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Cloning of a cytosolic ascorbate peroxidase gene from *Lycium chinense* Mill. and enhanced salt tolerance by overexpressing in tobacco

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ABSTRACT

To evaluate the physiological importance of cytosolic ascorbate peroxidase (APX) in the reactive oxygen species (ROS)-scavenging system, a full-length cDNA clone, named LmAPX, encoding a cytosolic ascorbate peroxidase was isolated from *Lycium chinense* Mill. using homologous cloning, then the expression of LmAPX under salt stress was investigated. After sequencing and related analysis, the LmAPX cDNA sequence was 965 bp in length and had an open reading frame (ORF) of 750 bp coding for 250 amino acids. Furthermore, the LmAPX sequence was sub-cloned into prokaryotic expression vector pET28a and the recombinant proteins had a high expression level in *Escherichia coli*. Results from a southern blot analysis indicated that three inserts of this gene existed in the tobacco genome encoding LmAPX. Compared with the control plants (wild-type and empty vector control), the transgenic plants expressing the LmAPX gene exhibited lower amount of hydrogen peroxide (H_2O_2) and relatively higher values of ascorbate peroxidase activity, proline content, and net photosynthetic rate (P_n) under the same salt stress. These results suggested that overexpression of the LmAPX gene could decrease ROS production caused by salt stress and protect plants from oxidative stress.

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1. Introduction

Plant cells produce a large number of reactive oxygen species (ROS) under biotic and abiotic stresses. Soil salinity is one of the most serious environmental stresses that disrupt the metabolic balance of cells and enhance the production of ROS (Wang et al., 2003). Under normal conditions, ROS have key functions in the oxidative signaling pathway and affect many cellular functions (Apel and Hirt, 2004). Therefore, the generation and removal of excessive ROS are of a dynamic balance. When plants grow under stress conditions, this balance may be destroyed. Excess ROS are generally toxic and can cause damage to nucleic acids, lipids and proteins (Møller, 2001). To scavenge these excess ROS, plants have developed a complex antioxidative defense system including antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT), as well as non-enzymatic compounds, such as ascorbic acid, betaine, proline and so on (Asada, 2006).

Hydrogen peroxide (H_2O_2), the most stable form among ROS, acts as a precursor of more cytotoxic reactive oxygen derivatives and can also severely damage plant growth and metabolism under stress conditions (Ashraf, 2009). Among antioxidant mechanisms, the ascorbate peroxidase plays an important role in the metabolism of H_2O_2, along with catalase, a central enzyme in hydrogen peroxide scavenging. Ascorbate peroxidases (APX; EC 1.1.1.11) are responsible for the reduction of H_2O_2 to water at the expense of ascorbate as a specific electron donor (Asada, 1997; Foyer and Noctor, 2000). In many higher plants, APX comprises a family of isoenzymes located in the cytosol, peroxisomes, mitochondria and the chloroplasts. The isoforms are thus named cytosolic APX (cAPX), peroxisomal or microbody APX (pAPX), mitochondrial APX and chloroplastic APX, such as thylakoidal and stromatic APX (tAPX and sAPX), respectively. The cDNA sequence encoding the APX isoenzymes has now been isolated and characterized from various plant species (Shigeoka et al., 2002). In recent years, cytosolic APX isoenzymes, taken as the front line of plant defense against H_2O_2, have been studied extensively. In rice, the transcripts of two ascorbate peroxidase (OsAPX1 and OsAPX2) genes were up-regulated upon diverse environmental cues, and they have a more general stress-protective function in stress-signaling pathways (Agrawal et al., 2003). In sweet

Abbreviations: APX, ascorbate peroxidase; cAPX, cytosolic APX; CAT, catalase; CTAB, N-cetyl-N, N, N-trimethylammonium bromide; H_2O_2, hydrogen peroxide; IPTG, isopropyl β-D-thiogalactopyranoside; MS, Murashige and Skoog; ORF, open reading frame; pAPX, peroxisomal APX; P_n, photosynthetic rate; PS II, photosystem II; ROS, reactive oxygen species; sAPX, stromatic APX; SOD, superoxide dismutase; tAPX, thylakoidal APX; UTR, untranslated region.

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potato, the cAPX gene is expressed in cultured cells and mature leaves, but not in stems, non-storage or storage roots. Also, it was highly induced in leaves by wounding and treatment with methyl viologen, hydrogen peroxide, abscisic acid or exposure to high temperature (Park et al., 2004). In muskmelons, the cAPX gene exhibited highly tissue-specific patterns of expression and enzyme activity assays showed that the cAPX might play an important role in the pathogenesis of powdery mildew (Cheng et al., 2009).

Lycium chinense Mill. (*L. chinense*) is a solanaceous defoliated shrub that grows in China and other parts of Asia. The fruit of *L. chinense*, known as *Fructus lycii* or Chinese wolfberry, has been regarded as an ingredient in the traditional herbal medicine with functions of nourishing the kidney and the liver (Amagase and Farnsworth, 2011). Valuable components of *L. chinense* are not limited to its high level of carotenoids, but include the polysaccharides and small molecules such as antioxidant enzymes and betaine. But until now, studies on the role of cAPX gene from *L. chinense* have not been reported.

To investigate the function of the ascorbate peroxidase in the scavenging of H_2O_2 and to further elucidate roles of APX in salt stress tolerance in plants, we isolated a cAPX cDNA from *L. chinense*, named LmAPX, and then generated transgenic tobacco plants with the LmAPX cDNA. Our results demonstrated that expression of LmAPX in tobacco led to an enhanced tolerance to salt stress due to the increase in APX activity and the decrease in the accumulation of H_2O_2.

2. Materials and methods

2.1. Cloning the full-length sequence of the LmAPX cDNA

Leaves of *L. chinense* were used for the total RNA isolation with the Trizol method (Invitrogen, Carlsbad, CA, USA). The first strand cDNA was synthesized from 1 μg total RNA using 3′-Full RACE Core Set Ver.2.0 (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instruction. 1 μL of the reversed transcription cDNA was used in the PCR with the following cycling steps: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; then an additional polymerization step at 72 °C for 10 min. Primers F1 and Q_{out} (in the 3′ RACE System Kit, TaKaRa Biotechnology, Dalian, China) were used to amplify the 3′-end of the LmAPX cDNA. The amplified products were cloned into pMD-18T vector (TaKaRa, Dalian, China) and sequenced. The specific primer F1 (5′-AGGATATTGTTGCACTCTCTGGTG-3′) and degenerate primer F2 (5′-CAATTA/GCTATGGGTAAGT-3′) were designed based on the conserved regions of the cytosolic APX cDNAs (*Nicotiana tabacum*, U15933; *Capsicum annum*, DQ002888; *Solanum lycopersicum*, DQ096286 and *Solanum tuberosum*, AB041343). It was worth mentioning that the sequences of primer F1 and primer F2 were in the coding region and the 5′ untranslated region (5′-UTR) of the conserved cytosolic APX cDNAs, respectively. Based on the sequence of the putative partial cDNA, additional reverse primer R1 (5′-GCCACT ACTCCCACCCT-3′) was designed to amplify the complete coding region of the APX cDNA. Finally the complete coding region cDNA (obtained by PCR using primers F2 and R1) was inserted into the pMD18-T, sequenced and named LmAPX.

2.2. Expression and purification of the recombinant LmAPX in Escherichia coli

The complete coding region of LmAPX was amplified with forward (LmAPX-MF: 5′-CGCGGATCCATGGGTAAGTGCTATCCTA-3′) and reverse (LmAPX-MR: 5′-ACGCGTCGACGTAGGATAGCACTTACCCAT-3′) primers. The sequences underlined are the recognition sites of the restriction enzymes *Bam*HI and *Sal*I, respectively. The amplified products were digested with *Bam*HI and *Sal*I, and inserted into the pET28a vector (TaKaRa, Dalian, China), which was treated with the same restriction enzymes. The successful transformation of the obtained plasmid was verified using DNA sequencing and named pET28a-LmAPX.

E. coli BL21 (DE3) was used for the expression of the pET28a-LmAPX. The cells were cultured at 37 °C in LB medium until OD_{600} reached 0.5. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 1 mM and incubation was continued for a further 16 h at 28 °C. The bacteria were harvested by centrifugation and lysed by sonication. The sample of soluble proteins after filtering through a 0.45 μm syringe filter was loaded into a Ni-Agarose affinity column to purify the crude proteins according to the operation manual of 6× His-Tagged Protein Purification Kit (Beijing CoWin Bioscience Co, Ltd., China). The total bacterial proteins and purified proteins were then subjected to 12% SDS-PAGE for protein separations.

2.3. Construction of plant transformation vector and tobacco transformation

The LmAPX gene was obtained from the pET28a-LmAPX using digestion with *Bam*HI/*Sal*I. The cleaved fragment was cloned in the pCAMBIA2300 binary vector to give rise to pCAMBIA2300-LmAPX, where the LmAPX gene was kept under control of CaMV 35S promoter with npt II as the selectable marker (Fig. 3A). Then the LmAPX expression cassette and the empty vector pCAMBIA2300 were transformed into tobacco (*N. tabacum* cv. SR-1) via Agrobacterium-mediated transformation as described by Hoekema et al. (1983).

2.4. PCR and southern blot analysis

The genomic DNA was extracted from young leaves of wild-type (WT) and empty vector (EV) control plants and from LmAPX-overexpressing lines by the CTAB (N-cetyl-N, N, N-trimethylammonium bromide) method (Porebski et al., 1997). Putative transformants were screened by the PCR analysis using the genomic DNA as the template. The amplification was carried out in the presence of 5′ and 3′ primers of LmAPX.

For the southern blot analysis, genomic DNA (20 μg) was digested with *Eco*RI, blotted, and probed using the LmAPX cDNA according to the manufacturer's instructions of the Dig Detection Kit II (Roche Co., Germany).

2.5. Evaluation of transgenic plants exposed to salt stress

Sterilized seeds of T0 generation transgenic lines (L6 and L8) were germinated in the presence of kanamycin (100 mg/L). The surviving seedlings (7 days old) were transferred to Murashige and Skoog (MS) medium supplemented with 0, 50, 100, 150 or 200 mM NaCl to simulate the salinity stress. WT and EV seeds which served as the experimental controls were germinated on the same medium without or with kanamycin added. The seedlings were maintained under the culture room conditions, and their growth was monitored for 25 days under this stress. At the end of this treatment, total chlorophylls were extracted with 96% ethanol and were estimated according to Wintermans and de Mots (1965).

To obtain further evidence that whether the overexpression of LmAPX could confer resistance to salt stress, another similar set of plants were examined by irrigation of two-month-old seedlings supplemented with or without 100 or 200 mM NaCl for 4 weeks at 3-day intervals. At the end of this salt treatment, seedlings were subjected to measurements of photosynthesis parameters, contents of H_2O_2, proline and the APX activity.

The net photosynthesis rate (P_n) was measured by a portable photosynthesis system (LI-6400, LI-COR, USA) in the fourth and fifth leaves from the apex. Measurements were made in the morning (600 photons μmol m^{-2} s^{-1} average PAR) on the attached leaves of all plants. The photosystem II (PS II) activity was determined using a portable modulated fluorometer, and expressed as F_v/F_m to assess the damage to leaf photosynthetic apparatuses. Hydrogen peroxide was measured as described by Alexieva et al. (2001). Proline content in leaves was determined following the protocol described by Bates et al. (1973). The APX activity was measured spectrophotometrically

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