Isolation and Transcriptional Expression of CuZn Superoxide Dismutase from *Codonopsis lanceolata*

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ABSTRACT

To investigate the defense mechanism against the abiotic stress, a cDNA clone encoding a CuZn superoxide dismutase (CuZnSOD) protein was isolated from a cDNA library prepared from tabroot mRNAs of *Codonopsis lanceolata*. The cDNA, designated ClSODCc, is 799 nucleotides long and has an open reading frame of 459 bp with a deduced amino acid sequence of 152 residues. The deduced amino acid sequence of ClSODCc matched to the previously reported CuZnSODs. Consensus amino acid residues (His-45, -47, -62, -70, -79, -119 and Asp-82) were involved in Cu-, Cu/Zn-, and Zn- binding ligands. The deduced amino acid sequence of ClSODCc showed high homologies (82%-86%) regardless of species. Expression of ClSODCc by oxidative stress was increased up to 1 h after treatment and declined gradually. Much earlier and stronger expression of ClSODCc was observed in the cold stress treatment.

Key words : abiotic stress, *Codonopsis lanceolata*, RT-PCR, superoxide dismutase

INTRODUCTION

During the life cycle, plants have to suffer from various environmental stresses. Abiotic stresses such as chilling and drought have been associated with the increased production of reactive oxygen species (ROS) (Price and Hendry, 1991; Leprince *et al.*, 1990). Although oxygen is essential for the aerobic life, toxic ROS, including the superoxide anion radical ($O2^{\cdot-}$), hydroxyl radical (OH.), and hydrogen peroxide (H_2O_2), are generated in all aerobic cells during metabolic processes (Asada, 1999). ROS can react very rapidly with DNA, lipids and proteins, which causes severe cellular damage (Van Breusegem *et al.*, 1999).

Tolerance to some abiotic stresses correlates with an increased capacity to scavenge or detoxify ROS (Malan *et al.*, 1990). Enzymes involved in the selective detoxification of ROS include superoxide dismutase

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(SOD), which catalyzes the dismutation of superoxide to hydrogen peroxide and molecular oxygen; catalase (CAT) that converts hydrogen peroxide to water; glutathione reductase (GR) and ascorbate peroxidase (APX), which scavenge H_2O_2 in the ascorbate-GSH cycle (Fig. 1). SOD initiates the defense system for removing superoxide (Beyer *et al.*, 1991). SOD enzymes are classified into three distinct types according to their metal cofactors: Cu/Zn, Mn, and Fe. CuZnSOD is present in the cytosol and chloroplast, whereas MnSOD and FeSOD are localized in the mitochondria and chloroplast, respectively (Bowler *et al.*, 1992). Several SOD cDNAs have been cloned from plants (Perl *et al.*, 1993), and transgenic plants have been produced that exhibit enhanced SOD activity.

Codonopsis lanceolata, a perennial herb, belongs to Campanulaceae. Its root is used a good source of a wild vegetable as well as a medicinal plant. To increase the abiotic stress-tolerance to *C. lanceolata*, we analyzed 1,000 ESTs (expressed sequence tags) from *C. lanceolata* and isolated superoxide dismutase gene (*ClSODCc*), which related in abiotic stress. In this study, we characterized the *ClSODCc* gene by sequence and quantitative RT-PCR analysis.

MATERIALS AND METHODS

Plant materials

Four-year taproot 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 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 library according to the manufacture's instruction manual (Clontech, PT3000-1, USA). Fractions containing cDNA greater than 500 bp were recovered and the library was amplified to yield a final titer of 2×10^9 pfu · ml^{-1}. Individual colonies were propagated and saved at -80 °C 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 using the 5' sequencing primer by an

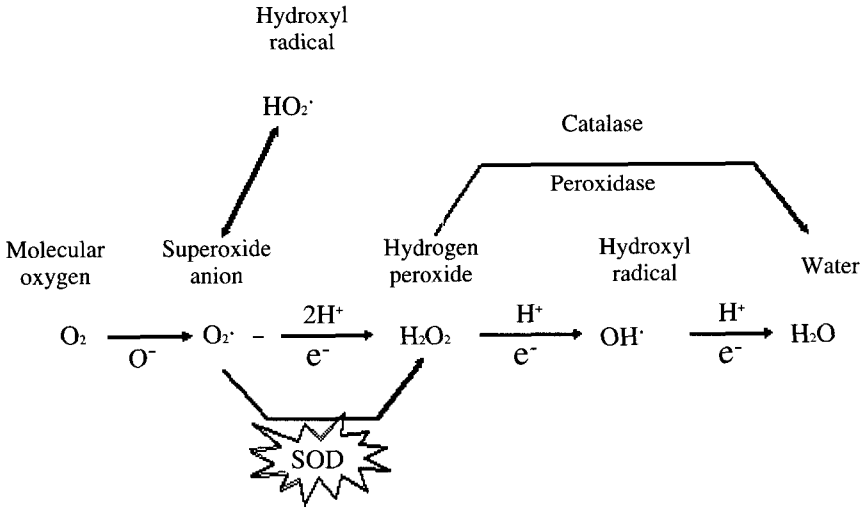

Fig. 1. General interconversion scheme of reactive oxygen species (ROS) derived from molecular oxygen.

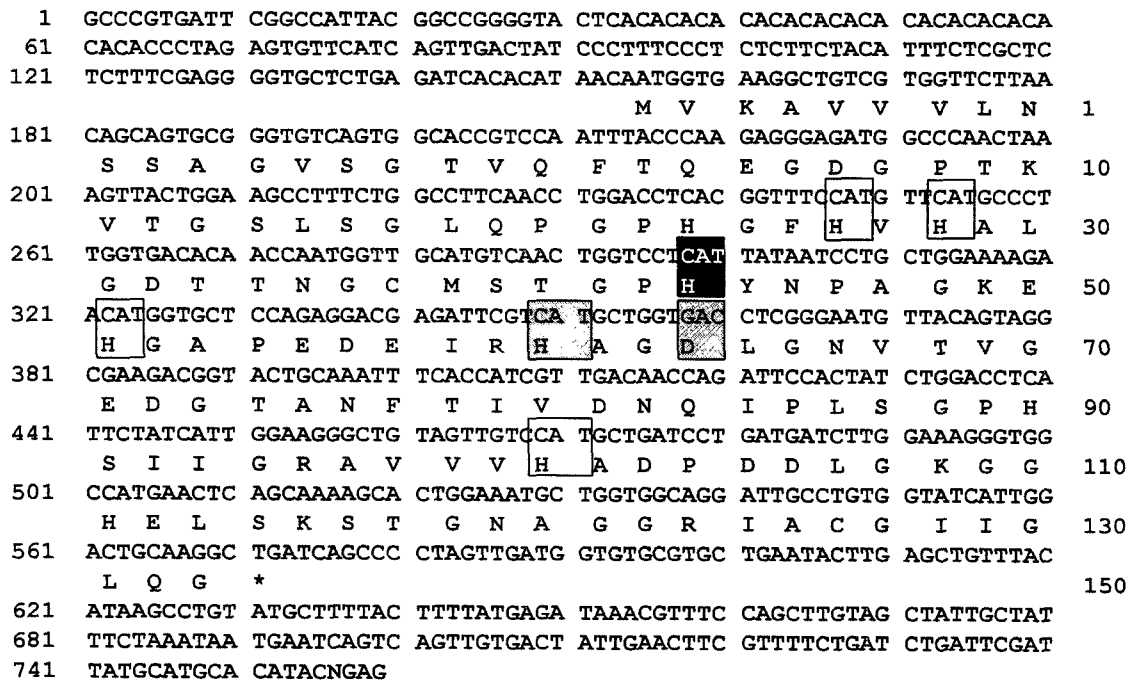

Fig. 2. **Nucleotide and deduced amino acid sequence of *ClSODCc*, CuZn superoxide dismutase cDNA, from *C. lanceolata*.** The positions of nucleotides are shown on the left and the positions of amino acids on the right. Asterisk shows the termination codon. Consensus amino acid residues (His-45, -47,-62, -70, -79, -119, and Asp-82) were involved in Cu-(black letters in white square), Cu/Zn- (white letters in black square), and Zn- (black letters in gray square) binding ligands are indicated.

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 clones 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.*, 2001). 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 wounding- and cold-stress treatments, roots were damaged by cutting them into slices and floating them on MS media for various intervals at either 26℃ or 4℃. The treatments for oxidative stress involved floating the root slices on MS media containing 10 mM H_2O_2 for various lengths of time. After the treatments, the tissues were immediately frozen in liquid N2 and stored at -80 ℃.

Quantitative RT-PCR analysis

The gene-specific primers of *ClSODCc* was designed and used for RT-PCR analysis. Specific primers for coding region of each gene included the following: (*ClSODCc*-forward) 5'-TCC ATG GTA ATG GTG AAG GCT GTC-3'; (*ClSODCc*-reverse) 5'-AGG ATC

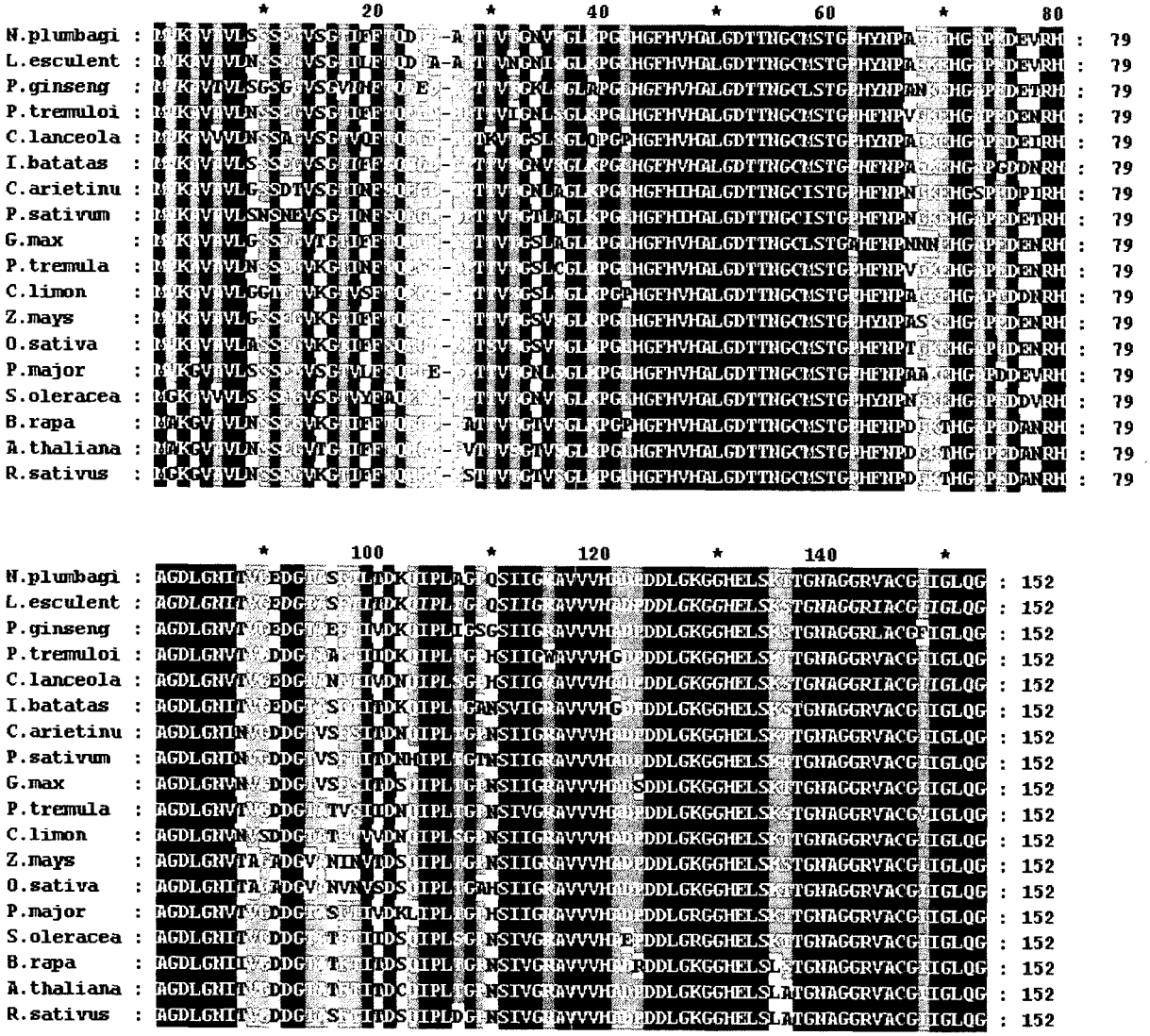

Fig. 3. **Multiple alignment of the deduced amino acid sequence of *ClSODCc* with those of CuZn SOD genes from other plants;** *N. plumbaginifolia*(CAA39444), *L. esculentum*(CAA60826), *P. ginseng*(022668), *P. tremuloides* (AAD01605), *I. batatas*(CAA51654), *C. arietinum*(CAA10160), *P. sativum*(BAC81657), *G. max (Q7M1R5), P. tremula*(CAC33845), *C. limon*(AAQ14591), *Z. mays*(CAB57993), *O. sativa*(P28757), *P. major*(CAH59422), *S. oleracea*(CAA37866), *B. rapa*(AAC25568), *A. thaliana*(AAM14107) and *R. sativus*(AAD05576). Sequence data were obtained from GeneBank and aligned using DDBJ ClustalW (Thompson *et al.*, 1994 and 1997) and GeneDoc (Nicholas *et al.*, 1997).

CAT CAG CCT TGC AGT C-3'. As a control, we used the primers specific to *C. lanceolata* actin gene ClAct1, 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 microgram of total RNA was used for the RT-PCR analysis, according to the method of Takakura *et al.* (2000). The PCR cycles numbered 35 for *ClSODCc* and 33 for *ClAct1*.

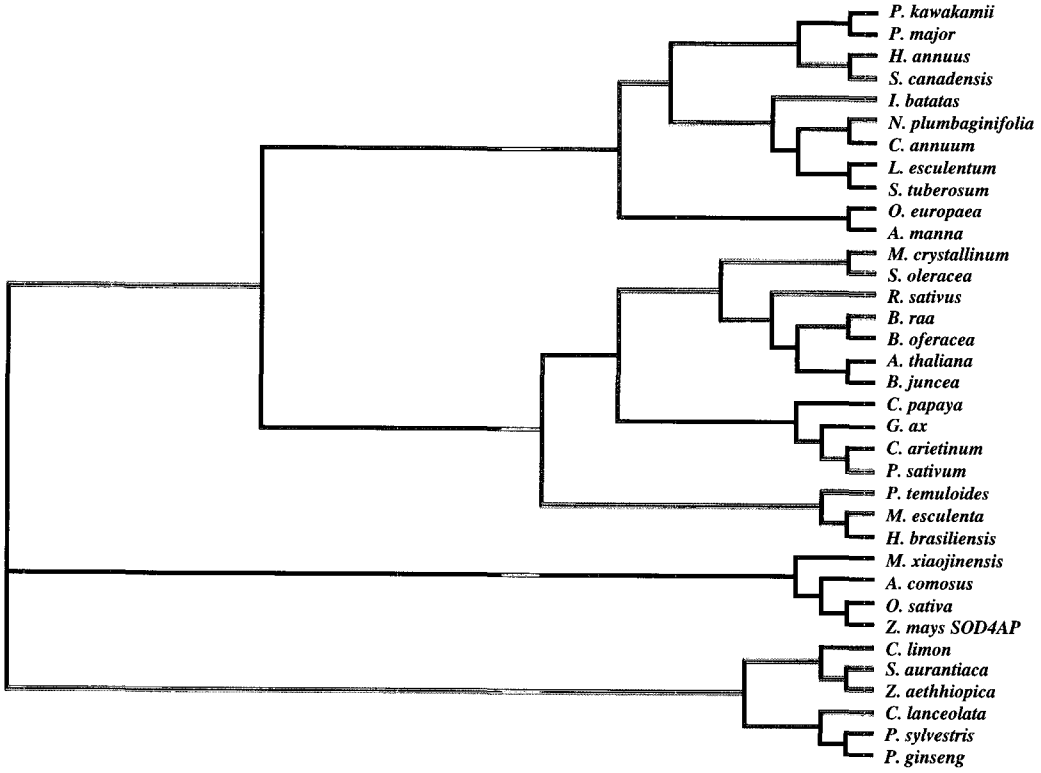

Fig. **4. Unrooted cladogram of the CuZn superoxide dismutase protein family from *C. lanceolata* and other plants.** Phylogenetic analysis is based on the deduced amino acid sequences of CuZnSOD genes from various plant species. The branch lengths are proportional to divergence, with the scale of 0.1 representing 10% change.

RESULTS AND DISCUSSION

The EST clones homologous to CuZn siperoxide dismutase genes were obtained from a cDNA library constructed with 4-year root of *C. lanceolata* and named *ClSODCc*. The *ClSODCc* clone was 799 nucleotides long and contains an open reading frame of 459 bp encoding a deduced 152 amino acid polypeptide (Fig. 2). The sequence context around the translation start site perfectly matched the consensus sequence reported from various plants (AACAATGG, Kaminaka *et al.*, 1997). The N-terminal regions of the SODs are generally divergent and the conserved regions exist in the C-terminus. The deduced amino acid sequences encoded by *ClSODCc* had a strong similarity to the previously described CuZn SODs of other plants. Consensus amino acid residues (His-45, -47,-62, -70, -79, -119, and Asp-82) were involved in Cu-(black letters in white square), Cu/Zn- (white letters in black square), and Zn- (black letters in gray square) binding ligands are indicated (Fig. 2). It has been suggested that the functional role of SOD in protecting cells involves several residues including metal binding sites (His and Asp) in the C-terminal part of the protein. Comparison of *ClSODCc* with other cytosolic CuZnSODs showed a higher homology (82% - 86%; Fig. 3) than chloroplastic CuZnSODs (~60%; data not shown) regardless of species. In the phylogenetic analysis, *ClSODCc* was closer with CuZnSODs of *P. ginseng* and *P. sylvestris* (Fig. 4).

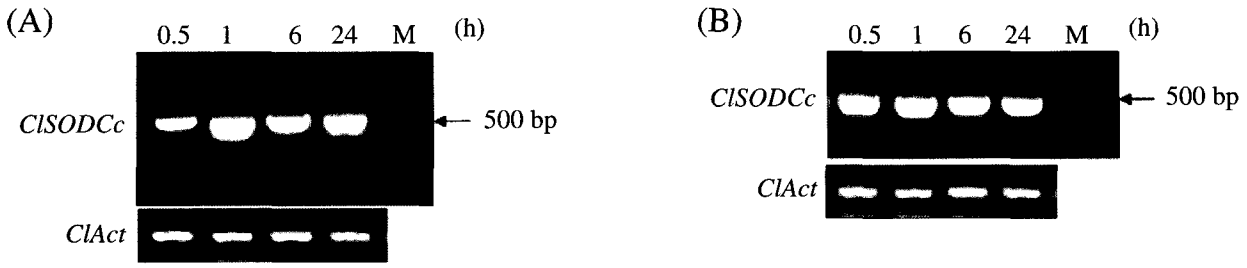

Fig. 5. Expression pattern of *ClSODCc* by oxidative (A) and cold (B) stress. Total RNA from stress-treated samples served as templates for quantitative RT-PCR with gene-specific primers. *ClAct* gene was used for PCR control.

We used semi-quantitative RT-PCR (QPCR) analysis to examine wether the expression of *ClSODCc* gene related with abiotic stresses. Identity of the PCR bands was confirmed by DNA sequence analysis. Expression of the *Codonopsis* actin gene, *ClAct1*, served as an internal control for PCR. Here, expression of *ClSODCc* by oxidative stress was increased up to 1 h after treatment and declined gradually (Fig. 5A). It is related that *ClSODCc* functions in the early stage of ROS detoxification.

In the cold stress treatment, much earlier and stronger expression of *ClSODCc* than in the oxidative stress was observed (Fig. 5B). Somehow the *ClSODCc* should protect the photosynthetic apparatus by efficiently removing the superoxide radicals generated from chloroplasts during the stress. But, the cytosolic form fo CuZnSOD could not scavenge the ROS exited in chloroplast directly because superoxide anion could not transit through the membrane. Thus, it could be presented that *ClSODCc* protected chloroplast external membrane against ROS existed in the cytosol According to the recent scavenging system model, cytosolic SOD changed superoxide anion (although it was not clear that produced from anywhere) into hydrogen peroxide. The hydrogen peroxide was considered as a possible signaling molecule in the signaling pathways (Shigeoka *et al.*, 2002). Therefore, we will further characterize the relations between *ClSODCc* and abiotic stress and then produce the abiotic stress-tolerant transformants by re-introduction of *ClSODCc* into *C. lanceolata*.

ACKNOWLEDGEMENTS

This work was supported by the financial support of the Rural Development Administration made in the BIOGREEN 21 Project.

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(Received Aug. 7, 2004)
(Accepted Sep. 1, 2004)