The Role of Membrane-Bound Ankyrin-Repeat Protein ACD6 in Programmed Cell Death and Plant Defense

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The *ACD6* gene was recently cloned by Lu *et al*. (*1*) from the gain-of-function accelerated cell death 6 (*acd6*) mutant of *Arabidopsis* (*2*). The molecular structure of the ACD6 protein and the phenotypes of both the gain-of-function as well as the loss-of-function of *acd6* mutants suggest a critical role for *ACD6* in regulating programmed cell death (PCD) and defense signaling in plants.

PCD is a general mechanism used by plants to defend against pathogen infection (*3*). The rapid death of cells under pathogen attack can often be observed as defined necrotic lesions at the site of infection. Appearance of such lesions, called the hypersensitive response (HR) by plant pathologists, is accompanied by a series of physiological reactions that include production of reactive oxygen species, accumulation of callose in cell walls, and synthesis of signal molecules such as salicylic acid (SA). Accumulation of SA in both local and systemic tissues leads to increased expression of pathogen-related (*PR*) genes and enhanced resistance against infection. The trigger for this cascade of events is the recognition of an avirulence (Avr) protein in the pathogen by a corresponding resistance (R) protein in the plant. Plant genomes encode hundreds of R proteins, which determine the innate immunity of each plant species or isolate against an array of pathogens (*4*).

To genetically dissect plant defense signaling pathways, a number of mutant screens have been performed in *Arabidopsis thaliana*, a reference organism for higher plants. Characterization of loss-of-resistance mutants has allowed identification of EDS1 and PAD4, two lipase-like proteins involved in *R* gene–mediated accumulation of SA (*5*–*9*); and NPR1, an ankyrin repeat–containing protein that is translocated to the nucleus and serves as a transducer of the SA signal in regulating *PR* gene expression (*10*, *11*). Screens for gain-of-resistance mutants have also been conducted in many laboratories (*12*–*14*). The lesion-simulating disease (*lsd*) and *acd* mutants form HR-like lesions in the absence of pathogen stress and have heightened disease resistance. The *LSD1*, *ACD2*, and *ACD5* genes have been cloned and shown to encode a zinc finger–containing GATA-type transcription factor [transcription factors that bind the consensus DNA sequence (A/T)GATA(A/G)], a red chlorophyll catabolite reductase, and a ceramide kinase, respectively (*15*–*17*). How these proteins affect PCD is still unknown.

Other lesion mimic mutants have also been identified. Among them, the dominant *ssi4* (suppressor of SA insensitivity 4) was found in a screen for suppressors of *npr1* (*18*). The *ssi4* mutant carries a missense mutation in an *R* gene. Evidently, this mutation constitutively activates the *R* protein in the absence of a signal from the pathogen. Defense-regulating genes were also isolated from recessive lesion mimic mutants. The *CPR5* gene encodes a protein with multiple transmembrane domains (*19*, *20*), whereas the product of the barley *MLO* gene is a founding member of the only family of seven transmembrane domain–containing proteins in plants (*21*). The resistance conferred by the recessive *mlo* mutation is suppressed in *ror1* and *ror2* mutants (*22*). The *ROR2* gene encodes a syntaxin that is involved in exocytosis of vesicles containing H_2O_2 (*23*). How MLO negatively controls cell death and ROR2-mediated vesicle transport has yet to be determined.

The recent cloning of *ACD6* adds another member to this list of plant PCD regulators. *ACD6* is member of a family of 34 proteins. These proteins have a short N-terminal region, nine ankyrin repeats in the middle, and five putative transmembrane domains in the C-terminal region (Fig. 1). A point mutation in the third putative transmembrane domain resulted in increased expression of the mutant *acd6* transcript and of *EDS1* and *PAD4*, which are regulatory genes upstream of SA synthesis. Consistent with this, amounts of SA in the *acd6* mutant are 10 times as high as those in the wild type, and SA-mediated *PR1* transcription is constitutively activated. This *acd6* phenotype was completely suppressed when SA accumulation was blocked by expression of the *nahG* gene in the mutant plants. In the presence of *NahG*, which is a bacterial gene encoding salicylate hydroxylase, SA is metabolized (*24*).

The expression of *ACD6* is enhanced in plants treated with benzothiadiazole S-methyl ester (BTH), a functional analog of SA. This is interesting because the gain-of-function *acd6* mutant produces increased amounts of SA, which puts the *acd6* gene upstream of SA synthesis. However, the responsiveness of the *ACD6* gene to BTH suggests that its expression is enhanced by SA, providing a feedback amplification loop. This pattern of regulation is similar to that of *EDS1* and *PAD4*, which are both regulators and effectors of SA signaling. In addition to controlling pathogen-mediated SA synthesis, the expression of *EDS1* and *PAD4* is also regulated by SA. Unlike expression of the *EDS1* and *PAD4* genes, however, *ACD6* gene expression is light-dependent. This is consistent with the lack of cell death in dark-grown *acd6-NahG* plants after BTH treatment. The photoreceptors phytochrome A and B were previously shown to be required for the onset of HR and also for SA-induced *PR* gene expression (*25*). It will be interesting to find out whether these phytochromes are involved in regulating the *ACD6* gene.

The *acd6* mutant phenotype could be completely suppressed when *acd6* was silenced with RNA interference. Conversely, the mutant phenotype was created when the genomic sequence of *acd6* was transformed into wild-type plants. However, overexpression of wild-type *ACD6* cDNA without the point mutation led only to moderate increases in SA concentrations and disease

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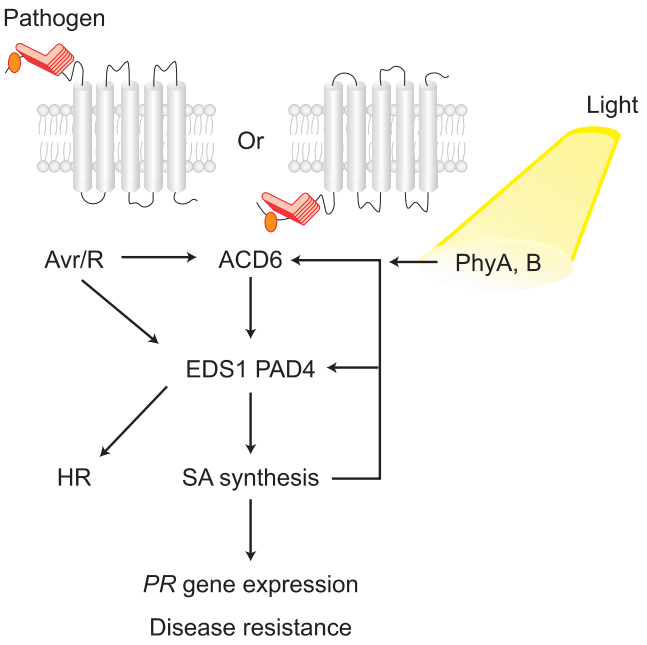

Fig. 1. A model for ACD6 function. The ACD6 protein is hypothesized to be a cytoplasmic membrane protein involved in perceiving a pathogen signal or in Avr/R-mediated signaling. The N-terminal region (oval) and ankyrin-repeat domain (L-shaped lines) could be either extracellular or intracellular. ACD6 is likely to function upstream of PAD4, EDS1, and SA synthesis. In the gain-of-function *acd6* mutant, the abundance of *PAD4* and *EDS1* transcripts is increased, whereas the *acd6* mutant phenotype (spontaneous HR, SA synthesis, *PR* gene expression, and disease resistance) is partially suppressed by *pad4*. Expression of *ACD6* as well as *PAD4* and *EDS1* is regulated by SA, forming a feedback signal amplification loop. In addition to SA, a light signal is required for *ACD6* expression, probably through the function of PhyA and PhyB.

resistance, suggesting that the *acd6* mutant phenotype is not caused solely by the increased gene expression. The point mutation in *acd6* therefore contributes to the phenotype by initiating the signal amplification cascade.

Another finding that requires further investigation is the inhibitory effect that the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* has on *ACD6* gene expression. Apparently, infection by *P. syringae maculicola* causes increased *ACD6* expression systemically but not in local tissue, suggesting that the pathogen may produce a signal that blocks SA-induced *ACD6* expression at the site of infection.

More evidence supporting a direct role of ACD6 in plant defense came from characterization of a transferred-DNA insertion mutant, *acd6-T*. In contrast to the gain-of-function *acd6* mutant, the *acd6-T* null mutant showed enhanced susceptibility to both virulent and avirulent pathogens. This mutation also delays pathogen-induced SA synthesis and partially reduces *PR1* gene expression. However, treatment of *acd6-T* plants with BTH does not completely restore resistance, indicating that ACD6 is probably involved in aspects of plant defense other than SA biosynthesis. Because the *acd6* gain-of-function phenotype can be partially suppressed by *pad4*, ACD6 is likely to function upstream of PAD4 and possibly of EDS1, which physically interacts with PAD4 (*26*).

The presumed membrane localization of ACD6 suggests a role in sensing or transducing extracellular signals, possibly from pathogens. Determining the topology of the protein on the membrane will be an important step toward understanding the molecular function of the protein. Is the ankyrin-repeat domain extracellular, serving as a ligand receptor, or intracellular, involved in transduction of a signal from the membrane? Ankyrin repeats are a common motif found in proteins with a wide range of functions: For example, ankyrin is a cytoskeleton organizer; Notch is a transmembrane protein important for *Drosophila* development; and IκB is involved in controlling the nuclear translocation of NF-κB in mammalian immune responses (*27*). With the same basic structure, ankyrin-repeat domains in different proteins interact with very different macromolecular targets. It will be interesting to examine the mutant phenotype of other *ACD6*-like genes in *Arabidopsis* to determine whether they all affect plant defense or have different biological functions. ACD6 might well be involved in the initial Avr-R interaction that triggers the HR, given that some Avr signals, R proteins, and signaling components such as RIN4, NDR1, and PBS1 are associated with cytoplasmic membranes (*28*–*33*). A role for ACD6 in defense against virulent pathogens is also possible. Identification of ACD6-interacting proteins will be very informative. A genetic screen for second-site suppressors of *acd6* may also be an effective way to identify other components in the ACD6 signaling pathway and to help us understand how ACD6 signals through PAD4 (EDS1) to induce SA synthesis and pathogen resistance.

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