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Expressional Characterization of Dehydroascorbate Reductase cDNA in Transgenic Potato Plants

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In plants, ascorbic acid (AsA) is a strong antioxidant or reductant that can be converted to dehydroascorbate (DHA) by oxidation. DHA, a very short-lived chemical, can either be hydrolyzed irreversibly to 2,3-diketogulonic acid or recycled to AsA by dehydroascorbate reductase (DHAR). *DHAR* cDNA, isolated from sesame hairy roots, was inserted into two plant expression vector systems with the *CaMV35S* promoter (CaMV35S::DHAR) and a potato tuber-specific promoter, *Patatin* (Patatin::DHAR). Southern and northern blot hybridization analyses indicated that *DHAR* cDNA was successfully integrated into the potato genome and actively transcribed. High levels of sesame *DHAR* transcript and DHAR enzyme activity were determined, by the *Patatin* promoter, in regenerated potato tubers, but their levels in leaves were very low. In contrast, much higher amounts of transcript were accumulated in the leaves of CaMV35S::DHAR regenerants than in the tubers while the activity of DHAR enzyme was higher in the latter. AsA content in the tubers of Patatin::DHAR transgenic lines was also increased (1.1- to 1.3-fold) compared with that of non-transgenic plants. However, this was not true for the transgenic leaves. In contrast, the *CaMV35S* promoter was associated with AsA accumulations in both the tubers (up to 1.6-fold) and the leaves (up to 1.5-fold). However, more detailed analyses indicated that this increased enzyme activity was not always accompanied by an elevation in AsA content from transgenic plants. This suggests that other factors may limit the accumulation of vitamin C via ascorbate-recycling in transgenic potato plants.

Keywords ascorbic acid, *CaMV35S* promoter, dehydroascorbate (DHA), dehydroascorbate reductase (DHAR), *Patatin* promoter, sesame hairy root, transgenic potato

Vitamin C (ascorbic acid; AsA) is synthesized in higher plants by one of two proposed metabolic pathways (Ishikawa et al., 2006). The first is the D-mannose/L-galactose pathway which occurs through a series of oxidative reactions of hexose sugars to L-galactono-1,4-lactone, a precursor to AsA (Wheeler et al., 1998). A second pathway is via two uronic acids -- galacturonic acid (Agius et al., 2003) and glucuronic acid (Lorence et al., 2004). However, L-gulono-1,4-lactone oxidoreductase, which is involved in the last step of the AsA biosynthetic pathway, is deficient in humans and must be supplied from other sources (Crawford and Crawford, 1980; Roig et al., 1993; Loewus, 1999).

Researchers have recently suggested increasing the current recommended dietary allowance for vitamin C from 75 or 90 mg to 200 mg (Levine et al., 1996). High levels of AsA are found naturally in several vegetables and fruits but not in such main staple foods as rice, wheat, or barley. In Europe, however, the potato tuber is a major source of vitamin C (Davey et al., 2000) because it contains relatively high levels of vitamin C (approximately 20 to 36 mg per 100 g FW), which is not easily degraded even when the potatoes are steamed or boiled because AsA is protected by the surrounding starch (Han et al., 2004). Although the potato tuber is an ideal sink organ, few details are known about the AsA mechanism, including its location of biosynthesis, transport to non-photosynthetic sink organs, and accumulation (Tedone et al., 2004).

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Several candidate genes have been introduced into heterologous plant species to increase their AsA contents. These include L-gulono-γ-lactone oxidase cDNA isolated from mouse (Jain and Nessler, 2000), a human *DHAR* gene (Kwon et al., 2001, 2003), NADPH-dependent D-galacturonic acid reductase (*GalUR*) cDNA of strawberry (Agius et al., 2003), and wheat *DHAR* cDNA (Chen et al., 2003). AsA, a strong anti-oxidant that protects cells from oxidative stresses, can be converted to monodehydroascorbate (MDA) and dehydroascorbate (DHA). The latter can be recycled to AsA by dehydroascorbate reductase (DHAR) or further and irreversibly degraded (Hossain and Asada, 1984; Hossain et al., 1984; Asada, 1999; Niyogi, 1999). DHAR (E.C. 1.8.5.1) exists in both plants and animals; in a recycling reaction, glutathione (GSH) serves as an electron donor to maintain the homeostasis of AsA, greatly influencing the maintenance of the redox balance (Vethanayagam et al., 1999; Foyer, 2000; Savini et al., 2003). *DHAR* genes have been cloned from rice (Kato et al., 1997; Urano et al., 2000), spinach (Shimaoka et al., 2000), and tomato (Zou et al., 2006).

Recently, we reported the isolation of cytosolic *DHAR* cDNA (GenBank Accession Number DQ289794) from sesame hairy root cultures, and showed that its transcript levels were, unexpectedly, 4.7-fold higher in stem tissue than in the hairy roots (Chun et al., 2006). Purified recombinant sesame DHAR from *E. coli* bearing sesame *DHAR* cDNA exhibited a different optimal pH (Chun et al., 2007). Here, our objective was to elevate the nutritional value of potato tubers by increasing their vitamin C content through the

overexpression of the sesame *DHAR* gene under the control of a potato tuber-specific promoter, *Patatin* (Mignery et al., 1988; Rocha-Sosa et al., 1989; Liu et al., 1990; Farran et al., 2002). We also compared the expressional efficiency of the *CaMV35S* and *Patatin* promoters in transgenic tubers.

MATERIALS AND METHODS

Plant Materials

Potato plants (*Solanum tuberosum* cv. Jowon) maintained *in vitro* were kindly provided from the National Institute of Highland Agriculture, RDA, South Korea. At 4-week intervals, they were sub-cultured, and were continuously propagated *in vitro* at 23°C under a 16-h photoperiod from lights at 4000 Lux (Visser et al., 1989; Lee et al., 2004). A solid medium included 4.4 g L^{-1} of basic MS salts, 30 g L^{-1} of sucrose, and 8 g L^{-1} of phyto agar. Leaves from 2- to 3-week-old *in vitro* plants were used for transformation. To analyze their DHAR enzyme activity and AsA contents, we collected leaf samples from 2- to 3-week-old transgenic plants growing in test tubes containing multiplication media. T_1 tuber samples also were harvested after the T_0 tubers obtained from regenerated transgenic plants were re-planted into pots.

Vector Construction

A *DHAR* cDNA fragment was originally isolated and cloned from the cDNA library of sesame hairy root cultures, as described by Chun et al. (2007). PCR-amplification was

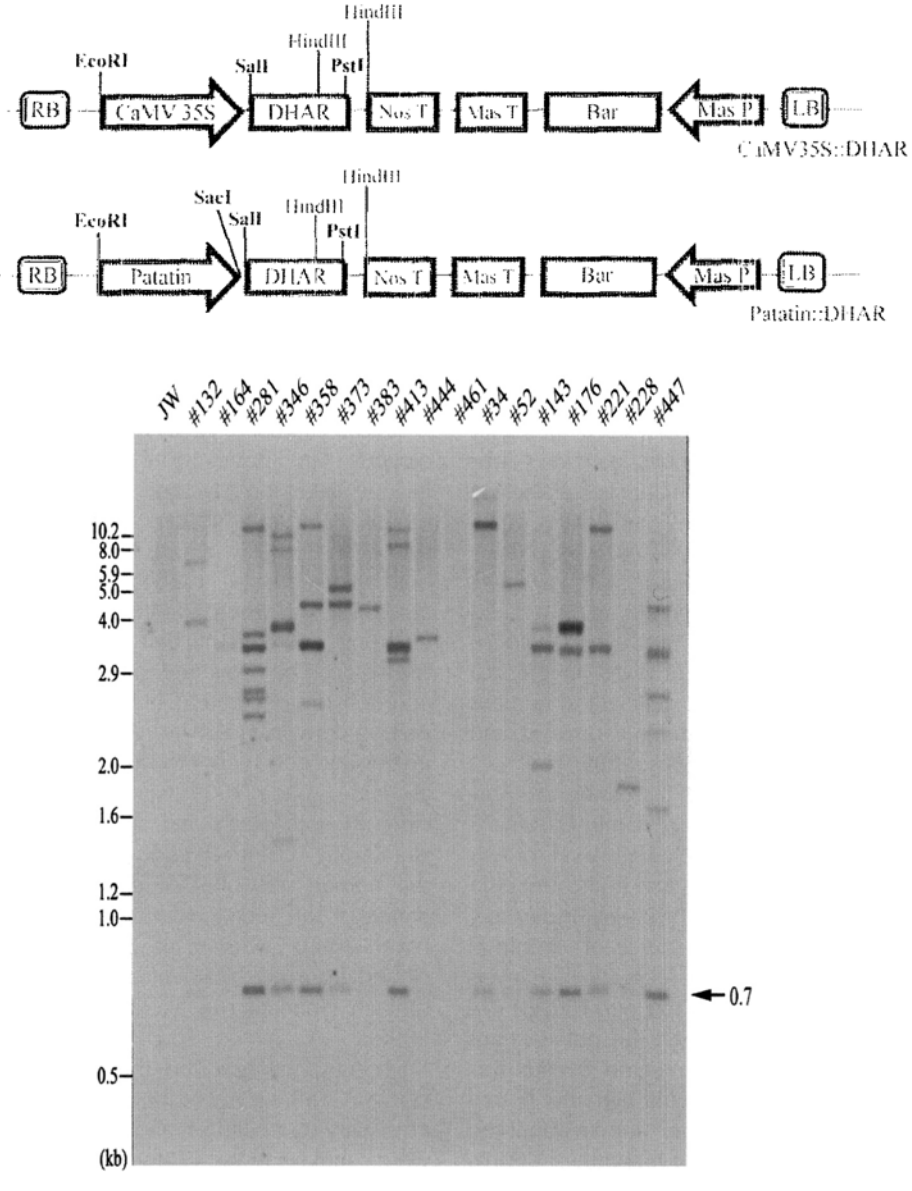

Figure 1. Schematic diagram of recombinant binary vectors (upper panel). Constructs carry left and right borders (LB, RB), and mannopine synthase (*Mas*) promoter followed by terminator of nopaline synthase (*Nos*) gene. Recombinant *DHAR* cDNA is controlled by either constitutive 35S cauliflower mosaic virus promoter (CaMV35S:: DHAR) or tuber-specific *Patatin* promoter (Patatin::DHAR). Genomic Southern blot analysis of transgenic potatoes with CaMV35S::DHAR construct (lower panel). JW indicates non-transgenic plant (*S. tuberosum* cv. Jowon). Ten μg of genomic DNA was digested with *Hind*III and fractionated on 0.8% agarose gel, transferred to nylon membrane, and hybridized with α-^{32}P dCTP-labeled full-length probe from sesame *DHAR* cDNA.

conducted with primers (forward, 5'-GCGTCGACATGGCTGTGGAAGTATGCGTC-3'; reverse, 5'-GGGCTGCAGTCATGCATTAACTTTGGGTG-3') that were designed to contain both *SalI* and *PstI* restriction endonuclease sites from the sesame hairy root library (Fig. 1). The identified *DHAR* cDNA fragment was inserted into the pSJ001 vector and designated as CaMV35S::DHAR. Dr. Ji-Hoon Ahn (School of Life Sciences and Biotechnology, Korea University, South Korea) kindly provided the pSJ001 vector, which was originally designed to contain the *Bar* gene (resistant to bialaphos herbicide) as a selective marker under the mannopine synthase (*Mas*) promoter, followed by the terminator of nopaline synthase (*Nos*) gene, and the target gene regulated by the 35S cauliflower mosaic virus promoter (*CaMV35S*) (Fig. 1).

As an alternative, the *CaMV35S* promoter was substituted with the *Patatin* promoter to direct tuber-specific expression of *DHAR*, as follows. The *Patatin* promoter fragment was directly amplified from the genomic DNA of potato leaves (*S. tuberosum* L. var. Dejima) by PCR, using a designed primer set (forward, 5'-ATGTTGCCATATAGAGTAGTTTGTGATGG-3'; reverse, 5'-TTTGCAAATGTTCAAAGTGTTTTTAAATTTTGTTGGTGCTTT-3') from known sequence information (*S. tuberosum* B 33 gene upstream region; GenBank Accession No. X14483). The amplified fragment was purified with a PCR purification system kit (Bioneer) and sub-cloned into the pGEM-T easy vector (Promega). To substitute with the *CaMV35S* promoter, the cloned *Patatin* promoter fragment was re-amplified with a primer set containing *Eco*RI and *Sac*I cloning sites (forward, 5'-GCGCGAATTCAAATCATTGTTTTATTTTCTC-3'; reverse, 5'-GGCGAGCTCTTTGCAAATGTTCAAAGTGTT-3'). The final vector was designated as Patatin::DHAR (Fig. 1). CaMV35S::DHAR and Patatin::DHAR were each transformed into *Agrobacterium tumefaciens* ASE.

Plant Transformation

Agrobacterium-mediated potato plant transformation was performed as described by Lee et al. (2004). Leaf or stem tissues propagated *in vitro* for 3 weeks were co-cultured for 15 min with *A. tumefaciens* bearing the recombinant vectors and 75 μM acetosyringone. After 8 weeks on a regeneration medium (MS plus 30 g L^{-1} of sucrose, 0.01 mg L^{-1} of NAA, 2.0 mg L^{-1} of zeatin, 0.1 mg L^{-1} of GA_3, and 500 mg L^{-1} of carbenicillin), the induced shoots were transferred to a selection medium [MS plus 30 g L^{-1} of sucrose, 0.5 mg L^{-1} of phosphionthricin (PPT), and 250 mg L^{-1} of carbenicillin]. After 4 weeks, the selected shoots were transferred to a rooting medium without PPT (MS plus 30 g L^{-1} of sucrose and 250 mg L^{-1} of carbenicillin) to produce completely regenerated, rooted plants. These new roots were cut with a knife and the plants were then re-rooted on a selection medium containing 2.5 mg L^{-1} of PPT to confirm that they were transgenic. They were then either acclimatized to pots for further experiments or maintained in a medium containing MS plus 90 g L^{-1} of sucrose and 8 g L^{-1} of phyto agar to obtain mini-tubers.

Southern Blot Hybridization

Genomic DNA was isolated via the CTAB method (Doyle and Doyle, 1987) from the leaves of candidate transgenic plants. Ten μg of genomic DNA was digested with *Hind*III restriction endonuclease and fractionated on a 0.8% (w/v) agarose gel before being transferred to a positively charged nylon membrane (Tropilon-plus™ Tropix Co., USA) according to the manufacturer's protocol. The [α-^{32}P] dCTP probe fragment was prepared by labeling the entire *DHAR* cDNA fragment with a HexaLabel™ DNA Labeling Kit (Fermentas Co., USA), then hybridized as described above.

Northern Blot Hybridization

Total RNA was purified with Trizol buffer (Invitrogen Co., USA) according to the manufacturer's directions. Twenty μg of total RNA was fractionated on a 1.5% (w/v) formaldehyde agarose gel in 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer (pH 5.3), then transferred to a positively charged nylon membrane, where it was UV cross-linked and hybridized with the same probe fragment used for our Southern blot hybridization.

Protein Extraction and Western Blot Hybridization

Total protein was purified from the tissues of transgenic plants as described by Chen et al. (2003). After the tuber (1.0 g) or the leaf (0.5 g) was macerated with liquid N_2 and 10% PVP (w/w), the powder was mixed with 50 mM potassium phosphate buffer (pH 6.5) and 5 mM EDTA, then centrifuged at 12,000 rpm for 15 min. The supernatant was kept at 4°C. Western blot hybridization was performed basically as described by Chun et al. (2007). The purified recombinant sesame DHAR enzyme from *E. coli* was injected into a rat to obtain antibodies (Chun et al., 2007). After the extracted total protein of transgenic plant tissues was fractionated on a 12% SDS polyacrylamide gel, it was transferred to a PVP (polyvinylidene difluoride) membrane (Amersham Biosciences, USA) using the Mini-Blot Cell (Bio-Rad, USA) system. The membrane was blocked overnight at 4°C with a Tris-buffered saline (TBS) buffer solution containing 5% fat-free skim milk and 0.1% Tween-20, then hybridized for 1.5 h with the diluted rat IgG (1:1000), thereby raising the purified DHAR protein as the primary antibody. Afterward, the hybridized membrane was washed 3 times (10 min each) with TBS buffer containing 0.1% Tween 20. The diluted secondary antibody, conjugated with horseradish peroxidase (Santacruz, goat anti-rat, 1:2000), was applied for 1.5 h, followed by washing, then exposure to X-ray film.

Measurement of DHAR Enzyme Activity

Total protein was extracted from the leaves or tuber tissues of transgenic plants, as described for our western blot hybridization. DHAR enzyme activity was assessed according to the methods of Hossain and Asada (1984) and Chen et al. (2003), with slight modification. Equal amounts of protein were adjusted in 50 mM potassium phosphate buffer (pH 6.5). At 1 min after the addition of GSH (5 mM) and DHA (0.5 mM) to this reaction buffer, DHAR enzyme activity was determined by measuring the increase in absorbance at 290 nm with a UV spectrophotometer (UV-2501PC, Shimadzu).

Ascorbic Acid Analysis

AsA content in the transgenic potatoes was determined by the method of Topuz and Ozdemir (2007), with slight modification. Briefly, fresh tubers (10 g) were homogenized in a hand blender (Shinil, Korea) with 15 mL of 3% metaphosphoric acid that contained 10^{-6} M EDTA and 10^{-5} M diethyldithiocarbamic acid. After sonication for 5 min, 10 mL of ethanol was added to the extract to precipitate the solubilized starch. For leaves (0.5 g), pestle, 3 mL of the metaphosphoric acid and no ethanol were used to extract the AsA. The slurry was centrifuged at 10,000 rpm for 10 min at 4°C, and the supernatant was filtered through a 0.45 μm membrane filter (13 mm PVDF; Whatman). Working calibration solutions (10 to 250 μg mL^{-1}) were prepared by diluting the stock solution of the external standard, ascorbic acid (Sigma).

An HPLC system (200 Series; Perkin Elmer, CT, USA) equipped with an autosampler, column oven, binary pump, and degasser was used for determining the levels of ascorbic acid in our tubers and leaves. A 10 μL volume of standard or sample solutions was directly injected on a YMC ODS C_{18} column (4 mm, 4.6 mm × 250 mm), using an isocratic potassium dihydrogen phosphate solution (0.2 M; adjusted to pH 2.2 with o-phosphoric acid) at 23°C. Flow rate was 0.7 mL min^{-1}, and the eluent was detected with a UV-Visible detector (254 nm). TotalChrom software (Perkin Elmer) was used to operate this HPLC system.

RESULTS AND DISCUSSION

Selection and Confirmation of Transgenic Potato Plants

Upon treatment with PPT, several candidate transgenic potato plants were selected during the shoot-induction and rooting procedures. Although a method for potato transformation has been well-established, the exact concentration of PPT has not been ascertained for selection pressure during regeneration. Here, we applied two steps to increase the fidelity of the transformants. During the induction of shoots from callus in the first stage, a low concentration of PPT (0.5 mg L^{-1}) was used, and the selected shoots were transferred onto rooting media without PPT to allow them to grow well to complete regenerants. Afterward, the new roots were removed and the plants re-rooted on a solid medium containing a high PPT concentration (2.5 mg L^{-1}). Final survival rates on the medium containing 2.5 mg L^{-1} of PPT were 6.38% for the CaMV35S :DHAR construct and 14.28% for Patatin::DHAR (Table 1).

The selected candidate transgenic plants were indirectly verified by PCR-amplification with primer sets for either the sesame *DHAR* cDNA or the *Bar* gene fragment (data not shown). These were further confirmed by genomic Southern blot hybridization. Because one *Hind*III site was located within the sesame *DHAR* cDNA and another site was found at the start point of the *Nos* terminator (see Fig. 1) within the recombinant vector cassettes, an approx. 700-bp fragment was expected to be presented in all transgenic plants. Accordingly, the other hybridized bands were assumed to be linked with the plant genomic DNA at the right border of the T-DNA, depending on where they were integrated (Fig. 1).

Our Southern blot hybridization verified that one to several copies of the sesame *DHAR* cDNA had been integrated into the potato genome – six transgenic lines (346, 383, 444, 34, 52, and 228) contained one copy whereas five lines (132, 373, 143, 176, and 221) contained two. Interestingly, at least seven and five copies were integrated into Lines 281 and 447, respectively. The intensity of the 700-bp fragment generated by the *Hind*III site within the sesame *DHAR* cDNA and at the start point of *Nos* terminator was correlated with increasing copy number as an internal control.

Tissue-specific Expression Pattern of Sesame *DHAR* cDNA in Transgenic Potato Plants

To further examine the correlation between copy number

Table 1. Frequency of transformation.

Vector	Variety	No. of explants	No. of regenerated shoots in PPT[1]	No. of resistant plants in PPT[2]	Efficiency (%)
CaMV35S::DHAR	Jowon	595	275	38	6.38
Patatin::DHAR	Jowon	245	197	35	14.28

[1]Number of shoots selected on 0.5 mg L^{-1} PPT
[2]Number of plants surviving on 2.5 mg L^{-1} PPT

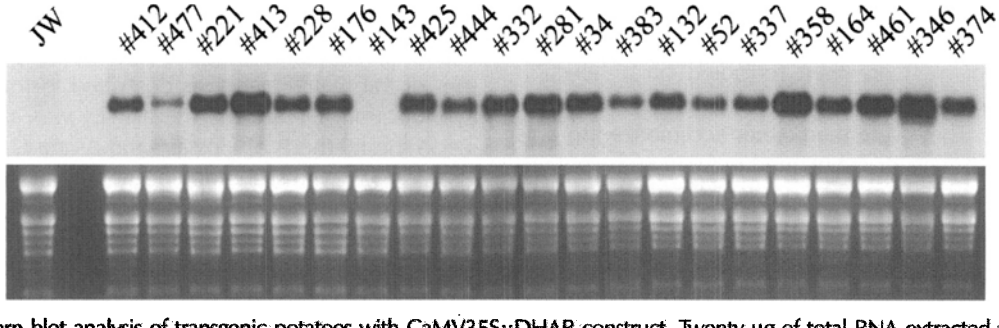

Figure 2. Northern blot analysis of transgenic potatoes with CaMV35S::DHAR construct. Twenty μg of total RNA extracted from leaves was fractionated on 1.5% formaldehyde agarose gel, transferred to positively charged nylon membrane, and hybridized with α-^{32}P dCTP-labeled full-length probe from sesame *DHAR* cDNA. Ribosomal rRNAs (18S, 28S, etc.) were stained with EtBr to normalize equal loading of total RNA.

and transcript levels of the introduced sesame *DHAR* cDNA, we conducted northern blot hybridization with total RNA isolated from leaves of the CaMV35S::DHAR transgenic lines. In general, more transcripts were accumulated as copy number increased; the exception was Line 346, which showed only one copy of sesame *DHAR* cDNA but had the highest amount of transcript (Fig. 2).

To examine their expression patterns, we chose five transgenic lines that expressed relatively high levels of sesame *DHAR* transcript for both CaMV35S::DHAR and Patatin::DHAR constructs. As expected, the *Patatin* promoter directed tissue-specific accumulation of transcripts in the tubers of the Patatin::DHAR transgenic lines, whereas almost no transcripts (or basal levels, if any) were observed in their leaves (Fig. 3). In contrast, the CaMV35S::DHAR transgenic lines accumulated high levels of transcript in both leaves and tubers. Moreover, much stronger expression was maintained in the leaves than in the tubers for all five lines; for both vector constructs, relatively low levels of transcripts were detected in the tubers than in the leaf tissues from the CaMV35S::DHAR transgenic lines.

Tuber-specific expression of sesame *DHAR* cDNA in our Patatin::DHAR transgenic lines was also confirmed via western blot hybridization using an antibody raised with the purified sesame DHAR protein. Higher levels of protein were accumulated only in the tubers whereas none was detectable in the leaves from any of the five Patatin::DHAR transgenic lines (Fig. 4). However, in both the leaves and the tubers of the CaMV35S::DHAR transgenic lines, levels of DHAR protein were relatively high. Interestingly, a very low amount of protein (basal readings) was detected in the tubers from Line 281, tissues from which almost no transcript had been detected by our northern blots even though very high levels of transcript and DHAR protein were maintained in the leaves from that same line (Fig. 3).

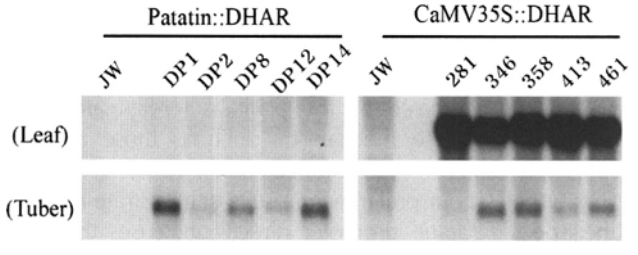

Figure 3. Tissue-specific expression pattern of sesame *DHAR* cDNA in transgenic potato lines. Total RNA (20 µg) was fractionated on 1.5% formaldehyde agarose gel, transferred to positively charged nylon membrane, and hybridized with α-^{32}P dCTP-labeled full-length probe from sesame *DHAR* cDNA. JW indicates non-transgenic plant (*S. tuberosum* cv. Jowon).

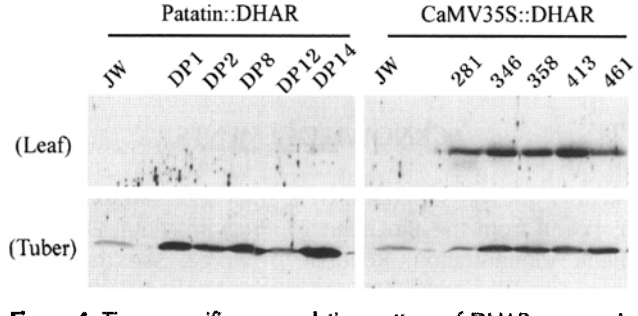

Figure 4. Tissue-specific accumulation pattern of DHAR enzyme in transgenic potato lines. Total proteins extracted from leaves and tubers were separated on 12% SDS polyacrylamide gel and electro-transferred to PVDF membrane. Western blot hybridization was carried out with antibody raised against sesame DHAR enzyme produced from recombinant *E. coli*.

Increased DHAR Enzyme Activity in Transgenic Potato Plants

For determining enzyme activity in *DHAR* over-expressing potato plants, DHA was used as a substrate for DHAR and the increased absorbance was converted to relative enzyme activity. Interestingly, in the non-transgenics, endogenous activity was 3.7-fold greater (3.4×10^{-4} mol min^{-1} mg^{-1} protein) in the tubers than in the leaves (9.1×10^{-5} mol min^{-1} mg^{-1} protein) (Fig. 5). Overexpression of sesame *DHAR* cDNA under the CaMV35S promoter elevated the level of activity by up to 3.5-fold in the leaves from Line 346, which carried only one copy of that cDNA (Fig. 5), whereas Lines No. 281, 413, and 358, which had more than two copies, showed an increase in activity of less than 1.8-fold compared with the non-transgenic plants. These results were consistent with those obtained from our northern and western blots (Figs. 3, 4). As expected, all transgenics with the Patatin::DHAR construct exhibited low levels of enzyme activity compared with those measured in the non-transgenic leaves.

Both the CaMV35S::DHAR and the Patatin::DHAR transgenic lines showed a slight elevation in DHAR enzyme activity within their tubers (Fig. 5). Furthermore, no such dramatic increase found in the leaves from Line 346 was observed in the tubers from these transgenic lines. For example, the DP1 line from our Patatin::DHAR construct had the highest enzyme activity (5.4×10^{-4} mol min^{-1} mg^{-1} protein), but that increase was only 1.6-fold greater than that found with the non-transgenic line (3.4×10^{-4} mol

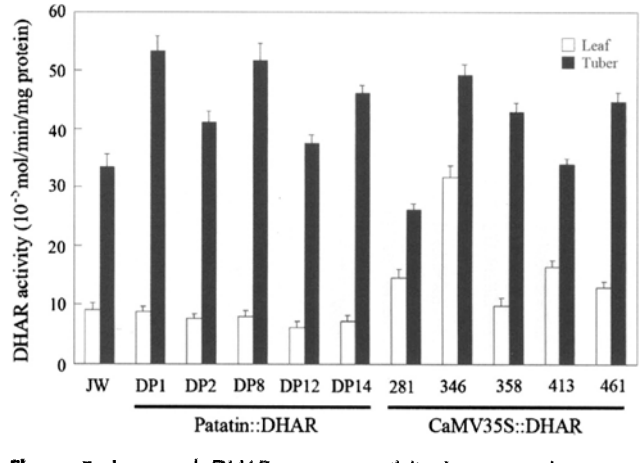

Figure 5. Increased DHAR enzyme activity in transgenic potato leaves (white histograms) and tubers (black histograms). JW indicates non-transgenic plant (*S. tuberosum* cv. Jowon). DP1, DP2, DP8, DP12, and DP14 are transgenic lines with Patatin::DHAR; Lines 281, 346, 358, 413, and 461 contain CaMV35S::DHAR construct. DHAR enzyme activity was defined as increase in absorbance at 290 nm upon converting DHA to AsA by DHAR enzyme, with GSH as an electron donor. Means and SDs are reported for 3 independent measurements.

min^{-1} mg^{-1} protein). Activity in the CaMV35S::DHAR transgenic lines also was enhanced (by 1.1- to 1.4-fold) in the tubers. However, Line 281, with more than seven copies of sesame *DHAR* cDNA, exhibited lower enzyme activity in its tubers compared with the non-transgenics, a result consistent with those from the northern and western blots.

Except for Line 346, enzyme activities in the leaves were 2- to 5-fold lower than in the tubers; however, much higher levels of sesame *DHAR* cDNA transcript were accumulated in the leaves of the CaMV35S::DHAR lines than in the tubers of the Patatin::DHAR lines. Although this enhancement of activity in No. 346 leaves, relative to the non-transgenic plants, was by more than 3-fold, actual activity was approximately 3.0×10^{-4} mol min^{-1} mg^{-1} protein, a level similar to that measured in the non-transgenic tubers.

Likewise, our non-transgenic tubers contained much higher levels of DHAR enzyme activity (3.4×10^{-4} mol min^{-1} mg^{-1} protein) compared with other crop species (Fig. 5). This tuber content, which was 3-fold greater than that of the potato leaves (9.1×10^{-5} mol min^{-1} mg^{-1} protein), suggests that the high level of DHAR enzyme in potato tubers is correlated with their high vitamin C content relative to other staple food crops, such as rice, barley and wheat. For instance, DHAR enzyme activity in rice bran (4.9×10^{-5} mol min^{-1} mg^{-1} protein) is 6-fold lower than in potato tubers or spinach leaves, the latter accumulating a relatively high amount of vitamin C while exhibiting a level of activity (3.6 to 4.0×10^{-4} mol min^{-1} mg^{-1} protein) similar to that in potato tuber tissues (Shimaoka et al., 2000).

Increased Ascorbic Acid Content in Transgenic Potato Plants

To determine the efficiency of ascorbate-recycling through the overexpression of exogenous *DHAR*, we finally measured the AsA content in transgenic potato tubers and leaves (Fig. 6). Of the five lines with the Patatin::DHAR construct, four (DP 1, DP 2, DP 8, and DP 14) showed increased contents in their tubers (1.1- to 1.3-fold), but not in their leaves, compared with the non-transgenics; Line DP1 accumulated the most AsA (30.9 mg per 100 g FW). These trends are very consistent with our data for DHAR enzyme activity. In contrast, the *CaMV35S* promoter enhanced AsA contents in the tubers and the leaves, although no lines showed a simultaneous increase in both tissue types. For instance, Lines 281 and 346 accumulated high amounts of AsA (1.3- to 1.6-fold) in their tubers relative to the control plants, but no increases were observed in their leaf levels. In contrast, Lines 358, 413, and 461 had elevated AsA contents in their leaves (1.2- to 1.5-fold) but not in their tubers. Previous reports also have noted that the strong expression of exogenous *DHAR* (3.2- to 100-fold) results in a 2- to 4-fold increase in AsA content, suggesting that other limiting factors may be involved in ascorbate accumulation in higher plants (Chen et al., 2003; Kwon et al., 2003).

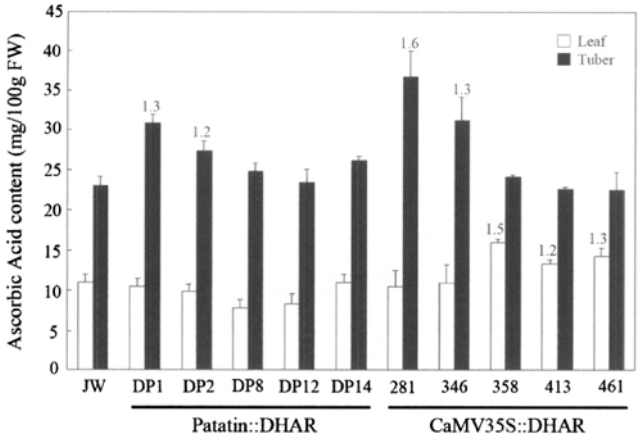

Figure 6. Increased AsA content in transgenic potato leaves (white histograms) and tubers (black histograms). JW indicates non-transgenic plant (*S. tuberosum* cv. Jowon). DP1, DP2, DP8, DP12, and DP14 lines are transgenic lines with Patatin::DHAR; Lines 281, 346, 358, 413, and 461 contain CaMV35S::DHAR construct. AsA contents were determined by HPLC. Fold increases are stated relative to control and non-transgenic plants, as indicated above histograms. Means and SDs are reported for 3 independent measurements.

The increased AsA content in several of our transgenic lines was not exactly correlated with the elevated levels of their transcripts, activity, or accumulated DHAR enzyme, as determined by northern and western blot hybridizations. For example, Line 281 had up to 1.6-fold more AsA in its tuber tissues, but transcript levels and DHAR enzyme activity were very low. In fact, the lowest activity was observed in that line (Figs. 4, 5). In contrast, Line 346 maintained a very high level of activity in its leaf tissues. Given that this line contained only one copy of the sesame DHAR cDNA (Fig. 2) while exhibiting the greatest amount of leaf enzyme activity (Fig. 5), it would seem to be the best candidate when developing an abiotic stress-resistant potato plant. However, the actual AsA content was not enhanced as much in the leaves while it was significantly elevated (1.3-fold) in the tubers. Although the reason for this discrepancy remains obscure, it has been suggested that a strong constitutive promoter, e.g., *CaMV35S*, initially leads to high levels of expression that are detected by the plant as foreign and then silenced by defense mechanisms such as those recently reported to depend on siRNAs (Lee et al., 2004).

Nevertheless, our preliminary results demonstrate that the tuber-specific *Patatin* promoter directly increases the content of AsA in potato tubers by up to 1.3-fold, making them useful as a food source with improved nutritional value. Here, we also obtained several transgenic lines that accumulated high amounts of AsA in their leaves as directed by the *CaMV35S* promoter. These have potential in the development of abiotic stress-resistant crop plants. Further experimentation, including agronomic performance, will undoubtedly be required when exploring these possibilities.

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