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# Molecular Characterization of a Dehydroascorbate Reductase from *Pinus bungeana*

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## Abstract

**Dehydroascorbate reductase (DHAR) plays a critical role in the ascorbate-glutathione recycling reaction for most higher plants. To date, studies on DHAR in higher plants have focused largely on *Arabidopsis* and agricultural plants, and there is virtually no information on the molecular characteristics of DHAR in gymnosperms. The present study reports the cloning and characteristics of a DHAR (*PbDHAR*) from a pine, *Pinus bungeana Zucc. ex Endl*. The *PbDHAR* gene encodes a protein of 215 amino acid residues with a calculated molecular mass of 24.26 kDa. The predicted 3-D structure of PbDHAR showed a typical glutathione S-transferase fold. Reverse transcription-polymerase chain reaction revealed that the *PbDHAR* was a constitutive expression gene in *P. bungeana*. The expression level of *PbDHAR* mRNA in *P. bungeana* seedlings did not show significant change under high temperature stress. The recombinant PbDHAR was overexpressed in *Escherichia coli* following purification with affinity chromatography. The recombinant PbDHAR exhibited enzymatic activity (19.84 μmol/min per mg) and high affinity (a $K_m$ of 0.08 mM) towards the substrates dehydroascorbate (DHA). Moreover, the recombinant PbDHAR was a thermostable enzyme, and retained 77% of its initial activity at 55 °C. The present study is the first to provide a detailed molecular characterization of the DHAR in *P. bungeana*.**

**Key words:** cloning; dehydroascorbate reductase; enzyme activity; gymnosperm.

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Ascorbic acid is a major antioxidant that serves many functions in plants. Ascorbic acid is involved in the detoxification of reactive oxygen species, for example, superoxide, singlet oxygen, ozone, and hydrogen peroxide, which are produced during aerobic metabolic processes such as photosynthesis or respiration (Chen and Gallie 2006). These physiological functions of ascorbic acid are associated with its univalent or divalent oxidation. The univalent oxidation of ascorbic acid leads to the formation of monodehydroascorbate that is converted to the divalent oxidation product dehydroascorbic acid (DHA) through spontaneous disproportionation or further oxidation (Ishikawa et al. 1998). DHA is then reduced to ascorbic acid by dehydroascorbate reductase (DHAR) in a reaction requiring glutathione (GSH). Because the apoplast contains little GSH or DHAR, DHA, which predominates the apoplast, must reenter the cell for reduction to ascorbic acid (Chen and Gallie 2006). In the absence of sufficient DHAR, however, DHA undergoes irreversible hydrolysis to 2,3-diketogulonic acid. Thus, DHAR is a physiologically important reducing enzyme in the ascorbate-glutathione recycling reaction for most higher plants. DHAR also plays important roles in plant adaptation to environmental stresses. Enhanced tolerance to ozone and drought stress was observed in transgenic tobacco overexpressing DHAR in the cytosol (Eltayeb et al. 2006). In another study, transgenic tobacco expressing a human DHAR gene showed high stress-tolerance to methyl viologen and hydrogen peroxide. Furthermore, transgenic seedlings showed enhanced tolerance to low temperature and high concentration of NaCl (Kwon et al. 2003). Recently, one case study showed transgenic *Arabidopsis* expressing the rice DHAR gene could be resistant to salt stress (Ushimaru et al. 2006). In addition, DHAR plays an important role in the regulation of growth, differentiation, and various metabolisms in higher plants. Suppression of DHAR expression in tobacco

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resulted in a slower rate of leaf expansion and reduced foliar dry weight (Chen and Gallie 2006). Moreover, an accelerated rate of loss of chlorophyll, RbcL, light-harvesting complex II, and photosynthetic functioning was observed in mature leaves (Chen and Gallie 2006). To date, DHAR studies on higher plants have concentrated mainly on *Arabidopsis*, tobacco and agricultural crops such as spinach and rice (Urano et al. 2000; Shimaoka et al. 2003; Eltayeb et al. 2006; Ushimaru et al. 2006). In contrast, there is virtually no information on the molecular characteristics of DHAR in gymnosperms. Gymnosperms represent a large group of plants with a long evolutionary history. Most of gymnosperms such as conifers are important forest-forming species. However, data on DHAR in conifer trees are very scarce. The present study cloned a full-length DHAR gene (*PbDHAR*) from a gymnosperm, *Pinus bungeana*. The PbDHAR was overexpressed in *Escherichia coli*, and the correspondent protein was characterized in detail as well.

## Results

### Cloning and sequence analysis of the *PbDHAR* gene

A 722 bp fragment of *P. bungeana* DHAR cDNA was amplified by reverse transcription-polymerase chain reaction (RT-PCR). The PCR products were sequenced in both directions and found to be identical. The full nucleotide and deduced amino acid sequence of this cDNA (GenBank accession no. EU665680) are shown in Figure 1. This cDNA sequence was named *PbDHAR*. The *PbDHAR* cDNA contained a 645 bp open reading frame (excluding the stop codon), 14 bp of 5′ the non-coding sequence and 18 bp of the 3′ non-coding sequence. This cDNA encodes a peptide of 215 residues that has a predicted molecular mass of 24.26 kDa.

The *PbDHAR* protein sequence analysis was carried out with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The protein sequence showed a high sequence identity to other plant DHAR proteins. A multiple alignment of the PbDHAR protein with other plant DHAR proteins is shown in Figure 2. The percent identity of the *PbDHAR* with other plant DHARs is summarized in Table 1. *PbDHAR* showed 58–71% sequence identity to other plant DHARs listed in Table 1. Much higher sequence identity was observed in the protein N-terminal than in the C-terminal (Figure 2). High sequence identity to other plants DHAR proteins suggested that the cDNA cloned in the present study was DHAR.

### Detection of mRNA transcript of *PbDHAR* in *P. bungeana*

To determine the expression pattern of *PbDHAR* in different *P. bungeana* tissues, RT-PCR was carried out on total RNA isolated from top bud, needles, phloem in the stem and root tissues. The results showed that the *PbDHAR* mRNA was expressed in all tissues (Figure 3, lanes 2–5), indicating that it

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                                  agaggaggaagattggcagtacacgaattgtgagc
ATGGCTGAAGCACTGGAAGTCTTTGTTAAGGCTGCCACGGGTGCACCTGACAAGCTTGGG
 M  A  E  A  L  E  V  F  V  K  A  A  T  G  A  P  D  K  L  G
GACTGTCCCTTTAGCCAAAGGGTTTTGCTTACTCTCGAGGAAAAACAGGTCCCATACAAC
 D  C  P  F  S  Q  R  V  L  L  T  L  E  E  K  Q  V  P  Y  N
ATGAAGTTGATTGATACCAGTAACAAACCTGAATGGTTTTTGCAGATAAACCCTGAAGGC
 M  K  L  I  D  T  S  N  K  P  E  W  F  L  Q  I  N  P  E  G
AAAGTTCCTGTCATCAAGATCGATGATAAGTGGATTCCAGACTCCGATGTTATAACCCAG
 K  V  P  V  I  K  I  D  D  K  W  I  P  D  S  D  V  I  T  Q
ATATTGGAGGAAAAGTACCCGGAACCACCTTTGGCAACCCCACCTGAGAAGGCAACTGTT
 I  L  E  E  K  Y  P  E  P  P  L  A  T  P  P  E  K  A  T  V
GGGTCAAAGATTTTCTCAACATTTATTGGCTTCTTGAAAAGCAAGGACCCTAATGATGGC
 G  S  K  I  F  S  T  F  I  G  F  L  K  S  K  D  P  N  D  G
ACAGAGCAAGCCTTATTGAATGAATTGCGTGCATTCGACGAATACCTAAAAGACAACGGG
 T  E  Q  A  L  L  N  E  L  R  A  F  D  E  Y  L  K  D  N  G
CCATTTATTAATGGGGAGAAAATATCTGCAGCGGACCTCAGCCTAGCGCCAAAGCTTTAC
 P  F  I  N  G  E  K  I  S  A  A  D  L  S  L  A  P  K  L  Y
CATATGAAGGTTGCCTTAGGTCACTTCAAAAAGTGGTCTGTTCCCGAAGAGTTCACTTAT
 H  M  K  V  A  L  G  H  F  K  K  W  S  V  P  E  E  F  T  Y
GTCCAGAGTTACATGAAGGCTGTCTTCTCTCGAGAATCATTTCAGCGTACAAACGCTGCA
 V  Q  S  Y  M  K  A  V  F  S  R  E  S  F  Q  R  T  N  A  A
GATGAGCAATATATTATTGCGGGTTGGTTTCCAAAGGTCAATGCTTAAaatgattagata
 D  E  Q  Y  I  I  A  G  W  F  P  K  V  N  A  *
taggatataaatggcctcacaaagcat
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**Figure 1.** cDNA sequence and deduced amino acid sequence of *PbDHAR*.

The stop codon is denoted by an asterix. The underlined sequences are primers PtaDHAR-CL1/2.

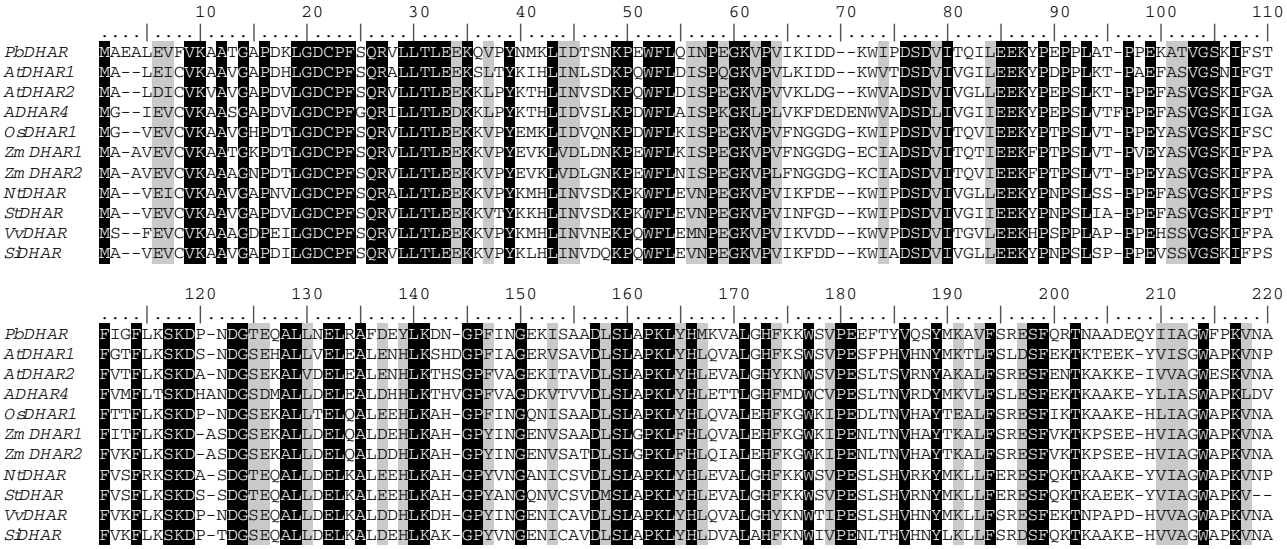


**Figure 2.** Multiple sequence alignment of plant dehydroascorbate reductases (DHARs).

Conserved residues in all plant DHARs are marked in black. This alignment was created using the sequences listed in Table 1.

**Table 1.** Pairwise amino acid sequence identities among the plant dehydroascorbate reductases (DHARs)

| DHARs | DHARs | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| 1 (*PbDHAR*) | | 66 | 66 | 58 | 71 | 67 | 65 | 68 | 67 | 68 | 68 |
| 2 (*AtDHAR1*) | | | 75 | 65 | 65 | 63 | 62 | 70 | 72 | 68 | 69 |
| 3 (*AtDHAR2*) | | | | 71 | 69 | 64 | 64 | 75 | 71 | 71 | 75 |
| 4 (*AtDHAR4*) | | | | | 63 | 60 | 61 | 66 | 65 | 61 | 63 |
| 5 (*OsDHAR1*) | | | | | | 84 | 82 | 70 | 70 | 71 | 73 |
| 6 (*ZmDHAR1*) | | | | | | | 94 | 65 | 68 | 68 | 71 |
| 7 (*ZmDHAR2*) | | | | | | | | 65 | 68 | 70 | 71 |
| 8 (*NtDHAR*) | | | | | | | | | 88 | 75 | 83 |
| 9 (*StDHAR*) | | | | | | | | | | 73 | 79 |
| 10 (*VvDHAR*) | | | | | | | | | | | 82 |
| 11 (*SiDHAR*) | | | | | | | | | | | |

Sequence identities were determined using BIOEDIT software. The values represent the percentage identity. 1, *PbDHAR* (GenBank accession no. EU665680); 2, *AtDHAR1* (GenBank accession no. AY039590); 3, *AtDHAR2* (GenBank accession no. AY087460); 4, *AtDHAR4* (GenBank accession no. NM_123018); 5, *OsDHAR1* (GenBank accession no. AAL71856); 6, *ZmDHAR1* (GenBank accession no. EE172277); 7, *ZmDHAR2* (GenBank accession no. EE188217); 8, *NtDHAR* (GenBank accession no. AAL71857); 9, *StDHAR* (GenBank accession no. ABA46750); 10, *VvDHAR* (GenBank accession no. ABX79343); 11, *SiDHAR* (GenBank accession no. DQ287974).

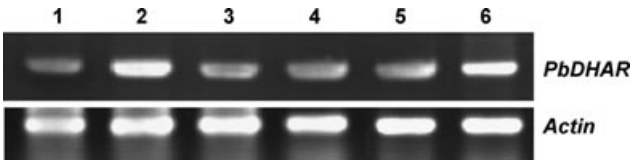


**Figure 3.** Expression of the *PbDHAR* gene in various tissues of *Pinus bungeana*.

Lanes 1–6: positive control, top bud, needle, phloem from the stem, root tissues and seedling. The *Actin* was used as an internal standard.

has a wide distribution and expression in the plant, both above and below ground. RT-PCR also detected highly expressed *PbDHAR* mRNA in seedling tissues (Figure 3, lane 6), indicating the *PbDHAR* was expressed in a different developmental stage.

To determine whether the *PbDHAR* was involved in a response to high temperature stress, one-month-old seedlings were treated in 40 °C, 60 °C and 80 °C. The RT-PCR did not detect expression of the *PbDHAR* gene in *P. bungeana* seedlings under temperature stress of 80 °C. Although the expression of the *PbDHAR* gene was detected by RT-PCR, compared with control plants, no significant change of *PbDHAR* mRNA level was detected by qPCR under stress at 40 °C and 60 °C (data not shown). Thus, these results indicated that the *PbDHAR* might not play an important role in the response to high temperature stress.

### Expression, purification and characterization of recombinant PbDHAR

Induction of the expression construct, pET30a/PbDHAR, resulted in the production of high yields of recombinant protein (Figure 4A). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the bacterial cells induced by isopropylthio-$\beta$-D-galactopyranoside (IPTG) at 37 °C revealed that most of the expressed protein was present in the supernatant (Figure 4A, lane 4). Because the *PbDHAR* gene was inserted into the *Bam*HI and *Sal*I site in the pET30a expression vector (Novagen Inc., Madison, WI, USA), which provides the correct reading frame and a 6× His-tag at the N-terminus, the theoretical molecular weight of the recombinant PbDHAR is 29.69 kDa. SDS-PAGE and matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) showed that the denatured PbDHAR consisted of one subunit of 29.69 kDa (Figure 4A, lane 6 and Figure 4B). Native PAGE showed that the molecular weight of the recombinant PbDHAR was less than 43 kDa (Figure 4C). These results strongly indicate that the recombinant PbDHAR is a monomer protein of 29.69 kDa, which is similar to other plant DHARs (Shimaoka et al. 2000; Dixon et al. 2002; Chun et al. 2007).

Plant DHAR was considered as a member of the plant glutathione S-transferase family (Basantani and Srivastava 2007). Thus, in the present study, a broad range of substrates specific to glutathione S-transferase was examined, including CDNB, NBD-Cl, NBC, 4-NPA and DCNB. As a result, PbDHAR did not show any activity towards these substrates. DHA was considered as a classical DHAR substrate. PbDHAR showed

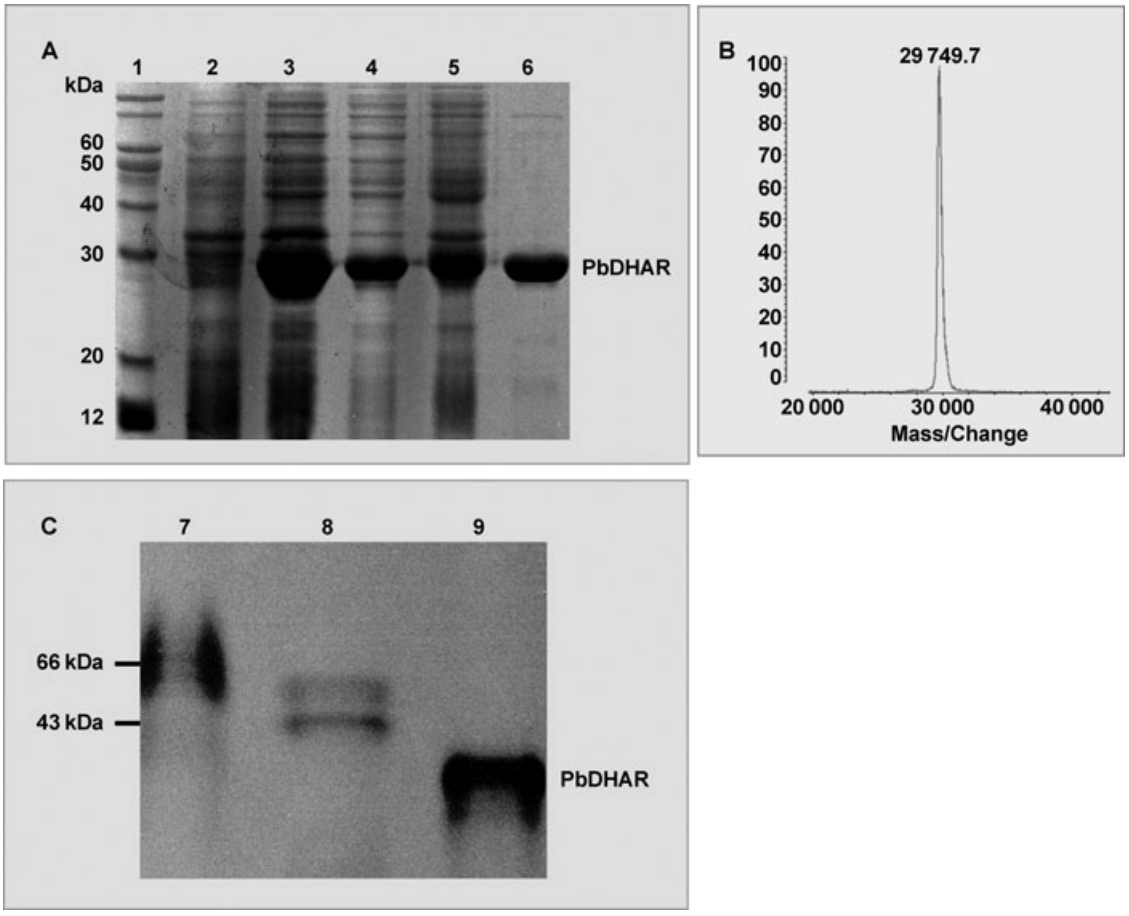


**Figure 4.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (**A**) matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (**B**) and native PAGE (**C**) analysis of the recombinant PbDHAR.

Lane 1, molecular mass markers with the sizes shown on the left in kilodaltons; lane 2, crude extract from *Escherichia coli* BL21; lane 3, crude extract from induced bacteria containing pET30a/PbDHAR; lane 4, supernatant after ultrasonication and centrifugation of cells expressing recombinant PbDHAR; lane 5, cell pellet after ultrasonication and centrifugation of cells expressing recombinant PbDHAR; lane 6 and 9, the purified recombinant PbDHAR; lane 7, bovine albumin; lane 8, egg albumin.

enzymatic activity (19.84 μmol/min per mg protein) toward substrate DHA. These results confirm that the PbDHAR was DHAR.

The enzyme's steady state kinetics were studied in assays with various concentrations of DHA and GSH (Figure 5). At fixed GSH concentration, the $K_m^{DHA}$ and $V_{max}^{DHA}$ values were 0.08 ± 0.01 mM and 34.12 ± 0.79 μmol/min per mg of protein, respectively. At fixed DHA concentrations, the $K_m^{GSH}$ and $V_{max}^{GSH}$ values were 1.03 ± 0.05 mM and 136.20 ± 6.85 μmol/min per mg of protein, respectively (Table 2).

Thermal stability of the PbDHAR is shown in Figure 6. It retained 77% of its initial activity at 55 °C, indicating that PbDHAR is stable below 55 °C. At 70 °C, PbDHAR still retained 46% of its initial activity. Thus, PbDHAR is a thermostable enzyme.

### Structure prediction and homology modeling of the PbDHAR protein

The 3-D structure of PbDHAR was modeled, based on the X-ray structure of the intracellular chloride ion channel protein 1 (Protein Data Bank code No. 1K0N). The structure modeled by the protein structure modeling program, Modeler, is displayed in Figure 7A. This structure was further checked by the Profile-3D program. Figure 7C showed that almost all residues of PbDHAR scored positive, which means that these residues are reasonable. Only the nine residues in the N-terminal of the PbDHAR were built poorly and should be considered to be an unreliable fold. The typical glutathione S-transferases structure contains a smaller thioredoxin-like N-terminal domain and a larger helical C-terminal domain (Figure 7B). The PbDHAR showed a typical glutathione S-transferase (GST) structure, and constituted two distinct domains; a smaller thioredoxin-like N-terminal domain (residues 1–86) and a larger helical C-terminal domain (residues 96–215). N-terminal domain and C-terminal domain were connected by 10-residue linker regions (residues 87 to 95). The N-terminal domain (domain I) constitutes roughly one-third of the protein and consists of a $\beta\alpha\beta\alpha\beta\beta\alpha$ structural motif in which $\beta$3 is antiparallel with respect to the other $\beta$-strands (Figure 7A). The six helices of the C-terminal domain (domain II) make an $\alpha$-helical bundle.

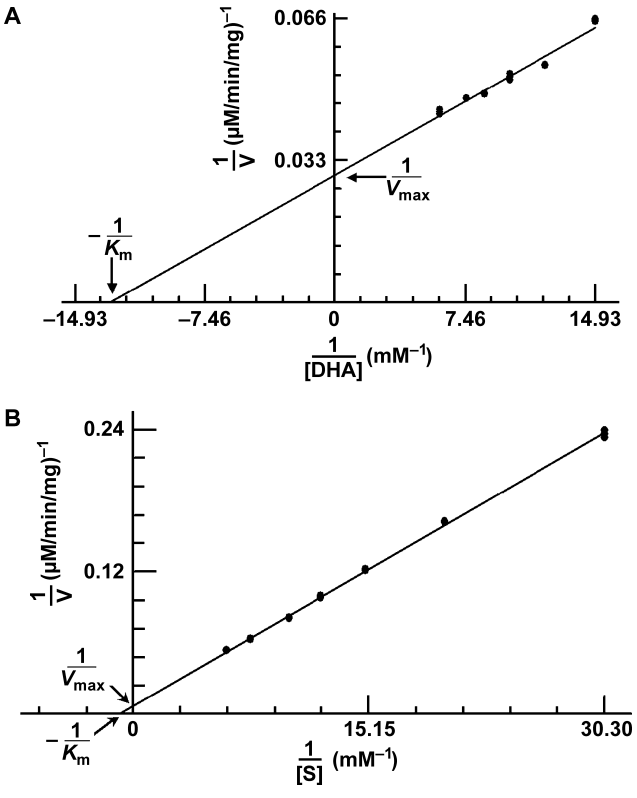


**Figure 5.** Kinetic analysis of the recombinant PbDHAR.

Dehydroascorbate (DHA) **(A)** and glutathione (GSH) **(B)** data are presented as double-reciprocal plots.

**Table 2.** Kinetic constant values for the recombinant PbDHAR to the substrates dehydroascorbate (DHA) and glutathione (GSH)

| Substrates | $K_m$ (mM) | $V_{max}$ (μM/min/mg) | $K_{cat}$ (/min) | $K_{cat}/K_m$ (/mM$^{per}$ min) |
|---|---|---|---|---|
| DHA | 0.08 ± 0.01 | 34.12 ± 0.79 | 609.28 | 7 616.00 |
| GSH | 1.03 ± 0.05 | 136.20 ± 6.85 | 2 432.14 | 2 361.30 |

Data are means ± *SD*; ($n = 3$)

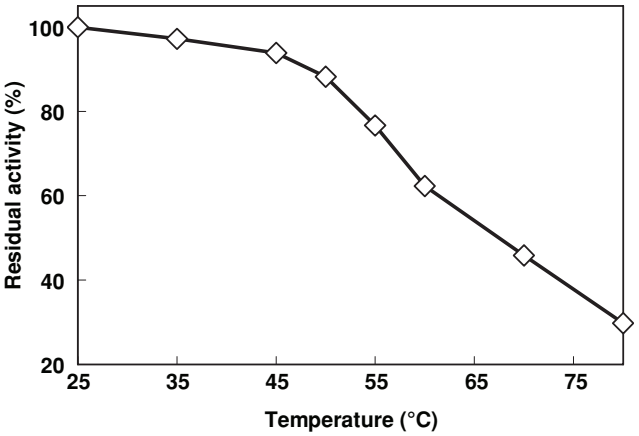


**Figure 6.** Thermal stability of PbDHAR based on the retention of enzymatic activity towards the substrate dehydroascorbate (DHA) and glutathione (GSH) following heat treatment.

## Discussion

Although some plant DHARs had been well characterized in several angiosperm species (Urano et al. 2000; Shimaoka et al.

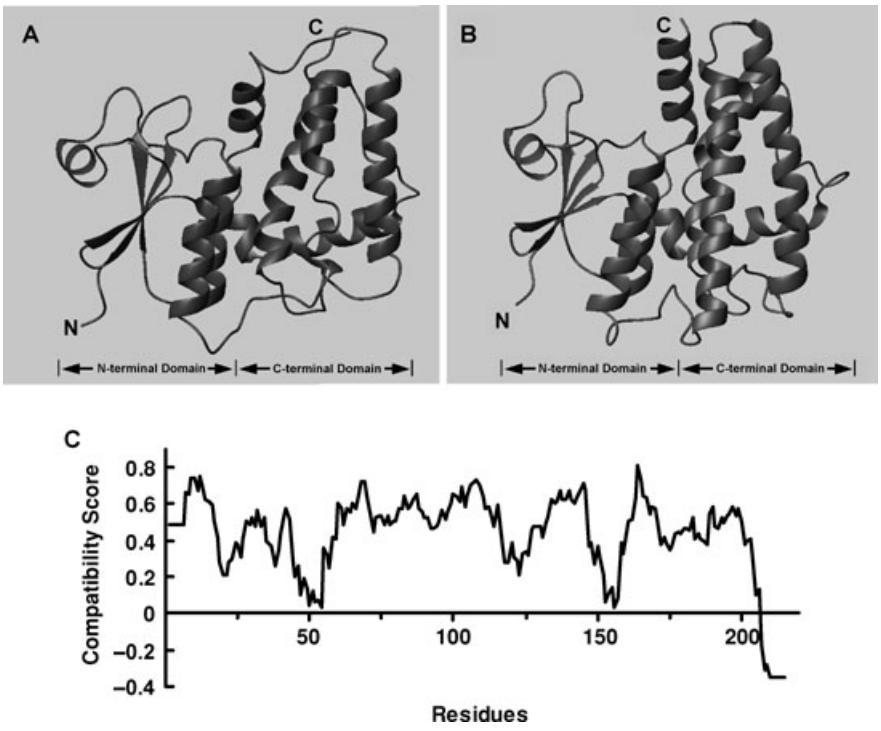


**Figure 7.** Structure modeling of PbDHAR.

**(A)** Predicted 3-D structure of PbDHAR.

**(B)** The crystal structure of wheat tau glutathione S-transferase (GST) (PDB: 1GWC).

**(C)** The evaluation of the structure of PbDHAR by Profile-3D program.

C, C-terminus; N, N-terminus. The 3-D structural image was generated using the molmol program.

2003; Eltayeb et al. 2006; Ushimaru et al. 2006), little is known about the molecular characteristics of DHAR in gymnosperms. In the present study, we cloned and characterized a new DHAR from gymnosperm *P. bungeana*. The cDNA sequence of *PbDHAR* showed little similarity to any other angiosperm DHAR gene sequences in the GenBank (data not shown), indicating that DHARs cDNA sequences between gymnosperms and angiosperm contain high levels of sequence variation. Nevertheless, protein sequences of the DHARs gene between gymnosperms and angiosperm exhibit high similarity, indicating all plant DHARs may have similar functions. In addition, we detected that *PbDHAR* mRNA was expressed in all of the tissues of adult and young trees, indicating that *PbDHAR* may play an important role in the growth and development processes in *P. bungeana*. In fact, all DHARs were considered to play important functions in plant photosynthesis. Increasing DHAR expression maintained higher levels of chlorophyll, RbcL, light-harvesting complex II, and photosynthetic functioning, resulting in delayed leaf aging (Chen and Gallie 2006).

Plant DHAR was considered as a member of the plant glutathione S-transferase family (Basantani and Srivastava 2007). Multiple sequence alignment of the DHAR protein sequences showed the N-terminal domain of DHAR protein was more conserved than the C-terminal domain (Figure 2). Similar results were observed when plant GST protein sequences were aligned (Zeng et al. 2005; Zeng and Wang 2006). To date, crystal structure data of DHAR are not available. The predicted 3-D structure of PbDHAR showed a typical GSH S-transferases structure (Figure 7). Thus, the data in the present study supported the conclusion that DHAR is a member of the plant glutathione S-transferase family.

A comparison of the enzymatic activities and kinetic constants of the plant DHARs toward the substrates DHA and GSH are summarized in Table 3. PbDHAR showed the lowest activity towards DHA among these plant DHARs. The low activity of the recombinant PbDHAR implies that it probably has some special biological functions in *P. bungeana*. For plant DHARs, the apparent $K_m$ values for DHA vary from 0.07 to 0.35 mM (Table 3), indicating different plant DHARs have different affinities to DHA. The PbDHAR and spinach DHARs showed the highest affinities to DHA among these plant DHARs. In addition, except for rice bran DHAR, the apparent $K_m^{GSH}$ value of PbDHAR was found

**Table 3.** Comparison of the specific activities and kinetic constants of PbDHAR and other plant dehydroascorbate reductases (DHARs) towards the substrates dehydroascorbate (DHA) and glutathione (GSH)

| DHARs | 6×His-tag | Activities (μmol/min per mg) | Kinetic constants | | Reference |
|---|---|---|---|---|---|
| | | | $K_m^{DHA}$ | $K_m^{GSH}$ | |
| PbDHAR | Yes | 19.84 | 0.08 | 1.03 | The present study |
| AtDHAR1 | Yes | 936.00 | 0.26 | 10.00 | (Dixon et al. 2002) |
| AtDHAR2 | Yes | 264.00 | ND | ND | (Dixon et al. 2002) |
| AtDHAR3 | Yes | 120.00 | 0.50 | 10.00 | (Dixon et al. 2002) |
| Spinach DHAR-a | No | 360.00 | 0.07 | 1.10 | (Shimaoka et al. 2000) |
| Spinach DHAR-b | No | 400.00 | 0.08 | 2.50 | (Shimaoka et al. 2000) |
| Rice bran DHAR | No | 49.10 | 0.35 | 0.84 | (Kato et al. 1997) |

ND, not determined.

to be lower than other plant DHARs, which indicated that the enzymatic characterization of DHARs between gymnosperms and angiosperm may be different.

The present study showed that the PbDHAR is a thermostable enzyme, and still retained 77% of its initial activity at 55 °C (Figure 6). Similar results were observed for the spinach DHAR-a protein (Shimaoka et al. 2000). Another study showed rice bran DHAR still retained 80% of its initial activity at 80 °C (Kato et al. 1997). Although PbDHAR is a thermostable enzyme, the expression level of *PbDHAR* mRNA in *P. bungeana* seedlings did not show significant change under high temperature stress, indicating that the *PbDHAR* might not play an important role in response to high temperature stress. Although DHARs were proposed to have protection roles under some abiotic stresses, such as salt, high temperature and hydrogen peroxide stresses (Urano et al. 2000; Kwon et al. 2003; Ushimaru et al. 2006), further studies are needed to determine whether DHAR plays an important role against high temperature stress.

In the *Arabidopsis* genome, four DHAR copies were identified (Dixon et al. 2002). To date, no complete genome of gymnosperms has been sequenced. Thus, it is difficult to know accurate copy numbers of DHAR in *P. bungeana*. Fortunately, approximately 320,000 expressed sequence tag (EST) sequences of *Pinus taeda* L. are available in the National Center for Biotechnology Information (NCBI) EST database. We identified three different DHAR copies in the *P. taeda* EST database based on a TBLASTN search (GenBank accession nos. DR117529, DN614482 and DN608778). *P. bungeana* and *P. taeda* belong to the family Pinaceae. So, the *P. bungeana* genome may contain more than one DHAR copy. Although the present study attempted to draw this functional association, further investigations on all of the members of this gene family in *P. bungeana* are necessary before any functional implications can be properly established. Nonetheless, the detailed molecular properties of the first DHAR from gymnosperm *P. bungeana* are in their own right interesting enough for further gene family evolution and function examinations.

## Materials and Methods

### EST identification

The GenBank EST database was searched using the TBLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/) with *Arabidopsis thaliana* DHAR protein (GenBank accession no. AY039590) as the query sequence. Two *Pinus taeda* L. EST sequences (GenBank accession nos. DR744716 and DR117529) encoding an unknown protein with high similarity to DHAR were identified. Based on these two EST sequences, two primers (PtaDHAR-CL1: 5′-AGAGGAGGAAGATTGGCAGTA-3′, PtaDHAR-CL2: 5′-ATGCTTTGTGAGGCCATTTAT-3′) were designed to amplify *Pinus bungeana Zucc. ex Endl.* DHAR cDNA.

### Molecular cloning

Top buds of *P. bungeana* collected from the Botanical Garden of the Institute of Botany, the Chinese Academy of Sciences were used for total RNA isolation using an Aurum Total RNA Kit (Bio-Rad, Hercules, CA, USA). The first strand cDNA was synthesized using a TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa, Shigo, Japan). PCR was carried out in a volume of 30 μL containing about 2 μL of the first strand cDNA, 25 μL TIANGEN 2×*Taq* PCR MasterMix (Beijing, China) and 10 pmol of each primer (PtaDHAR-CL1/2). PCR conditions were optimized to consist of an initial denaturation of 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 45 °C and 90 s at 72 °C, and a final extension of 3 min at 72 °C. A PCR product (approximately 750 bp) was recovered from the agarose gel using a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and was cloned into the pGEM-T Vector (Promega, Madison, WI, USA) and sequenced in both directions. The DHAR cDNA from *P. bungeana* was termed *PbDHAR* (GenBank accession no. EU665680).

### Expression of *PbDHAR* gene in *P. bungeana* tissues

To investigate whether (and where) this gene is functionally active, a specific primer pair 5′-TGGATCCATGG CTGAAGCACTGGAAG-3′ and 5′-TGTCGACTTAAGCATT GACCTTTGGA-3′ was designed, based on the cDNA of PbDHAR, and was used in RT-PCR. Total RNA was isolated from the top bud, needle, phloem in the stem and root tissues of 10-year-old *P. bungeana* using an TIANGEN PLANT RNA Kit (Tiangen Biotech, Beijing, China). In addition, to investigate whether this gene is functionally expressed in young seedling, one-month-old seedlings were used to isolate total RNA. All total RNAs were reverse transcribed into cDNA using TaKaRa cDNA Synthesis kit (Takara). Two microliters of the RT-PCR mixtures were used as a template for amplification of the *PbDHAR* gene. Amplification was carried out in a volume of 50 μL containing 10 pmol of each primer, 10 μL 5×PCR buffer, and 0.25 μL TaKaRa ExTaq (Takara). PCR conditions were optimized to comprise an initial denaturation of 3 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 60 °C for 40 s and 72 °C for 60 s, followed by a final extension of 3 min at 72 °C. PCR products were analyzed by electrophoresis on 1% agarose gels in 1× TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) buffer.

To determine whether the *PbDHAR* was involved in response to high temperature stress, one-month-old seedlings were treated in 40 °C, 60 °C and 80 °C. Four hours after the treatments, total RNA was isolated from whole seedlings, and reverse-transcribed into cDNA using a TaKaRa cDNA Synthesis kit (Takara). Quantitative RT-PCR (qPCR) was carried out on an Mx3000P real-time PCR system (Stratagene , La Jolla, CA, USA) using the RT-PCR products as templates. Brilliant SYBR Green QPCR Master Mix (Stratagene) was used in all qPCR reactions. Two specific primers for *PbDHAR* were used: 5′-TTCCTGTCATCAAGATCGATGATA-3′ and 5′-TTCATTCAATAAGGCTTGCTCTGT-3′. The *Actin* gene was used as an internal control in qPCR analysis. The qPCR conditions were optimized to comprise an initial denaturation step of 10 min at 95 °C followed by 40 cycles of 95 °C for 30 s, 60 °C for 45 s and 72 °C for 30 s. A melt-curve analysis immediately followed amplification at 94 °C for 60 s, cooling to 60 °C for 30 s, and a slow rise in temperature to 95 °C with continuous acquisition of fluorescence decline. The relative expressions of specific genes were quantified using $2^{-\Delta\Delta Ct}$ calculation, where $\Delta\Delta Ct$ is the difference in the threshold cycles of the test and housekeeping gene *Actin*. $\Delta Ct$ is the threshold cycle of the target gene subtracted from the threshold cycle of the housekeeping gene.

### Expression and purification of recombinant PbDHAR

The open reading frame (including the stop codon) of the cDNA encoding *PbDHAR* was amplified by PCR with the upstream primer 5′-TGGATCCATGGCTGAAGCACTGGAAG-3′ and the downstream primer 5′-TGTCGACTTAAGCAT TGACCTTTGGA-3′ (*Bam*HI and *Sal*I sites underlined, respectively). The PCR product was digested with *Bam*HI and *Sal*I and subcloned into the plasmid expression vector pET30a (Novagen, Inc., Madison, WI, USA), which provides the correct reading frame and a 6× His-tag at the N-terminus. The resultant plasmids were used to transform *E. coli* BL21. Colonies containing the appropriate insert were identified by sequencing.

An overnight culture of *E. coli* BL21 transformed with plasmid pET30a/PbDHAR was diluted 1:100 and grown until the optical density ($A_{600}$) reached 0.5. IPTG was added to the culture at a final concentration of 0.1 mM and incubation was continued overnight at 37 °C. Bacteria were harvested by centrifugation at 8 000*g* for 3 min at 4 °C, resuspended in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4), and disrupted by cold sonication. The homogenate was then subjected to centrifugation at 10 000*g* for 10 min at 4 °C. The resultant particulate material and a small portion of the supernatant were analyzed by SDS-PAGE. The rest of the supernatant was loaded onto a Ni Sepharose High Performance column (Amersham Pharmacia Biotech) that had been pre-equilibrated with binding buffer. The overexpressed protein that bound to the Ni Sepharose High Performance column was eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4). The purified recombinant protein was desalted using a PD-10 column (Amersham Pharmacia Biotech) in 10 mM Tris-HCl buffer, pH 7.5.

### Enzyme assays, protein assays and kinetic studies

PbDHAR activity towards DHA was measured as described by Edwards and Dixon (2005). Plant DHAR was considered as a member of the plant GSH S-transferase family (Basantani and Srivastava 2007). Thus, in the present study, a broad range of substrates specific to GSH S-transferase were examined, including 1-chloro-2,4-dinitrobenzene (CDNB), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), nitrobutyl chloride (NBC), p-nitrophenyl acetate (4-NPA), 1,2-dichloro-4-nitrobenzene (DCNB) and ethacrynic acid (ECA). CDNB, DCNB, ECA, NBC and 4-NPA were measured as described by Habig et al. (1974), and activity towards NBD-Cl as described by Ricci et al. (1994). All assays were carried out at 25 °C. SDS-PAGE was carried out on 10% separating gel and 5% stacking gel. To accurately determine the enzyme mass, the purified recombinant PbDHAR was denatured in 8.0 M urea and then analyzed on a Bruker Biflex-III MALDI/TOF mass spectrometer (Bruker Analytical Systems). The molecular weight of the PbDHAR was estimated by native PAGE. Thermal stability of PbDHAR was measured by incubating the enzyme for 15 min at various temperatures from 25 °C to 85 °C at 10 °C increments. PbDHAR activity towards DHA was determined at the end of each incubation. Protein concentrations were determined by measuring the absorbance at 280 nm.

The apparent $K_m$ and $V_{max}$ values for GSH were determined using a GSH range from 0.2 to 1.0 mM and a fixed DHA concentration of 0.17 mM. The apparent $K_m$ and $V_{max}$ values for DHA were determined using a DHA range from 0.05 to 0.17 mM and a fixed GSH concentration of 1 mM. Data were plotted as double-reciprocal Lineweaver-Burk plots to determine the apparent $K_m$ and $V_{max}$ values.

### Homology modeling

The intracellular chloride ion channel protein 1 (Protein Data Bank code No. 1K0N) from *Homo sapiens* was used as templates for constructing a structure model of PbDHAR. Sequences were aligned using the Align 2D structure alignment program (Homology Module in Insight II software, Accelrys, San Diego, CA, USA). Structures were built using the modeler module of Insight II. All structures were verified by a profile-3D program in Insight II. The models were selected according to the model evaluation score calculated by profile-3D.

## References

**Basantani M, Srivastava A** (2007). Plant glutathione transferases – a decade falls short. *Can. J. Bot.* **85**, 443–456.

**Chen Z, Gallie DR** (2006). Dehydroascorbate reductase affects leaf growth, development, and function. *Plant Physiol.* **142**, 775–787.

**Chun JA, Lee WH, Han MO, Lee JW, Yi YB, Goo YM et al.** (2007). Molecular and biochemical characterizations of dehydroascorbate reductase from sesame (*Sesamum indicum* L.) hairy root cultures. *J. Agric. Food Chem.* **55**, 6067–6073.

**Dixon DP, Davis BG, Edwards R** (2002). Functional divergence in the glutathione transferase superfamily in plants. Identification of two classes with putative functions in redox homeostasis in *Arabidopsis thaliana*. *J. Biol. Chem*. **277**, 30859–30869.

**Edwards R, Dixon DP** (2005). Plant glutathione transferases. *Methods Enzymol.* **401**, 169–186.

**Eltayeb AE, Kawano N, Badawi G, Kaminaka H, Sanekata T, Morishima I et al.** (2006). Enhanced tolerance to ozone and drought stresses in transgenic tobacco overexpressing dehydroascorbate reductase in cytosol. *Physiol. Plant*. **127**, 57–65.

**Habig WH, Pabst MJ, Jakoby WB** (1974). Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **249**, 7130–7139.

**Ishikawa T, Casini AF, Nishikimi M** (1998). Molecular cloning and functional expression of rat liver glutathione-dependent dehydroascorbate reductase. *J. Biol. Chem.* **273**, 28708–28712.

**Kato Y, Urano J, Maki Y, Ushimaru T** (1997). Purification and characterization of dehydroascorbate reductase from rice. *Plant Cell Physiol.* **38**, 173–178.

**Kwon SY, Choi SM, Ahn YO, Lee HS, Lee HB, Park YM et al.** (2003). Enhanced stress-tolerance of transgenic tobacco plants expressing a human dehydroascorbate reductase gene. *J. Plant Physiol.* **160**, 347–353.

**Ricci G, Caccuri AM, Lo Bello M, Pastore A, Piemonte F, Federici G** (1994). Colorimetric and fluorometric assays of glutathione transferase based on 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. *Anal. Biochem.* **218**, 463–465.

**Shimaoka T, Yokota A, Miyake C** (2000). Purification and characterization of chloroplast dehydroascorbate reductase from spinach leaves. *Plant Cell Physiol.* **41**, 1110–1118.

**Shimaoka T, Miyake C, Yokota A** (2003). Mechanism of the reaction catalyzed by dehydroascorbate reductase from spinach chloroplasts. *Eur. J. Biochem.* **270**, 921–928.

**Urano J, Nakagawa T, Maki Y, Masumura T, Tanaka K, Murata N et al.** (2000). Molecular cloning and characterization of a rice dehydroascorbate reductase. *FEBS Lett.* **466**, 107–111.

**Ushimaru T, Nakagawa T, Fujioka Y, Daicho K, Naito M, Yamauchi Y et al.** (2006). Transgenic *Arabidopsis* plants expressing the rice dehydroascorbate reductase gene are resistant to salt stress. *J. Plant Physiol.* **163**, 1179–1184.

**Zeng QY, Lu H, Wang XR** (2005). Molecular characterization of a glutathione transferase from *Pinus tabulaeformis* (Pinaceae). *Biochimie* **87**, 445–455.

**Zeng QY, Wang XR** (2006). Divergence in structure and function of tau class glutathione transferase from *Pinus tabulaeformis*, *P. yunnanensis* and *P. densata*. *Biochem. Syst. Ecol.* **34**, 678–690.

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