Molecular Cloning and Characterization of Soybean Cinnamoyl CoA Reductase Induced by Abiotic Stresses

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(Received on August 10, 2010; Accepted on October 26, 2010)

Suppression subtractive hybridization was used to isolate wound-induced genes from soybean. One of the wound-induced genes, *gmwil43* designated as *GmCCR*, showed high homology with genes encoding cinnamoyl-CoA reductase (CCR; EC 1.2.1.44). Deduced amino acid sequences encoded by *GmCCR* showed the highest identity (77%) with those of *Acacia* CCR. There are 2 CCR genes highly homologous to *GmCCR* in soybean genome based on Phytozome DB analysis. RNA expression of *GmCCR* was specifically induced by local and systemic wounding, drought, high salinity or by ultra-violet stress. Our study suggests that *GmCCR* may be involved in resistance mechanism during abiotic stresses in plants.

Keywords : cinnamoyl-coA reductase, lignin, soybean, wounding, UV

Lignin, a major component of secondary cell walls, is deposited mainly in the vascular tissues during plant development and environmental signals (Boerjan et al., 2003). Lignins contribute to major functions in plants, such as stem rigidity, water transport in xylem and are also involved in defense reactions against wounding, predators or pathogens (Vance et al., 1980). It has been reported that lignin biosynthesis is regulated by developmental signal in the vascular tissues and by defense responses in plant (Lauvergeat et al., 2001).

The biosynthesis of lignins begins with the common phenylpropanoid pathway, starting from phenylalanine and leading to cinnamoyl CoAs which are the common precursors of a wide range of phenolic compounds. These CoA esters subsequently are changed to monolignols via two reductive steps catalyzed by cinnamoyl CoA:NADP oxidoreductase (CCR, EC 1.2.1.44) and cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195). Lignins result from the oxidative polymerization of monolignols.

Several genes encoding CCR have been shown to be transcriptionally induced by developing xylem tissues or by biotic stress (Kim et al., 2000; Lacombe et al., 1997; Lauvergeat et al., 2001). Lacombe et al. (1997) showed that *Eucalyptus* CCR transcript was expressed in lignified organs such as leaves, stems and roots. By *in situ* hybridization technique, *EuCCR* was strongly expressed in the differentiating xylem zone but was not present in the secondary phloem fibers or in the young periderm (Lacombe et al., 1997). Rice *OsCCR1* RNA expression was induced in suspension cell cultures treated with sphingolipid elicitor purified from rice blast *Magnaporthe grisea* (Kawasaki et al., 2006). Previously, soybean chip analysis showed that RNA expression of CCR involved in phenylpropanoid pathway were induced in the syncytia laser microdissected soybean roots infected with soybean cyst nematode (Klink et al., 2009).

There are 11 putative CCR homologues sharing homology identities ranging from 82.8 to 32.8% in *Arabidopsis* genome (Costa et al., 2003). *Arabidopsis AtCCR1* transcript was expressed in the stem and floral tissues (Lauvergeat et al., 2001) and the *irregular xylem4* mutant (*irx4*) defective in the *AtCCR1* gene showed severely reduced lignin contents (50%) compared to the wild type (Jones et al., 2001). Lauvergeat et al. (2001) suggested that *AtCCR2* responsive to pathogen infection of *Xanthomonas campestris* pv. *campestris* may play a role in the formation of phenolic compounds associated with HR in *Arabidopsis* (Lauvergeat et al., 2001).

In the present study, we have isolated the full-length cDNA of *GmCCR* encoding CCR-like protein regulated by abiotic stresses. We propose that the *GmCCR* is involved in defense responses during environmental stresses in plants.

Materials and Methods

Plant materials and treatments. Soybean plants (*Glycine max* cv. 'Sinpaldal 2') were grown in a growth chamber with 16 h/day light supply (22,000 lux) at 28°C for 3-4 weeks. Soybean leaves from the whole plant were cut with scissors as a wounding stress. The wounded leaves were

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sampled for the local wounding and the normal leaves far from the wounded leaves were for the systemic wounding sample at the indicated time. For drought stress, whole plants were removed from the soil and dehydrated at 28°C upto 24 h. To induce high salinity stress, plants were soaked in a solution of NaCl (250 mM) at 28°C for 24 h. For the UV-B treatment, whole plants were irradiated with UV light at the indicated time. Plant samples were immediately frozen in liquid nitrogen, and stored at -75°C.

Suppression subtractive hybridization (SSH). SSH was performed using PCR-Select cDNA Subtraction kit (Clontech Laboratories Inc., USA) as described by the manufacturer. For PCR-based cDNA subtraction method, all of the procedure was carried out according to the manufacture instructions. The cDNA synthesized from extracted mRNA at untreated leaves was used for driver. Tester was prepared at cDNA from combined mRNA that was extracted wounding treated leaves. First and second strand cDNA synthesis and blunt ending of DNA ends were carried out according to manufacture instructions (Clontech, USA). Product from the second PCR was inserted into pCR 2.1 vector using a TA Cloning Kit (Invitrogen, USA). Positively selected clones were sequenced (Genotech, Korea) and the sequences were analyzed using BLAST program by comparing with GenBank/EMBL database.

Rapid amplification of cDNA ends (RACE). Complementary DNA synthesis was carried out using reverse transcription kit (Promega). The full-length *GmCCR* cDNA was obtained using SMART RACE cDNA amplification kit (Clontech) according to the manufacturer's instruction. Gene-specific 5' primer FSP1-*GmCCR* (5'-ATCGACGAA-GTCGCCACCACGCG-3') and 3' primer RSP1-*GmCCR* (5'-CTTGAGAACAAGAAAGCAGCAGGAAGGCAC-3') were used for RACE. The full-length cDNA of *GmCCR* was PCR-amplified with a 5'-*GmCCR* primer (5'-CAAAG-CAACCCTATGTCGAAGGTGG-3') and the 3'-*GmCCR* primer (5'-ACTCAGTCCCTCTGCAGCAG-3'). The resulting PCR product was cloned to pGEMT-Easy (Promega) for the sequencing.

Northern blot and Southern blot analysis. Total RNA sequences were isolated using RNA extraction Kit (Ambion). Total RNA (20 mg) was fractionated on a 1% formaldehyde agarose gel, and transferred to a nylon membrane. The *GmCCR* probe was PCR-amplified using a 5'-*GmCCR* primer (5'-CAAAGCAACCCTATGTCGAAG-GTGG-3') and the 3'-*GmCCR* primer (5'-ACTCAGTCCC-TCTGCAGCAG-3') covering the entire cDNA region of *GmCCR* gene (966 bp). The probe was labeled by a random priming method using the Ladderman labeling kit (Takara).

The membrane was hybridized to a ³²P-dCTP labeled probe using fragments encompassing *GmCCR* cDNA and washed, as described by Church and Gilbert (1984) prehybridization for 2 h at 42°C (0.5 M sodium phosphate pH 7.0, 1 mM EDTA, 7% SDS), hybridization overnight at 42°C, washes at room temperature (0.5 X SSC, 0.1% SDS) and then 65°C (0.1 X SSC, 0.1% SDS). The membrane was exposed to X-ray film (Kodak) for detection of signals.

Genomic DNA was extracted from the leaf of soybean (*Glycine max.* cv Sinpaldal 2) as described by Shure et al. (Shure et al., 1983). Genomic DNA from the leaf of soybean was digested with *Xba*I, *Eco*RI and *Hind*III and then electrophoresed through 1.0% agarose gel. The fractionated agarose gel was denatured with a denaturation solution (0.5 M NaOH, 1.5 M NaCl and then renatured in a neutralization solution (0.5 M Tris-HCl, 1.5 M NaCl, pH 7.5). The DNA was transferred onto a nylon membrane. To detect *GmCCR*, 966-bp DNA fragments covering the full-length cDNA of *GmCCR* was used for probe labeling. Probe labeling, prehybridization, hybridization and washing were conducted as the same as Northern blot analysis.

Results and Discussion

Cloning and sequence analyses of a gene encoding cinnamoyl-CoA reductase from soybean. In this study, we report molecular characterization of *GmCCR* cDNA from soybean obtained from SSH to isolate genes differentially expressed during wounding stress (Chung et al., 2007). One of wound-inducible cDNA encoding CCR family protein was designated as *GmCCR* (*Glycine max* Cinnamoyl-CoA Reductase) (Fig. 1A). The full-length cDNA of *GmCCR* induced by wounding stress was obtained by 5' and 3' RACE using cDNA of soybean leaf with a pair of specific primer as described in materials and methods (Genbank number; ABY81885). The full-length cDNA sequence of *GmCCR* is composed of an open reading frame (966 bp) encoding a protein of 321 amino acids (MW, 35.2 kDa; pI 5.66).

A multiple sequence alignment of the deduced protein sequence of *GmCCR* gene with other CCR protein sequences is shown in Fig. 1A. The *GmCCR* showed 77% protein sequence identity to CCR of *Acacia* (AAY86360), 66% to that of *A. thaliana* (*AtCCR12*), 67% to that of grapevine (Fig. 1A). Multiple alignment of *GmCCR* and other CCR proteins showed that in the N-terminal region (pos. 7-27 aa) a highly conserved motif is found: VTG(A/G)(A/G)G(F/Y)IAS(G/W)(L/M)VK(I/L/R)LL(D/E)(K/R)GY (Fig. 1A). This conserved sequence represents a dinucleotide binding domain of NADP(H) in NADH-dependent reductases and dehydrogenases (Lacombe et al. 1997) (Fig. 1A). This suggests that *GmCCR* and other CCRs have the amino acids

Fig. 1. Multiple alignment and phylogenetic tree of the deduced amino acid sequences of GmCCR with CCRs from other plant species. (A) Alignments of sequences were performed using the Clustal W program on the EBI WWW molecular biology server. The N-terminal signal peptide predicted for vacuole targeting sequence is marked by arrow and the putative NAD (P) binding domain found in other CCRs is underlined. The Accession numbers of the sequences used in the comparison are: *Acacia mangium* x *A. auriculiformis*, AAY86360; *Arabidopsis thaliana*, AAO22571; *Glycine max*, ABY81885 (in this study); *Vitis vinifera*, CAO21401. (B) The tree was constructed using the Clustal method based on amino acid similarities of the full sequences. The Accession numbers of the sequences used in the comparison are: *Arabidopsis thaliana*, AAO22571 (AtCCR12); *Glycine max*, ABY81885 (GmCCR); *Oryza sativa*, NP_001051706 (OsCCR); *Ricinus communis*, EEF35783 (RcCCR); *Camellia oleifera*, ACQ41893 (CoCCR); *Solanum tuberosum*, ABA46761 (StCCR); *Zea mays*, X98083 (ZmCCR); *Nicotiana tabacum*, A47101 (NtCCR); *Eucalyptus gunnii*, X79566 (EgCCR); *Petunia hybrida*, X15537 (PhDFR); *V. vinifera*, X75964 (VvDFR); *Antirrhinum majus*, X15536 (AmDFR).

residues strictly conserved in the mammalian 3 β -hydroxysteroid dehydrogenase/CCR superfamily (Lacombe et al. 1997) (Fig. 1A). However, there is no catalytic site of CCR, which is a conserved motif, NWYCY found in CCRs (Fig. 1A) (Lacombe et al., 2000; Lauvergeat et al., 2001).

A phylogenetic analysis of 12 different plant CCRs and

DFRs was carried out using the Clustal method (Fig. 1B). As seen in the evolutionary tree of amino acid sequences, GmCCR lies between CCR and DFR (Fig. 1B). Protein sequence identity of GmCCR ranges from 56% with OsCCR to 66% to AtCCR12 (Fig. 1B). Another CCR group such as *Eucalyptus* EgCCR shows lower identities between 40%

and 46% with GmCCR (Fig. 1B). The CCR group more similar to GmCCR lacks the conserved catalytic domain NWYCY found in other functional CCRs with biochemical activities (Lacombe et al., 1997; Lauvergeat et al., 2001). The overall identities between GmCCR and dihydroflavonol-4-reductase (DFR), the first specific enzyme of anthocyanin biosynthesis proteins was much lower from 31% to 36% (Fig. 1B). It was found that CCR and DFRs, share a striking homology due to the presence of the conserved domain such as NADPH-dependent reductases (Lacombe et al., 1997).

Copy number and RNA expression of *GmCCR*. To estimate the copy number of *GmCCR* gene in the soybean genome, southern blot analysis was performed (Fig. 2). It was shown that there was one major band in *EcoRI*- or *HindIII*-lane but two bands in the *XbaI*-digested sample (Fig. 2). However, blast search reveals that there are two CCR genes sharing high homology (100 and 95%) and two genes having large difference with *GmCCR* in soybean genome (Phytozome; www.phytozome.net) (Fig. 2). This may be natural because soybean genome is comprised of tetraploid ($2n = 4\times$). It has been proven that *AtCCR2* and *EUCCR* were present as single copy in *Arabidopsis* and *Eucalyptus* genome (Lacombe et al., 1997; Lauvergeat et al., 2001). Gene expression and the biochemical functions of each CCR were shown to be different despite the annotated CCR. Therefore, Costa et al. (2003) proposed that it should remain to characterize gene expression and also their precise metabolic roles *in vivo* (Costa et al., 2003).

To investigate *GmCCR* RNA expression in soybean plants with abiotic stresses, Northern blot was carried out (Fig. 3). Since *GmCCR* was isolated from wound-inducible

Fig. 2. Southern blot analysis of soybean genomic DNA for *GmCCR*. Twenty microgram of genomic DNA was digested with *XbaI*, *EcoRI*, or *HindIII* and loaded on each lane. PCR products corresponding to the full-length cDNA of *GmCCR* was used for probe labeling.

cDNAs, *GmCCR* RNA expression was estimated in the wound-treated leaf tissues (Fig. 3A). *GmCCR* RNA level showed maximum after 6 h by wounding stress and its RNA level was reduced after that (Fig. 3A). *GmCCR* RNA

Fig. 3. Expression of *GmCCR* gene by wounding, drought, high salinity or UV-B stresses. (A) Soybean leaves were wounded for 0, 3, 6, 12, or 24 h. (B) Soybean plants were treated with drought (Dr), high salinity (S; 250 mM NaCl) local wounding (LW) or systemic wounding (SW) for 24 h. (C) Soybean seedlings grown under dark (D) or light (L) conditions, were exposed to UV for 8 h. (D) Soybean seedlings were exposed to UV upto 12 h under light conditions or to dark (D). Twenty microgram of total RNAs was loaded on each lane. RNA blot analysis was carried out as described in Materials and Methods.

expression was monitored by other abiotic stresses such as drought, local and systemic wounding and high salinity (NaCl) stresses (Fig. 3B). *GmCCR* RNA was strongly induced by drought or high salinity stress (Fig. 3B). Both local and systemic wounding stresses slightly induced *GmCCR* RNA expression (Fig. 3B).

UV-B radiation induces secondary metabolite productions including phenolic compounds such as lignin in plants (Rozema et al., 1997; Yamasaki et al., 2007). This prompted us to investigate *GmCCR* RNA expression by UV-B irradiation (Fig. 3C, D). It was shown that *GmCCR* RNA expression gradually increased and reached the maximum level at 8 h under UV-B radiation (Fig. 3C). UV-B was irradiated to the etiolated seedlings in either dark or light conditions (Fig. 3D). Without UV-B, *GmCCR* RNA was not expressed in dark-treated samples, but weakly expressed in light-illuminated samples (Fig. 3D). There was no significant difference in *GmCCR* RNA expression in the UV-B irradiated seedlings under either dark or visible light conditions (Fig. 3D). Taken together, RNA expression of *GmCCR* gene is regulated by wounding, drought, high salinity or UV-B stresses in plants. Induction of *GmCCR* RNA expression may contribute to defense responses to abiotic stresses in plants.

There are accumulating evidences that CCR expression is correlated with lignin accumulation in plants (Jones et al., 2001; Kawasaki et al., 2006; Piquemal et al., 1998; van der Rest et al., 2006). However, it has been suggested that CCR-mediated reaction products monolignols are not only destined for polymerization into lignins but into others such as antioxidants or biocides (Lacombe et al., 1997; Lewis and Davin, 1994). It is not clear if *GmCCR* might be involved in lignin deposition or in production of polyphenols or antioxidants at this point.

Here, we have shown for the first time, implication of CCR-like protein, *GmCCR* in defense responses to wounding, drought, high salinity and UV-B stresses.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (KRF) funded by the Ministry of Education, Science and Technology (KRF-2009-0068370 and -2009-0074413) and by Biogreen 21 (2008-0401034040) funded by the Korean Government.

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