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

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Abstract The full-length gene of an ascorbate peroxidase (PutAPx) was isolated from *Puccinellia tenuiflora* OhwicDNA library. The gene is 1 125 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 closdest 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 levle, 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), EF095710), 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 (O^{2-}), hydrogen peroxide (H_2O_2), 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 H_2O_2, catalyzing H_2O_2 to reduce into H_2O by the reductive ascorbic acid substrate, produces dehydroascorbic acid, and the acid can be reduced to ascorbic acid through many different pathways coupling with H_2O_2 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 H_2O_2 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 *PutA Px* sequence

Plasmid PSK−46 in cDNA library was PCR by universal primers F1 and R1 PutA Px 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 tranformed 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 *Hin*dIII/*Eco*RI, and the results displayed that three bandsof 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.

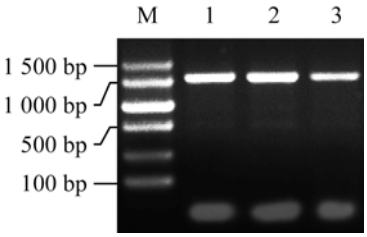


Figure 1 PCR product of *PutAPx* gene

Note: M: Marker− λ /*Hin*dIII; 1~3: PCR products

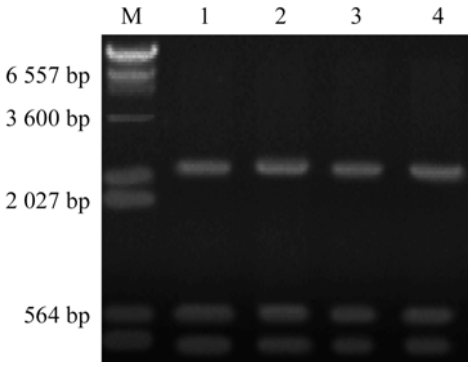


Figure 2 Identification of pMD18−T-PutApx confirmed by enzyme digestion

Note: M: Marker− λ /*Hin*dIII; 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 *PutA Px*

1.2.1 Analysis of *PutA Px* 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 *PutA Px* gene (Figure 3).

Table 1 Sequences homology alignments of nucleotide sequence by NCBI BlAST

Accession No.	Description	Max score	Total score	Similarity (%)
AK251405.1	*Hordeum vulgare* subsp. vulgare cDNA clone: FLbaf108f14, mRNA sequence	1 285	1 285	86
AB063117.1	*Hordeum vulgare* HvAPX1 mRNA for peroxisome type ascorbate peroxidase, complete cds	1 285	1 285	89
EF555121.1	*Triticum aestivum* peroxisomal ascorbate peroxidase (APX) mRNA, complete cds	1 279	1 279	86
NM_001068974.1	*Oryza sativa* (japonica cultivar-group) Os08g0549100 (Os08g0549100) mRNA, complete cds	1 137	1 137	91
CT841589.1	*Oryza rufipogon* (W1943) cDNA clone: ORW1943C103I13, full insert sequence	1 131	1 131	91
CT832438.1	*Oryza sativa* (indica cultivar-group) cDNA clone: OSIGCSN015H07, full insert sequence	1 131	1 131	91
CT832436.1	*Oryza sativa* (indica cultivar-group) cDNA clone: OSIGCRA102O15, full insert sequence	1 131	1 131	91
AK104490.1	*Oryza sativa* Japonica Group cDNA clone: 006-302-B09, full insert sequence	1 131	1 131	91
AK070842.1	*Oryza sativa* Japonica Group cDNA clone: J023074O14, full insert sequence	1 131	1 131	91
CU405801.1	*Oryza rufipogon* (W1943) cDNA clone: ORW1943C004J17, full insert sequence	1 126	1 126	91
EU976229.1	*Zea mays* clone 688596 APx4-Peroxisomal Ascorbate Peroxidase mRNA, complete cds	957	957	88

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

Location	Score	Location	Score
Nucleus	0.000	Endoplasmic reticulum	0.000
Cytoplasm	0.671	Peroxisome	0.700
Plasma membrane	0.000	Chloroplast thylakoid	0.432
Mitochondrion	0.218		

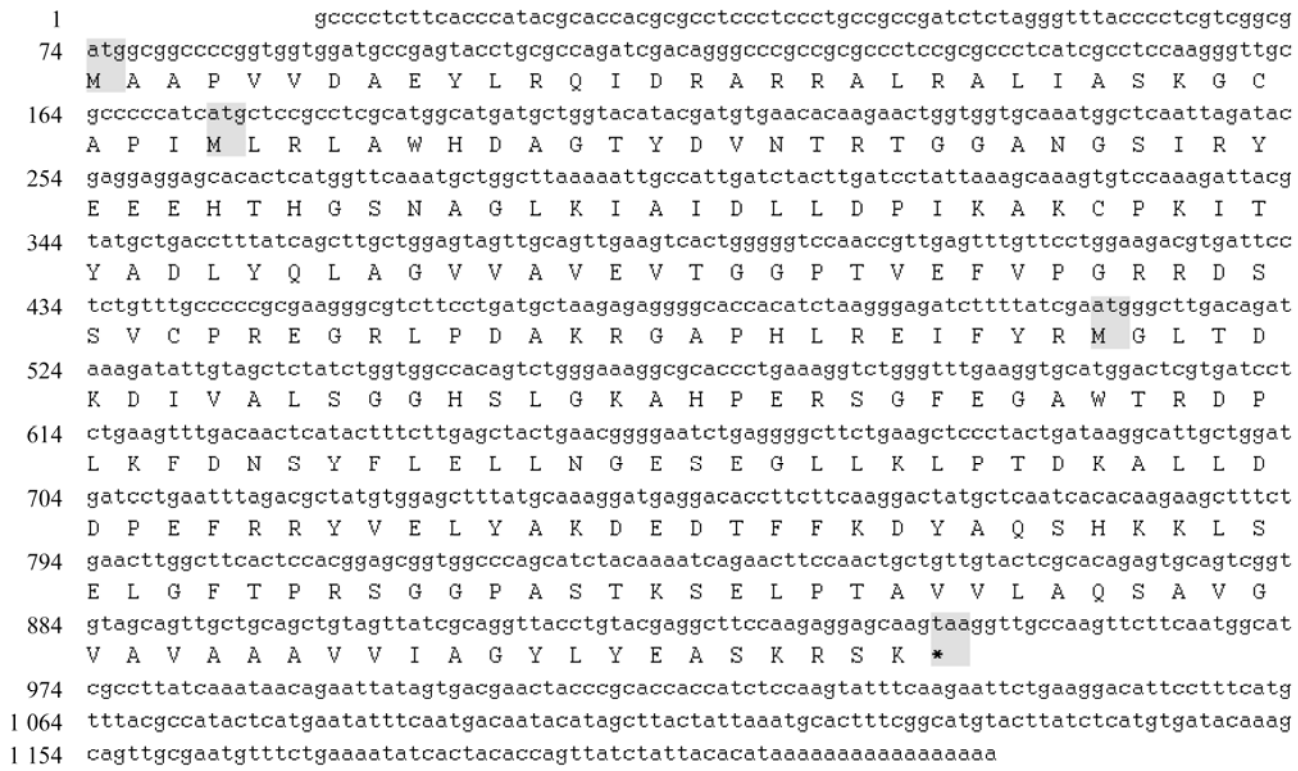


Figure 3 Open reading frame and amino acid sequence

1.2.3.2 Analysis of *PutA Px* transmembrane domain

Analysis 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 (H_2O_2) 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 H_2O_2 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 *Bam*HⅠ 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 detectedby *Bam*HⅠ 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 H_2O_2) has no obvious difference on SD medium. Overexpression *PutA Px* of yeasts colonies represented antioxidation differentia under 8 mmol/L H_2O_2 (Figure 8). Furthermore, with the increase of dilution times, the growth status of overexpression *PutA Px* of yeasts 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,

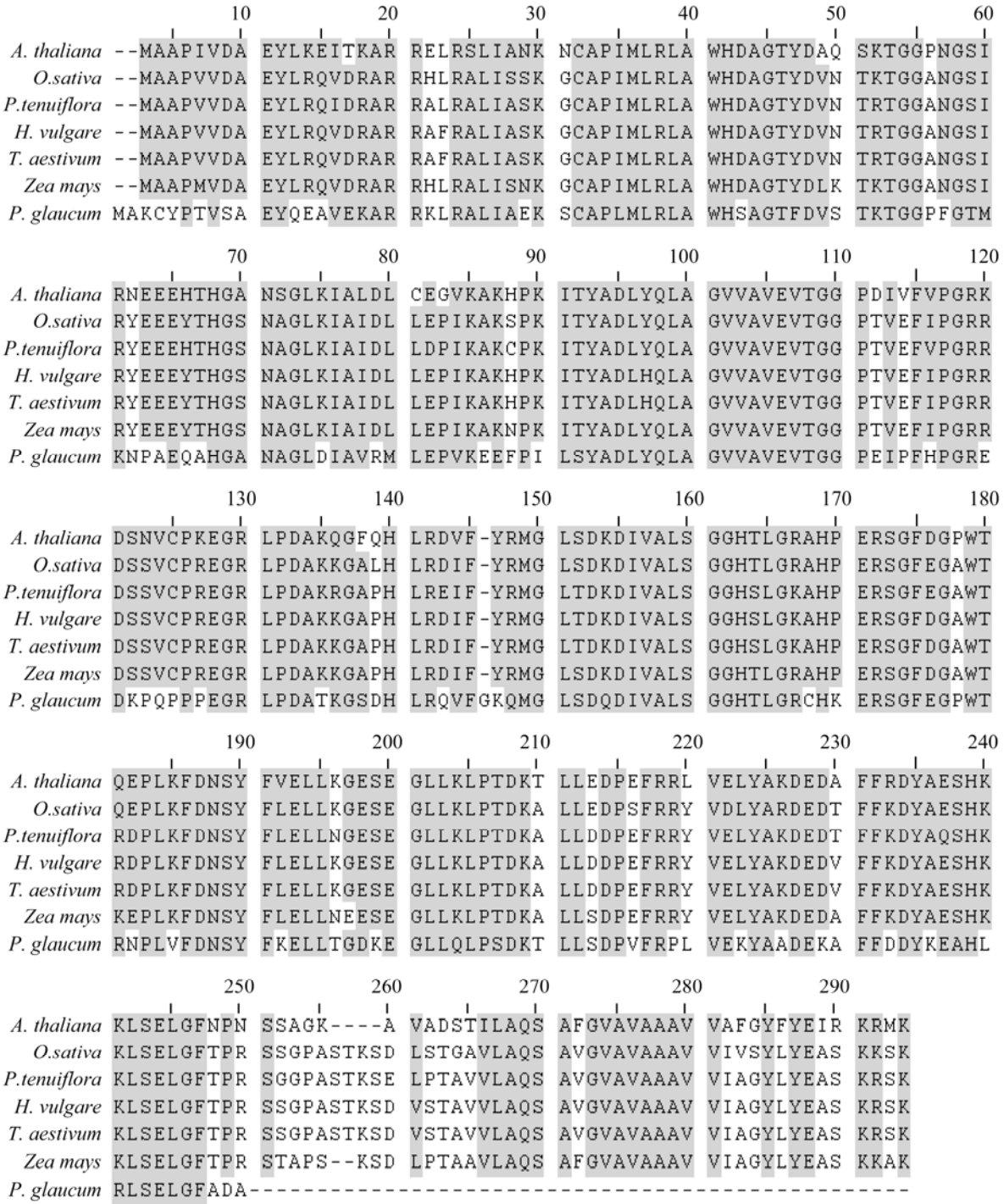


Figure 4 Homologous analysis of amino acid sequence of *PutAPx* gene with amino acid sequence of *Oryza sativa* L., *Hordeum vulgare* L., *Triticum aestivum*, *Lolium perenne* L., *Zea mays* and *Puccinellia tenuiflora*

protecting tissues and cells, thus which would enhance the antioxidation of yeasts and suggest that *PutA Px* possess good antioxidation.

1.4 RT-PCR analysis on *PutA Px* gene mRNA expression in different tissues

RT-PCR analysis on *PutA Px* mRNA expression in different tissues during the growth stage, the results indicated that *PutA Px* gene expression is obviously specific in tissues, and the expression level was ear >

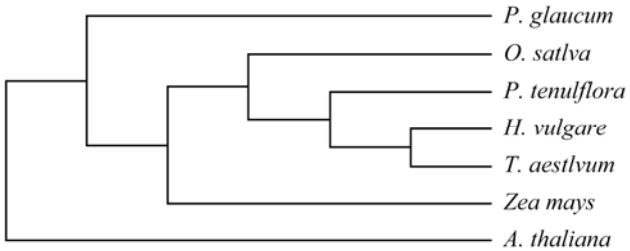


Figure 5 Evolutionary tress analysis of *PutAPx*

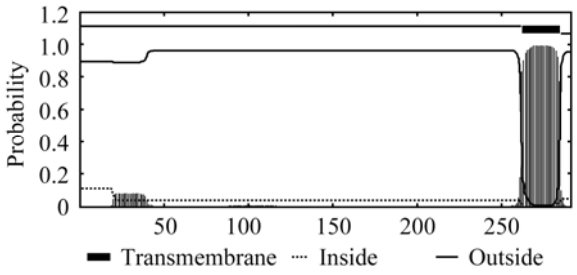


Figure 6 Prediction of *PutAPx* transmembrane domain

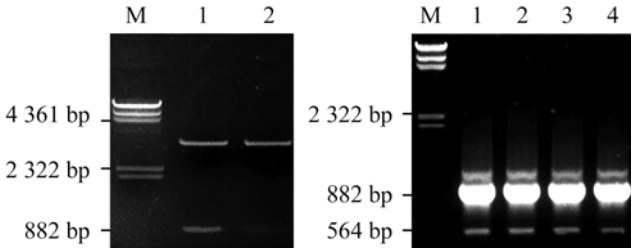
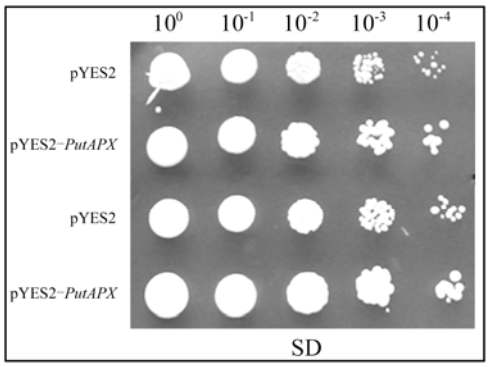
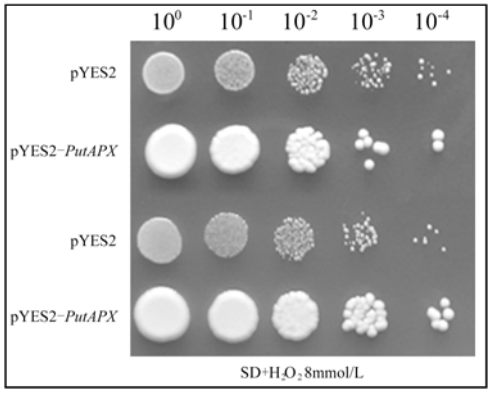


Figure 7 pYES2−PutAPx confirmed by enzyme digestion and PCR detection of transformant of yeast

Note: M: Marker-λ/*Hin*dⅢ; A: 1,2: pYES2−PutAPx fusion plasmid confirmed byenzyme (*BamH* Ⅰ/*Xba* Ⅰ); B: 1~4: PCR detection of transformant of yeast with pYES2−PutAPx



A



B

Figure 8 Hyper-resistance of *PutAPx* overexpression cells to H_2O_2 stresses

Note: SD is Ura supplement media; A: *PutAPx* over expressing cells pYES2-PutAPx and the pYES2 cellswere grown on SD media (two repeat); B: *PutAPx* over expressing cells pYES2-PutAPx and the pYES2 cells were grown on SD media with 8 mmol/L H_2O_2 (two repeats)

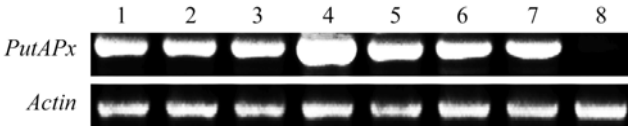


Figure 9 *PutAPx* gene expression in different organ

Note: 1~8: *PutAPx* gene and Actin gene expression in leaf, roots, stem, ear, ear stem, sheath, anther and flower; Actin: Constitutive gene (control)

ear stem > anther > sheath > stem > leaf > root > female flower in order (Figure 9). Therefore, the expression of *PutA Px* 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 Disscussion

Stress, such as drought, saline-alkali, hypothermy and hyperthemy, will destroy the active oxygen detoxification system, accumulated the active oxygen, damaged the cytomembrane, prevected H_2O_2 reducing to H_2O 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 H_2O_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

(Ma et al., 2002).

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 weightb 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 INYSc1inducing by galactose suggested that overexpression *PutA Px* yeasts colonies showed better antioxidative stress than the control under 8 mmol/L H_2O_2, which meant that the *PutA Px* catalysed H_2O_2 to reduce into H_2O and protected the tissues and cells, thus it made *PutA Px* transformed yeast strains grow better on SD medium with H_2O_2. All these evidence demonstrated that the effects of acsorbate peroxidase on resisting oxidative stress.

In this research, we successfully cloned acsorbate 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 Zhuzhe.

3.2 Reagents

cDNA library of *Puccinellia tenuiflora* (deal with 150 mmol/L $NaHCO_3$) was constructed by our team. *Escherichia coli* JM109 and yeast strain IVSc1 were conserved in our lab. DNA extraction kit, pMD18−T vector, *Taq* polymerase, T4 DNA ligase and restriction endonucleases were bought from TAKARA. DNA sequencing and primers were synthesized by Invitrogen, Shanghai branch. Other reagents were all domestic analytically pure.

3.3 Acquiring DNA fragment of target gene

Universal primers of vector PSK were designed by Primer 5.0 (F1: 5'−GGATCCGGGCCCTTTATTCTA−3', R1: 5'−GAGCTCGGGCCCTTACTTGCT−3'). The full-length gene of ascorbate peroxidase of *Puccinellia tenuiflora Chinampoensis* Ohwi: PutAPx was amplified used the cDNA library of plasmid PSK−46 as template.

PCR reaction system was 20 μL, containing ddH_2O 12.0 μL, Pn 1.0 μL, Pm 1.0 μL, 10×Buffer 2.0 μL, dNTP 2.0 μL, cDNA 1.0 μL and *Taq* polymerase 1.0 μL. The PCR reaction conditions was following: an initial denaturation step at 94°C for 2 min, followed by 30 cycles of 94℃ for 30 s, 55℃ for 30 s and 72℃ for 1 min, and ending with a final extension at 72°C for 10 min, finishing at 4℃.

3.4 Extration and identification of recombinant plasmid DNA

Plasmid DNA was extracted by boiling method according to the Molecular Cloning (Sambrook et al., 2002).

The extraction plasmid DNA was identified by restriction endonuclease digestion *Hin*dIII/*Eco*RI, and the digestion products were detected the by agarose gel electrophoresis. After sequencing the positives clone, it would be sent to sequence and named as pT-PutA Px. The strain would be stored at −70℃.

3.5 Sequence analysis of *PutAPx* gene

By blasting the GenBank nucleotide data on NCBI (http://www.ncbi.Nlm.Nih.gov/gorf/gorf.html), we analyzed ORF of the initiation site and terminator domain and compared the similarities of *PutA Px* genes from *Puccinellia tenuiflora*, *Arabidopsis thaliana* NP195226, *Oryza sativa* AK070842, barley BAB6253, *Triticum aestivum* Linn.EF555121,

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 *PutAPx* gene was analysed and predicted respectively by PSORT and ProtComp Version 611 softwares (http://www.softberry.Compberry.phtml).

3.6 Congstruction of yeast expression vector pYES2-PutAPx and its antioxidation analysis transforming into yeast

PCR the recombination plasmid pT-PutA Px and add two restriction enzyme sites of *Bam*H Ⅰ and *Xba* Ⅰ to ORF of pT-PutA Px, and then isolate and recover pYES2 vector and target fragment after digesting by *Bam*H Ⅰ and *Xba* Ⅰ, then linke the two fragments by T4 ligase to transforme into *Escherichia coli* JM109. After identifing by the above enzymes, we assigned the recombination plasmid as pYES2−PutAPx.

the pYES2−PutAPx was tranformed into yeast strain INVScI by lithium acetate (Goetz et al., 1995), and the clones were detected by PCR (F2: 5'−GGATCCATGGCGGCCCCGGT−3'; R2: 5'−TCTAGATTACTTGCTCCTCTTG−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 H_2O_2 respectively. the OD_{600} was 2 and the yeast solution was dilute 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} respectively, and were cultivated at 30℃ 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 mateials 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'−CGATGGCGGCCCCGGTGGTG−3', R3: 5'−CCTTACTTGCTCCTCTTGGA−3', annealed at 56℃, 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.

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