Journal of Plant Physiology 175 (2015) 183–191

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Molecular Biology

A peroxisomal APX from *Puccinellia tenuiflora* improves the abiotic stress tolerance of transgenic *Arabidopsis thaliana* through decreasing of H_2O_2 accumulation

Qingjie Guan[a,b], Zhenjuan Wang[a], Xuhui Wang[a], Tetsuo Takano[c], Shenkui Liu[a,*]

[a] *Key Laboratory of Saline-Alkali Vegetation Ecology Restoration in Oil Field (SAVER), Ministry of Education, Alkali Soil Natural Environmental Science Center (ASNESC), Northeast Forestry University, No. 26 Hexing Road, Nangang District, Harbin 150040, China*
[b] *Laboratory of Soybean Molecular Biology and Molecular Breeding, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, No. 138 Haping Road, Nangang District, Harbin 150081, China*
[c] *Asian Natural Environmental Science Center, The University of Tokyo, Nishitokyo, Tokyo 188-0002, Japan*

ARTICLE INFO

Article history:
Received 5 May 2014
Received in revised form 29 October 2014
Accepted 30 October 2014
Available online 29 November 2014

Keywords:
Puccinellia tenuiflora
Arabidopsis thaliana
APX (ascorbate peroxidase)
Transgenic plant
Salt-alkali and oxidative stresses

ABSTRACT

Ascorbate peroxidase (*APX*, EC 1.11.1.11) is one of the major members of the ROS scavenging system that plays an important role in improving saline-alkali tolerance. *Puccinellia tenuiflora*, as a perennial wild grass, is able to grow in extreme saline-alkali soil environments. In this study, we investigated the relationship between the *P. tenuiflora* ascorbate peroxidase (*PutAPX*) gene and saline-alkali tolerance. A phylogenetic analysis indicated that *PutAPX* is closely related to *AtAPX3* and *OsAPX4* and that these genes are on the same branch. The *PutAPX*-GFP fusion protein is located in the peroxisome in onion epidermal cells. The transcriptional expression of *PutAPX* increased with prolonged exposure to NaCl, $NaHCO_3$, PEG6000 and H_2O_2 stresses in *P. tenuiflora*. The overexpression of *PutAPX* in *Arabidopsis thaliana* significantly increased the tolerance of plants treated with 150 and 175 mM NaCl and decreased the extent of lipid peroxidation. The transgenic seedlings presented higher chlorophyll content than wild type (WT) seedlings treated with 1, 3, and 5 mM $NaHCO_3$ and 3 mM H_2O_2. The DAB staining results revealed that the H_2O_2 content in transgenic seedlings was significantly lower than that in WT plants under both normal conditions and 200 mM NaCl stress. Moreover, the expression of APX proteins and enzyme activity in the transgenic seedlings increased to level that were greater than twofold higher than those found in WT plants exposed to 200 mM NaCl. The saline-alkali tolerance conferred by the *PutAPX* gene may provide a reliable basis for the use of molecular breeding techniques to improve plant tolerance and obtain a better understanding of the physiological mechanism of anti-oxidative and ROS stresses.

Introduction

Reactive oxygen species (ROS), such as O^{2-}, hydrogen peroxide (H_2O_2) and OH^-, are formed as a natural byproduct of the normal metabolism of oxygen (Foyer and Noctor, 2005). Several researchers have shown that a low concentration of ROS can act as redox signal molecules involving signal transduction in plant cells (Foyer and Noctor, 2005; Mittler, 2002; Agarwal et al., 2003). However, environmental stresses (e.g., saline-alkali stress and heat exposure) may lead to a marked increase in the ROS level and cause oxidative damage in plant cells, such as DNA mutation, protein oxidation, and lipid peroxidation (Mittler, 2002; Agarwal et al., 2003). Over the long course of evolution, plants have formed a potent antioxidant detoxification system to protect themselves against oxidative damage (Asada, 1999; Mittler et al., 2004). To date, some anti-oxidases, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), and Peroxiredoxin (Prx) have been discovered in all cells. In general, there is more than one type of enzyme in a cell for scavenging a certain type of ROS (Mittler et al., 2004;

Abbreviations: APX, ascorbate peroxidase; H_2O_2, hydrogen peroxide; ASA, ascorbic acid; PutAPX, *Puccinellia tenuiflora* ascorbate peroxidase; DAB, 3,3′-diaminobenzidine staining; MDA, Malondialdehyde; NCBI, National Center for Biotechnology Information; NBT, nitro-blue tetrazolium; PCR, polymerase chain reaction; WT, wild type; 35SCaMV, Promoter 35S from Cauliflower Mosaic Virus; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; Prx, Peroxiredoxin; ROS, reactive oxygen species; CDP-Star, Disodium 2-chloro-5-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1]decan}-4-yl) phenyl phosphate Ultra-sensitive and fast chemiluminescent substrate for alkaline phosphatase; Dig, digoxin.

* Corresponding author. Tel.: +86 0451 82191402; fax: +86 0451 82191394.
E-mail address: shenkuiliu@nefu.edu.cn (S. Liu).

http://dx.doi.org/10.1016/j.jplph.2014.10.020
0176-1617/

Scandalios, 2005). APX is one of the major members in the antioxidative system that scavenges H_2O_2 caused by stresses (Noctor and Foyer, 1998; Lu et al., 2007; Gill and Tuteja, 2010). Using ascorbic acid (ASA) as the electron provider, APX changes H_2O_2 into H_2O. Begara-Morales et al. (2014) observed increased APX activity and Malondialdehyde (MDA) and hydrogen peroxide levels in pea leaves under salinity stress. The importance of APX and the ascorbate-GSH cycle is, not only their scavenging of ROS in chloroplast, but also their functions in the cytoplasm, mitochondria, and peroxisome (Noctor and Foyer, 1998; Asada, 1999; Shigeoka et al., 2002; Mittler et al., 2004; Caverzan et al., 2012). Studies on APX have been performed in *A. thaliana*, *Oryza sativa* L., *Glycine max*, *Zea mays* L., tomato, and *Spinacia oleracea* (Ishikawa and Shigeoka, 2008), which shows that APXs are isozymes that are expressed either in the cytoplasmic matrix (sAPX) or in the thylakoid (tAPX). Eight members of the ascorbic acid peroxidase gene family in rice (OsAPX) have been identified and are mainly located in the cytoplasm, peroxisome, chloroplast, and mitochondria (Bunkelmann and Trelease, 1996; Teixeira et al., 2006). Under normal conditions, the *AtAPX3* found in the peroxisome of *A. thaliana* is not necessary for growth and development (Narendra et al., 2006), but may have an effect on the accumulation of ROS caused by stress.

Saline and alkaline are two stresses that may severely damage plants. In contrast to many other plant species that are unable to survive in saline-alkali soil, *Puccinellia tenuiflora* can grow in bare alkali-saline patches with pH values greater than 10, coupled with surface salt contents greater than 5%. Researchers have paid much attention to *P. tenuiflora* due to its saline-alkaline resistance. As a pioneer species that is extremely tolerant to stress, *P. tenuiflora* is an annual herbaceous graminoid that is distributed throughout the sodic saline-alkali soil of the Songnen Plain in northeastern China (Li and Yang, 2004). It can be used as a high quality forage and can be explored as a type of precious halophyte Germplasm resource. Transgenic yeast overexpressing *PutAPX* gene can grow in the presence of 8 mM H_2O_2, which implies that it exhibits extreme tolerance to oxidative stress (Guan et al., 2011). This finding indicates that the anti- oxidation ROS scavenging system plays an important role in improving the tolerance of plants to saline-alkaline stress. Our research focuses on the evolution, subcellular location, and saline-alkaline tolerance of *PutAPX*. By studying the mechanism of *PutAPX*, we aimed to clarify the secret of its effects on improving the tolerance of plants to saline-alkaline stress. As a result, we hope to provide a theoretical basis for the future exploration of *PutAPX* as a saline-alkaline gene resource.

Materials and methods

Materials

Plant materials and stress treatments

Puccinellia tenuiflora (Turcz.) Scribn. et Merr. was identified by Prof. Zhuzhe Jin (College of Agriculture, Yanbian University, China). The *P. tenuiflora* seeds were collected from the Anda experimental fields at the Alkali Soil Natural Environmental Science Center (ASNESC) of Northeast Forestry University.

After surface sterilization, the seeds of *P. tenuiflora* were planted, hydroponically cultured for 3 weeks, and subjected to various stress treatments, namely 100 mM NaCl, 60 mM $NaHCO_3$ and 5 mM H_2O_2, for 6, 12, 24, or 48 h. Deionized water was used as the control. All of the treatments were performed in triplicate. The roots and leaves of *P. tenuiflora* seedlings were harvested, immediately frozen in liquid nitrogen, and stored in a freezer at −80 °C for analysis.

Reagents and strains

The EX-Taq DNA polymerase, pMD18-T vector, and a reverse transcriptase kit (AMV) were purchased from TaKaRa (Da-lian, China). T4 DNA ligase, restriction enzymes, and a DNA gel extraction kit were purchased from MBI (Fermentas). Trizol was obtained from Invitrogen, digoxin (DIG) and disodium 2-chloro-5-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1]decan}-4-yl) phenyl phosphate Ultra-sensitive and fast chemiluminescent substrate for alkaline phosphatase (CDP-Star) were purchased from Roche, and 3,3′-diaminobenzidine (DAB) was purchased from Sigma. The other chemicals were of analytical grade.

The *Escherichia coli* (JM109 and BL21) and *Agrobacterium tumefaciens* (EHA105) strains and the pBS-*MCS-GFP* and pBI121-*MCS-GFP* plasmids were maintained in our laboratory.

Methods

Phylogenetic tree and subcellular localization analysis of PutAPX gene

The ascorbate peroxidase (APX) sequences of rice, maize, soybean, and *Arabidopsis thaliana* were aligned using Clustal W (v1.83) (Thompson et al., 1994). The phylogenetic tree analysis was based on alignments of all of the sequences using the MEGA v4.0 program with the neighbor-joining method (bootstrap = 1000) (Tamura et al., 2007) to provide theoretical support for the evolutionary relationship of *P. tenuiflora* ascorbate peroxidase (*PutAPX*).

The *PutAPX* gene was cloned, and its nucleotide sequence was submitted to the GenBank database (code: KF214536). To determine the subcellular localization of the *PutAPX* gene, a KpnI site was designed in front of the 5′-end ATG of the *PutAPX* gene with an upstream primer (P1: 5′-actagtatggcggccccggtggtg-3′), and a SpeI site was added after the 3′-end with a downstream primer (P2: 5′-actagtcttgctcctcttggaag-3′). The plasmid DNA from pMD18-T::*PutAPX* (Guan et al., 2011) was used as the template to amplify *PutAPX* with KpnI and SpeI sites via polymerase chain reaction (PCR), and the PBI121-*PutAPX::GFP* expression vector was constructed. The PBI121-*PutAPX::GFP* plasmid DNA and peroxisome marker DNA (kind gift from Dr. Tsugama at the University of Tokyo, ABRC stock number: CD3-984, (http://www.arabidopsis.org) were then co-introduced into onion epidermal cells via gene gun transformation (Wang et al., 2011; Alinsug et al., 2012). The green fluorescence, which demonstrated the location of the expressed gene, was observed by confocal laser scanning microscopy (Olympus).

PutAPX gene expression analysis

For the qRT-PCR analysis of the gene expression of *PutAPX* in *P. tenuiflora*, RNA was separately extracted from various tissues using the Trizol reagent (Sambrook and Russell, 2001). cDNA was prepared using the SuperScript III reverse transcriptase (Invitrogen, USA) from 1 μg of total RNA. qRT-PCR was performed on an MxPro-Mx3000P Real-Time Thermal Cycling System using 2× Brilliant III SYBR Green QPCR Master Mix (Agilent) as the fluorescence probe reaction system, according to the manufacturer's instructions. The transcript levels of *PutAPX* were determined by the following primers: RT-F1, 5′- cgatggcggccccggtggtg-3′; RT-R1, 5′-ccttacttgctcctcttgga-3′. In addition, the transcript levels were normalized with the actin transcript abundance, which was taken as a reference, using the following primers: RT-F2, 5′-gtgtcagccatactgtgccaatc-3′; RT-R2, 5′-ttgctcatgcggtcagcaatacc-3′.

A RNA blot analysis (Sambrook and Russell, 2001) was performed to examine the transcription of the *PutAPX* gene, which was marked with a probe labeled with DIG and detected with CDP-Star. Ten micrograms of total RNA were denatured at 65 °C for 10 min, subjected to 1.5% agarose formaldehyde denaturing