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Biochemical Characterization of Transgenic Tobacco Plants Expressing a Human Dehydroascorbate Reductase Gene

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Dehydroascorbate (DHA) reductase (DHAR, EC 1.8.5.1) catalyzes the reduction of DHA to reduced ascorbate (AsA) using glutathione (GSH) as the electron donor in order to maintain an appropriate level of ascorbate in plant cells. To analyze the physiological role of DHAR in environmental stress adaptation, we developed transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) plants that express a human DHAR gene isolated from the human fetal liver cDNA library in the chloroplasts. We also investigated the DHAR activity, levels of ascorbate, and GSH. Two transgenic plants were successfully developed by *Agrobacterium*-mediated transformation and were confirmed by PCR and Southern blot analysis. DHAR activity and AsA content in mature leaves of transgenic plants were approximately 1.41 and 1.95 times higher than in the non-transgenic (NT) plants, respectively. In addition, the content of oxidized glutathione (GSSG) in transgenic plants was approximately 2.95 times higher than in the NT plants. The ratios of AsA to DHA and GSSG to GSH were changed by overexpression of DHAR, as expected, even though the total content of ascorbate and glutathione was not significantly changed. When tobacco leaf discs were subjected to methyl viologen at 5 μM, T_0 transgenic plants showed about a 50% reduction in membrane damage compared to the NT plants.

Keywords: Ascorbic acid, Dehydroascorbate reductase, Glutathione, Oxidative stress, Transgenic tobacco plant

Introduction

Oxidative stress, derived from reactive oxygen species (ROS) under stressful environmental conditions, is associated with a number of physiological disorders in plants. Plants have developed an antioxidative defense mechanism in order to cope with these ROS, including antioxidant enzymes such as superoxide dismutase (SOD), peroxidase, catalase, as well as low molecular mass antioxidants such as ascorbate (AsA), α-tocopherol (vitamin E) (Alscher and Hess, 1995; Inze and van Montagu, 1995; Lee and Kim, 1998; Noctor and Foyer, 1998; Ahn *et al.*, 1999; Lee *et al.*, 1999).

The ascorbate-glutathione pathway, recently renamed as the water-water pathway by Asada (1999), might be the most important antioxidative mechanism in plant chloroplasts (Asada, 1994). Superoxide anion radicals are dismuted by SOD that is associated with PSI. The resulting hydrogen peroxide is scavenged by thylakoid-bound ascorbate peroxidase (APX). ROS that escape destruction at the thylakoid are scavenged by stromal SOD and stromal APX. Monodehydroascorbate radicals (MDA), which are produced by APX, are converted to AsA through reactions with Fd or monodehydroascorbate reductase (MDHAR). Reduction of dehydroascorbate (DHA) to AsA is catalyzed by DHA reductase (DHAR) via the ascorbate-glutathione pathway (Fig. 1). Genes that encode antioxidant enzymes (such as SOD, APX, MDHAR involved in this pathway) have been reported and characterized from plant species (Allen, 1995).

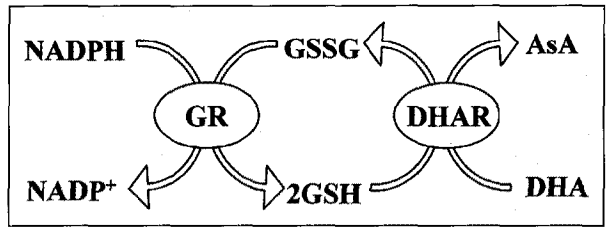

Fig. 1. Partial diagram of ascorbate-glutathione pathway. Oxidized ascorbate (dehydroascorbate, DHA) is converted to reduced ascorbate (AsA) by DHA reductase (DHAR) using reduced GSH as an electron donor. In turn, the reaction of oxidized glutathione (glutathione disulfide, GSSG) to reduced GSH is catalyzed by glutathione reductase (GR) using NADPH as an electron donor.

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DHARs from spinach leaves (Hossain and Asada, 1984), potato tubers (Dipierro and Borranccino, 1991), and rice (Kato *et al.*, 1997) were purified and characterized on the basis of the enzyme. The rat DHAR cDNA was isolated from a rat liver cDNA library for the first time as a DHAR gene (Ishikawa *et al.*, 1998). Recently, plant DHAR genes were cloned from rice (Urano *et al.*, 2000), spinach (Shimaoka *et al.*, 2000), and Arabidopsis (Shimaoka *et al.*, 2000). However, there is no report on the transgenic plants that express a DHAR gene in the chloroplasts. Such a report would assist in understanding the physiological role of DHAR in response to oxidative stress.

Manipulation of the expression of enzymes that are involved in scavenging ROS by gene transfer technology has provided new insights into the role of these enzymes in the chloroplasts by allowing direct investigation of their functions and interactions (Foyer *et al.*, 1994; Allen 1995; Allen *et al.*, 1997). Ascorbate acts as an important antioxidant in plant cells (Conklin *et al.*, 1996; Smirnoff 1996; Smirnoff and Pallanca, 1996). For maintenance of the antioxidative activity of ascorbate, its regeneration from DHA to AsA is necessary, as well as its biosynthesis (Wheeler *et al.*, 1998). In this report, we describe the isolation of a DHAR gene from the human fetal liver, the development of transgenic tobacco plants that express a human DHAR gene in the chloroplasts, and their biochemical analysis.

Material and Methods

Cloning of DHAR gene DHAR cDNA was cloned from the cDNA library of a human fetal liver (Kim *et al.*, 1995). For the cloning of the DHAR gene, and construction of the plant expression vector, PCR was conducted to get the cDNA for the human DHAR using primers (5'-TCCGAGTCGACAGCATGAGGTTCT-3' and 5'-CTGGATCCTCAGAGCCCATAAT-3') and the human fetal cDNA library as a template (94°C, 1 min; 60°C, 1 min; 72°C, 1 min). The amplified fragment was cloned into pGEM T-easy (Promega, Madison, USA) and sequenced. The amplified fragments were digested with *Sal*I and *Bam*HI (underlined parts of these primers) and subcloned into the corresponding sites of pRW20 (Allen *et al.*, 1997), which enhanced the CaMV 35S promoter, TEV leader, transit peptide for chloroplast, and CaMV 35S terminator (Fig. 3A). The chimeric gene construct was digested with *Pst*I and ligated into the corresponding site of pCGN1578 (McBride and Summerfelt, 1990) for plant transformations.

Development of transgenic plants DHAR transgenic plants were developed by *Agrobacteria*-mediated transformation (Horsch *et al.*, 1985). The recombinant pCGN1578 expression vector, carrying the DHAR cDNA, was transferred into *A. tumefaciens* (strain EHA 101) and introduced into the tobacco genome using a leaf disc transformation procedure. The regenerated tobacco shoots were selected by growth on a MS (Murashige and Skoog, 1962) medium that was supplemented with 100 mg/l kanamycin and 250 mg/l carbenicillin. Kanamycin resistant shoots were directly formed on the cut edges of the leaf discs. Rooting of the kanamycin resistant plantlets was carried out in a selectable MS medium without growth regulators. After acclimation, the plants were transplanted to potting soil and maintained in a greenhouse. The plants were grown in 10 cm diameter pots that contained mineral-mixed soils. Three-month-old plants at the ten-leaf stage were used for this experiment. The seventh leaf from the top was harvested as a mature leaf, whereas the third leaf from the top was harvested as an immature leaf.

Analysis of enzyme activity Fresh leaf tissues (0.2 g) were homogenized on liquid nitrogen with a mortar and pestle, and 50 mM potassium phosphate buffer (pH 7.0, 400 µl) was added to the powder sample. The homogenate was centrifuged at 12,000 × *g* for 15 min at 4°C. The resulting supernatant is referred to as the crude extract and was used immediately for enzyme assays. The DHAR activity was measured by the method of Nakano and Asada (1981). The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0) that contained 0.1 mM EDTA and 2.5 mM GSH, and the crude extract. The enzyme activity was determined by the formation of AsA at 265 nm. One unit of DHAR activity was defined as oxidizing the 1 µM AsA to DHA per min. GR activity was measured by the method of Cakmak *et al.* (1993). The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0) that contained 0.1 mM EDTA, 0.5 mM NADPH, and 15 mM GSSG, as well as the crude extract. The enzyme activity was determined from the rate of NADPH oxidation, as measured by the decrease in OD at 340 nm for 1 min. One unit of GR activity was defined as reduction of the 1 mM GSSG to GSH per min.

Analysis of AsA and GSH Endogenous AsA was extracted and analyzed by the method of Graham and Annette (1992) with a slight modification (Ahn *et al.*, 1999). The leaves were homogenized on ice with a mortar and a pestle in 62.5 mM metaphosphoric acid (1 : 2, w/v). The homogenate was centrifuged at 12,000 × *g* for 20 min at 4°C. For the analysis of total AsA, the oxidized AsA (DHA) in the sample was reduced by a slightly modified method of Law *et al.* (1983). A solution of sixty mM DTT was added to the extract and incubated for 24 h at 25°C. Samples with or without DTT treatment were filtered through a 0.5 µm FH-type Millipore filter. The filtrate was loaded onto an aminex HPX-87 H ion exclusion column (300 × 7.8 mm, Bio-rad) that was connected to a Spectra-Physics HPLC system and eluted with 4.5 mM H_2SO_4 at a flow rate of 0.5 ml per min. The elution of AsA was detected at 245 nm. The retention times (min) of AsA and DHA were found to be 12.3 and 10.9, respectively. A quantitative analysis was carried out by comparing the peak areas of the samples with those of the authentic AsA. DHA was determined as the difference between the total AsA contents after DHA reduction and the AsA contents of the original sample.

GSH was assayed according to the method of Griffith (1985). Endogenous GSH was extracted in the same potassium buffer that was used for the ascorbate. The supernatant was used for total GSH. For the assay of GSSG, 2-vinylpyridine was added to mask GSH, the tube mixed until an emulsion formed, then incubated more than 1 h at 25°C. The standard reaction mixture contained 100 ml potassium phosphate buffer (pH 7.5), containing 5 mM EDTA, 0.2 mM NADPH, 0.6 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and GR (10 units). The reaction was monitored by measuring the change in OD at 412 nm per min. GSH was

determined as the difference between the total GSH contents and GSSG.

MV treatment and cell leakage analysis Methyl viologen (MV, paraquat) damage, using leaf discs, was analyzed, as described by Yun *et al.* (2000). Leaf discs (6 mm diameter), collected from the mature leaves, were transferred to 5.0 cm Petri dishes containing 17 ml of MV solutions at various concentrations. Each Petri dish contained fifty leaf discs and was incubated at 25°C for 12 h in darkness to allow diffusion of the MV into the leaf. After preincubation, the leaf discs were illuminated until samplings were obtained for a cell leakage analysis. The conductivity of the decanted MV solution was measured with an Orion model 162 conductivity meter.

Results and Discussion

Cloning of the DHAR gene from a human fetal liver DHAR cDNA (639 bp) was cloned from the human fetal liver cDNA library. The human DHAR cDNA, cloned in this study, was identical to the EST clone (GenBank Accession Number U90313) reported. Fig. 2 shows the comparison of amino acid sequences of the human DHAR gene in this study and other DHAR genes, such as rats (Ishikawa *et al.*, 1998), spinach (Shimaoka *et al.*, 2000), rice (Urano *et al.*, 2000), *Arabidopsis* (Shimaoka *et al.*, 2000). There is a high amino acid identity

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Human       ------------------------------------------------------------
Rat         ------------------------------------------------------------
Spinach     MSTVKITPVAYSLSSSTLKRQLIPNPQFHRRNRTIFTQHFKNGTQRNLTVSMSSSDPLQI 60
Rice        ------------------------------------------------------MGVEV 5
Arabidopsis -----QPSTTAGVLSAS-------VSRAGFIKR------CGSTKPGRVGRFVTMATAASPLEI 45

Human       ----------MRFCPFAERTRLVLKAKGIRHEVININLKNKPEWFFKKNPFGLVPVLEN 49
Rat         ----------MRFCPFAQRTLMVLKAKGIRHEIININLKNKPEWFFEKNPFGLVPVLEN 49
Spinach     CVKESVTTPNKLGDCPFCQRVLLTLEEKHLPYDMKLVDLSNKPEWFTNINPDGKVPVVK- 119
Rice        CVKAAVGHPDTLGDCPFSQRVLLTLEEKKVPYEMKLIDVQNNPDWFLKISPEGKVPVFNG 65
Arabidopsis CVKASITTPNKLGDCPFCQKVLLTMEEKNVPYDMKMVDLSNKPEWFLKISPEGKVPVVK- 104
                       ***          *          * * **    * * ***

Human       SQGQLIYESAITCEYLDEAYPGKKLLPDDPYEKACQKMILELFSKVPSLVGSFIRSQNKE 109
Rat         TQGHLITESVITCEYLDEAYPEKKLFPDDPYEKACQKMTFELFSKVPSLVTSFIRAKRKE 109
Spinach     FDENWVADSDIIAKSLEERYPNPPLATPDEKSSVG---------SKIFPAFVGFIKSKDPS 171
Rice        GDGKWIPDSDVITQVIEEKYPTPSLVTPPEYASVG---------SKIFSCFTTFLKSKDPN 117
Arabidopsis FDEKWVPDSDVITQALEEKYPEPPLATPPEKASVG---------SKIFSTFVGFLKSKDSG 156
                  *        * **  *                    **        *

Human       DYAGLKEEFRKEFTKLEEVLTNKKTTFFGGNSISMIDYLIWPWFERLEAMKLNECVDHTP 169
Rat         DHPGIKEELKKEFSKLEEAMANKRTAFFGGNSLSMIDYLIWPWFQRLEALELNECIDHTP 169
Spinach     D---GKEQGLLNELSSFNDYLK-ENGPFINGEKISAADLALGPKLYHMEIALGHYKNWSVP 228
Rice        D---GSEKALLTELQALEEHLK-AHGPFINGQNISAADLSLAPKLYHLQVALEHFKGWKIP 174
Arabidopsis D---GTEQVLLDELTTFNDYIK-DNGPFINGEKISAADLSLAPKLYHMKIALGHYKNWSVP 213
            * *       *              * *  * *   *                 *

Human       KLKLWMAAMKEDPTVSALLTSEKDWQGFLELYLQNSPEACDYGL 213
Rat         KLKLWMATMQEDPVASSHFIDAKTYRDYLSLYLQDSPEACDYGL 213 (76%)
Spinach     ESLPYVKSYMKNIFSRDSFVKTIASTEDVIAGWAKHTS------- 266 (29%)
Rice        EDLTNVHAYTEALFSRESFIKTKAAKEHLIAGWAPKVNA------ 213 (29%)
Arabidopsis DSLPFVKSYMENVFSRESFTNTRAETEDVIAGWRPKVMG------ 252 (29%)
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Fig. 2. Alignment of amino acid sequences of a human DHAR that was isolated in this study with rat DHAR (Ishikawa *et al.*, 1998), spinach DHAR (Shimaoka *et al.*, 2000), rice DHAR (Urano *et al.*, 2000), and Arabidopsis DHAR (Shimaoka *et al.*, 2000). A gap, introduced to optimize the alignment, is indicated by a hyphen. Amino acids, conserved in all of the proteins, are indicated by *, and amino acid identities of human DHAR with other DHAR are in parenthesis.

(about 76%) between human and rat DHAR genes. However, there is a low amino acid homology (about 29%) between human and plant DHARs. The activity of isolated DHAR was confirmed in *E. coli* using pET19b (data not shown here). The sequence data and expression in *E. coli* suggested that the cDNA, cloned from the human fetal liver cDNA library, might code the DHAR by converting DHA to reduced ascorbate (AsA).

Development of transgenic plants Two transgenic tobacco plants were developed by *Agrobacterium*-mediated transformation. To examine the integration of the DHAR gene in the transformed plants, PCR and a Southern blot analysis were performed. Genomic DNA was prepared from mature leaves of transformed and non-transformed plants. Both NPTII and DHAR genes were detected by a PCR analysis in the two transformed plants (Fig. 3B). Genomic DNA was digested with *Pst*I, *Eco*RI, and *Bam*HI. It was then subjected to a Southern blot analysis (Fig. 3C). A single band was detected, implying that there is one gene that corresponded to DHAR in tobacco. These results confirmed the presence of the foreign DHAR gene in transformed tobacco plants.

Increased DHAR activity in transgenic plants Immature and mature leaves of the first generation (T_0) tobacco were used for the analysis of DHAR activity using one transformant 1 plant in Fig. 3. The DHAR transgenic plant

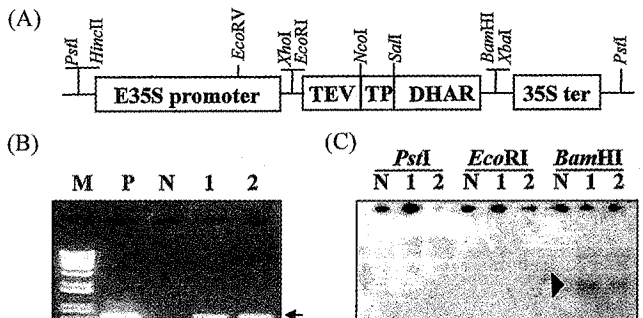

Fig. 3. Identification of transformation of DHAR transgenic tobacco plants by genomic PCR and Southern blot analysis. A, Partial map of DHAR expression vector, B, Agarose gel electrophoresis of PCR products, and C, Southern blot analysis in DHAR transgenic tobacco plants. N and numbers on the gel indicate non-transgenic and independent transgenic lines, whereas M and P indicate molecular marker and plasmid DNA as a positive control, respectively.

Table 1. DHAR and GR activity (units/g fresh wt) in DHAR transgenic and non-transgenic (NT) tobacco plants. The data are means ± SE of the three replicates.

Enzyme activity	DHAR plants	NT plants	DHAR plants/ NT plants
DHAR activity			
Immature leaf	19,410 ± 1,667	14,570 ± 952	1.33
Mature leaf	31,760 ± 476	22,520 ± 1,809	1.41
GR activity			
Immature leaf	1,580 ± 292	1,365 ± 44	1.16
Mature leaf	1,434 ± 234	1,244 ± 175	1.15

showed a significant increase in DHAR activity, compared to non-transgenic (NT) plants, regardless of their leaf development stage. The mature leaves of both transgenic and NT plants showed a 1.6 times higher DHAR activity than immature leaves. DHAR activity (31,760 units/g fresh wt) in the mature leaves of transgenic plants showed approximately 1.41 times higher than in the non-transgenic plants.

In addition, DHAR plants induced GR activity, showing that GR activity (1,434 units/g fresh wt) in the mature leaves of transgenic plants was about 1.2 times higher than in the NT plants. The same results were obtained in the immature leaves of both transgenic and NT plants. These results indicate that enhanced DHAR activity in transgenic plants resulted from the introduced DHAR gene. Biochemical data in the transformant 2 plant showed a similar result with that in transformant 1 plants in Fig. 3 (data not shown).

Increased or reduced AsA and oxidized GSSG content in transgenic plants The reduced and oxidized ascorbate levels were investigated in the immature and mature leaves of transgenic plants (Table 2). As expected, the reduced ascorbate (AsA) level in transgenic plants was markedly increased, showing that the AsA content of mature leaves in the transgenic plant (215 μg/g fresh wt) was approximately 1.95 times higher than in the NT plants. The content of the oxidized DHA in the mature leaves of transgenic plants was decreased by 65% compared to NT plants. The total glutathione content showed a similar level in transgenic and NT plants. As a result, the AsA content in the total ascorbate (R/T) in mature transgenic leaves was significantly changed from 21.6% to 45.3%, whereas that in immature transgenic leaves was changed from 21.4% to 31.9%. Interestingly, DHAR transgenic plants altered the ratio of oxidized and reduced glutathione levels, regardless of the leaf development stage. The reduced glutathione (GSH) level (μg/g fresh wt) in the immature leaves of transgenic plants was markedly decreased from 123 to 59, whereas the oxidized glutathione (GSSG) level was increased from 25 to 64. Similar results were obtained in the mature leaves of transgenic plants. The total glutathione level was almost the same in the immature and mature leaves, regardless of whether it was transgenic or NT plants.

Tolerance to MV-induced oxidative stress Leaf discs of the T_0 DHAR transgenic plant showed reduced membrane damage to oxidative stress that was derived from methyl viologen (MV), a superoxide anion radical generation chemical, relative to NT plants (Babbs *et al.*, 1989; Donahue *et al.*, 1997). When tobacco leaf discs were subjected to MV at 5 μM and 10 μM, T_0 transgenic plants showed about 48% and 28% reduction in membrane damage relative to NT plants, respectively. At 0 and 100 mM MV treatments, there was no significant difference in the ion leakage between transgenic and NT plants. These results indicate that DHAR may contribute to the protection against the oxidative stresses in plant cells. Further characterization of DHAR transgenic plants using the next generation (T_1) is under study in terms of

Table 2. The content of ascorbate and glutathione (μg/g fresh wt) in DHAR transgenic and non-transgenic (NT) tobacco plants. The data are means ± SE of the three replicates.

Antioxidant contents	Reduced type (R)	Oxidized type (O)	Total content (T)	R/T (%)
<Ascorbate content>				
DHAR plants				
Immature leaf	145 ± 0	310 ± 15	455	31.9
Mature leaf	215 ± 10	260 ± 25	475	45.3
NT plants				
Immature leaf	90 ± 15	330 ± 30	420	21.4
Mature leaf	110 ± 25	400 ± 55	510	21.6
<Glutathione content>				
DHAR plants				
Immature leaf	59 ± 1	64 ± 3	123	48.0
Mature leaf	60 ± 2	65 ± 2	125	48.0
NT plants				
Immature leaf	123 ± 12	25 ± 2	148	83.1
Mature leaf	115 ± 2	22 ± 1	137	83.9

various oxidative stresses. This includes MV, hydrogen peroxide and chilling.

Transgenic tobacco plants that express a human DHAR gene in chloroplasts were successfully developed and biochemically characterized. As expected, DHAR transgenic plants showed high levels of reduced ascorbate (AsA) and low reduced glutathione (GSH), when compared to the NT plants. Furthermore, DHAR transgenic plants showed an enhanced stress tolerance to MV-mediated oxidative stress. These results indicate that DHAR cDNA, isolated from the cDNA library of the human fetal liver, properly worked in plant cells, even though there is a low amino acid homology between the animal and plant DHARs (Fig. 2). Further characterization of the DHAR transgenic plants using the next generation (T_1) is being studied in terms of various oxidative stresses.

Chloroplasts are especially sensitive to damage by ROS, because electrons that escape from the photosynthetic electron transfer system are able to react with a relatively high concentration of O_2 in chloroplasts (Foyer *et al.*, 1994). To maintain the productivity of plants under stress conditions, it is important to fortify the antioxidative mechanism in the chloroplasts by manipulating the antioxidant enzymes, including DHAR in this study, and small antioxidant molecules, which includes AsA in the chloroplasts. Ascorbate acts as an important antioxidant in plant cells, and particularly as a substrate of ascorbate peroxidase that converts hydrogen peroxide into water (Conklin *et al.*, 1996; Smirnoff 1996; Smirnoff and Pallanca, 1996). For maintenance of the antioxidative activity of ascorbate, its regeneration from DHA to AsA is necessary, as well as its biosynthesis (Wheeler *et al.*, 1998).

On the other hand, a strong constitutive promoter, such as the CaMV 35S promoter, is typically used for the expression of foreign genes in plants (for example, the DHAR plants in this study). But, a more precise regulation of the expression using an inducible promoter, especially a stress-inducible promoter, might be useful for the development of stress-tolerant plants. In this respect, transgenic plants that express multiple antioxidant enzymes, including DHAR under the control of an oxidative stress-inducible promoter (Kim, 2000) in chloroplasts, are under investigation.

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