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Molecular cloning and characterization of a rice dehydroascorbate reductase

Jun'ichi Urano[a], Tomofumi Nakagawa[a], Yasushi Maki[b], Takehiro Masumura[c,d], Kunisuke Tanaka[c,d], Norio Murata[e], Takashi Ushimaru[a,*]

[a] *Department of Biology, Faculty of Science, Shizuoka University, Shizuoka 422-8529, Japan*
[b] *Department of Physics, Osaka Medical Collage, Osaka 569-0084, Japan*
[c] *Laboratory of Genetic Engineering, Faculty of Agriculture, Kyoto Prefectural University, Kyoto 606-8522, Japan*
[d] *Kyoto Prefectural Institute of Agricultural Biotechnology, Kyoto 619-0244, Japan*
[e] *National Institute for Basic Biology, Okazaki 444-8585, Japan*

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Abstract Plant dehydroascorbate reductase (DHAR), which re-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.

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 μmol/m^2/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 μg 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].

*Corresponding author.
E-mail: sbtushi@ipc.shizuoka.ac.jp

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

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2.3. cDNA cloning and sequencing of DHAR1

A rice shoot cDNA library in λ 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 *Sac*I and *Sal*I digestion sites at the ends (forward, 5′-GGGGGAGCTCGGCGTGGAGGTGTGCGTC-AAGG-3′; reverse, 5′-GGGGGTCGACTTACGCATTCACTTTTG-GTGC-3′) was inserted into *Sac*I/*Sal*I-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 14 000×*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 μg 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, KYPTPSLVTPxEYAxVG (x is an unidentified amino acid); peptide 2, xVEVxxKAxxGxPD; peptide 3, LQALEExLKAxGPFINGQNI. 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.

```
        10        20        30        40        50        60
AAAATCTTCTCTTCCCGTACGTGAGAAGCGCCAGGTCGTCGTCGCCGCCATGGGCGTGGA
                                                 M  G  V  E

        70        80        90       100       110       120
GGTGTGCGTCAAGGCCGCCGTCGGCCACCCGGACACGCTCGGCGACTGTCCATTCTCGCA
 V  C  V  K  A  A  V  G  H  P  D  T  L  G  D  C  P  F  S  Q

       130       140       150       160       170       180
GAGGGTGCTGCTGACTCTGGAGGAGAAGAAGGTGCCCTACGAGATGAAGCTCATCGACGT
 R  V  L  L  T  L  E  E  K  K  V  P  Y  E  M  K  L  I  D  V

       190       200       210       220       230       240
CCAGAACAACCCCGACTGGTTTCTGAAGATCAGCCCAGAGGGGAAGGTGCCTGTGTTTAA
 Q  N  N  P  D  W  F  L  K  I  S  P  E  G  K  V  P  V  F  N

       250       260       270       280       290       300
CGGTGGTGATGGCAAATGGATTCCTGATTCTGATGTGATCACTCAAGTCATTGAGGAGAA
 G  G  D  G  K  W  I  P  D  S  D  V  I  T  Q  V  I  E  E  K

       310       320       330       340       350       360
GTACCCAACCCCGTCTCTTGTCACCCCTCCTGAGTATGCATCAGTGGGATCAAAAATTTT
 Y  P  T  P  S  L  V  T  P  P  E  Y  A  S  V  G  S  K  I  F

       370       380       390       400       410       420
CTCATGCTTCACAACGTTCTTGAAGAGCAAGGATCCAAATGATGGTTCAGAGAAGGCACT
 S  C  F  T  T  F  L  K  S  K  D  P  N  D  G  S  E  K  A  L

       430       440       450       460       470       480
TCTTACTGAACTGCAGGCACTCGAGGAGCATCTGAAAGCTCATGGCCCCTTTATCAACGG
 L  T  E  L  Q  A  L  E  E  H  L  K  A  H  G  P  F  I  N  G

       490       500       510       520       530       540
GCAGAACATTTCAGCTGCTGACCTTAGCCTGGCACCAAAGCTCTACCATCTCCAGGTTGC
 Q  N  I  S  A  A  D  L  S  L  A  P  K  L  Y  H  L  Q  V  A

       550       560       570       580       590       600
TCTGGAGCATTTCAAAGGCTGGAAGATCCCGGAAGACCTAACCAATGTTCATGCTTACAC
 L  E  H  F  K  G  W  K  I  P  E  D  L  T  N  V  H  A  Y  T

       610       620       630       640       650       660
AGAGGCTCTGTTTAGCCGCGAATCTTTCATCAAGACGAAGGCAGCTAAGGAGCACCTGAT
 E  A  L  F  S  R  E  S  F  I  K  T  K  A  A  K  E  H  L  I

       670       680       690       700       710       720
TGCTGGATGGGCACCAAAAGTGAATGCGTAAGAGCCTGCCCTTATGCTCTGGTGCTGCTT
 A  G  W  A  P  K  V  N  A  *

       730       740       750       760       770       780
GGACACCATGCTGTTTATCTGATCGGTCCATGTCAGTGGTGGGCACTACTACTACTCTTG
       790       800       810       820       830       840
TGTAGCTTGGGTGCATGATTGGGTTGGAATAATGTAGCCTCATCCGTTGAGTACCTTGAT
       850       860       870       880       890       900
ATGGTTGTTGCAAGTGTGCACTTTTTCTATGAACTATCTCCTGCTGGCTTAAGTCGAAAC
       910       920       930       940       950       960
CGTGGGTCGGTTTGGCCTTATGTTCAACTAAGAGAGTGCATATACTGTAATGGAACCTTT
       970       980       990      1000      1010      1020
GCTAGTACAATATGTTATATGAATAATGGAGATGCAGCCTGCAGCTGCTCTTGCTTGGAG
      1030      1040      1050
CTTAAAAAAAAAAAAAAAAAAAAA
```

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.

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

```
Rice      1:MGVEVCVKAAVGHPDTLGD...S..V.LT.EE.KVPY.MKL.DVQ.N.D..LKIS.E.K. 60
Rat       1:                MRFCPFAQRTLMVLKAKGIRHEIININLKNKPEWFFEKNPFGLV 44
Human     1:                ......................V.................L... 44
            *                  *** ** *  *  *    *   *   * * **    * * *

Rice     61:..FNGGD.KW.PD.DVITQVIE.K..TPS.VTPPE.ASVGS.I----..---CF-.T.LK 112
Rat      45:PVLENTQGHLITESVITCEYLDEAYPEKKLFPDDPYEKACQKMTFELFSKVPSLVTSFIR 104
Human    45:.....S....V.........................K..R....L.S.....P.IA.... 104
            **     *     *        * **   *     *     *     **        *

Rice    113:S.DPN.GS--EKA.LTELQA...HL-KAHGP.IN.QNI.AA.LSLA.KLYHLQV...HFK 169
Rat     105:AKRKEDHPGIKEELKKEFSKLEEAMANKRTAFFGGNSLSMIDYLIWPWFQRLE-ALELNE 163
Human   105:S.....S.NLR.A.EN..K....G.D.YK-S.L..D....V...T........-....K. 162
             *   *       *  *   ***        *  *   *  *    *    *  ***

Rice    170:GWKIPED.TN-VHAYT.ALFSRES..KT.AAKEH.IAGWAPKVN.               213
Rat     164:CIDHTPKLKLWMATMQEDPVASSHFIDAKTYRDYLSLYLQDSPEACDYGL          213
Human   163:.LA..........A..Q.......K.......E..N..............          212
                   *                 *  *     *         *

            Identity (%)  Similarity (%)
Rice           26              65
Rat            -               -
Human          81              97
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Fig. 2. Alignment of amino acid sequences of rice Dhar1p and rat DHAR and a hypothetical protein from human. A gap introduced to optimize the alignments is indicated by a hyphen. The conserved residues between three proteins are marked by asterisks and residues identical to those of rat DHAR are indicated by dots. The identity scores and similarity scores for the deduced amino acids of hypothetical proteins compared to the sequence of rat DHAR are shown. GenBank accession number: rat DHAR, AB008807; a hypothetical protein from human, U90313.

pression sequence tag (EST) database, were isolated from green shoots (GenBank accession number D45966), immature seeds (AA751997) and callus (AU068682), respectively.

DHAR isozymes resident in chloroplasts are reported in spinach [13], but we had already suggested that the purified rice Dhar1p may be localized in the cytosol, from the existence in tissues without chloroplasts [19]. Dhar1p has no sequences characteristic of proteins transported to plastids, in addition to mitochondria and endoplasmic reticulum, indicating that Dhar1p is resident in the cytosol. No rice homolog with an obvious transit peptide for targeting to plastids was found in the EST database.

Computer-assisted comparison revealed that many hypothetical genes, which are registered in the EST database, have considerable similarity to *DHAR1* in amino acid sequence (data not shown): *Arabidopsis thaliana*, AA394395; soybean, AI441026; tomato, AI486002; maize, AI649593. It strongly suggests that Dhar1p homologs are widely spread in various plants. They all have no predicted plastid-targeting peptide. The rice DHAR was different from plastidic enzyme in terms of heat stability and antigenicity (for details, see Section 1). It is possible that plastidic DHAR isozyme might be structurally distinct from the cytosolic one.

On the other hand, Dhar1p showed a low similarity to a rat GSH-dependent DHAR and its human hypothetical homolog [16] in amino acid sequences (Fig. 2), while these mammalian genes are quite similar to one another. These results suggest a wide spread of DHAR family proteins among various organisms. Since plant DHARs possess a more than 10-fold higher specific activity relative to the rat one [13,15,19], it is an interesting problem which residues specific to plant enzymes result in the difference in activity. On the other hand, Dhar1p did not resemble proteins having a slight DHAR activity, such as, glutaredoxin (thioltransferase), PDI and plant trypsin inhibitor (data not shown).

3.3. Expression and purification of recombinant Dhar1p

To confirm that the *DHAR1* gene encodes a functional DHAR enzyme, we expressed a recombinant His6-tagged version of Dhar1p (H6-Dhar1p) in *E. coli*. The recombinant protein was almost recovered as a soluble protein. Like the native protein, its apparent molecular mass determined by SDS-PAGE (about 28 kDa) was higher than that of the predicted one (24 997 Da) (Fig. 3A). H6-Dhar1p was clearly recognized by anti-DHAR antiserum when the *DHAR1* gene was

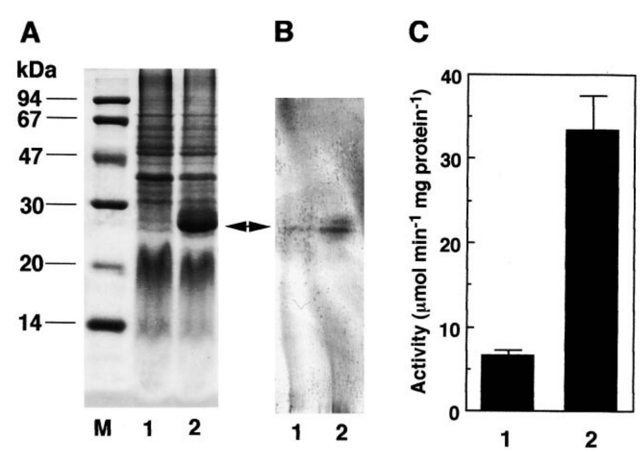

Fig. 3. Expression of a recombinant DHAR protein in *E. coli*. (A) Extract from *E. coli* harboring pQE-DHAR1 for expression of amino-terminal His6-tagged Dhar1p was subjected to SDS-PAGE followed by Coomassie brilliant blue staining. Lane M, molecular weight markers; lane 1, a lysate from cells before induction of *DHAR1*; lane 2, a lysate from cells after induction. (B) Western blot analysis of lysates from *E. coli* harboring pQE-DHAR1. Lane 1, a lysate from cells before induction; lane 2, a lysate from cells after induction. (C) The DHAR activity in *E. coli* extracts. Column 1, a lysate from cells before induction; column 2, a lysate from cells after induction. Each column represents the mean of two measurements with the range indicated by a vertical line.

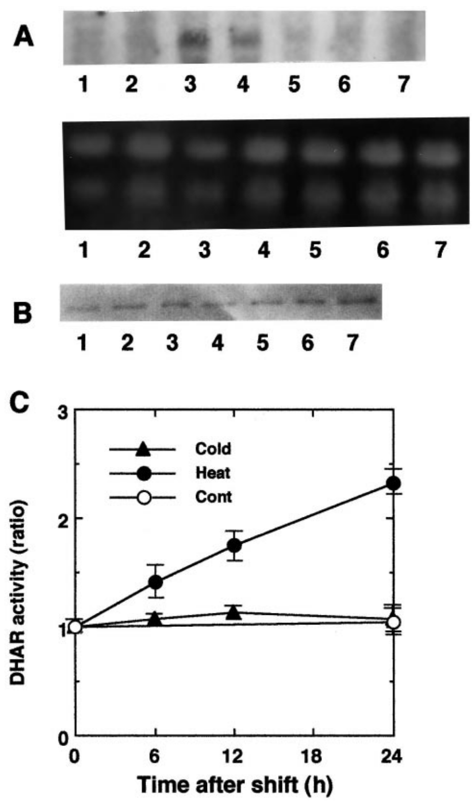

Fig. 4. Induction of expression of rice *DHAR1* gene at high temperature. One-week-old rice seedlings were transferred from 30°C to 5°C or 40°C at time 0. Shoots of seedlings were used as samples. (A) Northern blot of rice *DHAR1*. Lanes 1, initial; lanes 2–4: 6, 12 and 24 h after transfer to 40°C; lanes 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 ± 0.04 μmol/min/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 μmol/min/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 μmol/min/mg protein) [19], albeit it was still lower than that of purified spinach leaves (370 μmol/min/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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