Dehydroascorbate reductase (DHAR) is an enzyme which mediates a regeneration of ascorbate from dehydroascorbate using GSH as the electron donor. Here we report a tropical fig lacking a heat-stable DHAR activity in leaves. The leaves of Ficus microcarpa L. f. cv. Golden Leaves are normally green under dim light but are yellow under full sunlight in the field. We compared DHAR activity from the leaves of Golden Leaves to that of the wild type using two distinct assay methods. Total leaf DHAR activity of Golden Leaves, determined in the presence of 2-mercaptoethanol and EDTA, was 0.3 μmol min⁻¹ (mg protein)⁻¹ which was comparable to that of the wild type or spinach leaves. This activity was completely eliminated by heat treatment at 80°C, whereas 40% of the activity was resistant to this treatment in the wild type leaves. Similar results were obtained with another assay method employing dithiothreitol and gel filtration. We conclude that the absence of heat-stable DHAR activity is a significant biochemical marker that distinguishes Golden Leaves from the wild type. A physiological role for DHAR in adaptation to high light stress is proposed.

Key words: Active oxygen — Ascorbate metabolism — Ascorbate-glutathione cycle — Dehydroascorbate — Oxidative stress.

The ascorbate-glutathione cycle is a series of redox reactions of ascorbate and glutathione. The basic concept of the cycle was originally proposed by Foyer and Halliwell (1976). They proposed that ascorbate, which along with glutathione is abundant in plant cells, participates in decomposition of superoxide (O₂⁻) and hydrogen peroxide (H₂O₂). In the original proposal (Foyer and Halliwell 1976), glutathione reductase (GR, EC 1.6.4.2) was supposed to be the only enzyme that mediates the cycle. The findings of ascorbate peroxidase (APX, EC 1.11.1.11) specific for the H₂O₂ scavenging and of two pathways for ascorbate regeneration near PSI of thylakoid membranes have indicated that in vivo operation of the ascorbate-glutathione cycle involves several enzymatic components (Asada 1997). These studies have shown that ascorbate plays a central role in scavenging of harmful H₂O₂ in plant tissues, especially in leaf cells (Asada et al. 1998). Recently, Asada and co-workers have expanded the concept and proposed the linkage of the water-water cycle and the ascorbate-glutathione cycle by the Mehler peroxidase process on thylakoid membranes (Asada et al. 1998).

In chloroplasts, ascorbate is used as the electron donor for the APX reaction and thus monodehydroascorbate (MDA) radical is produced as the primary oxidation product of ascorbate. If MDA radical is not reduced to ascorbate immediately by monodehydroascorbate reductase (MDAR, EC 1.6.5.4) or reduced ferredoxin, two MDA molecules spontaneously disproportionate to DHA and ascorbate (Asada 1997). Dehydroascorbate reductase (DHAR, EC 1.8.5.1) is an enzyme that regenerates ascorbate from DHA in conjunction with GSH. Nakano and Asada (1981) and Jablonski and Anderson (1981) independently found that DHAR is involved in the ascorbate-glutathione cycle of chloroplasts. They characterized and partially purified the enzymes from spinach (Hossain and Asada 1984) and pea (Jablonski and Anderson 1981). The series of redox reactions for the ascorbate regeneration in the ascorbate-glutathione cycle is summarized in Figure 2A.

Because GSH can reduce DHA to form ascorbate by a spontaneous reaction even in the absence of the enzyme, there has been controversy regarding the association of DHAR with the ascorbate-glutathione cycle. This spontaneous reaction is favored at alkaline pHs which may be obtained in the stroma of illuminated chloroplasts. Although no direct evidence is available, DHA contained in human cells is considered to be directly converted to ascorbate by GSH rather than by an enzymatic reaction (Vera et al. 1995).

Recent biochemical studies have raised additional new questions concerning the enzymatic nature of proteins exhibiting DHAR activities. Both plant and human cells appear to have a range of enzymes (or proteins) that exhibit DHAR activities including protein disulfide isomerase (Wells and Xu 1994), glutaredoxin (Sha et al. 1997) and trypsin inhibitor (Trumper et al. 1994). Recently, May et al. (1997) found that the selenoenzyme thioredoxin reduc-
tase purified from a rat liver functions as a NADPH-dependent DHAR. Moreover, stromal thioredoxins can also reduce DHA (Morell et al. 1997). Thus, the mechanism of DHA reduction in vivo is still in debate for both plant (Morell et al. 1997) and mammalian cells (May et al. 1997, Vera et al. 1995). Although it has been more than 40 years since the first purification of DHAR from peas (Yamaguchi and Joslyn 1952), the physiological significance of this enzyme has not been yet revealed.

We previously reported that the tropical fig *Ficus microcarpa* L. f. cv. Golden Leaves lacks the DHAR activity whereas its wild type possesses the activity (Yamasaki et al. 1995b). Golden Leaves shows normal green leaves under shade conditions but exhibits the characteristic yellow leaves which are produced only under the high-light environment in the field (Fig. 1B) (Yamasaki et al. 1995a). We have shown that the light-sensitive phenotype of Golden Leaves is associated with a loss of DHAR activity in leaves (Yamasaki et al. 1995b). Using an improved DHAR assay, we report here that Golden Leaves lacks a leaf-specific heat-stable DHAR activity but retains a heat-sensitive activity.

**Materials and Methods**

**General procedure for enzyme activity assays**—Fully expanded leaves were harvested from field grown *Ficus microcarpa* L. f. (wild type) and *F. microcarpa* L. f. cv. Golden Leaves (Golden Leaves) on Okinawa Island, Japan. The extract for the enzyme assays was prepared as follows. Leaves were disrupted with a Polytron homogenizer (Kinematica, Luzern, Switzerland) for 30 s at 0°C in a 30-fold grinding medium containing 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, 10 mM sodium ascorbate and 5% (w/v) polyvinylpolypyrrolidone (PVPP). For SOD assay, sodium ascorbate was omitted from the grinding medium. Sodium ascorbate, EDTA and PVPP were omitted from the grinding medium for MDAR and DHAR assays. The homogenate was filtered through four layers of cheesecloth and centrifuged at 20,000 x g for 30 s. The supernatant was used for the assay.

**Sample preparation for the assay of DHAR activity using 2-mercaptoethanol (ME method)**—A soluble fraction was prepared by the method described above using a grinding medium containing 100 mM potassium phosphate (pH 7.8), 2 mM 2-mercaptoethanol, 8 mM EDTA and 5% (w/v) PVPP. The soluble fraction was used to determine total DHAR activity. Heat treatment of the soluble fraction was carried out as follows. An aliquot (2 ml) of the soluble fraction was incubated at 80°C for 2 min. After cooling the sample with a tap water, aggregates caused by heat treatment were removed by centrifugation at 1,500 x g for 3 min at a room temperature (25°C). The supernatant was used for the assay as the heat stable fraction.

**Sample preparation for the assay of DHAR activity using dithiothreitol (DTT method)**—Sample preparation was similar to the ME method described above, except that 5 mM dithiothreitol (DTT) was present instead of 2-mercaptoethanol and EDTA. The soluble fraction that passed through a cellulose acetate syringe filter was subjected to a gel chromatography to remove excess DTT. Aliquots (3 ml) of the soluble fraction prepared with DTT were applied to a column (Econo-Pac 10 DG) which had been equilibrated with 100 mM potassium phosphate (pH 7.0). A void volume (first 3 ml) was discarded and the next eluted 4 ml was collected as a protein fraction which was used for the determination of total DHAR activity. A heat stable fraction was obtained by incubating the sample at 80°C for 5 min. Denatured proteins were removed by a centrifugation at 1,500 x g for 3 min and the supernatant was used for the assay.

**DHAR activity measurement**—The method for measurement of DHAR activities of the soluble fractions was similar to the previous method of Hossain and Asada (1984). DHAR activity was measured as a rate of ascorbate formation monitored by an increase of absorbance at 265 nm, assuming an absorption coefficient of 14 mM⁻¹·cm⁻¹. Absorbance changes were measured with a double beam spectrophotometer (UV 160A, Shimadzu, Kyoto, Japan) at a room temperature (25°C). The reaction medium contained 100 mM potassium phosphate (pH 7.0) and 0.5 mM DHA. The reaction was initiated by the addition of 5 mM GSH and the activity was determined with an initial rate of ascorbate formation of the first 30 s. A background activity due to nonenzymatic reactions by GSH or 2-mercaptoethanol was subtracted.

**Chemicals**—DHA was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.), GSH and PVPP from Nacalai Tesque (Kyoto, Japan), and DTT and 2-mercaptoethanol from Kanto Chemical (Tokyo, Japan). Protein was determined by the Bradford method with a Protein Assay (Bio-Rad).

**Results**

**Dysfunction of active oxygen scavenging system in Golden Leaves—*Ficus microcarpa* L. f. cv. Golden Leaves shows characteristic yellow leaves (Fig. 1B). This coloration is due to the high flavonoid and low chlorophyll contents in the yellow leaves of Golden Leaves (Yamasaki et al. 1995a). These yellow leaves change to apparently normal green leaves (Fig. 1C) when they are shaded from direct sunlight (Yamasaki et al. 1995a). As shown in Figure 1, we can see a color gradation in one branch of Golden Leaves. To distinguish them from conventional etiolated-yellow leaves we have designated this yellowing as the goldenning phenomenon (Yamasaki et al. 1995a).

Using a rapid assay method to measure DHAR activity, we previously showed that Golden Leaves may have an incomplete ascorbate regenerating system associated with DHAR (Yamasaki et al. 1995b). This assay allows for a quick sample preparation and activity measurement within 300 s to minimize the inactivation of DHAR activities by unknown factors. Figure 2B shows the antioxidant enzyme activities of the green leaves of Golden Leaves in the field.
Tropical fig lacking heat-stable DHAR

Fig. 1 Leaf colors of *Ficus microcarpa* L. f. cv. Golden leaves. Left photograph shows a branch of Golden Leaves. Leaves in positions exposed to direct sunlight exhibit yellow color (B) whereas those in shade show apparently normal green (C). A, a leaf of *F. microcarpa* (wild type); B, a yellow leaf of Golden Leaves; C, a green leaf of Golden Leaves.

Fig. 6 Restoration of the heat stable DHAR activity in a revertant of Golden Leaves. A, green leaves appeared on a canopy of Golden Leaves. B, Golden Leaves branching out green leaves. C, DHAR activities determined with the ME method. Specific activities are represented on the basis of the protein concentration of the extract before heat treatment (80°C for 2 min). Data are the averages of four experiments.
results similar to an earlier report (Yamasaki et al. 1995b). Although stromal APX activity was significantly lower in a daytime, activities of the antioxidant enzymes SOD, stromal APX, MDAR and GR in Golden Leaves were comparable to or higher than those of the wild type. However, only the DHAR activities of Golden Leaves were below detectable limits. DHAR activity was reproducibly absent from Golden Leaves when the assay was carried out with the rapid assay method. To confirm the absence of DHAR activity in Golden Leaves plant, we further examined procedures for the DHAR assay.

**DHAR activity determined with the ME method**—DHAR is extremely labile and easy to lose its activity (Hossain and Asada 1984). One contributing factor to DHAR inactivation could be an oxidation of the enzyme by endogenous oxidants probably formed during preparation. An addition of appropriate reductant is generally effective to avoid unfavorable formation of oxidants after disruption of cells. Ascorbate is preferentially used for this purpose because it can directly reduce active oxygen species (Asada 1997) and phenoxyl radicals (Yamasaki and Grace 1998) in addition to the enzymatic decomposition of H$_2$O$_2$ by APXs. In *F. microcarpa*, however, ascorbate even at 1 mM was not sufficient to protect the DHAR activity (not shown). Higher concentrations of ascorbate interferes with the DHAR assay. Thus, ascorbate was not practical for use in the present study.

Thiols like DTT or 2-mercaptoethanol could be used, but such reagents are capable of forming ascorbate from DHA. As with GSH, this nonenzymatic reduction of DHA strongly interferes with the assay. Figure 3 shows the nonenzymatic reduction of DHA by the two thiols. Both 2-mercaptoethanol and DTT showed DHA reducing activities but DTT was 200-fold more potent than 2-mercaptoethanol. Thus, DTT in the DHAR assay system even at micromolar concentrations is a potent interference factor. In comparison, 1 mM 2-mercaptoethanol has practically no significant effect on the nonenzymatic ascorbate formation. Based on these results, we prepared a soluble fraction to be assayed for DHAR activities from *F. microcarpa* using the two distinct methods as described in Materials and Methods, i.e. the ME and DTT methods. In contrast to the rapid assay method (Fig. 2), by use of these modified methods we have detected the DHAR activity from Golden Leaves and a higher DHAR activity from wild type.

Figure 4 shows DHAR activities determined with the ME method. This method is simple and suitable for a rapid assay of a large number of samples compared with the DTT method described later. The total DHAR activity of spinach leaves determined by the ME method was 0.71 μmol min$^{-1}$ (mg protein)$^{-1}$, which is twice as high as the value reported previously (Hossain and Asada 1984). On a protein basis there was no significant difference in the levels of

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**Fig. 2** Antioxidant enzyme activities of Golden Leaves. A, schematic illustration for the outline of the ascorbate-glutathione cycle in conjunction with the Mehler peroxidase reaction. E.T., photosynthetic electron transport chain. B, enzyme activities of Golden Leaves grown in the field. The green leaves of Golden Leaves harvested at 9:00 (day) and 22:00 (night) were used to determine the enzyme activities. The activities are represented as% of the wild type activities. Numbers in the figure show each enzyme activity (μmol (mg Chl)$^{-1}$ h$^{-1}$). The DHAR activities were determined with the rapid assay method. Data are the averages of five different days. sAPX, stromal ascorbate peroxidase.

**Fig. 3** Nonenzymatic reduction of DHA by thiols. Ascorbate formation was monitored by an increase in absorbance at 265 nm. The reaction mixture contained 100 mM potassium phosphate (pH 7.0) and 0.5 mM DHA.
Tropical fig lacking heat-stable DHAR

Fig. 4 DHAR activities determined with the ME method. Closed area represents heat sensitive DHAR activity which can be inactivated by a heat treatment at 80°C for 2 min. Dotted area shows the residual activity obtained after the heat treatment. Fractions of heat stable DHAR activities are shown as % beside each bar. All activities were determined with the ME method and represented as the specific activity on the protein basis of the extract before the heat treatment. Data are the averages of five to six experiments. WT, wild type (F. microcarpa L. f.); GL, Golden Leaves (F. microcarpa L. f. cv. Golden Leaves).

The ME method clearly shows that the leaves of Golden Leaves lack the heat stable DHAR activity. We verified this by the DTT method (Fig. 5). DTT is an effective reductant to protect thiol enzymes but it is virtually impossible to measure the activity in the presence of DTT because of an extremely high nonenzymatic reduction of DHA as shown in Figure 3. Thus, we removed excess DTT by gel chromatography. Results obtained with the DTT method were essentially the same to those with the ME method. Green leaves of Golden Leaves had a comparable amount of DHAR activity to that of the wild type leaves, but a heat treatment at 80°C for 5 min completely abolished the activity. The heat stable activity detected in the wild type leaves was not changed by a further heat treatment for 15 min.

Recovery of heat stable DHAR activity in a revertant of Golden Leaves—Results obtained by both the ME and DTT methods indicate that Golden Leaves completely lacks a heat stable DHAR activity. We further confirmed this by assaying the DHAR activity of a revertant of Golden Leaves. Green leaves are sometimes produced from Golden Leaves by spontaneous somatic mutation (Fig. 6A, B, see p. 642). These green leaves are tolerant to high light, therefore, they retain green coloration as seen in patches of a canopy (Fig. 6A). Interestingly, the heat stable DHAR activity in revertant leaves was restored to a level similar to the wild type leaves (Fig. 6C). These results strongly indicate that the heat stable DHAR activity is a determinant of phenotype of Golden Leaves.

Discussion

In an earlier study, heat stable DHAR activity was not detected in leaf extracts of spinach (Foyer and Halliwell 1977). By improving the assay for DHAR activity, we have successfully measured the heat stable DHAR activity in...
a soluble fraction prepared from leaves of spinach and F. microcarpa (Fig. 4). Because the specific activity of spinach leaf extracts obtained with the ME method is reproducible and similar to the previously reported values (Hossain and Asada 1984), we consider that the method is reliable for determining the DHAR activity of leaf extracts. Using this method, Golden Leaves showed no DHAR activity after the heat treatment whereas the wild type had the heat stable activity (Fig. 4). This is also confirmed by the results obtained with the DTT method (Fig. 5). From these results, we conclude that Golden Leaves lacks the heat stable DHAR activity in a soluble fraction.

The leaf-goldenning phenomenon in Golden Leaves is induced by a long term exposure to a direct sunlight, suggesting that high-light stress participates in the phenomenon (Yamasaki et al. 1995a). High-light (or excess light energy) causes an inhibition of electron transport in PSII and increases photoreduction of O₂ at PSI, ultimately destroying the photosynthetic apparatus. The xanthophyll cycle is involved in the photoprotection of PSII by dissipating excess photon energy as a heat (Gilmore 1997). Violaxanthin deepoxidase (VDE), a xanthophyll cycle enzyme, located in the thylakoid lumen requires ascorbate to form zeaxanthin and antheraxanthin (Gilmore and Yamasaki 1998). Because there is no MDAR in the lumen, DHA is produced via a spontaneous MDA disproportionation by the VDE reaction or by the electron donation to PSI (Asada 1997). The produced DHA is probably reduced to ascorbate by DHAR in the stroma (Asada 1997). Lack of the ascorbate regeneration through this pathway would cause a gradual decrease in the concentration of ascorbate in the stroma under the high-light, which may also result in the inactivation of APXs (Amako et al. 1994) as seen in Figure 2B. Thus, it is plausible that DHAR contained in photosynthetic tissues is important for growth under stress conditions in the field where excess light energy is irradiated on leaves. Mano et al. (1997) clearly demonstrated that the DHAR regenerates ascorbate from the DHA leaking from the lumen, a result supporting the hypothesis that DHAR protects thylakoids from photoinhibition by reducing the DHA produced in the lumen. In this context, Golden Leaves would be a mutant defective in a mechanism for high-light tolerance owing to a lack of DHAR.

Recently, plant mitochondria and peroxisomes were found to have the ascorbate-glutathione enzymes APX, MDAR and GR (Jiménez et al. 1997). DHAR activity was also detected in those organelle fractions. Co-localization of DHAR with these enzymes may support the idea that DHAR is involved in the ascorbate-glutathione cycle to detoxify H₂O₂. However, there is a possibility that DHAR participates in ascorbate metabolism other than the ascorbate-glutathione cycle.

Ascorbate synthesis occurs in mitochondria (Wheeler et al. 1998) and therefore ascorbate must be translocated to other organelles or compartments. In plasma membranes, ascorbate is translocated back to the cytosol from apoplasts in the form of DHA and must be regenerated by DHAR (Córdoba and González-Reyes 1994, Horemans et al. 1998). This mechanism is also required for phenolic-dependent H₂O₂ scavenging in the vacuoles (Yamasaki and Grace 1998, Yamasaki et al. 1997). Although the mechanism involved in the reduction of DHA has been controversial, the intracellular regeneration of ascorbate from DHA is the part of the mechanism for the ascorbate accumulation in the mammalian cells (May et al. 1995, Vera et al. 1995). Therefore, in addition to its H₂O₂ scavenging function, it is necessary to consider that DHAR participates in the ascorbate accumulating mechanism by reducing DHA transported into the stroma. Because chloroplasts contain millimolar concentrations of ascorbate to detoxify H₂O₂, in the absence of ascorbate synthesis, a uni-directional transport mechanism is required to accumulate ascorbate in the stroma. In fact, the DHA that is taken up by intact chloroplasts in the dark may be converted to ascorbate in the stroma under illumination (K. Amako, personal communication).

The issue of the mechanism for the intracellular reduction of DHA is controversial in both plant and animal systems. No direct evidence is available to show what component is responsible for the DHA reduction, or whether it occurs through enzymatic or nonenzymatic pathways. No heat stable DHAR has been reported in dicotyledons (Kato et al. 1997). Although the intracellular localization and molecular and enzymatic properties of the heat stable DHAR remain to be clarified, Golden Leaves plant offers a unique opportunity to address these issues since no other mutant defective in DHAR activity has been available in either plants or animals. Assays to distinguish DHAR activities by heat sensitivity used in the present study provide a useful experimental tool to characterize the proteins that exhibit DHAR activity. Together with its unique phenotype, we consider a lack of the heat stable DHAR activity of Golden Leaves to be an important route to explore the physiological role of DHAR in higher plants.

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