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# Genetic analysis of *acd6-1* reveals complex defense networks and leads to identification of novel defense genes in Arabidopsis

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## Summary

Pathogen infection leads to the activation of defense signaling networks in plants. To study these networks and the relationships between their components, we introduced various defense mutations into *acd6-1*, a constitutive gain-of-function Arabidopsis mutant that is highly disease resistant. *acd6-1* plants show spontaneous cell death, reduced stature, and accumulate high levels of camalexin (an anti-fungal compound) and salicylic acid (SA, a signal molecule). Disruption of several defense genes revealed that in *acd6-1*, SA levels/signaling was positively correlated with the degree of disease resistance and defense gene expression. SA also modulates the severity of cell death. However, camalexin accumulation in *acd6-1* is largely unaffected by reducing SA levels. In addition, *acd6-1* shows ethylene- and jasmonic acid-mediated signaling that is antagonized and therefore masked by the presence of SA. Mutant analysis revealed a new relationship between the signaling components NPR1 and PAD4 and also indicated that multiple defense pathways were required for *acd6-1*-conferred phenotypes. In addition, our data confirmed that the size of *acd6-1* was inversely correlated with SA levels/signaling. We exploited this unique feature of *acd6-1* to identify two genes disrupted in *acd6-1 suppressor* (*sup*) mutants: one encodes a known SA biosynthetic component (SID2) and the other encodes an uncharacterized putative metalloprotease (At5g20660). Taken together, *acd6-1* is a powerful tool not only for dissecting defense regulatory networks but also for discovering novel defense genes.

### Keywords

salicylic acid; suppressor; disease resistance; signal transduction; *Pseudomonas syringae*; camalexin

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## Introduction

Pathogen infection evokes sophisticated responses in plants. Plants have evolved surveillance systems that detect various pathogen-derived molecules and subsequently activate defense responses. In the absence of this recognition and/or preformed defenses, plants are highly susceptible to pathogen attacks and disease ensues (Flor, 1971; Gomez-Gomez and Boller, 2002; Jones and Dangl, 2006; Zipfel, 2008). An array of genes works together to detect pathogens, transduce defense signaling, and activate defense responses. Therefore, it is critical to identify such defense genes, understand their mechanisms of action, and delineate the defense signaling networks.

Salicylic acid (SA), a small phenolic compound, is a key signaling molecule for plant disease resistance. Accumulation of SA is induced by pathogen attacks and other stress conditions (Schenk *et al.*, 2000; Uknes *et al.*, 1992). Exogenous application of SA or SA analogs such as benzo (1,2,3) thiadiazole-7-carothioic acid and 2,6-dichloroisonicotinic acid induces enhanced disease resistance in plants (Gorlach *et al.*, 1996; Lawton *et al.*, 1996; Metraux *et al.*, 1991; White, 1979). In addition, mutations that reduce SA accumulation or block SA signaling lead to compromised defense responses. At least three types of SA regulators have been described. The type I regulators include enzymes involved in SA biosynthesis. SA INDUCTION-DEFICIENT 2 (SID2) converts chorismate to isochorismate for SA biosynthesis (Wildermuth *et al.*, 2001). Although alternative SA biosynthetic pathways have been proposed (Verberne *et al.*, 1999), the highly reduced SA levels in the *sid2* mutants suggests that SID2 is part of the major pathway for SA biosynthesis. The type II regulators affect SA accumulation, but may not be biosynthetic enzymes. Examples of such SA regulators include ACCELERATED CELL DEATH 6 (ACD6), AGD2-LIKE DEFENSE 1 (ALD1), ENHANCED DISEASE SUSCEPTBILITY 1 (EDS1), PHYTOALEXIN DEFICIENT 4 (PAD4), and SID1/EDS5 (Falk *et al.*, 1999; Jirage *et al.*, 1999; Lu *et al.*, 2003; Nawrath *et al.*, 2002; Song *et al.*, 2004). Although how these proteins act is not fully understood, it is conceivable that they affect the availability of SA precursors, the activities of enzymes involved in SA biosynthesis, and/or SA catabolism. The type III regulators transduce defense signaling downstream of SA. NONEXPRESSOR OF PR GENES 1 (NPR1) is critical for SA-mediated defense signaling (Cao *et al.*, 1997; Ryals *et al.*, 1997; Shah *et al.*, 1997). There are also NPR1-independent SA signaling pathways, some of which have not yet been molecularly identified (Bowling *et al.*, 1997;Desveaux *et al.*, 2004; Rate and Greenberg, 2001; Shah *et al.*, 2001). The identification of many SA regulators suggests that there are multiple pathways feeding into the regulation of SA signaling.

In contrast to plants with compromised defense, there are mutants that display constitutive defense, often associated with cell death (also called a “lesion mimic phenotype”) and elevated SA accumulation (for review, see (Lorrain *et al.*, 2003)). Interestingly, the phenotypes of such mutants are often differentially affected by mutations in various SA regulators (Clarke *et al.*, 2000; Jirage *et al.*, 2001; Zhang *et al.*, 2003a). Observations from these studies support the view that SA-mediated signaling is important for plant defense and cell death formation and also suggest that some SA regulators likely act in parallel pathways to independently affect SA-mediated responses. Moreover, some lesion mimic mutants have been used to dissect interactions between SA and two other defense signaling pathways mediated by ethylene (ET) or jasmonic acid (JA) (Clarke *et al.*, 2000; Kachroo *et al.*, 2003; Kachroo *et al.*, 2001; Reymond and Farmer, 1998). Some studies indicate that ET and JA can either synergistically or antagonistically affect SA signaling, and that such intricate interactions fine-tune plant defense responses (reviewed in (Feys and Parker, 2000; Kunkel and Brooks, 2002)).

We previously characterized a type II SA regulator ACD6 in Arabidopsis. ACD6 is a plasma membrane protein with a cytoplasmic ankyrin repeat motif (Lu *et al.*, 2005; Lu *et al.*, 2003). ACD6 and SA-derived signals interact mutually to amplify defense responses in Arabidopsis. *acd6-1* is a gain-of-function mutant caused by a leucine to phenylalanine substitution in the transmembrane domain of ACD6. The hallmarks of *acd6-1* include extreme dwarfism, punctate cell death, accumulation of high levels of SA and camalexin (an anti-fungal metabolite) (Schuhegger *et al.*, 2006), and constitutive resistance to *Pseudomonas syringae* and *Hyaloperonospora parasitica* (Lu *et al.*, 2003; Rate *et al.*, 1999; Song *et al.*, 2004). Therefore, dissecting signaling pathways that regulate *acd6-1*-conferred phenotypes will give insight into the mechanisms of plant disease resistance and cell death formation.

We showed previously that some *acd6-1*-conferred phenotypes are partially dependent on SA regulators, such as PAD4, ALD1, and NPR1. In addition, depletion of SA by salicylate hydroxylase (NahG), encoded by a bacterial-originated transgene (Gaffney *et al.*, 1993), completely suppressed *acd6-1*-conferred phenotypes (Lu *et al.*, 2003; Rate *et al.*, 1999; Song *et al.*, 2004). Based on these observations, we hypothesized that SA is required for *acd6-1*-conferred phenotypes. However, some SA regulators may also affect other defense signals and NahG can have SA-independent effects (Heck *et al.*, 2003; van Wees and Glazebrook, 2003). Therefore, there are still many open questions. Do *acd6-1*-conferred phenotypes truly require SA? If so, how do different SA regulators interact with each other to affect *acd6-1*-conferred phenotypes and defense signaling? Are SA-independent pathways also involved in regulating *acd6-1*-conferred phenotypes?

To begin answering these questions, we performed a genetic analysis by introducing mutations causing defects in various defense pathways into the *acd6-1* background. Our study suggests that the defense signaling networks are complex, involving multiple factors that may act either independently in different pathways or together in the same pathway. Increased SA signaling antagonizes ET and JA signaling in the *acd6-1* background, but genetically blocking ET or JA signaling has no detectable effect on *acd6-1*-conferred phenotypes. Importantly, we show that *acd6-1*-conferred defense phenotypes indeed are largely dependent on SA. The finding that the small size of *acd6-1* is largely SA-dependent enabled us to employ a facile genetic screen to isolate *acd6-1 suppressor* (*sup*) mutants that affect SA levels and/or signaling. Among the *SUP* genes cloned, *SUP1* is *SID2* and *SUP6* encodes an uncharacterized putative metalloprotease (At5g20660). SUP6 is important for basal resistance in otherwise wild-type Arabidopsis, highlighting the utility of *acd6-1* for uncovering novel defense components as well as the novel features of the defense networks.

## Results

### Multiple regulators affect SA accumulation in *acd6-1*

We sought to determine whether several known SA regulators affect *acd6-1*-conferred phenotypes and possibly show genetic interactions. Therefore, we compared the phenotypes of newly constructed *acd6-1eds5-1*, *acd6-1sid2-1* and *acd6-1npr1-1pad4-1* mutants to the respective single and previously constructed double mutant parents. We first determined effects of mutations in these SA regulators on the levels of SA and its glucoside conjugate SAG.

Similar to disruption of the *PAD4* gene (Figure 1a and (Lu *et al.*, 2003)), an *EDS5* mutation also partially suppressed both SA and SAG accumulation in *acd6-1*. In contrast, *acd6-1npr1-1* accumulated three-fold more free SA than *acd6-1*, although total SA levels were comparable in these two genotypes. This effect of *npr1-1* on SA accumulation was previously observed in other constitutive defense mutants, and suggests that NPR1

participates in negative feedback regulation of SA (Clarke *et al.*, 2000; Delaney *et al.*, 1995; Zhang *et al.*, 2003b; Zhou *et al.*, 1998). The high production of SA in *acd6-1npr1-1* was largely PAD4-dependent (Figure 1a, compare SA levels in *acd6-1npr1-1* and *acd6-1npr1-1pad4-1*). Additionally, *acd6-1npr1-1pad4-1* produced lower SA levels than *acd6-1pad4-1* (Figure 1a), suggesting NPR1 can positively regulate SA in the absence of PAD4. Finally, of all the double and triple mutants analyzed, *acd6-1sid2-1* had the lowest overall levels of SA. However, the SA and SAG levels were still slightly higher in *acd6-1sid2-1* than *sid2-1*, indicating a small SID2-independent biosynthetic source of SA is produced in *acd6-1* (Figure 1a). These data indicate that all three types of SA regulators contribute to SA accumulation, and that SID2 is the major contributor.

### Multiple regulators affect disease resistance and defense signaling in *acd6-1*

To examine whether changes in SA levels and/or signaling were correlated with *acd6-1*-conferred disease resistance, we infected our double and triple mutant plants with *P. syringae* pv. *maculicola* ES4326 strain *Pma*DG3. The resistance of acd6-*1eds5-1* and *acd6-1sid2-1* was positively correlated with the SA levels in these plants, similar to what we previously found in *acd6-1pad4-1* ((Lu *et al.*, 2003) and Figure 1b). The *npr1-1* mutation was previously shown to partially suppress disease resistance in *acd6-1* (Rate *et al.*, 1999). The presence of *pad4-1* did not significantly enhance the susceptibility of the *acd6-1npr1-1* mutant. Additionally, the fact that *acd6-1sid2-1*, *acd6-1npr1-1*, and *acd6-1npr1-1pad4-1* were significantly more resistant than the single mutants *sid2-1*, *npr1-1* and *pad4-1* suggests that the small amount of SA, additional SA regulators and/or an SA-independent pathway in these plants are responsible for the residual disease resistance (Figure 1b).

To further study how defenses are affected by the SA regulators in *acd6-1*, we examined the expression of several defense-related genes in these plants. *PR1* transcript levels are usually highly correlated with SA-mediated signaling (Glazebrook *et al.*, 1997). We previously found that *npr1-1* and *pad4-1* partially suppressed *PR1* expression in *acd6-1* (Rate *et al.*, 1999). Consistent with the observations on SA levels, the presence of both *npr1-1* and *pad4-1* further suppressed *PR1* transcript levels in *acd6-1pad4-1npr1-1* (Figure 1c). Compared with that in *acd6-1*, expression of *PR1* was also much reduced in *acd6-1eds5-1*, but was completely abolished in *acd6-1sid2-1*. Since *acd6-1sid2-1* had the lowest total SA levels (4.4 μg/g fresh weight) among all the plants in the *acd6-1* background that were tested in this study (Table S1), our results suggest that a threshold level of SA is necessary to induce *PR1* expression. ALD1 and PAD4 are type II SA regulators whose transcript levels are highly induced in *acd6-1* and in wild-type plants after *P. syringae* infection or SA (or SA agonist) treatment (Jirage *et al.*, 1999; Song *et al.*, 2004). Compared with that of *PR1*, expression of *ALD1* and *PAD4* was not appreciably affected in *eds5-1* and *sid2-1*, but was drastically reduced in *acd6-1npr1-1pad4-1*. One possible explanation for these results is that expression of these two genes can be induced by low SA levels and such a high sensitivity to SA is NPR1- and PAD4-dependent. In addition, an NPR1- and PAD4-independent pathway is also attributable to the residual expression of both *ALD1* and *PAD4* in *acd6-1npr1-1pad4-1*.

Taken together, these data indicate that *acd6-1*-conferred constitutive defenses were largely SA-dependent. Both PAD4-/NPR1-dependent and –independent pathways are required for *acd6-1* disease resistance and defense gene expression. In addition, a SID2-independent pathway is also activated in the *acd6-1* background and contributes to its constitutive defense.

### Accumulation of camalexin is largely SID2-independent

To test whether SA regulators affect camalexin accumulation in *acd6-1*, we measured camalexin levels in the plants used in Figures 1. As shown in Figure 2, most mutants, except *acd6-1sid2-1*, did not significantly alter camalexin accumulation in *acd6-1*. Compared with that of the SA levels (27-fold reduction), the reduction of camalexin (<2-fold) caused by *sid2-1* was quite small in *acd6-1* (Table S1). Therefore, such a decrease in camalexin likely reflects an indirect role of SID2 in affecting camalexin accumulation in *acd6-1*.

To further investigate how camalexin production is regulated in *acd6-1*, we made a double mutant between *acd6-1* and *pad3-1*, a mutant defective in pathogen-induced camalexin accumulation (Glazebrook and Ausubel, 1994; Zhou *et al.*, 1999). Whereas the SA levels were only slightly decreased, camalexin accumulation was greatly reduced in *acd6-1pad3-1* compared with *acd6-1*. However, the rosette size and bacterial growth remained similar in *acd6-1pad3-1* and *acd6-1* (Table 1). Taken together, these data suggest that camalexin accumulation in *acd6-1* is largely independent of SA and can be uncoupled from plant size, bacterial disease resistance, and SA accumulation.

### SA regulators modulate cell death in *acd6-1*

One of the hallmarks of *acd6-1* is spontaneous cell death patches on its leaves. A close examination of *acd6-1* leaves revealed three categories of cell death: single foci, intermediate clusters (2–20 cells), and very large clusters (>20 cells) (Figure 3). Cell death usually starts with single cells and spreads to neighboring cells to different extents. The cell death pattern of *acd6-1* suggests a loss of control of both cell death initiation and spreading. We previously showed that the cell number in the *acd6-1* leaf was similar to that in wild type, although cell size of *acd6-1* was much smaller (Vanacker *et al.*, 2001). Thus, in order to quantify the degree of cell death in the *acd6-1* mutants, we measured the number of dead cells on quarter leaf basis. Figure 3a shows that all double or triple mutants abolished or reduced the very large clusters of dead cells in *acd6-1*. *acd6-1pad4-1* harbored a similar number of single cell death foci as *acd6-1*, possibly due to the relatively high levels of SA in this mutant. These observations suggest that high SA levels stimulate both initiation and spreading of cell death. However, since SA treatment of wild type does not induce cell death, a second signal(s) from *acd6-1* that perhaps is SA-independent is required to act with SA in regulating these processes. Interestingly, although the number of single death foci was much reduced, some very large clusters of dead cells were found in *acd6-1sid2-1*, often adjacent to the vascular tissue (Figure 3b). It is possible that the low SA levels in *acd6-1sid2-1* not only lead to reduced cell death initiation, but also render the plant more sensitized to the signal that controls death spreading.

### ET and JA pathways are dispensable for *acd6-1*-conferred dwarfism and defense

To determine whether SA-independent pathways affect *acd6-1*-conferred phenotypes, we examined defense signaling mediated by ET and JA, which are known to cooperate with or antagonize SA during defense responses, depending on the types of pathogens and plants tested (Feys and Parker, 2000; Kunkel and Brooks, 2002). Figure 1c shows that expression of *PDF1.2*, a marker for activation of ET and JA pathways (Penninckx *et al.*, 1998; Reymond and Farmer, 1998), was highly induced as the SA levels were decreased in *acd6-1pad4-1*, *acd6-1eds5-1*, and *acd6-1sid2-1*, relative to *acd6-1*. In addition, *acd6-1npr1-1* expressed much more *PDF1.2* transcripts than *acd6-1*, whose *PDF1.2* expression was only detectable with a longer exposure and comparable to that in wild type (Figure 1c and data not shown). In addition, *PDF1.2* expression was highest in *acd6-1npr1-1pad4-1*. The expression pattern of *PDF1.2* was opposite to that of *PR1*, a marker of the SA signaling pathway. These data clearly demonstrate the antagonistic effect

of SA on ET and JA: the reduction in SA levels and/or signaling resulted in the induction of the ET and JA signaling that leads to *PDF1.2* expression in *acd6-1*.

To test if disruption of ET and JA signaling interferes with *acd6-1*-conferred phenotypes, we crossed *acd6-1* to *ethylene response 1-1* (*etr1-1*), a mutant defective in ET reception (Chang *et al*., 1993), or *jasmonic acid resistant 1-1* (*jar1-1*), a mutant defective in JA signaling (Staswick *et al*., 1998). The double mutants, *acd6-1etr1-1* and *acd6-1jar1-1*, were indistinguishable from *acd6-1* in SA accumulation, rosette size, and resistance to *P. syringae* (Table 1). These results indicate that disruption of the JA and ET-mediated signaling does not affect *acd6-1*-conferred dwarfism and defense.

### *acd6-1*-conferred dwarfism is largely SA-dependent

An obvious phenotypic difference among these mutants was the plant size, which appeared to be inversely correlated with SA levels and/or signaling (Figure 4 and Table S1). The rosette sizes of *acd6-1*, *acd6-1pad4-1*, *acd6-1eds5-1* and *acd6-1sid2-1* increased as the levels of SA in these plants decreased. The *acd6-1sid2-1* plants differed in their appearance from other double mutants, as the leaves had a wavy appearance. As reported previously, blocking SA signaling with the *npr1-1* mutation also slightly suppressed *acd6-1* dwarfism and resulted in the bleaching of some leaves (Rate *et al*., 1999). Consistent with its low SA levels, the triple mutant *acd6-1npr1-1pad4-1* was much larger than either *acd6-1npr1-1* or *acd6-1pad4-1*. Interestingly, although it had about four times more total SA levels than *acd6-1sid2-1*, *acd6-1npr1-1pad4-1* was much larger than *acd6-1sid2-1*. It is possible that residual SA in *acd6-1sid2-1* affects plant size due to the activities of NPR1 and/or PAD4. Alternatively, SA-independent signaling in *acd6-1sid2-1* may also affect the stature of *acd6-1*.

### A suppressor of *acd6-1*-conferred dwarfism identifies a novel defense component

Since mutations that reduce *acd6-1*-conferred dwarfism showed increased disease susceptibility, we used T-DNA insertional mutagenesis to identify suppressors of *acd6-1* with increased stature. Mutants that appear to be completely suppressed might carry mutations in the *ACD6* gene (Lu *et al*., 2005). Therefore, we focused on mutations that conferred partial suppression. Compared with *acd6-1*, partial *sup* mutants were easily identified by the naked eye (Figure 5a). As a proof of concept, we characterized two of the 20 *sup* mutants, *sup1-1* and *sup6-1*, which we have obtained to date.

The wavy rosette phenotype of *acd6-1sup1-1* mutant strongly resembled *acd6-1sid2-1* plants. Therefore, we sought to amplify the *SID2* gene by PCR, but failed to detect the expected band in *sup1-1*, indicating that a T-DNA fragment likely inserted in the *SID2* gene. To further test whether the mutation in *SID2* was responsible for *sup1-1*-conferred phenotypes, we crossed *sup1-1* to *sid2-1* and infected the $F_1$ plants with *Pma*DG3. We found that the *sup1-1* × *sid2-1* $F_1$ plants showed similar susceptibility as *sup1-1* and *sid2-1* (Figure 5b and 5c). These data strongly suggest that *sup1-1* is allelic to *SID2*.

The $F_2$ progeny of a cross between *acd6-1sup6-1* and Col showed 80 resistant: 25 plants that were sensitive to the herbicide glufosinate (conferred by the T-DNA), suggesting a single T-DNA insertion in the genome ($X^2$=0.051, P> 0.8211). *sup6-1* has a T-DNA insertion in the eighth exon of At5g20660, a single copy gene that encodes a protein with a predicted N-terminal peptidase domain commonly found in metalloproteases and a C-terminal transmembrane domain (Figure 6a). *In silico* analysis of gene expression profile with publicly available microarray database (Genevesitgator, (Zimmermann *et al*., 2004)) indicated that *SUP6* was constitutively expressed during Arabidopsis development and in different organs and was not much affected under various conditions that lead to induction

of numerous defense-related genes (data not shown). Proteins with high sequence similarity to SUP6 were found in many plants, as determined by the analysis of pBLAST data (http://blast.ncbi.nlm.nih.gov/Blast.cgi). However, the only experimentally characterized SUP6 homologue was a 24-kd vacuolar protein (VP24), which was enriched in the anthocyanin-containing vacuoles of cultured sweet potato (*Ipomoea batatas*) cells (Nozue *et al.*, 1997; Xu *et al.*, 2001). VP24 is derived from a larger precursor peptide that shares 50% identity to *SUP6*. The physiological functions of VP24 and its precursor are not well understood.

A second allele, *sup6-2* (salk_072469), also harbors a T-DNA insertion in the eighth exon of *SUP6*. Both mutant alleles abolished expression of the *SUP6* gene and possibly led to truncated *sup6* transcripts before the T-DNA insertion sites (Figure 6b). Like *sup6-1*, *sup6-2* also suppressed *acd6-1* dwarfism (Figure 6c and 6d). Therefore, the suppression of *acd6-1* dwarfism was caused by the mutations in the *SUP6* gene.

As a secondary screen to test the effects of *sup6* mutations on defense, we measured SA levels in *acd6-1sup6-1* and *acd6-1sup6-2*. Both mutants caused a reduction of the total SA levels in *acd6-1* (Figure 7a). In addition, both *PR1* expression and cell death formation were much reduced in the *acd6-1sup6* mutants (Figure 7b and c). To assess disease resistance, we infected both *sup6* alleles (in the absence of the *acd6-1* mutation) with *Pma*DG3 and found more bacterial growth in the mutants compared with the wild-type control (Figure 7d). Together, these data suggest that SUP6 is likely a type II SA regulator important for basal defense responses in Arabidopsis.

## Discussion

Pathogen infection induces multifaceted defense responses in plants, including activation of signaling pathways, accumulation of antimicrobial compounds, and promotion of cell death. The Arabidopsis mutant *acd6-1* constitutively exhibits these defense responses in the absence of pathogens. Therefore, dissecting signaling pathways activated in *acd6-1* and required for *acd6-1*-conferred phenotypes will help in the determination of disease resistance mechanisms. From the genetic analysis described here, we have gained a better understanding of the factors regulating *acd6-1*-conferred phenotypes and came to the following conclusions: 1) Defense responses with respect to SA accumulation, disease resistance, and expression of some defense genes are positively correlated in *acd6-1*. The small size of *acd6-1*, however, is inversely related to the strength of defense signaling; 2) SA is neither necessary nor sufficient for camalexin accumulation in *acd6-1*; 3) the severity of cell death in *acd6-1* is affected by SA and another signal(s); 4) ET and JA mediated signaling are not required for *acd6-1*-conferred dwarfism and resistance; 5) suppression of *acd6-1*-conferred dwarfism can be used to dissect the interactions among SA regulators and to identify novel defense components.

### Multiple components are activated to regulate SA-mediated defense in *acd6-1*

Our studies showed that *acd6-1*-conferred disease resistance and defense gene expression are largely SA-dependent and require the activities of multiple SA regulators (Lu *et al.*, 2003; Rate *et al.*, 1999; Song *et al.*, 2004). The residual SA levels in *acd6-1sid2-1* suggest that whereas SID2 mediates the synthesis of the majority of SA, a SID2-independent pathway is activated in *acd6*-1, which contributes to the partial induction of many defenses *in acd6-1* (Figure S1). Hence, the *acd6-1sid2-1* double mutant will provide a useful tool for studies of SID2-independent SA synthesis.

Interestingly, *nahG* had a greater impact in lowering SA levels in *acd6-1* (Vanacker *et al.*, 2001) than *sid2-1* and also suppressed *acd6-1*-conferred phenotypes to a much greater extent

(Rate *et al.*, 1999). Differences in *acd6-1sid2-1* and *acd6-1nahG* phenotypes are likely due to the fact that *ACD6-1* transcript levels are undetectable in *acd6-1nahG*, whereas *acd6-1sid2-1* plants still express *ACD6-1* (our unpublished data).

Our studies also indicate that at least three type II SA regulators, ALD1, PAD4, and EDS5, are required for *acd6-1*-conferred phenotypes (this study and (Song *et al.*, 2004)). However, compared with *sid2-1*, disease resistance and defense gene expression were not as greatly affected by mutations in these genes. This suggests that each of these type II SA regulators may only represent one branch of the networks regulating the full accumulation of SA. Consistent with this notion, no single mutant affecting SA levels tested so far completely suppressed SA accumulation and constitutive defense in *acd6-1*. It is conceivable that additional known and yet-to-be-discovered type II SA regulators also contribute to the regulation of *acd6-1*-conferred phenotypes (Figure S1). However, although a mutation in the *NON-RACE-SPECIFIC DISEASE RESISTANCE* gene compromises pathogen-induced SA accumulation (Shapiro and Zhang, 2001), the *acd6-1ndr1-1* mutant did not have altered defenses and plant size, compared with *acd6-1* (our unpublished observations). This suggests that only a subset of SA regulators is required for *acd6-1*-conferred phenotypes.

Phenotypic analysis of the triple mutant *acd6-1npr1-1pad4-1* revealed potentially novel roles of the type III regulator NPR1 and the type II regulator PAD4 in defense signaling. Mutations in both NPR1 and PAD4 additively affected SA levels, defense responses, and plant size in the *acd6-1* background. This suggests a positive role for NPR1 in affecting SA accumulation, in addition to NPR1's ability to negatively regulate SA as well as positively transduce SA signaling. The negative function of NPR1 may require PAD4. When PAD4 is mutated, the negative role of NPR1 is abolished but its positive role is not affected. Therefore, the *acd6-1pad4-1* mutant should still have relatively high levels of SA, which is corroborated by our results (Figure 1a and (Lu *et al.*, 2003)). However, when both PAD4 and NPR1 are mutated, both the negative and positive effects of NPR1 are eliminated, leading to much reduced SA and defense levels. PAD4 activity or a component upstream of PAD4 may be the direct target of NPR1, forming a negative feedback loop with NPR1 to regulate SA levels (Figure S1). An interesting open question is how additional SA regulators interact with each other to form the complex defense signaling networks.

## SID2-independent defenses are activated in *acd6-1*

Disruption of SID2 in *acd6-1* did not completely suppress several *acd6-1*-conferred phenotypes. Since *acd6-1sid2-1* plants have slightly higher SA levels than *sid2-1*, the phenotypes of *acd6-1sid2-1* plants could result from the residual SA or SA-independent signaling. The strongest indication of a SID2-and SA-independent pathway being activated is the high accumulation of camalexin in *acd6-1sid2-1* (Figure 2). Camalexin induction during pathogen infection is known to be SID2-independent (Nawrath and Metraux, 1999). Importantly, camalexin levels are unaffected by SA treatment of wild type (Zhou *et al.*, 1998). Our data are consistent with ACD6 regulating SA levels/signaling and camalexin accumulation (Lu *et al.*, 2003) through separate pathways.

Analysis of *PDF1.2* expression suggests that ET/JA signaling has the potential to be highly activated in *acd6-1* plants. However, this only occurs when SA levels/signaling is blocked, consistent with the known antagonizing effect of SA on ET/JA signaling (reviewed in (Feys and Parker, 2000; Kunkel and Brooks, 2002)). ET/JA signaling does not significantly impact the size, bacterial disease resistance, or SA accumulation in *acd6-1*, indicating that these two pathways are dispensable for *acd6-1*-conferred phenotypes. Possibly, activation of ET/JA signaling results from the cell death conferred by *acd6-1*.

### *acd6-1* is a useful tool to identify novel defense genes

In addition to its usefulness in studying the interactions among the known defense components, *acd6-1* can be further employed to identify additional defense genes. The potential of the suppressor genetics was validated by the isolation of the known SA regulator SID2 and the novel defense component SUP6. A high conservation between SUP6 and the precursor peptide of the vacuolar protein VP24 suggests that SUP6 is a potential vacuolar protease and possibly plays a role in plant defense and/or cell death as some reported vacuolar proteases do (Hara-Nishimura *et al.*, 2005). We have presented evidence to show that SUP6 regulates basal defense in Arabidopsis. Additional studies are necessary to fully discern how SUP6 acts to regulate plant defense and to place SUP6 in the complex defense signaling networks. Further characterization of SUP6 and additional novel SUP proteins will facilitate an understanding of the fundamental mechanisms of plant defense responses, expand our knowledge of the defense regulatory networks, and eventually provide molecular targets for genetic engineering to produce crops with enhanced disease resistance.

## Experimental procedures

### Plant Materials

Plants used in this report were in the Columbia (Col) background. The mutants *acd6-1*, *acd6-1npr1-1* and *acd6-1pad4-1*, *etr1-1*, *jar1-1*, *npr1-1*, *pad3-1*, *pad4-1*, *eds5-1*, *sid2*-1 were described previously (Chang *et al.*, 1993; Lu *et al.*, 2003; Nawrath *et al.*, 2002; Rate *et al.*, 1999; ; Song *et al.*, 2004; Staswick *et al.*, 1998; Thomma *et al.*, 1999). Double mutants (*acd6-1eds5-1*, *acd6-1sid2-1*, *acd6-1etr-1*, *acd6-1jar-1*, and *acd6-1pad3-1*) were constructed by crossing *acd6-1* to each corresponding single mutant and screening for the respective double mutant from each $F_2$ population using relevant derived cleaved amplified polymorphic sequence (dCAPS) markers. The triple mutant *acd6-1npr1-1pad4-1* was made by crossing *acd6-1npr1-1* to *acd6-1pad4-1* and screening for homozygous $F_2$ants using dCAPS markers for *acd6-1*, *npr1-1* and *pad4-1*, respectively. Primers for all dCAPS markers used in this study were listed in Table S2.

To detect the mutation in the *SID2* gene in *sup1-1*, we used the primer set *sup1-1(sid2)* (Table S2) and expect a 836-bp band in wild type, but no amplification in *sup1-1*. *sup6-1* is a suppressor identified from *acd6-1* suppressor screen (see below). *sup6-2* (salk_072496) was ordered from the Arabidopsis Biological Resource Center. The double mutant *acd6-1sup6-2* was made by crossing *acd6-1 to sup6-2* and homozygous F2 plants were selected by using the dCAPS markers for *acd6-1* and the primer sets for the *sup6-2* mutant (Table S2).

### Bacterial culture and infection

*Pseudomonas syringae* pv. m*aculicola (Pma)* DG3 is a derivative of *Pma*ES4326 (Guttman and Greenberg, 2001). For the bacterial growth assay, the fifth to seventh leaves of 20-day old plants grown in growth chambers with 16h light/8h dark were infected with *Pma*DG3 for 3 days before leaf discs were taken for measurement of the number of bacteria. Bacterial culturing, infection, and growth analysis were performed as described previously (Greenberg *et al.*, 2000; Lu *et al.*, 2003).

### Cell Death Staining and cell death quantitation

Fifth to seventh leaves of each genotype were collected and stained with trypan blue as described (Rate *et al.*, 1999) and were examined using a Stemi SV 1.1 stereomicroscope (Zeiss, Inc., Germany). To quantify cell death, dead cell foci (single dead cell, 2–20 dead cell cluster, and over 20-dead cell cluster) were counted in an area equivalent to a quarter

region of a whole leaf on the tip area. Pictures of stained leaves were taken with an AxioCam MRc5 camera (Zeiss, Inc., Germany).

### RNA analysis

Extraction of total RNA and Northern blot analysis were performed as previously described (Lu *et al.*, 2003). The DNA fragment specific to each gene was amplified by PCR from the genomic DNA, confirmed by sequencing, and labeled with $^{32}$P-dCTP as a probe for Northern blot analysis. For RT-PCR, one microgram of the total RNA extracted from each sample was reverse transcribed with the First Strand cDNA Synthesis kit (Fermentas International, Glen Burnie, MD) according to the manufacturer's instructions. PCR reactions were carried out with gene specific primers and samples were taken at 25, 30, and 35 cycles. All primers used for RNA analysis were listed in Table S2.

### SA and Camalexin Measurement

SA and camalexin were extracted and analyzed by HPLC as previously described (Lu *et al.*, 2003; Song *et al.*, 2004).

### Screen T-DNA insertion mutagenized *acd6-1*

*acd6-1* plants ($T_0$) were transformed with *Agrobacteria* carrying the pAOV1.3 vector, which harbors the BAR gene to confer resistance to herbicide glufosinate (Mylne and Botella, 1998). The $T_1$ seeds collected from the primary transformation were planted on soil and selected for T1 transgenic plants by spraying Liberty®, a glufosinate containing product (Bayer Crop Science, VA). $T_2$ seeds from each 500 $T_1$ plants were collected in a pool and a total of 70 pools containing about 35,000 lines were harvested. At least 5,000 $T_2$ seeds from each pool were planted for selection for transgenic plants resistant to glufosinate and visually screened for *acd6-1sups* with a larger size. *acd6-1sups* were crossed back to *acd6-1* to confirm the suppression phenotypes and also to Col to separate a *sup* mutation from *acd6-1*. For *acd6-1sups* with a confirmed larger size, we performed TAIL-PCR (Liu *et al.*, 1995) to identify the junction region between a T-DNA insertion and an Arabidopsis chromosome. Twenty putative *acd6-1sups* were isolated from the screen for further analysis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## References

Bowling SA, Clarke JD, Liu Y, Klessig DF, Dong X. The *cpr5* mutant of Arabidopsis expresses both *NPR1*-dependent and *NPR1*- independent resistance. Plant Cell. 1997; 9:1573–1584. [PubMed: 9338960]

Cao H, Glazebrook J, Clarke JD, Volko S, Dong X. The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell. 1997; 88:57–63. [PubMed: 9019406]

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM. Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators. Science. 1993; 262:539–544. [PubMed: 8211181]

Clarke JD, Volko SM, Ledford H, Ausubel FM, Dong X. Roles of salicylic acid, jasmonic acid, and ethylene in cpr-induced resistance in Arabidopsis. Plant Cell. 2000; 12:2175–2190. [PubMed: 11090217]

Delaney TP, Friedrich L, Ryals JA. Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. Proc. Natl. Acad. Sci. USA. 1995; 92:6602–6606. [PubMed: 11607555]

Desveaux D, Subramaniam R, Despres C, Mess JN, Levesque C, Fobert PR, Dangl JL, Brisson N. A "Whirly" transcription factor is required for salicylic acid-dependent disease resistance in Arabidopsis. Dev. Cell. 2004; 6:229–240. [PubMed: 14960277]

Falk A, Feys BJ, Frost LN, Jones JD, Daniels MJ, Parker JE. EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. Proc. Natl. Acad. Sci. USA. 1999; 96:3292–3297. [PubMed: 10077677]

Feys BJ, Parker JE. Interplay of signaling pathways in plant disease resistance. Trends Genet. 2000; 16:449–455. [PubMed: 11050331]

Flor HH. Current status of the gene-for-gene concept. Annu. Rev. of Phytopathol. 1971; 9:275–296.

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J. Requirement of salicylic acid for the induction systemic acquired resistance. Science. 1993; 261:754–756. [PubMed: 17757215]

Glazebrook J, Ausubel FM. Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. Proc. Natl. Acad. Sci. USA. 1994; 91:8955–8959. [PubMed: 8090752]

Glazebrook J, Zook M, Mert F, Kagan I, Rogers EE, Crute IR, Holub EB, Hammerschmidt R, Ausubel FM. Phytoalexin-deficient mutants of Arabidopsis reveal that *PAD4* encodes a regulatory factor and that four *PAD* genes contribute to downy mildew resistance. Genetics. 1997; 146:381–392. [PubMed: 9136026]

Gomez-Gomez L, Boller T. Flagellin perception: a paradigm for innate immunity. Trends Plant Sci. 2002; 7:251–256. [PubMed: 12049921]

Gorlach J, Volrath S, Knauf-Beiter G, Hengy G, Beckhove U, Kogel KH, Oostendorp M, Staub T, Ward E, Kessmann H, Ryals J. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell. 1996; 8:629–643. [PubMed: 8624439]

Greenberg JT, Silverman FP, Liang H. Uncoupling salicylic acid-dependent cell death and defense-related responses from disease resistance in the Arabidopsis mutant *acd5*. Genetics. 2000; 156:341–350. [PubMed: 10978297]

Guttman DS, Greenberg JT. Functional analysis of type III effectors AvrRpt2 and AvrRpm1 of *Pseudomonas syringae* with the use of a single copy genomic integration system. Mol. Plant Microbe Interact. 2001; 14:145–155. [PubMed: 11204777]

Hara-Nishimura I, Hatsugai N, Nakaune S, Kuroyanagi M, Nishimura M. Vacuolar processing enzyme: an executor of plant cell death. Curr. Opin. Plant Biol. 2005; 8:404–408. [PubMed: 15939660]

Heck S, Grau T, Buchala A, Metraux JP, Nawrath C. Genetic evidence that expression of NahG modifies defence pathways independent of salicylic acid biosynthesis in the Arabidopsis-Pseudomonas syringae pv. tomato interaction. Plant J. 2003; 36:342–352. [PubMed: 14617091]

Jirage D, Tootle TL, Reuber TL, Frost LN, Feys BJ, Parker JE, Ausubel FM, Glazebrook J. *Arabidopsis thaliana* PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. Proc. Natl. Acad. Sci. USA. 1999; 96:13583–13588. [PubMed: 10557364]

Jirage D, Zhou N, Cooper B, Clarke JD, Dong X, Glazebrook J. Constitutive salicylic acid-dependent signaling in cpr1 and cpr6 mutants requires PAD4. Plant J. 2001; 26:395–407. [PubMed: 11439127]

Jones JD, Dangl JL. The plant immune system. Nature. 2006; 444:323–329. [PubMed: 17108957]

Kachroo A, Lapchyk L, Fukushige H, Hildebrand D, Klessig D, Kachroo P. Plastidial fatty acid signaling modulates salicylic acid- and jasmonic acid-mediated defense pathways in the Arabidopsis ssi2 mutant. Plant Cell. 2003; 15:2952–2965. [PubMed: 14615603]

Kachroo P, Shanklin J, Shah J, Whittle E, Klessig D. A fatty acid desaturase modulates the activation of defense signaling pathways in plants. Proc Natl Acad Sci USA. 2001; 98:9448–9453. [PubMed: 11481500]

Kunkel BN, Brooks DM. Cross talk between signaling pathways in pathogen defense. Curr. Opin. Plant Biol. 2002; 5:325–331. [PubMed: 12179966]

Lawton KA, Friedrich L, Hunt M, Weymann K, Delaney T, Kessmann H, Staub T, Ryals J. Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. Plant J. 1996; 10:71–82. [PubMed: 8758979]

Liu YG, Mitsukawa N, Oosumi T, Whittier RF. Efficient isolation and mapping of Arabidopsis thaliana T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J. 1995; 8:457–463. [PubMed: 7550382]

Lorrain S, Vailleau F, Balague C, Roby D. Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants? Trends Plant Sci. 2003; 8:263–271. [PubMed: 12818660]

Lu H, Liu Y, Greenberg JT. Structure-function analysis of the plasma membrane- localized Arabidopsis defense component ACD6. Plant J. 2005; 44:798–809. [PubMed: 16297071]

Lu H, Rate DN, Song JT, Greenberg JT. ACD6, a novel ankyrin protein, is a regulator and an effector of salicylic acid signaling in the Arabidopsis defense response. Plant Cell. 2003; 15:2408–2420. [PubMed: 14507999]

Metraux, JP.; Ahl-Goy, P.; Staub, T.; Speich, J.; Steinemann, A.; Ryals, J.; Ward, E. Induced resistance in cucumber in response to 2,6-dichloroisonicotinic acid and pathogens Dordrecht . The Netherlands: Kluwer Academic Publishers; 1991.

Mylne J, Botella JR. Binary Vectors for Sense and Antisense Expression of Arabidopsis ESTs. Plant Mol. Biol. Rep. 1998; 16:257–262.

Nawrath C, Heck S, Parinthawong N, Metraux JP. EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in Arabidopsis, is a member of the MATE transporter family. Plant Cell. 2002; 14:275–286. [PubMed: 11826312]

Nawrath C, Metraux JP. Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. Plant Cell. 1999; 11:1393–1404. [PubMed: 10449575]

Nozue M, Yamada K, Nakamura T, Kubo H, Kondo M, Nishimura M. Expression of a vacuolar protein (VP24) in anthocyanin-producing cells of sweet potato in suspension culture. Plant Physiol. 1997; 115:1065–1072. [PubMed: 9390439]

Penninckx IA, Thomma BP, Buchala A, JP M, Broekaert WF. Concomitant Activation of Jasmonate and Ethylene Response Pathways Is Required for Induction of a Plant Defensin Gene in *Arabidopsis*. Plant Cell. 1998; 10:2103–2114. [PubMed: 9836748]

Rate DN, Cuenca JV, Bowman GR, Guttman DS, Greenberg JT. 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. Plant Cell. 1999; 11:1695–1708. [PubMed: 10488236]

Rate DN, Greenberg JT. The Arabidopsis *aberrant growth and death2* mutant shows resistance to *Pseudomonas syringae* and reveals a role for NPR1 in suppressing hypersensitive cell death. Plant J. 2001; 27:203–211. [PubMed: 11532166]

Reymond P, Farmer EE. Jasmonate and salicylate as global signals for defense gene expression. Curr. Opin. Plant Biol. 1998; 1:404–411. [PubMed: 10066616]

Ryals J, Weymann K, Lawton K, Friedrich L, Ellis D, Steiner HY, Johnson J, Delaney TP, Jesse T, Vos P, Uknes S. The *Arabidopsis* NIM1 protein shows homology to the mammalian transcription factor inhibitor I kappa B. Plant Cell. 1997; 9:425–439. [PubMed: 9090885]

Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, Somerville SC, Manners JM. Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proc. Natl. Acad. Sci. USA. 2000; 97:11655–11660. [PubMed: 11027363]

Schuhegger R, Rauhut T, Glawischnig E. Regulatory variability of camalexin biosynthesis. J. Plant Physiol. 2006

Shah J, Kachroo P, Nandi A, Klessig DF. A recessive mutation in the Arabidopsis SSI2 gene confers SA- and NPR1- independent expression of PR genes and resistance against bacterial and oomycete pathogens. Plant J. 2001; 25:563–574. [PubMed: 11309146]

Shah J, Tsui F, Klessig DF. Characterization of a salicylic acid-insensitive mutant (sai1) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA- inducible expression of the *tms2* gene. Mol. Plant-Microbe Interact. 1997; 10:69–78. [PubMed: 9002272]

Shapiro AD, Zhang C. The role of NDR1 in avirulence gene-directed signaling and control of programmed cell death in Arabidopsis. Plant Physiol. 2001; 127:1089–1101. [PubMed: 11706189]

Song J, Lu H, McDowell J, Greenberg J. A key role for ALD1 in activation of local and systemic defenses in Arabidopsis. Plant J. 2004; 40:200–212. [PubMed: 15447647]

Staswick PE, Yuen GY, Lehman CC. Jasmonate signaling mutants of Arabidopsis are susceptible to the soil fungus Pythium irregulare. Plant J. 1998; 15:747–754. [PubMed: 9807813]

Thomma BP, Nelissen I, Eggermont K, Broekaert WF. Deficiency in phytoalexin production causes enhanced susceptibility of Arabidopsis thaliana to the fungus Alternaria brassicicola. Plant J. 1999; 19:163–171. [PubMed: 10476063]

Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, Chandler D, Slusarenko A, Ward E, Ryals J. Acquired resistance in *Arabidopsis*. Plant Cell. 1992; 4:645–656. [PubMed: 1392589]

van Wees SC, Glazebrook J. Loss of non-host resistance of Arabidopsis NahG to Pseudomonas syringae pv. phaseolicola is due to degradation products of salicylic acid. Plant J. 2003; 33:733–742. [PubMed: 12609045]

Vanacker H, Lu H, Rate DN, Greenberg JT. A role for salicylic acid and NPR1 in regulating cell growth in Arabidopsis. Plant J. 2001; 28:209–216. [PubMed: 11722764]

Verberne, M.; Budi Muljono, A.; R, V. Salicylic acid biosynthesis. In: K, L.; M, H.; PJJ, H., editors. Biochemistry and Molecular Biology of Plant Hormones. London: Elsevier; 1999. p. 295-312.

White RF. Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. Virology. 1979; 99:410–412. [PubMed: 18631626]

Wildermuth MC, Dewdney J, Wu G, Ausubel FM. Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature. 2001; 414:562–565. [PubMed: 11734859]

Xu W, Shioiri H, Kojima M, Nozue M. Primary structure and expression of a 24-kD vacuolar protein (VP24) precursor in anthocyanin-producing cells of sweet potato in suspension culture. Plant Physiol. 2001; 125:447–455. [PubMed: 11154352]

Zhang Y, Goritschnig S, Dong X, Li X. A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in suppressor of npr1-1, constitutive 1. Plant Cell. 2003a; 15:2636–2646. [PubMed: 14576290]

Zhang Y, Tessaro MJ, Lassner M, Li X. Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. Plant Cell. 2003b; 15:2647–2653. [PubMed: 14576289]

Zhou N, Tootle T, Glazebrook J. PAD3, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monoxygenase. Plant Cell. 1999; 11:2419–2428. [PubMed: 10590168]

Zhou N, Tootle TL, Tsui F, Klessig DF, Glazebrook J. PAD4 functions upstream from salicylic acid to control defense responses in *Arabidopsis*. Plant Cell. 1998; 10:1021–1030. [PubMed: 9634589]

Zipfel C. Pattern-recognition receptors in plant innate immunity. Curr. Opin. Immunol. 2008; 20:10–16. [PubMed: 18206360]

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W. GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol. 2004; 136:2621–2632. [PubMed: 15375207]

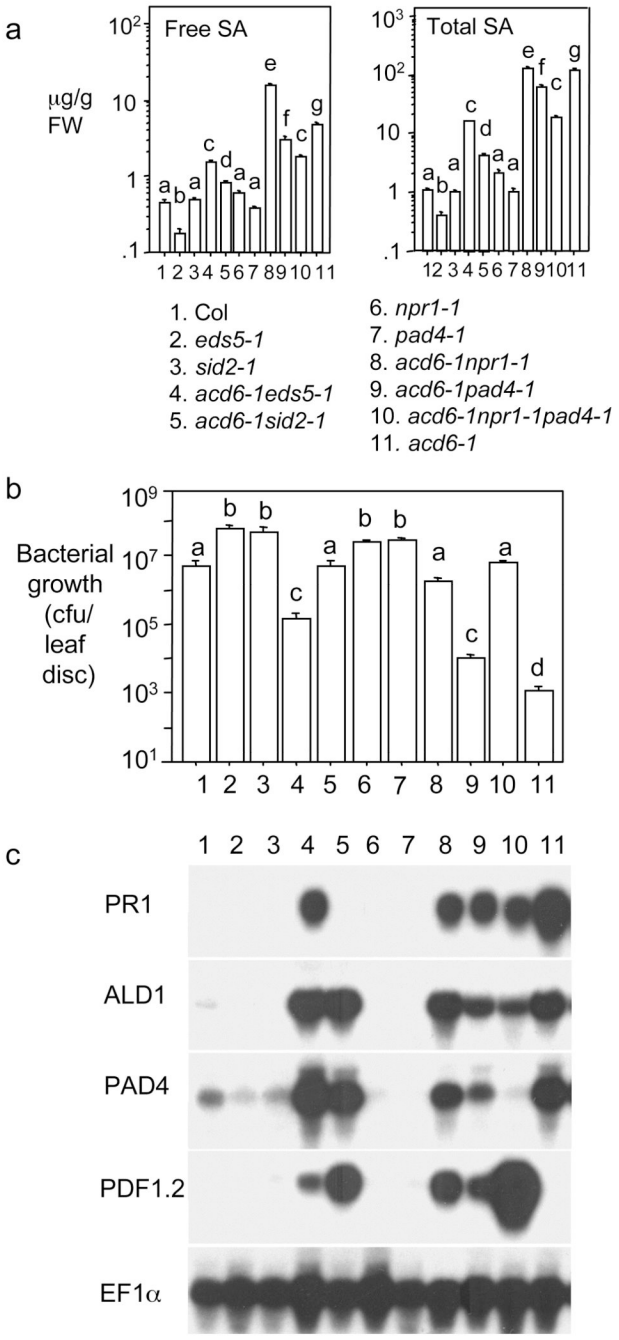


**Figure 1.**

Effects of various mutations on SA accumulation, disease resistance, and defense gene expression in *acd6-1*. (a) SA quantification. SA was extracted from the indicated plants at 20 days of age and analyzed by HPLC. P< 0.01 (n=3). (b) Bacterial growth. The 5$^{th}$ to 7$^{th}$ leaves of each plant were infected with *P. syringae maculicola* (*Pma*) ES4326 DG3 ($OD_{600}$ = 0.0001) for 3 days before bacterial measurement. P < 0.05 (n=6). (c) Defense gene expression. Total RNA was extracted from each plant and subject to Northern blot analysis with the indicated probes. The blots for *ALD1* and *PAD4* were exposed overnight and those for *PR1*, *PDF1.2*, and *EF1a* were exposed for 1 hour. No *PR1* expression was detected in the *acd6-1sid2-1* mutant in an overnight exposed film (data not shown). Similarly low levels

of *PDF1.2* expression were detected in Col, *eds5-1*, *sid2-1*, *npr1-1*, *pad4-1*, and *acd6-1* in an overnight exposed film (data not shown). For (a) and (b), statistical differences among the samples were labeled with different letters. These experiments were repeated two times with similar results.

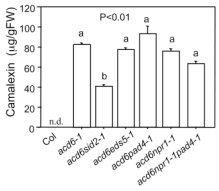


**Figure 2.**

Camalexin accumulation in *acd6-1* is SA-independent. Same samples used in Figure 1 were quantified for camalexin by HPLC analysis. Col, *eds5-1*, *sid2-1*, *npr1-1*, and *pad4-1* did not accumulate detectable camalexin (not shown). $P < 0.001$ (n=3). Statistical differences among the samples were labeled with different letters. These experiments were repeated three times with similar results.

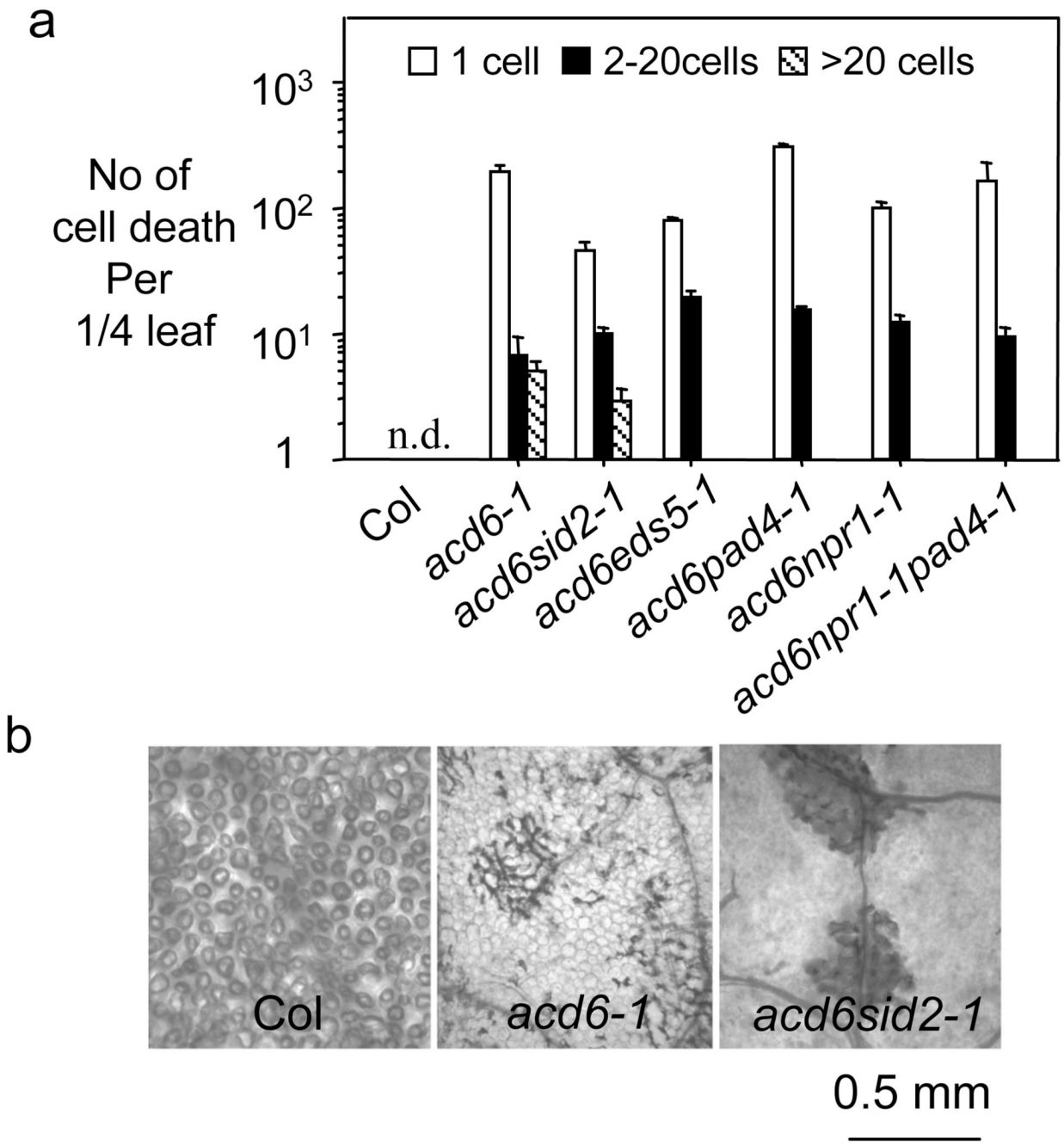


**Figure 3.**

Effect of various SA-regulatory mutations on cell death formation in *acd6-1*. (a) Cell death quantification of trypan blue-stained leaves. The number of dead cells was counted from a quarter region of a leaf (n=5) at the same position in each genotype and data were plotted in a logarithmic scale. Three classes of cell death (single foci, 2–20 cell clusters, and >20 cell clusters) were recorded. Col, *npr1-1*, *pad4-1*, *eds5-1*, and *sid2-1* did not have cell death (not shown). (b) Pictures of leaf tissue after trypan blue staining. These experiments were repeated two times with similar results.

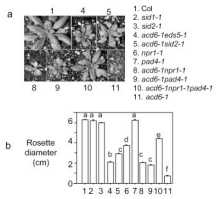


**Figure 4.**

Effect of various SA-regulatory and novel mutations on *acd6-1*-conferred dwarfism. (a) Pictures and (b) rosette diameters of 20-day old plants of the indicated genotypes. Statistical differences with a $P<0.0001$ among the samples ($n>10$) were labeled with different letters in (b). These experiments were repeated two times with similar results.

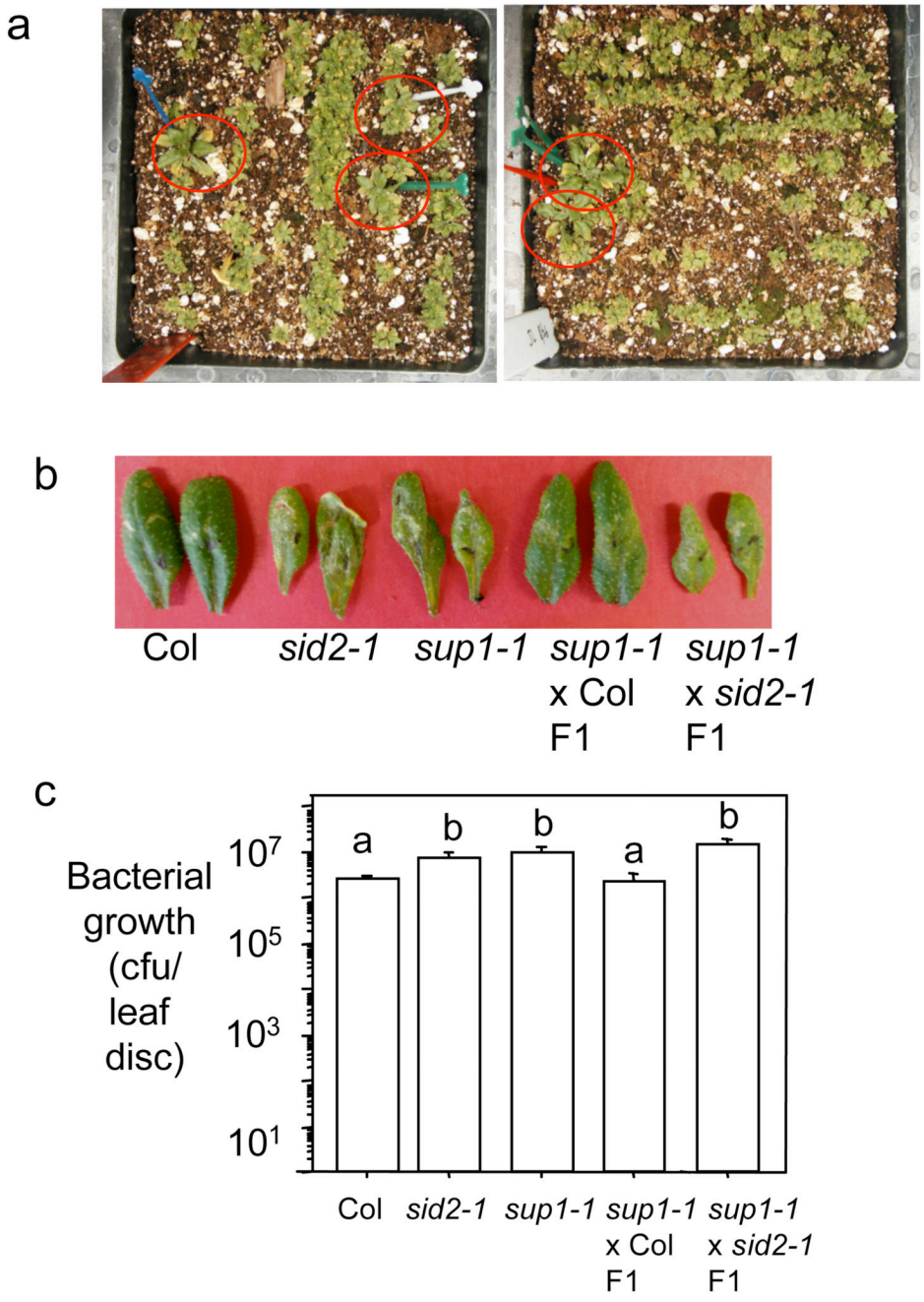


**Figure 5.**

Identification of *sup1-1* as an allele of *SID2* in *acd6-1* mutant screen. (a) Mutant screen for *acd6-1* suppressors. Circles indicate a known suppressor, *acd6-1pad4-1* (left), and novel suppressors (right) that were visually distinguishable from the densely planted *acd6-1* 20 days after being sown. (b) Disease symptoms. The 5$^{th}$ to 7$^{th}$ leaves of 20-day-old plants were infected with *Pma*DG3 ($OD_{600}$ = 0.0001) for 3 days before being photographed. (c) Bacterial growth. Data of bacterial growth represented the average of six samples (n=6, $P < 0.05$). Statistical differences among the samples were labeled with different letters. These experiments were repeated twice with similar results.

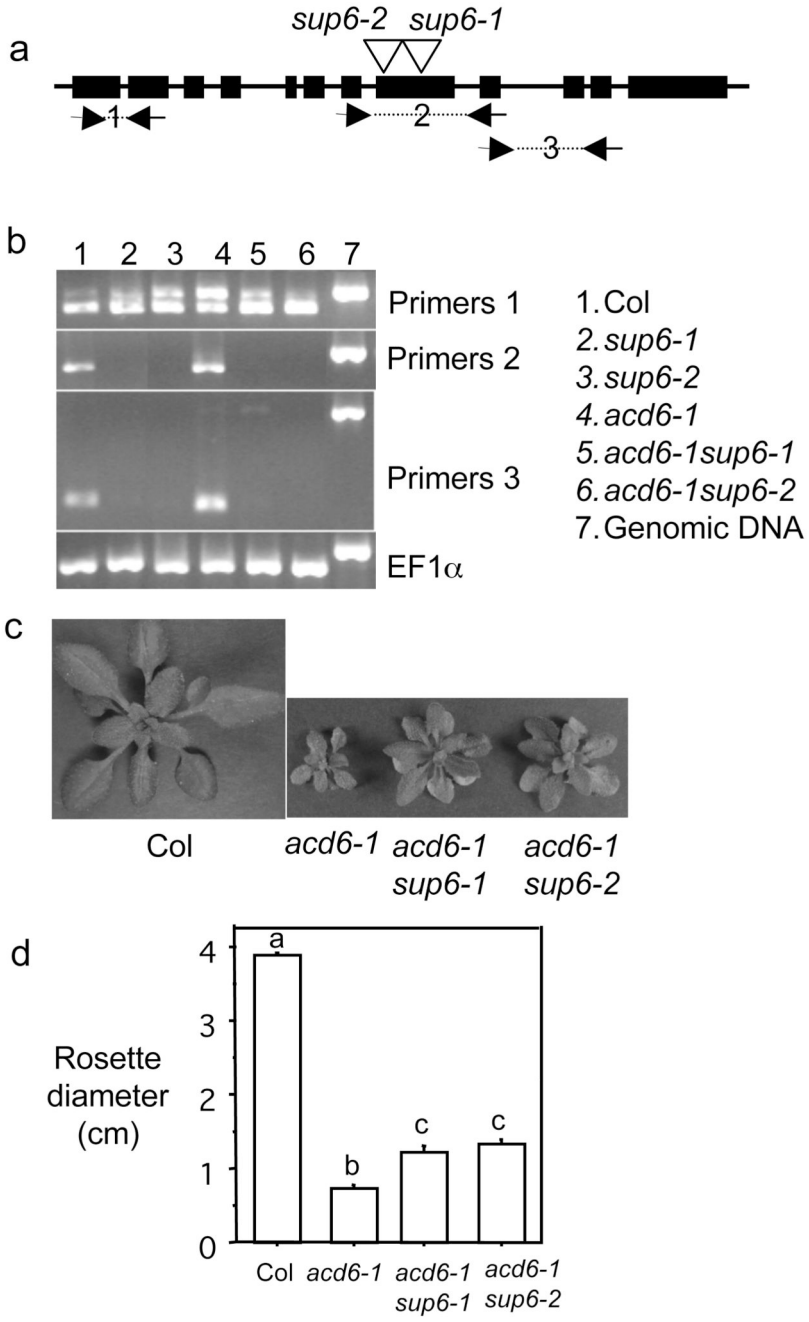


**Figure 6.**

Identification of *SUP6* and characterization of its effects on the size of *acd6-1*. (a) *SUP6* gene structure and positions of the two mutant alleles. Filled boxes indicate exons and lines indicate introns and untranslated regions. Open triangles indicate the T-DNA insertion sites in the two *sup6* alleles. (b) RT-PCR analysis of *SUP6* expression. The positions of the three sets of primers were illustrated in (a). (c) Picture of 20-day old plants. (d) Rosette diameters of plants shown in (c). At least 20 individual plants were measured for the rosette diameter. Asterisks indicate the significant difference between the two double mutants and *acd6-1* ($P<0.0001$). Experiments for (b), (c), and (d) were repeated two times and similar results were obtained.

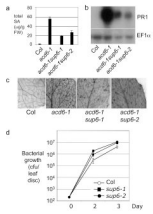

**Figure 7.**

Characterization of the defense phenotypes of *acd6-1sup6* and *sup6* mutants. (a) SA quantification. (b) *PR1* expression by Northern blot analysis. (c) Cell death assay by trypan blue staining. (d) Bacterial growth. The 5$^{th}$ to 7$^{th}$ leaves of 20-day old plants were infected with *Pma*DG3 ($OD_{600}$ = 0.0001) and assayed for bacterial growth at the indicated times (n=6). Statistical difference was observed between the two *sup6* alleles and Col at day 2 and day 3 post infection with a P value < 0.1. These experiments were repeated twice with similar results.

**Table 1**

Effect of PAD3, ETR1, and JAR1 on *acd6-1*-conferred phenotypes. 20-day-old plants were harvested for camalexin (n=3) and/or SA (n=3) analyses by HPLC and size measurement (n=15). For bacterial growth, the 5$^{th}$ to 7$^{th}$ leaves of the indicated plants were infected with *Pma*DG3 ($OD_{600}$ = 0.0001). Three days after infection, leaf discs were taken for quantifying bacterial growth. n: sample size. Each data represents the average result of n samples ± standard error. N/A: data not available.

| Genotype | Camalexin (μg/gFW) n=3 | Free SA (μg/gFW) n=3 | Total SA (μg/gFW) n=3 | Rosette diameter (cm) n=15 | Bacterial growth (×1 $0^4$ cfu /leaf disc) n=6 |
|---|---|---|---|---|---|
| *acd6-1* | 98.1 ± 3.9* | 6.4 ± 0.3 | 148.1 ± 5.2 | 0.8 ± 0.1 | 2.9 ± 1.4 |
| *acd6-1pad3-1* | 3.0 ± 0.5 | 3.9 ± 0.3 | 121.5 ± 5.0 | 0.8 ± 0.1 | 1.7 ± 0.8 |
| *acd6-1etr1-1* | N/A | 5.5 ± 0.7 | 139.6 ± 12.7 | 0.8 ± 0.1 | 6.6 ± 2.9 |
| *acd6-1jar1-1* | N/A | 4.8 ± 0.1 | 130.8 ± 3.2 | 1.0 ± 0.1 | 3.6 ± 1.0 |

* indicates statistical difference between one sample and other samples in the same column with a P value < 0.001. FW is fresh weight.