Isolation and Characterization of Cinnamoyl CoA Reductase Gene from *Codonopsis lanceolata*

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ABSTRACT

A cinnamoyl CoA reductase (CCR) cDNA (*ClCCR*) was isolated from tabroot mRNAs of *Codonopsis lanceolata* by cDNA library construction, and its expression was investigated in relation to abiotic stresses. The *ClCCR* is 1008 bp in length with an open reading frame (ORF) of 336 amino acids. The deduced amino acid sequence was showed high similarity with cinnamoyl-CoA reductases of P. *tremuloides (AAF43141)* 87%, F.×aranassa (AAP46143) 83%, *L. album* (CAD29427) 80%, *E. gunnii* (CAA66063) 72%, S. *tuberosum* (AAN71761) 83%. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was revealed that the *ClCCR* expression was regulated by abiotic stresses.

Key words : Abiotic stress, *Codonopsis lanceolata*, RT-PCR, cinnamoyl CoA reductase.

INTRODUCTION

Plants respond to various environmental stimuli, which often enables them to endure at the conditions of their local environments. Lignification is known to the plant defense response to environmental stimuli such as wounding, mechanical stress or pathogen attack (Davin and Lewis, 1992, Chang *et al.*, 1995, *Baucher et al.*, 1998, 2003).

Lignification is the process of forming the collective of phenylpropanoid macromolecules termed lignin. Lignin is the second most abundant compound on the earth after cellulose. The strength of wood is a result of lignin, which makes up about one-quarter to one-third of the mass of dry wood (Harkin, 1967). It is removed from wood pulp before it is turned into paper, and the extracted lignin is used as a binder in particleboard, adhesive for linoleum, and raw material for processing into chemicals (such as DMSO, vanillin) (Campbell and Sederoff, 1996; Ben-Haj-Salah and Tardieu, 1995). Deposition of lignin occurs in the walls of certain specialised cells such as tracheary elements allowing water and nutrient conduction as well as structural support (Scobbie *et al.*, 1993; Vance, 1980). From the functional point of view, lignin impart strength to cell walls, facilitate water transport and impede the

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degradation of wall polysaccharides thus acting as a major line of defense against pathogens, insects and other hervivores.

The biosynthesis of lignin initiates from the common phenylpropanoid pathway, beginning with phenylalanine and leading to cinnamoyl CoAs which are the common precursors of a wide range of phenolic compounds (Collazo *et al.*, 1992; Costa *et al.*, 1998; Piquemal *et al.*, 1998.) These-CoA esters can be led into the lignin branch pathway to produce monolignols via two reductive steps catalyzed by cinnamoyl CoA reductase and cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) (Schwarz-Sommer *et al.*, 1987). In this study, we isolated the cinnamoyl CoA (*ClCCR*) gene from *C. lanceolata* and investigated the correlation between the *ClCCR* expression and abiotic stress by quantitative RT-PCR.

MATERIALS AND METHODS

Plant materials

Four-year old taproots of *Codonopsis lanceolata* cultivated in field were used for the cDNA library construction and the gene expression analysis.

RNA isolation and construction of a cDNA library

Total RNA was isolated from four-year old taproot from *C. lanceolata* by an aqueous phenol extraction procedure as described by Morris *et al.* (1990). A commercial cDNA synthesis kit was used to construct a library according to the manufacture's instruction manual (Clontech, PT3000-1, USA). Fractions containing cDNA greater than 500 bp were recovered. The cDNA library was amplified to yield a final titer of 2×10^9 pfu · ml^{-1}. Individual colonies were propagated and saved at -80℃ until further use.

Nucleotide sequencing and sequence analysis

pTriplEx phagemids were excised from the Uni-ZAP XR library and used as templates for sequence analysis. The 5′ ends of randomly selected cDNA inserts were sequenced by an automatic DNA sequencer (ABI prism 3700). Nucleotide and amino acid sequence analyses were performed using DNASIS program (Hitachi). Comparison of sequences to DNA and protein databases at NCBI was performed using the blast algorithm of *Altschul et al.* (1990).

The functional classification of EST clone was based on the results of a comparison to the non-redundant protein database of GenBank using the blastx algorithm. EST clone was annotated manually following the Munich Information Center for Protein Sequences (MIPS) role categorization (Frishman *et al.*, 2000). ExPasy (http://www.expasy.org/ tools/) and PSORT (http://psort.ims.u-tokyo.ac.jp) were used for the prediction of pI, MW and signal peptide of protein.

Stress treatments

For abiotic stress treatments, roots were slices and floated on MS media containing 1 mM H_2O_2 and 100 μM NaCl (Murashige and Skoog, 1962). After the treatments, the tissues were immediately frozen in liquid N_2 and stored at -80℃

Quantitative RT-PCR analysis

The gene-specific primers of *ClCCR* were designed and used for RT-PCR analysis. Specific primers for coding region of each gene included the following: (*ClCCR*-forward) 5′-CTT CAT GGA TGG TTA AAT TGC TC-3′; (*ClCCR*-reverse) 5′-ATC ACA TTC TTC GTT CCA ATC AC-3′. As a control, we used the primers specific to *C. lanceolata* actin gene, 5′-CGA GAA GAG CTA CGA GCT ACC CGA TGG-3'(forward) and 5′-CTC GGT GCT AGG GCA GTG ATC TCT TTG CT-3′ (reverse). Ten micrograms of total RNA were used for the RT-PCR analysis. The PCR cycles was numbered 40 for *ClCCR* and the actin.

RESULT AND DISCUSSION

The EST clones homologous to cinnamoyl-CoA reductase genes were obtained from a cDNA library constructed with 4-year old root of *C. lanceolata* and named *ClCCR*. The *ClCCR* clone is 1232 nucleotides long and contains an open reading frame composed of 336 amino acid polypeptides (Fig. 1). The deduced

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TTCATTATTCTTTATTTCCTACTCCACCTGTCAAAACCCCATCACCAACCATTTTTTCCT    60
TTTAAAATCTCTTCCATTTGCGTTGCGCTGTTGAGTAGTGATTCGGACTAGGAATTGGGA   120
AGCCATTTGAGGAGTTTGCGTCAAAAGGAAGCAAATATTAGAAGTGACCAATTTGTCAAT   180
TCAACTGAAGAATGCCGCCAGTCTCCAACCAAGTCATATGTGTCACCGGTGCCGGCGGAT   240
            M  P  P  V  S  N  Q  V  I  C  V  T  G  A  G  G  F      17

TTATAGCTTCATGGATGGTTAAATTGCTCCTGGAAAAAGGCTATTCCGTTCGAGGAACTG   300
 I  A  S  W  M  V  K  L  L  L  E  K  G  Y  S  V  R  G  T  V      37

TCAGAAATCCTGATGATCCGAAGAATAGCCATTTGAGGGACCTTGAAGGAGCAAAGGATA   360
 R  N  P  D  D  P  K  N  S  H  L  R  D  L  E  G  A  K  D  R      57

GATTAACTCTCTGCAAAGCTGATCTCCTCGATTATCAGAGTTTGCTTGAAGCTATTATTG   420
 L  T  L  C  K  A  D  L  L  D  Y  Q  S  L  L  E  A  I  I  G      77

GGTGTGACGGCGTTTTCCACACTGCATCACCCGTTACCGATGATCCTGAACAAATGGTGG   480
 C  D  G  V  F  H  T  A  S  P  V  T  D  D  P  E  Q  M  V  E      97

AGCCGGCAGTGATTGGAACGAAGAATGTGATAGTAGCGGCAGCAGAAGCCAAATGCCGGC   540
 P  A  V  I  G  T  K  N  V  I  V  A  A  A  E  A  K  C  R  R     117

GAGTGGTTTTCACTTCATCAATTGGTGCGGTCTACATGGACCCTAACAGGAGCCCTGATG   600
 V  V  F  T  S  S  I  G  A  V  Y  M  D  P  N  R  S  P  D  A     137

CTGTTGTTGATGAAACTTGCTGGAGCGATCTCGAGTTTTGCAAGAACACTAAGAATTGGT   660
 V  V  D  E  T  C  W  S  D  L  E  F  C  K  N  T  K  N  W  Y     157

ACTGTTACGGAAAGGCAGTGGCGGAGCAGGCAGCGTGGGATGAGGCCAAGGTGAGAGGAG   720
 C  Y  G  K  A  V  A  E  Q  A  A  W  D  E  A  K  V  R  G  V     177

TGGACCTAGTGGTAGTCAACCCAGTGTTGGTGCTTGGTCCACTGTTACAACACACCGTAA   780
 D  L  V  V  V  N  P  V  L  V  L  G  P  L  L  Q  H  T  V  N     197

ATGCCAGCATTGTTCATGTCCAGAAGTACCTCACTGGCTCCGCTAAGACCTATGCCAACT   840
 A  S  I  V  H  V  Q  K  Y  L  T  G  S  A  K  T  Y  A  N  S     217

CGGTTCAGGCCTATGTCCACGTTAGGGACGTGGCGTTGGCACATATTCTTCTCTTCGAGA   900
 V  Q  A  Y  V  H  V  R  D  V  A  L  A  H  I  L  L  F  E  T     237

CTCCTTCAGCGTCTGGCCGGTACCTCTGCGCTGAGAGCGTGCTTCACCGTGGGGAGGTTG   960
 P  S  A  S  G  R  Y  L  C  A  E  S  V  L  H  R  G  E  V  V     257

TCGAAATCCTGGCCAAGTTTTTCCCTGAATACCCAATTCCTACCAAGTGCAAGGATGACG  1020
 E  I  L  A  K  F  F  P  E  Y  P  I  P  T  K  C  K  D  D  G     277

GCAAGCCAAGAGCAAAACCATACAAGTTTTCGAATCAAAAGCTAAAAGATTTGGGGCTGG  1080
 K  P  R  A  K  P  Y  K  F  S  N  Q  K  L  K  D  L  G  L  E     297

AATTCACACCAGTTAAACAAGGCTTATACGAAACCGTAAAGAGCCTGCAGGAGAAAGGTC  1140
 F  T  P  V  K  Q  G  L  Y  E  T  V  K  S  L  Q  E  K  G  H     317

ACCTCCCAGTTCTTTCTCCTCCTCCCCAACAAACTGATGACTCTATTCGTATTCAGTCTT  1200
 L  P  V  L  S  P  P  P  Q  Q  T  D  D  S  I  R  I  Q  S  *     336

AAGTCAAGAATTCACAACCTAATTAGTCCCAG                               1232
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Fig. 1. **Nucleotide and deduced amino acid sequence of *ClCCR*, cinnamoyl CoA cDNA, from *C. lanceolata*.** The amino acid sequence deduced from an open reading frame is shown below the nucleotide sequences. Asterisk shows the termination codon. The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequencing Database under the accession number AB243011.

amino acid sequences has high homology with plant cinnamoyl CoA reductase (Fig. 2). Multiple alignment among these CCR sequence revealed the presence of a highly conserved motif, NWYCY, in all sequences, which was proposed to be involved in the catalytic site of CCR. By comparison with 5 previously identified

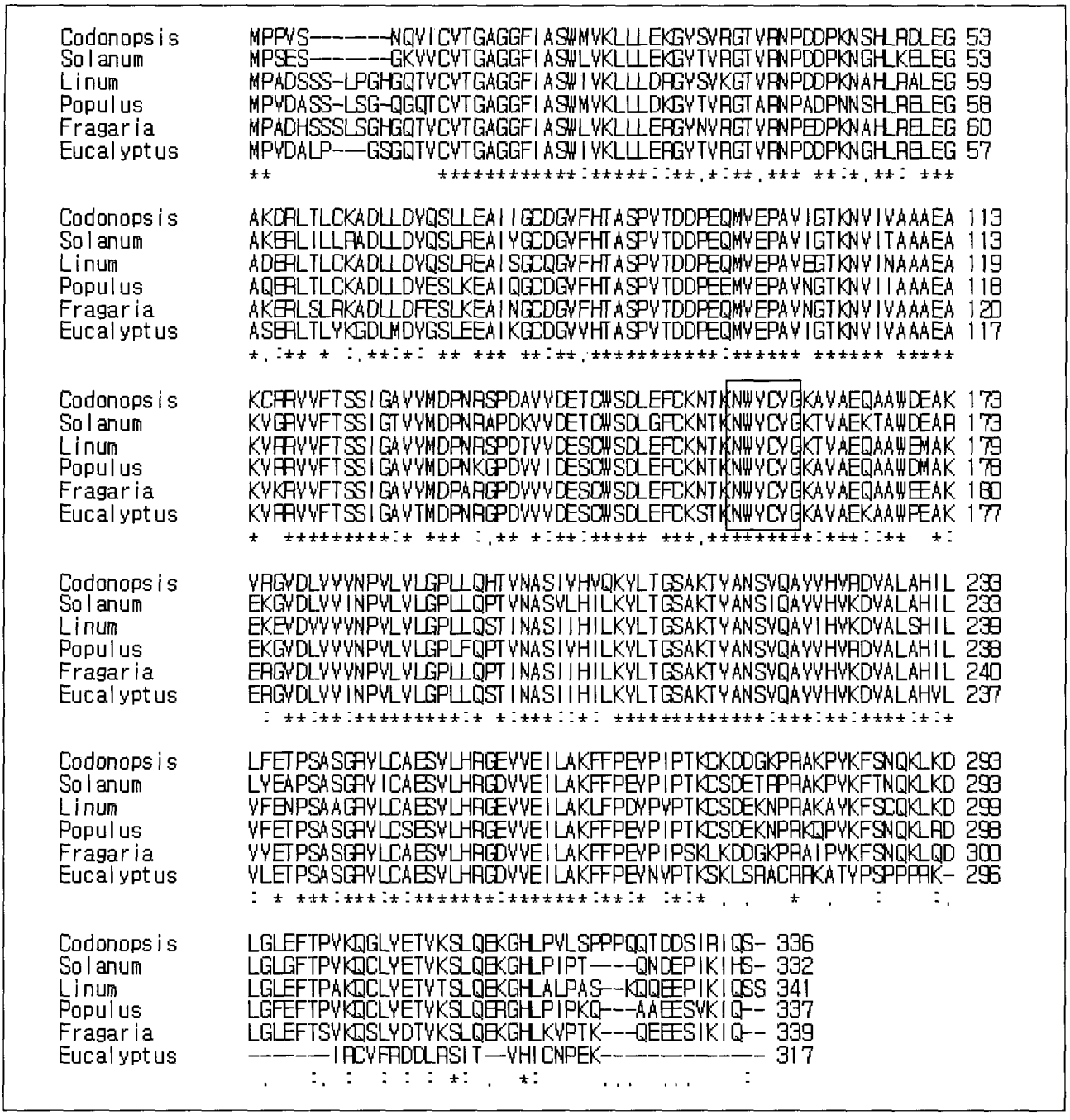

Fig. 2. **Amino acid sequence alignment of ClCCR with cinnamoyl-CoA reductase from other plant species;** *P. tremuloides* (AAF43141), *F.×aranassa* (AAP46143), *L. album* (CAD29427), *E. gunnii* (CAA66063), and *S. tuberosum* (AAN71761). Alignment of sequences was performed using the Clustal X programe on EBI molecular biology server (Thompson *et al.*, 1997). The box indicated the putative specific motif of CCRs.

CCRs of other species, the phylogenetic relationship of *ClCCR* was established (Fig. 3). Of them, *L. esculentum* (AAY41880) had very close genetic relationship with the *ClCCR*.

We performed RT-PCR analysis to investigate the expression pattern of *ClCCR* gene against abiotic stresses. Expression of the actin gene in *Codonopsis lanceolata* served as an internal control for PCR (Fig. 4). The transcriptional expresssion of *ClCCR* gene, encoding cinnamoyl CoA reductase, was strongly expressed at 12 h, and then reduced dramatically at 2 day after treatment under 1 mM H_2O_2. And *ClCCR* transcript level increased at 2h, and reduced dramatically at 4 h after treatment under 100 μM NaCl. These results suggest that the *ClCCR* gene responds to oxidative and salt stresses.

In the biosynthetic pathway leading from phenylalanine to lignin, cinnamoyl CoA reductase (CCR) is the key enzyme in the biosynthesis of monolignols, and catalyzes the conversion of cinnamoyl CoA esters to their corresponding cinnamaldehydes. Since lignification has been reported as a part of the active mechanisms induced during resistance (Dixon and Paivia, 1995), the question whether the *ClCCR* genes are regulated in response to abiotic stresses has been addressed.

We report here the first isolation of a cDNA encoding cinnamoyl CoA reductase (CCR) in *C. lanceolata*. Also We identified the expression pattern of *ClCCR* gene against abiotic stresses. The expression level of *ClCCR* is significantly induced by oxidative and salt stresses. Therefore, we will further characterize

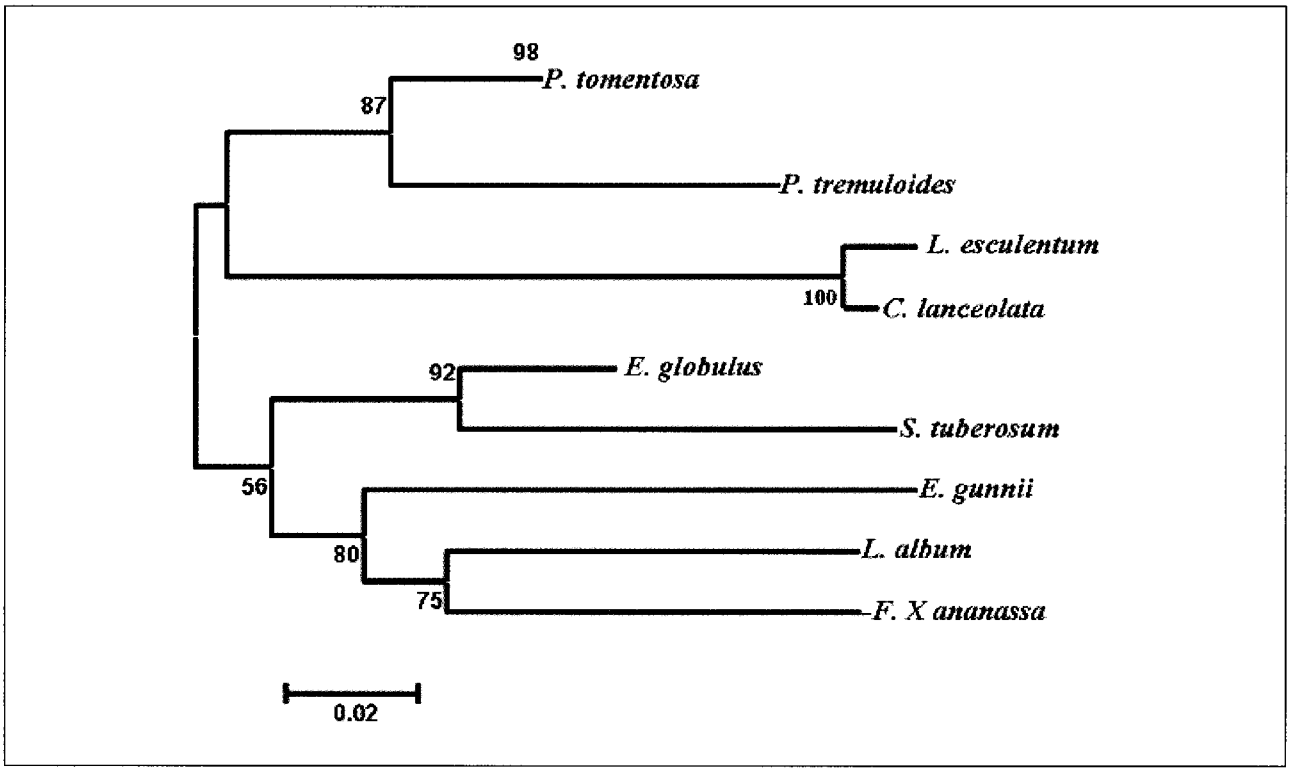

Fig. 3. **Phylogenetic tree illustrating the genetic relationships between plant ClCCRs.** The tree was built using the sequences indexed in EMBL as follows : *P. tomentosa* (AAR83344), *P. tremuloides* (AAF43141), *L. esculentum* (AAY41880), *E. globulus* (AAT74877), *F. X aranassa* (AAP46143), *L. album* (CAD29427), *E. gunnii* (CAA66063) , and *S. tuberosum* (AAN71761).

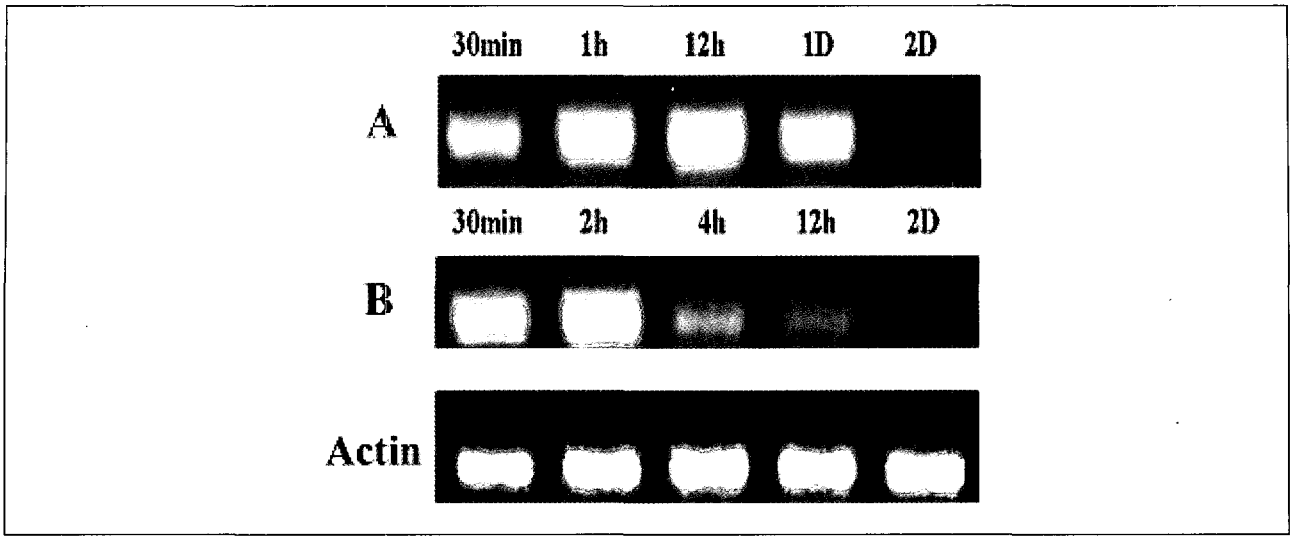

Fig. 4. RT-PCR analysis of the expression of the *ClCCR* gene in root of *C. lanceolata* under stress conditions. A: 1 mM H_2O_2, B: 100 μM NaCl. The lower panel shows loading control of actin transcripts in each sample.

the relations between *ClCCR* and various abiotic stress and then produce the abiotic stress-tolerant transformants by re-introduction of *ClCCR* into *C. lanceolata*.

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LITERATURE CITED

Altschul, S.F. W.Gish, W.Miller, E.W.Myers and D.J.Lipman. 1990. Basic local alignment search tool. J.Mol. Biol. 215:403-410.

Baucher, M. C.Halpin, M.Petit-Conil and W.Boerjan. 2003. Lignin: genetic engineering and impact on pulping. Crit. Rev. Biochem. Mol. Biol. 38: 305-350.

Baucher, M. B.Monties, M.Van Montagu and W.Boerjan. 1998. Biosynthesis and genetic engineering of lignin. Crit. Rev. Plant. Sci. 17: 125-197.

Ben-Haj-Salah, H. and F. Tardieu. 1995. Temperature affects expansion rate of maize leaves without change in spatial distribution of cell length (analysis of the coordination between cell division and cell expansion). Plant. Physiol. 109:861-870.

Campbell, M.M and R. Sederoff. 1996. Variation in lignin content and composition. Plant Physiol. 110:3-13.

Chang, S., J.D. Puryear, E.A. Funkhouser, R.J. Newton and J. Cairney. 1995. Gene expression under water deficit in loblolly pine (*Pinus taeda L.*): Isolation and characterization of cDNA clones. Physiol. Plant. 95:1-10.

Collazo, P., L. Montoliu, P. Puigdomenech and J. Rigau. 1992. Structure and expression of the lignin O-methyltransferase gene from *Zea mays* L. Plant. Mol. Biol. 20:857-867.

Costa P., N. Bahrman, J.M. Frigerio, A. Kremer and C. Plomion. 1998. Waterdeficit responsive proteins in maritime pine. Plant. Mol. Biol. 38:587-596.

Davin, L.B. and N.G. Lewis. 1992. Phenylpropanoid metabolism: biosynthesis of monolignols, lignans and neolignans, lignins and suberins. In HA Stafford, RKIbrahim, eds, Phenolic Metabolism in Plants.

Plenum Press, New York, pp 325-375.

Dixon, R.A. and N.L. Paiva. 1995. Stress-induced phenylpropanoid metabolism. Plant Cell 7:1085-1097.

Frishman, D., H.W. Mewes, C. Gruber, B. Geier, D. Haase, A. Kaps, K. Lemcke, G. Mannhaupt, F. Pfeiffer, C. Schuller, S. Stocker and B. Weil. 2000. MIPS: A database for genomes and protein sequences. Nucleic Acids Res. 28:37-40.

Murashige, M. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Harkin, J.M. 1967. Lignin: A natural polymeric product of phenol oxidation. In WI Taylor, AR Battersby, eds, Oxidative Coupling of Phenols. Marcel Dekker, New York, pp. 243-321.

Piquemal, J., C. Lapierre, K. Myton, A. O'Connell, W. Schuch, J. Grima-Pettenati and A.M. Boudet. 1998. Down-regulation of cinnamoyl-CoA reductase induces significant changes of lignins profiles in transgenic tobacco plants. Plant. J. 13:71-83.

Schwarz-Sommer, Z., N. Shepherd, E. Tacke, A. Gierl, W. Rohde, L. Leclercq, M. Mattes, R. Berndtgen, P.A. Peterson and H. Saedler. 1987. Influence of transposable elements on the structure and function of the A1 gene of *Zea mays*. EMBO J. 6:287-294.

Scobbie, L., W. Russell, J. Provan and A. Chesson. 1993. The newly extended maize internode: a model for the study of secondary cell wall formation and consequences for ingestibility. J. Sci. Food. Agric. 61:217-225.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876-4882.

Vance, C.P., T.K. Kirk and R.T. Sherwood. 1980. Lignification as a mechanism of disease resistance. Annu. Rev. Phytopath. 18: 259-288.

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