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Molecular Characterization of an Apple cDNA Encoding Cinnamyl Alcohol Dehydrogenase

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Abstract The study of lignin, a major component of secondary cell wall, has been partly focused on its removal from the woody part in the kraft pulping industry. Cinnamyl alcohol dehydrogenase (CAD; EC1.1.195) catalyzes the synthesis of cinnamyl alcohols from corresponding cinnamaldehydes. A cDNA clone, *MdCAD1*, encoding putative CAD from apples (*Malus domestica* Borkh. cv Fuji) was characterized in this study. The clone contains an open reading frame of 325 amino acid residues, which shows a greater than 80% identity with *Eucalyptus* CAD1. *MdCAD1* mRNA was detectable in vegetative tissues and was strongly expressed in the fruit. The expression pattern of *MdCAD1* mRNA in the fruit peel after light exposure was also examined. The mRNA was rapidly increased until 1 day after light exposure and remained stable thereafter, suggesting that *MdCAD1* is light inducible. The inducibility of the *MdCAD1* gene was examined using several environmental stresses. Mechanical wounding of leaves increased the *MdCAD1* mRNA level and the induction was further increased by salicylic acid. Southern blot hybridization showed that there is either one or a few copies of *CAD* genes in apples. To our knowledge, it is believed that *MdCAD1* is the first *CAD* clone expressed predominantly in fruit.

Key words: lignin, cinnamyl alcohol dehydrogenase, cDNA, light, wounding, salicylic acid

Lignin is a major structural component, composed of monomeric units, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, in the secondary walls of plant cells such as tracheary elements that provides mechanical support and disease resistance [8]. Lignin comprises 18–36% of the dry weight of wood in trees; as a result, the delignification of wood has been a strong focus of the kraft pulping industry [30].

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Lignin biosynthesis occurs via a multi-step metabolic pathway starting from the shikimate pathway. Cinnamyl alcohol dehydrogenase (CAD) catalyzes the synthesis of the cinnamyl alcohols, the precursors of lignins, from the corresponding cinnamaldehydes. The genes encoding CAD have been obtained from beans (*Phaseolus vulgaris* L.), tobacco (*Nicotiana tabacum*), *Eucalyptus gunnii*, *Aralia cordata*, the Norway spruce (*Picea abies* L.), loblolly pine (*Pinus taeda* L.), and zinnia (*Zinnia elegans*) [37, 18, 14, 11, 16, 20, 31]. The cDNAs encoding the CAD were either isolated from stem tissues or cultured cells of leaves. The expression of *CAD* has been studied for several species. In *E. gunnii*, *CAD1* mRNA was more abundant in the leaves (poorly lignified organs) than in the stems and not detected in the roots [12]. However, *CAD2* mRNA was found to be equally abundant in the stems and leaves, especially in xylem, with small amount in the roots [14]. These tissue- and cell-specific expressions of CAD were further characterized by a promoter analysis of poplar [10]. The *CAD* gene has been a target for the modification of the lignin content in plants through genetic engineering. The down-regulation of CAD using an antisense approach in tobacco and poplar failed to reduce the amount of lignin. However, the composition of lignin in transgenic plants was changed, suggesting that the lignin biosynthetic pathway is a rather complex combinatorial network [4, 15]. Recently, a report on the mutant loblolly pine [25] has heightened the possible success of the antisense approaches in the delignification of wood in the kraft pulping industry. The mutant pine was viable and apparently normal, although its CAD activity was severely depleted.

CAD-encoding genes have been studied for inducibility using various phytohormones and environmental stimuli. In Zinnia, the level of *ZCAD1* mRNA was high in the D cells (culture cells grown in the medium which contained 0.1 mg/l NAA and 0.2 mg/l BA), yet in the absence of NAA or BA, the level of mRNA was very low indicating that the *ZCAD1* gene could be induced by a combination

of NAA and BA [31]. In spruce, the addition of an elicitor to the cell cultures and ozone treatment of seedlings strongly increased the level of *CAD* mRNA [11]. The treatment of bean cell cultures with an elicitor also increased the synthesis and the activity of the CAD enzyme [13]. The elicitor-responsive increase of the *CAD* expression has been related to the defensive role of some CAD enzymes. It is known that a stress-induced lignin deposition provides a mechanism for sealing off sites of pathogen infection and wounding [7]. This report presents the results of molecular characterization of a putative CAD-encoding cDNA clone from apple fruits. In addition, a study on the expression pattern of the *CAD* gene caused by various stimuli such as light is also presented.

MATERIALS AND METHODS

Plant Materials and Bacterial Strains

Various tissues of apple were either harvested from the National Horticultural Research Institute experimental station or grown in the greenhouse of Sogang University. The samples were transferred to the laboratory in an icebox and immediately stored in liquid nitrogen until used. For the wounding experiments, leaves were wounded by being cut into pieces and floated on sterilized deionized water at room temperature for 3 h. SA was added to the final concentration of 1 mM.

E. coli strains, MC1000 [F^-, *araD139, (araABC-leu)7679, galU, galK, (lac)X74, thi*$^-$, *rpsL*(Str^r)], and XL-1 Blue MRF' [F'::Tn*10 proA*$^+$*B*$^+$, *lacIq*, *(lacZ)M15/recA1, endA1, gyrA96*(Nal^r), *thi*$^-$, *hsdR17*(rk^-mk^+), *supE44, relA1, lac*] were used as hosts for the molecular clonings. The f1 helper phage, R408, was used for the excision of the pBluescript phagemid from the λ UNI-Zap XR vector (Stratagene).

Sequence Analysis of *MdCAD1*

Plasmid DNA was isolated according to the method developed by Murray and Thompson [22]. Both strands of the cDNA were sequenced using the dideoxynucleotide chain termination method with double-stranded DNA as a template [29]. The computer software programs, DNAsis and PROsis (Hitachi), were used for a sequence analysis. The GenBank, EMBL, and Swissprot databases were searched for a sequence homology using the BLAST algorithm [1].

Southern and Northern Analyses

Genomic DNA was prepared from young leaves using the CTAB (cethyltrimethylammonium bromide) method [26]. The quantity and quality of the DNA were determined by measuring the optical density at 220–320 nm. Ten μg of DNA was digested with appropriate restriction enzymes (20 unit/μg DNA) for 6 h at 37°C, separated on a 0.8% agarose gel, and then transferred to Hybond-N (Amersham) using a vacuum transfer system (TransVac™, Hoefer). Ten to thirty μg of total RNA isolated using the guanidium thiocyanate method was resolved on a 1.2% formaldehyde agarose gel, blotted onto a nylon membrane, and then hybridized with a ^{32}P-labeled probe [6]. Southern and Northern hybridizations were both performed for 16 to 24 h at 42°C [28]. The membrane was washed in 2× SSC and 0.1% SDS at room temperature. When necessary, the membrane was further washed in 0.1× SSC, 0.1% SDS, at 65°C and exposed to Kodak XAR-5 film or a phosphoimage plate (BAS 1500, Fuji Co., Japan).

Preparation of Labeled Probes

After the digestion of the DNA with the *Eco*RI and *Xho*I restriction enzymes, the DNA fragments were purified by electroelution. The DNA was radioactively labeled with [α-^{32}P] dCTP (3,000 ci/mmole, Dupont) using the random priming method [9]. Any unincorporated nucleotides were removed using G-50 Sephadex column chromatography.

RESULTS AND DISCUSSION

Sequence Analysis of *MdCAD1*

During the analysis of the Expressed Sequence Tags (ESTs) from the young fruit library, a clone F19 was obtained, which had high homology with *Eucalyptus* cinnamyl alcohol dehydrogenase (CAD1). An initial partial analysis of F19 indicated a homology to dihydroflavonol reductase (DFR) [32]. A nucleotide sequence analysis of the full-length EST clone revealed that the clone was 1,262 bp in length, and contained an open reading frame of 325 amino acid residues between nucleotides 16 and 990 (Fig. 1). A deduced amino acid sequence of the clone showed an 82% identity with *Eucalyptus* CAD1 and included 33 matching amino acid residues among the 37 positions conserved between other CAD enzymes (Fig. 2). Accordingly, the clone was designated as *MdCAD1* (*Malus domestica* Cinnamyl Alcohol Dehydrogenase 1). The calculated molecular mass of MdCAD1 was 35.6 kDa, similar to the mass of *Eucalyptus* CAD1, which is 35.8 kDa [12]. The pI value of MdCAD1 was 6.7, which is different from the pI value 8.1 of *Eucalyptus* CAD1 [12]. The deduced amino acid sequence of MdCAD1 had 70% and 56% identities with CPRD14, a drought-responsive clone of cowpea [17], and *Arabidopsis* CAD homolog (GenBank accession no. AC003970), respectively. MdCAD1 also exhibited a homology to several cinnamoyl-CoA reductases (CCRs) and many DFRs (Fig. 3). Computer-aided database searches with MdCAD1 revealed that it had a 38% identity with DFR in *Callistephus*. It is known that DFRs belong to the 3β-hydroxysteroid dehydrogenase/

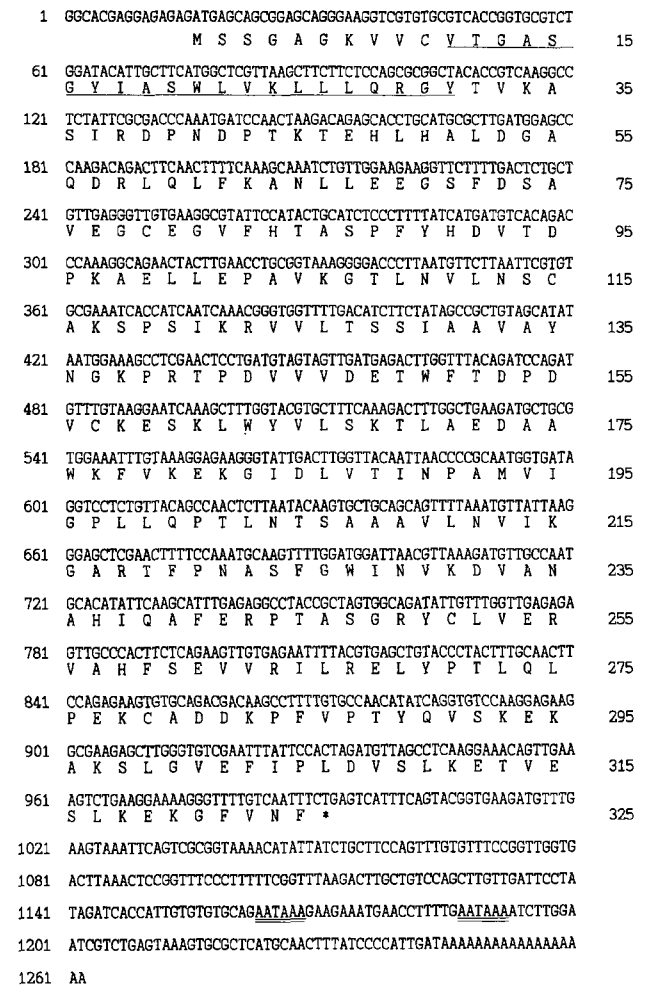

Fig. 1. Nucleotide sequence of *MdCAD1*.
The deduced amino acid sequence of *MdCAD1* is shown below the nucleotide sequence. The left and right numbers indicate the position of the nucleotide and amino acid, respectively. The putative NAD/NADP (H) binding domain located in the N-terminal region is underlined. The putative polyadenylation signal sites are double-underlined. The nucleotide sequence was deposited in the GenBank database under accession number AF053084.

DFR superfamily [2]. The high homology between MdCAD1 and DFRs indicates that MdCAD1 may be a new member of the superfamily as in the case of *Eucalyptus* CAD1 [12].

Southern Analysis of *MdCAD1*

Southern analysis was conducted to assess the copy number of the *MdCAD1* gene. The hybridization pattern with the radiolabeled *MdCAD1* probe revealed that the gene is either a single copy or a member of a small gene family (Fig. 4). Two *Hin*dIII restriction sites present in the *MdCAD1* cDNA support this result. It is known that each *CAD* gene isolated in many species is either single or a member of a small gene family. Southern analysis revealed that the loblolly pine *CAD* is single [20] as is the bean *CAD* gene [37]. In the case of *Eucalyptus*, the gene is composed

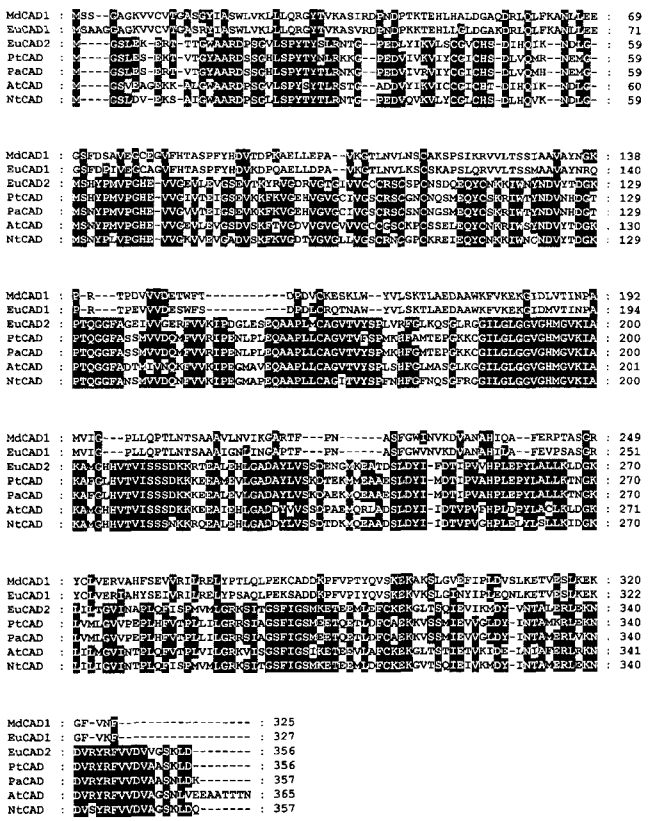

Fig. 2. Comparison of the deduced MdCAD1 protein with corresponding sequences of other CAD proteins.
The deduced MdCAD1 protein was compared with EuCAD1of *E. gunnii* [12], PtCAD of *P. taeda* [20], PaCAD of *P. abies* [11], AtCAD of *A. thaliana* [3], and NtCAD of *N. tabacum* [18]. A multiple sequence alignment was performed using the Clustal W program version 1.6 [34] and displayed using GeneDoc [23]. Identical residues are highlighted in black. The numbers indicate the position of the amino acid.

of a small multigene family [12]. Therefore, the result of *MdCAD1* is consistent with those of other *CAD* genes.

Northern Analysis of *MdCAD1*

The expression level of the *CAD* gene in various organs or tissues was studied using an RNA blot analysis (Fig. 5). The *MdCAD1* mRNA was highly present in the fruit tissues. The mRNA was also detected in the seedlings and leaves although at a low level (Fig. 5A). Expression in the 'lignified' roots and in floral buds was also confirmed (data not shown). A high abundance of *MdCAD1* mRNA in the reproductive organ has not been previously reported. Interestingly, the lignification level and relative expression of *CAD* is not always strictly related. In *E. gunnii, CAD1* transcripts were detected in abundance in the leaves (poorly lignified organs) yet hardly detected in the stems (lignifying organs) [12].

So far, *MdCAD1* is the first cDNA clone obtained from fruit. In fruit, lignin is a secondary wall component of fruit cells. Lignin fiber comprises about 0.6% of apple fruit

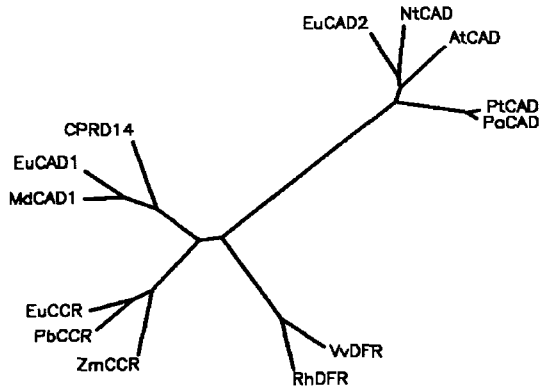

Fig. 3. Phylogenetic relationships between MdCAD1, other CADs, cinnamoyl CoA-reductases (CCR), dihydroflavonol-4-reductases (DFR), and a cowpea clone responsive to dehydration (CPRD14) from various species.

An unrooted phylogenetic tree was constructed with the amino acid sequences indicated below, using PHYLIP program version 3.57c as the computer software. MdCAD1 (in this paper), EuCAD1 [12], and EuCAD2 [14] of *E. gunnii*, PtCAD of *P. taeda* [20], PaCAD of *P. abies* [11], AtCAD of *A. thaliana* [3], NtCAD of *N. tabacum* [18], EuCCR of *Zea mays* [24], PbCCR of *Populus balsamifera* [19], RhDFR of *Rosa hybrida* [35], VvDFR of *Vitis vinifera* (EMBL accession no. Y11749), and CPRD14 of cowpea [17]. The length of each branch is proportional to the evolutionary distance.

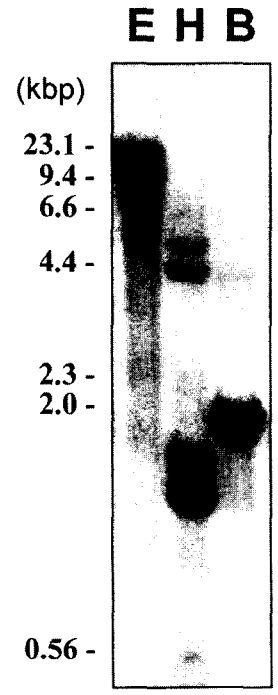

Fig. 4. Southern blot of *MdCAD1*.

Thirty five mg of the apple genomic DNA isolated from young leaves was digested with *Eco*RI (E), *Hin*dIII (H), and *Bam*HI (B), blotted onto a nylon membrane, and probed with *MdCAD1* cDNA. The numbers indicate the size of the λ DNA cut with *Hin*dIII in kbp.

[27]. In terms of fiber content and fruit quality, the clone obtained in this study may be useful in future study.

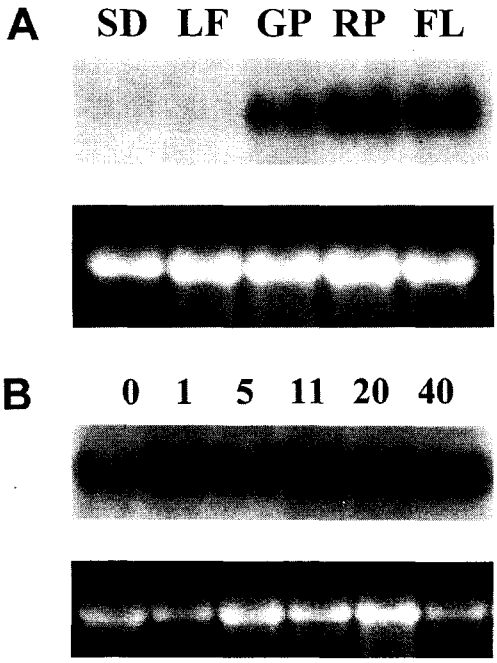

Fig. 5. Northern analysis of *MdCAD1*.

A. Expression of *MdCAD1* in various tissues or organs. Ten μg of total RNA isolated from seedlings (SD), leaves (LF), green peels (GP) of 0 DABR (days after bag removal), red peels of 5 DABR (RP), and flesh of 5 DABR (FL) fruit were loaded on each lane. B. Expression of *MdCAD1* with light exposure. Ten μg of total RNA isolated from peels of 0, 1, 5, 11, 20, and 40 DABR were loaded on each lane. Lower part of each panel shows EtBr stained 25S rRNAs.

Induction of *MdCAD1* Expression by Light Exposure

The expression pattern of *MdCAD1* mRNA was examined in fruit after light exposure. On a farm, young apples are usually bagged for protection from pathogen attacks, and for a better and even coloration. The fruit is bagged with two layers of paper envelops in early June and the bags are removed around late September. The expression of *MdCAD1* in the fruit peel was examined after the bags were removed. The mRNA rapidly increased until 1 day after light exposure and remained stable thereafter, suggesting that *MdCAD1* is light inducible (Fig. 5B).

Since *MdCAD1* belongs to a *DFR* superfamily, which is an anthocyanin biosynthetic enzyme, the *DFR* and *CAD* expression levels were compared. *MdDFR* was also induced by light although the induction kinetics were different from that of *MdCAD1* (unpublished data). Whether the regulatory mechanism of the *MdCAD1* gene is similar to that of *MdDFR* should be studied further. It would be interesting to investigate the existence of an I-box and GT-1 box [33] found in the promoters of many light-regulated genes. The *cis*-element TAACGT, present in the anthocyanin biosynthetic genes in maize, was also found in the regulatory region of the *Arabidopsis Cad* gene [3].

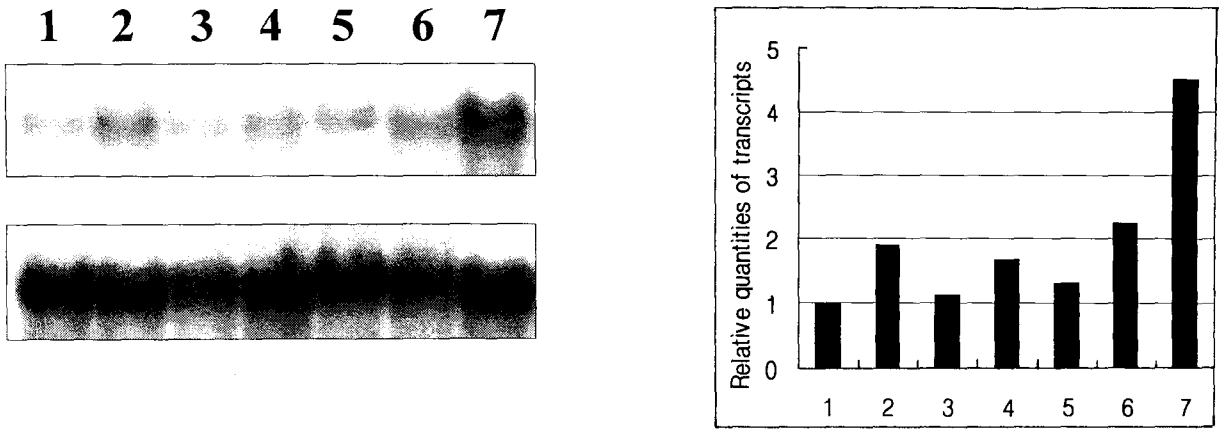

Fig. 6. Induction of *MdCAD1* mRNA in apple leaves by various stimuli.
Left. Wounded leaf fragments were floated on water (2) containing 3% sucrose (3), 1 μM 2,4-D (4), 10 μM MJ (5), 4 mM ethephon (6), and 1 mM SA (7) for 3 h. After incubation, total RNA was prepared and 30 μg of RNA was loaded on each lane and hybridized with a radioactively labeled MdCAD1 probe. 1, control. Lower part of panel shows hybridized bands with radiolabeled 25S rRNAs. Right. Relative quantities of the transcripts induced by various stimuli are shown as a bar graph. The values have been corrected based on the quantities of 25S rRNAs in each lane.

Induction of *MdCAD1* by Other Environmental Stimuli

To understand what environmental stresses other than light trigger *MdCAD1* expression, the gene was investigated for inducibility using several environmental stimuli, including wounding and salicylic acid (Fig. 6). The expression of *MdCAD1* was induced in leaves by mechanical wounding with a level of about 2-fold higher than that of the control leaves. The mRNA level was further increased to 4.5-fold by the addition of 1 mM of salicylic acid, the phytohormone related to a defense response in plants. Salicylic acid (SA) is known to be a product of the phenylpropanoid pathway [38] and to induce resistance in plants. These results reveal that the *MdCAD1* gene may participate in synthesizing defense-related lignins like *Eucalyptus CAD1* [12]. In *Arabidopsis* leaves, SA treatment induced pathogenesis-related (PR)-1, 2, and 5 genes, indicating that SA could be an endogenous signal that evokes the induction of the genes and a systemic acquired resistance (SAR) [36]. Wounding mimics a pathogenic attack and SA could be a signal to induce resistance in plants. The transcription level of *MdCAD1* initially increases with mechanical wounding only when a pathogen attacks the plant and thereafter endogenous SA is produced in the pathogen-induced necrotic lesion [36]. The increased level of SA may strongly induce the expression of *MdCAD1* to catalyze the synthesis of cinnamyl alcohol. The increased lignin content in the attacked tissue will thus be a barrier against any pathogenic invasion. There have been some previous reports that showed the relationship between CAD and plant defense. In experiments on the *R* gene-mediated incompatible interaction between wheat and stem rust fungus (*Puccinia graminis* f sp *tritici*), the application of CAD inhibitors significantly decreased the frequency of lignified host cells and concomitantly allowed an increase in fungal growth [21]. This indicates that the hypersensitive cell death is related to cellular lignification which restricts further fungal growth. The treatment of cell cultures of bean induced CAD transcription with a fungal elicitor [37] and the addition of an elicitor to spruce cell cultures stimulated CAD transcription [11]. The induction of *MdCAD1* expression by wounding and the further induction by SA, yet not by ethylene or jasmonic acid (Fig. 6), suggests that the induction of *MdCAD1* transcripts may follow the SA-dependent pathway of plant defense.

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