Knockout of *Arabidopsis* ***ACCELERATED-CELL-DEATH11*** encoding a sphingosine transfer protein causes activation of programmed cell death and defense

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We describe the lethal, recessive *accelerated-cell-death11* *Arabidopsis* mutant (*acd11*). Cell death in *acd11* exhibits characteristics of animal apoptosis monitored by flow cytometry, and *acd11* constitutively expresses defense-related genes that accompany the hypersensitive response normally triggered by avirulent pathogens. Global transcriptional changes during programmed cell death (PCD) and defense activation in *acd11* were monitored by cDNA microarray hybridization. The PCD and defense pathways activated in *acd11* are salicylic acid (SA) dependent, but do not require intact jasmonic acid or ethylene signaling pathways. Light is required for PCD execution in *acd11*, as application of an SA-analog to SA-deficient *acd11* induced death in the light, but not in the dark. Epistatic analysis showed that the SA-dependent pathways require two regulators of SA-mediated resistance responses, PAD4 and EDS1. Furthermore, *acd11* *PR1* gene expression, but not cell death, depends on the SA signal transducer NPR1, suggesting that the *npr1-1* mutation uncouples resistance responses and cell death in *acd11*. The *acd11* phenotype is caused by deletion of the *ACD11* gene encoding a protein homologous to a mammalian glycolipid transfer protein (GLTP). In contrast to GLTP, ACD11 accelerates the transfer of sphingosine, but not of glycosphingolipids, between membranes in vitro.

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In multicellular organisms, cell death occurs as necrosis or genetically defined programmed cell death (PCD). In necrosis, cells die in response to trauma and have little or no control over degenerative processes leading to death. In contrast, PCD is an active process that involves signaling pathways controlling the dismantling of the cell. In animals, the most common form of PCD is termed apoptosis. PCD in plants may share morphological characteristics with animal apoptosis such as condensation of nuclei, fragmentation of nuclear DNA, and cell shrinkage (Pennell and Lamb 1997).

PCD occurs in plants during various developmental processes and during interactions with pathogens. Examples of developmentally controlled PCD include senescence, sex determination in unisexual flowers, and

the formation of the vascular system (Pennell and Lamb 1997). PCD in plant pathogen interactions is observed both during the hypersensitive response (HR) to avirulent infections and during the development of disease symptoms following virulent infections (Morel and Dangel 1997).

Cell death during HR is elicited by specific recognition of pathogen-derived molecules from avirulent pathogens by plant proteins encoded by resistance (*R*) genes. Recognition is followed by local accumulation of reactive oxygen species (ROS), nitric oxide (NO), and salicylic acid (SA), that all potentiate resistance and pro-death pathways (Shirasu and Schultze-Lefert 2000). HR is accompanied by fortification of cell walls and accumulation of pathogenesis-related (*PR*) mRNAs and antimicrobial phytoalexins. These responses serve to protect the plant by restricting the growth of pathogens.

The control of HR cell death appears to involve the concerted action of both ROS and NO (Delledonne et al. 1998). The importance of SA has also been demonstrated

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in transgenic *Arabidopsis* expressing the bacterial SA hydroxylase (NahG), as depletion of SA abolishes an *R* gene-mediated HR (Delaney et al. 1994). Although SA plays a crucial role in HR and is sufficient for *PR* gene accumulation and resistance (Ward et al. 1991), SA alone is insufficient to induce HR. The execution of HR cell death may involve proteolysis, as inhibitors of animal caspases reduce HR cell death (Poza and Lam 1998).

Genetic approaches in *Arabidopsis* have been used to identify signaling components involved in HR control. Broadly, two different classes of mutants have been identified. The first includes mutants that fail to develop HR and resistance in response to infection by normally avirulent pathogens. Although many of these mutations identify *R*-genes (Ellis and Jones 1998), four different downstream regulators have also been identified by this approach. The lipase-like EDS1 and PAD4, and the putative 2TM protein NDR1 function at or downstream of the convergence of several different R-proteins (Aarts et al. 1998; Jirage et al. 1999), whereas the PBS1 protein kinase appears to be required specifically for AvrPphB-elicited HR and resistance (Swiderski and Innes 2001).

The second group consists of mutants that develop HR-like lesions and activate defense responses in the absence of pathogens. These so-called lesion mimic mutants are represented by the *acd*- and *lsd*-mutants (Dietrich et al. 1994; Greenberg et al. 1994; Weymann et al. 1995). Because of their constitutive HR/defense phenotype, lesion mimic mutants may define genes with regulatory roles in HR. Although this may not be correct in all cases (Molina et al. 1999), they serve as important tools to study PCD in plants. For example, much has been learned about SA and SA signal transducers in the death and defense pathways turned on in lesion mimic mutants. Thus, expression of the SA hydroxylase NahG rescues several lesion mimic mutants, whereas application of the SA-analogs benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH) or 2,6-dichloroisonicotinic acid (INA) restores lesioning in all but the *lsd7* mutant (Weymann et al. 1995; Greenberg et al. 2000). These results demonstrate that PCD can be SA dependent. However, SA-independent cell death pathways also exist, as NahG expression does not abate lesions in *lsd2* and *lsd4* (Hunt et al. 1997) and exacerbates lesions in *acd2* (Mach et al. 2001).

The role of the NPR1 ankyrin repeat protein in SA-dependent cell death pathways has also been analyzed by use of *acd*-type mutants. NPR1 is required for responsiveness to exogenous SA (Cao et al. 1997), but, although loss of NPR1 function reduces lesion development in the *acd5* mutant (Greenberg et al. 2000), cell death and defense activation occur independently of NPR1 in most lesion mimic mutants.

Of the many *Arabidopsis* cell death mutants described, six corresponding genes have been isolated. These genes fall into two general classes. The first defines genes involved in porphyrin metabolism (Molina et al. 1999; Ishikawa et al. 2001; Mach et al. 2001), whereas genes in the second class are involved in fatty acid metabolism (Mou et al. 2000; Kachroo et al. 2001). In addition, *LSD1* encodes a zinc-finger protein proposed to negatively regulate a cell death pathway by monitoring a superoxide-dependent signal (Jabs et al. 1996; Dietrich et al. 1997).

Here, we report the characterization of the lethal, recessive *acd11* *Arabidopsis* mutant, which exhibits characteristics of animal apoptosis and defense-related responses that accompany HR. The *acd11* phenotype is SA dependent, as *acd11* is rescued by *nahG*, and application of BTH to *acd11/nahG* restores cell death. This SA-mediated death pathway requires both functional PAD4 and EDS1, as the *acd11* phenotype is suppressed by the *pad4-2* and *eds1-2* mutations. Molecular cloning, complementation, and lipid transfer assays revealed that *ACD11* encodes a homolog of mammalian glycolipid transfer protein and has sphingosine transfer activity in vitro.

Results

Identification and phenotype of the *acd11* mutant

The *acd11* mutant was identified among stable transposant lines generated with a modified maize *Ds* element (Fig. 1A; Sundaresan et al. 1995). *acd11* seed germinated with normal cotyledons, but developed chlorotic leaf margins at the two- to six-leaf stage. The chlorosis progressed and eventually engulfed the rosette before new leaves had fully expanded (Fig. 1A). This phenotype was equally strong in soil-grown plants at 16- and

Figure 1. Phenotype of *acd11* mutant. (A) *Ler* and *acd11*. (B) Cross sections of *Ler* and *acd11* rosette leaves. Bar, 25 μ m. (C) Trypan blue staining of *Ler* and *acd11* rosette leaves. Bar, 25 mm.

8-h light cycles and in sterile-grown plants under continuous light. These results indicate that the phenotype was neither dependent on day length nor due to hypersensitivity to infection. The phenotype of *acd11* segregated as a recessive trait. When *acd11* plantlets survived long enough to initiate reproductive growth, flowers formed directly at the rosette, indicating that shoot formation is inhibited in *acd11*. In contrast, no obvious effect was observed on root growth in *acd11* (data not shown).

Microscopic examination of *acd11* rosette leaves revealed that overall cell size was normal. However, the plasma membrane of individual cells was separated from the cell wall (Fig. 1B). Compared with *Ler* controls, the *acd11* mesophyll appeared disorganized and numerous cells contained clusters of wall material (Fig. 1B). In addition, numerous dead cells were seen microscopically by trypan blue staining (Fig. 1C).

PCD in *acd11*

The type of cell death observed in *acd11* could be necrosis due to basic cellular dysfunction. Alternatively, it might result from the execution of a cell death program activated as a consequence of the *acd11* mutation. To distinguish between these two possibilities, we examined *acd11* cell function in several ways. First, PCD is often preceded by a transient burst of reactive oxygen species (ROS; Pierce et al. 1991). To examine whether an oxidative burst is generated in *acd11*, mutants showing early symptoms were stained with nitro blue tetrazolium (NBT). The purple formazan precipitate produced by the reaction of NBT with superoxide was clearly visible throughout the leaves of *acd11*, but was absent in the *Ler* control (Fig. 2A).

Second, it is possible to measure apoptosis by flow cytometry through the use of the fluorescent dye

7-amino actinomycin D (7-AAD), which binds DNA. A selective decrease in DNA stainability is seen in apoptotic cells because of DNA condensation and/or cleavage. In addition, as *acd11* cells are normal in size, apoptotic cell shrinkage can be detected because light scatter in the forward direction is proportional to cell size (Skov et al. 1997; Gómez-Angelats et al. 2000). Figure 2B shows 7-AAD staining and cell sizes of *Ler* and *acd11* protoplasts. A clear decrease in DNA stainability was observed in protoplasts isolated from *acd11* compared with the *Ler* controls. Furthermore, a major fraction of *acd11* protoplasts displayed low forward scatter values (Fig. 2B). This result suggests that the *acd11* phenotype results from an active cell death program akin to animal apoptosis, rather than from necrosis.

Third, diphenylene iodonium (DPI), an inhibitor of the neutrophil NADPH oxidase, reduces lesion formation in *lsd1* when shifted to nonpermissive long-day conditions (Jabs et al. 1996), probably as a consequence of reduced production of ROS and NO (Delledonne et al. 1998). Accordingly, *acd11* plants were treated with DPI for 48 h prior to protoplast isolation. Flow cytometry revealed a more than twofold decrease in the apparent apoptosis of *acd11* compared to untreated controls (Fig. 2C).

Fourth, we examined the effects of the protein kinase inhibitor staurosporine and the phosphatase inhibitor okadaic acid on cell death in *acd11* by treatments 48 h before protoplast isolation. Flow cytometry showed that both staurosporine and okadaic acid reduced *acd11* cell death compared with untreated controls (Fig. 2C), further demonstrating that active participation of the cell regulatory machinery is required for *acd11* cell death.

Fifth, accelerated cell death in *acd11* might be the outcome of an early senescence program. Because cytokinins can inhibit the progression of leaf senescence (for review, see Gan and Amasino 1997), we examined whether cytokinin could block the progression of *acd11*

Figure 2. ROS and apoptosis measurements. (A) NBT staining of *Ler* and *acd11* seedlings. (B) Flow cytometry of 7-AAD stained protoplasts isolated from *Ler* and *acd11* rosette leaves showing number of cells (events) vs. 7-AAD intensity. Bars indicate fluorescence intensity intervals, which define apoptotic cells. (Inset) Side vs. forward scatter (cell size). Each experiment was repeated three times. (C) As in B with diphenylene iodonium (DPI), staurosporine (STA), okadaic acid (OKA), or N(6)-benzyladenine (BA).

cell death. Protoplasts were isolated from *acd11* plants 48 h after treatment with the cytokinin N(6)-benzyladenine (BA) and from mock-treated *acd11* plants. No discrepancy in the 7-AAD staining of DNA was observed between protoplasts from BA- and mock-treated *acd11* plants (Fig. 2C). Therefore, the PCD pathway activated in *acd11* is not likely to be related to senescence.

acd11 expresses markers associated with HR

Plant cells undergoing hypersensitive cell death produce and deposit secondary metabolites that are visible by their fluorescence. In addition, cell wall materials including callose and aromatic polymers are deposited at infection sites (Dietrich et al. 1994). Therefore, we examined *acd11* leaves for the presence of autofluorescent material and stained for callose with aniline blue. Figure 3A demonstrates that leaves of *acd11* accumulate substantial amounts of autofluorescent products. Whole mounts of *acd11* leaves showed a massive abundance of callose, whereas *Ler* controls only showed small patches of callose, primarily along the vein (Fig. 3A).

We also examined the expression of *PR* genes in *acd11*, as other lesion mimic mutants constitutively express defense-related *PR* genes (Dietrich et al. 1994; Greenberg et al. 1994; Bowling et al. 1997). RNA blots verified that *PR1*, *PR2*, and *PR5* were expressed constitutively in *acd11* (Fig. 3B). In addition, transcripts of genes encoding proteins with roles in oxygen metabolism (peroxidase C, *PRXc*), detoxification (glutathione-S-transferase, *GST*) and synthesis of phenylpropanoid products (chalcone synthase, *CHS*) accumulate during HR and in lesion mimic mutants (Greenberg et al. 1994; Jabs et al. 1996). These transcripts also accumulated in *acd11* (Fig. 3B).

Several genes are known to accumulate during senes-

cence including *SAG12*, encoding a cysteine protease, and *SAG13*, encoding a short-chain alcohol dehydrogenase (Lohman et al. 1994). Intriguingly, requirement for a short-chain alcohol dehydrogenase in plant PCD is observed during sex determination in the maize *tassel-seed2* mutant (DeLong et al. 1993). Therefore, we examined the expression of *SAG12* and *SAG13* in *acd11*. *SAG13* mRNA was strongly expressed in *acd11*, whereas *SAG12* mRNA did not accumulate (Fig. 3B). The absence of *SAG12* mRNA confirms that cell death in *acd11* does not result from activation of a senescence program, whereas the strong expression of *SAG13* suggests that this gene may serve as a PCD marker.

Consistent with their normal appearance, roots of *acd11* did not have constitutive *PR1*- or *SAG13*-expression (data not shown).

Patterns of global gene expression in *acd11*

To expand our understanding of gene expression in *acd11*, we compared mRNA levels in *acd11* and wild-type seedlings by cDNA hybridization to a microarray of 9861 cDNAs expressed throughout *Arabidopsis* development (Ruan et al. 1998). Of the 6820 (70%) cDNAs that hybridized, 6374 hybridized at approximately equivalent levels in *acd11* and wild type (93% between 2.5-fold over- and underexpressed). Genes exhibiting significantly higher expression levels in *acd11* (see Materials and Methods) were assigned to groups according to known or putative functions (Table 1). Although this list is not comprehensive, it clearly illustrates different patterns of gene expression.

The first group includes genes related to oxidative burst/stress and apoptosis. It includes mRNAs that accumulate during HR, such as *PRX* and *GSTs*, that were also detected by Northern hybridization (Fig. 3B). The overexpression of a flavin-containing monooxygenase (FMO) in *acd11* is notable and was confirmed by Northern blotting (Fig. 3C). FMOs catalyze diverse reactions including the oxidation of N- and S-containing compounds (Ziegler 1993) and have not previously been associated with cell death and defense responses in plants.

The second group includes defense-related mRNAs that accumulate during resistance responses. For example, *PR2* mRNA accumulated ninefold higher in *acd11*, consistent with the Northern results (Fig. 3B). In addition, the *PAD3* cytochrome P450 accumulates rapidly in infected tissue of wild type and is 15-fold induced in *acd11*. *PAD3* is required for synthesis of the antimicrobial phytoalexin camalexin (Zhou et al. 1999). The production of camalexin also requires anthranilate synthase (*ASA*; Niyogi and Fink 1992), and *ASA1* mRNA accumulation is up-regulated about fourfold in *acd11*. This group also includes the lipase-like gene *EDS1* required for signaling from certain R-genes, and for accumulation of SA following virulent infection (Parker et al. 1996; Aarts et al. 1998). Constitutive expression of *EDS1* was confirmed by Northern blotting (Fig. 3C).

The third group includes genes with putative signaling and regulatory functions. It includes the WRKY70 tran-

Figure 3. Accumulation of defense-related markers. (A) Autofluorescence (top) and callose staining (bottom) of *Ler* and *acd11* rosette leaves. Bar, 100 µm. (B,C) RNA gel blots of 10 µg total RNA hybridized with radiolabeled probes as indicated. The *SAG12* probe detected ~1 pg of the 1200-bp fragment used as a probe template spotted onto a filter. Equal loading of RNA was checked on all blots by hybridization with *EF-1α*. A representative blot is shown.

Table 1. *Genes >3.8-fold up-regulated in acd11*

G1	Protein or homolog	Fold up-regulation
Oxidative stress/burst and apoptosis		
8954062	flavin-containing monooxygenase	17.9
443697	glutathione-S-transferase (ERD11)	16.7
16202	blue copper protein	15.4
407089	glutathione-S-transferase	8.7
1402905	peroxidase (PRXr2)	6.6
7270886	DNA damage-inducible protein-like	4.9
8778278	DNA break/repair protein-like	4.7
4432856	dioxygenase	4.0
Defense-related		
2288989	chitinase	16.2
1167960	extensin (EXT1)	15.8
15231632	cytochrome P450 (PAD3)	15.1
903895	pectin methyltransferase (PME1)	9.0
166636	β -(1,3)-glucanase 2 (PR2)	8.7
8777408	myrosinase bd protein	5.7
457716	CXc750	5.4
4115363	NBS-LRR R protein-like	5.2
3482931	oxalate oxidase-like (GLP5)	4.3
166604	anthranilate synthase (ASA1)	4.3
7269612	stomatin-like	4.2
8954056	pto responsive-like	4.0
4454567	lipase/esterase (EDS1)	3.8
166638	β -(1,3)-glucanase 3 (BGL3)	3.8
Signal transduction and regulation		
8671834	protein kinase/U-box	11.2
2244792	methyl transferase/ankyrin repeat	7.4
4887748	LRR receptor-like kinase	7.1
7594518	transcription factor (WRKY70)	6.9
13430790	calmodulin-like	5.3
598067	calmodulin (TCH3)	5.2
7573467	RING finger/VWA copine-like	4.1
7573430	lipase/esterase	4.1
9957238	ARF-GAP/C2 domains (ZAC)	4.0
1209633	ARF-GEF (GNOM)	4.0
Housekeeping		
2098712	pectin esterase	9.0
13877696	AAA ATPase	8.4
1805365	xyloglucan endo-transglycosylase	8.2
^a	phosphoglycerate mutase-like	7.7
7529275	FH bd-/ABA induced protein like	7.3
3319354	hexose transporter-like	5.4
6017105	cytochrome P450	5.2
4587550	exocyst subunit Exo 70-like	5.0
507946	Asn synthetase (ASN1)	5.0
4544456	cytochrome P450	4.6
13374872	mannosyl transferase-like	4.3
6650523	RRM-motif RNA bd protein (MEI2)	4.0
1336083	Glu dehydrogenase (GDH2)	3.9
Others		
15219011	unknown	18.7
6041793	unknown	13.1
7269940	unknown	11.5
8778226	flavonol-induced protein like	8.5
4185135	unknown	7.8
6850886	contains C2 motif	6.6
10177080	unknown	6.4
4432837	unknown	4.6
3688186	unknown	4.5
13430466	acyl transferase-like	3.9
10177423	unknown	3.8
3075398	unknown	3.8

^aNonannotated ORF on BAC F21M12, positions 118956–119026, 119363–119385, 119554–119664, 119849–119971.

scription factor, a calmodulin, a receptor like-kinase (RLK), a class IV U-box motif (Azevedo et al. 2001), and a RING finger motif-containing protein. WRKY transcription factors are plant-specific and regulate genes associated with pathogen defense. WRKY70 is closely related to two tobacco proteins, NtWRKY3 and NtWRKY4, that are induced by SA and pathogen infection (Chen and Chen 2000). Calmodulins may mediate HR-associated Ca^{2+} signals, as overexpression of calmodulin isoforms can induce lesions (Heo et al. 1999). Similarly, RLKs are rapidly induced in response to avirulent infections and may be involved in HR signaling (Du and Chen 2000), and this particular RLK also accumulates in *mpk4*, which constitutively expresses SA-dependent defenses (Petersen et al. 2000). Both the U-box and the RING finger domains are involved in ubiquitination of target proteins for proteasomal degradation (Freemont 2000; Azevedo et al. 2001). In this respect, the up-regulation of an AAA motif ATPase is notable, as members of a large subgroup of this family function as components of the 26S proteasome (Patel and Latterich 1998).

The fourth group includes mRNAs that normally function in cellular housekeeping. Some encode enzymes involved in wall modifications, such as pectin esterase and xyloglucan endo-transglycosylases (XET), not previously implicated in HR-like responses, whereas the accumulation of a sugar transporter in *acd11* is consistent with former reports (Schenk et al. 2000).

Table 2 lists mRNAs that accumulate to significantly lower levels in *acd11* than *Ler*. The largest group includes genes involved in photosynthesis, which is not surprising given the chlorosis of *acd11*. The significance of the genes in the remaining three groups is speculative. However, the down-regulation of the cyclic nucleotide regulated ion channel is notable, because loss-of-function mutations in the homologous *DND1* gene produce mutants with constitutive defense activation and conditional lesion formation (Clough et al. 2000). In addition, the repression of a MAP kinase phosphatase is consistent with reports of activation of MPK3 and MPK6 following oxidative stress (Kovtun et al. 2000) and of sustained MAP kinase activation after an HR inducing R-Avr recognition (Romeis et al. 1999).

The acd11 phenotype is SA and light dependent

The development of PCD in lesion mimic mutants may be SA dependent (Weymann et al. 1995) or SA independent (Hunt et al. 1997; Mach et al. 2001). To examine whether SA accumulation is important for *acd11* cell death, *acd11* was crossed to *nahG*. Expression of *nahG* completely rescued the *acd11* mutant, and although application of the SA analog BTH to *acd11/nahG* restored runaway cell death within 5 d, wild-type *Ler* was unaffected by this treatment (Fig. 4A; data not shown).

To characterize further this SA-dependent pathway, *acd11* was crossed to the *pad4-2*, *eds1-2*, and *npr1-1* mutants. *PAD4* and *EDS1* encode lipase-like proteins that may function in an SA-amplification loop, as they are

Table 2. Genes >3.8-fold repressed in *acd11*

GI	Protein or homolog	Fold down-regulation
Photosynthesis-related		
5734518	PSI subunit III precursor	5.7
4741950	LHC II antenna protein	4.9
9759044	LipA-like	4.8
16367	chlorophyll a/b bd protein	4.7
4741958	PSII chlorophyll bd protein (Lhcb5)	4.5
3157944	GCV subunit T (aminomethyltransferase)	4.3
968976	protochlorophyllide reductase	4.1
12083257	chlorophyll a/b bd protein	3.9
1465365	PS-II subunit T	4.0
16446	PSII 10 kDa polypeptide	4.0
430946	PSI chlorophyll bd protein (Lhca3*1)	3.9
4741959	PSII chlorophyll bd protein (Lhcb6)	3.9
Signal transduction and regulation		
217853	DNA-bd HMG-box protein	5.1
435428	GTP-bd protein	4.3
4581207	cyclic nucleotide/calmodulin-reg. ion chl.	4.1
499161	homeobox leucine zipper (HB-6)	3.8
6862915	MAP kinase phosphatase	3.8
Housekeeping and development		
166923	ubiquitin carrier protein	4.9
9758220	(+)- δ -cadinene synthase	4.6
13877603	contains EF1G and GST domains	4.6
4240120	cytochrome b-5	3.9
12323431	metal-bd isoprenylated protein	3.8
16930501	thiazole biosynthesis enzyme (ARA6)	3.8
Other		
9758251	unknown	5.0
^a	unknown	4.8

^aNonannotated ORF on BAC T1E4; positions 3665–3895, 4748–4857.

transcriptionally induced by SA, but loss-of-function *pad4* and *eds1* mutants fail to accumulate SA after virulent and certain avirulent pathogen infections (Zhou et al. 1998; Falk et al. 1999; Jirage et al. 1999). Both *pad4-2* and *eds1-2* completely suppressed the *acd11* phenotype (Fig. 4A). In addition, both *EDS1* and *PAD4* mRNAs accumulate in *acd11* (Fig. 3C). However, although BTH application had an inhibitory effect on the growth of the shoot and leaf petioles, it did not restore lesion formation in *acd11/eds1-2* and resulted in only sporadic cell death in *acd11/pad4-2* (Fig. 4A). The difference between the effects of the *eds1-2* and *pad4-2* mutations on *acd11* was confirmed by monitoring the expression of the *SAG13* and *PR1* markers 6 d after BTH application. Although *acd11/pad4-2* expressed these markers almost as strongly as *acd11/nahG*, only weak expression was detected in *acd11/eds1-2* (Fig. 4B).

In contrast to *PAD4* and *EDS1*, the NPR1 ankyrin repeat protein acts downstream of SA (Cao et al. 1997). The size of *acd11/npr1-1* double mutants was intermediate between *acd11* and wild type, and although *acd11/npr1-1* developed chlorosis at about the same stage as *acd11*, *acd11/npr1-1* leaves appeared paler than *acd11*

leaves (Fig. 4C). As in *acd11*, chlorosis spread and eventually engulfed the entire plant before seed set. Consistent with this death phenotype, expression of *SAG13* in *acd11/npr1-1* was as strong as in *acd11* (Fig. 4D). In contrast, expression of the defense marker *PR1* was nearly completely suppressed in *acd11/npr1-1* (Fig. 4D). This result suggests that separable pathways leading to NPR1-dependent *PR* gene expression and NPR1-independent cell death are activated in *acd11*.

Apart from SA, the phytohormones ethylene and jasmonic acid (JA) have been shown to modulate PCD pathways (Asai et al. 2000). To examine JA and ethylene signaling in *acd11* cell death, we constructed *acd11/jar1-1* and *acd11/ein2-1* double mutants. These double mutants fully retained the *acd11* cell death phenotype (data not shown) and expressed *PR1* and *SAG13* to the same level as *acd11* (Fig. 4E), indicating that intact ethylene and JA pathways are not required for *acd11* cell death.

Light is a trigger of the *lsd1* phenotype and is required for cell death in mutants defective in porphyrin metabolic pathways (Dietrich et al. 1994; Mach et al. 2001) and for PCD induced by the mycotoxin fumonisin B1 (FB1; Asai et al. 2000). To examine the effect of light on the *acd11* phenotype, detached leaves of *acd11/nahG* were treated with BTH and kept in the light or in darkness for 4 d. Although propagating cell death was absent in BTH-treated *acd11/nahG* leaves in the dark, PCD developed rapidly in the light (Fig. 4F).

Cloning of ACD11

Inverse PCR was used to isolate chromosomal sequences flanking both ends of the transposon in *acd11*. This approach showed that *Ds* had integrated on chromosome 2 in a position corresponding to BAC T29F13. Surprisingly, there was a 30-kb gap between the matching chromosomal sequences, indicating that the transposon had created a 30-kb chromosomal deletion upon insertion. This event was confirmed by PCR with *acd11* and *Ler* genomic DNA templates and primer sets either outside or within the 30-kb region. Although a fragment outside the 30-kb region was amplified equally well from *acd11* and *Ler*, no DNA could be amplified from *acd11* with any of four different primer sets within the 30-kb region (data not shown). Moreover, one end of the *acd11 Ds* locus (GI 3132469, pos. 50406) is identical to that of the T-DNA insertion in the original *DsG1* donor line (Sundaresan et al. 1995; Parinov et al. 1999).

Linkage analysis indicated that the *ACD11* gene is closely linked to, and possibly contained within, the 30-kb deleted region (data not shown). This region contains five annotated genes (T29F13.7–T29F13.11). T29F13.11 is disrupted by the *DsG1* donor T-DNA insertion, and, therefore, is not a candidate for *ACD11*. For each of the four remaining genes, DNA fragments including 1500 bp of 5' upstream and 500 bp of 3' downstream sequence were transformed into *acd11* heterozygotes. Only T29F13.10 complemented the *acd11* mutant, consistent with BAC subclone transformations that showed that

only a 15-kb fragment spanning T29F13.10 complemented *acd11* (data not shown). A full-length cDNA corresponding to T29F13.10 was obtained (EST 166P10T7) and cloned behind the strong viral 35S promoter. This construct also complemented *acd11*, demonstrating that T29F13.10 is the *ACD11* gene.

Sequence analysis of ACD11

The coding region of the *ACD11* cDNA confirmed the T29F13.10 annotation. *ACD11* encodes a 22.7-kD protein with no predicted transmembrane domains or localization motifs, suggesting that it is a cytosolic protein. *ACD11* has two closely related homologs in rice and *Arabidopsis*, both of unknown function (Fig. 5A). Therefore, *ACD11* belongs to a small family of proteins conserved in plants. More distantly related proteins were found in *Drosophila melanogaster*, *Caenorhabditis elegans*, different mammals, and in the ascomycete *Podospira anserina* (Fig. 5A). Most of these proteins are similar in length to *ACD11*, and their sequence similarities are found throughout the *ACD11* sequence. In other cases, the *ACD11*-like sequence was part of a larger protein. Only two of the identified *ACD11* homologs have been characterized. First, the nearly identical pig and bovine homologs catalyze the transfer of glycosphingo- and glycolipids between membranes in vitro, and therefore, are called glycolipid transfer proteins (GLTP; Lin et al. 2000). However, the in vivo function of these proteins has not been rigorously established. The second is the *P. anserina* Het-C protein (Saupe et al. 1994). The multiallelic *het-c* locus is involved in specifying vegetative incompatibility, a process that impairs hyphal fu-

sions of genetically different strains by triggering a lytic process reminiscent of animal PCD (Bégueret 1972).

ACD11 has sphingosine transfer activity in vitro

The homology of *ACD11* to GLTP suggested that *ACD11* may have lipid transfer or binding activity. Because sphingolipid-derived signals have been implicated in the regulation of PCD (Schmelz et al. 1998), we tested the ability of heterologously expressed *ACD11* protein to catalyze the transfer of three different sphingolipids between vesicles in vitro, the GLTP substrate galactosylceramide (GalCer), ceramide (Cer), and sphingosine (Sph). Parallel transfer assays were carried out with recombinant GLTP protein purified by use of the same procedure as for *ACD11*. None of the proteins catalyzed Cer transfer, and although GLTP catalyzed GalCer transfer as expected, no such activity was observed for *ACD11*. In contrast, *ACD11* had a small, but reproducible catalytic effect on the transfer of Sph, whereas Sph transfer in the presence of GLTP was equal to the background level (Fig. 5B,C). *ACD11*-stimulated Sph transfer was linearly dependent on *ACD11* input in the range of 0.5–2.5 μ g, but further addition of *ACD11* did not result in further stimulation of Sph transfer. To confirm *ACD11* Sph transfer selectivity, transfer assays were repeated in the presence of bovine serum albumin (BSA), which has multiple binding sites for hydrophobic ligands. BSA did not affect *ACD11* sphingolipid transfer specificity and only slightly increased *ACD11* activity toward Sph (data not shown). Thus, the homologous *ACD11* and GLTP proteins appear to differ in their sphingolipid transfer catalytic activities: GLTP acceler-

Figure 5. ACD11 is homologous to glycolipid transfer proteins and accelerates the transfer of sphingosine between membranes in vitro. (A) Alignment of ACD11 protein homologs of the same length as ACD11 ordered according to their similarity to ACD11. (Os) *Oryza sativa*; (At) *A. thaliana*; (Mm) *Mus musculus*; (Ce) *C. elegans*; (Bt) *Bos taurus*; (Pa) *P. anserina*. GI numbers corresponding to the proteins are OSJNBa0018H01.3, 13702819; T19P19.60, 7487218; MGC7473, 13277382; K08E4.2, 7505546; GLTP, 6959680; Het-C2, 2133321. (B) Sphingosine transfer activity as a function of recombinant ACD11 input. (C) ACD11 and GLTP sphingolipid transfer specificity. The background transfer levels are subtracted from transfer in the presence of ACD11 or GLTP.

ates transfer of the complex sphingolipid GalCer, whereas ACD11 accelerates the transfer of the sphingoid base Sph.

Discussion

Both defense-associated and senescence-related pathways may lead to PCD in plant leaves. Cell death in

acd11 probably does not proceed via a senescence-related pathway, because *acd11* cell death could not be inhibited by cytokinin, and because mRNA of *SAG12*, a reliable senescence marker, was not detected in *acd11*. These results and the defense activation data indicate that *acd11* cell death occurs via a PCD pathway that is coordinated with the activation of defense responses. Such coordination of PCD and defense responses occurs both

during HR and after virulent infections. In the plant-pathogen system, these two types are readily separated by the speed, amplitude, and localization of activation. In contrast, it is difficult to distinguish between the two responses in a mutant such as *acd11* with constitutive PCD/defense activation. Nonetheless, the independence of EIN2 argues that the PCD pathway activated in *acd11* is different from that used during at least some susceptible pathogen interactions, as *ein2* mutants showed a normal HR but developed less severe cell death symptoms upon virulent bacterial infections than wild type (Bent et al. 1992).

The clear PCD and defense activation phenotypes of the *acd11* mutant prompted us to use this system to elucidate global transcriptional reprogramming during PCD and defense activation by microarray hybridization. As might be expected, this approach showed that photosynthesis-related genes were repressed, whereas numerous, well-known PCD and defense-associated transcripts were up-regulated in *acd11*. This observation argues that the changes detected by the microarray hybridization are reliable. Other genes with likely roles in PCD and defense, including FMO, XET, and pectin esterase, were also identified. In addition, previously uncharacterized genes were found to be differentially expressed in *acd11*. For example, the up-regulation of genes potentially involved in the ubiquitin-proteasome pathway raises interesting questions as to how their activities may contribute to PCD and defense activation and execution. Also, the correlation of sustained activation of the NtWIPK/AtMPK3 and NtSIPK/AtMPK6 MAP kinases during HR activation with the transcriptional down-regulation of a MAPK phosphatase in *acd11* suggests that one or both of these MAPKs could be in vivo substrates of this phosphatase.

In more general terms, the microarray results provide a list of genes related either causally or symptomatically to PCD/defense activation. Our genetic analysis with the *eds1* and *pad4* mutants indicates clearly that genes causally linked to PCD/defense are included in the list of up-regulated genes, but assignment of the remainder of the genes awaits studies by targeted gene disruption or identification through lesion mimic suppressor screens. Likewise, some of the genes repressed in *acd11* may turn out to be *LSD* or other *ACD* genes, as indicated by the down-regulation of a cyclic nucleotide regulated ion channel homologous to the *LSD* gene *DND1*.

As in most other lesion mimic mutants, SA is a requisite modulator of the PCD pathways activated in *acd11*. Previous work has shown that the accumulation of SA in some *R*-gene dependent responses, such as *RPP2* and *RPP4* dependent responses, requires functional forms of both PAD4 and EDS1, whereas resistance triggered by other *Arabidopsis* *R* genes does not (Glazebrook et al. 1997; Aarts et al. 1998;). Similarly, accumulation of SA and resistance in *cpr1* require PAD4 and EDS1, whereas lesion formation and SA accumulation in *cpr5* are largely PAD4 and EDS1 independent (Clarke et al. 2001; Jirage et al. 2001). These data indicate that PAD4 and EDS1 act at similar positions in specific pathways

controlling SA accumulation. Our epistatic analyses suggest that loss of ACD11 turns on such a pathway upstream of PAD4 and EDS1, as *acd11* phenotypes depend on SA accumulation as well as functional PAD4 and EDS1. Although restoration of massive cell death by treatment with the SA-analog BTH occurs in SA-deficient *acd11/nahG*, *acd11/pad4-2* only exhibits sporadic cell death, and *acd11/eds1-2* is nearly unaffected. Thus, analysis of the *acd11* mutant clearly indicates that both EDS1 and PAD4 not only function in SA accumulation, but also control other signals that work in conjunction with SA to potentiate cell death. Two models may explain the different effects of *pad4* and *eds1* mutations. First, compared with PAD4, EDS1 may control a larger subset of other pro-death signals than SA, such that PCD activation in *acd11/eds1-2* is more efficiently blocked than in *acd11/pad4-2*. Second, EDS1 or another homolog may partially compensate for loss of PAD4 function in the control of pro-death signals other than SA. Lastly, we note that because application of SA is sufficient to activate resistance responses in *pad4* and *eds1* (Falk et al. 1999; Jirage et al. 1999), there may be different requirements for PAD4 and EDS1 in cell death and resistance responses.

What may be the function of ACD11? ACD11 does not appear to have an indispensable cellular function, such that loss of *ACD11* function causes cells to collapse, because *acd11* is suppressed by *nahG*. Thus, the *acd11* phenotype is due to activation of signaling pathways dependent on SA that lead to PCD and defense activation. A major question is then whether the *Acid*⁻ phenotype arises as a misinterpretation of an imbalanced biochemical state in the *acd11* mutant, or whether ACD11 actually functions to negatively regulate PCD and defense in vivo. Although we are unable to distinguish clearly between these two possibilities, this question is pertinent to all lesion mimic mutants and has not been rigorously addressed in any case. Nonetheless, we note that large groups of genes differentially regulated in *acd11* and wild type are related to death and defense activation and that well-defined groups of target genes for other hormonal or cellular responses could not be identified, indicating that the *acd11* mutant is not excessively pleiotropic.

The in vitro sphingosine transfer activity of ACD11 provides a link between ACD11 function and sphingolipid metabolism. Sphingolipid biochemistry is connected to both plant and animal PCD, as the sphinganine-analog mycotoxins fumonisin B1 (FB1) and AAL toxin induce PCD in both plant and animal systems and competitively inhibit ceramide biosynthesis in vitro (Gilchrist 1997). In a colon-derived cell line, the PCD-inducing effect of FB1 was shown to be mediated by the accumulation of the free sphingoid bases sphinganine and sphingosine (Schmelz et al. 1998). Also, AAL-susceptible, but not resistant tomato leaves accumulated high levels of free sphingoid bases when treated with AAL toxins (Abbas et al. 1994), presumably as a consequence of ceramide synthase inhibition.

In contrast to complex sphingolipids, sphingosine is able to move through the aqueous phase between mem-

branes at appreciable rates on its own (Hannun et al. 2001), and, therefore, the physiological need for a sphingosine transfer protein is unclear. Nonetheless, a transfer protein may be required to rapidly sort sphingosine or other sphingoid bases in membrane compartments. Another possibility is that ACD11 functions as a cofactor for sphingoid base-modifying enzymes. This type of action is seen for other lipid transfer proteins. For example, the mammalian phosphoinositide transfer protein may function to supply substrates to phosphoinositide kinases (Cockcroft 1998). Elimination of such functions could lead to sphingoid base-mediated PCD activation. However, given that our survey of potential ACD11 sphingolipid substrates is not exhaustive and that the *in vitro* sphingosine transfer catalytic activity of ACD11 is relatively modest, we cannot rule out the possibility that physiological ACD11 substrates include sphingolipids other than sphingosine. Finally, we note that ACD11 homologs are found in multicellular eukaryotes capable of undergoing PCD, but not in the unicellular yeast *Saccharomyces cerevisiae*, arguing that ACD11 does not have a function in basic sphingolipid metabolism shared among all eukaryotes.

acd11 shares certain characteristics with PCD activation by FB1 in *Arabidopsis*, including dependence on light, SA accumulation, and functional PAD4, but not NPR1 (Asai et al. 2000). However, the genetic requirements for PCD activation are not identical, as FB1-induced cell death requires functional ethylene and JA signaling pathways, whereas both *acd11/ein2-1* and *acd11/jar1-1* were *Acd*⁻. This observation suggests that either separate PCD pathways are activated in *acd11* and upon FB1 treatment or that *acd11* acts genetically downstream of *JAR1* and *EIN2*. Preliminary results indicate that sphingosine levels are significantly higher in *acd11* than in wild type, so both FB1 and ACD11 may lead to alterations in sphingoid base levels. If so, a biochemical interpretation would be that ethylene and jasmonate signaling are required to maintain perturbed sphingoid base metabolism upon FB1 treatment, but not in *acd11*. In any event, and regardless of whether ACD11 activity is used *in vivo* directly to regulate PCD, our results indicate that sphingolipid signaling is used to regulate PCD and defenses in plants.

Materials and methods

Isolation of *acd11*

F₃ progeny of transposant lines, generated in ecotype *Ler* according to Sundaresan et al. (1995), were examined phenotypically by growing 12 plants per line in soil.

Histochemistry

Leaf sections were fixed and embedded in Spurr's resin and stained with toluidine blue for cell walls and nucleic acids. Nitro blue tetrazolium and trypan blue staining were performed after Jabs et al. (1996) and Bowling et al. (1997). Callose and

autofluorescence detections were performed after Dietrich et al. (1994).

Apoptosis analysis

Protoplasts were isolated from 16-day-old sterile-grown *Ler* and *acd11* according to standard procedures. Protoplasts were pelleted, washed once in 2 mL of 0.03% saponin (S7900; Sigma) in PBS, and treated with 1 mL of 0.4 mg/mL 7-AAD (A9400; Sigma) in 0.03% saponin for 25 min at room temperature in the dark. Samples were analyzed immediately by flow cytometry in a FACScan (Becton Dickinson) using a logarithmic fluorescence scale. Treatments with either 5 μ M DPI, 0.1 μ M BA, 0.5 μ M staurosporine, or 0.1 μ M okadaic acid were performed by spraying plantlets 48 h prior to protoplasting.

RNA analyses

Total RNA was prepared for RNA gel blot hybridizations by use of standard protocols (RNAagents Total RNA, Promega). Probe templates were amplified by PCR from cDNAs or genomic DNA with primer sequences from *PR1* (GI 3810599), *PR2* (166636), *PR5* (2435405), *CHS* (22983), *SAG13* (6457348), *FMO* (8954062), *EDS1* (4454566), *PAD4* (6457330), *EF-1 α* (16260), *PRXc* (EST B25XP), and *GST* (EST 139F9T7). For cDNA microarray analysis, total RNA from 2 g of 14-day-old, sterile-grown wild type and *acd11* was extracted by use of Trizol Reagent (Life Technologies). Poly(A)⁺ RNA was purified from 200 μ g of total RNA with 2 μ g of Dynabeads Oligo(dT)₂₅ (Dynal). cDNA microarray production, preparation of fluorescent probes, and microarray hybridization and scanning have been described (Ruan et al. 1998). In two independent hybridizations, 70% (1) and 14% (2) of the cDNAs on the microarray hybridized with intensities within the dynamic range. We attribute the relatively low total hybridization percentage in hybridization 1 to the fact that RNA from seedling rosette leaves was analyzed, and most (80%) of the nonhybridizing clones were from root, stem, bud, or flower libraries. Although hybridization 2 was of low quality, it identified a set of differentially expressed genes contained within the larger set revealed in hybridization 1. Only hybridization 1 was used for further analysis, and data reported in Tables 1 and 2 are from this experiment.

After normalization of hybridization signals, the average (0.00) and standard deviation (0.20) of the log₁₀-fold balanced difference between *Ler* and *acd11* were calculated. This step enabled calculation of the frequency of random occurrences beyond a given log-fold balanced difference. In our case, the estimated frequency beyond ± 0.58 log-fold (3.8-fold up-regulated/repressed, or 2.9 standard deviations) is 3.9×10^{-3} .

Genetic analyses

Homozygous *nahG* in ecotype Col-0 was crossed to heterozygous *acd11* and F₁ Ds heterozygotes identified by PCR. Of 255 F₂ progeny, 20 (1/13) exhibited the *acd11* phenotype, indicating that the dominant *nahG* transgene suppresses *acd11*. This event was confirmed by PCR identification of *acd11* homozygotes carrying *nahG*.

For analysis of crosses to *eds1-2* and *pad4-2*, PCR-genotyped Ds-heterozygous F₁ plants were self-pollinated and 60 F₂ seeds from each cross germinated in soil. Of these, 30 were sprayed with 100 μ M BTH. Plants exhibiting shoot inhibition after treatment were confirmed by PCR as homozygous for the *acd11* and *eds1-2* or *pad4-2* alleles.

acd11/npr1-1 was constructed by CAPS-selection of *npr1-1* homozygous F₃ families by use of the *Nla*III polymorphism of

npr1-1. Families segregating putative *acd11/npr1-1* were identified, and *acd11/npr1-1* plants were shown by PCR and sequencing to be homozygous for *npr1-1* and *acd11*.

acd11/ein2-1 and *acd11/jar1-1* were constructed by selection of kanamycin-resistant F₂ seedlings insensitive to 500 μ M ACC or 50 μ M MeJA, respectively. Roughly one-third of such plants had a typical *acd11* phenotype, and surviving plants were heterozygous for KanR. Sequencing of *EIN2* in *acd11/ein2-1* confirmed that it was homozygous for the *ein2-1* allele. *jar1-1* homozygosity was confirmed in several lines by rechecking MeJA insensitivity of F₃ families. Double mutants segregating from *ACD11/acd11 ein2-1/ein2-1* and *ACD11/acd11 jar1-1/jar1-1* families were used for expression analysis.

Isolation and analysis of the ACD11 gene

DNA from BAC T29F13 was prepared by Wizard Maxiprep (Promega) and digested with *Bam*HI or *Sna*BI. The 21-kb *Bam*HI fragment was cloned into pBIBACII (Hamilton 1997) whereas the 15-kb *Sna*BI fragment was cloned into pCambia3300. Single annotated gene fragments were PCR amplified from BAC T29F13 DNA template and cloned into pCambia3300. To isolate the ACD11 cDNA, EST clones 166P10T7, 166I22T7, 215G14T7, and 162J7T7 were sequenced. 166P10T7 contained a full-length cDNA that was excised from pSPORT1 and cloned into VIP26 (Benfey et al. 1990). This cassette was subcloned into pCambia3300 for transformation.

All constructs were transformed into *ACD11/acd11* plants. Roughly 25% of transgenic T1 plants exhibited the *acd11* phenotype except for those containing the 15-kb *Sna*BI fragment (8 out of 135), T29F13.10 (3 out of 181), and 35S-ACD11 (0 out of 31). For these three constructs, several phenotypically wild-type T2 lines homozygous for *Ds*-carried kanamycin resistance were identified. Each was shown by PCR with primers specific for the four annotated genes deleted in *acd11* to be *acd11* homozygous, but to have the gene(s) carried on the respective T-DNAs. These lines segregated *acd11* mutants, and such *acd11* T2 plants were shown by PCR to lack the relevant transgene. ACD11 homologs were identified by BLASTP search of GenBank, aligned with ClustalW, and formatted using BOXSHADE.

In vitro assay of sphingolipid intervesicular transfer

The ACD11 cDNA was cloned into pGEX-5X-1 for protein expression. Both recombinant ACD11 and GLTP were produced as described previously (Lin et al. 2000), except that ACD11 was cleaved from GST with Factor-Xa (Pharmacia). A charged vesicle assay was used to monitor protein-mediated intermembrane transfer of radiolabeled sphingolipids (Brown et al. 1990; Mattjus et al. 2000). By the inclusion of negatively charged phospholipid in the donor vesicles, separation from neutral acceptor vesicles could be achieved after incubation with protein by elution through DEAE-Sephacel minicolumns. Both donors and acceptors were small unilamellar vesicles prepared by sonication (Barenholz et al. 1977) in transfer assay buffer (10 mM Na₃PO₄ at pH 7.4 containing 1 mM EDTA, 1 mM DTT, and 0.02% NaN₃). Donors consisted of 88 mol% 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), 2 mol% [³H]sphingolipid, 10 mol% negatively charged dipalmitoyl phosphatidic acid, and a trace of nontransferable [¹⁴C]tripalmitin. Acceptor vesicles were comprised entirely of POPC. Each assay (0.5 mL) contained 0.2 μ mole of donors, 2 μ mole of acceptors, and protein. Incubations were typically performed at 37°C for 40 min. prior to separation of donor and acceptor vesicles. [³H]sphingolipid transfer to recovered acceptor vesicles was quantitated by liquid scintillation counting as described by Brown and Thompson

(1987). Phospholipids and sphingolipids were obtained from Avanti Polar Lipids (Alabaster, MO) and Sigma-Aldrich (St. Louis, MO). Tritiated D-sphingosine was purchased from American Radiolabeled Chemicals (St. Louis, MO), tritiated galactosylceramide (galactocerebrosides, Sigma C1516) was synthesized as described by Brown and Thompson (1987), and tritiated ceramide (*N*-palmitoyl-D-sphingosine) was produced by hydrogenation (Schwarzmann 1978).

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Knockout of *Arabidopsis* *ACCELERATED-CELL-DEATH11* encoding a sphingosine transfer protein causes activation of programmed cell death and defense

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