Salicylic Acid and a Chitin Elicitor Both Control Expression of the CAD1 Gene Involved in the Plant Immunity of Arabidopsis

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The Arabidopsis mutant cad1 (constitutively activated cell death 1) shows a phenotype that mimics hypersensitive response (HR)-like cell death. The CAD1 gene, which encodes a protein containing a domain with significant homology to the MACPF (membrane attack complex and perforin) domain of complement components and perforin, is likely to control plant immunity negatively and has a W-box cis-element in its promoter region. We found that expression of the CAD1 gene and other W-box containing genes, such as NPR1 and PR2, was promoted by salicylic acid (SA) and benzothiadiazole (BTH) as a SA agonist. The CAD1 gene was also stimulated by a purified chitin oligosaccharide elicitor (degree of polymerization = 8). This latter control was not under SA, because CAD1 expression was not suppressed in 35S
nahG transgenic plants, which are unable to accumulate SA. These expression profiles were confirmed by promoter analysis using pCAD1::GUS transgenic plants. The CAD1 expression promoted by BTH and the chitin elicitor was not suppressed in the npr1 mutant, which is insensitive to SA signaling. These results indicate that the CAD1 gene is regulated by two distinct pathways involving SA and a chitin elicitor: viz., SA signaling mediated through an NPR1-independent pathway, and chitin elicitor signaling, through an SA-independent pathway. Three CAD1 homologs that have multiple W-box elements in their promoters were also found to be under the control of SA.

Key words: chitin oligosaccharide elicitor; WRKY domain; W-box; membrane attack complex and perforin (MACPF) domain; salicylic acid

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 restricted growth and restricted spread of the pathogen.1–3 Little is known, however, about the regulatory mechanism of programmed cell death (PCD) in plant immunity.

Salicylic acid (SA) has emerged as a key signaling component that activates both HR and pathogenesis-related (PR) gene expression. Indeed, SA levels increase in conjunction with activation of PR gene expression and disease resistance in many plant species.4,5 SA-insensitive mutants such as npr1 (nonexpressor of PR genes 1), which do not express the PR genes or show systemic acquired resistance (SAR) and cannot be rescued by exogenous SA, exhibit compromised resistance to pathogen infection.5–8

The control of gene expression by SA mainly involves cis-acting W-box elements in the promoter. The W-box element contains a TGAC core, which is preferentially bound by WRKY proteins containing either one or two WRKY domains. The WRKY proteins comprise a superfamily of transcription factors with up to 100 members in Arabidopsis.9 Current data indicate that many WRKY proteins have a regulatory function in the response to pathogen infection and other stresses.

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 has a phenotype that mimics the lesions seen in the HR.10 This mutant shows spontaneously
activated expression of PR genes, leading to a 32-fold increase in SA. Inoculation of cad1 mutant plants with *Pseudomonas syringae* pv. *tomato* DC3000 shows that the cad1 mutation results in a 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. Thus the CAD1 protein appears negatively to control the SA-mediated pathway of programmed cell death in plant immunity.10)

Chitin, a polysaccharide composed of β-1→4-linked N-acetyl-d-glucosamine, is a structural element found in fungal cell walls, insect exoskeletons, and the hard carapace of crustaceans. Increasing evidence indicates that chitin and chitin oligomers can regulate defensive, symbiotic, and developmental processes in plants.11) It has been shown, mainly by using suspension cultured cells of rice, that N-acetylchitooligosaccharide of a certain size can induce various cellular responses, including phytoalexin production, transient depolarization of membrane potential, ion flux, transient generation of reactive oxygen species, and jasmonic acid production.12) Recently, Arabidopsis was used to study chitin signaling, indicating that certain mitogen-activated protein kinases (MAPKs) and WRKY transcription factors might be important components of a pathway involved in chitin signaling.13)

In this study, to clarify the physiological roles of CAD1 in plant immunity, we examined the expression profile of CAD1 and its gene homologs in terms of their response to SA and a chitin oligosaccharide elicitor. Because the CAD1 gene has putative W-box elements in its promoter, functional promoter analysis was also done. The results indicate that CAD1 is regulated by two distinct pathways involving SA and a chitin oligosaccharide elicitor.

### Materials and Methods

**Plant materials and growth conditions.** Arabidopsis thaliana (Columbia-0 and all plants used in this study) were grown at 22°C. For germination, seeds were surface-sterilized and placed on Murashige and Skoog medium supplemented with 20 g/l sucrose. After an overnight cold treatment to synchronize germination, the seeds were grown at 22°C at 50% relative humidity under a 16/8 h light/dark cycle.

**Plant treatments.** Two-week-old Arabidopsis plants were sprayed with 1 ml/plant of 100 μM Acibenzolar-S-methyl ester (benzo(thio)diazole or BTH), and 10 μg/ml chitin oligosaccharide (dp = 8). The control plants were similarly treated with an equivalent amount of distilled water. BTH and chitin oligosaccharides were dissolved in distilled water plus 0.05% Tween-20 and distilled water respectively.

**RNA isolation, RT-PCR, and Northern bolt analysis.** Total RNA was isolated from whole plants with an RNaseasy Plant RNA isolation kit (Qiagen, Valencia, CA). A single-stranded cDNA was synthesized from total RNA and oligo (dT) primer (RNA PCR kit, TaKaRa Shuzo, Siga, Japan). PCR (total volume, 20μl) was performed using 0.2 units of Taq DNA polymerase (Ex Taq, TaKaRa Shuzo). Gene-specific primers were designed to produce DNA fragments of the PR2 and EF1α genes. The primers used (Table 1) were designated CAD1-1 and CAD1-2 (set 1), CAD1L1-1 and CAD1L1-2 (set 2), CAD1L2-1 and CAD1L2-2 (set 3), CAD1L3-1 and CAD1L3-2 (set 4), PR2-1 and PR2-2 (set 5), and EF1α-1 and EF1α-2 (set 6), and were used to amplify DNA fragments of CAD1 (At1g29690), CAD1L1 (At1g14780), CAD1L2 (At1g28380), CAD1L3 (At4g24920), PR2, 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. Twelve, 9, and 8 cycles of amplification were used for CAD1 and related genes, the PR2 and EF1α genes respectively. The amplified DNA products (10 μl of each reaction) and 10μg of total RNA were separated on 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 was 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 start site of the CAD1 gene that is transcriptionally fused to the GUS reporter gene. GUS activity was assayed as described
by Jefferson et al.14) 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.

Results and Discussion

The CAD1 gene is up-regulated by SA

A previous study10) indicates that the CAD1 protein negatively controls the SA-mediated pathway of programmed cell death in plant immunity. We examined the expression pattern of the CAD1 gene in this study.

The SA agonist BTH promotes both the activation of SA-mediated plant immunity and the expression of PR genes such as PR2. The CAD1 transcript was detected to a small extent in the water-treated wild-type plant (Fig. 1A), but, it accumulated at 1 h and disappeared at 6 h after treatment with 1 mM SA, and the extent of accumulation was promoted by 100 μM BTH. These results suggest that the CAD1 gene is upregulated by SA.

SA signaling is mediated by at least two mechanisms: one requires the NPR1 gene, whereas the other is independent of this gene.15,16) Hence, to evaluate the detailed pathway of SA signaling, we examined CAD1 expression in the npr1-1 mutant, which is insensitive to SA signaling. We found a lack of PR2 gene expression, whereas the CAD1 transcript accumulated, in the npr1-1 mutant (Fig. 1B). These results suggest that CAD1 expression is controlled mainly by SA signaling that is independent of NPR1, whereas the PR2 gene is regulated by a NPR1-dependent pathway.

The CAD1 gene is up-regulated by a chitin oligosaccharide elicitor

Chitin and chitin oligomers have been found to function as an elicitor signal in plant immunity.17) To study the response of CAD1 to such an elicitor, plants were exposed to 10 μg/ml chitin oligosaccharide (dp = 8) (Fig. 2). The CAD1 transcript was detectable at 20 min, reached a plateau at 40–60 min (Fig. 2A) and disappeared at 4 h (data not shown) after treatment with chitin oligosaccharide, whereas the PR2 transcript was detectable at 60 min (Fig. 2A), indicating that the CAD1

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Fig. 1. BTH Induces CAD1 mRNA Accumulation.
Quantitative RT-PCR and Southern blot hybridization analysis was performed for CAD1 and PR2 in 2-week-old wild-type Col (A) and npr1-1 (B) plants treated with distilled water (Water), 1 mM SA, and 100 μM BTH. Distilled water (C) was used as a control. To verify equal RNA amplification in the various lanes, the gel was stained with ethidium bromide and analyzed for rRNA.

Fig. 2. A Chitin Oligosaccharide Elicitor Induces CAD1 mRNA Accumulation.
Quantitative RT-PCR and Southern blot hybridization analysis was performed for CAD1 and PR2 in 2-week-old wild-type Col (A), npr1-1 (B) and 35S:ubG (C) plants treated with 10 μg/ml chitin oligosaccharide (dp = 8). Distilled water (C) was used as a control. To verify equal RNA amplification in the various lanes, the gel was stained with ethidium bromide and analyzed for rRNA.
gene is stimulated by a chitin oligosaccharide elicitor. Because the CAD1 gene is also up-regulated by BTH and SA (Fig. 1), we evaluated the possibility that the chitin elicitor activates CAD1 expression via the SA-signaling pathway. In the npr1-1 mutant (Fig. 2B), expression of CAD1 was induced by the chitin oligosaccharide to the same level as in the wild-type plant (Fig. 2A), whereas SA-mediated PR2 expression was greatly reduced. Similar results were obtained in 35SnahG transgenic plants, which are engineered to express the nahG gene and are unable to accumulate SA owing to degradation (Fig. 2C). These results indicate that the expression of CAD1 triggered by the chitin oligosaccharide elicitor does not involve the SA signaling pathway.

CAD1 homologs comprise a small MACPF family

The CAD1 gene encodes a protein containing a domain with significant homology to the MACPF (membrane attach complex and perforin) domain of complement components and perforin. There are three CAD1 homologs that also contain the MACPF domain. In an amino acid alignment, we found that CAD1L1 (CAD1-like 1; At1g14780), CAD1L2 (At1g28380), and CAD1L3 (At4g24290) share 42.2%, 43.7% and 43.9% identity with CAD1 (At1g29690) respectively (Fig. 3A). In the MACPF domain, amino acids are mostly conserved among the family, but, CAD1 has a deletion of two regions (see arrowheads) that are more weakly conserved among the other family members (Fig. 3A). The cad1 mutant, which shows a cell death phenotype, is caused by a loss-of-function mutation. The cad1l1
(SAIL-512_C01) and cad1l3 (SALK_040186, SALK_052845) mutants, which have T-DNA inserted in their exons, do not show any cad1 phenotypes, whereas the cad1l2 mutant is not available from the public T-DNA knockout lines. Therefore, the CAD1 homologs are likely to have functions that are distinct from those of CAD1.

The MACPF domain has also been identified in many proteins from diverse species including plants, insects, and mammals; so far, CAD1 is the first MACPF-containing protein identified in the plant kingdom.\(^{10}\) The MACPF domain of CAD1 is closely related to those of the C6–C9 components of the mammalian complement system. Complement provides a critical and multifaceted defense system against infection. After activation, complement can function in the clearance of invading microorganisms either by opsonization, which promotes recognition by phagocyte complement receptors, or directly by lysis. The complement system consists of about 20 components (C1 to C9) in which the sequential assembly of C6, C7, C8, and (C9)\(_n\) is mediated through their MACPF domains.\(^{19}\) Thus, CAD1 might function in association with the other CAD1 homologs to control plant immunity.

**All CAD family genes are under SA control**

The SA inducibility of the CAD1 homologs was examined (Fig. 3B). Transcripts of CAD1L1, CAD1L2, and CAD1L3, as well as CAD1, accumulated 1 h after treatment with 100 \(\mu\)M BTH, indicating that all the CAD1 homologs are induced by SA.

The CAD1 genes and all of their homologs have putative cis-acting W-box elements within their promoter regions that have high-binding affinity for the WRKY protein (Fig. 3C). There are several SA-induced WRKY genes in the Arabidopsis genome. In addition, a specific WRKY protein active upstream of NPR1 and positively regulates its expression during the activation of plant immunity.\(^{20}\)

To evaluate the SA and chitin elicitor response of the CAD1 gene, we generated a pCAD1::GUS transgenic Arabidopsis plant. This CAD1 promoter \(\beta\)-glucuronidase (GUS) construction contains 2 kb of sequence lying directly upstream of the translation site of the CAD1 gene transcriptionally fused to the GUS reporter gene. Histochemical observation showed that GUS activity was promoted by BTH and the chitin oligosaccharide elicitor (Fig. 4), indicating that the CAD1 promoter can respond to signaling by SA and a chitin elicitor. The GUS activity promoted was observed in all organs, but especially in the vascular system, indicating that the CAD1 protein is associated with a systemic response.

**SA and elicitor inducibility on CAD1 expression**

The cad1 mutant, which has a phenotype that mimics the lesions seen in the HR, shows spontaneously activated expression of PR genes, leading to a 32-fold increase in SA.\(^{10}\) In this study, we found that the CAD1 gene is under the control of SA and a chitin elicitor. Gathering all lines of evidence together, we propose the following model of transcriptional regulation of the CAD1 gene in plant immunity (Fig. 5). The CAD1 protein which encodes the gene is constitutively expressed to a small extent, and negatively controls the endogenous SA level and the SA-mediated defense pathway. The CAD1 gene itself is also under SA control. Since CAD1 expression is controlled by SA signaling, that is independent of NPR1, this protein is likely to function SA signaling at a relatively upstream site. Additionally, the CAD1 gene is under the control of other factors such as chitin elicitor signaling which is mediated by the MAP kinase cascade and WRKY proteins.\(^{13}\) Further experiments are needed to clarify the transcriptional regulation of the CAD1 gene.

Transcriptional regulation of the CAD1 gene might be associated preferentially with cis-acting W-box elements in the promoter. The W-box is defined as a (T)(T)TGAC(C/T) sequence element that contains an invariant TGAC core, which is essential for function and WRKY binding. The CAD1 gene has three of these elements within its 500-bp promoter region and an additional three within the 1,500-bp flanking region (Fig. 3C). WRKY proteins are a superfamily of transcription factors that has up to 100 members in Arabidopsis.\(^{9}\) It has been reported that SA-induced expression of most of these WRKY proteins is independent of NPR1.\(^{21}\) Therefore, the WRKY proteins that are involved in regulation of CAD1 are also likely to be independent of NPR1, because the npr1 mutation did not affect the expression of CAD1 (Fig. 1B). Northern
analysis has revealed that the SA-inducible WRKY proteins such as WRKY7 (At4g24240), WRKY15 (At2g23320), WRKY21 (At2g30590), WRKY25 (At2g30250), WRKY39 (At3g04670), and WRKY60 (At2g25000) are controlled in an NPR1-independent manner.\(^2\)\(^3\) Hence, the \textit{CAD1} gene is likely to be under SA control mediated by such WRKY proteins.

With regard to the chitin elicitor, its signals are likely to be mediated by a specific receptor in the microsomal/plasma membrane\(^2\)\(^3\) and transduced through MAPK cascades.\(^2\)\(^3\) In \textit{Arabidopsis}, the chitin-inducible WRKY proteins such as WRKY22 (At4g01250), WRKY29 (At4g23550), WRKY33 (At2g38470), and WRKY53 (At4g23810),\(^2\)\(^0\) which are similar to those associated with PAMPs recognition such as bacterial flagellin,\(^2\)\(^4\) are preferentially involved in elicitor signaling.\(^1\) Thus these WRKY proteins might also regulate \textit{CAD1} expression in plant immunity, but, it cannot be ruled out that the other combinations of cis- and trans-acting factors, except for the W-box and WRKY proteins, are responsible for regulation of \textit{CAD1} gene expression. Further experiments are needed to clarify the regulatory pathways involved in expression of the \textit{CAD1} gene.

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