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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 attach 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 p*CAD1*::*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.[6–8]

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

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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$\rightarrow$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 (benzothiadiazole 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.

Table 1. Sequences of Primers Used to Analyze Gene Expression

Primer name	Sequence (5′-3′)
CAD1-1	TAAGCCGGTCACAGGTCTCAGA
CAD1-2	GAAACGTGTCACGAAACGGGCA
CAD1L1-1	GCTATACACCTACAACACCTAG
CAD1L1-2	ACAAGAAGACGTTGGCGATGTA
CAD1L2-1	ACTACGCCGCTCGAAACAAAGT
CAD1L2-2	GACCAGTGAGTCTTCCGATATG
CAD1L3-1	GGTTTTCATCTCATTATACTCG
CAD1L3-2	CACTAGTATGAAGAATAACTCC
PR2-1	TTAGTTGAAATTAACTTCATAC
PR2-2	TGATCTGAATCAAGGAGCTTAG
EF1α-1	GCTGTCCTTATCATTGACTCCACC
EF1α-2	TCATACCAGTCTCAACACGTCC

RNA isolation, RT-PCR, and Northern bolt analysis. Total RNA was isolated from whole plants with an RNeasy Plant RNA isolation kit (Qiagen, Valencia, CA). 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, Siga, Japan). PCR (total volume, 20 µl) 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 (At4g24290), 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 10 µg 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 [^{32}P]-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 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 study[10] 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*

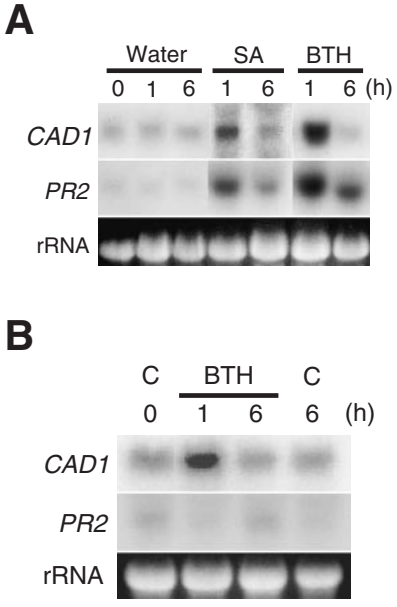

Fig. 1. BTH Induces *CAD1* mRNA Accumulation.

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.

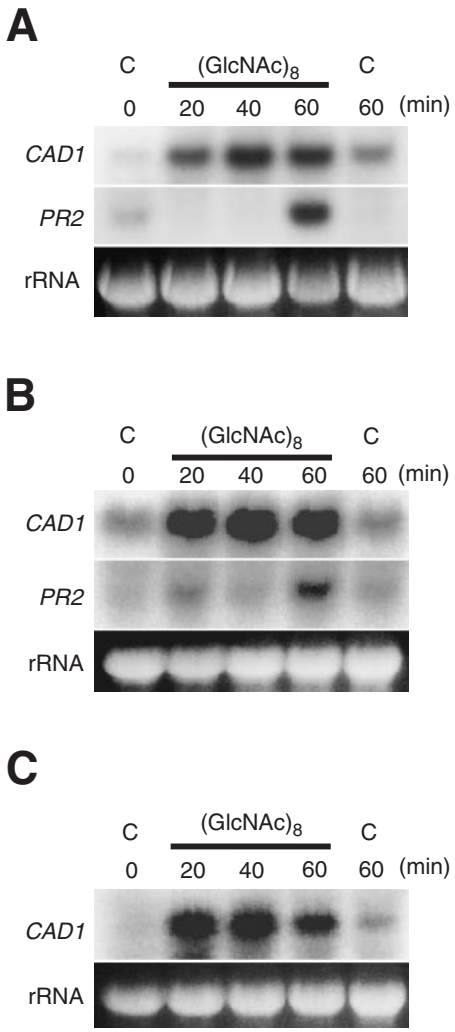

Fig. 2. A Chitin Oligosaccharide Elicitor Induces *CAD1* mRNA Accumulation.

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 *35SnahG* (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 examined for rRNA.

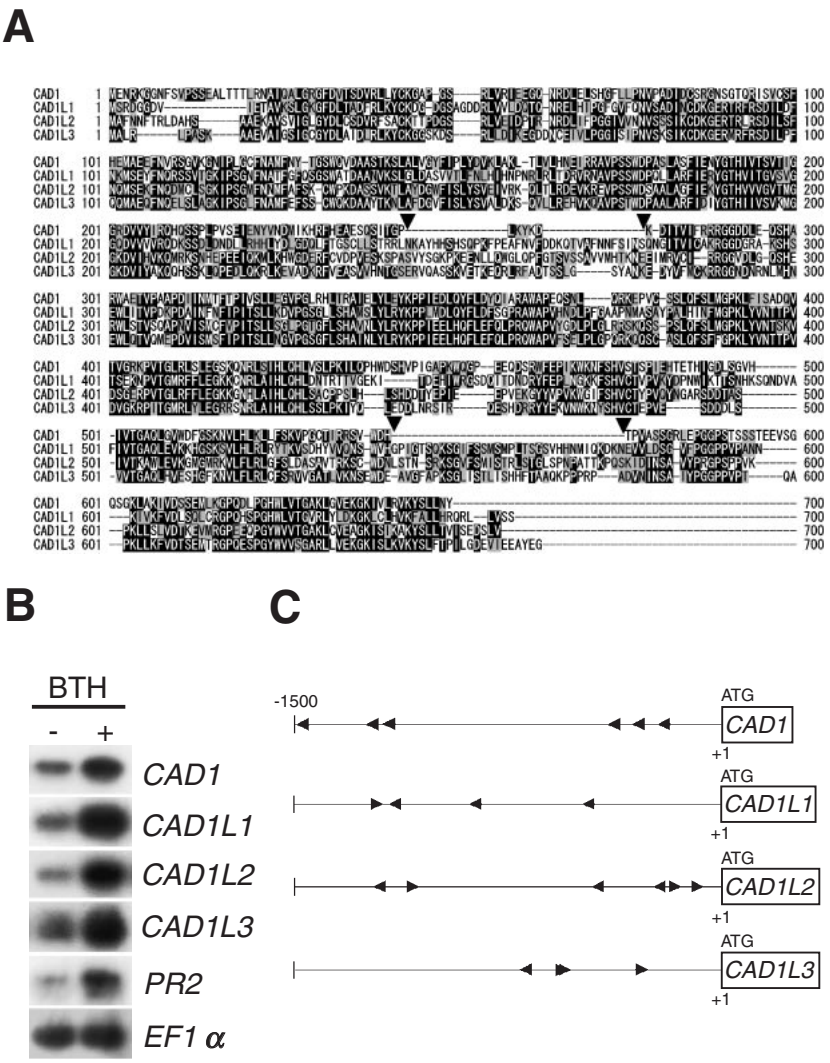

Fig. 3. Alignments, BTH Inducibility and Promoter Motifs in the CAD Family.

A, Amino acid alignment of the MACPF domain in CAD1 (At1g29690), CAD1L1 (At1g14780), CAD1L2 (At1g28380), and CAD1L3 (At4g24290). Black shading indicates identical residues, and gray shading indicates a conservative substitution between CAD1 and the other proteins. Arrowheads delineate deleted regions of CAD1. B, BTH induces mRNA accumulation in CAD family members. Quantitative RT-PCR and Southern blot hybridization analysis was done for *CAD1*, *CAD1L1*, *CAD1L2*, *CAD1L3*, *PR2*, and *EF1α* (control) in 2-week-old wild-type Col plants treated with water (−) and 100 μM BTH (+) for 1 h. C, Scheme showing alignment of the promoter regions of the CAD family. Triangles indicate W-box motifs.[9)]

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 35S*nahG* 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.[18)] 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.[10)] 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 μM 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 acts 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 p*CAD1::GUS* transgenic *Arabidopsis* plant. This *CAD1* promoter β-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

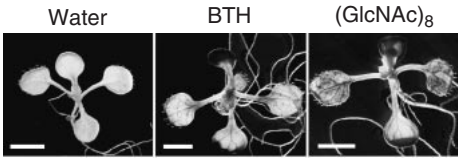

Fig. 4. Histochemical Localization of p*CAD1*::*GUS* Expression after Treatment with BTH and a Chitin Oligosaccharide Elicitor.

Seedlings (10 d old) of p*CAD1*::*GUS* transgenic plants were treated with water, 100 μM BTH, or 10 μg/ml chitin oligosaccharide (dp = 8) for 2 h and observed by GUS staining.

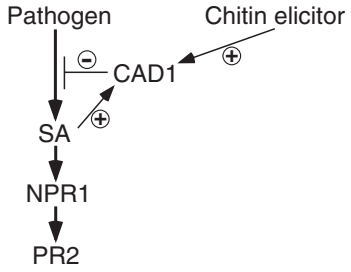

Fig. 5. Hypothetical Model of Transcriptional Regulation of *CAD1* Gene in the Plant Immune System.

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.[20,21] Hence, the *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[22] and transduced through MAPK cascades.[23] In *Arabidopsis*, the chitin-inducible WRKY proteins such as WRKY22 (At4g01250), WRKY29 (At4g23550), WRKY33 (At2g38470), and WRKY53 (At4g23810),[20] which are similar to those associated with PAMPs recognition such as bacterial flagellin,[24] are preferentially involved in elicitor signaling.[13] Thus these WRKY proteins might also regulate *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 *CAD1* gene expression. Further experiments are needed to clarify the regulatory pathways involved in expression of the *CAD1* gene.

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