The *Arabidopsis*-accelerated cell death gene *ACD2* encodes red chlorophyll catabolite reductase and suppresses the spread of disease symptoms

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***accelerated cell death 2* (*acd2*) mutants of *Arabidopsis* have spontaneous spreading cell death lesions and constitutive activation of defenses in the absence of pathogen infection. Lesion formation in *acd2* plants can be triggered by the bacterial toxin coronatine through a light-dependent process. Coronatine-triggered and spontaneous lesion spreading in *acd2* plants also requires protein translation, indicating that cell death occurs by an active process. We have cloned the *ACD2* gene; its predicted product shows significant and extensive similarity to red chlorophyll catabolite reductase, which catalyzes one step in the breakdown of the porphyrin component of chlorophyll [Wüthrich, K. L., Bovet, L., Hunziger, P. E., Donnison, I. S. & Hörtensteiner, S. (2000) *Plant J.* 21, 189–198]. Consistent with this, ACD2 protein contains a predicted chloroplast transit peptide, is processed *in vivo*, and purifies with the chloroplast fraction in subcellular fractionation experiments. At some stages of development, ACD2 protein also purifies with the mitochondrial fraction. We hypothesize that cell death in *acd2* plants is caused by the accumulation of chlorophyll breakdown products. Such catabolites might be specific triggers for cell death or they might induce cellular damage through their ability to absorb light and emit electrons that generate free radicals. In response to infection by *Pseudomonas syringae*, transgenic plants expressing excess ACD2 protein show reduced disease symptoms but not reduced growth of bacteria. Thus, breakdown products of chlorophyll may act to amplify the symptoms of disease, including cell death and yellowing. We suggest that economically important plants overexpressing ACD2 might also show increased tolerance to pathogens and might be useful for increasing crop yields.**

Plant senescence and disease are linked by common features including cell death and chlorophyll breakdown. Although chlorophyll degradation may *a priori* seem to be a peripheral consequence of cell death in disease resistance, several observations link both light and chlorophyll degradation to disease resistance. As part of cell death in disease, or programmed cell death in disease resistance [hypersensitive response (HR)], chlorophyll degradation happens as the lesion turns yellow or brown. Chlorophyll breakdown causes both a decrease in photosynthetic capacity and production of light-absorbing chlorophyll breakdown products. Intriguingly, light is required for the HR in the inoculation of rice with *Xanthomonas* (1). In other reports, the lack of light can also cause a stronger HR (2). Light may be required in the HR for the production of adequate ATP to allow programmed cell death, or it might activate phototoxic porphyrin chlorophyll breakdown products, which accelerate cell death.

Porphyrin compounds, the precursors and breakdown products of both chlorophyll and heme, are extremely phototoxic; thus, their synthesis and degradation are highly compartmentalized and regulated (3, 4). As chlorophyll can absorb light and donate active electrons, so can its porphyrin relatives. In the absence of productive outlets for these active electrons, they can be donated to other compounds, including molecular oxygen, forming free radicals that can be toxic and can act as cellular signals (5–7). Accumulation of porphyrin compounds, caused by inactivation of enzymes in the porphyrin synthesis pathway, can cause cell death in animals and plants (8). For example, *Les22* mutant maize accumulate uroporphyrin III and form light-dependent cell death lesions on their leaves (9). Interestingly, in mammalian cells, protoporphyrin IX specifically triggers the mitochondrial permeability transition (10, 11) and thus apoptosis (12). The phenotype of porphyrin precursor accumulation in plants appears similar to the induction of defenses in pathogen resistance, including cell death and defense gene transcription. This is termed a lesion mimic phenotype (13, 14). Lesion mimic mutants have been isolated in many species, including the *accelerated cell death* (*acd*) and *lesions simulating disease* (*lsd*) series of *Arabidopsis* mutants (13–16). Many have been cloned, including the *LSD1* gene, which encodes a putative zinc finger protein (17).

Reactive oxygen species and light can affect the phenotype of some lesion mimic mutants. In *lsd1* mutants, superoxide can trigger the lesion mimic phenotype (18); also, copper–zinc superoxide dismutase is not properly induced in response to analogs of the defense signal salicylic acid (SA; ref. 19), indicating that a major perturbation of antioxidant defenses may cause the *lsd1* phenotype of spreading lesions. A lesion mimic phenotype associated with *Cladosporum fulvum* resistance in tomato showed reduced symptoms in partial shade (20). Also, *lsd1* and *lsd3* mutants are suppressed under short day conditions, but *lsd2* and, to a lesser extent, *lsd5*, are suppressed under long day conditions (14). Some light-sensitive lesion phenotypes, such as that of antisense catalase (21), may be caused by excess reactive oxygen produced by chloroplast metabolism. In contrast, the *Arabidopsis psi2* mutant shows red light-dependent cell death that requires the photoreceptors *phyA* and *phyB* (22). The effects of reactive oxygen and light indicate that some lesion mimic phenotypes may require light energy, light signaling, or active chloroplast metabolism.

We describe here the cloning and characterization of the *Accelerated Cell Death 2* (*ACD2*) gene, which encodes the *Arabidopsis* homologue of red chlorophyll catabolite (RCC) reductase (RCCR). We hypothesize that accumulation of chlorophyll breakdown products in the mutant causes the *acd2* phenotype and show that light induces the cell death phenotype.

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Abbreviations: HR, hypersensitive response; Pa, pheophorbide a; RCC, red chlorophyll catabolite; RCCR, RCC reductase; SA, salicylic acid.

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Moreover, we show that ACD2 protein localizes to chloroplasts and mitochondria and that the removal of SA by the transgene *nahG* exacerbates the *acd2* mutant phenotype. Plants that overexpress ACD2 protein show reduced yellowing and cell death in disease, indicating that chlorophyll breakdown products may amplify cell death, affecting disease symptoms.

Materials and Methods

***Arabidopsis* Strains and Plant Growth Conditions.** *acd2* alleles were described previously (15). Additional alleles were derived from further screening of ethyl methane sulfonate-mutagenized Columbia seeds, except *acd2*-12E13, which was a gift from S. Y. He (Michigan State University, East Lansing). *nahG* line B15 was a gift from Novartis (Research Triangle Park, NC). Plants were grown in 16-h day conditions, as described (23). Seedlings for organelle purification were grown from sterilized seed spread to confluence on sterile cheesecloth over sterile soil for 7 days.

Recombination Mapping. Recombinants between *ACD2* and the flanking markers *AP2* or *CP3* (15) were obtained by selecting *acd2*, *ap2*, or *cp3* plants from an F_2 population and scoring segregation of the other mutant phenotype in the F_3 progeny. Additional recombinants were obtained by crossing an *ap2 acd2* recombinant with *cp3* to find plants in which *acd2* did not segregate with *ap2*. We obtained two plants recombinant between *ACD2* and *AP2* and 82 plants recombinant between *ACD2* and *CP3*. For fine mapping, we used the Cleaved Amplified Polymorphic Sequences (CAPS) method (24). The marker order is: *AP2*, JM142, JM127, JM110, JM411, and *CP3*. CAPS information was deposited at www.arabidopsis.org. One of the plants that was recombinant between *AP2* and *ACD2* was also recombinant at JM142 but not at JM127. One of the plants that was recombinant between *ACD2* and *CP3* was also recombinant at JM411 but not at JM110. Thus, the *ACD2* locus mapped between JM142 and JM411, a 50-kb interval with 11 predicted ORFs.

Allele Sequence and Complementation Constructs. PCR analysis of the six *acd2* mutant alleles showed a small deletion in the AT4 g37000 transcript (Munich Information Center for Protein Sequences designation) in *acd2–7*, an allele generated during T-DNA transformation (15). Sequencing showed an in-frame deletion of amino acids 181–192 in *acd2–7*. Sequencing of pooled PCR products from mutant genomic DNA also showed alterations in this transcript in the other *acd2* mutant alleles: both *acd2–2* and *acd2–5* have a G to A change in the 3′ splice acceptor site of the intron. Conceptual translation of the unspliced mRNA shows a stop codon 10 amino acids after the 5′ splice site. *acd2–12E13* has a glycine-to-valine change at amino acid 140. Both *acd2–6* and *acd2–8* have an arginine-to-lysine change at amino acid 279. Sequence changes were confirmed by generation and scoring of a directed CAPS (dCAPS) marker (25) for each allele.

ACD2 cDNAs were derived from a Columbia library (26). The two longest cDNAs stopped short of the first two amino acids. For *in vitro* translation, we used PCR to restore the first two amino acids to the cDNA. Reverse transcription–PCR with primers 130 nucleotides upstream of the first AUG gave a specific product, indicating that the transcript extends upstream of the first methionine. The genomic complementation construct included the complete AT4 g37000 ORF plus approximately 1 kb upstream and 0.5 kb downstream, but none of the adjacent predicted genes. We used Pfu polymerase (Stratagene) to amplify (5′-aacaaaaccgatgaacaaagtagtcg-3′; 5′-ccgggatgaaaagaattatgtgg-3′), cloned the product into the *Hin*dIII site of pBI121 (CLONTECH), and introduced it into plants by *Agrobacterium*-mediated transformation (27). Four independent transformants of *acd2–2* with this construct showed a wild-type phenotype.

Sense and Antisense Constructs. For sense and antisense expression constructs of ACD2, the longest cDNA insert was cloned into the *Sma*I site of pBI121, downstream of the CaMV ^{35}S promoter. Of 17 transformants with the antisense construct, 10 showed a cell death phenotype resembling *acd2* mutants. Two independent transformants of the sense construct into wild type showed increased levels of ACD2 protein on Western blots and decreased disease symptoms. Six transformants of the sense construct into *acd2–2* complemented the cell death phenotype.

Western Blotting. Polyclonal anti-ACD2 antibodies were generated in two rabbits by using inclusion body-purified bacterially expressed ACD2 protein (Covance, Denver, PA). The bacterial expression construct fused the second exon of ACD2, beginning with the amino acids IDFV, in-frame into the *Bam*HI site of pET24b (Novagen). Anti-ACD2 antiserum was used for Western blotting at a concentration of 1:5,000 in BLOTTO (20 mM Tris, pH 7.5/154 mM NaCl/0.1% Tween 20/5% Carnation nonfat dry milk) and detected by donkey anti-rabbit horseradish peroxidase (Pierce) followed by chemiluminescent detection, per the manufacturer's instructions (Pierce).

Mitochondrial and Chloroplast Purification. Chloroplasts were purified as in ref. 28; mitochondria were purified as in ref. 29. Organelle purity was assessed by chlorophyll content, by staining with the mitochondria-specific dye Mitotracker CMX-Ros (Molecular Probes), by Western blotting with antibody to the mitochondrial protease AtLON (ref. 30; gracious gift of S. Mackenzie, University of Nebraska, Lincoln), and by activity gels (31) for cytoplasmic, chloroplast, and mitochondrial isoenzymes of aspartate aminotransferase (32). This experiment was repeated three times with similar results.

Bacterial Strains, Pathogenicity Tests, and Growth Curves. Derivatives of *Pseudomonas syringae* pv. *maculicola* strain PsmES4326 with vector (PsmDG3), *avrRpm1* (PsmDG34), or *avrRpt2* (PsmDG6) integrated at the *recA* locus are described in ref. 33. For Western blot analysis, *avr* genes cloned into the plasmid pLAFR were used; these constructs were a gift of F. M. Ausubel (Harvard University and Massachusetts General Hospital, Boston, MA). Hand inoculations, ion leakage, and growth curves were performed as described (23, 33). Growth curves were repeated twice with similar results.

Coronatine and Cycloheximide Treatments. Fifteen microliters of 10 μg/ml coronatine (kind gift from R. Mitchell, Horticultural and Food Research Institute, Aukland, New Zealand), 0.1% methanol, or 20 μg/ml cycloheximide (34) was pipetted on top of the leaf. Dark-incubated leaves were covered in aluminum foil. This experiment was repeated three times with similar results.

Results

Cloning *ACD2*. We cloned the *ACD2* locus by standard *Arabidopsis* positional cloning methods, recombination mapping the locus to a 50-kb interval between two CAPS markers (see *Materials and Methods*). To identify the locus, we examined the putative transcripts in the region by PCR and found a small deletion in *acd2–7*. We isolated and sequenced cDNA clones corresponding to the *ACD2* mRNA and used reverse transcription–PCR to determine the extent of the ORF. We proved that the affected ORF encodes the *ACD2* gene. First, all of the previously isolated *acd2* alleles had lesions in this ORF (see *Materials and Methods*). Second, either the genomic region covering the *ACD2* locus or a full-length cDNA under the control of CaMV 35S promoter, introduced into mutant plants, fully complemented the *acd2* phenotype (Fig. 1 and data not shown). Third, Western blot analysis showed that ACD2 protein is reduced or absent in *acd2* mutants (Fig. 2*A*). Additionally, the mutant phenotype was

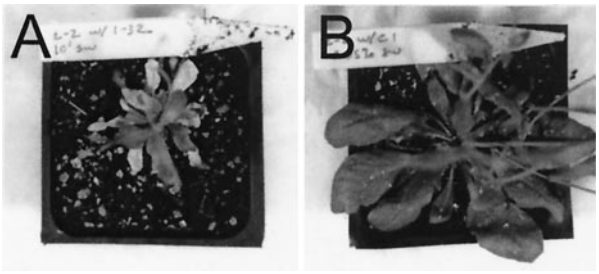

Fig. 1. Complementation of the *acd2* mutant phenotype. *acd2–2* plants with a control transgene containing the gene adjacent to *ACD2* (AT4 g36990) (*A*) and the *ACD2* (AT4 g37000) genomic transgene (*B*).

induced by antisense expression of the ACD2 cDNA (data not shown). The predicted ACD2 protein shows extensive homology to RCCR (35). Indeed, the gene we isolated as *ACD2* was designated *AtRCCR*, and its gene product catalyzes the reduction of RCC *in vitro* (35). We will refer to this gene as *ACD2* or *ACD2/RCCR*.

ACD2 Protein Localizes to the Chloroplast and Mitochondrion. We had several lines of evidence that ACD2 localized to chloroplasts or mitochondria. First, ACD2 protein from leaves migrates at a smaller size than ACD2 protein *in vitro* translated from a full-length cDNA on a Western blot, consistent with cleavage of a transit peptide (data not shown). Second, analysis with the PSORT algorithm (36) predicts that ACD2 protein localizes to chloroplasts and mitochondria. Third, *in vitro* translated AtRCCR can be imported into isolated chloroplasts (35). To address the subcellular localization of ACD2, we purified chloroplasts and mitochondria from 7-day-old seedlings and analyzed the purified organelles by Western blot analysis. The mitochondrial fraction was substantially free of chloroplast contamination, as indicated by chlorophyll content (Fig. 2*B*). Also, the chloroplast fraction was substantially free of mitochondrial contamination, as indicated by staining with the mitochondrial-specific dye Mitotracker Red CMXRos (Fig. 2*B*) and by abundance of the mitochondrial isoenzyme of aspartate aminotransferase (32). ACD2 protein was substantially enriched in purified chloroplasts and mitochondria in *Arabidopsis* seedlings. The two organellar forms migrated at slightly different sizes, both smaller than *in vitro* translated product (data not shown), indicating that transit peptide cleavage may occur at different residues or that there is another differential modification (Fig. 2*B*). The two forms migrated as a doublet on Western blots, allowing us to monitor their presence and ratio. The mitochondrial form was less abundant than the chloroplast form (Fig. 2*B*, lane 1). Furthermore, the mitochondrial form was found only in 7-day-old seedlings, not in plants older than 2 weeks. Thus, ACD2 localizes mainly to the chloroplast.

ACD2 Protein Abundance in Senescence and Pathogen Attack. ACD2/RCCR should function during the breakdown of chlorophyll in senescence and pathogen attack. To determine whether ACD2 protein levels change during senescence, we collected the ninth true leaf from wild-type plants, from a day 3 leaf that was expanding to a day 28 leaf that was yellowing and collapsed. Western blot analysis of equal amounts of protein from leaf extracts showed constant levels of ACD2 protein (Fig. 3*A*).

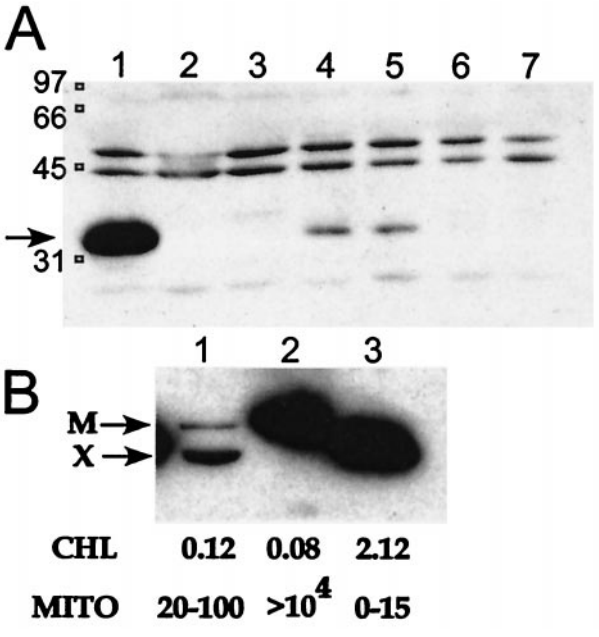

Fig. 2. (*A*) Western blot of *acd2* mutant plants with anti-ACD2 antiserum. Lanes contain protein from approximately equal weights of leaf tissue. Lanes: 1, Columbia; 2, *acd2–2*; 3, *acd2–5*; 4, *acd2–6*; 5, *acd2–8*; 6, *acd2–12E13*; 7, *acd2–7*. The doublet above the ACD2 band is not detected by antiserum from an independently inoculated rabbit or by affinity-purified antiserum (data not shown); thus, we conclude that it is not specific to the ACD2 protein. (*B*) ACD2 localizes to chloroplasts and mitochondria. Lanes: 1, crude extract; 2, purified mitochondria; 3, purified chloroplasts. M, mitochondrial form of ACD2; X, chloroplast form of ACD2; CHL, chlorophyll in mg/ml; MITO, mitochondria/microliter.

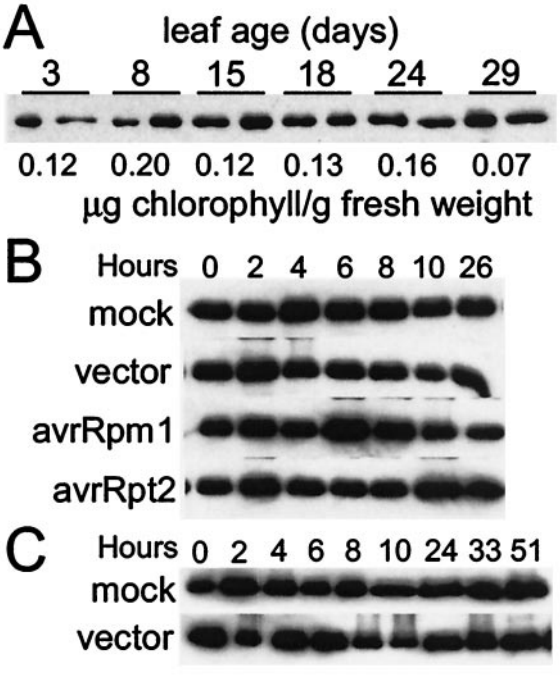

Fig. 3. Western blot analysis of ACD2 protein abundance in senescence and pathogenesis. Each lane contains equal amounts of protein by Bradford assay (Bio-Rad). (*A*) ACD2 in senescence. Duplicate samples of the ninth true leaf of wild-type plants through its development. At day 3, the leaf was approximately 3 mm long. The chlorophyll measurement is the average of the two leaves. (*B*) ACD2 in the HR. Panels show wild-type leaves mock inoculated ($MgSO_4$) or inoculated with 0.01 A_{600} of *P. syringae* containing vector only (pLAFR) or containing pLAFR with *avrRpm1* or *avrRpt2*. (*C*) ACD2 in disease. As in *B*, infection with *P. syringae* containing pLAFR only compared with mock inoculation. Strong disease symptoms were seen at 48 h.

To determine whether ACD2 protein levels change during pathogen attack, we inoculated wild-type plants with derivatives of *P. syringae* strain PsmES4326 containing either the pLAFR vector only (virulent) or pLAFR containing either *avrRpm1* or *avrRpt2* (avirulent). Infection with strains bearing *avrRpm1* and *avrRpt2* provoked an HR 6 and 16 h after inoculation, respectively. Virulent bacteria showed disease symptoms after 48 h. Western blot analysis showed no significant differences in ACD2 protein levels in any of the infections (Fig. 3 *B* and *C*). Thus, although ACD2 likely plays a key role in chlorophyll catabolism, its levels do not change during pathogen attack or senescence.

Light Activates the *acd2* Mutant Phenotype. A predicted phenotype of a mutation in ACD2/RCCR is an accumulation of porphyrin compounds such as RCC and, possibly, its precursor pheophorbide a. The phototoxic properties of these compounds should depend on light. To determine whether the *acd2* phenotype requires light, we induced the phenotype in mutant leaves by treating with the *P. syringae* toxin coronatine. In *Arabidopsis*, this toxin partially mimics the hormone methyl jasmonate, alters chloroplast functions, and induces the expression of chlorophyllase, the first enzyme in the chlorophyll breakdown pathway (37, 38). In wild-type *Arabidopsis*, 1.5–150 ng of coronatine induces anthocyanin accumulation but no significant cell death (ref. 39 and data not shown). In *acd2* mutants, as little as 1.5 ng induced cell death within 24 h (data not shown). *acd2* mutant leaves were treated with 15 ng of coronatine and either covered or exposed to light for 24 h. The absence of light completely suppressed cell death in *acd2* (Fig. 4*A*).

The *acd2* Mutant Phenotype Requires Active Translation. To determine whether the cell death in *acd2* is an active process, we applied cycloheximide, an inhibitor of translation, to *acd2* mutant leaves along with coronatine. If *acd2* cells die by passive toxic cell death, then cycloheximide should not affect the formation and spread of the lesion. Instead, we found that application of cycloheximide inhibited cell death in response to coronatine (Fig. 4*B*) and the spread of cell death in spontaneous lesions (data not shown).

Depletion of SA Exacerbates the *acd2* Mutant Phenotype. The defense signal SA is required for cell death induced by some pathogens (40), accumulates in *acd2* plants (15), and activates many of the defenses expressed in *acd2* mutants. To determine whether the *acd2* cell death phenotype requires SA, we crossed *acd2–2* plants with *nahG* plants, in which SA is depleted (41). We isolated *acd2 nahG* plants by screening for F_2 progeny containing the *nptII* gene of the *nahG* transgene and lacking ACD2 protein. *acd2 nahG* plants showed waves of cell death that initiated at approximately the same time of development as *acd2* single mutants but spread to consume young leaves as well as old (Fig. 4 *C* and *D*). Thus, depletion of SA amplified the cell death phenotype of *acd2*, producing a systemic cell death phenotype.

Overexpression of ACD2 Protein Suppresses Cell Death in Bacterial Infection. If ACD2 acts to suppress cell death, then overexpression of ACD2 might prevent cell death. We engineered plants that produce excess ACD2 protein (Fig. 5*A*) from a CaMV 35S-driven ACD2 cDNA (35S-ACD2). To test the cell death responses of these plants, we inoculated leaves with avirulent derivatives of PsmES4326 (containing the *avrRpm1* or *avrRpt2* genes) or the cognate virulent strain PsmDG3. The 35S-ACD2 plants showed no detectable reduction of the HR with the avirulent pathogens but did show reduced yellowing and cell death, as measured by ion leakage, with the virulent pathogen (Fig. 5*B*). The reduced disease symptoms of 35S-ACD2 plants, if correlated with reduced growth of the pathogen, could indicate activation of the defense response. However, the growth of PsmDG3 was indistinguishable in wild type and 35S-ACD2 (Fig. 5*C*). Thus, overexpression of ACD2 protein made the plants tolerant but not resistant to bacterial infection.

Discussion

Chlorophyll breakdown during senescence detoxifies photoreactive molecules so that other components of the chloroplast can be recycled. Catabolism of chlorophyll (reviewed in ref. 3) begins in the chloroplast, with the removal of the phytol tail and the chlelated magnesium ion from the chlorophyll porphyrin, forming pheophorbide a (Pa). Next, the porphyrin ring is broken by the enzyme Pa oxidase, forming RCC. Plants defective in this step accumulate Pa and its precursors and do not yellow as they age, instead exhibiting a "stay-green" phenotype (42–44). RCC is broken down by ACD2/RCCR, eventually forming fluorescent chlorophyll catabolites, which are moved to the vacuole for storage. RCCR activity is found throughout terrestrial plants (45). *acd2* is the first mutant shown to affect the gene for an enzyme of chlorophyll catabolism.

Disruption of chlorophyll catabolism by the *acd2* mutant is likely to cause the accumulation of RCC and Pa. This could cause spreading cell death by one of two mechanisms. In the first mechanism, accumulation of phototoxic porphyrin molecules causes cell death by the light-dependent production of free radicals. SA-depleted *acd2* plants show enhanced cell death; this may be caused by a lowered antioxidant capacity of the plants, as SA can be important for maintaining the redox capacity of plants in some situations (46). Also, the application of SA analogs produces an increase of copper–zinc superoxide dismutase, an antioxidant enzyme (19). Depletion of SA also exacerbates the phenotype of the lesion mimic mutants *lsd2* and *lsd4* (47) and the HR in response to some pathogens (ref. 46 and D. N. Rate and J.T.G., unpublished work).

Accumulation of photoactive porphyrin compounds has been shown to be toxic to both plants and animals (8, 48). In plants, accumulation of porphyrin precursors causes a lesion mimic phenotype and induction of defense gene expression (49–51). Interestingly, *Les22* mutant maize leaves, which accumulate uroporphyrin III (9), show punctate discrete lesions, in contrast to the spreading lesions of *acd2* mutant leaves. This may reflect a difference in porphyrin localization, or porphyrin accumulation alone may not be sufficient to cause cell death. In support of the latter possibility, some strains of grass and legumes accumulate Pa but do not show a cell death phenotype (42–44).

In a second possible mechanism to account for the *acd2* cell death phenotype, accumulation of RCC may cause a specific signal that triggers cell death. Interestingly, in mammalian systems, protoporphyrin IX, but not other porphyrins such as porphobilinogen, triggers apoptosis by induction of the mitochondrial permeability transition (10, 11). RCC may specifically trigger cell death in plants, possibly by affecting mitochondria and/or chloroplasts. In mammalian systems, mitochondria integrate cell death signals and trigger cell death (12). Both mitochondrial (52) and chloroplast functions may affect the life–death decision in plants. For example, perturbation of the levels of the chloroplast protease *FtsH* affects cell death in response to tobacco mosaic virus (53).

What causes the rapid spread of cell death in *acd2* plants? We hypothesize that cell death in this mutant activates chlorophyll breakdown, which then causes more cell death. This process requires both active translation and light, which are required for cell death in *acd2* mutant protoplasts (54). Translation may be involved in the activation of cell death, in the initiation of chlorophyll breakdown, or in other aspects of the process. Blocking translation inhibited degreening and accumulation of RCC in the alga *Chlorella* (55). Light may be absorbed by accumulated porphyrins, it may activate photosensitive signaling

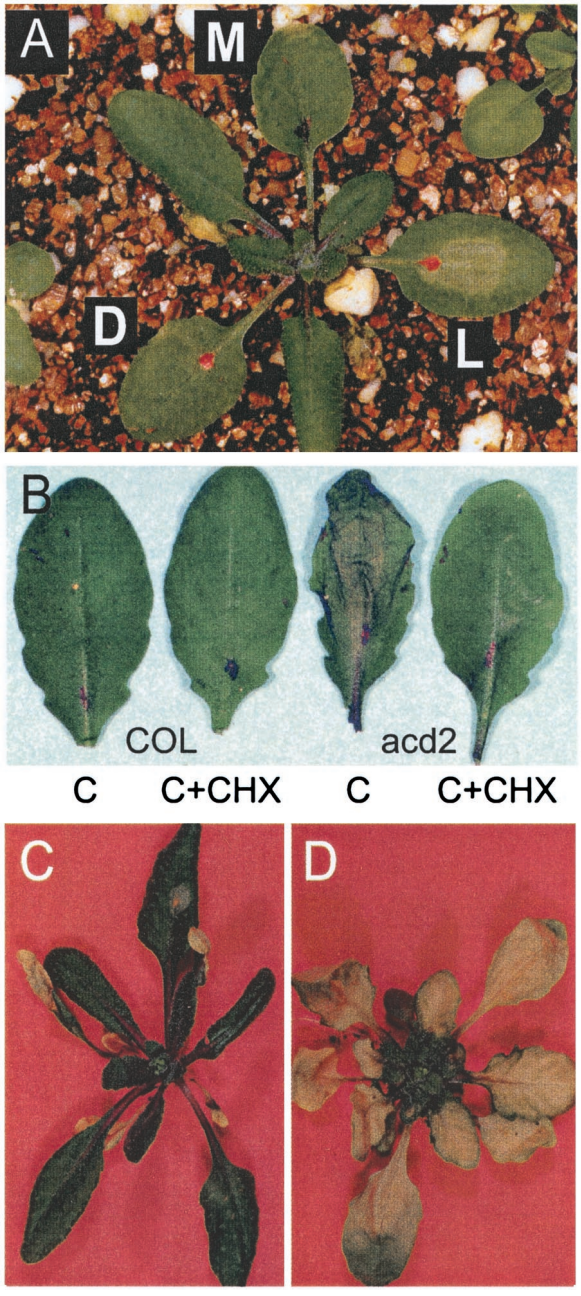

Fig. 4. Effects of light, translation, and SA on *acd2* cell death. (*A*) Light activates the *acd2* mutant phenotype. The center of each indicated *acd2–2* leaf was spotted with either 0.01% methanol as a control (M) or coronatine (D and L). The leaf marked D (dark) was covered. The other leaves (including L, light) remained in the light for 24 h. Similar treatment of the wild-type control did not induce any cell death or chlorosis. (*B*) The *acd2* phenotype requires active translation. Wild-type (COL) or *acd2–2* mutant (acd2) plants were spotted with either coronatine (C) or coronatine and cycloheximide (C + CHX) and were left in the light for 24 h. Depletion of SA exacerbates the *acd2* mutant phenotype. *acd2–2* (*C*) or *acd2–2 nahG* (*D*).

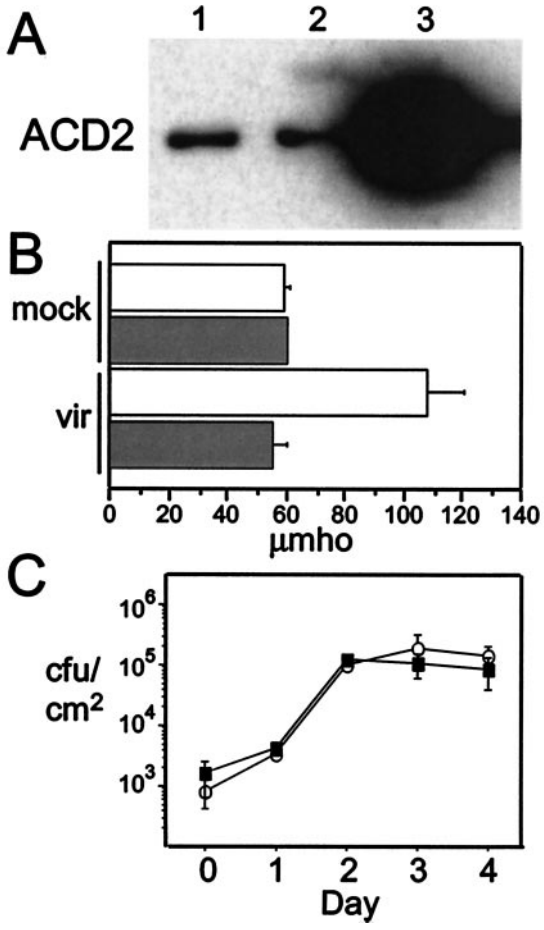

Fig. 5. Overexpression of ACD2 protein suppresses symptoms of pathogen infection. (*A*) Western blot of plant extracts, with equal amounts of protein loaded. Lanes 1 and 2, wild type; lane 3, 35S-ACD2. (*B*) Ion leakage 48 h after inoculation with 0.003 A_{600} of *P. syringae* strain PsmDG3. White bars are wild type; shaded bars are 35S-ACD2. (*C*) Growth, in colony-forming units/cm^2, of virulent PsmDG3 on wild type (○) or 35S-ACD2 (■).

pathways, or it may be used for photosynthesis, providing energy required for active cell death.

Although we have concentrated on the likely effect of the *acd2* mutation on chlorophyll catabolism, the *ACD2* gene may have additional, possibly redundant, functions. ACD2 protein is present at all times of development and in all tissues tested, including roots (ref. 35 and data not shown). The mutant phenotype of defense gene transcription and cell death activation begins at the same time as chlorophyll breakdown in senescence. However, *acd2* plants show some elevated resistance to *P. syringae* as early as the four-leaf stage (15). The mitochondrial localization of ACD2 protein, although transient, may also reflect additional functions. Protein localization to either chloroplasts or mitochondria is usually highly specific (56). However, glutathione reductase produces forms that localize to the chloroplast, mitochondrion, and cytoplasm from a single nuclear gene (57). Examination of the temporal and spatial requirement for ACD2 may prove instructive.

Plants with no ACD2 show spreading cell death. Conversely, plants expressing high levels of the ACD2 protein show increased tolerance to infection with virulent *P. syringae.* An increase in ACD2/RCCR protein levels may alter the flux of chlorophyll catabolites that may normally accumulate during disease and trigger cell death. The ethylene-insensitive mutant *ein2* shows similar tolerance to bacterial infection (39). The ethylene-insensitive *etr1* and *ein2* mutants show delayed senescence (ref. 58 and J.T.G., unpublished observations). Ethylene induces the synthesis of chlorophyllase in *Citrus* fruit (59). It is unknown whether *ein2* alters the flux of chlorophyll catabolites, but it will be interesting to determine whether there are shared mechanisms of tolerance. Manipulation of chlorophyll breakdown may be useful in creating agronomically useful tolerance to pathogens.

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