The *Arabidopsis* Gene CAD1 Controls Programmed Cell Death in the Plant Immune System and Encodes a Protein Containing a MACPF Domain

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To clarify the processes involved in plant immunity, we have isolated and characterized a single recessive *Arabidopsis* mutant, *cad1* (constitutively activated cell death 1), which shows a phenotype that mimics the lesions seen in the hypersensitive response (HR). This mutant shows spontaneously activated expression of pathogenesis-related (PR) genes, and leading to a 32-fold increase in salicylic acid (SA). Inoculation of *cad1* mutant plants with *Pseudomonas syringae pv tomato* DC3000 shows that the *cad1* mutation results in the restriction of bacterial growth. Cloning of *CAD1* reveals that this gene encodes a protein containing a domain with significant homology to the MACPF (membrane attack complex and perforin) domain of complement components and perforin proteins that are involved in innate immunity in animals. Furthermore, cell death is suppressed in transgenic *cad1* plants expressing *nahG*, which encodes an SA-degrading enzyme. We therefore conclude that the *CAD1* protein negatively controls the SA-mediated pathway of programmed cell death in plant immunity.

Keywords: Hypersensitive response — Membrane attack complex and perforin — Plant immunity — Programmed cell death — Salicylic acid.

Abbreviations: CFP, cyan fluorescent protein; CIYVV, clover yellow vein virus; GFP, green fluorescent protein; GUS, β-glucuronidase; HR, hypersensitive response; MACPF, membrane attack complex and perforin; *nahG*, salicylate hydroxylase; NPR1, NONEXPRESSOR OF PR GENES 1; PCD, programmed cell death; PCR, polymerase chain reaction; PR genes, pathogenesis-related genes; SA, salicylic acid; TIR, Toll/interleukin-1 receptor.

Introduction

Plants respond to pathogen infection by activating a defense mechanism known as plant immunity. One of the most efficient and immediate resistance reactions against pathogen attack in plants is the hypersensitive response (HR), which leads to rapid local cell death at the site of pathogen entry that is characterized by the restricted growth and spread of the pathogen (Heath 2000a, Heath 2000b, Lam 2001). Little is known, however, about the regulatory mechanism of programmed cell death (PCD) in plant immunity. Several pathogenesis-related (PR) genes are also induced during HR. Most of the PR proteins have been shown to possess antimicrobial activity in vitro or an ability to enhance disease resistance when overexpressed in transgenic plants (Ryals et al. 1996). Control of this HR-mediated PCD seems to involve the concerted action of several signaling molecules.

Salicylic acid (SA) has emerged as a key signaling component for activating the HR and PR gene expression. Indeed, SA levels increase in conjunction with the activation of PR gene expression and disease resistance in many plant species (Malamy et al. 1990, Metraux et al. 1990, Ukness et al. 1993). Eliminating the accumulation of SA in transgenic plants by expressing the bacterial salicylate hydroxylase (*nahG*) gene, which encodes an SA-degrading enzyme, has been shown to prevent both PR gene expression and resistance to pathogen infection (Gaffney et al. 1993, Ryals et al. 1996). Furthermore, SA-insensitive mutants such as *npr1* (non-expressor of PR genes 1) or *nim1* (non-inducible immunity 1), which do not express the PR genes or show systemic acquired resistance (SAR) and cannot be rescued by added SA, exhibit compromised resistance to pathogen infection (Cao et al. 1994, Delaney et al. 1995, Glazebrook et al. 1996, Cao et al. 1997, Ryals et al. 1997).

Lesion mimic mutants (Lorrain et al. 2003) that result in constitutive misregulation of cell death are powerful tools with which to unravel the complex PCD pathway in relation to plant immune systems, and a few such mutants have been identified, including accelerated cell death (*acd*), lesion simulating disease (*lsd*), *gop* (*cpr*), constitutive expression of PR (*cpr*), suppressor of *SA* and insensitivity of *npr1* (*ssi*). Many lesion mimic mutants exhibit a state of increased disease resistance known as SAR with constitutive activation of PR gene expression and the SA signaling pathways (Lorrain et al. 2003). These studies have demonstrated that these lesion mimic mutants show dis-
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an eruption of a gene that seems to be a negative regulator of the PCD pathway in plant immunity. Despite the fact that powerful mutant screening in Arabidopsis has made significant contributions to identifying components of defense activation mechanisms, the overall view of these pathways remains unclear.

To clarify new components of the negative regulation of the PCD pathway in HR, here we have isolated and characterized a single recessive mutant, cad1, which shows a severe HR-like cell death phenotype in the absence of pathogens. Our studies show that the CAD1 gene controls the PCD pathway in a manner that depends on the SA-dependent defense pathways, and that CAD1 is a good candidate for a negative regulator of plant immunity. Furthermore, genetics and cloning analyses show that the CAD1 gene encodes a protein containing a MACPF (membrane attack complex and perforin) domain that is present in perforin and in complement components involved in animal innate immunity (Esser 1994, Trapani and Smyth 2002).

Results

cad1 shows an HR-like cell death phenotype

The cad1 mutant was isolated by screening mutant lines of Arabidopsis that had been mutagenized by T-DNA insertion for a lesion mimic phenotype showing spontaneously activated cell death. The germinating cad1-1 mutant showed a dwarf phenotype (Fig. 1A) with normal cotyledons, but dark brown- or black-colored cell death lesions on the true leaves (Fig. 1B). To investigate the spontaneous cell death phenotype of the cad1 mutant, the leaves were washed with ethanol to remove chlorophyll. The lesions, which were initially localized in cells of the vascular system, eventually spread to all of the rosette leaves (Fig. 1B) along the leaf veins (Fig. 1C). Leaf senescence with chlorophyll breakdown was also accelerated in this mutant (Fig. 1B). The intensity of trypan blue staining showed that there is a high concentration of dead cells in these dark brown- or black-colored lesion areas (Fig. 1E). These phenotypes of cell death were similar to those that are seen during the HR.

The cad1 phenotype segregated as a recessive trait. The homozygous cad1 mutant could not produce seeds, indicating that normal plant developmental processes, such as flower development and seed maturation, are also disturbed by the mutation.

cad1 shows a defense activation phenotype

In plants, PCD occurs during the HR that results from interaction with avirulent pathogens. Expression of the PR1 and PR2 proteins is tightly correlated with development of the HR in response to infection by pathogens (Klessig and Malamy 1994, Shah 2003). To evaluate whether the cad1 mutant has an HR-like cell death phenotype, we analyzed expression of the PR genes by quantitative reverse transcription–polymerase chain reaction (RT–PCR). As shown in Fig. 2A, constitutive expression of PR1 and PR2 was detected in the cad1-1 mutant, suggesting that the cad1 mutant shows a constitutively activated HR-like cell death phenotype. Because constitutively activated cell death mutants show, in most cases, resistance to some compatible plant–pathogen interactions (Lorrain et al. 2003), these results raised the possibility that basal resistance to pathogens is constitutively activated in the cad1 mutant.

We therefore carried out bacterial infection in a well-established experiment to define pathogen resistance in Arabidopsis. Three-week-old cad1 mutant and wild-type (Col) plants were tested for disease tolerance to pathogen growth...
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Following challenge with *Pseudomonas syringae* pv *tomato* DC3000 (Fig. 2B). Bacterial numbers were determined at 0, 1 and 2 d after inoculation. A marked increase in bacterial numbers was detected in the wild type at 2 d after inoculation, resulting in disease symptoms such as cell death and chlorophyll loss (data not shown). In contrast, the cad1 mutant showed more limited bacterial growth than the wild type, indicating that the cad1 mutant does show a constitutively activated defense phenotype.

**cad1 constitutively accumulates high levels of SA**

Activation of the HR and of PR1 and PR2 gene expression generally requires SA signaling. To clarify the role of SA in the defense phenotype of the cad1 mutant, we directly measured the endogenous levels of SA by HPLC. The endogenous SA level was 32-fold higher in the cad1 mutant than in the wild type in 3-week-old plants (Fig. 3). The PR1 and PR2 genes were also activated in cad1 (Fig. 2A). Taken together, these results indicate that the SA-dependent defense pathway is activated in the cad1 mutant, resulting in the HR-like cell death phenotype.

**CAD1 negatively controls PCD by repressing SA signaling**

To evaluate the dependency of the cad1 phenotype on SA, transgenic plants engineered to express the nahG gene, which are unable to accumulate SA owing to degradation (Ryals et al. 1996), were crossed with the cad1-1 mutant. In 35S nahG cad1-1 plants, formation of the cell death lesions was markedly suppressed, such that light-colored lesions occurred only at the vascular bundle (Fig. 4A, panels c, g), indicating that SA is required for the cad1 phenotype.

SA signaling is mediated by at least two mechanisms: the first requires the NPR1 gene, whereas the other is independent of this gene (Lorrain et al. 2003, Shah 2003). The npr1-1 mutant is insensitive to SA signaling, leading to a lack of PR gene expression and the formation of cell death lesions following pathogen attack (Cao et al. 1994, Delaney et al. 1995, Glazebrook et al. 1996, Cao et al. 1997, Ryals et al. 1997). Thus, the NPR1 is a key transducer of SA-mediated plant immunity. We found that cad1-1 mutants also accumulated transcripts of NPR1 (Fig. 2A), suggesting that the NPR1-dependent SA signaling pathway is strongly activated in the cad1-1 mutant. To test whether the cad1 phenotype requires this NPR1-mediated signaling pathway, we generated an npr1-1 cad1-1 double mutant. The phenotype of the double mutant (Fig. 4A, panels d, h) was almost the same as that of cad1-1, except that the appearance of lesions was delayed by 7–10 d and the frequency of lesion formation was decreased compared with cad1-1 (Fig. 4A, panel f). Although 35S nahG and npr1-1
mutation restored the \textit{cad1} phenotype markedly and partially, respectively, the \textit{PRI} and \textit{PR2} genes were constitutively expressed in both of these double mutants (Fig. 4B, C), as well as in the single \textit{cad1} mutant (Fig. 2A).

Taking these results together, we conclude that the CAD1 protein functions at an early stage in the SA signaling pathway as a negative regulator of PCD. Furthermore, CAD1 function is followed not only by an NPR1-independent pathway but also by a pathway that is partially dependent on NPR1, because the \textit{npr1-1 cad1-1} mutant did not completely mimic the \textit{cad1-1} phenotype; namely, these double mutant plants were larger than \textit{cad1-1} mutant plants (Fig. 4A, panels b, d), but their size was not completely restored to that of the wild type (Fig. 4A, panels a, d).

\textbf{CAD1 encodes a novel protein containing a MACPF domain}

To elucidate the molecular function of CAD1, we cloned the \textit{CAD1} gene. The \textit{cad1-1} mutant shows a cell death phenotype only in the homozygous state. The \textit{T2} line was back-crossed to wild-type plants, and the segregation patterns of the \textit{F2} family showed that the \textit{cad1} phenotype co-segregated with a T-DNA insert. To isolate the sequence flanking the T-DNA insertion site in the \textit{cad1} mutant, thermal asymmetric interlace (TAIL)-PCR was performed (Liu et al. 1995). A single PCR fragment was isolated after three enrichment cycles as described in Materials and Methods. The PCR fragment was cloned and sequenced, which indicated that the left border of the T-DNA was perfectly matched and the novel sequence was identical to that of the gene At1g29690. A BLAST search of GenBank showed that the \textit{CAD1} gene encodes a previously uncharacterized protein containing a region with significant homology to a MACPF domain (Fig. 5A, B). The MACPF domain of CAD1 is highly related to those of the C6–C9 components of the mammalian complement system and to that of perforin (Fig. 5B); these proteins are well-characterized pore-forming proteins involved in innate immunity (Esser 1994, Trapani and Smyth 2002).

RT–PCR analysis using \textit{CAD1}-specific primers did not detect \textit{CAD1} mRNA in the \textit{cad1} mutant, indicating that the T-DNA insertion markedly destabilizes the \textit{CAD1} transcript (Fig. 5C). To verify that disruption of the \textit{CAD1} gene was responsible for the mutant phenotype in \textit{cad1-1} plants, we examined three more alleles, \textit{cad1-2}, \textit{cad1-3} and \textit{cad1-4}, that showed a single insertion of T-DNA in the third, sixth and sixth exon of the gene, respectively. As shown in Fig. 5A, we verified the T-DNA insertion site in these three mutant alleles. All of these mutants, as well as \textit{F1} plants of \textit{cad1-1} and \textit{cad1-2}, showed the
same lesion mimic phenotype as the cad1-1 mutant (data not shown), indicating that the insertion of T-DNA into At1g29690 is responsible for the cad1 mutant phenotype.

To investigate the organ-specific expression of the CAD1 gene, we carried out RNA gel blot analysis by using a DNA fragment corresponding to the full-length CAD1 cDNA as a probe. Although hybridization signals were found in RNA from various organs, the signals were very weak (data not shown). To evaluate the expression sites of the CAD1 gene in more detail, we generated a pCAD1::GUS transgenic Arabidopsis plant.
The *CAD1* promoter β-glucuronidase (GUS) construction contains 2 kb of sequence directly upstream from the translation site of the *CAD1* gene transcriptionally fused to the GUS reporter gene. Histochemical observation showed that GUS activity was present in various organs, but was strongest in newly produced leaves (Fig. 5D).

The *cad1* phenotype restricts long-distance movement of clover yellow vein virus

The enhanced SA signaling and expression of the PR genes (see Fig. 2A, 3) in the *cad1* mutant may restrict viral infection. To evaluate the functional significance of the *cad1* phenotype on viral infection, we monitored the spread of infection in *cad1* plants. Three-week-old *cad1-1* mutant and wild-type plants were inoculated with a modified clover yellow vein virus (ClYVV) encoding green fluorescent protein (GFP) as a reporter gene (Fig. 6A, Sato et al. 2003). Systemic viral infection of the plants was established after successful amplification of the viral genome in infected cells, followed by cell to cell and long-distance viral movement between organs. In wild-type plants, GFP expression spread through the vascular bundle and showed that the virus had systemically infected the whole plant 8 d after inoculation. For the site of inoculation, we used a rosette leaf because cell death lesions do not develop in these leaves in the *cad1* mutant. The rates of cell to cell movement in the inoculated leaves were nearly identical in the *cad1-1* mutant and the wild type, with similar cell numbers showing GFP fluorescence between 48 and 72 h after inoculation (Table 1). In contrast, long-distance viral movement from the inoculated leaf to uninoculated leaves (Fig. 6B, panels a–f, circles) was markedly restricted in the mutant (Fig. 6B, panel e) as compared with the wild type (Fig. 6B, panel b). Immunoblot analysis using an antibody against ClYVV showed that the coat protein was not detectable in uninoculated leaves of the

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**Fig. 5** Structure of the *CAD1* gene and location of the T-DNA insertions in *cad1* mutants. (A) Exon structure of the *CAD1* gene, and position of the T-DNA insertions and MACPF protein domain. Blue boxes represent the six exons. The *CAD1* gene encodes a protein of 561 amino acids containing a MACPF domain (red underline). (B) Alignment of the MACPF domain in *CAD1*, human complement components C6–C9 and perforin. Black shading indicates identical residues; gray shading indicates a conservative substitution between *CAD1* and the other proteins. Numbers on the left and right refer to the amino acid position in the deduced *CAD1* protein and in the other MACPF proteins. (C) Expression of the *CAD1* gene in the *cad1-1* mutant. Shown is an ethidium bromide-stained agarose gel of RT–PCR analysis of RNA from wild type (Col) and the *cad1-1* mutant. Total RNA from 3-week-old vegetative shoot tissue was analyzed by quantitative RT–PCR to monitor the accumulation of *CAD1* transcripts. Each PCR amplification of the cDNA was done in parallel with primers specific for *CAD1* (CAD1-1 and CAD1-2; see Table 2) and the control (*EF1α*). (D) Histochemical localization of p*CAD1::GUS* expression. Seedlings of p*CAD1::GUS* transgenic plants were grown on an agar plate containing MS media for 10 d. The bottom panel shows a close-up view of the image in the top panel. Bar = 0.2 cm.

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**Fig. 6** Long-distance movement of CIYVV/C3-S65T is restricted in the *cad1* mutant. (A) Schematic representation of the CIYVV/C3-S65T virus construct (Sato et al. 2003) used in this study. (B) (Panels a–c) Monitoring CIYVV/C3-S65T infection in an uninoculated leaf. Wild-type Col (panels a–c) and *cad1-1* (panels d–f) plants were inoculated with CIYVV/C3-S65T. Shown are the bright-field (panels a and d), the fluorescence (panels b and e) and the autofluorescence (panels c and f) images. Circles indicates an uninoculated leaf. Bar = 0.5 cm. (C) Analysis of uninoculated leaves 8 d after inoculation by Western blotting using an antibody against CIYVV/C3-S65T.
Table 1  Comparison of viral spread in inoculated leaves in cad1-1 and wild-type Col

<table>
<thead>
<tr>
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<th>No. of cells infected</th>
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<tr>
<td></td>
<td>48 h.p.i. a</td>
</tr>
<tr>
<td>Col</td>
<td>45</td>
</tr>
<tr>
<td>cad1-1</td>
<td>78</td>
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Eighteen plants were examined for each genotype.
a Hours post-inoculation.

Table 1  Comparison of viral spread in inoculated leaves in cad1-1 and wild-type Col

Discussion

Function of CAD1 in plant immunity

To clarify the mechanism that activates plant immunity, we have isolated and characterized the spontaneously activated cell death mutant cad1. The following observations from phenotypic analyses of a single recessive cad1 mutant show that loss of function of CAD1 leads to a phenotype of constitutively activated SA-mediated plant immunity. First, the cad1 mutant shows an HR-like cell death phenotype with high expression of the PR genes (Fig. 1, 2A). Secondly, the cad1 mutant shows a constitutively activated defense phenotype against challenge with P. syringae pv tomato DC3000 (Fig. 2B). Thirdly, the endogenous SA content is up-regulated in the cad1 mutant (Fig. 3). Finally, genetic analyses show that the cell death phenotype depends on a high level of endogenous SA, because overexpression of the nahG gene, which encodes an SA-degrading enzyme, almost completely restores the cell death phenotype (Fig. 4A, panels c, g). Taken together, these results strongly indicate that an accumulation of endogenous SA is required for the HR-like cell death phenotype, suggesting in turn that the CAD1 gene is a negative regulator of SA-mediated plant immunity. However, the activation of SA-mediated plant immunity in the cad1 mutant was accompanied by an accumulation of endogenous JA and expression of the JA/ethylene-inducible gene PDF1.2 (data not shown), suggesting that other signaling molecules such as JA are required to accelerate the HR-like cell death phenotype in the cad1 mutant.

Gathering all lines of evidence together, we propose the following model of CAD1 function in plant immunity. When the plant is not subject to pathogen attack, CAD1 functions as a negative regulator that represses the PCD pathway mediated by SA; thus, under conditions in which there is no pathogen infection, CAD1 negatively controls plant immunity. Once the plant recognizes pathogen infection, however, the negative regulatory function of CAD1 will be inhibited, leading to activation of the PCD pathway to restrict the growth and spread of the pathogen. Thus, the CAD1 protein is likely to act as a mediator that recognizes plant signals for pathogen infection.

Long-distance movement of CIYVV in cad1

Virus pathogens systemically infect plants by moving through the vascular system. The requirements for long-distance viral movement through the vascular system principally differ from the requirements for cell to cell movement. After cell to cell movement, the virus is transported into companion cells by a specific viral transport protein in order to enter the phloem (Oparka and Turgeon 1999). In this study, the long-distance movement of the CIYVV virus was markedly restricted by the cad1 mutation (Fig. 6B, panel e). This restriction of long-distance movement may be due to inhibition of the entry of viral components into the vasculature caused by the cad1 mutation, because the CIYVV coat protein was not detected in uninoculated leaves (Fig. 6C).

Interestingly, the local cell death phenotype is probably not involved in the restriction of long-distance viral movement seen in this study, because we observed an almost identical inhibition of movement in cad1 mutant leaves with severe cell death lesion and those with mild cell death lesion (data not shown). These results suggest that long-distance viral movement is associated with a cad1 phenotype other than the HR-like cell death phenotype; in other words, the CAD1 gene controls not only the SA-mediated cell death pathway but also long-distance movement during the process of viral infection. The mechanism underlying the restriction of viral movement by plant factors may be very complex and may not be explained by a single gene. Thus, the cad1 mutant is likely to be a valuable tool with which to clarify the mechanisms for long-distance viral movement.

CAD1 and MACPF proteins in the immune system of plants and animals

We have shown here that the Arabidopsis CAD1 protein contains the MACPF domain that is also found in human perforin. This protein is thought to be released from natural killer and/or killer T cells and undergoes self-polymerization to form a pore-like structure on the membrane of target cells to induce apoptosis by the activation of caspases through granzyme B (Trapani and Smyth 2002, Lieberman 2003). The MACPF domain has been also identified in many proteins from diverse species including plants, insects and mammals (Fig. 7); so far, however, a MACPF-containing protein has not been characterized in the plant kingdom. The MACPF protein family might have evolved from a common progenitor involved in a fundamental signaling pathway in cells of the last common ancestor. From a database analysis, there are four CAD1-like genes that encode a protein containing a MACPF domain in the Arabidopsis genome. All four of these Arabidopsis genes have cis-acting W-box elements within their promoter sequence that have high binding affinity for the WRKY protein. There are several SA-induced WRKY genes in the Arabidopsis genome;
a certain WRKY protein acts upstream of NPR1 and positively regulates its expression during the activation of plant immunity (Diqiu et al. 2001). In general, the products of gene duplication and members of a gene family often encode, or are assumed to encode, proteins with similar and/or overlapping function. In this study, we have demonstrated that CAD1 controls SA-mediated plant immunity. Thus, are these four Arabidopsis CAD1-like proteins also involved in plant immunity? Answering this question will require a more detailed evaluation of the biological function of CAD1 in plant immunity.

Numerous mediators of disease resistance signaling in plants have been found to share conserved motifs with proteins that are known to play similar roles in the defense response in animals (Staskawicz et al. 2001, Nurnberger and Brunner 2002, Parker 2003). Plant–pathogen interactions are governed by disease resistance (R) loci: the R genes encode proteins containing an N-terminal domain that resembles the Toll/interleukin-1 receptor (TIR) of Drosophila and mammals. One of the downstream targets of the R genes is NPR1, which has an ankyrin domain that is homologous to IκB, an inhibitor protein that regulates the expression of defense-related genes downstream of the TIR in animal innate immunity. Individuals with perforin deficiency have been reported to show not only a defect in cytotoxicity but also an acutely fatal deregulation of the immune system (Stepp et al. 1999). Interestingly, a deficiency in MACPF proteins results in symptoms or phenotypes similar to those caused by immune deregulation in humans (Stepp et al. 1999) and in Arabidopsis (this study). Further studies of the function of both perforin and CAD1 as negative regulators of the immune system will be very informative.
Table 2. Sequences of primers used to analyze gene expression.

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<th>Primer name</th>
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<tr>
<td>CAD1-2</td>
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Because plants lack the acquired immune system that has developed in animals, they have adapted a more general defense mechanism with analogy to the animal innate immune system, namely, strategic PCD, which leads to the restriction of pathogen growth and spread. It will be important to identify proteins that interact with CAD1 in order to elucidate the detailed molecular mechanism of CAD1 function in the PCD pathway in plant immunity.

Materials and Methods

Plants and growth conditions

*Arabidopsis thaliana* (Columbia-0 and WS) plants were grown at 22°C. For germination, seeds were surface sterilized and placed on Murashige and Skoog medium supplemented with 10 g l⁻¹ sucrose. After an overnight cold treatment to synchronize germination, seeds were grown at 22°C and 50% relative humidity under a 16/8 h light/dark cycle.

Infection of *Pseudomonas syringae pv tomato* DC3000

The virulent bacterial leaf pathogen *P. syringae pv tomato* DC3000, which causes bacterial speck disease, was grown overnight at 28°C in liquid NBY medium. Bacterial cells were collected by centrifugation and resuspended in 10 mM MgSO₄ to a final density of 10⁵ colony-forming units ml⁻¹. For the inoculation of plants by leaf dip, the surfactant Silwet-77 was added to a final concentration 0.01%. For each sample, three leaf discs were pooled three times per data point. Leaf discs were bored from the infiltrated area, ground in distilled water, and serially diluted to measure bacterial numbers.

Trypan blue staining

Leaves were submerged in lactic acid–phenol–trypan blue solution [2.5 mg ml⁻¹ trypan blue, 25% (v/v) lactic acid, 23% water-saturated phenol, 25% glycerol and H₂O₂] at 70°C, slow-release vacuum infiltrated for 2 min, and then reinfiltated. Leaves were then heated over boiling water for 2 min and cooled for 1 h before replacing the lactic acid–phenol–trypan blue solution with a chloral hydrate solution (25 g in 10 ml of H₂O₂) for destaining.

Gene expression analysis of the cad1 mutant

Total RNA was isolated from wild-type Col and cad1-1 mutant leaf tissue with the RNeasy Plant RNA isolation kit (Qiagen, Valencia, CA, USA). A single-stranded cDNA was synthesized from total RNA. First-strand cDNA synthesis was performed with reverse transcriptase using 1 µg of total RNA and oligo(dT) primer (RNA PCR kit, TAKARA SHUZO CO., LTD., Shiga, Japan). PCR (total volume 20 µl) was performed using 0.2 U of Taq DNA polymerase (Ex Taq, TAKARA SHUZO CO., LTD., Shiga, Japan). Gene-specific primers were designed to produce DNA fragments of the CAD1, PR1, PR2, NPR1 and EF1α genes. The primers used (Table 2) were designated CAD1-1 and CAD1-2 (set 1), PR1-1 and PR1-2 (set 2), PR2-1 and PR2-2 (set 3), NPR1-1 and NPR1-2 (set 4) and EF1α-1 and EF1α-2 (set 5), and were used to amplify DNA fragments of CAD1, PR1, PR2, NPR1 and EF1α, respectively, from their cDNAs by PCR. The amount of template cDNA required and the number of PCR cycles necessary were determined in preliminary experiments to ensure that amplification occurred in the linear range and allowed accurate quantification of the amplified products by Southern blot hybridization analysis. Ten cycles of amplification were used for each gene. The amplified DNA products (10 µl of each reaction) were separated on a 1.2% (w/v) agarose gel, transferred to a nylon membrane (Hybond-N⁺, Amersham Pharmacia Biotech, Buckinghamshire, UK), and hybridized with 32P-labeled cDNA fragments at 65°C. The filter was washed twice with 2× SSC containing 0.1% SDS at 65°C for 15 min and examined by autoradiography.

β-Glucuronidase (GUS) assays

The CAD1 promoter GUS construction contains a 2 kb fragment encompassing the 5’ region upstream from the translation site of the CAD1 gene that is transcriptionally fused to the GUS reporter gene. After the histochemical reaction, samples were fixed with 3% glutaraldehyde in phosphate buffer (50 mM, pH 7.0) for 1 h, washed twice with the same phosphate buffer, and passed through an ethanol series to remove chlorophyll before observation under a light microscope.

Crosses with the nprr-1 mutant and 35SnahG transgenic plants

The nprr-1 cad1-1 double mutant was generated by using pollen from homozygous nprr-1 plants to fertilize heterozygous cad1-1 plants. Seeds of transgenic 35SnahG in the Col ecotype were provided by Dr. Xinnian Dong at Duke University. The double mutant was confirmed by sequence analysis of the NPRI gene and PCR analysis of the CAD1 gene (see ‘Isolation of cad1 mutant alleles’). The 35SnahG cad1-1 line was generated by using pollen from homozygous 35SnahG transgenic plants to fertilize heterozygous cad1-1 plants. A double homozygous line was confirmed by PCR analysis of the nahG gene and the CAD1 gene. To amplify a region of the nahG gene, specific PCR primers designed nahG1 and nahG2 were designed (Table 2).

Molecular cloning of CAD1

The genomic sequence flanking the T-DNA insertion in cad1 was amplified using TAIL-PCR as described (Liu et al. 1995). We used the T-DNA left border-specific primers LB1, LB2 and LB3 (Table 2), and the previously described degenerate primer AD2 (Liu et al. 1995). PCR amplifications were performed by using genomic DNA isolated from the cad1-1 mutant as a template and recommended cycle settings (Liu et al. 1995). DNA fragments were cloned into the pCR2.1 vector and sequenced.

Isolation of cad1 mutant alleles

Four lines with T-DNA insertions in cad1 were obtained from the ABRC (Ohio State University, Columbus, OH, USA; stock number...
SA measurement

SA was extracted from 0.2 g of 4-week-old leaves. Each sample was extracted four times with 1.5 ml of methanol. A 5 µl aliquot of 1 mg ml⁻¹ m-hydroxybenzoic acid was added to the extract as an internal standard. The solution was dried out in an evaporator and the residue was dissolved in 150 µl of methanol and then 600 µl of 1 mM KOH was added. Lipophilic substances were removed by extraction with chloroform twice. The aqueous phase was transferred to a new tube, and then 10 µl of phosphoric acid and 500 µl of ethyl acetate were added. The solution was mixed and centrifuged at 17,000 g for 5 min. The supernatant was transferred to a new tube and the aqueous phase was extracted again with ethyl acetate. After centrifugation, the supernatant was dried out and the residue was dissolved in 50% methanol and analyzed by HPLC. SA was detected with a fluorescent detector set at Ex = 295 nm and Em = 370 nm. The mobile phase was 20 mM sodium acetate containing 20% methanol.

Monitoring of long-distance movement of ClYVV/C3-S65T

The leaves of seedlings were dusted with carborundum and then mechanically inoculated with ClYVV/C3-S65T in 0.01 M phosphate buffer (pH 7.0). For the cad1 mutant, viral inoculation was done in a rosette leaf, where cell death lesions do not develop. The inoculated plants were maintained under continuous light at 22°C.

The numbers of infected cells were monitored by GFP fluorescence. We observed GFP fluorescence in non-inoculated leaves 8 d after inoculation of the wild type and the cad1 mutant (Fig. 6B, panels b, e). Non-specific fluorescence was identified by comparing images showing GFP fluorescence (Fig. 6B, panels b, e) and autofluorescence, which was recorded as cyan fluorescent protein (CFP) fluorescence (Fig. 6B, panels c, f). GFP fluorescence was examined by an epifluorescence microscope (SZX-12; Olympus, Tokyo, Japan) equipped with a GFP cube (SZX-MGFP; Olympus), and CFP fluorescence was observed by using the SZX-12 microscope equipped with a CFP cube (SZX-MCFP; Olympus).

Western blot analysis

Total proteins were extracted from individual plants. Immunodetection was done by using a polyclonal antibody against ClYVV. An alkaline phosphatase-labeled secondary antibody was used to detect immunoreactive bands (Guglielminetti et al. 1995).

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