Comparative Expression and Characterization of Dehydroascorbate Reductase cDNA from Transformed Sesame Hairy Roots using Real-Time RT-PCR

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The differential transcription activity of dehydroascorbate reductase (DHAR) was scrutinized in the transformed hairy roots, leaves, stems, roots, and developing seeds of sesame (*Sesamum indicum* L.). Its relative levels of expression were compared via the threshold cycle (C_T) method, using real-time RT-PCR. Ubiquitous expression of DHAR in all organs was confirmed with both real-time and conventional RT-PCR. With the former, DHAR transcript levels were, unexpectedly, 4.7-fold higher in the stem tissue than in the hairy roots; the lowest levels were detected in the seeds. It was possible to determine the transcription activity of hairy root DHAR, with a low amount of total RNA (0.5 ng), using real-time RT-PCR but not with conventional RT-PCR gel analysis. This indicated that the former is more sensitive and efficient than the latter for the detection of gene expression. We also characterized DHAR cDNA cloned from transformed hairy roots, and found that sequence identity for the deduced amino acids of the DHAR enzyme was shared at 60 to 83% among plant species. The algorithm prediction and phylogenetic analysis suggested that the cloned cDNA polypeptide is cytosolic DHAR. Another feature of the cloned cDNA polypeptide was the presence of a CXXS instead of CXXC motif in the active center of the DHAR enzyme.

Keywords: Agrobacterium rhizogenes, dehydroascorbate reductase, differential expression, hairy roots, real-time RT-PCR, sesame

Dehydroascorbate reductase (DHAR) is a reducing enzyme that catalyzes the conversion of dehydroascorbate (DHA) to ascorbic acid (AsC), utilizing glutathione (GSH) as an electron donor in the ascorbate-glutathione cycle reaction of most higher plants (Hausladen and Alscher, 1993; Moon et al., 2005). This reaction is important for the regeneration of AsC in plants because DHA is rapidly lost after the oxidation of AsC if DHA is not quickly recycled to AsC by the enzyme. DHAR has some physiologically pivotal functions in higher plants. First, it plays an important role in regulating the redox state of DHA by its expression level in response to water stress, leading to the control of stomatal movement (Chen and Gallie, 2004). Second, DHAR can be used to increase AsC contents in crop plants by enhancing the ascorbate-glutathione cycle reaction. Chen et al. (2003) have shown that its overexpression in wheat, tobacco, or maize can result in a significant increase in AsC by improving the DHA recycling reaction. These observations demonstrate that DHAR not only has important physiological functions but also can be applied in efforts to improve the nutritional quality of crop plants.

Real-time RT-PCR is a very sensitive and convenient method for monitoring the expression patterns and levels of endogenous genes and multigene families in various plant tissues or organs (Bustin, 2000, 2002; Gachon et al., 2004). The main advantages of this technique over classical RT-PCR are its ease of operation, the capacity for high throughput, greater sensitivity with reliable specificity, and rapid data acquisition (Gachon et al., 2004). In particular, real-time RT-PCR can provide a very powerful assay when only limited starting material is available, or when the expression patterns of multigenes and very low levels of their transcription activities must be determined (Bustin, 2002). Moreover, this assay system can replace cumbersome techniques, e.g., Southern and northern hybridizations, that are now widely used for molecular confirmation of gene identification and expression (Gachon et al., 2004).

Plant DHAR genes have been isolated and characterized from several species. In this study, we used a comparative threshold cycle (C_T) method for relative quantification to assess the differential transcription levels of a DHAR cDNA isolated from sesame hairy roots transformed with *Agrobacterium rhizogenes*. Its transcription sensitivity was also differentially analyzed in other sesame organ tissues, and their expression patterns were compared using classical RT-PCR and real-time RT-PCR.

MATERIALS AND METHODS

Plant Materials and Initiation of Transformed Sesame Hairy Roots

Tissue samples were obtained from seedlings of sesame (*Sesamum indicum* L.) that were reared in a growth chamber at 26°C under a 16-h/8h photoperiod. Developing seeds were obtained from adult sesame plants. Hypocotyl explants were transformed according to the method of Jin et al. (2005), using seedlings that had been aseptically grown

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as described above. Transformed hairy roots were induced with *A. rhizogenes*, using an electroporatic micropulsor device (Bio-Rad, USA). The hairy roots that formed were then selected on MS (Murashige and Skoog, 1962) agar medium containing kanamycin sulfate (100 mg mL^{-1}), followed by three weeks of culturing. Afterward, 2- to 3-cm-long hairy roots were cultured in shaking flasks for isolation of the sesame DHAR cDNA, then immediately frozen in liquid nitrogen and stored at -80°C.

Isolation of RNA

All materials used for RNA isolation were RNase-free. Frozen samples (2 g) were pulverized with a pestle in a mortar containing liquid nitrogen and transferred to a beaker containing five volumes of a commercial RNA extraction solution (Invitrogen, USA). After incubation for 5 min at room temperature, this mixture was centrifuged and filtered through three layers of nylon mesh. Then, 5 M NaCl (0.2 volume) and chloroform (0.6 volume) were added to the filtrate, followed by centrifugation at 2600g for 30 min at 4°C. The aqueous phase was mixed with a 0.9 volume of isopropyl alcohol before the RNA pellet was precipitated by centrifugation, and finally washed with 75% absolute alcohol.

Assays with Real-Time RT-PCR and Conventional RT-PCR

To analyze the expression profiles of sesame DHAR in various organs, we employed a real-time quantitative C_T (threshold cycle) method, using the Applied Biosystems 7900HT Real-Time PCR System (Applied Biosystems, USA) and TaqMan probes and primers to compare relative transcription levels. As the minor groove-binder (MGB), 6-carboxyfluorescein (FAM) was labeled at the 5′ end of the probes as a reporter with a non-fluorescent quencher (NFQ). The primers and probes used for the DHAR target gene, those of the TaqMan, and 18S rRNA as an active reference, are listed in Table 1. Before performing the assay, we conducted a validation experiment to verify the efficiencies of the target gene (DHAR) and the reference (18S rRNA). Briefly, 1 μg each of total RNA, prepared from the transformed hairy roots, leaves, stems, roots, or developing seeds, was used to synthesize the first cDNA strand, using a commercial RT-PCR kit (Bioneer, Korea). Three separate tests were carried to compare the differential expression levels in those tissues. In Test 1, six different amounts (0.05, 0.25, 0.50, 0.75, 1.00, or 1.25 μL) of the first RT-PCR products (i.e., the first cDNA strand), which had been prepared from the transformed hairy roots, were independently incorporated in a reaction mixture (total 20 μL), and 2 μL of the final PCR products (total 20 μL) was resolved in an agarose gel. In Test 2, either 0.01 or 0.10 μL of the first RT-PCR products, prepared from the hairy roots, leaves, stems, roots, or seeds, was analyzed in the agarose gel. For Test 3, 0.1 μL (corresponding to 5 ng total RNA) of the first RT-PCR product prepared from each tissue type was added to a real-time RT-PCR reaction solution (total 20 μL). PCR was performed with a TaqMan Universal Master Mix (Applied Biosystems) by cycling 40 times according to the manufacturer's protocol. For an active reference, the 18S rRNA was used (Shimada et al., 2003) to normalize DHAR transcription levels in the samples because relatively high amounts of 18S rRNA are ubiquitous in the RNA pool of higher-plant cells.

Construction of cDNA Library from Hairy Roots

A cDNA library was constructed with mRNA isolated from the total RNA of transformed hairy roots. This mRNA was obtained with a commercial mRNA purification kit, using the NucleoTrap oligo(dT) latex beads mRNA isolation system (Macherey-Nagel, USA), and according to the manufacturer's instructions. To construct the cDNA library, we applied approximately 5 μg of mRNA with a commercial cDNA synthesis and cloning kit (Stratagene, USA). The synthesized cDNA was then cloned into ZAP expression vectors, packaged, and amplified according to the manufacturer's protocol.

Isolation and Cloning of DHAR cDNA

We performed a reverse transcription-polymerase chain reaction (RT-PCR) to obtain a DNA fragment for preparing our probe, using two degenerate primers: forward, 5′CT(C/T)GG(A/C) GACTGTCC(A/G)TT(C/T)(A/T)(C/G)(C/G)CA(A/G)(A/C)G (G/T)-3′; and reverse, 5′-GTA (A/C/G)AG(C/T) TT(C/T)GG(A/T)GC(C/T)A(A/G) (A/G)CT(A/C)A(A/G)(A/G)TC-3′. These were designed on the basis of the conserved amino acid regions commonly found in other plant DHAR genes. The 426-bp DNA fragment obtained with this RT-PCR was then used as a probe to screen a full-length DHAR cDNA of hairy roots from the cDNA library. We conducted plaque hybridization by applying a radio-labeled (α-^{32}p dCTP) probe. As a result, some positive plaques were selected and inserted into the cloning vectors, pBluescript SK- (Stratagene, USA).

DNA Sequence Analysis

The DHAR cDNA clone was sequenced with an automatic DNA sequencer, the ABI prism 3730xl DNA analyzer (Applied Biosystems). After the amplified DNA sample was purified with an AmpliCycle Sequencing Kit (Applied Biosystem) and dissolved in a Hi-Di formamide solution, sequencing reactions were conducted with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. The deduced amino acid

Table 1. Primer and probe sequences used for real-time RT-PCR.

	Primer (5′ to 3′)	TaqMan Probe (5′ to 3′)
DHAR	Forward; AGGAGAAATACCCGAACCCTTCT	CCACAGATGAGACTTC
	Reverse; GATCCTTGCTCTTCAAGAATTTGACAA	
18S rRNA	Forward; CGGCTACCACATCCAAGGAA	TGCTGGCACCAGACTTGCCCTC
	Reverse; GCTGGAATTACCGCGGCT	

sequences of the DHAR cDNA were identified and compared with those of other plant DHAR genes. For their alignment sequence analysis using ClustalW, 12 plant DHAR genes deposited in the GenBank database were taken under the accession numbers for sesame hairy root (DQ287974, this study), tobacco (AY074787), *Zinnia elegans* (AB158512), potato (DQ191638), *Lycopersicon esculentum* (AY971873), *Arabidopsis* (AY140019), rice (AY074786), wheat (AY074784), *Brassica juncea* (AF536329), spinach (AF195783), *Medicago truncatula* (DQ006811), and *Glycine max* (DQ006810).

RESULTS AND DISCUSSION

Comparison of Detection Capabilities between Real-Time RT-PCR and Conventional RT-PCR for Transcription Activity of the Cloned DHAR

We compared the detectability of transcription activity of sesame DHAR by employing conventional RT-PCR versus real-time RT-PCR analysis. When 0.01 μL of the RT product (20 μL RT reaction volume; 0.01 μL of the RT product corresponding to 0.5 ng of total RNA) was used for these PCR reactions (to synthesize double-stranded DHAR cDNA), their 636-bp cDNA fragments from the hairy roots, leaves, or seeds were not detectable on the gel, but could be detected from the stems and roots, where those products were more abundant (Fig. 1B). Moreover, as the amounts of the RT products increased, stronger band signals were observed, although at >0.5 μL it was difficult to precisely discriminate their transcription levels (Fig. 1A, C). The real-time RT-PCR quantitatively distinguishes very low transcription levels with relative differences in threshold cycle (CT) values. Using this method, some variations in their relative transcription levels were examined (data not shown). In the case of the sesame seeds, their band signal was not recognizable on the gel (Fig. 1C), even though we had used 2 μL (corresponding to 100 ng total RNA) of the first RT product. This implies that their DHAR transcription activity was very low compared with that recorded with other organs. Nevertheless, our real-time RT-PCR analysis revealed transcription activity from the seed tissue (Fig. 1D) even though those levels were very low, thereby demonstrating that real-time RT-PCR is a very sensitive technique for the detection of gene expression.

Comparison of DHAR Expression in Various Organ Tissues

To examine the differential expression of the cloned DHAR cDNA in various sesame organs, we tested a more convenient technique, real-time RT-PCR (Bustin, 2000, 2002; Gachon et al., 2004). Before performing this assay, we conducted a validation experiment for relative quantification, with an absolute value (-0.0173) of the slope (data not shown), thereby verifying the efficiencies of DHAR (the target gene) and 18S rRNA (the reference gene). To compare DHAR transcription activities between the two methods, we used 1 μg each of total RNAs prepared from different tissue types to synthesize the first cDNA strand with a commercial RT-PCR kit. Afterward, the comparative

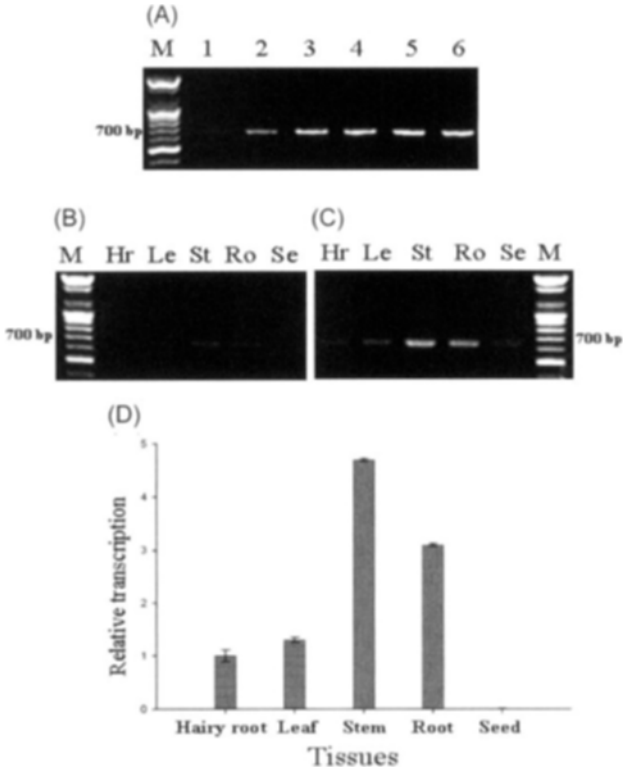

Figure 1. Comparative transcription activities of cloned DHAR cDNAs in sesame organs, as determined by conventional RT-PCR and real-time RT-PCR techniques. (**A**) Gel analysis of (2 μL) final PCR products (20 μL PCR reactions) prepared from total RNAs of transformed hairy roots. Lanes 1-6, 0.05, 0.25, 0.50, 0.75, 1.00, and 1.25 μL of RT products, respectively, were used for final products; M, size marker. (**B**) 0.01 μL each of RT products prepared from total RNAs of hairy roots (Hr), leaves (Le), stems (St), roots (Ro), and seeds (Se) was used for final PCR products. M, size marker. (**C**) 0.1 μL each of RT products was used as described for (**B**). (**D**) Relative transcription activities in various tissues. Data were calculated with differences in values analyzed by comparative C_T method for relative quantification using TaqMan real-time PCR. Transcription activity level from hairy roots was assigned a value of 1 (as relative values normalized to 18S rRNA transcription level). All data are presented with mean values ± SE.

threshold cycle (C_T) method for relative quantification of DHAR expression levels was employed with the TaqMan probes and primers (Table 1). The greatest amount of DHAR transcript was observed in the stem tissues, with a value 4.7-fold higher than that obtained from the hairy roots (value = 1), whereas the lowest value was found with the seed (Fig. 1D). Moreover, DHAR transcript levels from the roots and leaves were 3.1 and 1.3 times higher, respectively, than those from the hairy roots (Fig. 1D). Based on these data, we can draw two conclusions. First, DHAR is a housekeeping gene in the sesame plant. This inference agrees with that reported from an immunoblot analysis of spinach DHAR proteins by Kato et al. (1997). Second, DHAR transcript levels are unexpectedly more predominant in the stems and roots than in the leaf tissue, which implies more active transcription in the former two organs. However, we had initially anticipated that the highest DHAR transcription would occur in the leaves because, generally, this gene is actively

Figure 2. Alignment of deduced amino acid sequences for DHAR using ClustalW. *, Colon, and semicolon denote identical amino acid residues, conserved sequences, and semi-conserved sequences, respectively. Two or three cysteine residues are boxed in sequences, and the essential motif, CXXC or CXXS, is box-shaded or shaded, respectively. Accession numbers for plant sources: sesame (DQ287974), tobacco (AY074787), *Z. elegans* (AB158512), potato (DQ191638), *L. esculentum* ((AY971873), *Arabidopsis* (AY140019), wheat (AY074784), rice (AY074786), *M. truncatula* (DQ006811), *B. juncea* (AF536329), spinach (AF195783), and *G. max* (DQ006810).

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Sesame          GSEQALLDELKALDEHLKAK-GPYVNGENICAVDLSLAPKLYHLDVALAHFKNWIVPENL 176
Tobacco         GTEQALLDELKALEEHLKAH-GPYVNGANICSVDLSLAPKLYHLEVALGHFKKWSVPESL 176
Zinnia          GTEQALLDELNELEEHLKKN-GPYVNGEKISAVDLSLAPKLYHLKVALGHFKKWTVPESL 175
Potato          GTEQALLDELKALEEHLKAH-GPYANGQNVCSVDMSLAPKLYHLEVALGHFKKWSVPESL 176
Lycopersicon    STEQALLDELKALEEHLKAH-GPYINGQNVCSVDMSLAPKLYHLEVALGHFKKWSVPESL 176
Arabidopsis     GSEKALVDELEALENHLKTHSGPFVAGEKITAVDLSLAPKLYHLEVALGHYKNWSVPESL 177
Wheat           GSEKALVDELQALEEHLKAH-GPYINGANISAVDLSLAPKLYHLQVALEHFKGWKVPETL 176
Rice            GSEKALLTELQALEEHLKAH-GPFINGQNISAADLSLAPKLYHLQVALEHFKGWKIPEDL 177
Medicago        GTEQALLNELSSFNDYLKEN-GPFINGKDISAADLSLGPKLYHLEIALGHYKKWTVPDSL 228
Brassica        GTEQVLLDELSTFNDYLKEN-GPYINGEKISAADLSLAPKLYHMKIALGHFKNWSVPDSL 221
Spinacia        GKEQGLLNELSSFNDYLKEN-GPFINGEKISAADLALGPKLYHMEIALGHYKNWSVPESL 231
Glycine         GTEQALLSELSSFSDYIKEN-GPFINGSEISAADLSLGPKLYHLEIALGHYKKWTVPDSL 223
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Sesame          THVHNYLKLLFSRDSFQKTK-AAKEHVVAGWAPKVNA 212
Tobacco         SHVRKYMKLLFERESFQKTK-AAKEYVIAGWAPKVNP 212
Zinnia          THVHDYMKSLFSRESFEKTKPAKEEYVVAGWAPKVNA 212
Potato          SHVRNYMKLLFERESFQNTK-AEEKYVIAGWAPKV-- 210
Lycopersicon    SHVRNYMKLLFERESFQKTK-AEEKYVIAGWAPKV-- 210
Arabidopsis     TSVRNYAKALFSRESFENTK-AKKEIVVAGWESKVNA 213
Wheat           TSV-AYTEALFSRESFVKTK-ATKENLIAGWAPKVNP 211
Rice            TNVHAYTEALFSRESFIKTK-AAKEHLIAGWAPKVNA 213
Medicago        TFLKSYLKEIFSRESFINTR-AQPEDVIEGWRPKVEG 264
Brassica        PFLKSYMENVFSRESFKNTE-AQTEDVIAGWRPKVG- 256
Spinacia        PYVKSYMKNIFSRDSFVKTI-ASTEDVIAGWAKHTS- 266
Glycine         TSLKSYMKAIFSRESFVKTS-AQPQDVIEGWRPKVEG 259
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Figure 2. Continued.

expressed in plant chloroplasts (Hausladen and Alscher, 1993). Although it is difficult to precisely explain this result, we cannot ruled out the possibility that variations in transcription activity were due to differences between activities by cytosolic and plastidic DHAR, which depend on the type and age of the plant tissues (Hausladen and Alscher 1993; Zou et al., 2006). However, it remains to be further investigated for more detailed explanation.

Characterization of the Cloned Sesame DHAR cDNA

A cDNA library was constructed with mRNA isolated from hairy roots cultured for three weeks. DHAR cDNA (accession number DQ287974) was isolated and cloned using one degenerate primer set, whose design was based on the conserved amino acid regions frequently found in plant DHAR amino acid sequences. The complete nucleotide sequence of the cloned cDNA is comprised of 918 bp, which encods a 636-bp open reading frame of 212 amino acid residues, with an estimated molecular mass of 23,574 and a pI value of 6.17. The nucleotide sequences of this cDNA exhibited approximately 72 to 91% identity with those of other plant DHAR cDNAs. Accordingly, we could assume that this cDNA was a putative DHAR-encoding gene.

The deduced amino acid sequences of the cloned cDNA were compared with those of other plant DHAR cDNAs. Identities were highest (72 to 83%) among tobacco, *Z. elegans*, potato, tomato, *Arabidopsis*, rice and wheat, while relatively lower identities were found among *B. juncea* (60%), spinach (60%), *M. truncatula* (59%), and *G. max* (59%). The algorithm prediction (http:// cbs.dtu.dk/services) indicated no recognizable signal peptide sequences in the N-terminal region of the deduced polypeptide of the cloned cDNA (data not shown). This suggested that the cDNA polypeptide is cytosolic DHAR (Kato et al., 1997; Urano et al., 2000; Zou et al., 2006), although some DHAR isozymes do contain the plastid-targeting sequence (Shimaoka et al., 2000; Zou et al., 2006). Another feature of our cloned cDNA polypeptide was the presence of a CXXS motif (Fig. 2) rather than the CXXC motif that is commonly included in DHA-reducing thiol enzymes (Wells et al., 1990; Yang and Wells, 1991; Shimaoka et al., 2003). For example, in the active

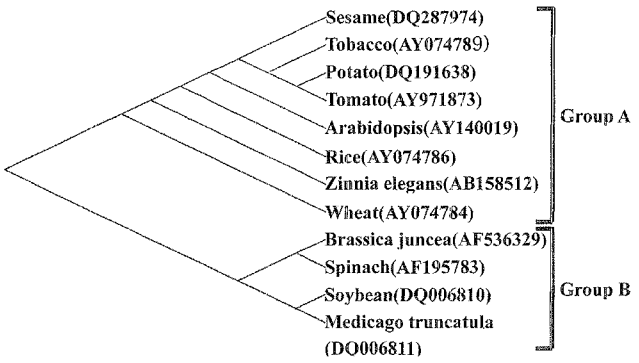

Figure 3. Phylogenetic tree of DHARs from various plant sources deposited in GenBank database, with accession numbers indicated in parentheses.

center of the spinach chloroplast-targeting DHAR isozyme, the CXXC amino acid sequence motif is essential to the catalytic reaction for converting dehydroascorbate to ascorbic acid (Shimaoka et al., 2003), whereas in other DHAR isozymes (including sesame DHAR), a cysteine is replaced by a serine residue, producing the CXXS motif. This sequence change in the catalytic motif may be due to the difference in redox function of DHAR isozymes present in various plant species as shown in Figure 2 (Shimaoka et al., 2003; Fomenko and Gladyshev, 2006; Iqbalsyah et al., 2006).

To elucidate the phylogenetic relationship of the cloned DHAR polypeptide, we applied a phylogeny software program and thus two groups were predicted for classifying the plant DHAR isozymes (Fig. 3). This implies that DHAR may have evolved into two DHAR families. Zou et al. (2006) also have reported, via similar analysis, two such groups, cytosolic and plastidic DHAR isozymes. Based on these results with sesame, we can conclude that the DHAR from our transformed hairy roots belongs to the cytosolic DHAR isozyme family group.

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