

# Molecular cloning and characterization of a rice dehydroascorbate reductase

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**Abstract** Plant dehydroascorbate reductase (DHAR), which reduces oxidized ascorbate to maintain an appropriate level of ascorbate, is very important, but no gene or cDNA for plant DHAR has been cloned yet. Here, we describe a cDNA for a rice glutathione-dependent DHAR (designated *DHAR1*). A recombinant Dhar1p produced in *Escherichia coli* was functional. The expression sequence tag database suggests that Dhar1p homologs exist in various plants. Furthermore, the rice Dhar1p has a low similarity to rat DHAR, although the rice enzyme has a considerably higher specific activity than the mammalian one. The mRNA level of *DHAR1*, the protein level of Dhar1p and the DHAR activity in rice seedlings were elevated by high temperature, suggesting the protection role of DHAR at high temperature.

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**Key words:** Ascorbate; Dehydroascorbate reductase; Rice

## 1. Introduction

Ascorbate acts as an important antioxidant in both enzymatic and non-enzymatic reactions in plant cells and is concomitantly oxidized to monodehydroascorbate. For maintenance of the antioxidative activity of ascorbate, its regeneration is necessary. Some monodehydroascorbate radicals are re-reduced by NAD(P)H in a reaction catalyzed by monodehydroascorbate reductase, but the remainder undergoes spontaneous dismutation to ascorbate and dehydroascorbate (DHA). DHA reductase (DHAR, EC 1.8.5.1) catalyzes the re-reduction of DHA to ascorbate by glutathione (GSH). Thus, DHAR, as well as monodehydroascorbate reductase, is critical for protection of cellular components against oxidative injury [1–4]. DHAR activity is enhanced in response to various environmental stresses [5–8].

On the other hand, it has been shown that exogenous ascorbate and its oxidized products, monodehydroascorbate and

DHA, differently affect cell elongation growth [9–12], suggesting that the redox state of ascorbate is strictly regulated. Accepting this, it is likely that DHAR and monodehydroascorbate reductase have different physiological roles.

DHARs from spinach leaves [13] and potato tubers [14] have been purified and characterized. In the case of mammalian DHAR, a rat GSH-dependent DHAR was purified [15], albeit it has a considerably lower specific activity as compared with plant ones. Also, a cDNA for the rat DHAR was cloned [16]. On the other hand, a variety of enzymes have slight DHAR activities: thioltransferase (glutaredoxin), protein disulfide isomerase (PDI) [17] and a Kunitz-type trypsin inhibitor purified from spinach chloroplasts [18]. However, no genes nor cDNAs for 'true' DHAR with a high specific activity have yet been cloned from plants.

We recently purified a GSH-dependent DHAR from rice [19]. The enzyme was a monomeric thiol enzyme, resembling DHARs purified from dicotyledonous plants. However, rice DHAR was considerably heat-resistant, unlike the spinach plastidic one. In addition, antiserum raised against the rice DHAR did not crossreact with spinach plastidic DHAR. Immunoprecipitation analysis showed that this enzyme was a major DHAR in etiolated seedlings. Western blot analysis indicated that this enzyme was distributed ubiquitously in rice tissues.

Here, we report a cDNA encoding the rice DHAR. This is the first report of the cDNA for plant DHAR. The rice DHAR gene was induced by heat shock, suggesting the protection role at high temperature.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Rice (*Oryza sativa* L. cv. Yamabiko) seedlings were continuously illuminated with white light from fluorescent lamps at a photon flux density of about 100  $\mu\text{mol}/\text{m}^2/\text{s}$  (400–700 nm) at 30°C. For stress experiments, seedlings were transferred to 5°C or 40°C.

### 2.2. Protein sequence analysis

50  $\mu\text{g}$  of the purified protein [19] was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with digesting by V8 protease, as described by Cleveland et al. [20]. Consequently, three separated peptides were clearly recognized by staining with Coomassie brilliant blue R-250. The digested peptides migrated as three bands of proteins during SDS-PAGE. The amino-terminal amino acid sequences of the digested peptides blotted on a polyvinylidene difluoride membrane were determined as described previously [19].

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**Abbreviations:** DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; EST, expression sequence tag; GSH, reduced glutathione; PDI, protein disulfide isomerase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

### 2.3. cDNA cloning and sequencing of DHAR1

A rice shoot cDNA library in  $\lambda$  ZIPLOX (Gibco BRL) [21] was screened, with antiserum raised against the purified rice DHAR [19]. After three rounds of screening, positive clones were isolated. The nucleotide sequence was determined with an automatic DNA sequencer (model 310; ABI).

### 2.4. Expression and purification of recombinant DHAR1 in *Escherichia coli*

Full-length open reading frame (ORF) of *DHAR1* amplified by PCR using primers with *SacI* and *SalI* digestion sites at the ends (forward, 5'-GGGGGAGCTCGGCGTGGAGGTGTGCGTC-AAGG-3'; reverse, 5'-GGGGGTGCACTTACGCATTCACTTTTG-GTGC-3') was inserted into *SacI/SalI*-digested pQE30 vector (Qiagen) to express N-terminal His6 tag-fused Dhar1p (H6-Dhar1p). The resulting plasmid (pQE-DHAR1) was transformed in *E. coli* JM109. Transformant cells were grown at 37°C until an  $A_{600}$  of 0.6 was reached, and the induction of *DHAR1* was started by the addition of isopropyl-D-thiogalactopyranoside to a final concentration of 1 mM. After incubation for another 4 h, cells were collected and the total soluble proteins were extracted by sonication. H6-Dhar1p was purified with Ni-NTA spin column (Qiagen) from the extract, according to the manufacturer's instruction.

### 2.5. Northern blot analysis

One gram of plant tissue was homogenized in 3.5 ml of an ice-cold extraction buffer (0.5 M NaCl, 0.2 M Tris-HCl, pH 7.5, 10 mM EDTA and 1% SDS) and 3.5 ml of phenol/chloroform with a Physcotron homogenizer (Nichion Irika, Funabashi, Japan). The homogenate was clarified by centrifugation at 14000×g for 10 min. Total RNA recovered in the upper aqueous phase was re-extracted with 3.5 ml of phenol/chloroform and was collected by precipitation with 2-propanol. 10  $\mu$ g of total RNA was subjected to agarose electrophoresis followed by Northern blot using Alkphos Direct (Amersham Pharmacia Biotech) with *DHAR1* ORF used for expression in *E. coli*, according to the manufacturer's instruction.

### 2.6. Other procedures

Assay of the DHAR activity, SDS-PAGE and Western blot analysis using anti-rice DHAR antiserum were done as described previously [19].

## 3. Results and discussion

### 3.1. Internal amino acid sequences of DHAR

Three V8 protease-digested peptides with apparent molecular masses of about 15 (peptide 1), 10 (peptide 2) and 9 kDa (peptide 3) were separated by SDS-PAGE (data not shown). Amino-terminal primary structure of each peptide was determined as follows: peptide 1, KYTPSLVTPx EYAxVG (x is an unidentified amino acid); peptide 2, xVEVxxKxGxPD; peptide 3, LQALExLKxGPFINGQNI. The amino acid sequence of peptide 2 completely matched to that of the undigested DHAR protein [19]. Hence, the internal sequences of the DHAR protein are those of peptides 1 and 3.

### 3.2. Isolation and characterization of rice DHAR1

Seven cDNA clones were isolated by immunoscreening with anti-rice DHAR antiserum. Among these, an ORF of one cDNA clone alone contained the three amino acid sequences identified by sequencing amino acids of the three V8 protease-digested peptides (Figs. 1 and 2). V8 protease cleaves peptides at the carboxyl side of glutamic acid. In fact, the residues of the carboxyl sides of peptides 1 and 3 were glutamic acid. These results clearly demonstrate that the clone encodes the rice DHAR. The gene is designated *DHAR1* and the purified DHAR protein is also called Dhar1p hereafter.

The *DHAR1* gene consists of 5' and 3' untranslated regions and the ORF encoding a polypeptide of 213 amino acids with

10	20	30	40	50	60
AAAATCTTCTCTCCCGTACGTGAGAAGCGCCAGGTCGTCTGTCGCGCATGGGGCGTGGGA					
				M	G V E
70	80	90	100	110	120
GGTGTGCGTCAAGGCCGCGTCGGCCACCCGGACACGCTCGGCGACTGTCCATTCTCGCA					
V	C	V	K	A	A
130	140	150	160	170	180
GAGGGTCTGCTGACTCTGGAGGAGAAGAAGGTGCCCTACGAGATGAAGCTCATCGACGT					
R	V	L	L	T	L
190	200	210	220	230	240
CCAGAACACCCCGACTGGTTTCTGAAGATCAGCCAGAGGGGAAGGTGCCTGTGTTTAA					
Q	N	N	P	D	W
250	260	270	280	290	300
CGGTGGTGATGGCAAAATGGATTCTGATTTCTGATGTGATCACTCAAGTCATTGAGGAGAA					
G	G	D	G	K	W
310	320	330	340	350	360
GTACCCAAACCCCGTCTCTGTGTCACCCCTCTCTGAGTATGTCATCAGTGGGATCAAAAATTTT					
Y	P	T	P	S	L
370	380	390	400	410	420
CTCATGCTTCACAAACGTTCTTGAAGAGCAAGGATCAAAATGATGGTTCAGAGAAGGCACT					
S	C	F	T	T	F
430	440	450	460	470	480
TCTTACTGAAGTGCAGGCACTCGAGGACATCTGAAAGCTCATGGCCCTTTATCAACGG					
L	T	E	L	Q	A
490	500	510	520	530	540
GCAGAACATTTTCAGCTGCTGACCTTAGCTGGCACCAGGCTTACCATCTCCAGGTTGC					
Q	N	I	S	A	A
550	560	570	580	590	600
TCTGGAGCATTTCAAAGGCTGGAAGATCCCGGAAGACCTAACCAATGTTTCATGCTTACAC					
L	E	H	F	K	G
610	620	630	640	650	660
AGAGGCTCTGTTTAGCCGCGAATCTTTTCATCAAGACGAAGGCAGCTAAGGAGCACCTGAT					
E	A	L	F	S	R
670	680	690	700	710	720
TGCTGGATGGGCACCAAAAGTGAATGCGTAAAGAGCCTGCCCTTATGCTCTGGTGTGCTT					
A	G	W	A	P	K
730	740	750	760	770	780
GGACACCATGCTGTTTATCTGATCGGTCCATGTGTCAGTGGTGGGCACTACTACTCTTG					
790	800	810	820	830	840
TGTAGCTTGGGTGTCATGATGGGTGGAATAATGTAGCCCTCATCCGTTGAGTACCTTGAT					
850	860	870	880	890	900
ATGGTGTGTGCAAGTGTGCACATTTTCTATGAACATATCTCTGCTGGCTTAAGTCGAAC					
910	920	930	940	950	960
CGTGGGTGGTTTGGCCTTATGTTCAAGTAAGAGAGTGCATATAGTCTGTAATGGAACCTTT					
970	980	990	1000	1010	1020
GCTAGTACAATATGTTATATGAATAATGGAGATGCAGCCTGCAGCTGCTCTTGTCTGGAG					
1030	1040	1050			
CTTAAAAA					

Fig. 1. Nucleotide sequence and deduced amino acid sequence of a cDNA clone for rice *DHAR1*. The underlined peptide sequence indicates those determined by peptide sequence.

a predicted molecular mass of 23 556 Da, although its apparent molecular weight determined by SDS-PAGE was about 26 kDa [19]. This difference was observed for a recombinant DHAR protein (see below). The amino-terminus of the native Dhar1p protein lacked the initiator methionine alone. It is most likely that Dhar1p becomes mature after the removal of the initiator methionine digested by methionine aminopeptidase, which excises the initial methionine if the penultimate residue has a small radius of gyration (glycine, alanine, serine, threonine, proline, valine and cysteine) [22]. In fact, the penultimate residue of Dhar1p is glycine.

We have shown that the rice Dhar1p ubiquitously exists in rice tissues examined, including shoots, roots and seeds [19]. Indeed, at least three rice cDNA clones of *DHAR1*, which had been registered as genes for hypothetical proteins in the ex-



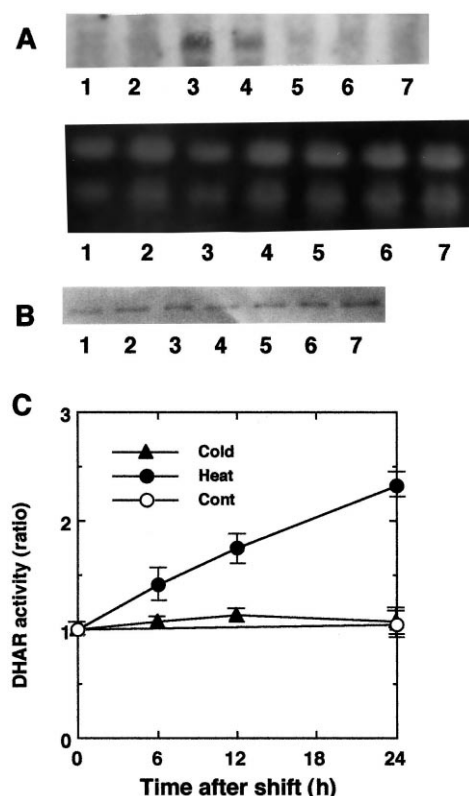


Fig. 4. Induction of expression of rice *DHAR1* gene at high temperature. One-week-old rice seedlings were transferred from 30°C to 5°C or 40°C at time 0. Shoots of seedlings were used as samples. (A) Northern blot of rice *DHAR1*. Lanes 1, initial; lanes 2–4: 6, 12 and 24 h after transfer to 40°C; lanes 5–7, 6, 12 and 24 h after transfer to 5°C. Upper panel, Northern blot of *DHAR1*; lower panel, total RNA stained by ethidium bromide for the loading control. (B) Western blot of rice Dhar1p. Lanes 1, initial; lanes 2–4: 6, 12 and 24 h after transfer to 5°C; lanes 5–7, 6, 12 and 24 h after transfer to 40°C. In each lane, an extract equivalent to 1 mg fresh weight of shoots was loaded on the gel. (C) The changes in the DHAR activity in rice seedlings after transfer to high (closed circles) or low (closed triangles) temperature. Control (open circle), non-transferred control. Each value represents the mean of three measurements with the S.D. indicated by a vertical line. The initial value:  $1.11 \pm 0.04$   $\mu\text{mol}/\text{min}/\text{g}$  fresh weight.

induced (Fig. 3B, lane 2). DHAR activity in *E. coli* cells increased when H6-Dhar1p was expressed (Fig. 3C). Although *E. coli* appears to have no *DHAR*-like gene, an extract from cells before induction contained a certain level of the enzymatic activity. From the lack of ascorbate in this organism, enzymes intrinsically involved in other metabolisms should have DHAR activity, like thioltransferase and PDI.

H6-Dhar1p purified by one-step affinity column chromatography using Ni-NTA agarose showed a high specific DHAR activity of 149  $\mu\text{mol}/\text{min}/\text{mg}$  protein. Thus, *DHAR1* encodes a functional DHAR protein. Its specific activity was rather higher than that of the native protein purified from rice (49  $\mu\text{mol}/\text{min}/\text{mg}$  protein) [19], albeit it was still lower than that of purified spinach leaves (370  $\mu\text{mol}/\text{min}/\text{mg}$  protein) [13]. It has been pointed out that some molecules of the purified rice Dhar1p may be inactivated as a result of the oxidation of thiol groups during purification, because treatment with 2-mercaptoethanol enhanced the enzymatic activity of the purified DHAR [19]. From this, it seems to be reasonable that

the activity of the recombinant protein was higher than that of the purified one.

### 3.4. Heat induction of the *DHAR1* gene

DHAR activity was reported to be enhanced in response to low temperature in some chilling-tolerant plants [5,7,8]. However, we could not detect induction of the transcript level of *DHAR1*, a protein level of Dhar1p or the DHAR activity by transfer of rice seedlings to low temperature (Fig. 4), although activities of some antioxidative enzymes, including ascorbate peroxidase, increased under the same conditions (our unpublished data).

In contrast, after transfer to high temperature, the *DHAR1* gene was transiently induced: the induction peaked at 12 h (Fig. 4A). The increase in the protein level was recognized at 24 h after the transfer (Fig. 4B). These findings suggest that *DHAR1* may be more needed for the antioxidative protection at high temperature. The induction of the *DHAR1* gene was recognized after 12 h, suggesting that this gene is not directly induced by heat shock. The *DHAR1* gene might be rather induced by a second signal, such as an increase in DHA or in a ratio of DHA relative to ascorbate under stress conditions. On the other hand, the overall activity of cellular DHAR gradually increases after the transfer, although the *DHAR1* induction appears to begin after 12 h. It is likely that other DHAR isozymes or proteins with DHAR activity are also induced by this stress.

### 3.5. Concluding remarks

The molecular cloning of a cDNA for plant DHAR is the first to be reported. It was found that homologs ubiquitously exist in various plants. These findings could prompt to elucidate the biological roles of plant DHAR and ascorbate in the antioxidative protection and the cell growth control. In this study, it was suggested that Dhar1p is involved in protection against high temperature. To obtain further insight into the biological role of plant DHAR, the construction of transgenic *A. thaliana* plants that overproduce a rice Dhar1p is in progress in our laboratory.

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