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Molecular and Biochemical Characterizations of Dehydroascorbate Reductase from Sesame (*Sesamum indicum* L.) Hairy Root Cultures

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Dehydroascorbate reductase (DHAR) is a biotechnologically or physiologically important reducing enzyme in the ascorbate–glutathione recycling reaction for most higher plants. A DHAR cDNA was isolated from sesame (*Sesamum indicum* L.) hairy roots, and its structure and biochemical properties were characterized to provide some information about its expressional and biochemical profiles in the hairy root cultures. The cDNA contained a catalytic motif CXXS, which may be indicative of a thiol-dependent redox function. A fusion DHAR expressed in an *Escherichia coli* expression system was purified with four purification steps until a homogeneous single band signal was seen in an acrylamide gel, and its antibody was prepared for Western blot analyses. The biochemical results showed that the purified recombinant DHAR had an optimal pH of around 6.0, which was different from those (pH 7.8–8.2) of other plant species. The temperature optimal for the DHAR activity was in a relatively wide range of 30–60 °C. It was proved by a real-time RT-PCR technique that the transcription activity of the DHAR was about 2–5-fold higher during the first 3 week cultures than during the latter 3 week ones. The highest activity of the sesame DHAR was detected in the 4 week cultures of the hairy roots, after which its activity was rapidly decreased to approximately 80%, suggesting that the most active DHAR occurred in this culture period. Western blot analyses confirmed that the presence of DHAR enzyme was identified in both cultures of the fused *E. coli* and the sesame hairy roots.

KEYWORDS: Dehydroascorbate reductase; differential expression; hairy root cultures; protein purification; real-time RT-PCR; recombinant DHAR; *Sesamum indicum* **L.**

INTRODUCTION

Ascorbic acid (vitamin C) is essential for the growth and physiological functions of humans and for animals as well. Because a lack of vitamin C causes some human diseases, it must be supplemented regularly for human health care. The ascorbic acid derived from plant species such as leafy vegetables, fruits, and other crop plants is a major source of vitamin C ingestion in the human diet. Although the biosynthetic pathways of vitamin C are somewhat complex (*1*, *2*), two pathways are proposed for its synthesis in higher plants (*3*). One is the D-mannose/L-galactose pathway, which occurs by oxidative reactions of hexose sugars to produce ascorbic acid.

Another is the pathway via two uronic acids, the galacturonic pathway (*4*) and the glucuronic pathway (*5*). These two pathways are currently the principal routes to the biosynthesis of ascorbic acid in plants.

It has been well-known that ascorbic acid plays some important roles in the regulation of growth, differentiation, and various metabolisms in higher plants (*6*–*8*). Moreover, its strong antioxidant ability scavenges free radicals such as H_2O_2 resulting from light reactions in the thylakoid membrane, which is inhibitory to photosynthetic CO_2 assimilation (*6*). In addition, recent works have indicated that the concentration of ascorbic acid in plant cells affected stomatal movement in response to water stress (*9*), transcriptional regulation of defense genes (*7*), and ozone tolerance (*10*). The homeostatic state of ascorbic acid has great influence on the maintenance of the redox balance in both plant and mammalian cells (*6*, *11*). The cellular redox balance between ascorbic acid and dehydroascorbate plays a pivotal role in normal cellular metabolisms and physiological

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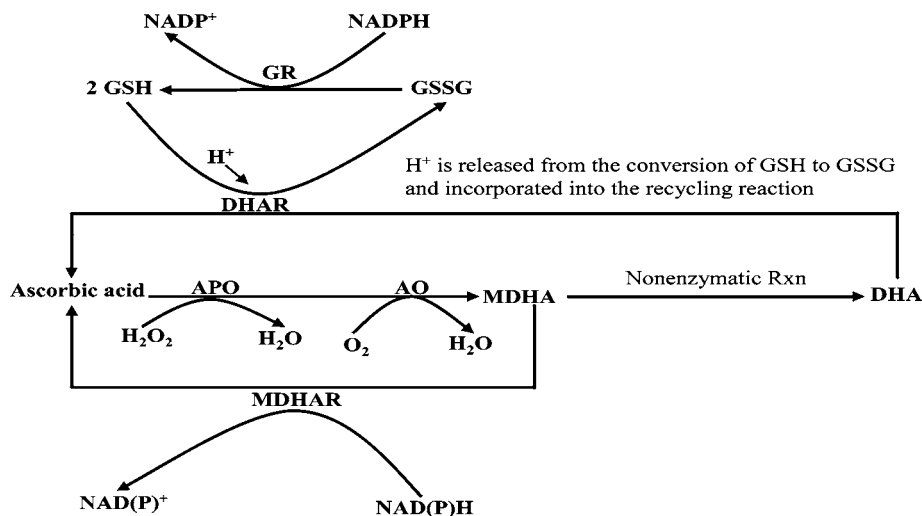§ National Institute of Agricultural Biotechnology.

Figure 1. Proposed ascorbate–glutathione recycling pathway: GSH, reduced glutathione; GR, glutathione reductase; GSSG, oxidized glutathione; DHAR, dehydroascorbate reductase; APO, ascorbate peroxidase; AO, ascorbate oxidase; MDHA, monodehydroascorbate; DHA, dehydroascorbate; MDHAR, monodehydroascorbate reductase; Rxn, reaction.

reactions such as transcriptional regulation of some genes, enzymatic actions, or function of electron transport (*6*).

In plant metabolism, the redox balance is regulated in the ascorbate–glutathione recycling reaction (**Figure 1**). This recycling reaction takes place in most plant cells and regulates their redox-related metabolisms (*12*). In this reaction, ascorbic acid is oxidized to monodehydroascorbate (MDHA) probably by ascorbate peroxidase (APO) or ascorbate oxidase (AO). The MDHA produced is labile and can be recycled to ascorbic acid by monodehydroascorbate reductase (MDHAR), but this recycling pathway is not predominant. The MDHA is thus immediately converted into dehydroascorbate (DHA) by nonenzymatic reaction (*12*), and the DHA is then recycled to ascorbic acid by dehydroascorbate reductase (DHAR). Moreover, DHAR converts reduced glutathione (GSH) to the oxidized form of glutathione (GSSG), liberating protons, which are incorporated into the recycling reaction of ascorbic acid (*12*). Accordingly, DHAR is responsible for maintenance of the homeostatic state of ascorbic acid (**Figure 1**) in vitamin C metabolism, by which it has influence on plant root development (*6*).

In particular, plant hairy roots have been used as a bioreactor for producing foreign proteins or secondary metabolites. Thus, their biomass growth pattern is directly involved with their yields (*13*). Plant DHAR genes have been cloned from various tissues of diverse plant species and characterized (*14–17*) and were used for molecular improvement of vitamin C content in crop plants such as wheat, corn, and other grain crops with low vitamin C (*18*). However, DHAR has not been reported in hairy root cultures. We have isolated a DHAR cDNA from sesame hairy root cultures obtained by transforming them with *Agrobacterium rhizogenes* and characterized its molecular structure. In this study, we provide some information about the expressional and biochemical profiles of the sesame DHAR during cultures of the sesame hairy roots. In addition, some biochemical properties of the transgenic sesame DHAR expressed in *Eschericha coli* are also reported and discussed here.

MATERIALS AND METHODS

Transformation, Initiation, and Cultures of Sesame Hairy Roots. The detailed procedures were performed with the method of Jin et al. (*13*). In brief, the wounded hypocotyls were transformed with *Agrobacterium rhizogenes* using an electroporatic micropulsor device (Bio-Rad, Hercules, CA) by applying a pulse of 12.5 kV/cm with a 25 μF

capacitor to obtain the transformed sesame hairy roots. The 3 week cultures in shaking flasks were used for isolation of the sesame hairy root DHAR cDNA (*13*).

Isolation of RNA and Construction of cDNA Library from Sesame Hairy Roots. All materials used for RNA isolation were RNase-free. Two grams of the hairy roots stored at −80 °C was pulverized with a pestle in a mortar containing liquid nitrogen, and the total RNA was extracted using a commercial RNA extraction solution (Invitrogen, Carlsbad, CA). A cDNA library was constructed with the commercial kits using a NucleoTrapoligo(dT) latex beads mRNA isolation system (Macherey-Nagel, Easton, PA) and the cDNA synthesis and cloning kit (Stratagene, La Jolla, CA). The detailed methods were performed according to the manufacturer's manuals.

RT-PCR and Cloning of DHAR cDNA. A reverse transcription–Polymerase Chain Reaction (RT-PCR) method was introduced to obtain a DNA fragment for preparing a probe using two degenerate primers: for forward primer, 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 for reverse primer, 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′, which are designed on the basis of the conserved amino acid regions commonly found in other plant DHAR genes. The DNA fragment (426 bp) obtained by the RT-PCR was then used as a probe to screen a full-length DHAR cDNA of the sesame hairy roots from the cDNA library. To screen the sesame hairy root DHAR cDNA, a plaque hybridization technique was conducted by applying the radiolabeled (α-^{32}P dCTP) probe. As a result, some positive plaques were selected and inserted into the cloning vectors (Stratagene).

DNA Sequencing and Data Analysis. The DHAR cDNA clone was sequenced with an automatic DNA sequencer, an ABI prism 373xl DNA analyzer (Applied Biosystems, Foster City, CA). After the DNA sample amplified was purified with AmpliCycle Sequencing Kit (Applied Biosystems, Foster City, CA) and dissolved in Hi-Di formamide solution, the sequencing reactions were conducted with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's manual.

Real-Time RT-PCR. A real-time quantitative C_T (threshold cycle) method was used with a model Applied Biosystems 7900HT Real-Time PCR System using TaqMan probes and primers for comparison of their relative transcription levels. 6-Carboxyfluorescein (FAM) was labeled at the 5′ end of the probes as a reporter with a nonfluorescent quencher (NFQ). For DHAR, the primers used were, forward primer, 5′-AGGAGAAATACCCGAAC CCTTCT-3′ and reverse primer, 5′-GATCCTTGCTCTTCAAGAATTTGACAA-3′, and the TaqMan probe used for the DHAR was 5′-CCACAGATGAGACTTC-3′. For 18S rRNA as an active reference, the primers used were, forward primer, 5′-CGGCTACCACATCCAAGGAA-3′ and reverse primer, 5′-GCTG-GAATTACCGCGGCT-3′, and the TaqMan probe used for 18S rRNA

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was 5′-TGCTGGCACCAGACTTGCCCTC-3′. In brief, approximately 200 mg of the subcultured sesame hairy root lines was cultured for the given culture periods, and 1 μg total RNA prepared from each of six different culture periods (from 1 to 6 weeks of culture) of the sesame hairy roots was used to synthesize the first cDNA strand using a commercial RT-PCR kit (Bioneer, DaeJeon, Korea). The first cDNA RT product (1 μL; corresponding to 50 ng total RNA) was then added to the PCR reaction solution (total 20 μL), and then 2 μL of the final PCR product was resolved for an agarose gel analysis. The PCR reaction was undergone with a TaqMan Universal Master Mix (Applied Biosystems) by cycling 40 times at the standard temperature conditions suggested in the manufacturer's manual. For an active reference, the 18S rRNA was used (*19*) to normalize DHAR transcription levels in the samples because a relatively high amount of 18S rRNA is ubiquitously present in the RNA pool of the highest plant cells.

Assay of DHAR Activity and Treatments of Temperature and pH. A spectrophotometric method (*20*) was used for assay of the DHAR activity of the sesame hairy roots using the root samples (1 g) collected at 1 week intervals from each of six different culture periods (from 1 to 6 weeks of culture). The crude proteins were extracted in 4 volumes of a buffer solution containing a solution of 10 mM potassium (pH 6.0) and 2 mM 2-mercaptoethanol by incubating the mixture at 4 °C for 4 h. The supernatant obtained after centrifugation at 6000g for 20 min was desalted with a PD-10 column (Millipore, Bedford, MA) and concentrated with an Amicon Ultra-4 centrifugal filter (Millipore). DHAR activity was measured at 265 nm using a spectrophotometer (Agilent 8453, Santa Clara, CA) in a reaction solution containing 50 mM potassium phosphate (pH 6.0), 5 mM DHA, 0.5 mM GSH, and 100 μg of the extracted protein (total 1 mL) by incubation at 30 °C for 10 min. The recombinant sesame DHAR activity from *E. coli* cell cultures obtained by disruption with a sonicator (Sonics & Materials, Newtown, CT) was initiated by the addition of the crude or purified protein (1 μg) to the same buffer solution (total 1 mL) as above. After the reaction mixture had been incubated at 30 °C for 2 min, the DHAR activity was measured by monitoring the absorbance changes at a wavelength of 265 nm. One unit of the DHAR activity was expressed as the amount of enzyme required to liberate 1 μmol of product (ascorbic acid) per minute for the recombinant DHAR or to liberate 1 μmol of product (ascorbic acid) per minute per gram of fresh weight for the nonrecombinant sesame DHAR.

To examine the effect of temperature on the recombinant sesame DHAR activity, nine different temperatures (10−90 °C at 10 °C intervals) were independently employed, and the assays were conducted at each temperature to be applied using the same procedure as above (before the incubation reactions were initiated, the temperatures to be treated were preconditioned at each temperature). To investigate the effect of pH on the recombinant sesame DHAR activity, eight different pH values (2−9 at 1 pH unit intervals) were used. For this experiment, four different buffers of 50 mM glycine−HCl (pH 2−3), 50 mM sodium acetate (pH 4−5), 50 mM potassium phosphate (pH 6−8), and 50 mM Tris-HCl (pH 8−9) were prepared, and the DHAR activity was assayed by adding the sample protein to each pH buffer to be applied using the same procedure mentioned above.

SDS-PAGE and Quantification of Protein. The protein−dye binding method of Bradford (*21*) was conducted to quantitate the amounts of protein from both samples of the recombinant cell cultures and the sesame hairy roots using a commercial protein assay kit (Bio-Rad). For SDS-PAGE analysis, appropriate amounts of the crude proteins were loaded and analyzed using the Bio-Rad mini-gel system according to the manufacturer's manual.

Expression of the Recombinant DHAR in *E. coli*. The coding sequence (639 bp) of the sesame hairy root DHAR was amplified with two cloning sites, *Eco*RI and *Sac*I, to clone it into the protein expression vector pET-32a (Novagen, Madison, WI) using two primers: forward primer, 5′-CCGAATTCATGGCTGTGGAAGTATGCGTC-3′; and reverse primer, 5′-CCGAGCTCTCATGCATTAACTTTGGGTG-3′. The DNA fragment was then ligated into the corresponding sites of the protein expression vector, and the resultant plasmid (pET-32a-DHAR) was transformed into *E. coli* strain BL21. The expression of the sesame hairy root DHAR was induced with isopropyl-β-D-thiogalactopyranoside

(IPTG). Fifty microliters of the transformed cells cultured overnight at 37 °C were transferred to 5 mL of LB broth and cultured until an OD value of 0.6 (OD$_{600}$) was reached (ca. 3 h), and then IPTG was added to the broth at the final concentration of 1 mM and another 3 h culture was performed. After the cells had been incubated in ice for 5 min and centrifuged at 5000g at 4 °C, the cell pellets were resuspended in a phosphate-buffered saline (PBS) solution and disrupted with a sonicator (Sonics & Materials). The mixture was then centrifuged at 13000g for 10 min, and the supernatant was used for further analysis.

Purification of the Recombinant DHAR from *E. coli*. The recombinant polyhistidine-tagged DHAR was purified with a commercial N-terminal histidine-tag fused protein purification system (HisLink Spin Protein Purification System, Promega). After the cells harboring the recombinant plasmids had been cultured with IPTG induction as mentioned above, 700 μL of the cell cultures was transferred into a 1.5 mL tube and mixed with a cell lysis reagent (FastBreak Reagent) provided by Promega. The protein isolated with a spin column was obtained with the HisLink Spin column according to the manufacturer's manual. For further purification of the His-tagged DHAR, the recombinant enterokinase (rEK) provided by a commercial company (Novagen, Madison, WI) was added to a cleavage solution (total 100 μL) containing the DHAR protein and incubated at 20 °C for 16 h. Thereafter, the rEK was removed from the mixture and then the DHAR enzyme recovered by spin filtration according to the manufacturer's recommendations. The recombinant sesame DHAR isolated from the rEK mixture was further purified with a fast performance liquid chromatography (FPLC). Before the isolated DHAR protein was subjected to an anion exchange column (Mono Q HR 5/5; Amersham Biosciences, Piscataway, NJ) using a FPLC system (ÄKTApurifier, Amersham Biosciences), the buffer change was done with PD-10 columns (Amersham Biosciences) and filtered through a 0.2 μm filter (Sartorius, Goettingen, Germany). After the column used had been equilibrated with 50 mM sodium phosphate buffer (pH 6.8), the DHAR protein sample (3 mL) was then injected into the column and eluted with the same buffer containing 1 M NaCl (20 mM) at flow rates of 1.0 mL/min. The pooled DHAR protein sample was finally concentrated with an Amicon Ultra-4 Centrifugal Filter system (Millipore).

Preparation of Antibody and Western Blot Analysis. The antigen (the purified DHAR protein) was eluted from a 12% acrylamide gel and its concentration adjusted to 1.0 mg/mL. Freund's complete and incomplete adjuvants were used for the first round of antigen injection and for the booster injections, respectively, to prepare its immunogen in rat. One microgram of the purified DHAR protein was resolved on an acrylamide gel and transferred to a poly(vinylidene difluoride) (PVDF) membrane (Amersham Biosciences) using a Mini-Blot Cell (Bio-Rad) system. The membrane was blocked with the PBS buffer solution containing 5% fat-free skim milk and 0.05% Tween-20 for 1 h. The diluted rat IgG (1:5000 for the recombinant DHAR in *E. coli*; 1:1000 for the DHAR in the hairy roots) raised against the purified DHAR protein as the primary antibody was hybridized overnight with the membrane prepared. After three washings with PBS, the diluted antibody (1:2000) conjugated with horseradish peroxidase (HRP) was applied for 1.5 h. Following three washings with the same buffer solution, the band signals were detected with enhanced chemiluminescence (ECL) Western blotting kits (Amersham Biosciences) by exposing it on an X-ray film (for 10 min for the recombinant DHAR in *E. coli*; for 30 min for the DHAR in the hairy roots).

RESULTS AND DISCUSSION

Structural Characterization of the Sesame DHAR cDNA. Sesame hairy roots transformed with a strain of *A. rhizogenes* (*22*) were initiated by wounding the hypocotyl surfaces of the sesame seedlings (*13*). After the transformed hairy roots had been cultured for 3 weeks and collected, RNA was isolated from them and a cDNA library was constructed as mentioned under Materials and Methods. As a consequence, a DHAR cDNA was cloned from the transformed sesame hairy roots by the plaque

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Table 1. Sequence Characterization of Deduced Amino Acids (AA) of DHAR and the Conserved CXXS or CXXC Motif

species (Genbank accession no.)	no. of AA residues	% identity (similarity)	site of CXXS or CXXC[a]
sesame (DQ287974)	212	100 (100)	20 CPFS
tobacco (AY074789)	212	83 (91)	20 CPFS
potato (DQ191638)	210	78 (87)	20 CPFS
tomato (AY971873)	210	77 (86)	20 CPFS
Zinnia elegans (AB158512)	212	77 (85)	20 CPFS
Arabidopsis (AY140019)	213	74 (85)	20 CPFS
rice (AY074786)	213	72 (83)	20 CPFS
wheat (AY074784)	211	72 (83)	19 CPFS
Brassica juncea (AF536329)	256	49 (64)	65 CPFC
soybean (DQ006810)	259	47 (62)	67 CPFC
Medicago truncatula (DQ006811)	264	47 (61)	72 CPFS
spinach (AF195783)	266	46 (61)	75 CPFC

[a] The number before CXXS or CXXC denotes the AA position corresponding to cysteine in the CXXS or CXXC motif sequence by calculating from the translation start.

hybridization method (data not shown). The nucleotide sequences of the sesame DHAR cDNA and its deduced amino acid compositions are deposited in the database (GenBank accession no. DQ287974). Their identity and the similarity between the deduced amino acid sequences of the sesame hairy root DHAR and those of other plant DHAR genes were characterized. As shown in **Table 1**, the sesame DHAR polypeptide has a motif peptide, CXXS, which is highly conserved in most DHAR polypeptide called a CXXS or CXXC motif (*23−27*). It is well-known that the motif of CXXS or CXXC peptide is involved in a redox function and corresponds to thiol-dependent redox sites in the thiol−disulfide oxidoreductase enzymes (*26, 27*). Shimaoka et al. (*25*) provided some information that the first positioned cysteine residue in the motif of CXXC plays a key role in forming a disulfide bond with GSH in the cysteinylthiohemiketal essential for the conversion reaction of DHA to ascorbic acid. Some other workers (*24, 26, 27*) also stated that the presence of cysteine residues in the DHAR is important for stable formation of intramolecular disulfide bonds in the native secondary structure of DHAR protein. The three cysteine residues contained in the DHAR sequences (data not shown), in which one is located in the N-terminal region, another is included in the CXXS motif, and the other is near the C terminus of the DHAR (data not shown), suggested that these cysteine residues may be conducive to the formation of its native secondary structure of the sesame DHAR protein (*24−27*). Another feature of the sesame hairy root DHAR polypeptide is that the putative catalytic site may be in the motif of CXXS, although a CXXC instead of the CXXS motif is always found in the active sites of thiol-dependent enzymes including DHAR. The reason for this is based on the fact that even though the original cysteine residue positioned at the fourth site of the CXXC motif was replaced with serine, the DHAR activity was rather increased, indicating that the disulfide bond between two cysteine residues in the CXXC motif is not the essential requirement for the catalytic reaction of the DHAR (*24*). Accordingly, the above description indirectly supports an assumption that the CXXS motif in the sesame hairy root DHAR may be responsible for its catalytic reaction. In addition, the sesame DHAR could be classified into the cytosolic DHAR isozyme family group because the sesame DHAR has higher homology with the DHAR, which is classified into the cytosolic DHAR group (*16, 28*), although no signal peptide is identified in their N-terminal sequence.

Table 2. Purification of the Recombinant Sesame DHAR Expressed in an *E. coli* Expression System

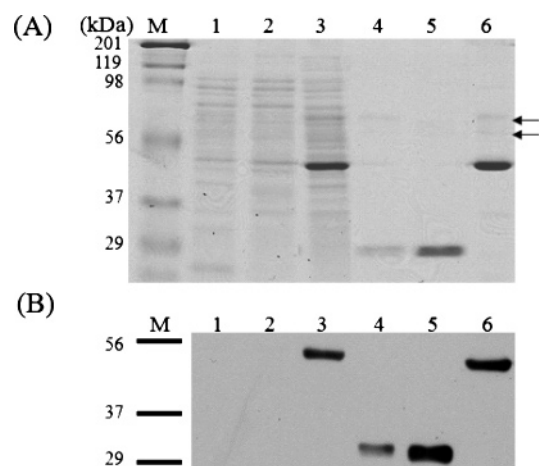| purification step	volume (μL)	total protein (μg)	total activity (μmol/min)	specific activity (μmol/min/μg of protein)	purification (fold)	yield (%)
crude extract	500	3260	120384	37	1	100
His-tag purification	200	990	101272	102	2.8	84.1
enterokinase treatment	200	530	74257	140	3.8	61.7
Mono Q HR	200	197	30084	153	4.1	25.0

Figure 2. Recombinant sesame DHAR protein expressed in an *E. coli* expression system, pET-32a: M, size marker (kilodaltons); lane 1, protein extract from the *E. coli* culture with no DHAR cDNA (control); lane 2, protein extract from the culture with DHAR cDNA (but no IPTG induction); lane 3, protein extract from the culture with DHAR cDNA by IPTG induction; lane 4, protein extract from the culture His-tag removed by treatment of rEK; lane 5, protein extract purified by a FPLC system; lane 6, His-tagged DHAR containing protein extract from the culture induced by IPTG; (**A**) SDS-PAGE analysis; (**B**) Western blot analysis. The arrows indicate that other proteins are still present in the extract.

Purification Characterization of the Recombinant Sesame DHAR in *E. coli*. The sesame DHAR cloned was confirmed and identified with the recombinant DHAR expressed in a commercial *E. coli* expression system, pET-32a (Novagen). For this study, the purity of the recombinant DHAR was quantitatively evaluated through four steps of purification procedure, and SDS-PAGE and Western analyses were carried. In the first purification step, the crude extract was prepared from the *E. coli* cells by centrifugation and used to estimate the overall purification parameters (**Table 2**). Following the second and third steps, increased purities of the recombinant DHAR were measured with 2.8- and 3.8-fold higher values (**Table 2**), respectively. When the DHAR protein treated with the rEK enzyme was finally subjected to an anion-exchange chromatography, the highest specific activity (153 μmol/min/μg of protein) was determined with the greatest purity (4.1-fold higher). Analysis by SDS-PAGE also revealed a difference in the degree of the DHAR purity in an acrylamide gel (**Figure 2A**). In the lanes (lanes 1−3 of **Figure 2A**) of the crude protein extracts with and without IPTG induction, a variety of polypeptides were observed. In contrast, three polypeptides (lane 6 of **Figure 2A**) were seen in the gel of the protein extract from His-tag purification. When the protein extract from the His-tag purification was then treated with rEK, its purity was further higher by 3.8-fold (**Table 2**). However, two or three other
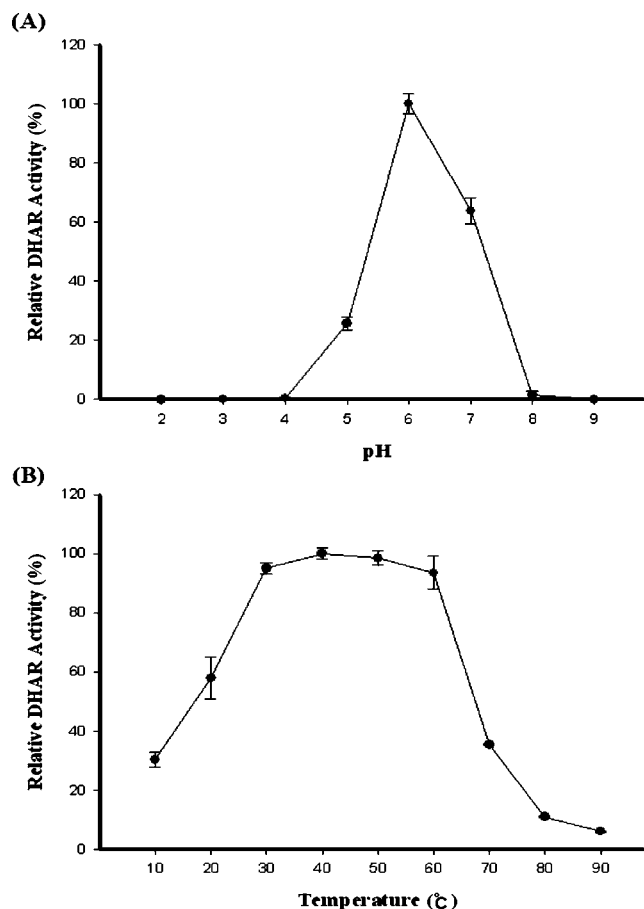
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(A)

(B)

Figure 3. Effects of pH and temperature on the recombinant sesame DHAR activity. (**A**) Four different buffers of glycine–HCl (pH 2–3), sodium acetate (pH 4–5), potassium phosphate (pH 6–8), and Tris-HCl (pH 8–9) were prepared, and the DHAR activity was assayed by adding the sample protein to each pH buffer to be applied. (**B**) Nine different temperatures (10, 20, 30, 40, 50 60, 70, 80, and 90 °C) were applied during incubation of the DHAR reaction.

polypeptides were still detected in the gel (arrows in lanes 4 and 6 of **Figure 2A**), indicating that the DHAR enzyme was not completely purified. Following these purification steps, one band signal was obtained in the gel (lane 5 of **Figure 2A**) with 4.1-fold higher purity (**Table 2**), indicating that the purified enzyme was homogeneous. The above results implied that the chromatographic step is necessary to gain the recombinant DHAR with its highest purity. The Western analysis also proved the presence of the DHAR enzyme in the gel (**Figure 2B**).

Effects of pH and Temperature on the Recombinant DHAR. To investigate the response of the recombinant DHAR activity to pH conditions during incubation of the DHAR reaction solution, different ranges of pH between 2 and 9 were introduced at an interval of 1 pH unit, and for their data analysis, the mean values were provided with standard deviation by calculation in triplicates for each experiment. As a consequence, it was evident that the recombinant DHAR would have an optimal reaction condition around pH 6 because one sharp pH peak was assayed at pH 6 (**Figure 3A**). This pH value for the purified recombinant sesame DHAR was different from those of other plant species having optimal pH between 7.8 and 8.2 depending on plant species or plant tissues isolated (*15*, *20*, *29*). This pH difference may be due to the difference of ionization properties in the thioredoxin-catalyzed reaction processes between them (*26*).
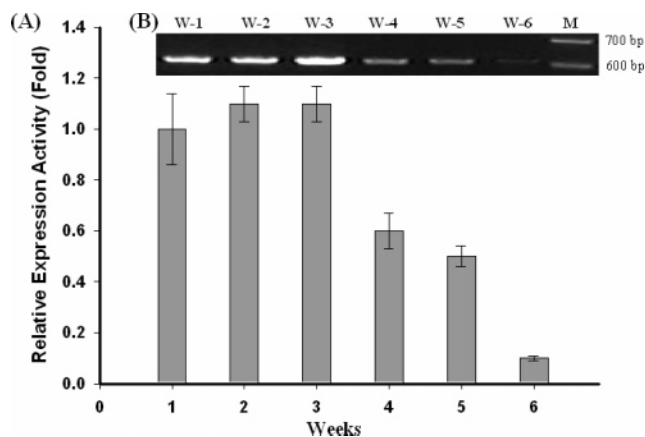

Figure 4. Profile of transcription activity of the sesame DHAR during the sesame hairy root cultures: (**A**) transcription activity by a real-time RT-PCR technique; (**B**) RT-PCR [M, size marker (bp); lanes W-1–W-6, culture period from 1 to 6 weeks, respectively].

For the optimal temperature of the DHAR, nine different reaction temperatures ranging from 10 to 90 °C were applied at intervals of 10 °C. **Figure 3B** shows that the temperatures optimal for the recombinant DHAR were in a range between 30 and 60 °C, suggesting that the DHAR has a relatively wide range of optimal temperatures. Below 30 °C and above 60 °C, its activity was rapidly decreased to 25−90%. The behavior of the DHAR activity responding to temperatures applied was similar to those of other plant species (*15*, *20*, *29*).

Analysis of Expression Profiles of DHAR cDNA during the Sesame Hairy Root Cultures. To characterize the expressional behaviors of the DHAR during the cultures of the transformed sesame hairy roots, a real-time RT-PCR and the conventional RT-PCR technique were employed. As shown in **Figure 4**, the transcription activities of the sesame DHAR were relatively greater during the first 3 week cultures. After the 3 week cultures, its transcription activity was rapidly diminished by approximately 50−90%. Its highest activity appeared in the 3 week cultures, whereas the lowest one was found in 6 week cultures (**Figure 4A**). This transcriptional pattern of the sesame DHAR during the cultures of the hairy roots was also supported by analysis of RT-PCR on the agarose gel, which shows relatively stronger band signals in their first 3 week culture than in the later 3 week one (**Figure 4B**). In particular, the band signal of the 6 week culture almost disappeared (**Figure 4B**). In contrast, using the real-time RT-PCR, its transcription activity was detected even in the 6 week culture, indicating that the real-time RT-PCR technique is a more sensitive method for the analysis of transcription activity (*28*). In general, it is well-known that gene expression levels can be assayed at RNA levels for comparison of differential transcription activity of specific genes. From the above results, two important findings were obtained. One is that during the cultures of the sesame hairy roots, change in the transcription activity of the sesame DHAR well reflected the behavior of gene expression of the sesame DHAR, demonstrating that the sesame DHAR would be a differentially expressed gene responding to culture periods of the sesame hairy roots. Although there has been no clear evidence in plants of a specific type of cell differentiation, our results support the DHAR being involved with major changes in gene expression, which is exclusively initiated during the development of hairy roots (*6*, *8*). Another is that the expression pattern of the sesame DHAR also suggested that the sesame DHAR would be a gene essential for the development of the sesame hairy roots because the DHAR gene is involved in
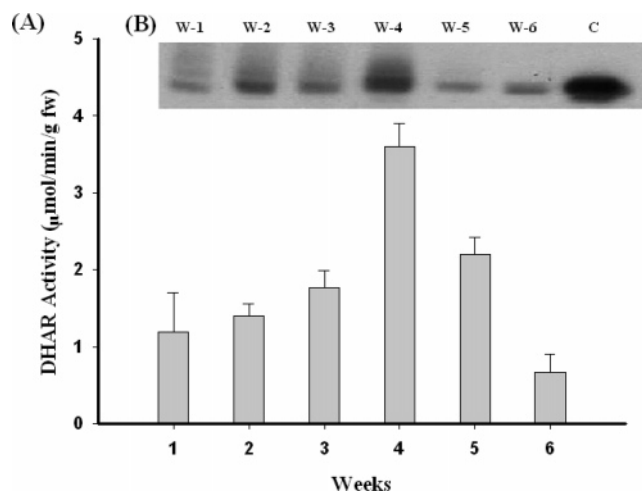
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Figure 5. Changes in the sesame DHAR activity during the sesame hairy root cultures: (**A**) comparative analysis of the sesame DHAR activity between the culture periods (C, control); (**B**) Western blot analysis [lanes W-1–W-6, culture period in weeks; lane C, control (purified recombinant sesame DHAR)].

vitamin C metabolism as a housekeeping gene expressed in all plant tissues during plant development (*6, 28*).

Behavioral Analyses of Protein and DHAR Activity during the Sesame Hairy Root Cultures. During the cultures of the sesame hairy roots, changes in the sesame DHAR activity were determined. To investigate the profile of the sesame DHAR activity during the cultures of the hairy roots, its activities were compared between their culture periods (**Figure 5A**), and Western blotting analysis was conducted for identification of its presence in the hairy roots (**Figure 5B**). **Figure 5A** shows that the sesame DHAR activity was gradually increased until the culture period reached 4 weeks. After 4 weeks of culture, the DHAR activity was rapidly decreased by ca. 40−80% (0.66 ± 0.23 to 2.19 ± 0.22 units) compared with that of the 4 week culture with 3.60 ± 0.29 units (**Figure 5A**). Analysis of the Western blot exemplified that the sesame DHAR enzyme was present in the hairy roots (**Figure 5B**). The intensities of the IgG band signals by the Western blotting analysis were different between the culture periods of the hairy roots. The highest level of IgG band signal intensity was found on the blot of the 4 week culture (**Figure 5B**), reflecting a correlation with the profile of the sesame DHAR activity during the cultures of the sesame hairy roots. These results suggested that a relatively greater amount of the DHAR enzyme was included in the hairy roots of the 4 week culture than in those of other cultures and also that the DHAR might more actively undergo a DHAR-related metabolism in the 4 week cultured hairy roots (*3, 8, 30, 31*).

A DHAR cDNA was isolated from sesame hairy roots and its molecular structure, and its expressional and biochemical properties were characterized. The functional active recombinant DHAR could be expressed using an *E. coli* expression system; it was purified through four purification steps of centrifugation, His-tag, rEK treatment, and a FPLC system, and its biochemical properties were determined. Using the purified DHAR protein, its antibody was prepared and used for Western blot analyses. During the sesame hairy root cultures, some expressional behaviors of the sesame DHAR by a real-time RT-PCT technique and protein profiles were investigated, and its biochemical properties were also characterized. Some information obtained from this study suggested that DHAR is present

in the hairy roots and could play a role during hairy root development.

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