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고려인삼으로부터 Cinnamyl Alcohol Dehydrogenase 유전자의 분리 및 특성

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Molecular Cloning and Characterization of the Gene Encoding Cinnamyl Alcohol Dehydrogenase in *Panax ginseng* C.A. Meyer

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ABSTRACT : Cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.95), catalyzes the reduction of hydroxycinnamaldehydes to give hydroxycinnamyl alcohols, or "monolignols," the monomeric precursors of lignin. Lignins are important components of cell walls and lignified secondary cell walls play crucial roles in long distance transport of water and nutrients during plant growth and development and in plant defense against biotic and abiotic stresses. Here a cDNA clone containing a CAD gene, named as *PgCAD*, was isolated from a commercial medicinal plant *Panax ginseng*. *PgCAD* is predicted to encode a precursor protein of 177 amino acid residues, and its sequence shares high homology with a number of other plant CADS. The expression of *PgCAD* in adventitious roots and hairy roots of *P. ginseng* was analyzed using reverse transcriptase (RT)-PCR under various abiotic stresses such as salt, salicylic acid, wounding and chilling treatment that triggered a significant induction of *PgCAD* at different time points within 2-48 h post-treatment. This study revealed that *PgCAD* may help the plants to survive against various abiotic stresses.

Key Words **:** Abiotic Stress, *Panax ginseng*, Cinnamyl Alcohol Dehydrogenase, Salicylic Acid, Semi-quantitative RT-PCR

INTRODUCTION

Cinnamyl alcohol dehydrogenase (CAD; E.C. 1.1.1.195) discovered by Zenk and coworkers in 1973 (Kim *et al.*, 2004), CAD are zinc dependent dehydrogenases and are among the least studied of the alcohol dehydrogenase enzymes. The function of CADs in plants has been well characterized, where they have been shown to catalyze the reversible conversion of *p*-hydroxycinnamaldehydes to their corresponding alcohols leading to lignin biosynthesis. CAD was one of the first enzymes studied in the lignin synthesis pathway (Wyrambik and Grisebach, 1975). *CAD* cDNAs have been isolated in different plant species (Dixon *et al.*, 2001).The discovery of isozymes in *Eucalyptus gunii* (Grima- Pettenati *et al.*, 1993), *Medicago sativa* (Brill *et al.*, 1999), and *Populus tremuloides* (Li *et al.*, 2001) with different affinities for various substrates has lead to the hypothesis that multiple substrate specificities were related to various physiological roles. In parallel, expression of *CAD* cDNAs in *Escherichia coli* or yeast (*Saccharomyces cerevisiae*) was carried out to determine substrate specificity of some cloned *CAD* cDNA, which shared high sequence similarity to known CAD proteins. However, in most cases, these experiments led to conflicting results. (Sibout *et al.*, 2003).

CAD is a dimeric enzyme and in Arabidopsis is encoded by a nine-member gene family (Tavares *et al.*, 2000; Sibout *et al.*, 2003). Analysis of the nine Arabidopsis CADs at the amino acid level revealed a diversified small family with highly conserved clusters. Only 26% of the amino acids are conserved on an overall total length of 383. However, some *At*CADs are rather closely related, such as *At*CAD-B1/ *At*CAD-B2 (85% identity), *AtCAD-C*/*AtCAD-D* (75% identity), and *At*CAD-E /*At*CAD-F (98% identity). In this family,

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*At*CAD-G is the most distant protein when compared with the others and shares less than 50% identity with the closest groups. When CADs previously identified and studied in other plant species were taken into consideration, phylogenetic analysis based on amino acid sequence comparison showed that Arabidopsis CADs are divided into four subfamilies. Among the nine CAD of *Arabidopsis, AtCAD-C* and *AtCAD-D* are likely to be involved in lignin biosynthesis (Sibout *et al*., 2003).

CAD enzyme activity or mRNA level is increased in response to fungal elicitor, ozone, wind-induced mechanical stimulation and wounding (Grand *et al.*, 1987). Studies of brown-midrib mutants of sorghum have shown that the activity of CAD is significantly reduced (Bucholtz *et al.*, 1980). Recently, it has been shown that the brown-midrib mutant *bm1* of maize has decreased CAD activity associated with a 20% reduction in lignin content (Halpin *et al.*, 1998). A loblolly pine mutant has also been identified (*cad-n1*) that has decreased CAD activity and altered lignin composition (MacKay *et al.*, 1997). In tobacco, lucerne and poplar it has been demonstrated that antisense expression of a *cad* cDNA results in modified lignin composition, making the lignin more accessible to chemical degradation (Baucher *et al.*, 1998). These observations indicate that lignin composition can be altered by the manipulation of *cad* gene expression. Till now there was no data on CAD in *Panax ginseng*. Thus, isolation and characterization of *CAD* from *P. ginseng* are essential for the further understanding of molecular biological mechanisms to protect the plants either by classical plant breeding or transformation-mediated genetic modification. Here we focused on the isolation of *PgCAD* and investigated its expression profile against various abiotic stresses.

MATERIALS AND METHODS

1. Plant Materials

Panax ginseng adventitious roots and hairy roots (Kim *et al.,* 2008) were collected from Korean Ginseng Center and Ginseng Genetic Resource Bank, Kyung Hee University and cultured in Gamborg's medium (B5 medium) supplemented with 3% sucrose and 2 ㎎/L Indole Butyric Acid (IBA) and Murashige & Skoog (MS) suspension medium without any hormone respectively (http://www.ginsengbank.org/). Both the roots were maintained by regular subculture in every 4 weeks. Abiotic stress treatment was carried out with one month-subcultured roots.

2. RNA isolation and construction of a cDNA library

The total RNA was isolated from the *P. ginseng* root tissue frozen and ground in liquid nitrogen. The poly $(A)^+$ RNA was isolated via oligo-dT-cellulose chromatography using an mRNA isolation kit (Stratagene, http://www.stratagene.com). A commercial cDNA synthesis kit (TriplEx2, Clontech) and a GigapackIII Gold packaging extract (Stratagene, http://www.stratagene.com) were utilized in the construction of the cDNA library, in accordance with the instruction manual provided by the manufacturer (Clontech, http://www.clontech.com) (Kim *et al.,* 2008). The fractions containing cDNAs larger than 500 bp were recovered, and used as a cDNA library construction.

3. Sequence analyses

The *PgCAD* gene was analyzed using softwares BioEdit, Clustal X, Mega3 and the other databases listed bellow; NCBI (http://www.ncbi.nlm.nih.gov/BLAST), ProtParam (http://us.expasy.org/tools/protparam.html), HMMTOP (http://www.enzim.hu/hmmtop), SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html) and Prosite (http://www.expasy.ch/prosite/).

4. Stress treatment

To investigate the response of *PgCAD* gene to various stresses, one month subcultured adventitious roots and hairy roots were used. For treatment with 10 mM salicylic acid (SA), 100 mM salt (NaCl), ginseng roots were cultured in suspension media containing each stress compound at 25℃ for 48 h. Chilling stress was applied by exposing the roots to a temperature of 4℃ (Pulla *et al*., 2008). Excised roots were wounded with a needle puncher (Huh *et al*., 1997). Each treatment sample was collected at time points 0, 2, 4, 8, 12, 24 and 48 hr post treatment, respectively. All treated plant materials were immediately frozen in liquid nitrogen and stored at –70℃ until required.

5. Semi-quantitative RT-PCR analysis

Total RNA was extracted from stress treated adventitious roots and hairy roots of *P. ginseng* using RNeasy mini kit (Qiagen, Valencia, CA, USA). For RT-PCR, 2 ug of total RNA was used as a template for reverse transcription.

Oligo $(dT)_{15}$ primer (0.2 mM) (INTRON Biotechnology, Inc., South Korea) was added and the mixture was heated for 5 min at 75℃. Then reaction mixture was incubated with AMV Reverse Transcriptase (10 U/ul) (INTRON Biotechology, Inc., South Korea) for 60 min at 42℃. The reaction was inactivated by heating the mixture at 94℃ for 5 min. PCR was then performed using a 1 ul aliquot of the first stand cDNA in a final volume of 25 ul containing 10 pmol of specific primers for coding of *PgCAD* gene (5'-TCCGAAAGCAAGAAAAAGGAAG -3'; (reverse) 5'- AAA CGGTATCGAACGTCAGC -3' were used. As a control, the primers specific to *P. ginseng* actin gene were used. (5'-CGT GAT CTT ACA GAT AGC TTG ATG-3'; reverse, 5'-AGA GAA GCT AAG ATT GAT CCT CC-3'). PCR was carried out using 1 U of taq DNA polymerase (Solgent Co., South Korea) in a thermal cycler programmed as follows: an initial denaturation for 5 min at 95℃, 30 amplification cycles [30s at 95℃ (denaturation), 30 s at 58℃ (annealing), and 90 s at 72℃ (polymerization)], followed by a final elongation for 10 min at 72℃. Actin was used as an internal control to normalize each sample for variations in the amount of RNA used.

RESULTS AND DISCUSSTION

1. Isolation and Characterization of a cDNA encoding *PgCAD* Gene

As part of a genomic project to identify genes of the

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1   ACAGCCCCTTGAGACACTTTGGACTAGACAAGCCCGGTATGCATGTGGGTGTTGTAGGAT 60
                                             M  H  V  G  V  V  G

61  TAGGTGGACTTGGTCATGTAGCTGTTAAAATGGCAAAGGCTTTTGGTACTAAAGTTACAG 120
    L  G  G  L  G  H  V  A  V  K  M  A  K  A  F  G  T  K  V  T

121 TCATCAGTACTTCCGAAAGCAAGAAAAAGGAAGCTATTGAACAACTTGGTGCTGATTCAT 180
    V  I  S  T  S  E  S  K  K  K  E  A  I  E  Q  L  G  A  D  S

181 TTGTGGTCAGTCGTGACCCTGAGCAATTGCAGGCTGCAGTGAACTCGCTGGATGGTATCA 240
    F  V  V  S  R  D  P  E  Q  L  Q  A  A  V  N  S  L  D  G  I

241 TTGATACTGTCTCTGCTACTCACCCTGTTTTACCATTGCTCGGAATGTTAAAGCCTCAAG 300
    I  D  T  V  S  A  T  H  P  V  L  P  L  L  G  M  L  K  P  Q

301 GAAAGCTTGTTATGGTTGGTGCACCTGAGAAGCCTCTTGAGCTGCCAGTGTTTCCTTTAA 360
    G  K  L  V  M  V  G  A  P  E  K  P  L  E  L  P  V  F  P  L

361 TTATGGGGAGGAAGATTCTTGCTGGGAGTGCCATCGGTGGGTTGAAGGAAACACAGGAAA 420
    I  M  G  R  K  I  L  A  G  S  A  I  G  G  L  K  E  T  Q  E

421 TGCTCGATTTTGCAGCAAAACACAACATAACAGCAGATGTCGAGATTATCCCTATAGACT 480
    M  L  D  F  A  A  K  H  N  I  T  A  D  V  E  I  I  P  I  D

481 ATGTAAACACTGCAATGGAGAGGCTCGTGAAAGCTGACGTTCGATACCGTTTTGTCATCG 540
    Y  V  N  T  A  M  E  R  L  V  K  A  D  V  R  Y  R  F  V  I

541 ACATAGCGAAAACCTTGAAAGCCGAGTAATTGACCCTTGTCATTTTTGAACAAGTATTAT 600
    D  I  A  K  T  L  K  A  E  *

601 TTACCTCGAGAATGTTTTAGTTGCTGGCATTGGTGGTTAATAGAGTCTTAGTATTTTGGN 660

661 TTTGGANG 668
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of a *PgCAD* cDNA isolated from *P.ginseng*. Numbers on the left and right represent nucleotide positions. The deduced amino acid sequence is shown in single-letter code below the nucleotide sequence. The asterisk denotes the translation stop signal.

medicinal plant *P. ginseng*, a cDNA library consisting about 20,000 cDNAs were previously constructed. A cDNA encoding Cinnamyl Alcohol Dehydrogenase (CAD), designated *PgCAD*, was isolated and sequenced. The sequence of *PgCAD* has been deposited in GenBank under accession number (FJ002429). As shown in Fig. 1, *PgCAD* is 668 bp in length, and it has an open reading frame (ORF) of 531 bp nucleotides with a 38 nucleotide upstream sequence and a 99 nucleotide downstream sequence. The ORF of *PgCAD* starts at nucleotide position 39 and ends at position 569. *PgCAD* encodes a precursor protein of 177 amino acids residues. The calculated molecular mass of the matured protein is approximately 18.79 kDa with a predicated isoelectric point of 6.92. In the deduced amino acid sequence of PgCAD protein, the total number of negatively charged residues (Asp + Glu) was 20 while the total number of positively charged residues (Arg + Lys) was 20 (ProtParam).

2. Homology and Secondary structure Analysis of protein PgCAD

A GenBank Blastp search revealed that PgCAD has the highest sequence homology to the Tobacco (*Nicotiana tabacum*) CAD (CAO99126) with 76% identity and 92% similarity. Fig. 2 shows a amino acid sequence alignment of PgCAD and other closely related CADs. PgCAD also shares sequence homology to Black cottonwood (*Populus trichocarpa*) CAD (ACC63874) 75% identity and 86% similarity; Tomato (*Solanum lycopersicum*) Eli3 protein (AAF72100) 73% identity and 93% similarity; Mouse-ear cress (*Arabidopsis thaliana*) CAD (AAA99511) 63% identity and 85% similarity and Norway spruce (*Picea abies*) CAD (CAI30877) 54% identity and 77% similarity. Secondary structure analysis and molecular modeling for PgCAD were performed by SOPMA. The secondary structure analysis revealed that PgCAD consists of 70 α-helices, 15 β-turns jointed by 43 extended strands, and 48 random coils.

Neighbor-joining method (Saitou *et al.,* 1987) was used for construction of phylogenetic tree based on CADs amino acid sequences to show relationships between PgCAD and other plant CADS. Phylogenetic analysis of plant CADs has been carried out using the Clustal X program (Fig. 3). According to phylogenetic tree *PgCAD* was closely related with *CAD* of Tobacco and Tomato.

3. Differential expressions of the *PgCAD* under various abiotic stresses

The expression patterns of the *PgCAD* under various stresses, such as stress-related chemicals, SA (10 mM), NaCl (100 mM), and chilling and also wounding were investigated by RT-PCR. In adventitious roots salicylic acid (SA) treatment was induced (Fig. 4A, 5A) *PgCAD* expression strongly at 2 hrs post treatment and expression pattern remains same until 24 hrs and remarkably increased at 48

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panax ginseng          1    MHVGVVGLGGLGHVAVKMAKAFGTKVTVISTSESKKKEAIEQLGADSFVVSRDPEQLQAA  60
Nicotiana tabacum      1    MHIGVVGLGGLGHMAVKFAKAFGTKVTVISTSASKKQEAIGRLGADSFLVSRDPDQMQAA  60
Populus trichocarpa    1    KHIGIVGLGGLGHVAVKFAKAFGSKVTVISTSPSKKEEALKNLGADSFLVSRDQEQMQAA  60
Picea abies            1    KKCGILGLGGVGHMGVKIAKAFGLHVTVISSSDKKKEEALEVLGADAYLVSKDAEKMQEA  60
Arabidopsis thaliana   1    KHLGVAGLGGLGHVAVKIGKAFGLKVTVISSSSTKAEEAINHLGADSFLVTTDPQKMKAA  60
Phleum pratense        1    KHLGVVGLGGLGHMAVKFGKAFGMTVTVISSSPRKREEAVERLGADAFLVSQDPDQMKAA  60

panax ginseng          61   VNSLDGIIDTVSATHPVLPLLGMLKPQGKLVMVGAPEKPLELPVFPLIMGRKILAGSAIG 120
Nicotiana tabacum      61   AGSLDGIIDTVSAIHPLLPLINLLKTHGKLVMVGAPEKPLELPVFPLLLGRKLVAGSAIG 120
Populus trichocarpa    61   AGTLDGIIDTVSAVHPLLPLFGLLKSHGKLILVGAPEKPLELPAFSLIAGRKTVAGSGIG 120
Picea abies            61   AESLDYIMDTIPVAHPLEPYLALLKTNGKLVMLGVVPEPLHFVTPLLILGRRSIAGSFIG 120
Arabidopsis thaliana   61   IGTMDYIIDTISAVHALYPLLGLLKVNGKLIALGLPEKPLELPMFPLVLGRKMVGGSDVG 120
Phleum pratense        61   AGTMDGIIDTVSAFHPIVPLLDLLKPMGQMVVVGAPSKPLELPAFAIIGGGKRLAGSGTG 120

panax ginseng          121  GLKETQEMLDFAAKHNITADVEIIPIDY--VNTAMERLVKADVRYRFVIDIAKTLKAE 176
Nicotiana tabacum      121  GIKETQEMVDFAAKHNITPDVEVVPMDY--VNTALDRLLKSDVKYRFVLDVGNTLNKN 176
Populus trichocarpa    121  GMKETQEMIDFAAKHNITADIEVISTDY--LNTAMERLAKNDVRYRFVIDVGNTLAAT 176
Picea abies            121  SMEETQETLDFCAEKKVSSMIEVVGLDY--INTAMERLVKNDVRYRFVVDVARSNLDK 176
Arabidopsis thaliana   121  GMKETQEMLDFCAKHNITADIELIKMDE--INTAMERLAKSDVRYRFVIDVANSLSPP 176
Phleum pratense        121  SVADCQAMLDFAGKHGITADVEVVKMDY--VNTAIERLEKNDVTYRFV---------- 166
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Fig. 2. Multiple alignment of the deduced amino acid sequence of *PgCAD* with those of CAD genes from other plants; *Nicotiana tabacum* (CAO99126), *Populus trichocarpa* (ACC63874), *Picea abies* (CAI30877), *Arabidopsis thaliana* (AAA99511) and *Phleum pratense* (ABC88229). Sequence data was obtained from GeneBank listed and aligned using DDBJ ClustalW and GeneDoc. Gaps are marked with dashes

Fig. 3. Phylogenetic tree based on amino acid sequence, showing the Phylogenetic relation between *PgCAD* and other plant CADs. The tree was constructed using the Clustal X method (Neighbor-joining method) and a bar represents 0.01 substitutions per amino acid position.

Fig. 4. RT-PCR analyses of the expressions of *PgCAD* gene in adventitious roots of *P. ginseng* at various time points (Hrs) with various stresses. A, 10 mM SA; B, 100 mM NaCl; C, chilling (4℃) and D, wounding. Actin was used as an internal control.

hrs post treatment. In hairy root also SA induced the *PgCAD* expression at 4 hrs and remains same until 48 hrs post treatment. *Eli3* genes, a novel type of pathogen induced benzyl alcohol dehydrogenase, BADs, have been recently described in several higher plants (Trezzini *et al.*, 1993; Somssich *et al.*, 1996). The *eli3* genes are strongly activated both in elicitor-treated cells and in fungal infection sites (Schmelzer *et al.*, 1989). These enzymes exhibit high amino acid sequence similarity with CADs from various higher plants. The *PgCAD* sequence show high homology with that of the *eli3* genes, this might be one of the reason for higher expression of *PgCAD* against SA stress. Mur *et al.*, (1996) also explained that SA enhances pathogen and wound-responsive transcription (Elizabeth *et al.*, 1999). Moreover, He *et al.*, (2005) explained that SA activated lignifications in asparagus. Several studies have shown that genes involved in the early or later steps of lignifications (phenylpropanoidand monolignols synthesis) are up-regulated in response to pathogen infection (Buell and Somerville 1995). Lignin imparts strength to cell walls and impedes the degradation of wall polysaccharides thus acting as a major line of defense against pathogens, insects and other herbivores (Kawalleck *et al.*, 1992).

Under salt stress, a transcript level of *PgCAD* was induced at 2 hrs post treatment and expression pattern remains same until 24 hrs and remarkably increased at 48 hrs post treatment in adventitious roots, where as in salt treated hairy roots *PgCAD* was strongly expressed at 4 hrs and expression pattern remains same until 48 hrs (Fig. 4B,

5B). Plants respond to unfavorable environmental condition (i.e., alteration of the water status) accumulating polyamines (PAs). PAs are commonly associated with responses to biotic, abiotic stresses and lignifications. PAs most likely rigidify microsomal membrane surfaces, stabilizing them against NaCl and osmotic stress damage. Moreover, the higher levels of PAs bound to microsomal membranes most likely minimize the damaging effect of NaCl (Groppa and Benavides, 2008).

In adventitious root chilling stress samples *PgCAD* was expression level induced at 2 hrs post treatment, strongly expressed at 24 hrs and remains same until 48 hrs post treatment. In case of excised hairy root samples, *PgCAD* was strongly induced at 2 hrs post reaction and reached maximum at 4 hrs, expression pattern was gradually decreased until 24 hrs and then expression was disappeared at 48 hrs post treatment. The major malicious effect of freezing is that it induces severe membrane damage (Mahajan *et al*., 2005). Overall, cold acclimation results in protection and stabilization of the integrity of cellular membranes, enhancement of the antioxidative mechanisms, increased intercellular sugar levels as well as accumulation of other cryoprotectants including polyamines that protect the intracellular proteins by inducing the genes encoding molecular chaperones (Fig. 4C, 5C). PAs are commonly associated with responses to biotic and abiotic stresses and have been shown to function in drought and chilling tolerance in some situations (Bae *et al*., 2008).

In adventitious root wounding stress samples *PgCAD* was strongly expressed at 2 hrs post treatment and gradually increased until 48 hrs where as in hairy root samples *PgCAD* was strongly expressed at 2 hrs and expression was remained same until 48 hrs (Fig. 4D, 5D). Bernards and Lewis (1992), reported that wounding enhances the biosynthesis of phenylpropanoid compounds, including lignin precursors. MacKay *et al.* (1997) explained that lignin composition used to alter the CAD Activity. Moreover, Brill *et al.* (1999) explained that in *Medicago sativa,* both *MsaCad1* and*MsaCad2 genes* expression were induced, after 4 hrs wounding stress. Expression levels of *PgCAD* to all the stress reagents reveals that CADs gene may play protective role in plants again to abiotic stresses. We will continuously study further to find relations between *PgCAD* and abiotic stress then produce the transformant by over expression of *PgCAD* in *P. ginseng.* These approaches will improve our understanding of the role of CAD in plant-environment interactions.

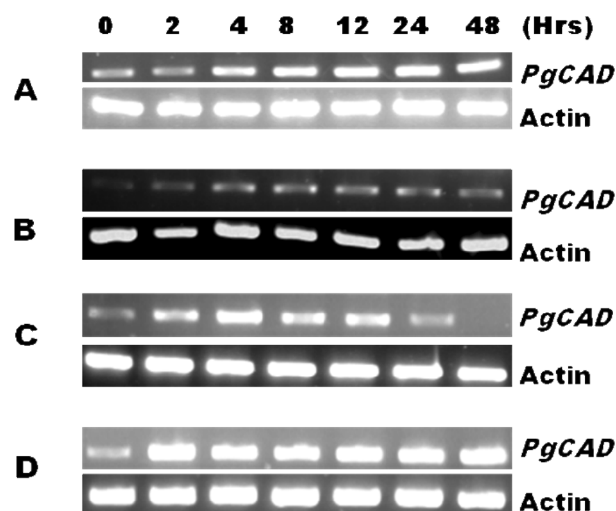

Fig. 5. RT-PCR analyses of the expressions of *PgCAD* gene in hairy roots of *P. ginseng* at various time points (Hrs) with various stresses. A, 10 mM SA; B, 100 mM NaCl; C, chilling (4℃) and D, wounding. Actin was used as an internal control.

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