SA or SA signaling is required for the basal ACD6 mRNA accumulation, which can be induced further when SA levels are increased.

ACD6 Expression Is Induced Systemically but Not Locally during Pathogen Infection

Because the steady state ACD6 mRNA level was under SA regulation, we further examined the expression of the *ACD6* gene during *P. syringae* infection, a condition known to induce SA accumulation (Zhou et al., 1998). In a 48-h time course of wild-type plants infected with *Pma* DG3 (virulent) and *Pma* DG34 (avirulent, carrying *avrRpm1*), no changes in the steady state level of *ACD6* transcript were observed. However, PR1 transcript levels were increased upon infection (data not shown). Because SA levels also increase in systemic tissue of *P. syringae*–infected plants (Delaney et al., 1995; Summermatter et al., 1995), we tested the steady state ACD6 mRNA level in the uninfected leaves adjacent to the infected leaves. The abundance of the ACD6 mRNA was higher in the systemic tissue of infected plants than in the tissue from mock-treated plants. As expected, the level of the PR1 mRNA also was induced systemically (Figure 5). Thus, *ACD6* is a systemically induced gene.

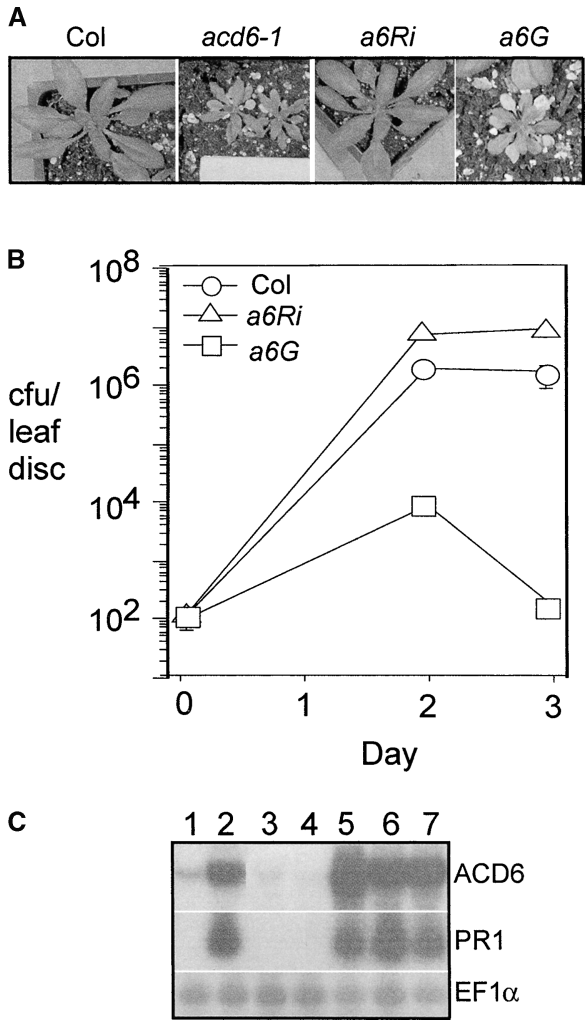

Figure 2. Suppression and Recapitulation of *acd6-1* Mutant Phenotypes in Transgenic Plants.

(A) Three-week-old wild-type and transgenic plants. *a6Ri*, *acd6-1* transformed with an ACD6RNAi construct; *a6G*, Col transformed with an *ACD6-1* genomic clone. At least 25 independent *a6Ri* and *a6G* lines behaved similarly to the lines shown.

(B) *P. syringae* growth curve. Col, *a6Ri*, and *a6G* plants were infected with *Pma* DG3 (OD_{600} = 0.0001). Bars indicate standard errors; in some cases, the symbol obscures the error bars. The growth of bacteria in the three hosts was significantly different on days 2 and 3 ($P < 0.001$ [*t* test], $n = 6$). Similar results were obtained with several additional independent transformants (data not shown). This experiment was repeated three times with similar results. cfu, colony-forming units.

(C) RNA gel blot analysis of the steady state accumulation of ACD6 and PR1 mRNAs. EF1α was used as a loading control. Total RNA was extracted from Col (lane 1), *acd6-1* (lane 2), two *a6Ri* lines (lanes 3 and 4), and three *a6G* lines (lanes 5 to 7).

ACD6 Expression Requires Light

Light is important for at least some aspects of SA signal transduction (Genoud and Metraux, 1999; Genoud et al., 2002). To test the effect of light on the expression of ACD6, wild-type plants maintained in the 16-h-light/8-h-dark cycle were moved to constant dark 4 h after the lights came on in the morning. Within 1 h, the abundance of the ACD6 mRNA was reduced significantly, and the decrease continued for up to 48 h. Interestingly, when the 48-h dark-treated plants were exposed to light again for only 4 h, the steady state level of ACD6 mRNA was restored back to the level seen in the control plants maintained in the normal light/dark cycle (Figure 6A). In agreement with these findings, the normal dark period also resulted in the downregulation of ACD6 (data not shown).

BTH-induced ACD6 mRNA accumulation in wild-type plants was reduced greatly in the dark (Figure 6B). A similar suppression by dark was found with the PR1 transcript. However, for EDS1 and PAD4 mRNAs, only the steady state basal but not the BTH-induced transcripts were reduced slightly during the 24-h dark treatment (Figure 6B). In addition, the level of NPR1 mRNA under basal or BTH induction conditions was unaffected by light. Thus, light differentially regulated defense gene expression.

Consistent with the requirement of light for ACD6 transcript accumulation, it also was required for the *acd6-1*–conferred cell death phenotypes. Under normal light conditions (16 h of light/8 h of dark), SA-depleted *acd6-1-nahG* plants that lacked cell death showed cell death upon BTH treatment (Rate et al., 1999). However, in constant dark, cell death was suppressed completely (Figure 6C).

ACD6 Loss-of-Function Plants Are More Susceptible to Disease and Have Attenuated Defenses

In *a6Ri* plants, suppression of the steady state ACD6 mRNA levels correlated well with a modest increase in the susceptibility of the plants to *Pma* DG3 (Figures 2B and 2C) as well as to the congenic avirulent strains *Pma* DG6 and *Pma* DG34 carrying *avrRpt2* and *avrRpm1*, respectively (data not shown). Wild-type plants harboring the *ACD6* RNAi construct behaved similarly to *a6Ri* plants (data not shown). The expression of genes with the closest similarity to *ACD6* (At4g14390 [*ACL1*] and At4g05040 [*ACL2*]) was not affected by the RNAi transgene (data not shown). These genes had 86 and 79% similarity, respectively, at the DNA level with *ACD6* in the region of the RNAi. Other genes related to ACD6 showed <55% similarity at the DNA level in this region, making it less likely that the ACD6 RNAi would affect their expression.

However, to be certain that the RNAi results are attributable to the specific downregulation of ACD6, we isolated and characterized a loss-of-function *acd6* mutant with a T-DNA insertion in the fourth exon of *ACD6* (*acd6-T*) in the Wassilewskija (Ws) ecotype background (Figure 3). Using an ACD6-specific probe complementary to the 3′ region of ACD6, no ACD6 transcript was detectable by RNA gel blot analysis in *acd6-T*, whereas a 2.0-kb band was visible in Ws (Figure 7A, left blot). It seemed possible that the T-DNA insertion could lead to the accumulation of a truncated *ACD6* transcript predicted to be 1.2 kb, shorter than any of the predicted transcripts of *ACD6*-like genes. However, with the full-length *ACD6* cDNA as a probe on a duplicate blot, neither the 1.2-kb truncated transcript nor the full-length ACD6 mRNA was observed in *acd6-T* plants (Figure 7A, right blot). A transcript of ~1.8 kb, which also was detected with an *ACL2* (At4g05040)-specific probe (data not shown), was visible in both wild-type

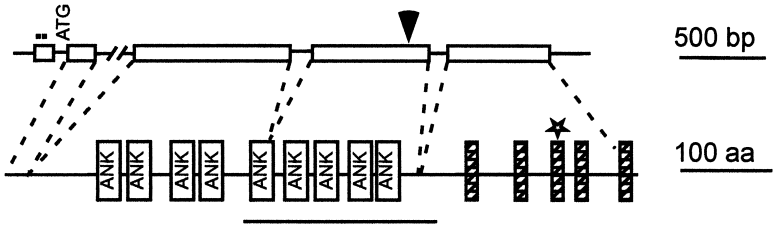

Figure 3. *ACD6* Encodes a Putative Protein with Ankyrin and Transmembrane Regions.

Structures of the *ACD6* genomic DNA (top) and the predicted ACD6 protein (bottom). In the DNA structure, the boxes indicate exons, lines indicate untranslated regions and introns, and dots indicate the in-frame stop codons in exon 1. ATG is the putative translation start site. The arrowhead indicates the T-DNA insertion site in *acd6-T*. In the protein structure, boxes labeled ANK indicate ankyrin repeats, hatched boxes indicate transmembrane helices, and the star indicates the Leu-to-Phe mutation in *acd6-1*. The line below the structure indicates the fragment used for the *ACD6* RNAi construct. The broken lines between the two structures connect the exons to their encoded protein regions. aa, amino acids.

and the *acd6-T* plants. This band was not induced significantly by treatments that induced ACD6 transcript accumulation, such as with the SA agonist BTH (Figure 7A). Thus, *acd6-T* appears to be a null mutant.

The *acd6-T* mutant was morphologically normal and displayed modestly increased susceptibility to *P. syringae* pv *tomato* strain DC3000 (*Pto* DC3000) as well as to *Pto* DC3000 carrying the *avrRpt2* avirulence gene (Figures 7B to 7D) (*Pma* DG3 was not used because it does not cause disease on the parental Ws ecotype). *acd6-T*/*ACD6* heterozygotes showed wild-type disease susceptibility, suggesting that the *acd6-T* mutation was recessive (data not shown). Furthermore, the susceptibility of *acd6-T* plants to *Pto* DC3000 was complemented by the *ACD6* genomic clone (Figure 7B).

Interestingly, the *acd6-T* plants were less responsive to the SA agonist BTH. Thus, *acd6-T* plants treated with BTH showed impaired disease resistance early during infection (on day 2) with *Pto* DC3000, relative to the wild-type parent Ws (Figure 7D). This impaired resistance of BTH-treated *acd6-T* plants was accompanied by an increase in the severity of disease symptoms elicited by *Pto* DC3000 (Figure 7D, inset). Consistent with these observations, *acd6-T* plants showed compromised induction of PR1 mRNA accumulation upon BTH treatment relative to that seen in Ws (Figure 7E). In addition to reduced responsiveness to BTH, *acd6-T* plants had transiently reduced SA production and PR1 expression after *P. syringae* infection (Figure 8; note levels at 12 h). In summary, ACD6 is important for disease resistance and the timely activation of defenses against virulent *P. syringae*.

Plants Overexpressing ACD6 Are More Resistant to Disease and Have Increased Defenses

To determine whether ACD6 was sufficient to confer disease resistance, we introduced extra copies of *ACD6* genomic clones into wild-type Col plants. Strikingly, 13 independent transformants carrying at least one extra copy of *ACD6* were more resistant to *Pto* DC3000. Examples of the growth of *Pto* DC3000 in two such lines are shown in Figure 9. Plants with extra copies of ACD6 also showed increased steady state ACD6 mRNA (Table 2). These plants all had modestly increased SA levels, although only some lines had statistically significant increases in SA (Table 2). Additionally, 2 of the 13 independent transgenic lines showed spontaneous microscopic cell death, whereas the rest showed cell death after treatment with BTH (Table 2 and data not shown). All of the transgenic plants were similar in size to

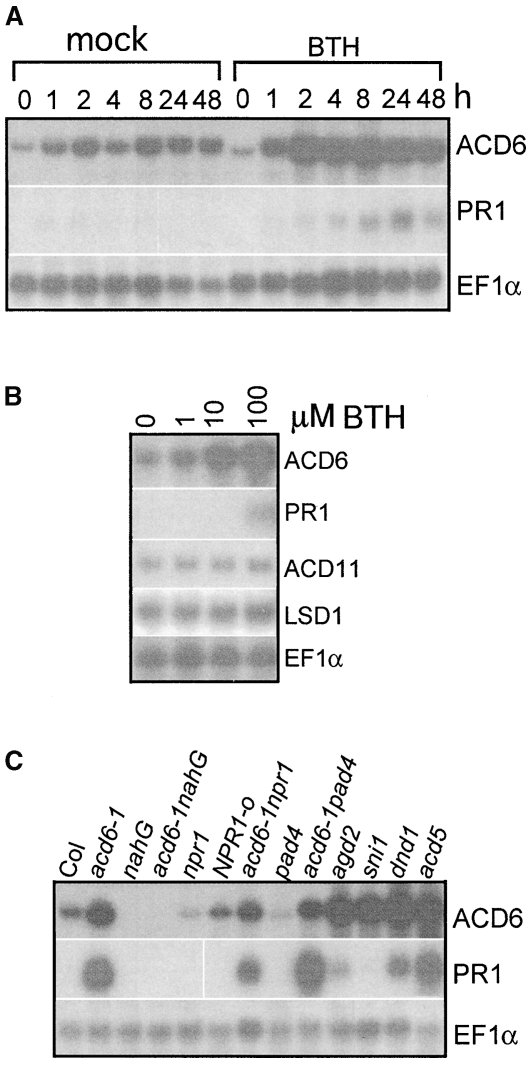

Figure 4. SA-Dependent *ACD6* Gene Expression.

(A) Time-course induction by 100 μM BTH. This experiment was repeated three times with similar results.

(B) BTH dose-dependent gene induction. Col leaves were collected 24 h after treatment with BTH at the indicated concentrations. This experiment was repeated three times with similar results.

(C) Gene expression in different genotypes in the Col background. RNA samples were extracted from 20-day-old plants. This experiment was repeated twice with similar results.

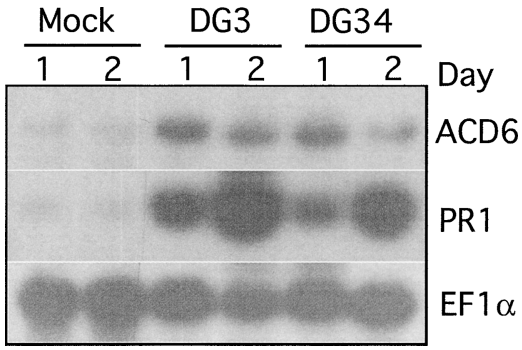

Figure 5. *ACD6* Gene Expression during Pathogen Infection.

Col leaves were inoculated with 10 mM $MgSO_4$ (mock treatment), *Pma* DG3, and *Pma* DG34 (carrying *avrRpm1*) at $OD_{600} = 0.01$. RNA was extracted at the indicated times from the uninfected leaves of inoculated plants. This experiment was repeated three times with similar results.

wild-type plants. Collectively, these data support a role for ACD6 as a necessary and dose-dependent component of the defense response against virulent *P. syringae* and suggest a role for ACD6 in activating SA-dependent cell death.

DISCUSSION

SA has been known for many years to be a key signal molecule that mediates plant disease resistance. However, the mechanism by which SA is regulated, perceived, and transduced is understood only in outline. In particular, only a few SA-regulated signaling components are known to be important for disease resistance. Here, we showed that the Arabidopsis *ACD6* gene encodes a novel component of the light-dependent branch of the SA signaling pathway. The abundance of the ACD6 mRNA is regulated by both light and SA. Furthermore, ACD6 transcript is induced in the systemic tissue of *P. syringae*–infected plants. Finally, we showed that plants lacking *ACD6* transiently produce less SA, are less responsive to the SA agonist BTH, and are more susceptible to virulent *P. syringae*. By contrast, plants with an extra copy(s) of ACD6 have modestly increased SA levels, are more resistant to virulent *P. syringae*, and show BTH-inducible and/or spontaneous cell death. These observations are consistent with our previous findings that SA-depleted *acd6-1* gain-of-function plants are more responsive to the SA agonist BTH and that *acd6-1* plants show SA-dependent disease resistance and defenses (Rate et al., 1999). Thus, ACD6 appears to be a regulator and an effector of the SA pathway.

ACD6 encodes a novel protein with ankyrin repeats and transmembrane regions, which are hallmarks of some signal transduction proteins. Ankyrin repeats are involved in diverse processes but share the property that they often are involved in protein–protein interactions (Sedgwick and Smerdon, 1999). The best characterized ankyrin protein from plants is NPR1/NIM1, which is involved in SA-dependent disease resistance and in an SA-independent resistance response elicited by certain root-associated bacteria (Pieterse et al., 1998). NPR1 localizes to the nucleus during SA signaling (Kinkema et al., 2000) and functions as a transcriptional coactivator to regulate the defense response (Zhang et al., 1999; Fan and Dong, 2002). ACD6 does not show significant similarity to NPR1, although both proteins have repeat regions in the ankyrin family. The possible transmembrane helices in ACD6 suggest that ACD6 may act at the membrane to activate defenses, as has been shown or suggested for other defense signaling components (Century et al., 1997; Boyes et al., 1998; Falk et al., 1999; Jirage et al., 1999; Mackey et al., 2002). Although the mutant ACD6-1 protein is not predicted to have altered membrane topological properties, it may have an altered

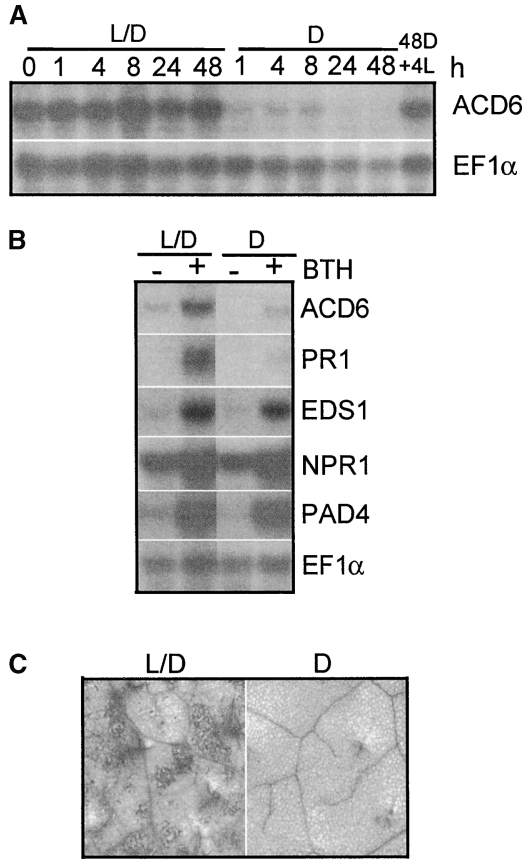

Figure 6. Light Is Required for *ACD6* Expression and the *acd6-1* Phenotype.

(A) A time course of steady state ACD6 mRNA accumulation. Twenty-day-old Col plants grown in a 16-h-light/8-h-dark cycle were kept in these conditions (L/D) or shifted to continuous dark (D) 4 h after the lights normally came on (time 0) for the indicated times and then switched back to light for 4 h (4L).

(B) Effect of light on BTH-induced defense gene expression. Col plants were treated with 100 μM BTH and subjected to the normal 16-h-light/8-h-dark cycle (L/D) or dark treatment (D) for 24 h.

(C) Cell death staining. *acd6-1-nahG* plants were treated with 100 μM BTH or water and grown in the normal 16-h-light/8-h-dark (L/D; left) or 24-h dark (D; right) condition for 1 day. The fourth leaves were stained with trypan blue to detect cell death. Wild-type control and water-treated *acd6-1-nahG* tissue showed no cell death under the conditions used here (data not shown). These experiments were repeated three times with similar results.

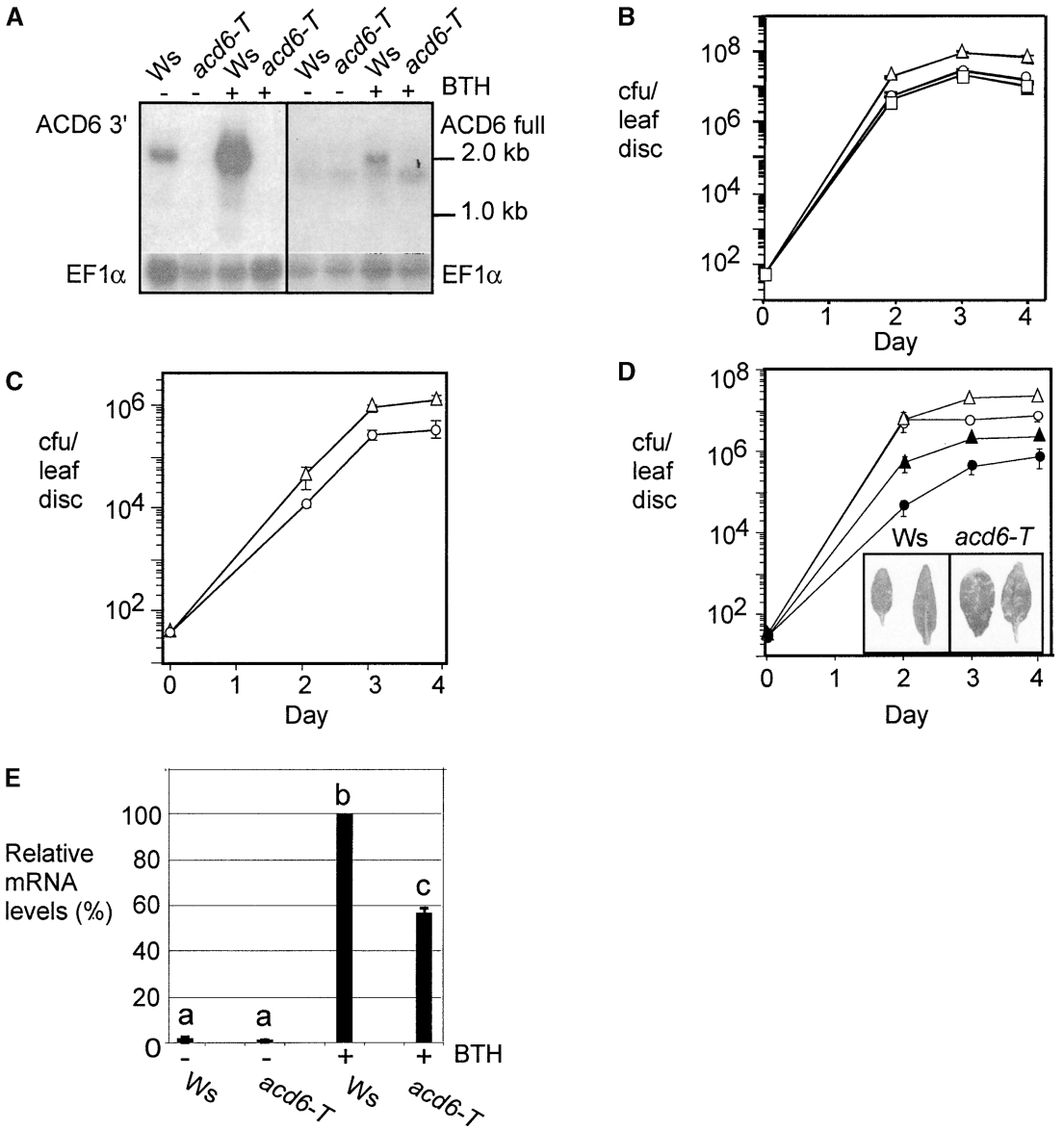

Figure 7. Defense Response and Disease Susceptibility of an *acd6-T* Loss-of-Function Mutant.

(A) Steady state accumulation of ACD6 mRNA using an *ACD6*-specific probe (ACD6 3′) (left) and the full-length *ACD6* cDNA probe (ACD6 full) (right). EF1α served as a loading control. Wild-type Ws and the *acd6-T* mutant were treated with 300 μM BTH or water for 1 day.

(B) and **(C)** Disease susceptibility and complementation of *acd6-T* plants. Wild-type Ws (circles), *acd6-T* (triangles), and/or *acd6-T* complemented with genomic ACD6 (squares) were infected with *Pto* DC3000 **(B)** or *Pto* DC3000 carrying *avrRpt2* **(C)** at an OD_{600} = 0.0001. The growth of *Pto* DC3000 in *acd6-T* was significantly different from that in the wild type and complemented on days 2, 3, and 4 ($P < 0.02$ [*t* test], $n = 6$). The growth of *Pto* DC3000 carrying *avrRpt2* in *acd6-T* was significantly different from that in the wild type on days 3 and 4 ($P < 0.007$ [*t* test], $n = 6$). These experiments were repeated twice with similar results. cfu, colony-forming units.

(D) Reduced disease resistance of *acd6-T* treated with BTH. Wild-type Ws and *acd6-T* were pretreated with 300 μM BTH or water for 2 days and then subjected to infection by *Pto* DC3000 (OD_{600} = 0.0001). Open circles, water-treated Ws; closed circles, BTH-treated Ws; open triangles, water-treated *acd6-T*; closed triangles, BTH-treated *acd6-T*. Bars indicate standard errors; in some cases, the error bars are obscured by the symbols. For water-treated plants, the growth of bacteria in *acd6-T* was significantly different from that in the wild type on days 3 and 4 ($P < 0.001$ [*t* test], $n = 6$). For BTH-treated plants, the growth of bacteria in *acd6-T* was significantly different from that in the wild type on days 2, 3, and 4 ($P < 0.001$ [*t* test]). The inset shows BTH-treated and infected leaves taken 3 days after the infection. Note the increased lesion numbers on the *acd6-T* plants. This experiment was repeated three times with similar results.

(E) Quantitation of the relative abundance of PR1 transcript in Ws and *acd6-T* plants after BTH treatment. Plants were treated as in **(A)**. PR1 mRNA levels were normalized to the level of EF1α mRNA. Different letters indicate that the values are significantly different from each other ($P < 0.01$). The data were averaged from four independent experiments.

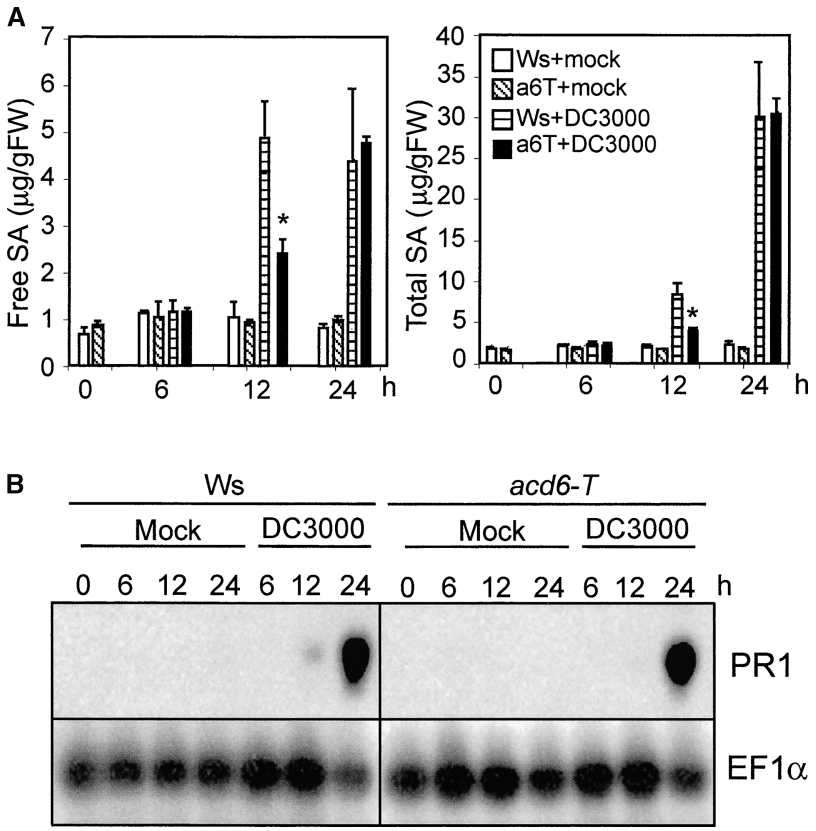

Figure 8. Reduced Defenses in *acd6-T* Plants.

(A) Reduced SA levels in *P. syringae*–infected *acd6-T* plants. Plants were inoculated with 10 mM $MgSO_4$ or *Pto* DC3000 at $OD_{600} = 0.01$. Samples were analyzed in triplicate. Asterisks indicate $P < 0.05$ at 12 h. FW, fresh weight.

(B) Reduced PR1 expression in *P. syringae*–infected *acd6-T* plants. Plants treated as in **(A)** were used for RNA isolation. This experiment was repeated twice with similar results.

conformation that switches it to a constitutively active form to amplify SA signaling.

The positive activators of SA accumulation, EDS1 and PAD4, have been proposed to form a signal-amplification loop with SA (Falk et al., 1999; Jirage et al., 1999). Like *eds1* and *pad4*, *acd6-T* is compromised for SA production upon infection. However, the effect of *acd6-T* on SA production after virulent *P. syringae* infection was more transient than that reported for *pad4* and *eds1* (Jirage et al., 1999; Feys et al., 2001). Consistent with a role for ACD6 in the regulation of SA production, ACD6-overexpressing plants have modestly increased SA levels in uninfected plants. Unlike ACD6, the regulation of EDS1 and PAD4 appears to be largely independent of light. The expression of *PAD4* and *EDS1* is much higher in the *acd6-1* gain-of-function mutant, whereas the loss of PAD4 function results in lower expression of *ACD6* (Figures 1A and 4C). In addition, *pad4* partially suppresses the *acd6-1* disease resistance and dwarf phenotypes shown previously to be SA dependent (Table 1). Thus, this suppression is likely the result of reduced SA levels in *acd6-1 pad4* plants relative to *acd6-1* alone. These results collectively suggest an interaction of the signaling pathways mediated by EDS1, PAD4, and ACD6, possibly by regulating SA and SA signaling, the key common molecule in the Arabidopsis defense response.

The regulation of ACD6 by SA and light is likely to occur at the level of transcriptional activation, because the promoter region of ACD6 has many predicted SA and light *cis*-regulatory elements (data not shown). Other possible modes of regulation, such as those that affect mRNA and/or protein stability, also are possible. Although we have not explored the molecular basis of the light requirement for ACD6 transcript accumulation, there is precedent for a role for the photoreceptor phytochrome in amplifying SA signaling to induce the transcription of some genes and to control cell death during HR (Genoud et al., 2002). The regulation of ACD6 by both light and SA suggests one way that plants can integrate external and internal signals to effect the amplification of the defense response.

ACD6 is a member of a large plant-specific gene family. Of the 158 Arabidopsis proteins with ankyrin repeat regions, 34 have the same overall organization as ACD6 and also are similar to ACD6 at the amino acid level in both the putative ankyrin repeat regions and in the transmembrane regions. None of the ACD6-like proteins have been assigned a function in Arabidopsis. A

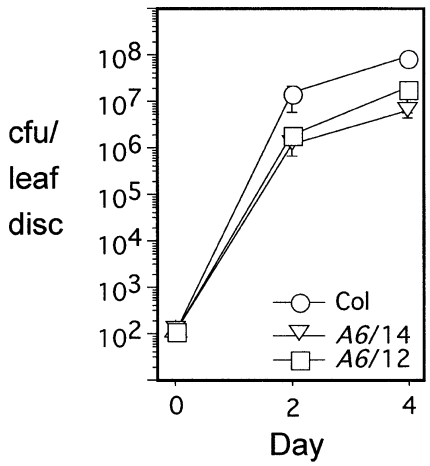

Figure 9. Growth of *P. syringae* in Plants with Extra Copies of *ACD6*.

Wild-type (Col) and T_2 plants from two independent transformants with extra copies of ACD6 (lines 12 and 14) were infected with *Pto* DC3000 at $OD_{600} = 0.0001$. Bars indicate standard errors ($n = 6$); in some cases, the error bars are obscured by the symbols. Bacteria grew significantly more in the wild type than in all of the lines carrying extra copies of ACD6 ($P < 0.01$). This experiment was repeated twice with similar results. cfu, colony-forming units.

number of the *ACD6*-like genes are located in a large cluster on chromosome IV, which may make them susceptible to intergenic recombination, similar to some disease resistance (*R*) loci containing clustered genes (Ellis et al., 2000). It is intriguing that some ecotypes of Arabidopsis also have a duplicated copy of *ACD6* lacking introns, although whether the additional copies have functional promoters is unclear (Rate, 2000). This observation could indicate that ACD6 is under adaptive selection. Because ACD6 and its related proteins represent one of the largest protein families in Arabidopsis, it is striking that no other loss-of-function mutants in the ACD6 family have been reported. Possibly, *ACD6* and some of the *ACD6*-like genes have overlapping and/or redundant functions that have been difficult to detect in loss-of-function mutant screens. It also is possible that some of the *eds* mutants that could not be mapped, such as *eds4* (Gupta et al., 2000), have defects in *ACD6* or *ACD6*-like genes. This possibility highlights the utility of obtaining gain-of-function mutants to identify the components of signaling pathways.

In some host–pathogen interactions, SA is important for cell death during the HR (Rate et al., 1999) and may play a role in micro-HR formation during systemic acquired resistance (Alvarez et al., 1998). Does ACD6 play a role in cell death control? The *acd6-1* gain-of-function mutant shows spontaneous SA- and light-dependent cell death (Rate et al., 1999). This cell death is localized, possibly as a result of the action of the cell death–suppressor genes *ACD11* and *LSD1*, whose transcripts are upregulated in *acd6-1*. The loss-of-function mutant (*acd6-T*), although showing enhanced disease susceptibility and increased disease symptoms (especially in BTH-treated plants; Figure 7D), did not show an obvious difference in the HR in dose-response experiments with two different avirulent pathogens (data not shown). However, other genes in the *ACD6* family may act redundantly to control SA-dependent cell death. Indeed, a number of *ACD6* family members were found in stress-induced cDNA libraries (H. Lu and J.T. Greenberg, unpublished data). Interestingly, BTH treatment of ACD6-overexpressing plants elicits localized cell death, suggesting that ACD6 does play a role in cell death control. The isolation of additional loss- and gain-of-function mutants in ACD6 family members should be valuable for discerning their possible functions in mediating both disease resistance and/or cell death.

METHODS

Plant Materials, Treatments, and Pathogen Infection

Seeds of *Arabidopsis thaliana acd6-1*, *npr1-1*, *acd6-1 npr1*, *acd6-1-nahG*, *nahG*, *NPR1-o* (an NPR1-overexpressing line), *acd5*, *agd2*, *pad4-1*, *sni1*, and *dnd1* were in the Columbia (Col) background. Isolation and/or construction of *acd6-1*, *acd6-1 npr1*, *acd6-1-nahG*, *acd5*, and *agd2* were described previously (Rate et al., 1999; Greenberg et al., 2000; Rate and Greenberg, 2001). *npr1-1*, *NPR1-o*, and *sni1* (Cao et al., 1994, 1998; Li et al., 1999) were from Xinnian Dong (Duke University, Durham, NC). *nahG* B15 (Delaney et al., 1995) seeds were from Syngenta (Research Triangle Park, NC). *dnd1* (Yu et al., 1998) was from Andrew Bent (University of Wisconsin, Madison). *pad4-1* (Glazebrook et al., 1997) was from Jane Glazebrook (University of Minnesota, St. Paul). *acd6-1 pad4* was constructed by crossing *acd6-1* with *pad4-1*. Double homozygous mutants were identified using cleaved amplified polymorphic sequence markers

Table 2. Relative ACD6 mRNA Accumulation, Cell Death, and SA Levels in Plants with Extra Copies of ACD6

Line	Transgene Copy Number	Relative ACD6 mRNA Level ($n = 2$)	BTH-Induced Cell Death	Free SA (μg/g Fresh Weight; $n = 3$)	Total SA[a] (μg/g Fresh Weight; $n = 3$)
Untransformed	0	1.0	−	0.8 ± 0.4	1.1 ± 0.6
3	1	3.2 ± 0.5	+	1.0 ± 0.2	2.2 ± 0.7 ($P = 0.114$)
4	1	5.4 ± 0.3	+	1.3 ± 0.05	2.7 ± 0.6 ($P = 0.030$)
12	2	2.4 ± 0.1	+	1.2 ± 0.2	2.9 ± 0.6 ($P = 0.020$)
14	≥3	5.8 ± 0.1	+[b]	0.7 ± 0.1	2.5 ± 0.7 ($P = 0.054$)

The copy number of the representative homozygotic ACD6 overexpression lines was determined by germinating the T_1 seeds on agar medium containing Murashige and Skoog (1962) salts and 40 μg/mL BASTA. The relative ACD6 mRNA level was normalized to the level of EF1α mRNA. To score cell death on leaf tissue, plants were treated with 100 μM BTH or water for 4 days and stained with trypan blue.

[a] *t* test results shown are for the wild type compared with each overexpression line. This experiment was repeated twice with similar results.

[b] This line also showed some cell death before BTH treatment.

in the F2 generation. An *acd6* T-DNA insertion mutant (*acd6-T*) in the Wassilewskija (Ws) background was obtained by screening the T-DNA insertion library at the University of Wisconsin (www.biotech.wisc.edu).

Arabidopsis plants were grown as described (Rate et al., 1999) in a 16-h-light (6 AM to 10 PM)/8-h-dark cycle. Unless specified otherwise, each treatment of 20-day-old Arabidopsis plants was started at ~10 AM. Benzo(1,2,3)thiadiazole-7-carbothioic acid (benzothiazole [BTH]) was a gift from Robert Dietrich (Syngenta). For BTH treatment, plants were sprayed with BTH at the concentrations indicated in the legends to Figures 4, 6, and 7 and Table 2 until all of the leaves were wet. BTH treatment of Ws and *acd6-T* was performed on 16- to 18-day-old plants. During dark treatments, plants were covered with an aluminum foil–wrapped plastic dome. The top of the dome was cut off to minimize the increase of humidity. *Pseudomonas syringae* pv *maculicola* strain DG3 (a *recA* derivative of ES4326), *P. syringae* pv *maculicola* strain DG6 (an ES4326 *recA* derivative expressing the type-III effector *avrRpt2*), and *P. syringae* pv *maculicola* strain DG34 (an ES4326 *recA* derivative expressing the type-III effector *avrRpm1*) were described previously (Guttman and Greenberg, 2001). *P. syringae* pv *tomato* strain DC3000 (*Pto* DC3000) was obtained from F.M. Ausubel (Massachusetts General Hospital and Harvard University, Boston, MA). *Pto* DC3000 carrying *avrRpt2* was constructed by transforming *Pto* DC3000 with pMMXR1 (Dong et al., 1991). Bacterial culturing, infection, and growth curve analysis were performed as described previously (Greenberg et al., 1994), except that *Pto* DC3000 was plated on Luria-Bertani agar (Sambrook et al., 1989) containing 100 μg/mL rifampicin.

RNA Gel Blot Analysis

The fourth and fifth leaves from treated and control plants were used for total RNA extraction as described (Rate et al., 1999). For probes, each DNA fragment shown in Table 3 was amplified by PCR from Col genomic DNA or ACD6 cDNA as indicated in the table, confirmed by sequencing, and labeled with α-^{32}P-dCTP by primer extension using a corresponding reverse primer. Unless specified otherwise, the expression of ACD6 was detected with ACD6 3′, an ACD6-specific probe. The blots were hybridized as described (Rate et al., 1999). Each experiment was repeated at least twice.

Salicylic Acid Measurements

The fourth and fifth leaves from treated and control plants were used for salicylic acid extractions and analysis as described (Vanacker et al., 2001).

Histochemical Staining

Autofluorescence and callose staining with 0.01% aniline blue of leaf tissue was performed as described (Adam and Somerville, 1996) and examined with epifluorescence illumination from an Axioskop microscope (Zeiss, Jena, Germany). All tissues were photographed with the same exposure time. Trypan blue staining for cell death detection was described previously (Rate et al., 1999).

Positional Cloning of *ACD6*

Recombinant plants were created by crossing the homozygous *acd6-1* mutant with ecotype Landsberg. The F_2 plants showing the *acd6-1* homozygous phenotype (dwarfed and harboring cell death patches on the leaves) in 25% of the population were used for mapping. After scoring 1384 homozygous *acd6-1* recombinant plants with 42 cleaved amplified polymorphic sequence or simple sequence length polymorphism markers, *acd6-1* was placed between two markers, PG1-2 and PG19 (Table 3), on chromosome IV, spanning 93.1 kb of sequence and containing 22 putative genes. The coding regions of 15 of the 22 genes (~40 kb) from both the wild type and the mutant were amplified by PCR and sequenced. The genes not sequenced comprised retrotransposons and

Table 3. DNA Fragments Used in This Report

Fragment Name	Primer Names	Primer Sequence
ACD6-full*	ACD6-full (rev); ACD6-full (for)	5′-TTCGGAACACGCCACACAACCA-3′; 5′-GCGATGGACAGTTCTGGAGCAG-3′
ACD6 3′ (specific)*	ACD6 3′ (rev); ACD6 3′ (for)	5′-CTTGTGAAGTATCGTCTATATTGCTCTTG-3′; 5′-AAGCTTGTTTTGGTTGTGTGG-3′
ACD6-RNAi*	ACD6-RNAi (rev); ACD6-RNAi (for)	5′-AAACGGATCCATTTAAATAAGAGCCGCTACCACGAGAAGAG-3′; 5′-ATCTACTAGTGGCGCGCCTGAAGGCCAAAAGTATAGGTGTC-3′
ACD6-screen	ACD6-screen (rev); ACD6-screen (for)	5′-TCTCTTCGAATGATATACTTTTCACGCTT-3′; 5′-CAATCGTAAGTGATACAAAATAGTTAGAAGTG-3′
ACD11	ACD11 (rev); ACD11 (for)	5′-TTTTTCAGGCTCTTCACCAATCA-3′; 5′-CATTAGCCGTGCCAGTTTGTAGGATG-3′
ACL1-specific	ACL1-specific (rev); ACL1-specific (for)	5′-TGTCATCATCATCAAAGGAAATACGAAAC-3′; 5′-AGTACGCTGGTGACTTTCTCG-3′
ACL2-specific	ACL2-specific (rev); ACL2-specific (for)	5′-GTCAATGATACCAAGAAGAGGGGG-3′; 5′-AAGTGAGTTTCTTCCCGAATCCCT-3′
EF1α	EF1α (for); EF1α (rev)	5′-GCTGTCCTTATCATTGACTCCACC-3′; 5′-TCATACCAGTCTCAACACGTCC-3′
LSD1	LSD1 (rev); LSD1 (for)	5′-CAACGGACATGGGGTTTTCTAC-3′; 5′-GGTTGCCCATGCTCCTTCCAG-3′
NPR1	NPR1 (rev); NPR1 (for)	5′-CACAATTGATACTATCTCTATTGG-3′; 5′-TTTCGGCGATCTCCATTGCAGC-3′
PAD4	PAD4 (rev); PAD4 (for)	5′-GCGATGCATCAGAAGAG-3′; 5′-TTAGCCCAAAAGCAAGTATC-3′
PG1-2	PG1-2 (for); PG1-2 (rev)	5′-GCCCAAGGAAAGCGTCGT-3′; 5′-GCAGACTCATCACCCCCATACTTA-3′
PG19	PG19 (for); PG19 (rev)	5′-CGGCTTTGGCTTGCTTTCACC-3′; 5′-CCCGTTGTCTCGCATATTCCATTG-3′
PR1	PR1 (rev); PR1 (for)	5′-CACATAATTCCCACGAGGATC-3′; 5′-GTAGGTGCTCTTGTTCTTCCC-3′

Asterisks indicate fragments amplified from an ACD6 cDNA by PCR. The rest of the fragments were amplified from the Col genome. for, forward; rev, reverse.

reverse transcriptase or were at the borders of the region defined by recombination.

To obtain an *ACD6-1* genomic clone, 15 μg of genomic DNA from the *acd6-1* mutant was digested with BamHI and SacI and separated on a 10 to 40% sucrose gradient. Fractions with 10 to 15 kb of DNA were pooled and ligated into binary vector pBIN19 by electroporating into *Escherichia coli* strain DH5α. The resulting library was plated and screened by colony hybridization using an α-^{32}P-dCTP–labeled 2663-bp DNA fragment called ACD6-screen as a probe (Table 3). One positive clone was found and was confirmed to contain the *ACD6-1* genomic region by sequencing and restriction map analysis. A full-length *ACD6* cDNA was isolated by screening a Col cDNA library (a kind gift from Fumiaki Katagiri, University of Minnesota, St. Paul) using colony hybridization.

DNA Construction and Plant Transformation

The 7670-bp EcoRV-SacI fragment of the *ACD6-1* gene was further subcloned into pBIN19 and used to transform Col using the dipping method (Clough and Bent, 1998). To construct an *ACD6* RNA interference clone, a 691-bp DNA fragment from *ACD6* cDNA was amplified by PCR (Table 3) and cloned as two inverted repeats into the binary vector pFGC1008 (http://ag.arizona.edu/chromatin/fgc1008.html). This construct was used to transform Col and *acd6-1* plants. A wild-type genomic clone of *ACD6* was subcloned as a 7670-bp EcoRV-SacI fragment from BAC clone F14B19 (ABRC Stock Center, Ohio State University, Columbus) into the pBin19 vector. The transformants were selected on agar plates containing Murashige and Skoog (1962) salts and appropriate antibiotics or on soil by spraying with BASTA at a dilution of 1:2000 (AgrEvo USA, Wilmington, DE).

Sequence Analysis

The putative functional regions of ACD6 were predicted by SMART (http://smart.embl-heidelberg.de/). The prediction of the transmembrane domain was made using several programs found at the following World Wide Web sites: http://www.ch.embnet.org/software/TMPRED_form.html, http://www.cbs.dtu.dk/services/TMHMM, and http://kr.expasy.org. CLUSTAL W (version 1.81) (http://www.ebi.ac.uk/clustalw/) was used for multiple sequence alignments.

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact Jean T. Greenberg, jgreenbe@midway.uchicago.edu.

Accession Number

The GenBank accession number for *ACD6* is AY344843.

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