Cloning of an Ascorbate Peroxidase Gene from *Puccinellia tenuiflora* and its Expression Analysis

Qingjie Guan 1,2  Lin Li 1  Takano Tetsuo 2  Shenkui Liu 1  
1. Alkali Soil Natural Environmental Science Center (ASNESC), Northeast Forestry University, Harbin, 150040; 2. Asian Natural Environment Science Center (ANESC), The University of Tokyo, Tokyo, 1880002, Japan

Corresponding author, shenkui@nefu.edu.cn;  

**Abstract** The full-length gene of an ascorbate peroxidase (PutAPx) was isolated from *Puccinellia tenuiflora* Ohwi's DNA library. The gene is 1125 bp in length and it has an ORF of 876 bp, which encoded a protein of 291 amino-acid with an estimated molecular weight 32 kD and an isoelectric point of 7.71. Blasting at NCBI, we found that PutAPx showed high similarity (89.7%, 94.3%, 94.2%, 50.7% and 79.6%) to 5 different gramineae species (*Oryza sativa* L., *Hordeum vulgare* L., *Triticum aestivum*, *Lolium perenne* L., and *Zea mays*). The result of phylogenetic tree showed that PutAPx has the closest genetic distance with *Hordeum vulgare* L. and *Triticum aestivum*. Transgenic yeast (InVSC1), expressing PutAPx gene, under the inducement with β-galactose showed higher stress resistance in oxidation stress than control. In this study, we successfully cloned an ascorbate peroxidase (PutAPx) and studied in primary level, which laid the foundation for the future study in the mechanism of oxidative stress mechanism of the role of the foundation establish.

**Keywords** *Puccinellia tenuiflora* Chinampoensis Ohwi; Ascorbate peroxidase; Gene cloning

**Background**

*Puccinellia tenuiflora* Chinampoensis Ohwi which grows in meadow steppe, saliferous soil of North China, is a perennial gramineous herbage, with strong resistance to saline-alkali. Because of long-term evolution and selection, the young seedlings with only five leaves can grow well in soil where alkalinity exceeds pH 10 and the salt content of surface soil overrides 5% (Li and Yang, 2004). Therefore, Puccinellia tenuiflora is not only a superior herbage, but also a precious halobiotic germplasm resource, saline-alkali-tolerant gene resource.

Nowadays, isolating saline-alkali-resistant gene from halophytes are the mechanism of plant resistance to saline-alkali and the hinge of molecular selective breeding saline-alkali-resistant cultivars. *Puccinellia tenuiflora* is now paid widely attention by the researchers who engage in saline-alkali-stress resistance and its genes also have been cloned and published on NCBI GenBank database one after another, including betaine aldehyde dehydrogenase (EF095710, EF095711), H+-ATPase (DQ090006), NADH-glutamine synthetic protein (DQ093360), heatshock protein (DQ093361), glutathione transferase (DQ093362), Na+/H+ pump (EF440291), H+ pump PutCAX1 (AB472071), ferritin (DQ090999), PutPMP3-1 (AB363567), PutPMP3-2 (AB363568), Actin (FJ545 641), Put-R40g3 (AB465547), dehydroascorbate reductase protein (DQ090998), Put-Cu/Zn-SOD and so on. Under salt stress, cell could produce active oxygen (ROS) such as oxyradical (O2·⁻), hydrogen peroxide (H2O2), hydroxyl radical (OH·), which could cause the oxidative stress (Shan et al., 2006). With high specificity and affinity to ascorbic acid, APX (ascorbate peroxidase, APX, EC1.11.1.11), which is the main enzymes for eliminating H2O2, catalyzing H2O2 to reduce into H2O by the reductive ascorbic acid substrate, produces dehydroascorbic acid, and the acid can be reduced to ascorbic acid through many different pathways coupling with H2O2 consumption (Asada, 1992;
Shigeoka et al., 1980a; 1980b). In the past decade, APX homologous genes of many plant has been cloned and investigated. Ishikawa et al (1995) and Kubo et al (1992) proved that cytoplasmic APXs of spinach and Arabidopsis displayed inverse correlation to strong light and MV, respectively. Lu et al (2007), Wang et al (2009) and Ma et al (2002) also had found that the expression of cytoplasmic APXs in rice, white birch nursery stock and suaeda salsa enhanced under salt stress induction. The results of searching nucleic acid database showed that many APXs, such as grape (Lin et al., 2006), cayenne pepper (Yoo et al., 2002), pea (Mittler and Zilinskas, 1991) and so on, have the same enzymatic characteristics and higher specificity to ascorbic acid substrate but more prone to inactive without substrate by comparing investigation of their structure and enzyme kinetics (Yoshimura et al., 1998; Nishikawa et al., 2003). Ascorbate peroxidase gene, as the main enzymes for eliminating H₂O₂ generated from salt stress in antioxidant system, has hitherto not been reported in Puccinellia tenuiflora.

In present study, we cloned ascorbate peroxidase gene from Puccinellia tenuiflora, preliminarily analyzed the sequence, the tissue-specific expression and the antioxidant capability by biology software, RT-PCR and yeast over-expression, respectively, which would pave the way for further investigating the mechanism of antioxidation. It will engender great economic, social and ecological benefits if it is used to breed novel varieties of herbage with saline-alkali-resistance and applied in developing saline and alkaline land.

1 Results and Analysis

1.1 Abtain PutAPx sequence

Plasmid PSK-46 in cDNA library was PCR by universal primers F1 and R1 PutAPx vetor and the products were validated in agarose gel. The results showed that there was a fragment approximate 1000 bp (Figure 1). And the target DNA fragment was recycled by using DNA qiaquick gel extraction kit, was linked to T-vector at 16℃. 16 hours later, the linked products were transformed into E. coli JM109 with Ampicillin 50 mg/L. The plasmid DNA of the positive clones T#1, T#2, T#3 and T#4 were extracted by boiling method. The DNA were identified by HindIII/EcoRI, and the results displayed that three bands of pMD18-T, about 2.7 kb, T#1,T#2,T#4, 700 bp and insert fragment, 400 bp, respectively, which were consistent with the target gene fragment, however, the insert fragment of T#3 is slightly smaller than others (Figure 2). The T#2 strain was sequenced, and the recombination plasmid named as pT-PutAPx.

![Figure 1](image1)

**Figure 1** PCR product of PutAPx gene

**Note:** M: Marker; λ/HindIII; 1~3: PCR products

![Figure 2](image2)

**Figure 2** Identification of pMD18-T-PutApx confirmed by enzyme digestion

**Note:** M: Marker; λ/HindIII; 1~4: pMD18-T-PutApx positive clones (T#1, T#2, T#3 and T#4) confirmed by enzyme digestion

1.2 Sequence analysis of PutAPx

1.2.1 Analysis of PutAPx promoter and terminator sites and its deduced amino acids

Blasting the target gene sequence, 1,024 bp, in GenBank at NCBI, the result showed that target gene sequence was highly homologous with PutAPx gene (>86%), and the score was up to 1,285. We presumed that its encoded protein was very likely the ascorbate peroxidase (Table 1). And then we analyzed the 1,024 bp fragment via network (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), the results revealed that promoter site was at +74, terminator site was at +949 and ORF is 876 bp, which contained 291 aa, and we estimated that it was indeed PutAPx gene (Figure 3).

![Figure 3](image3)

**Figure 3** PutAPx protein sequence analyzed by Enzyme digestion

**Note:** M: Marker; λ/HindIII; 1~4: pMD18-T-PutApx positive clones (T#1, T#2, T#3 and T#4) confirmed by enzyme digestion
### Table 1 Sequences homology alignments of nucleotide sequence by NCBI BIAST

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Description</th>
<th>Max score</th>
<th>Total score</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK251405.1</td>
<td><em>Hordeum vulgare</em> subsp. vulgare cDNA clone: FLba108f14, mRNA sequence</td>
<td>1285</td>
<td>1285</td>
<td>86</td>
</tr>
<tr>
<td>AB063117.1</td>
<td><em>Hordeum vulgare</em> HvAPX1 mRNA for peroxisome type ascorbate peroxidase, complete cds</td>
<td>1285</td>
<td>1285</td>
<td>89</td>
</tr>
<tr>
<td>EF555121.1</td>
<td><em>Triticum aestivum</em> peroxisomal ascorbate peroxidase (APX) mRNA, complete cds</td>
<td>1279</td>
<td>1279</td>
<td>86</td>
</tr>
<tr>
<td>NM_001068974.1</td>
<td><em>Oryza sativa</em> (japonica cultivar-group) Os08g0549100 mRNA, complete cds</td>
<td>1137</td>
<td>1137</td>
<td>91</td>
</tr>
<tr>
<td>CT841589.1</td>
<td><em>Oryza sativa</em> (indica cultivar-group) Os08g0549100 mRNA, complete cds</td>
<td>1131</td>
<td>1131</td>
<td>91</td>
</tr>
<tr>
<td>CT832438.1</td>
<td><em>Oryza sativa</em> (indica cultivar-group) OSIGCSN015H07, full insert sequence</td>
<td>1131</td>
<td>1131</td>
<td>91</td>
</tr>
<tr>
<td>CT832436.1</td>
<td><em>Oryza sativa</em> (indica cultivar-group) OSIGCRA102O15, full insert sequence</td>
<td>1131</td>
<td>1131</td>
<td>91</td>
</tr>
<tr>
<td>AK104490.1</td>
<td><em>Oryza sativa</em> Japonica Group cDNA clone: 006-302-B09, full insert sequence</td>
<td>1131</td>
<td>1131</td>
<td>91</td>
</tr>
<tr>
<td>AK070842.1</td>
<td><em>Oryza sativa</em> Japonica Group cDNA clone: J023074O14, full insert sequence</td>
<td>1131</td>
<td>1131</td>
<td>91</td>
</tr>
<tr>
<td>CU405801.1</td>
<td><em>Oryza rufipogon</em> (W1943) cDNA clone: ORW1943C004J17, full insert sequence</td>
<td>1126</td>
<td>1126</td>
<td>91</td>
</tr>
<tr>
<td>EU976229.1</td>
<td><em>Zea mays</em> clone 688596 APx4-Peroxisomal Ascorbate Peroxidase mRNA, complete cds</td>
<td>957</td>
<td>957</td>
<td>88</td>
</tr>
</tbody>
</table>

#### 1.2.2 Homology comparison and phylogenetic tree of the presumed amino acids

Comparing the *PutA Px* genes from Puccinellia tenuiflora with the *APX* gene of Arabidopsis NP195226, Rice AK070842, Barley BAB6253, Wheat EF555121, Ryegrass EF495352, Corn BT016732 by DNAStar software, we found that they had high similarities, which were 70.7%, 89.7%, 94.3%, 94.2%, 73.7%, 79.6% at amino acid level, respectively (Figure 4). The phylogenetic tree displayed higher homology of *PutA Px* with the gramineae plants barley, wheat and rice on amino acid level (Figure 5). The high similarities between *PutA Px* and five other species demonstrated that the *APX* genes of gramineae are highly conserved. All these results indicated that *PutA Px* encoded protein had ascorbate peroxidase activities.

#### 1.2.3 Prediction of *PutA Px* subcellular localization and analysis of *PutA Px* transmembrane structure

**1.2.3.1 Prediction of *PutA Px* subcellular localization**

Using PSORT, subcellular localization was analyzed based on the amino acid sequence of *PutA Px*, the results indicated that the possibility of *PutA Px* was localization in cytoplasm (0.70) and peroxisome (0.671) was extremely large (Table 2).

#### Table 2 Analysis of subcellular location

<table>
<thead>
<tr>
<th>Location</th>
<th>Score</th>
<th>Location</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus</td>
<td>0.000</td>
<td>Endoplasm reticulum</td>
<td>0.000</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>0.671</td>
<td>Peroxisome</td>
<td>0.700</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>0.000</td>
<td>Chloroplast thylakoid</td>
<td>0.432</td>
</tr>
<tr>
<td>Mitochondrion</td>
<td>0.218</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.2.3.2 Analysis of PutA Px transmembrane domain

Analysis of the PutA Px encoded transmembrane domain by using ProtParam on line (http://www.cbs.dtu.dk/cgi-bin/nph), it demonstrated that this protein had one transmembrane domain, peroxisome, which was consistent with barley HvAPx. PutA Px protein was a secreted protein encoded by cytoplasmic peroxisome gene, which was synthesized in cytoplasm and located on PutA Px gene of peroxisome by protein transport system (Figure 6).

1.3 Expression analysis of PutA Px under oxidative stress (H₂O₂) in yeast

In this work, we designed to confirm the antioxidative function of PutA Px by detecting the growth status of PutAPx-overexpressed yeast under H₂O₂ oxidative stress.

1.3.1 Construction of yeast expression vector

The plasmid pT-PutA Px and vector pYES2 which both have restriction enzyme sites of BamH I and Xba I were digested by these two restriction enzymes, linked and transformed the products into E. coli JM109, selected the positive clones, extracted the plasmid DNA and detected by BamH I and Xba I digestion. It showed that a target fragment of 882 bp was gained (Figure 7A), named it pYES2-PutA Px, which indicated that we have constructed the yeast expression vector successfully. The pYES2-PutA Px was transformed into yeast strain INYSc1 by lithium acetate, and the clones were detected by PCR with primers F3 and R3. It showed that four positive clones with a target band of 882 bp were selected (Figure 7B), which proved that the target gene had been transformed.

Antioxidative stress detection of transformed yeast strain INYSc1 showed that the growth status of different oxidative stress (0, 2 mmol/L and 4 mmol/L H₂O₂) has no obvious difference on SD medium. Overexpression PutA Px of yeasts colonies represented antioxidation differentia under 8 mmol/L H₂O₂ (Figure 8). Furthermore, with the increase of dilution times, the growth status of overexpression PutA Px colonies were better than that of the control. When the dilution was 10⁻⁴, the control yeasts could hardly grow, while the overexpression PutA Px clones grew well. The result indicted that overexpression of PutA Px in yeast could improve the growth status of yeast under oxidative stress.
Figure 4 Homologous analysis of amino acid sequence of PutAPx gene with amino acid sequence of *Oryza sativa* L., *Hordeum vulgare* L., *Triticum aestivum*, *Lolium perenne* L., *Zea mays* and *Puccinellia tenuiflora* protecting tissues and cells, thus which would enhance the antioxidation of yeasts and suggest that PutAPx possess good antioxidation.

1.4 RT-PCR analysis on PutAPx gene mRNA expression in different tissues

RT-PCR analysis on PutAPx mRNA expression in different tissues during the growth stage, the results indicated that PutAPx gene expression is obviously specific in tissues, and the expression level was ear >
Figure 5 Evolutionary trees analysis of PutAPx

Figure 6 Prediction of PutAPx transmembrane domain

Figure 7 pYES2–PutAPx confirmed by enzyme digestion and PCR detection of transformant of yeast

Note: M: Marker-λHindIII; A: 1,2: pYES2–PutAPx fusion plasmid confirmed by enzyme (BamHI/XbaI); B: 1–4: PCR detection of transformant of yeast with pYES2–PutAPx

ear stem > anther > sheath > stem > leaf > root > female flower in order (Figure 9). Therefore, the expression of PutAPx gene is universal in different tissues, its expression of ear is highest, ear stem, anther and sheath are higher, female flower, however, is lowest.

2 Discussion

Stress, such as drought, saline-alkali, hypothermy and hyperthemy, will destroy the active oxygen detoxification system, accumulated the active oxygen, damaged the cytomembrane, prevented \( \text{H}_2\text{O}_2 \) reducing to \( \text{H}_2\text{O} \) by ascorbate peroxidase (APX, EC1.11.1.11, active-oxygen-eliminated enzymes) which were of high specificity and affinity to ascorbic acid. The reaction consumed \( \text{H}_2\text{O}_2 \) and produced dehydroascorbic acid which could be reduced to ascorbic acid through many different pathways (Lu et al., 1998). Northern blot analysis showed the expression of APX gene of suaeda salsa increased under salt stress (400 mmol/L NaCl) and the enzyme activity also increased remarkably. It demonstrated that this gene was induced by salt. Therefore APX might play a certain role in protecting suaeda salsa from oxidative damage.
Puccinellia tenuiflora, an important salt-tolerant herbage, is the main constructive species and pioneer species of salinized grassland in North China; because of its excellent cold-resistance, drought tolerance and salt tolerance, it has become the precious material of plant stress tolerant research (Li and Yang, 2004). In this article, we cloned the PutA Px gene from Puccinellia tenuiflora cDNA library, determined the formula weight (32 kD) and isoelectric point (7.71) of the encoded protein by using biosoftwares, which showed the same number of nucleotide and formula weight as those of rice OsAPx3 and OsAPx4 (Teixeira et al., 2004; Miyazaki et al., 2003). And the homology of amino acid sequence indicated that it had a high similarity to graminea plants rice (89.7%), barley (94.3%), wheat (94.2%), ryegrass (73.7%), corn (79.6%), respectively. The PutA Px encoded protein had only one transmembrane domain and its protein located on peroxisome, which was consistent with the report of barley HvAPX1 (Shi et al., 2001).

PutA Px was universally expressed in different tissues (highest in ear, ear stem, anther and sheath, but lowest in female flower). Antioxidative stress results of yeast strain INYSc1 inducing by galactose suggested that overexpression PutA Px yeasts colonies showed better antioxidative stress than the control under 8 mmol/L H$_2$O$_2$, which meant that the PutA Px catalysed H$_2$O$_2$ to reduce into H$_2$O and protected the tissues and cells, thus it made PutA Px transformed yeast strains grow better on SD medium with H$_2$O$_2$. All these evidence demonstrated that the effects of ascorbate peroxidase on resisting oxidative stress.

In this research, we successfully cloned ascorbate peroxidase gene PutA Px and conducted preliminary investigation on it, which would pave the way for further understanding the mechanism of antioxidation.

3 Methods and Materials

3.1 Tested plant materials

Puccinellia tenuiflora adult plants were obtained from Anda Practice Base, Alkali Soil Natural Environmental Science Center (ASNESC), Northeast Forestry University, and identified by professor Jin Zhzhuhe.
Lolium perenne L. EF495352, Zea mays BT016732 at amino acid level by DNASTar software. On the other hand, we assayed the protein transmembrane structure of Put Px on line (http://www.cbs.dtu.dk/cgi-bin-nph) by ProtParam soft. At last, the subcellular localization of Put APx gene was analysed and predicted respectively by PSORT and ProtComp Version 611 softwares (http://www.softberry.com/TypSoft.html).

3.6 Congstruction of yeast expression vector pYES2-PuTApx and its antioxidation analysis transmorfing into yeast

PCR the recombinam plasmid pT-PuTA Px and add two restriction enzyme sites of BamH I and Xba I to ORF of pT-PuTA Px, and then isolate and recove pYES2 vector and target fragment after digesting by BamH I and Xba I, then linke the two fragments by T4 ligase to transforme into Escherichia coli JM109. After identifying by the above enzymes, we assigned the recombination plasmid as pYES2–PuTApx.

the pYES2–PuTApx was transformed into yeast strain INVScI by lithium acetate (Goetz et al., 1995), and the clones were detected by PCR (F2: 5′-GGCGGCCGCGTCCTTGGTG-3′; R2: 5′-TCTAGATTACTTGC-3′). The positive clones were cultivated on the SD medium. Diluted the yeast transformants pYES2–PuTApx and pYES2 into 100 different concentration, and cultivated on the SD medium with 0 mmol/L, 2 mmol/L, 4 mmol/L, 8 mmol/L, 16 mmol/L H2O2 respectively, the OD600 was 2 and the yeast solution was dilute 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} respectively, and were cultivated at 30°C to observe the growth status directly. Finally, comparing the adaptive capability of recombination transformed yeasts under oxidative stress.

3.7 Tissue specific expression analysis of PuTA Px

The tested materials about leaves, roots, stems, sheaths, anthers, female flowers, scapes, pollinated ears of Puccinellia tenuiflora were collected at flowering phase and total RNA of them were extracted respectively using TRIzol. And then cDNA were obtained by reverse transcription kit. PCR cDNA by two specific primers (F3: 5′-CGATGGGCGGCCCG GTGGTG-3′; R3: 5′-CCTTACTTGCTCCTTGGGA-3′, annealed at 56°C, 30 cycles) to analyse the Tissue specific expression of PuTA Px in different tissues via detecting with 0.8% agarose gel electrophoresis.

Author Contributions
Qingjie Guan and Shenkui Liu conceived and conducted this research and prepared the manuscript. Qingjie Guan, Lin Li and Takano Tetsuo involved this research and collected data. All of them had read the final version of this paper and agreed with the authors’ credits.

Acknowledgements
This work was initiated by the Youth Science Fund of Northeast Forestry University (07049) and Harbin Youth Science and Technology Innovation Fund Project (RC2007QN002079). We also thank two anonymous reviewers for their strict criticism on this paper.

References

http://gab.sophiapublisher.com


