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Molecular Characterization and Expression Analysis of Ascorbate Peroxidase in *Codonopsis lanceolata* (S. et Z.) Trautv

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ABSTRACT

A cytosolic ascorbate peroxidase, hydrogen peroxide-scavenging enzyme, was characterized from *Codonopsis lanceolata*. The cytosolic ascorbate peroxidase cDNA (*CAPX*) was 983 nucleotides long and possess an open reading frame of 753 bp with 251 amino acids (MW 27.9 kDa) with pI 5.61. The deduced amino acid sequence of *CAPX* shows high homology to other known cytosolic APXs (78~83%), but the *CAPX* was clustered independently from compared ten plant APXs. The *CAPX* gene was highly expressed in leaf and stem tissues, but not in root. When *Codonopsis* leaves cut using scalpel were soaked in 1 mM hydorgen peroxide, the expression of *CAPX* gene was suppressed.

Key words : Ascorbate peroxidase, *CAPX*, *Codonopsis lanceolata*, root, RT-PCR

INTRODUCTION

Ascorbate is one of the most important components in both plants and animals. There have been several investigations on ascorbate metabolism and its function in plants which provide the major source of dietary vitamin C for humans (Noctor and Foyer, 1998; Smirnoff *et al.*, 2001). Ascorbate is oxidized by enzymatic or non-enzymatic reactions. A well-recognized enzyme consuming ascorbate is ascorbate peroxidase (APX), which catalyses the reduction of hydrogen peroxide to water with the simultaneous oxidation of ascorbate with a high specificity.

APX isoenzymes play an important role in eliminating H_2O_2 and are distributed in at least four distinct cell compartments, the stroma (sAPX) and thylakoid membraine (tAPX) in chloroplasts, the microbody (mAPX), and the cytosol (cAPX) (Asada, 1992; Miyake and Asada, 1992; Ishikawa *et al.*, 1998). Plant ascorbate peroxidases are intracellular enzymes encoded in the nucleus (Mittler and Zilinskas, 1991). They function as scavengers of H_2O_2, thereby protecting plant cells from the deleterious effects of H_2O_2 generated as a by-product during photosynthesis and respiration (Dalton, 1991). The enzyme catalyses the reduction of H_2O_2 to water by using ascorbate as an

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electron donor.

The cAPX encoding the APX isozymes were isolated in various plant species and have been well characterized by many research groups (Ishikawa *et al.*, 1995; Santos *et al.*, 1996; Jespersen *et al.*, 1997; Mano *et al.*, 1997; Caldwell *et al.*, 1998). Recent studies have focused on the changes in the cAPX expression level under environmental stresses such as ozone, UV-B radiation, high-light stress, water stress including drought, and pathogen infection (Tanaka *et al.*, 1985; Mishra *et al.*, 1993; Willekens *et al.*, 1994; Conklin and Last, 1995; Kubo *et al.*, 1995; Rao *et al.*, 1996; Mittler *et al.*, 1998). Considering the specific distributions and roles of the APX isoenzymes and the potential for active oxygen species production in each organelle of higher plants, it seems likely that the APX isoenzymes are expressed by distinct regulatory mechanisms. In the present study, to obtain clues for possible diverse roles and functions in *C. lanceolata* root, a ascorbate peroxidase cDNA (*CAPX*) was isolated for the first time and molecular characterization and expression analysis were investigated.

MATERIALS AND METHODS

Plant materials

C. lanceolata cultured during 5 weeks *in vitro* and leaves grown at field were used. Leaves attached in the upper region cut with a scalpel. Cut leaves were soaked in 1 mM H_2O_2 for the oxidative stress during 30 min, 1 hr, 2 hr, and 4 hr, respectively. After the stress treatments, the leaves were immediately frozen in liquid nitrogen and stored at -80℃.

Sequence analysis

The pTriplEx phagemid for ascorbate peroxidase cDNA from cDNA library of *C. lanceolata* root was excised from the λTriplEx2 and used as templates for sequence analysis. The cDNA insert was sequenced using the 5' and 3' sequencing primer by an automatic DNA sequencer (ABI prism 3700). Nucleotide and amino acid sequence analyses were performed using DNASIS program (Hitachi, Japan). Comparison of sequences to DNA and protein databases at NCBI was performed using the blast algorithm of Altschul *et al.* (1990).

Sequence alignment and phylogenetic analysis

We used Clustal W (1.82) with default gap penealties to perform multiple alignment of cytosolic ascorbate peroxidases isolated from *C. lanceolata* and previously registered in other plants (Thompson *et al.*, 1994). Based on this alignment, a phylogenetic tree was constructed according to the UPGMA method.

Total RNA preparation

Total RNA was isolated from the stress treated *C. lanceolata* leaves grown at the field and tissues cultured *in vitro* using the method of guanidine isothiocyanate (TRIzol, Gibco BRL, USA). Each tissue was frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. Extraction reagent was added 1 ml and ground more than 3 min. The mixture was centrifuged for 5 min at 4℃ The supernatant was removed to a new tube, added 0.2 ml chloroform, and vigorously mixed. Following centrifugation, total RNA was precipitated with isopropanol. The pellet was washed once with 75% ethanol, dried in vacuum for 3 min and dissolved in DEPC treated DDW. The total RNA solution was stored at -80℃ for the RT-PCR analysis.

Quantitative RT-PCR analysis

A pair of gene-specific primers of the *CAPX* gene were designed and used for RT-PCR analysis. Specific primers included the following: (*CAPX*-forward) 5'-GTG CTC CTC TGA TAC TGC GAC TCG C-3'; (*CAPX*-reverse) 5'-GAT GAG AGG GTT GAA GGT

CCA CGG C-3'. As a control, we used a pair of the specific primers to *C. lanceolata* actin gene (*CAct*), 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). Two micrograms of total RNA were used for the RT-PCR analysis. The PCR cycles for quantitative RT-PCR numbered 30 for the *CAPX* and the *CAct* genes. RT-PCR products were run on 1.2% (W/V) agarose gel in 0.5×TAE buffer and then photograped for the expression analysis.

RESULTS AND DISCUSSION

Ascorbate peroxidase (APX) is a hydrogen peroxide-scavenging enzyme whose presumed function in to protect the cell from hydrogen peroxide accumulation, particularly under stress condiditons. A ascorbate peroxidase homolog was isolated from the root cDNA library of *C. lanceolata* and named as *CAPX*. The cDNA insert was sequenced using the 5' and 3' sequencing primer by an automatic DNA sequencer. The *CAPX* cDNA was 983 nucleotides long and possess an open reading frame of 753 bp with 31 bp 5'-untranslated region (5'UTR) and 196 bp 3' UTR (Fig. 1). BLASTX database searches with the *CAPX* sequence gave cytosolic ascorbate peroxidase from various organism as the top 100 best matches. The *CAPX* cDNA encodes a protein of 251 amino acids (MW 27.9 kDa) with pI 5.61 (Fig. 2).

The deduced amino acid sequence of *CAPX* cDNA was compared to other related cytosolic ascorbate peroxidase gene products registered previously (Fig. 2). The CAPX amino acid sequence shows high homology to other known cytosolic APXs (78~83%), using the BLAST algorithm, with the closet match being that to *C. annuum* cDNA (AAL83708) (Altschul *et al.*, 1990). Fig. 2 shows a multiple alignment of the *C. lanceolata* CAPX protein and other plant APX homologous molecules available in the GenBank.

A phylogenetic tree was constructed with the *CAPX* and other plant APXs. The *CAPX* showed 83% high similarity with the APX of *C. annuum* (AAL83708), but the *CAPX* was clustered independently from compared ten plant APXs (Fig. 3).

We performed quantitative RT-PCR analysis for the analysis of *CAPX* gene expression in various tissues and against the hydrogen peroxide (H_2O_2). Total RNA was isolated from Leaf, stem, and root of *C. lanceolata* cultured *in vitro*. Two microgram total RNA were used for cDNA synthesis and used for the *CAPX* specific RT-PCR analysis. To identification of the PCR product amplified with *CAPX* specific primers, we were cloned and sequenced for the confirmation of the *CAPX* gene. Expression of the actin gene isolated from *C. lanceolata* served as an internal control for quantitative RT-PCR analysis. The *CAPX* gene was highly expressed in leaf and stem tissues, but not in root (Fig. 4).

C. lanceolata leaves attached petiole were soaked in 1 mM hydrogen peroxide. Total RNA was extracted from each samples and performed the RT-PCR analysis (Fig. 5). We used distilled water as a control. The *CAPX* gene was suppressed by 1 mM hydorgen peroxide than control. When *Codonopsis* leaves cut using scalpel were soaked in water, the expression of *CAPX* gene was increased at 1 hr to 4 hr after treatment (Fig. 5). Hydrogen peroxide functions as a second messenger to regulate the gene expression of some antioxidative enzymes in plant cells, and a transient increase of H_2O_2 is observed during the early stages under oxidative stress conditions (Foyer *et al.*, 1997). The H_2O_2 is controlled by cAPX suppression during pathogen infection (Mittler *et al.*, 1998). It suggests the possibility that the instability of cAPX might have functions related to stress signalling.

APX has been identified in many higher plants and comprises a family of isoenzymes with different characterics. Many isoenzymes of guaiacol peroxidase (GP) in plant tissues are localized in vacuoles, the cell

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CCAATCTGCTCAAAGGAACTAGTTGATTACCATGGGAAAGTGTTATCCAACTGTGACTGA      60
                                  M  G  K  C  Y  P  T  V  T  E      10

GGAATACGAAAAAGCAGTTGAAAAGTGTAAGAAGAAGCTCAGGGGTCTCATTGCTGAGAA     120
 E  Y  E  K  A  V  E  K  C  K  K  K  L  R  G  L  I  A  E  K      30

GAAATGTGCTCCTCTGATACTGCGACTCGCATGGCACGCAGCAGGTACTTATGACTACAA     180
 K  C  A  P  L  I  L  R  L  A  W  H  A  A  G  T  Y  D  Y  K      50

GACTAAGACCGGAGGGCCATTTGGGACAATTAGAAGTCCAGAGGAGCTTTCTCATGCAGC     240
 T  K  T  G  G  P  F  G  T  I  R  S  P  E  E  L  S  H  A  A      70

CAACAACGGCCTTGATATTGCCGTGAGGTTGTTGGAGCCAATCAAGCAGCAGTTCCCTAT     300
 N  N  G  L  D  I  A  V  R  L  L  E  P  I  K  Q  Q  F  P  I      90

CCTCTCATATGCAGACTTTGACCAATTGGCTGGAATTGTTGCTGTGGAAGTTACAGGTGG     360
 L  S  Y  A  D  F  D  Q  L  A  G  I  V  A  V  E  V  T  G  G     110

GCCGGAGATCCCTTTTCATCCTGGGAGAGAGGACAAAACTAAACCACCTCCCGAAGGCCG     420
 P  E  I  P  F  H  P  G  R  E  D  K  T  K  P  P  P  E  G  R     130

GTTGCCTAATGCCACTAAAGGCACAGACCATCTAAGACAAGTATTTGGCCACCAGATGGG     480
 L  P  N  A  T  K  G  T  D  H  L  R  Q  V  F  G  H  Q  M  G     150

CCTTAGTGATCAAGATATTGTTACTCTATCCGGTGGTCACACTCTGGGAAGGTGCCACAA     540
 L  S  D  Q  D  I  V  T  L  S  G  G  H  T  L  G  R  C  H  K     170

GGAAAGGTCTGGATTCGAAGGGCCGTGGACCTTCAACCCTCTCATCTTTGATAACTCATA     600
 E  R  S  G  F  E  G  P  W  T  F  N  P  L  I  F  D  N  S  Y     190

CTTTAAGGAACTTCTTGCTGGAGAAAAGGAAGGGTTGCTTCAGCTGCCAACAGACAAGGT     660
 F  K  E  L  L  A  G  E  K  E  G  L  L  Q  L  P  T  D  K  V     210

TCTTTTAGAGGATCCAGTTTTTCGTCCTCTTGTTGAGAAGTACGCAGCGGATGAGGAAGC     720
 L  L  E  D  P  V  F  R  P  L  V  E  K  Y  A  A  D  E  E  A     230

TTTCTTCAGAGACTATGCTGAATCTCACTTGAAGCTGTCAGAGCTCGGATTCGCGGAGGC     780
 F  F  R  D  Y  A  E  S  H  L  K  L  S  E  L  G  F  A  E  A     250

TGAGTAAGGGGATGGAAGAAGGCCGTATTTGTATTTTAAGAAATCTTTAGAATGCTGGTA     840
 E  *

TTTCTATACTATATTTGATATGTTATTGTGCTATTAACAATTATATGCAAGTCTATAGCC     900
TGAATATTTGATGTTCCCATGTGTGGNAATGTGTCTAAACAGAATGTATTACAATTACAT     960
AATCTTTACCCGTATTATGATTT                                          983
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Fig. 1. **Nucleotide sequence of *CAPX* and deduced amino acid sequences.** 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 AB243015.

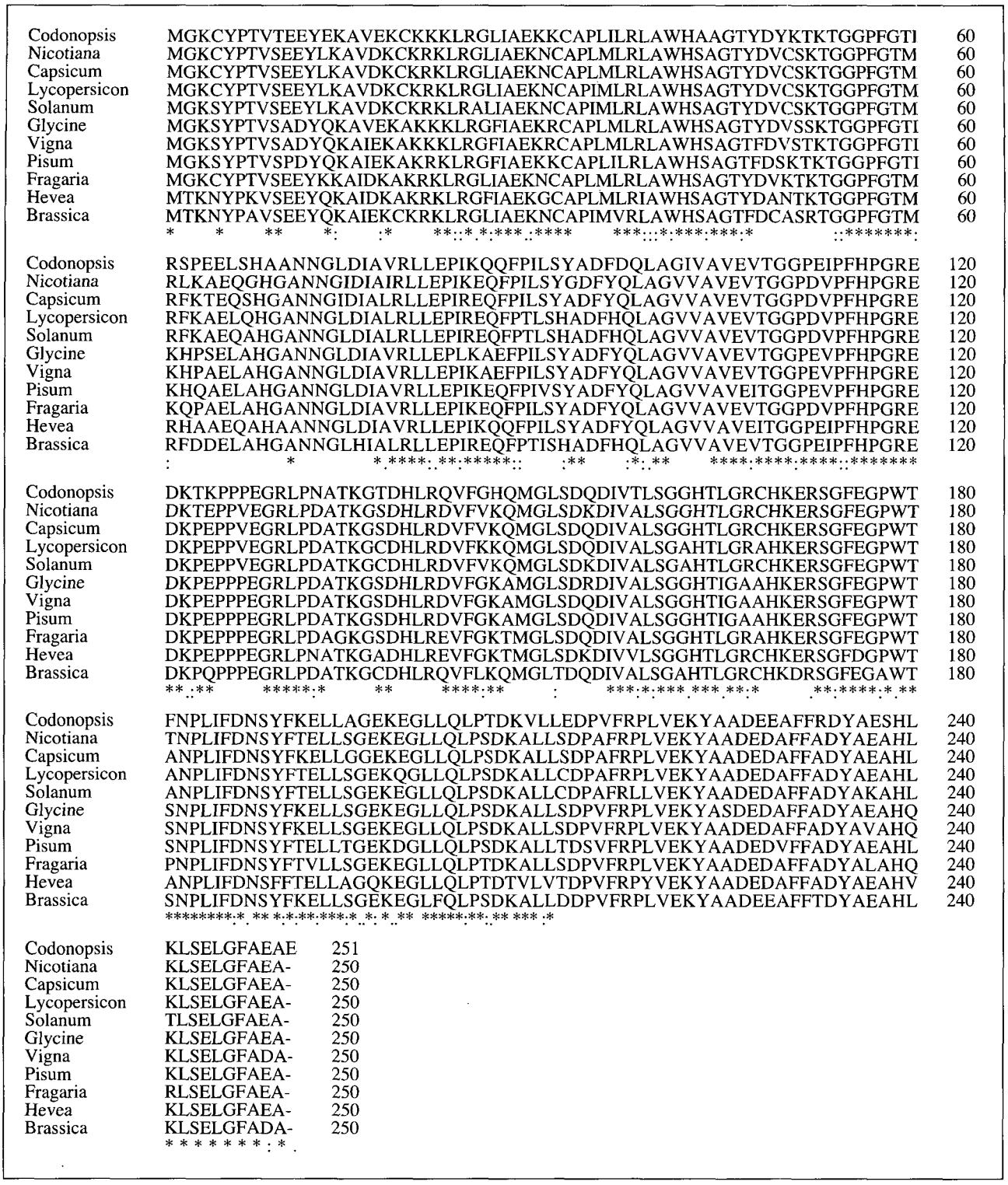

Fig. 2. **Alignment of the deduced amon acids sequences of ascorbate peroxidase from *C. lanceolata* (AB232686) with cytosolic ascorbate peroxidase from other plants;** *F. x ananassa* (AAD41405), *N. tabacum* (AAA86689), *C. annuum* (AAL83708), *P. sativum* (CAA43992), *G. max* (BAC92740), *H. brasiliensis* (AAO14118), *V. unguiculata* (AAB03844), *L. esculentum* (CAB58361), *B. oleracea* (BAB84008), *S. tuberosum* (ABB02650).

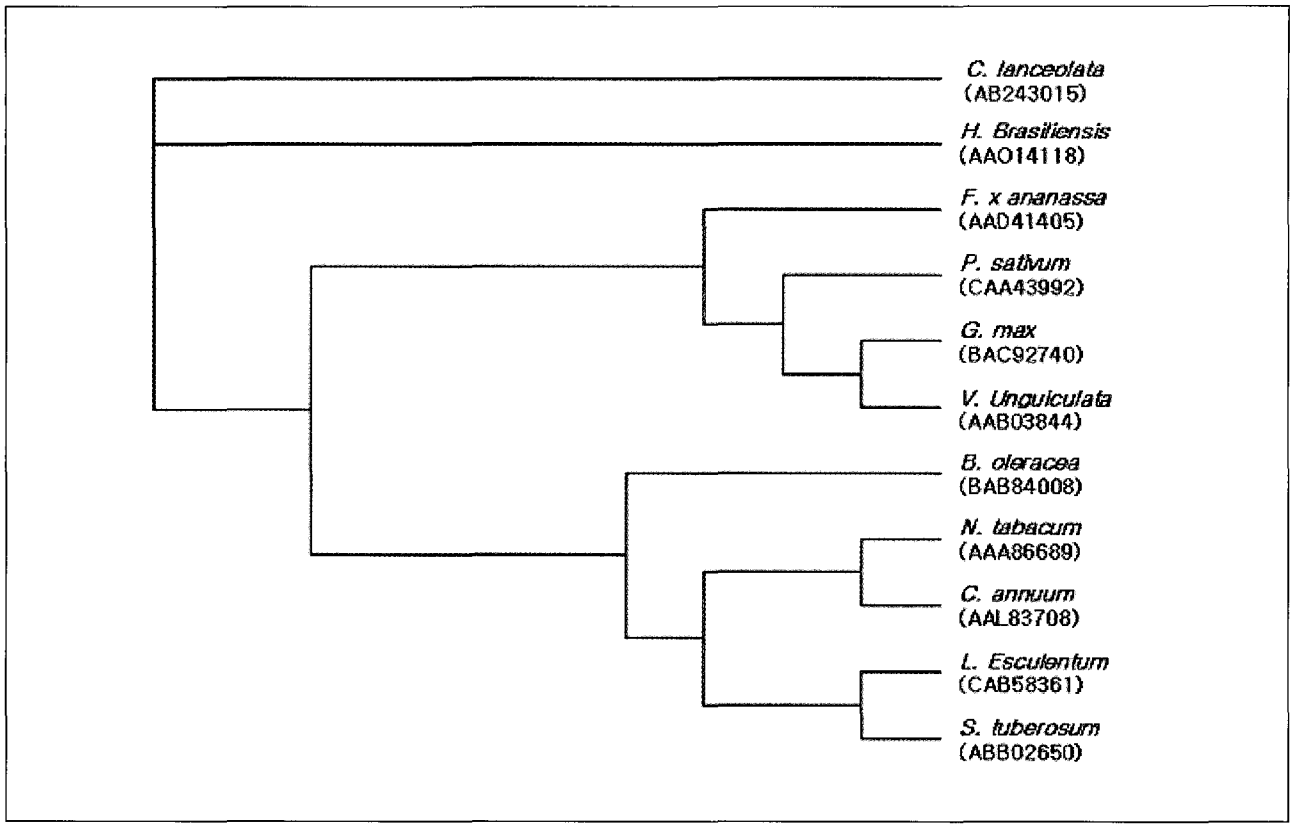

Fig. 3. **Phylogenetic relationship of the cytosolic ascorbate peroxidase protein family from *C. lanceolata* and other plants.** Phylogenetic analysis is based on the deduced amino acid sequences of peroxidase genes from various plant species. The branch lengths are proportional to divergence, with the scale of 0.1 representing 10% change.

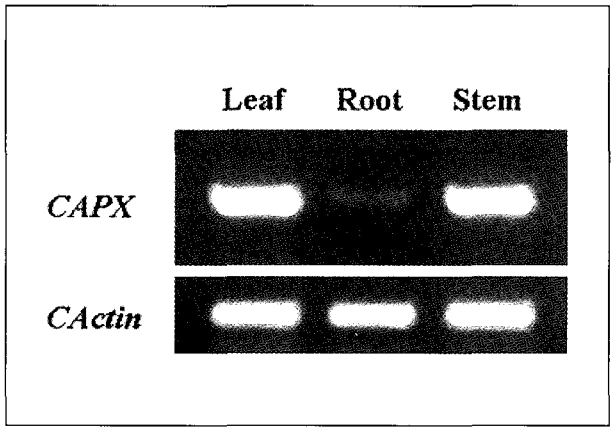

Fig. 4. **RT-PCR analysis of *C. lanceolata* leaf, stem and root.** Total RNA from each tissue served as templates for quantitative RT-PCR with *CAPX* specific primers. Actin gene isolated from *C. laceolata* was used for RT-PCR control.

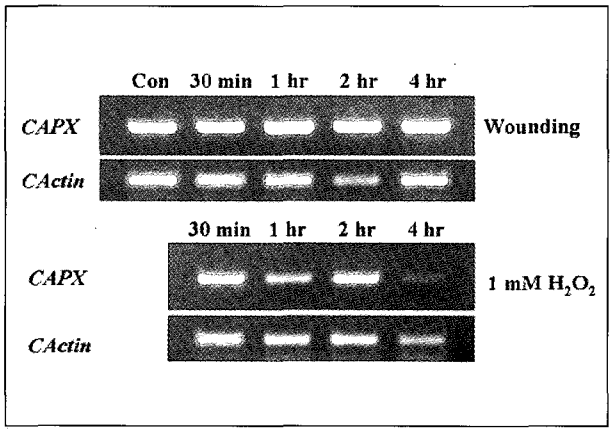

Fig. 5. **Expression pattern of *CAPX* under the stress of 1 mM hydrogen peroxide.** Total RNA from each sample served as templates for quantitative RT-PCR with *CAPX* specific primers. Actin gene isolated from *C. laceolata* was used for RT-PCR control.

wall, and the cytosol, but not in organelles (Asada, 1992). Generally, APX activities increase along with activities of other antioxidant enzymes like catalase, SOD, and GSH reductase in response to various environmental stress factors, suggesting that the components of AOS-scavenging systems greater induction of APX activity than sensitive plants, suggesting that the components of AOS-scavenging systems are co-regulated. Drought-resistant maize shows greater induction of APX activity than sensitive plants, in addition to a significant increase in GSH reductase activity (Pastori and Trippi, 1992).

In the present study, we described the isolation and characterization of ascorbate peroxidase (*CAPX*) gene from *C. lanceolata*. The CAPX gene was abundantly expressed in leaf and stem tissues. When leaves of *C. lanceolata* was treated with 1 mM H_2O_2, the CAPX was suppressed. The instability of cAPX might have functions related to oxidative stress signalling. The functions of cAPX in plants are still largely unknown. Therefore, we will study continuously to understand the physiological function of *CAPX* regulation in *C. lanceolata*.

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