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 (acd5), 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 damaging 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 mutants 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.

Because 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 PCDo 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 PCDo-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-
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 2. CERK activity of ACD5. (A) Autoradiographs of TLC plates detecting $^{32}$P incorporation from [$\gamma$-$^{32}$P]ATP (3000 Ci/m mole) 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-phosphate 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.
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-32P]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 F2 and acd5 × Cvi F2 populations using standard approaches. Approximately 3000 F2 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 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).
harbored a mutation in atractylodinigenin synthase (AGS), a key enzyme in the biosynthesis of cyclic AMP (cAMP) and cyclic guanosine monophosphate (cGMP) in plants. To rescue the cell death phenotype in the mutant, the authors performed transgenic studies using different binary vectors. The results showed that the ACD5 cDNA is a strong candidate for rescuing the phenotype.

**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.  

The data suggest that ACD5 plays a role in the defense response against *P. syringae* infection, possibly by influencing the levels of cyclic nucleotides, which are known to regulate various cellular processes including the immune response.

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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 *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). 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 (GCAGACGTGCCAGATTTGGA) and used as a probe. EF1α was used as a loading control.

**Plant infections**

Inoculations at OD<sub>600</sub> = 0.01 of strains DG3 (virulent), DG34 (carrying avrRpm1) or 10 mM MgSO<sub>4</sub> [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 E45326 (Guttman and Greenberg 2001).

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