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Transcriptional regulation of the cinnamyl alcohol dehydrogenase gene from sweetpotato in response to plant developmental stage and environmental stress

Young-Hwa Kim · Jung Myung Bae · Gyung-Hye Huh

Abstract Cinnamyl alcohol dehydrogenase (CAD) is a key enzyme in the biosynthesis of lignin. We have isolated full length of a cDNA encoding CAD (*IbCAD1*) that was previously identified as the most abundant gene in an EST library of sweetpotato suspension cells. Phylogenetic analysis revealed that IbCAD1 belongs to the family of defense-related CADs. High levels of *IbCAD1* mRNA were found in the roots of sweetpotato, but not in the leaves and petioles. The *IbCAD1* gene transcripts were highly induced by cold, wounding, and reactive oxygen species. Analyses of transcriptional regulation of the *IbCAD1* gene in transgenic tobacco plants carrying the *IbCAD1* promoter–GUS revealed that *IbCAD1* promoter expression was strong in the roots, but barely detectable in the cotyledons. *IbCAD1* promoter activity increased with increasing root age, and strong promoter expression was observed in the lateral root emergence sites and in root tips. Weak GUS expression was observed in lignified tissues of vascular system of mature leaves and stems. *IbCAD1* promoter activity was strongly induced in response to the biotic and abiotic stresses, with the strongest inducer being wounding, and was also induced by salicylic acid (SA) and jasmonic acid (JA) as well as by abscisic acid (ABA) and 6-benzylaminopurine. Taken together, our data suggest that *IbCAD1* can be involved in JA- and SA-mediated wounding response and ABA-mediated cold response, respectively. The *IbCAD1* gene may play a role in the resistance mechanism to biotic and abiotic stresses as well as in tissue-specific developmental lignification.

Keywords Cinnamyl alcohol dehydrogenase · Promoter activity · Biotic and abiotic stresses · Hormone response · Lignification

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Introduction

Lignin is the second most abundant biopolymers in nature after cellulose. It forms an integral part of the secondary cell walls of specialized conducting and supporting tissues of plants, facilitating water transport and providing mechanical strength, respectively (Boudet 2000). The lignification of tissues is also thought to play a role as a defense barrier to limit pathogen invasion (Boerjan et al. 2003; Goujon et al. 2003). The biosynthesis of lignin proceeds via the general phenylpropanoid pathway, which generates a pool of hydroxycinnamyl-CoA esters. These hydroxycinnamyl-CoA esters are then channeled into the lignin-specific pathway to produce monolignols through the action of two enzymes, cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD). CCR catalyzes the conversion of hydroxycinnamyl-CoA esters to their corresponding cinnamaldehydes, and these are subsequently converted to their corresponding alcohols (*p*-coumaryl, coniferyl, and sinapyl alcohol) by CAD (Walter et al. 1988; Goujon et al. 2003).

In angiosperm species, CAD is encoded by a multigene family. Nine *CAD* genes (*AtCAD1* to *AtCAD9*) have been identified in *Arabidopsis* and classified into three groups based on both homology and biochemical properties in vitro (Sibout et al. 2003; Kim et al. 2004, 2007). The first group consists of *AtCAD4* (AY302081) and *AtCAD5* (AY302082). These genes are associated with the highest CAD enzyme activity and homology (74–83% similarity) with respect to the bona fide CADs, such as Eucalyptus *EgCAD2* and rice *OsCAD2*, and act as primary genes in lignin biosynthesis in *Arabidopsis*. *EgCAD2* is the best characterized CAD involved in developmental lignin synthesis (Goffner et al. 1992). The second group of *Arabidopsis* CADs consists of *AtCAD2* (AY302077), *AtCAD3* (AY302078), *AtCAD7* (AY302079) and *AtCAD8* (AY302080). These all have a relatively lower CAD catalytic activity. *AtCAD7* (AY302079) and *AtCAD8* (AY302080) show the highest homology ($\sim$78%) to poplar sinapyl alcohol dehydrogenase (*PtSAD*) (Li et al. 2001). *PtSAD* has been shown to be highly specific for sinapaldehyde substrates and is possibly responsible for syringyl unit deposition in poplar. *AtCAD7* and *AtCAD8* are induced in response to pathogen infection (Kiedrowski et al. 1992). The members of the last group, *AtCAD1* (AY288079), *AtCAD6* (AY302075) and *AtCAD9* (AY302076), lack detectable CAD catalytic activities in vitro, but they are expressed predominantly in vascular (lignin deposition) tissues. The expression of *AtCAD1* was found to be induced by bacterial pathogen (Tronchet et al. 2010). The true biochemical roles of these CAD enzymes still need to be elucidated, particularly so as differences in the lignin composition of tissues in different plant species result from the different activities of several CAD isoenzymes, each with a different specificity.

Detailed knowledge of the role of CADs in relation to the biosynthesis of developmental lignins would contribute extensively to our understanding of the in vivo enzyme activity of CADs and subsequent lignin composition in tissues. In comparison to wild-type *Arabidopsis* plants, the content of lignin in the stem of double mutant *Atcad4/ Atcad5* plants is reduced by 60% and the level of coniferyl and sinapyl alcohol is reduced by 94% (Sibout et al. 2003, 2005). The rice *gold hull and internode2* (*gh2*) mutant, which results from a mutation in the *OsCAD2* gene, has reduced levels of *p*-hydroxyphenol, guaiacyl and syringyl monomers—in almost the same ratio—compared to the wild type (Zhang et al. 2006). Transgenic plants having reduced levels of lignins are more readily attacked by fungi and bacteria, as shown by double mutant *Atcad4/Atcad5* plants, whose disease symptoms are distinctly more severe than those of the wild type (Tronchet et al. 2010). The relationship between CAD and biotic stress has been well-established. The mRNA level of alfafa *MsaCADs* and ryegrass *LpCADs* was observed to increase following

wounding treatments (Brill et al. 1999; Lynch et al. 2002), and the addition of fungal elicitor to bean cell cultures leads to the rapid accumulation of CAD transcripts (Walter et al. 1988). In addition, syringyl lignin was found to be accumulated during the hypersensitive resistance response in wheat (Menden et al. 2007). All of these results indicate that the amount and unit composition of lignin is influenced by both developmental and environmental conditions. However, little information is yet available on the role of CAD in relation to abiotic stress.

We have isolated a cDNA (*IbCAD1*) encoding CAD and the upstream region of the *IbCAD1* gene from sweetpotato and have investigated this *IbCAD1* gene transcript and the activity of its promoter in response to various development stages. To gain an understanding of the regulation mechanism of *IbCAD1* gene by biotic and abiotic stresses, we subjected wild type and transgenic plants to various stresses and then monitored promoter activity and tissue-specific expression. The effect of various plant hormones on *IbCAD1* promoter activity was also examined. We report here the results of these analyses and suggest that the *Ib-CAD1* gene may be involved in lignification induced by both abiotic and biotic stresses and in tissue-specific developmental lignification.

Materials and methods

Plant materials

Sweetpotato plants (*Ipomoea batatas* cv. Yulmi) and transgenic tobacco plants were maintained in pots in a greenhouse at 25°C under a 16/8-h light/dark photoperiod. They were propagated from cuttings taken from the top growing tip and grown for 3 weeks for the stress treatments. For the *Agrobacterium*-mediated transformation procedure, tobacco (*Nicotiana tabacum* cv. Xanthi) seeds were surface sterilized and sown on MS medium (Murashige and Skoog 1962) containing 3% sucrose and 0.8% agar.

Southern blot analysis

Whole cells of sweetpotato cultured in cell suspension were frozen and ground to a fine powder under liquid nitrogen, and genomic DNA was isolated according to the manufacturer's instructions (QIAGEN, Germany). The genomic DNA (10 µg) was then digested with *Eco*RI and *Hin*dIII independently, followed by electrophoretic separation in a 0.8% agarose gel. These restriction enzymes do not have any recognition sequences within the *IbCAD1* cDNA sequence. After a complete denaturation and subsequent renaturation, the gel was blotted onto a positively charged nylon membrane (Hybond-N$^+$, Amersham

Biosciences, UK). Biotin (biotin-14-dCTP, Invitrogen, USA) was used as a probe and labeled by PCR amplification using *IbCAD1* cDNA as a template. The PCR analysis was performed in a 20 µl volume containing 1.25 U *ExTaq* DNA polymerase (Takara, Japan), 2 µl of 10× *Taq* buffer, 4 µl of 5× dNTPs mix (0.25 mM biotin-14-dCTP, 0.25 mM dCTP, 0.5 mM dATP, 0.5 mM dGTP and 0.5 mM dTTP), and 10 pmol of T3 and T7 primers. The PCR cycling conditions consisted of 94°C for 5 min, 30 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, with a final extension step of 7 min at 72°C. The labeled probe was purified using a PCR Purification kit (QIAGEN). The membrane was hybridized at 65°C for 16 h and then washed twice with 2× SSC/1% SDS at room temperature for 10 min, twice with 0.1× SSC/1% SDS at 65°C for 20 min, and twice with 1× SSC at room temperature for 10 min. Hybridized signals were detected using the Southern-Star™ System (Tropix, USA).

Reverse transcription-PCR analysis

Semi-quantitative reverse transcription (RT)-PCR was performed to analyze the expression pattern of *IbCAD1*. Total RNA (2 µg) was utilized for the synthesis of first strand cDNA, and the RT was performed using oligo (dT)$_{20}$ (Invitrogen, USA) as a primer and Superscript™ II reverse transcriptase (Invitrogen). Aliquots consisting of one-tenth of the RT product were used as a template for each of the subsequent PCR amplifications. To amplify the *IbCAD1*, we designed PCR primers for gene-specific amplification in the 5′- and 3′-untranslated region (UTR) of the *IbCAD1* cDNA. Sequences of the forward (F) and reverse (R) primers used to amplify cDNA were: F, 5′-ATCTTGATT GTCTCAATCTA3′, R, 5′-GGACATTATTACATTAC AC-3′. Tubulin was used as the internal control (F, 5′-CAA CTACCAGCCACCAACTGT-3′; R, 5′-CAAGATCCTCA CGAGCTTCAC-3′). PCR amplification was carried out in a 20 µl reaction mixture containing 0.2 mM of each dNTPs, 1 pmol of each forward and reverse primers, and 0.125 U of *Taq* polymerase (NEB, USA), including cDNA products as a template. After an initial denaturation at 94°C for 5 min, amplification was carried out at 94°C for 30 s, 60°C for 1 min, 72°C for 1 min, with a final incubation for 7 min at 72°C. There were 30 and 20 PCR cycles for *IbCAD1* and tubulin, respectively. The amplified PCR products were separated in a 1% agarose gel.

Construction of genomic library and isolation of the *IbCAD1* promoter

The 5′-flanking region of *IbCAD1* was isolated from the genomic DNA of sweetpotato cells grown in suspension culture following the instructions of the Universal GenomeWalker kit (Clontech, USA), which is a simple method for cloning unknown genomic sequences adjacent to a DNA fragment of known sequence. The first step consisted of constructing adaptor-ligated sweetpotato genomic DNA libraries. This was accomplished by completely digesting separated aliquots of sweetpotato genomic DNA with different restriction enzymes, namely, *Nae*I, *Nru*I, *Hpa*I, and *Sca*I. Each batch of digested product was then ligated separately to the GenomeWalker Adaptor. In the primary PCR, the four adaptor-ligated genomic libraries were used as a template. The PCR was performed in 50 µl aliquots, each containing 1 µl of each template, 5 µl of 10× PCR reaction buffer, 4 µl of dNTPs (2.5 mM each), 1 µl of outer adaptor primer (AP1, 5′-GTAATACGACT CACTATAGGGC-3′), 1 µl of gene-specific primer (GSP1, 5′-AAAGGAGAAAGAACTCCAGAGGTGTCC-3′), and 0.5 µl of *ExTaq* polymerase (Takara, Japan). The PCR cycling conditions consisted of seven cycles at 94°C for 25 s and 72°C for 3 min; 32 cycles of 94°C for 25 s and 67°C for 3 min; one final cycle of 67°C for 7 min. The primary PCR products were then diluted and used as a template in a second PCR using a nested adaptor primer (AP2, 5′-ACTATAGGGCACGCGTGGT-3′) and a nested gene-specific primer (GSP2, 5′-CCACAGGCCTTCACT GGGTGCTCGTTT-3′). After the amplification step, the major PCR product was isolated and cloned into pGEM-T Easy vector (Promega, USA) for sequencing.

Construction of the *IbCAD1* promoter–GUS fusion and plant transformation

To generate a binary vector for the transformation of tobacco plants, the *IbCAD1* promoter region was amplified with gene-specific primers (F, 5′-ACGC<u>GTCGAC</u>CTGG TAACAAAACTATTGGAT-3′; R, 5′-CG<u>GGATCC</u>TTTC CTTGTTGCAGGGGGAT-3′). The reverse primers were designed in front of the ATG start codon of the *IbCAD1* gene. The underlined sequences, which are *Sal*I and *Bam*HI sites, were introduced at the end of primers in order to facilitate subcloning. The PCR product was then inserted into the *Sal*I/*Bam*HI sites of pBI101 carrying the GUS reporter gene, and the resulting construct was utilized to transform tobacco by *Agrobacterium tumefaciens* (GV3101) infection (Martinez-Trujillo et al. 2004). The transformants were identified by genomic DNA PCR. Primary transformants were grown to maturity in soil in a greenhouse to produce seeds. The seeds were germinated on MS medium after sterilization in distilled water:chlorox:0.5% Tween 20 (3:2:1). The *IbCAD1* promoter activity in transgenic tobacco was analyzed using T2 plants.

Stress and hormone treatments

Sweetpotato plants were grown in a greenhouse at 25°C for 3 weeks and subjected to several stresses. As cold stress, sweetpotato plants in a pot were exposed to 15°C for 24, 48, and 72 h, respectively. For the methyl viologen (MV) treatment, 50 μM of a solution containing 0.125% Triton X-100 was sprayed onto whole plants and the plants incubated for 6, 12, and 24 h, respectively. For the H_2O_2 treatment, the second and third leaves from the top were removed from plants and incubated in 440 mM hydrogen peroxide (H_2O_2) solution at 25°C for 12 and 24 h, respectively; sterile water was used as a control for the H_2O_2 treatment. Wounding stress was performed according to Sasaki et al. (2002). Fully expanded leaves were detached from the plant and immediately cut into pieces with a razor blade. After the midrib had been removed, six pieces from each leaf, from six different leaves ($n = 36$ leaf pieces), were placed on filter paper moistened with distilled water and incubated for 1, 4, 8, 12, and 24 h, respectively, at 25°C under continuous illumination. Untreated and stress-treated plant samples were collected, frozen in liquid nitrogen, and stored at −70°C for RNA purification.

Four-day-old seedlings were used for the stress treatments of transgenic tobacco plants containing the *IbCAD1* promoter. For the cold stress, 4-day-old seedlings in MS medium were exposed to 4°C for 48 h. For the H_2O_2 stress, transgenic seedlings were transferred to MS medium containing 0.1 M H_2O_2 at 25°C for 24 h. For the wounding treatment, detached leaves and stems of mature plants were cut into slices and incubated for 24 h at 25°C. For the hormone treatments, 4-day-old tobacco seedlings were transferred to MS medium containing 100 μM abscisic acid (ABA), 1 mM salicylic acid (SA), or 50 μM jasmonic acid (JA) for 24 h or to MS medium containing 10 μM 6-benzylaminopurine (BA) for 72 h. Untreated and hormone-treated plant samples were collected, and crude extracts were prepared for the analysis of GUS activity.

Fluorometric and histochemical GUS assay

The crude plant extract was prepared and used for the quantitative fluorometric assays of GUS activity according to Jefferson et al. (1987) using 4-methylumbelliferyl glucuronide (MUG; Sigma, USA). GUS values were expressed as nmoles of MUG per minute per milligram protein. Protein concentrations were determined by the method of Bradford (1976). The histochemical analysis of GUS activity was performed by incubating plant tissues with 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc,

Gold Biotechnology, USA) for 16–24 h at 37°C (Jefferson et al. 1987). Chlorophyll was removed from the tissues by immersion in 70% ethanol. The samples were observed and photographed under a SZ61 zoom stereo microscope (Olympus, Japan) equipped with digital camera system (Canon, USA).

Results

Isolation and sequence analysis of *IbCAD1* cDNA

In an earlier study, we constructed an expressed sequence tag (EST) library from sweetpotato cells grown in suspension culture and identified highly abundant and cell culture-specific genes, of which the most abundant and unique gene was *CAD* (Kim et al. 2006). Here, we have isolated and characterized the full length of the cDNA clone (*IbCAD1*, GU380306). The *IbCAD1* cDNA consisted of 1,355 nucleotides, and the largest open reading frame (ORF) encoded a polypeptide of 357 amino acids. It encoded a 38.5-kDa protein with an isoelectric point of 6.26. The putative polyadenylation signal AATAAT was located 94 bases upstream from the start of the poly(A)$^+$ tail. We found no clear targeting domains within the sequence, suggesting a cytoplasmic location of the protein (WoLF PSORT Prediction; probability cytoplasm 12.0, chloroplast 1.0).

The deduced protein sequence of *IbCAD1* was highly homologous to that of other plant *CADs* identified to date, including strawberry *FxaCAD* (AF320110, 73.5% identity), parsley *ELI-3* (X67817, 71.2% identity), poplar *PtSAD* (AF273256, 70.2% identity), alfafa *MsaCAD1* (AF083332, 67.8% identity), Arabidopsis *AtCAD8* (AY302080, 67.6% identity) and *AtCAD7* (AY302079, 66.9% identity). All of these CADs clustered with defense-related proteins compared to classical development-related lignifications CADs. The sequence alignment indicated that the IbCAD1 protein possessed all of the structural characteristics of CADs, including the catalytic Zn1 binding sequence $[GHE(X)_2G(X)_5G(X)_2V]$, the Zn2 binding sequence $[GD(X)_{9,10}C(X)_2C(X)_2C(X)_7C]$, and the coenzyme (NADPH) binding domain $GXG(X)_2G$ (McKie et al. 1993) (Fig. 1). The Zn1 and Zn2 binding sequences in IbCAD1 were located at amino acid residues 72–86 and 92–118, respectively. A coenzyme binding site located at residues 188–193 and structural zinc ion coordinating residues, Cys 104, Cys 107, Cys 110, and Cys 118, have also been identified (Vallee and Aulds 1990; Grima-Pettenati et al. 1993). His 73 and Cys 163 may be involved as a catalytic zinc ion coordinating residue and thus play an

Fig. 1 Alignment of amino acid sequences of IbCAD1 and other plant CADs. Conserved important regions identified previously are marked as follows: *inverted filled triangle* denotes catalytic zinc ion coordinating residue, *filled circle* denotes structural Zn ion coordinating residue, *star* denotes key residues for substrate specificity. Locations of the Zn1, Zn2, and NADPH binding domains are shown in *boxes*. The alignment was performed with the ClustalX and GENEDOC software programs

important role in active zinc binding (Vallee and Aulds 1990; Knight et al. 1992). Gly 299 is a key determinant residue of substrate specificity (Bomati and Noel 2005).

To estimate the genomic constitution of the *IbCAD1* gene, we analyzed genomic DNA isolated from sweetpotato cells grown in suspension culture by Southern blot analysis (Fig. 2). Three major bands and several minor ones were identified on the Southern blots, indicating that *IbCAD1* belongs to a member of a multigenic family. This was an expected result as, with the exception of gymnosperm species, all of the *CAD* genes isolated from plant species to date have been present as members of a multigene family (MacKay et al. 1995).

Effects of environmental stresses on the transcription levels of the *IbCAD1* gene

In an earlier publication, we reported that *IbCAD1* was highly expressed in the log and stationary phases of sweetpotato suspension-cultured cells (Kim et al. 2006). Here, we investigated the tissue specificity and stress response of *IbCAD1* in sweetpotato plants using a comparative quantitative RT-PCR analysis. The *IbCAD1* gene was most strongly expressed in the roots, less actively in the petioles, and only rarely expressed in the leaves of sweetpotato plants (Fig. 3a). To study the response to environmental stresses, we subjected 3-week-old sweetpotato

Fig. 2 Southern hybridization analysis of the *IbCAD1* gene. Genomic DNA (10 μg) purified from sweetpotato suspension cells was digested with *Eco*RI and *Hin*dIII, respectively, and then separated in a 0.8% agarose gel. The full length of the *IbCAD1* cDNA was used as a probe. Migration position of the DNA size standards are shown at *left*
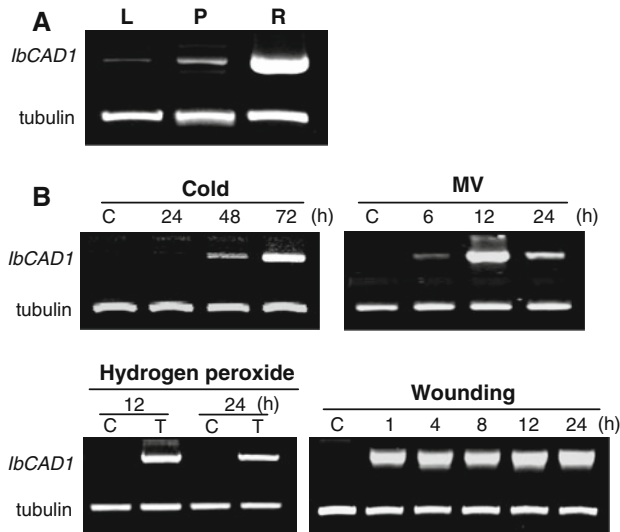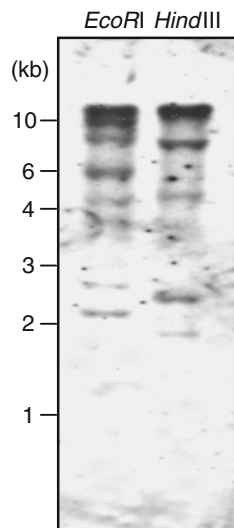

Fig. 3 Expression of the *IbCAD1* gene in sweetpotato. **a** Tissue-specific expression of *IbCAD1*. Total RNA was isolated from leaves (*L*), petioles (*P*) and roots (*R*) of sweetpotato, and RT-PCR was performed. Tubulin was used as an internal control. **b** *IbCAD1* expression in response to various stresses. Sweetpotato plants were subjected to cold, methyl viologen (*MV*), wounding, and H_2O_2 treatments, respectively. The cold treatment consisted of exposing the plants to 15°C. For the MV treatment, a 50 μM solution containing 0.125% Triton X-100 was sprayed onto whole plants. The wounding treatment consisted of cutting sweetpotato leaves into small pieces and incubating these at 25°C. For the H_2O_2 treatment, two or three leaves from the top of the growing tip were removed from sweetpotato plants and incubated in a 440 mM H_2O_2 solution. RNA was isolated from leaves and subjected to RT-PCR analysis. Tubulin was used as an internal control

plants to cold, MV of a superoxide-generating herbicide, H_2O_2, and wounding and then analyzed the time-course accumulations of the *IbCAD1* transcript in sweetpotato leaves. The *IbCAD1* transcript was highly induced at 72 and 12 h after the start of the cold stress and MV treatments,

respectively, and at 12 and 24 h after the start of the H_2O_2 treatment. The response of the *IbCAD1* gene to wounding was very fast (1 h after wounding), and the transcription levels remained high until 24 h post-treatment initiation (Fig. 3b). Taken together, these results show a root-specific expression of the *IbCAD1* gene and that its transcription can be highly induced by various stress treatments.

Nucleotide sequence analysis of the *IbCAD1* promoter

To isolate a 5′ flanking region of the *IbCAD1* gene, we designed specific primers in the ORF region of the *IbCAD1* cDNA and performed PCR-based genome walking. A 1,260-bp product was obtained, and the nucleotide sequence of the product was compared with that of the *IbCAD1* cDNA. This PCR product encoded 16 amino acids, including an ATG start codon, and corresponded exactly with the 5′-UTR and N-terminal region of the *IbCAD1* cDNA. The nucleotide site that corresponded in sequence to the end of 5′-UTR of the *IbCAD1* cDNA was designated as +1. A putative TATA box (−65 to −58) and CAAT box (−1043 to −1040, −831 to −828, −138 to −135, and −27 to −24), which act as general signals for eukaryotic gene expression, were observed in *IbCAD1* promoter.

A promoter motif search carried out using the software program PLACE to define putative *cis*-elements (Higo et al. 1999) (Fig. 4) revealed that the *IbCAD1* promoter contained putative regulatory elements corresponding to known *cis*-elements of eukaryotic genes. One of the most abundant motifs detected was the nuclear response regulator (ARR) binding site (NGATT), which is involved in cytokinin-mediated regulation during root differentiation (Yokoyama et al. 2007). Many DOF (AAAG) binding sites were also found. DOF has been found to influence phenylpropanoid metabolism in an environmental and tissue-specific manner (Skirycz et al. 2007). ACGT (ACGT) and GT-1 (GAAAAA/GRWAAW) motifs were also present, both of which are required for gene expression during dehydration stress (Simpson et al. 2003). The stress-responsive MYB and MYC elements (Abe et al. 1997) and the W box (TGACY) and WRKY binding sites (TGAC), all of which are involved in pathogen and wounding, were also present. These results indicate that the transcriptional regulation of *IbCAD1* gene may be involved in root development and in the response to various stresses.

Spatial and temporal regulation of the *IbCAD1* gene during development

Transgenic tobacco plants harboring the GUS reporter gene fused to the *IbCAD1* promoter were constructed and three independent transgenic lines analyzed. *IbCAD1* promoter
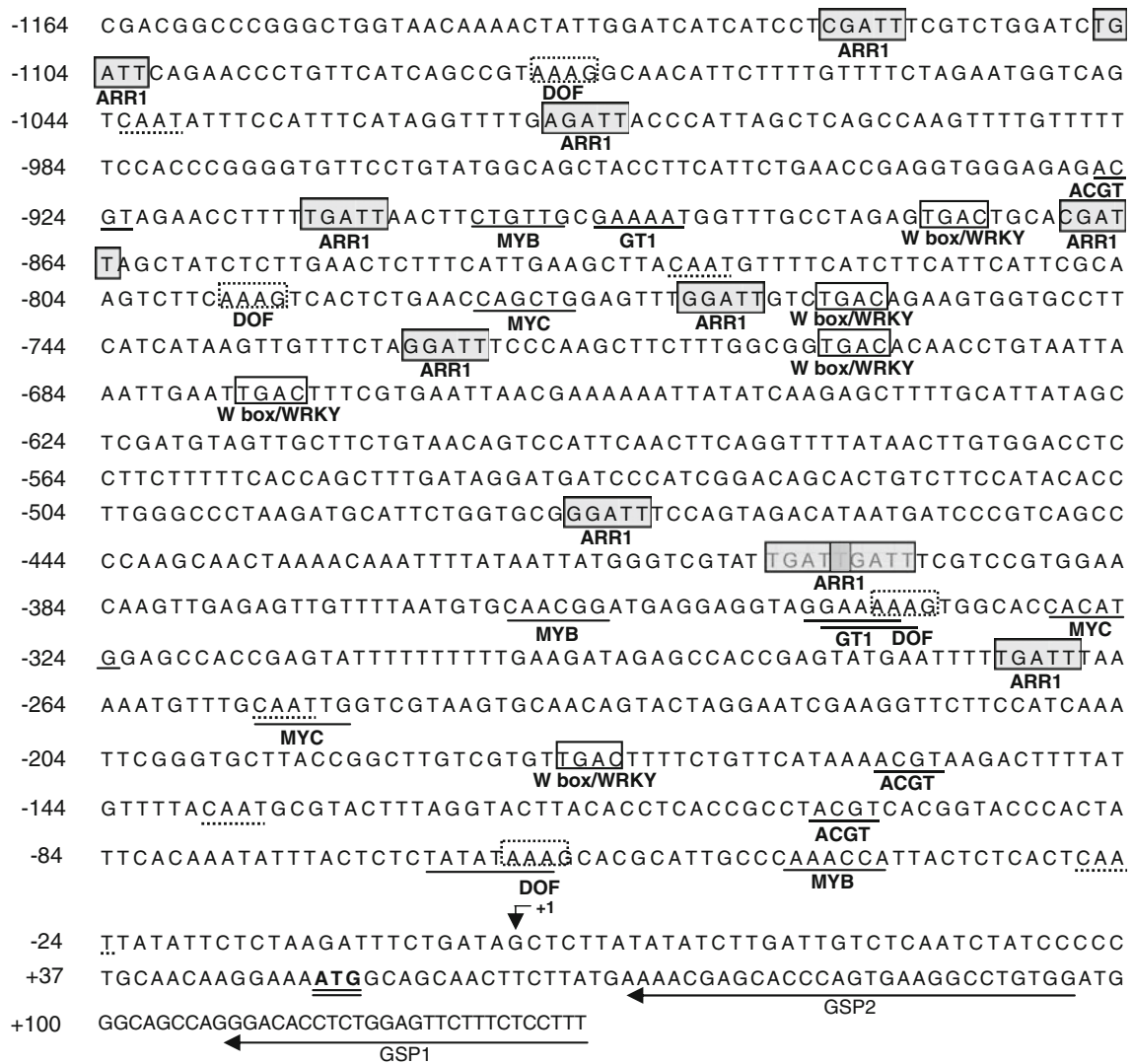
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-1164   CGACGGCCCGGGCTGGTAACAAAACTATTGGATCATCATCCTCGATTTCGTCTGGATCTG
                                                   ARR1
-1104   ATTCAGAACCCTGTTCATCAGCCGTAAAGGCAACATTCTTTTGTTTTCTAGAATGGTCAG
        ARR1                         DOF
-1044   TCAATATTTCCATTTCATAGGTTTTGAGATTACCCATTAGCTCAGCCAAGTTTTGTTTTT
                                      ARR1
-984    TCCACCCGGGGTGTTCCTGTATGGCAGCTACCTTCATTCTGAACCGAGGTGGGAGAGAC
                                                                 ACGT
-924    GTAGAACCTTTTTGATTAACTTCTGTTGCGAAAATGGTTTGCCTAGAGTGACTGCACGAT
                 ARR1        MYB      GT1             W box/WRKY   ARR1
-864    TAGCTATCTCTTGAACTCTTTCATTGAAGCTTACAATGTTTTCATCTTCATTCATTCGCA
-804    AGTCTTCAAAGTCACTCTGAACCAGCTGGAGTTTGGATTGTCTGACAGAAGTGGTGCCTT
              DOF                MYC        ARR1   W box/WRKY
-744    CATCATAAGTTGTTTCTAGGATTTCCCAAGCTTCTTTGGCGGTGACACAACCTGTAATTA
                              ARR1                   W box/WRKY
-684    AATTGAATTGACTTTCGTGAATTAACGAAAAAATTATATCAAGAGCTTTTGCATTATAGC
                 W box/WRKY
-624    TCGATGTAGTTGCTTCTGTAACAGTCCATTCAACTTCAGGTTTTATAACTTGTGGACCTC
-564    CTTCTTTTTCACCAGCTTTGATAGGATGATCCCATCGGACAGCACTGTCTTCCATACACC
-504    TTGGGCCCTAAGATGCATTCTGGTGCGGGATTTCCAGTAGACATAATGATCCCGTCAGCC
                                     ARR1
-444    CCAAGCAACTAAAACAAATTTTATAATTATGGGTCGTATTGATTGATTTCGTCCGTGGAA
                                              ARR1
-384    CAAGTTGAGAGTTGTTTTAATGTGCAACGGATGAGGAGGTAGGAAAAGTGGCACCACAT
                                 MYB                 GT1 DOF        MYC
-324    GGAGCCACCGAGTATTTTTTTTTTGAAGATAGAGCCACCGAGTATGAATTTTTGATTTAA
                                                              ARR1
-264    AAATGTTTGCAATTGGTCGTAAGTGCAACAGTACTAGGAATCGAAGGTTCTTCCATCAAA
                 MYC
-204    TTCGGGTGCTTACCGGCTTGTCGTGTTGACTTTTCTGTTCATAAAACGTAAGACTTTTAT
                                W box/WRKY              ACGT
-144    GTTTTACAATGCGTACTTTAGGTACTTACACCTCACCGCCTACGTCACGGTACCCACTA
              MYC                              ACGT
-84     TTCACAAATATTTACTCTCTATATAAAGCACGCATTGCCCAAACCATTACTCTCACTCAA
                             DOF                  MYB                MYC
                                    ┌─ +1
-24     TTATATTCTCTAAGATTTCTGATAGCTCTTATATATCTTGATTGTCTCAATCTATCCCCC
+37     TGCAACAAGGAAAATGGCAGCAACTTCTTATGAAAACGAGCACCCAGTGAAGGCCTGTGGATG
                         ←─────────────────────────────────
                                                  GSP2
+100    GGCAGCCAGGGACACCTCTGGAGTTCTTTCTCCTTT
                ←───────────────────
                       GSP1
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Fig. 4 Nucleotide sequence of the promoter region of *IbCAD1* gene. The nucleotide site that corresponded in sequence to the end of 5′-UTR of the *IbCAD1* cDNA was designated as +1. The TATA box (TATATAAA) is *underlined* and the start codon is *double underlined*. The *dotted underline* indicates the CAAT box. Several regulatory *cis*-elements are represented. GSP1 and GSP2 were primers used for PCR-based genome walking

activity in various tissues during different growth stages was investigated in in vitro-grown seedlings and in mature plants grown in soil using the quantitative GUS assay (Fig. 5A) and by histochemical GUS staining (Fig. 5B). These tests showed that the activity of the *IbCAD1* promoter did not change significantly in the cotyledons during seedling development, while that in the roots increased gradually with root maturation (Fig. 5A). Promoter activity was much higher in the roots than in the cotyledons. The results of the histochemical assay revealed that GUS staining was absent in the cotyledons but that it became stronger in the root tips with advancing seedling development (Fig. 5Ba–d). Strong GUS staining was observed around the sites where lateral roots were initiated from the primary roots (Fig. 5Be).

IbCAD1 promoter activity was investigated in the leaves, petioles, stems, and roots of mature plants grown in soil. It was relatively low in 4-week-old mature plants, except for the roots. GUS activity increased only slightly in the leaves, petioles, and stems with increasing plant age. In contrast, there was a dramatic increase in GUS activity in the roots as the plant matured (Fig. 5A), with GUS activity in the roots of 8-week-old plant roots being 2.9- to 6.8-fold higher than that of 4-week-old plant roots. The lateral root initiation site was strongly stained in 4-week-old plants (Fig. 5f–g), while much stronger staining was observed in lateral roots and primary roots of 8-week-old plants (Fig. 5Bh). Weak GUS staining was observed in the fibers, phloem, and xylem of the stem and in the midrib and veins of leaves (Fig. 5Bi–k). In the reproductive tissues, the
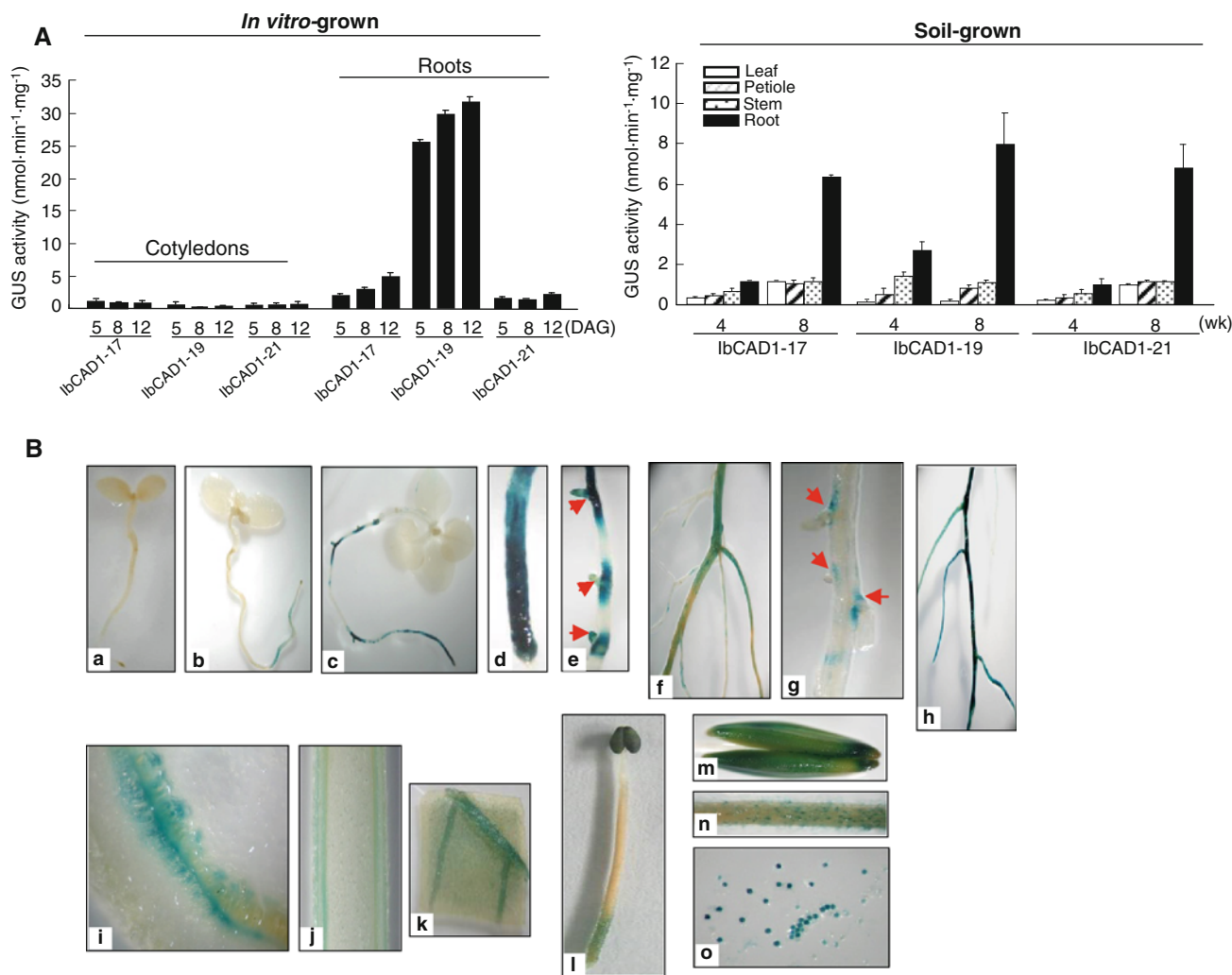
Fig. 5 GUS activity and histochemical localization of the *IbCAD1* promoter at different developmental stages of transgenic tobacco plants. **A** Transgenic tobacco seedlings were germinated on MS medium and collected at 5, 8, and 12 days after germination (*DAG*). GUS activity was measured in the cotyledons and roots, respectively. The data represent the average value ± SD from independent experiments (*n* = 40). Fully developed leaves, petioles, upper stems, and root materials were collected from 4- to 8-week-old mature plants grown in soil and analyzed for GUS activity. The data represent the average value ± SD from independent experiments (*n* = 6). **B** (*a*) Seedling with fully developed cotyledon at 5 DAG; (*b*) seedling with fully matured cotyledons at 8 DAG; (*c*) seedling with expanding secondary leaves and initiating lateral roots at 12 DAG; (*d*) root tip of seedling at 12 DAG; (*e*) initiation regions of lateral roots at 12 DAG; (*f*, *g*) root of 4-week-old plant; (*h*) root of 8-week-old plant; (*i*) transverse section of stem; (*j*) longitudinal section of stem; (*k*) leaf of 4-week-old plant; (*l*) stigma; (*m*) anther; (*n*) filament; (*o*) pollen grains

anther, trichomes in the filament, and pollen grains all showed strong GUS staining, while the stigma showed weak GUS staining (Fig. 5B*l–o*). Based on these results, the *IbCAD1* promoter directs strong expression in the meristematic tissue of the root, but weak expression in the lignified tissue of the leaves and stem.

Up-regulation of *IbCAD1* gene expression by both biotic and abiotic stresses

The *IbCAD1* promoter has Myb and Myc responsive elements that respond to dehydration stress as well as the W

box and WRKY recognition sequence of the wounding responsive element. To investigate the transcriptional regulation of the *IbCAD1* gene in response to various stresses, we examined the expression of the GUS gene driven by the *IbCAD1* promoter under conditions of cold (abiotic stress), wounding (biotic stress), and H_2O_2 (reactive oxygen species, ROS). The production of ROS is a common signal which triggers the downstream responses of plants to biotic and abiotic stresses. The cold treatment (4°C) induced an increase in promoter activity of the cotyledons (2.2- to 13.2-fold) and roots (1.2- to 3.8-fold) compared with control plants (Fig. 6A). Although GUS activity was higher
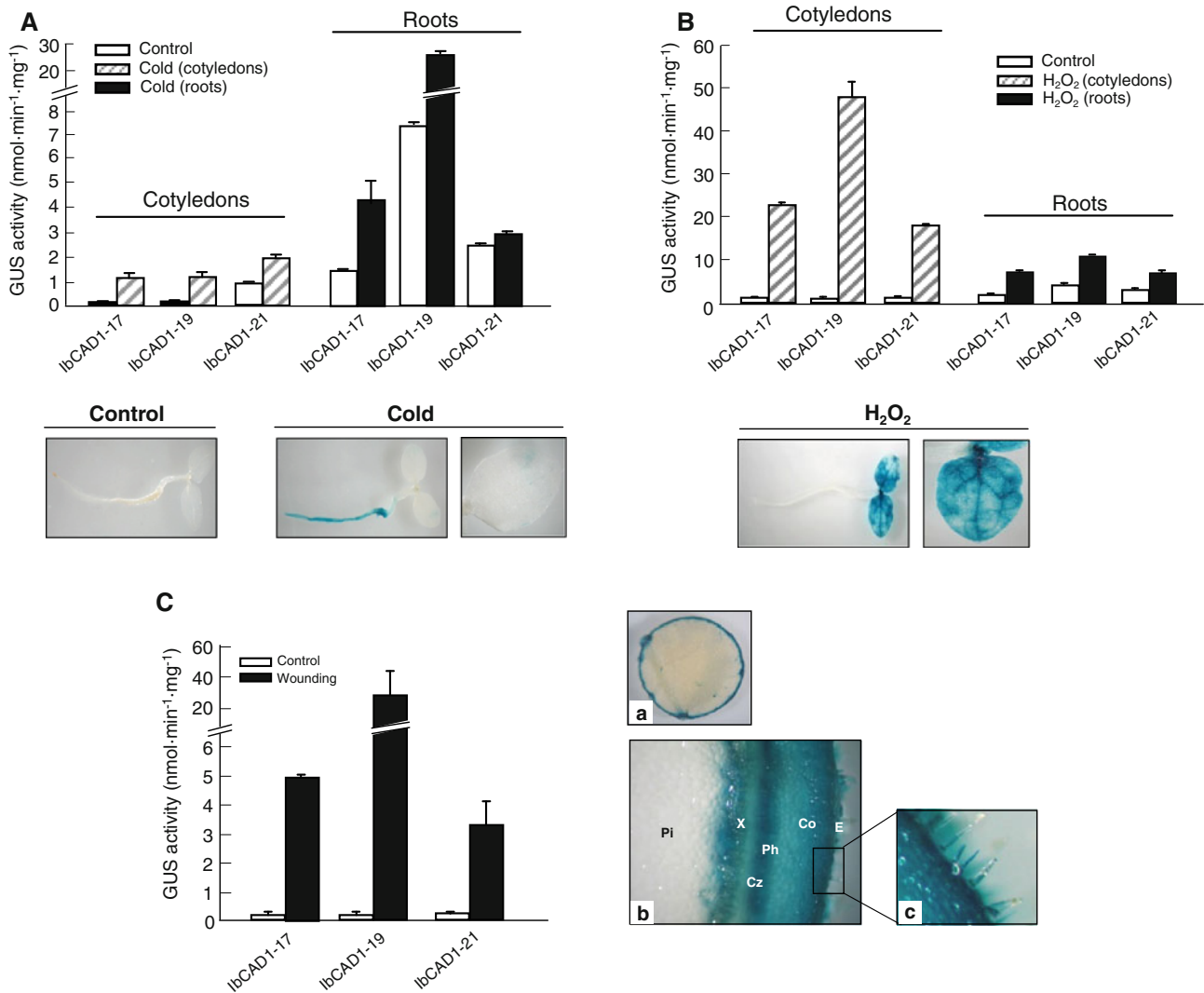
Fig. 6 *IbCAD1* promoter activity in transgenic tobacco plants following treatment with cold, H_2O_2, and wounding stresses. Four-day-old seedlings were exposed to 4°C for 48 h in the cold treatment (**A**) or transferred to MS medium containing 0.1 M H_2O_2 for 24 h (**B**). Cotyledons and roots of each transgenic line were collected separately. After the respective stress treatment, the tissues were homogenized and measured for GUS activity. The data represent the average ± SD from independent experiments ($n = 40$). **C** For the wounding stress, the leaves of tobacco plantlets were injured by punching and incubated on moistened filter paper. After 24 h, the tissues were homogenized and the GUS activity measured. The data represent the average ± SD of six transgenic plants per line. For the histochemical analysis after the wounding stress, the leaves and stems of transgenic plants were cut and placed on moistened filter paper for 30 and 4 h, respectively, and then stained. (*a*) Wounded leaves; (*b*) wounded stem (hand-sectioned); (*c*) trichomes of stem. *Pi* Pith, *X* xylem, *Cz* cambial zone, *Ph* phloem, *Co* cortex, *E* epidermis

in the cotyledons than in the roots following the cold treatment, the GUS activity value in the cotyledons was below the detection limit of the analysis for GUS staining. Thus, following the cold stress treatment, strong GUS staining was only observed in the roots. When seedlings were treated with H_2O_2, *IbCAD1* promoter activity was highly induced compared to control plants—very strongly in the cotyledons (about 15.3- to 47.4-fold) and significantly less in the roots (2.5- to 4.2-fold) (Fig. 6B). Histochemical analysis showed strong GUS staining in cotyledons. These results show that the *IbCAD1* promoter was highly activated under these stress conditions, with the induction of promoter activity being much higher in the cotyledons than in the roots.

To provoke a wounding response, we wounded detached leaves and stems from 6-week-old mature plants grown in soil by punching or cutting and then incubated the leaf pieces on moistened filter paper. Relative to intact tissues, the GUS activity directed by the *IbCAD1* promoter was dramatically increased (15.2- to 174.1-fold) in wounded

tissues (Fig. 6C). As shown in Fig. 6C*a*, the wounding response was highly localized. Strong GUS staining was also observed at wounded site in the stem, and a histochemical study of cross-sections of the stem revealed that the *IbCAD1* promoter was strongly expressed in the xylem, phloem, cortex, and epidermis as well as in the trichomes (Fig. 6C*b, c*). Taken together, these results show that the *IbCAD1* promoter was highly activated by both biotic and abiotic stresses and that the stress response was tissue-specific.

Effects of hormones on the *IbCAD1* promoter activity

The presence of several hormone-responsive *cis*-regulatory elements suggested the possibility that *IbCAD1* gene expression may be controlled by hormones. Many wound-induced genes have been reported to be JA-inducible (Sasaki et al. 2002). To determine whether the *IbCAD1* gene mediates hormone-responsive expression, we investigated *IbCAD1* promoter activity in 4-day-old transgenic tobacco seedlings treated with ABA, BA, JA and SA by measuring the levels of GUS activity (Fig. 7). Following the ABA and BA treatments, *IbCAD1* promoter activity increased by approximately 1.1- to 2.6-fold and 1.1- to 2.5-fold,

respectively (Fig. 7a, b); in comparison, treatments with SA and JA separately resulted in a dramatic increase in *IbCAD1* promoter activity, 9.1- to 15.4-fold and 6.4- to 8.5-fold, respectively (Fig. 7c, d). These results clearly demonstrate that the expression of the *IbCAD1* gene is induced by various hormones. Among them, JA and SA are the strongest inducer of *IbCAD1* gene at the seedling stage.

Discussion

Cinnamyl alcohol dehydrogenase (EC 1.1.1.195) is the last enzyme in the lignin biosynthetic pathway, and it mediates the reduction of coniferaldehyde, *p*-coumaraldehyde, and sinapaldehyde into guaiacyl, *p*-coumaryl, and syringyl monolignols, respectively. Lignification is a tightly regulated and dynamic process subject to modulation at different levels during normal development and in response to different stresses (Boudet 2000). In this study, we isolated the CAD cDNA (*IbCAD1*) from sweetpotato, which can be phylogenetically categorized as belonging to the family of defense-related CADs. The Gly 299 residue in IbCAD1 is a key determinant of substrate specificity and is a sinapaldehyde-specific residue according to Bomati and Noel

(2005). These researchers reported that the Gly residue at the base of the active site of PtSAD and FxaCAD is responsible for the high specificity of these proteins for the substrate sinapaldehyde. PtSAD is required for the biosynthesis of syringyl lignin (Li et al. 2001). Classical CAD enzymes involved in developmental lignification, such as AtCAD4, AtCAD5, and EgCAD2, have Phe instead of Gly in the active site for substrate specificity. AtCAD5 is able to use all substrates effectively, while AtCAD4 shows a very poor affinity for sinapaldehyde (Kim et al. 2004). Thus, IbCAD1 may have high affinity for the substrate sinapaldehyde based on its structure and, consequently, be involved in the defense-related process.

Angiosperm CAD is encoded by a multigenic family consisting of members thought to have distinct roles, while gymnosperm CAD is encoded by a single gene (MacKay et al. 1995; Zinser et al. 1998). Different CAD genes in angiosperms may be expressed in different tissues or at different stages during growth and development (Kim et al. 2007; Barakat et al. 2009) in response to the type of lignin required. Several authors have suggested that the chemical composition of defense lignin is different to that of developmental lignin. For example, syringyl lignin is accumulated in the plant cell wall during the hypersensitive resistance response in wheat (Menden et al. 2007) and sinapaldehyde-specific CAD activity is activated in *F. oxysporum*-treated flax cells (Hano et al. 2006). While much is known on how CAD is induced by biotic stress, such as fungal elicitor (Dixon and Paiva 1995; Brill et al. 1999), relatively little information is available on the role of CAD in relation to lignification under conditions of abiotic stress. We have investigated the transcriptional regulation of the *IbCAD1* gene in response to various stresses. RT-PCR analysis shows that *IbCAD1* gene expression in sweetpotato was strongly induced by both abiotic stress (cold) and biotic stress (wounding), including ROS (MV and H_2O_2). ROS is a common signal that acts as a trigger of the downstream stress response. Fujita et al. (2006) reported that ROS may mediate crosstalk between biotic and abiotic stress-responsive gene-expression networks. Our RT-PCR results are consistent with those of our promoter analysis of *IbCAD1*. The activity of the *IbCAD1* promoter was induced by the cold and H_2O_2 treatments in this study, but the stress response was quite different in different tissues; for example, at the seedling stage, the cotyledons were more sensitive than the roots to *IbCAD1* promoter induction. Among the stressors tested, wounding was found to be the strongest inducer of *IbCAD1*, and the wounding response was highly localized. These data suggest that the expression of the *IbCAD1* gene is regulated in a tissue-specific manner under conditions of biotic and abiotic stresses.

Hormone signaling pathways regulated by ABA, SA, JA, and ethylene as well as the ROS signaling pathway all play key roles in the crosstalk between biotic and abiotic stress responses (Fujita et al. 2006). ABA is extensively involved in the plant's response to abiotic stresses, such as drought, low temperature and osmotic stress, and also regulates a variety of growth and developmental processes. In contrast, SA and JA play central roles in biotic stress signaling following pathogen infection and wounding. We identified several hormone-responsive *cis*-regulatory elements in the *IbCAD1* promoter region, such as ABRE (ABA) (Yamaguchi-Shinozaki and Shinozaki 2005), ARR binding site (cytokinin) (Yokoyama et al. 2007), as-1 element (auxin, SA) (Niggeweg et al. 2000), MYB responsive element, MYC responsive element (Yamaguchi-Shinozaki and Shinozaki 2005), and GARE (gibberellin) (Sutoh and Yamaguchi 2003). AtMYC2, which was identified as an activator involved in the ABA-mediated drought stress signaling pathway, upregulates the expression of genes that are involved in the JA-mediated wounding response stress signaling pathway (Abe et al. 2003; Boter et al. 2004; Lorenzo et al. 2004). Transgenic plants that overexpress both AtMYC2 and AtMYB2 show a greater sensitivity to ABA and enhanced osmotic stress tolerance (Abe et al. 2003). When we tested the GUS activity driven by the *IbCAD1* promoter, we found that *IbCAD1* promoter activity was enhanced when the plants were treated with various plant hormones, being most strongly induced by JA and SA, respectively. The *IbCAD1* promoter was also induced by ABA and cytokinin (BA), but to a lesser degree. Taken together, these results indicate that *IbCAD1* may function through a JA- and SA-mediated wounding response and an ABA-mediated cold response.

The expression of the *IbCAD1* gene was regulated during growth and development in a tissue-specific manner. Our histochemical analysis showed that weak GUS expression was observed in the xylem, phloem, and fiber of stems, and in the midrib and veins of mature leaves, which are lignified tissues of the plant vascular system. However, the *IbCAD1* gene was strongly expressed in the area of the initiation sites for lateral roots developing from primary roots as well as in root tips—but not in the vascular cylinder of lignified root tissue. It has been reported that there is no lignification in root meristem cells, which is primarily involved in the constant progression of cell division (de Obeso et al. 2003). GUS expression was also strong in anther and pollen. We have previously shown that *IbCAD1* is the most abundant gene in the EST library of cells grown in suspension culture and that it is highly expressed at the actively dividing-log phase. In light of these results, the expression of the *IbCAD1* promoter indicates a new role of CAD protein in lateral root formation, cell-wall synthesis, and the

promotion of cell division that is unrelated to constitutive lignin formation. We suggest that IbCAD1 may also be involved in lignifications induced by both abiotic and abiotic stresses and in tissue-specific developmental lignification. Further analysis of the *IbCAD1* gene will provide an insight into the signaling pathway involved in the regulation of biotic and abiotic stresses in lignin biosynthesis as well as into the role of CAD protein during cell division.

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