RESEARCH COMMUNICATION

Ceramides modulate programmed cell death in plants

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The balance between the bioactive sphingolipid ceramide and its phosphorylated derivative has been proposed to modulate the amount of programmed cell death (PCD) in eukaryotes. We characterized the first ceramide kinase (CERK) mutant in any organism. The *Arabidopsis* CERK mutant, called *accelerated cell death 5*, accumulates CERK substrates and shows enhanced disease symptoms during pathogen attack and apoptotic-like cell death dependent on defense signaling late in development. ACD5 protein shows high specificity for ceramides in vitro. Strikingly, C2 ceramide induces, whereas its phosphorylated derivative partially blocks, plant PCD, supporting a role for ceramide phosphorylation in modulating cell death in plants.

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Ceramides and their related sphingolipid derivatives are bioactive lipids that play important roles as second messengers in animals (Hannun and Obeid 2002). This family of signal molecules can profoundly affect cell fate, eliciting apoptosis and/or altering differentiation or cell-cycle events (Hannun and Obeid 2002). A ceramide kinase (CERK) from humans was recently identified and shown to act on a number of ceramide substrates with much higher activity than the related sphingosine substrate (Sugiura et al. 2002). Although phosphorylated ceramide has been proposed to have novel functions promoting vesicle fusion (Bajjalieh et al. 1989; Hinkovska-Galcheva et al. 1998) and stimulating DNA synthesis (Gomez-Munoz et al. 1995, 1997), it has also been proposed to play a role in dampening signals for apoptosis in animals (Sugiura et al. 2002). Homologs of the human CERK are present in a number of higher eukaryotes, including plants, but yeast lack a clear homolog (Sugiura et al. 2002). This suggests a role for CERK in processes important for a multicellular lifestyle.

The role of sphingolipids in controlling plant cell fate is not well characterized. The fungal toxin fumonisin induces plant programmed cell death (PCD) through a process thought to involve disruption of sphingolipid metabolism, although the mechanism by which it acts in plants is still unclear (Asai et al. 2000). The ectopic PCD phenotype of *Arabidopsis* mutants lacking a sphingosine transfer protein also provides a hint that sphingolipids have a role in plant cell death control (Brodersen et al. 2002). However, a direct demonstration of the involvement of ceramides or other sphingolipids in plant PCD is still lacking.

We report here the first characterization of a CERK mutant in any organism, called *accelerated cell death 5* (*acd5*). We previously showed that *Arabidopsis acd5* mutant plants initially develop normally, but show excessive cell death upon infection with the bacterial pathogen *Pseudomonas syringae*. Furthermore, *acd5* mutant plants late in development show spontaneous cell death largely dependent on the stress and defense signaling pathways controlled by the hormone ethylene and the phenolic salicylic acid, respectively (Greenberg et al. 2000). *acd5* plants have a number of the characteristics of wild-type plants infected with *P. syringae* (Greenberg et al. 2000). This includes the accumulation of a number of defense-related compounds and markers (Greenberg et al. 2000). However, *acd5* plants support modestly increased growth of *P. syringae*, suggesting a role for ACD5 in controlling disease susceptibility (Greenberg et al. 2000).

The *Arabidopsis acd5* mutant hyperaccumulates the lipid substrates for recombinant ACD5 CERK and shows spontaneous cell death with some apoptotic features late in development. Furthermore, ceramide is sufficient to induce cell death in wild-type and *acd5* protoplasts, with effects on wild-type protoplasts that are weaker than those seen on *acd5*. Ceramide-1-phosphate was able to partially abrogate the cell death-inducing effects of ceramide. Our study provides strong evidence that the balance between ceramide and its phosphorylated derivative modulates the amount of PCD in plants. Furthermore, these data suggest a conserved mechanism for regulating PCD in plants and animals. The existence of a conserved PCD regulatory pathway will help to clarify the identity and evolutionary origin of the basal eukaryotic cell death machinery.

Results and Discussion

We used map-based cloning to isolate the *ACD5* gene (see Materials and Methods) and found it to encode a 608-amino acid-long protein with 31% identity and 43% similarity, respectively, to the biochemically characterized human CERK enzyme (Sugiura et al. 2002). This similarity occurred throughout most of the ACD5 region spanning amino acids 96–607. The *acd5* mutant harbored a missense mutation that in the predicted protein converted the glycine at amino acid 412 to arginine due to a G-to-A mutation in the first position of the codon. (Fig. 1A). This glycine is conserved in most CERK homologs found in the database (data not shown). The *acd5* cell death phenotype was complemented with a genomic clone of *ACD5* (Fig. 1B).

To determine whether ACD5 possessed CERK activity, we produced and purified recombinant enzyme and performed activity assays on ceramides and related substrates. ACD5 showed highest activity on synthetic C6 and C8 ceramides and 10- to 20-fold less activity on natural ceramides (a mixture of ceramides from bovine

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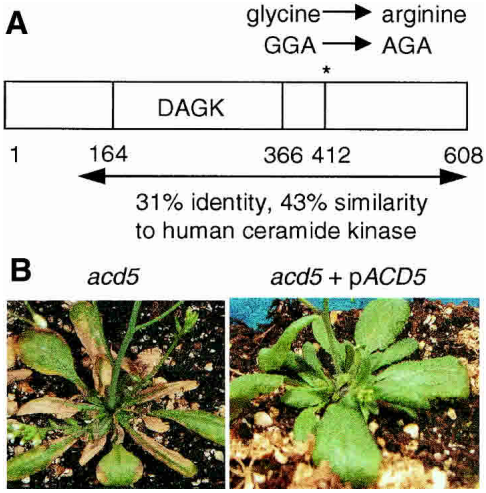

Figure 1. Identification and complementation of the *acd5* mutation. (*A*) Features of the wild-type and mutant ACD5 protein are shown. Numbers indicate the positions of amino acids. The region between amino acids 164 and 366 shows similarity to the diacylglycerol kinase (DAGK) family (E = 0.0073). Arrow indicates the region of similarity in ACD5 to human CERK. Asterisk indicates the position of the mutation in *acd5*. (*B*) Complementation of *acd5* with a genomic clone of the *ACD5* gene. Note the lack of cell death regions in the complemented plant.

brain) and the synthetic substrate C2 ceramide, respectively (Fig. 2A,B). ACD5 showed only weak activity on diacyl glycerol and sphingosine. As expected, the recombinant mutant enzyme had very little activity (Fig. 2A,B). Dihydroceramides were poorer substrates for ACD5 than the corresponding unsaturated ceramides (Fig. 2B). The ACD5 CERK showed typical Michaelis-Menton kinetics with C8 ceramide and had a K_m of 24.4 μM (Fig. 2C). Like the human CERK (Sugiura et al. 2002), ACD5 CERK activity was stimulated by Ca^{2+} (Fig. 2D). ACD5 CERK had an optimum pH of 8.2 and was most active at 30°C (data not shown). Importantly, *acd5* crude extracts had 10-fold less CERK activity than wild-type crude extracts in phosphorylating C8 ceramide (Fig. 2E). Furthermore, ceramide-enriched lipid extracts from *acd5* were phosphorylated by recombinant ACD5 three- to eightfold more than the ceramide-enriched lipid extracts from wild-type plants (Fig. 2F). This indicates that ACD5 CERK substrates accumulated in *acd5* plants to a greater extent than in wild-type plants. The phosphorylated ACD5 CERK substrates comigrated with the phosphorylated synthetic C6 (and to a lesser degree C8) ceramide marker on thin layer chromatographs (TLCs; Fig. 2F).

Because *acd5* accumulates CERK substrates and ceramides are known to induce apoptosis in animals, we examined *acd5* mutant cells for apoptotic features. *acd5* cells at the initiation of cell death (Fig. 3B) showed regions of condensed chromatin in their nuclei (Fig. 3D). These regions had a large number of DNA strand breaks generated by endonucleolytic cleavage as evidenced by the high degree of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeled (TUNEL) staining (Fig. 3F). *acd5* showed significantly more TUNEL staining than wild type at both 16 and 26 d (Fig. 3G, $P = 0.001$, Student's *t*-test). Other organelles, including mitochondria, in these TUNEL-staining cells did not show any obvious changes in their morphology or integrity (Supplementary Fig. 1), although cells at more advanced stages of cell death showed highly condensed chromatin and abundant DNA fragments typical of apoptotic cells (data not shown).

To further establish that ceramides are sufficient to induce cell death in plants, we treated wild-type protoplasts with C2 ceramide and found that it induced nuclear fragmentation characteristic of apoptotic dying cells (Fig. 4A). We also treated wild-type and *acd5* protoplasts with different doses of the soluble C2 ceramide or its biosynthetic precursor, C2 dihydroceramide. C2 ceramide was more potent than the dihydroceramide at eliciting cell death of both wild-type and *acd5* protoplasts at all concentrations tested (Fig. 4B; $P < 0.02$), similar to what has been reported in animal cells (for review, see Hannun and Obeid 2002). Furthermore, *acd5* protoplasts were significantly more sensitive to C2 ceramide than wild-type protoplasts at all concentrations tested (Fig. 4B; $P \leq 0.02$). Thus, ceramide is sufficient to induce cell death in *Arabidopsis*. Interestingly, the presence of C2 ceramide-1-phosphate partially blocked cell death induction by C2 ceramide (Fig. 4C), suggesting that differently modified ceramides have distinct biological activities.

As *acd5* mutants have increased disease symptoms upon *P. syringae* infection (Greenberg et al. 2000), we examined whether steady-state ACD5 mRNA levels were modulated during pathogenesis. We inoculated wild-type plants with disease-causing virulent *P. syringae* and a congenic strain to which the plants were resistant due to the presence of the *avrRpm1* gene in the bacteria (rendering the strain avirulent). We found only modest induction of ACD5 mRNA in response to *P. syringae/avrRpm1* and about fivefold induction of ACD5 mRNA in plants infected with virulent *P. syringae* (Fig. 5). The relatively high induction of ACD5 mRNA with virulent bacteria correlates well with the observation that more cell death is induced in *acd5* plants with virulent versus avirulent bacteria (Greenberg et al. 2000). It was recently shown that virulent *P. syringae* possess at least one protein that, when injected into host cells, suppresses PCD and promotes virulence (Abramovitch et al. 2003). The induction of ACD5 mRNA could be one way that such suppression is achieved. In avirulent bacteria, such a suppressive mechanism may be overridden by the PCD-promoting activity of other injected proteins such as AvrRpm1. In potato, the serine palmitoyltransferase gene, encoding the first committed step in the de novo sphingolipid biosynthesis pathway, was induced after infection with *Phytophthora infestans* (Birch et al. 1999). This indicates that multiple steps in the sphingolipid pathway may be activated by infection.

We propose that the balance between ceramides and their phosphorylated derivatives is important for modulating cell death in plants in a manner dependent on the defense and stress signals salicylic acid and ethylene (Greenberg et al. 2000). Our data support the view that phosphorylation of ceramides by the ACD5 CERK directly dampens the proapoptotic effects of unphosphorylated ceramides. A similar scenario likely exists in animals as well (Sugiura et al. 2002); the related sphingosine-1-phosphate has been shown to suppress PCD in animals (Cuvillier et al. 1996). Do plants and animals have a similar mechanism for the execution of ceramide-

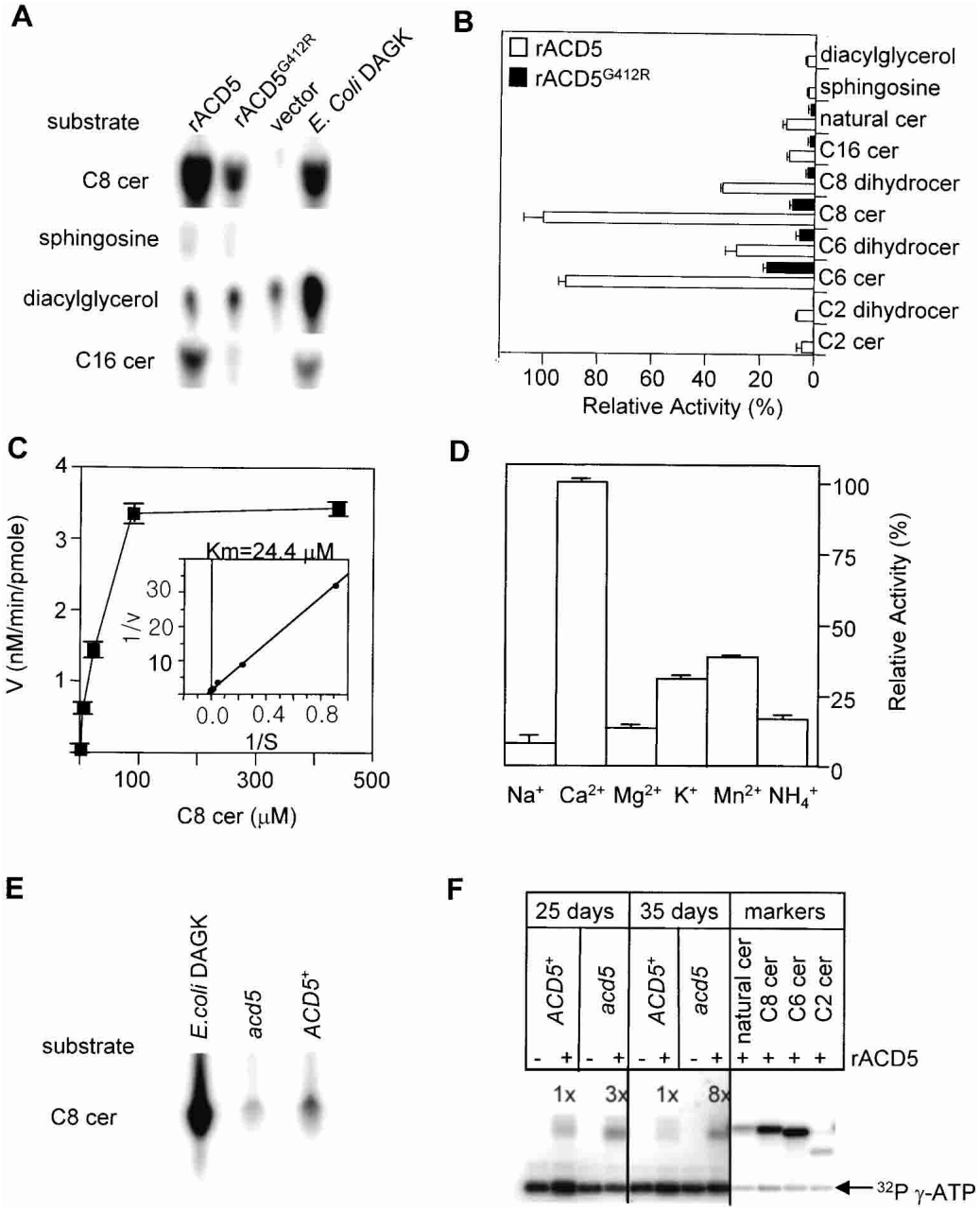

Figure 2. CERK activity of ACD5. (*A*) Autoradiographs of TLC plates detecting ^{32}P incorporation from [γ-^{32}P]ATP (3000 Ci/mmole) into lipid substrates. (rACD5) Recombinant ACD5; (DAGK) diacylglycerol kinase. (*B*) Quantitative analysis of relative ACD5 CERK activity. Standard deviations are indicated (n = 3). ACD5 activity was 344 and 36.1 fmole/min/pmole of enzyme, using C8 and natural ceramide (cer), respectively. (*C*) Kinetic analysis of recombinant ACD5. Standard deviations are indicated (n = 3). (*D*) Effect of various cations (100 mM) on recombinant ACD5 enzyme activity. (*E*) Conversion of C8 ceramide to C8 ceramide-1-phospate by *E. coli* DAGK and crude extracts from 33-day-old plants; 2.5-fold more *acd5* protein extract than wild-type (*ACD5*$^+$) extract was used in the assay. (*F*) Accumulation of ACD5 CERK substrates in *acd5* and wild-type lipid extracts. Recombinant ACD5 or vector control protein (–) was used to phosphorylate ceramide-enriched *acd5* and wild-type lipid extracts, and the phosphorylated products were run on TLC. Darker bands in *acd5* lanes indicate that more CERK substrate was present in the *acd5* lipid extracts than in the wild-type lipid extracts; 1.5-fold more lipids were loaded on the TLC plate from the 25-day-old vs. 35-day-old material.

mediated PCD? In animals, ceramide activates PCD in a process in which mitochondria play a prominent role (Birbes et al. 2002). Interestingly, ceramide-induced PCD in plant protoplasts requires the mitochondria to undergo a permeability transition much like that described in animals (N. Yao and J.T. Greenberg, unpubl.). This suggests that there are common elements in the regulation and execution of ceramide-mediated PCD in plants and animals. Determination of the plant targets of the different bioactive sphingolipids should provide tools for insightful comparative studies of their mechanisms of action in both plants and animals.

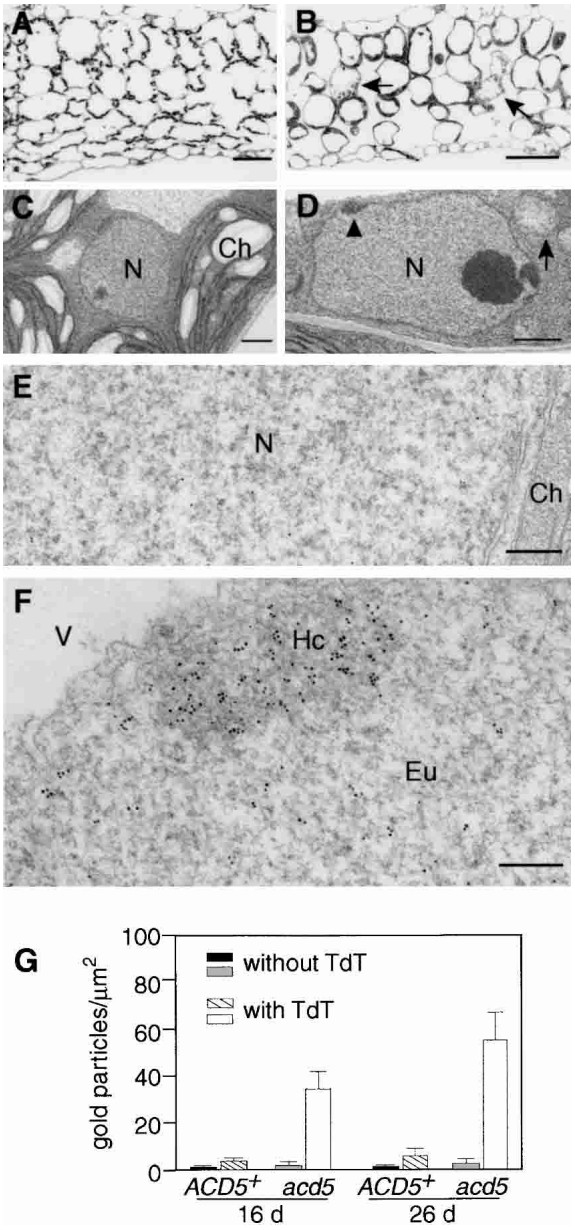

Figure 3. Apoptosis-like cell death in *acd5* mutant plants. (*A*,*B*) Light micrograph of cross-sections of 18-day-old wild-type (*A*) and *acd5* (*B*) leaves stained with toluidine blue. Arrows indicate dying cells. (*C*) Transmission electron micrograph (TEM) of one of the wild-type mesophyll cells from *A*. (*D*) TEM of one of the *acd5* mesophyll cells adjacent to the dying cell by the *left* arrow in *B*. Arrowhead and arrow indicate an aggregate of condensed chromatin and a morphologically intact mitochondrion, respectively. (*E*,*F*) EM-TUNEL analysis of the localization of DNA strand breaks in the mesophyll cell nuclei of wild-type (*E*) and *acd5* (*F*) leaf segments from the tissue shown in *A* and *B*. (*F*) Note that immunogold-labeled DNA fragments were abundantly localized in the condensed heterochromatin portion of the *acd5* mesophyll cell nucleus. Abbreviations used in *C–F* were as follows: (N) nucleus; (Ch) chloroplast; (V) vacuole; (Hc) heterochromatin; (Eu) euchromatin. (*G*) Statistical analysis of the density of endonucleolytic DNA strand breaks. Bars: *A*,*B*, 50 µm; *C*,*D*, 1 µm; *E*,*F*, 200 nm.

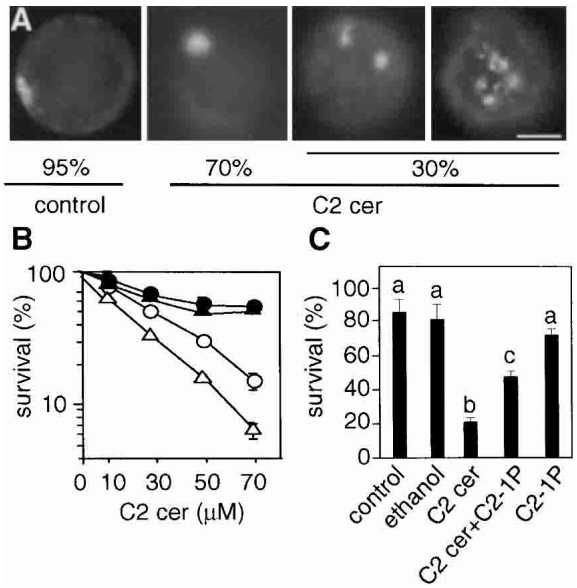

Figure 4. Cell death-modulating activities of ceramides. (*A*) Nuclear morphology of wild-type protoplasts after control or C2 ceramide treatments. Protoplasts were stained for nuclei using Hoechst33342 16 h after treatment with 30 µM C2 ceramide. Protoplasts showed 32% viability. Bar, 10 µm. The percentage of protoplasts of each genotype with the morphologies shown is indicated *below* each picture. (*B*) Ceramide-induced cell death in wild-type and *acd5* protoplasts. Protoplasts were treated with C2 ceramide (open symbols) or C2 dihydroceramide (closed symbols) for 10 h. (Circles) Wild-type protoplasts; (triangles) *acd5* protoplasts. Standard deviations are shown ($n = 3$). Some symbols obscure the error bars. (*C*) Cell death-blocking effects of C2 ceramide-1-phosphate (C2-1P). Protoplasts were left untreated or were treated with 0.2% ethanol (solvent in which ceramides were dissolved), 30 µM C2 ceramide, 30 µM C2 ceramide + 10 µM C2 ceramide-1-phosphate, or 10 µM C2 ceramide-1-phosphate for 18 h. Standard deviations are shown ($n = 3$). Letters indicate that values are different using Student's *t*-test ($P < 0.04$).

Materials and methods

Materials

Natural ceramide and sphingosine were purchased from Sigma. C2, C16 ceramides, and C2 dihydroceramide were from Matreya. C2 ceramide-1-phosphate was from Avanti Polar Lipids. Other ceramides were from BIOMOL Research Laboratory. Recombinant *Escherichia coli* diacylglycerol kinase was from Calbiochem. All other lipids were purchased from Avanti Polar Lipids. [γ-^{32}P]ATP (3000 Ci/mmole) was purchased from Amersham Biosciences. Iatrobeads (6RS 8060) were from Iatron Laboratory. TLC plates (Baker Si250) were from Mallickrodt Baker. Wild-type and *acd5* mutant *Arabidopsis thaliana* plants (ecotype Columbia) were grown in 16-hour-day conditions, as described (Greenberg et al. 2000).

Map-based cloning of ACD5

Fine mapping of the *acd5* mutation was conducted in both *acd5* × Ler F_2 and *acd5* × Cvi F_2 populations using standard approaches. Approximately 3000 F_2 plants showing the *acd5* phenotype (2000 from the cross to Ler and 1000 from the cross to Cvi) were analyzed. Markers were made based on the Cereon *Arabidopsis* polymorphism database (http://www.arabidopsis.org/cereon) and random sequencing of the Cvi genome in the region of *ACD5*. Markers are available upon request.

The *acd5* mutation mapped to a 81-kb region between nucleotides 20379600 and 20461201 on chromosome 5. BAC clone MWD22 (Ohio State Stock Center) that covers most of this region was subcloned into

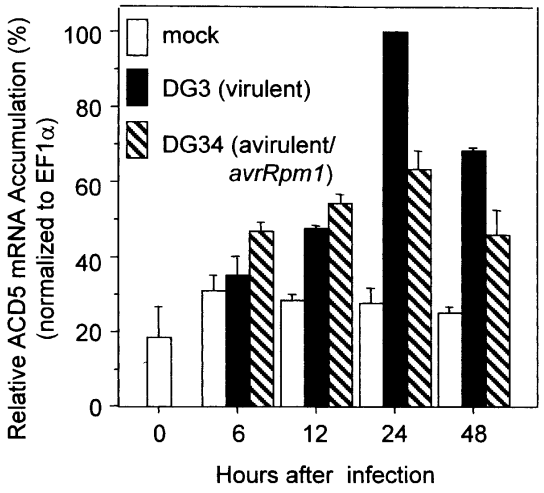

Figure 5. ACD5 mRNA induction during *P. syringae* infection. Relative levels of ACD5 mRNA transcript averaged from two experiments are shown. Bars indicate standard deviations.

binary vector pAOV-35 [a vector derived from pAOV (Mylne and Botella 1998) from which the CaMV 35S promoter was removed]. Nine clones with 10–15-kb inserts that overlapped for at least 5 kb were transformed into *acd5npr1* plants (which have better fertility than *acd5* alone; Greenberg et al. 2000) by the *Agrobacterium*-mediated floral dip method (Bechtold and Pelletier 1998) for complementation tests. T_1 seeds were selected by spraying the herbicide basta. Clone 1H3 with three predicted open reading frames (ORFs encoded by the genes At5g51280, At5g51290, At5g51300) not contained in any overlapping noncomplementing clones rescued the cell death phenotype in *acd5 npr1*. Only ORF At5g51290 harbored a mutation in *acd5* genomic DNA.

A 7-kb *Nco*I fragment from 1H3 encompassing the entire genomic gene At5g51290 was cloned into the pGR111 [a modified version of the pGreen binary vector (Hellens et al. 2000) and a gift from Dr. Daphne Preuss, University of Chicago, Chicago, IL]. Fifteen of seventeen transformed *acd5* plants were complemented by this pACD5 construct. An *ACD5* RNAi construct transformed into wild-type plants also recapitulated the *acd5* mutant phenotype (data not shown).

Isolation of ACD5 cDNA

Reverse transcriptase PCR (RT–PCR) was performed on wild-type and *acd5* poly(A)-RNA using Superscript II (Invitrogen) and Pfu DNA polymerase (Stratagene). Products were cloned into the pET14b *E. coli* expression vector (Novagen) in frame with a 6x His-tag at the C-terminal end.

The GenBank accession number for the *ACD5* cDNA is AY362552.

CERK activity assay

CERK activity was measured as described (Bajjalieh and Batchelor 2000) except that plant crude extract or 2 pmoles (142 ng) recombinant protein purified by NTA nickel agarose column (in 192 mM imidazole, 50 mM $NaPO_4$ at pH 8.0, and 300 mM NaCl) was used and 10 µM ATP was included in each reaction. Reactions were analyzed by TLC developed with chloroform:methanol:acetic acid (65:15:10), or counted with a scintillation counter (QC 4000, Bioscan) for kinetic studies.

To prepare crude extracts, 0.8 g of tissue was frozen in liquid N_2, homogenized, and placed in 1 mL extraction buffer (20 mM MOPS at pH 7.2, 2 mM EGTA, 1 mM DTT, 10% glycerol) to which 1 µL of plant protease inhibitor cocktail P9599 (Sigma) was added. The extract was centrifuged for 10 min at 14,000 *g*, and supernatant was assayed for CERK activity. The diacylglycerol kinase assay was the same as above except for the following different components in the reaction mixture: 888 µM diacylglycerol, 2 mM EDTA, and 5 mM $MgCl_2$.

Lipid profile analysis

Plant sphingolipids were extracted according to Fujiwaki et al. (1999) using one gram of tissue. Iatrabeads (Iatron Laboratory) were used to purify crude sphingolipids, following the method of Vance and Sweely (1967).

Protoplast preparation and treatment

Protoplasts were prepared using 14–18-day-old plants as described (Asai et al. 2000) except that 1.2% cellulase "Onozuka R-10" from *Trichoderma viride* and 0.3% macerozyme R-10 from *Rhizopus sp. lyophil.* (SERVA Electrophoresis) were used. C2 ceramide, C2 dihydroceramide, and C2 ceramide-1-phosphate were dissolved in ethanol.

The viability of protoplasts after treatments was determined by fluorescein diacetate (FDA) staining (500 ng/mL) using a Hemacytometer (Hausser Scientific). At least 300 cells per treatment were counted. Trials were performed in triplicate.

Ultrastructural analysis and EM-TUNEL assay

Leaf segments were fixed as described (Yao et al. 2001) and embedded in Epon (Electron Microscopy Sciences). The EM-TUNEL procedure using an ApopTag Plus Fluorescein in situ Apoptosis Detection Kit (Intergen) and the statistical analysis were done as described (Yao et al. 2001). Sheep anti-digoxigenin antibody conjugated to 10 nm colloidal gold was from Electron Microscopy Sciences. A Philips CM-120 transmission electron microscope was used at an accelerating voltage of 120 kV.

RNA blot analysis

RNA blot analysis was performed as described (Rate et al. 1999). A portion of the ACD5 cDNA (nt 687–1659) was amplified by PCR using the primer pairs pr24–1F-2 (CTTGCAATTTGCACGACATAG) and pr24–3R (GCAGACGTGCCAGAGTTGGA) and used as a probe. EF1α was used as a loading control.

Plant infections

Inoculations at OD_{600} = 0.01 of strains DG3 (virulent), DG34 (carrying *avrRpm1*) or 10 mM $MgSO_4$ (mock control) into 19–21-day-old plants was done as described (Rate et al. 1999). DG3 and DG34 are derived from *P. syringae* pv *maculicola* strain ES4326 (Guttman and Greenberg 2001).

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