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Research article

Cloning, heterologous expression and characterization of ascorbate peroxidase (APX) gene in laticifer cells of rubber tree (*Hevea brasiliensis* Muell. Arg.)

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ABSTRACT

Ascorbate peroxidases (APXs) are a kind of crucial enzymes for removing reactive oxygen species (ROS) in plant cell. In the present study, a full-length cDNA encoding an APX, designated *HbAPX*, was isolated from *Hevea brasiliensis* by the rapid amplification of cDNA ends (RACE) method. *HbAPX* was 1174-bp in length and contained a 912-bp open reading frame (ORF) encoding a putative protein of 304 amino acids. The predicted molecular mass of HbAPX was 27.6 kDa (kDa) with an isoelectric point (pI) of 6.73. The phylogenetic analysis showed that HbAPX belonged to the cytosolic subgroup and was more relative to PtAPX and MdAPX2. By using PlantCare online analysis, such *cis*-acting elements as W-box and MRE were detected in the promoter region of *HbAPX*. Overproduction of recombinant HbAPX protein either in *Escherichia coli* or yeast enhanced their tolerance to such abiotic stresses as Cu^{2+}, Zn^{2+}, Na^{2+} and hydrogen peroxide (H_2O_2). Ethrel application significantly down-regulated the expression of *HbAPX* and inhibited the activity of HbAPX *in vivo*. The ethrel-caused down-regulation of HbAPX may disturb the redox homeostasis in laticifer cells of rubber tree.

1. Introduction

Reactive oxygen species (ROS) are a kind of chemically reactive molecules such as hydroxide peroxide, superoxide and hydroxide radicals. ROS are generated in several biological processes and the accumulation of high level of ROS occurs upon various biotic and abiotic stresses (Apel and Hirt, 2004). Plant has evolved highly regulated mechanisms to keep the balance between ROS production and destruction (Halliwell, 2006). The ascorbate-glutathione (AsA-GSH) cycle has been regarded as the most important defense mechanism for the resistance of plants to oxidative stress (Nazar et al., 2015). Ascorbate peroxidases (APXs) are a kind of key enzymes in AsA-GSH cycle, which are highly specific to ascorbate and have a clear physiological role as an electron source for detoxifying H_2O_2 to water in plant cell (Lad et al., 2002; Nishikawa et al., 2005; Ishikawa and Shigeoka, 2008; Kovacs et al., 2013). Crystal structure of APX identifies two different substrate binding sites, one for ascorbate and another for aromatic substrates (Sharp et al., 2003). Genome-wide analysis shows APX isoenzymes are of a multigene family. For example, there are 9 members in Arabidopsis, 8 in rice, and 7 in tomato (Chew et al., 2003; Teixeira et al., 2004; Najami et al., 2008). The APXases specifically locate into chloroplasts, mitochondria, peroxisomes, cytosol and apoplast (Apel and Hirt, 2004). The compartmentalized isoforms have specific roles. For example, the chloroplastic APXs mainly protect the photosynthetic system from oxidative stress while the mitochondrial APXs detoxify H_2O_2 produced byβ-oxidation of fatty acids (Khanna-Chopra et al., 2011; Caverzan et al., 2014). The cytosolic APXs have a general stress-protective role in defending against biotic and abiotic stresses (Zhang et al., 2013; Wu et al., 2014).

Rubber tree (*Hevea brasiliensis*) is the main source of natural

Abbreviations: 5-ALA, 5-aminolevulinic acid; APX, Ascorbate peroxidase; AsA-GSH, Ascorbate-glutathione; EU, Extension unit; GS/GOGAT, Glutamine synthetase/glutamate synthase; H_2O_2, Hydrogen peroxide; INA, 2,6-dichloroisonicotinic acid; IPTG, Isopropyl-beta-D-thiogalactopyranoside; kDa, kilodalton; LB, Luria Bertani; ORF, Open reading frame; PBS, Phosphate buffer saline; pI, Isoelectric point; QRT-PCR, Quantitative real-time polymerase chain reaction; RACE, Rapid amplification of cDNA ends; ROS, Reactive oxygen species; SC, Synthetic complete medium; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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rubber due to the good yield and excellent physical properties of its rubber products (Eschbach and Lacrotte, 1989). Natural rubber biosynthesis takes place in the cytoplasm of highly specialized laticifer cells. In natural rubber production, latex (essentially the cytoplasm of laticifer cells) is obtained by recurrent tapping, a severe mechanical wounding (Tang et al., 2010). In addition to tapping, ethrel (an ethylene releaser) is often used to increase rubber yield per tapping, mainly contributing to its effect on prolonging the duration of latex flow (Zhu and Zhang, 2009). Available data show that mechanical wounding has effect on local and systemical ROS generation via the octadecanoid pathway in plant (Orozco-Cardenas and Ryan, 1999) and the generated ROS have a role in amplifying the ethylene signaling cascade (Xia et al., 2015; Steffens, 2014; El-Maarouf-Bouteau et al., 2015). Therefore, the redox homeostasis in laticifer cells of rubber tree maybe disturbed by successive tapping and ethrel application. In the present study, a novel ascorbate peroxidase gene (designated as *HbAPX*, GenBank Accession Number: KR080580.1) was cloned and characterized to understand the influence of ethrel application on the redox homeostasis of laticifer cells in rubber tree.

2. Materials and methods

2.1. Plant materials and treatments

Plantlets of rubber tree clone CATAS7-33-97 budded on rootstocks were grown on the Experimental Farm of Chinese Academy of Tropical Agricultural Sciences (CATAS) on Hainan Island. Both ten-years-old tapping tree and one-years-old epicormic shoots were used in current study. For epicormic shoots, the plantlets were pruned each year and epicormic shoots grew from the latent buds on the pruned trunks. The epicormic shoot flushes five to six times a year and therefore consists of a series of foliage clusters, separated by lengths of leafless stem (Hao and Wu, 2000). Each of these morphologically distinct growth increments represents a growth flush, and is referred to as an extension unit (EU) (Hao and Wu, 2000). 0.5% ethrel and water (as control) were applied either at the site of central part of epicormic shoot of EU2 (counting from top to bottom) or at the trunk of the trees of being tapped. The latex samples were respectively collected one hour (1 h), four hours (4 h), eight hours (8 h), one day (1 d), two days (2 d), three days (3 d), five days (5 d) after treatments for epicormic shoots, or two days (2 d) and three days (3 d) after treatments for the tapped trees. The latex samples from three of the epicormic shoots or tapping tree were mixed, and used to extract total RNA and determine activity of HbAPX.

2.2. Latex ultracentrifugation and C-serum isolation

The separation of latex into rubber and non-rubber zones was performed by ultracentrifuging at 53, 000 g for 1 h at 4 °C (Chow et al., 2012). The intermediate weight fraction was C-serum (Wang et al., 2013). The protein concentration of C-serum was determined according to the method described by Bradford (1976).

2.3. RNA, DNA isolation and cDNA synthesis

Total latex RNA was extracted as described (Tang et al., 2010) and genomic DNA from leaf was extracted according to the manufacturer instructions for the TIANcombi DNA Lyse&Det PCR Kit (TIANGEN BIOTECH, China). The quality and concentration of the extracted RNA and DNA were evaluated by NanoDrop 2000 (Thermo Scientific Inc., USA). Approximately 1 μg of RNA was used for reverse transcription based on the introduction of RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific Inc., USA).

2.4. Cloning of HbAPX

Based on the unigene18915 from a RNA-Seq library of latex (NCBI accession numbers: GSE59981), a pair of specific primers (5F: AAG CAG TGG TAT CAA CGC AGA GT; 5R: GTC GGC ACC ATC CTC TA) were designed for 5-RACE and another pair of primers (3F: ATT GCT GAT CCC GTC TT; 3R: TAC GTT TTT TTT TTT TT) for 3-RACE reaction by using SMARTer RACE 5′/3′ Kit (Clontech, Palo Alto, CA). A full-length cDNA was assembled by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and thereafter amplified with a pair of gene-specific primers (F: ATG ACC AAG AAC TAC CCA AAA; R: TTA GGC CTC AGC AAA TCC CAG C). The PCR was performed using PrimeSTAR® HS DNA Polymerase (TaKaRa Biotechnology, Japan) with the following conditions: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 3 min, with a final extension at 72 °C for 10 min. The PCR product was cloned into the pMD18-T vector (TaKaRa Biotechnology, Japan) and sequenced.

2.5. Cloning of promoter region of HbAPX

The promoter region of *HbAPX* was isolated by PCR-based Genome Walking according to the manufacturer instructions for the GenomeWalkerTM Universal Kit (Clontech, Palo Alto, CA).

2.6. Multiple sequence alignments and in-silico analyses

The sequence comparison was conducted through database search using BLAST program (http://www.ncbi.nlm.nih.gov). Multiple alignments were carried out using the MEGA software version 4.0 based on 14 protein sequences. The sequences were retrieved from 10 species such as *Malus domestica* (MdAPX2, XP_008350397.1; MdAPX3, XP_008394024.1), *Populus trichocarpa* (PtAPX, EEE87461.1), *H. brasiliensis* (HbAPX, KR080580.1), *Oryza sativa* (OsAPX1, Q10N21.1; OsAPX5, P0C0L0.1), *Zea mays* (ZmAPX1, NP_001150192.1; ZmAPX3, NP_001148710.1), *Arabidopsis thaliana* (AtAPX1, Q05431.2; AtAPX3, Q42564.1); *Solanum lycopersicum* (SlAPX2, NP_001234788.1; SlAPX3, XP_004232505.1), *Cucumis sativus* (CsAPX6, XP_004149799.1) and *Vitis vinifera* (VvAPX6, XP_002285865.1). The molecular phylogeny was constructed from a complete protein sequence alignment of APXs by the neighbor-joining method with bootstrapping analysis (1000 replicates). The molecular mass and isoelectric point (pI) of HbAPX was predicted by DNAMAN. The conserved domain of HbAPX was predicted using the online tool Conserved Domain Search Service of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). *Cis*-acting elements that were analyzed online using PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.7. Plasmid construction and transformation

Expression vectors for *HbAPX* ORF were constructed using both pET-28a for prokaryotic system and pYES2 for eukaryotic system. Briefly, restriction sites of BamHI and EcoRI were introduced by PCR at the 5- and 3-end of the *HbAPX* ORF. The amplified fragments were sequenced and digested with BamHI and EcoRI, and then ligated into the digested pET-28a and pYES2 vectors with the same restriction endonuclease, respectively. According to the standard methods, pET-HbAPX and pYES2-HbAPX expression plasmid were transformed into the *Escherichia coli* BL21 (DE3) strain and yeast INVSC1, respectively.

2.8. Purification of recombinant HbAPX

The BL21 cells with pET-HbAPX plasmid was grown at 37 °C in 20 mL of Luria Bertani (LB) medium containing 100 mg/L