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PHOTOSYNTHESIS AND METABOLITE LEVELS IN DEHYDRATING LEAVES OF *REAUMURIA SOONGORICA*

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Reaumuria soongorica (Pall.) Maxim., a perennial semishrub, is able to survive almost complete tissue dehydration when water is withheld from it, and then the stem can rehydrate on rewatering. In this work, a series of experiments were conducted to characterize the drought-induced changes in two-year-old *Reaumuria soongorica*. The plants were subjected to dehydration by withholding water for 15 days. Net photosynthetic rate (Pn), maximal photochemical efficiency of photosystem (Fv/Fm) and the activity of ribulose-1,5-bisphosphate carboxylase (RuBPCO) were significantly decreased under drought stress, but phosphoenolpyruvate carboxylase (PEPCase) activity increased in the leaf extracts. Content of chlorophylls and carotenoids had no marked variation. Zeaxanthin, the xanthophyll cycle pigment, increased during drying. Plants exposed to drought showed accumulation of sucrose, with lower soluble protein content than in the control plants. The SDS-PAGE protein profile showed a 52 kD polypeptide disappearing under progressive drought stress, but no drought-induced protein occurred. All these findings indicate that the metabolic network systems of *Reaumuria soongorica* have a robust regulation capability for management of severe drought stress.

Keywords: Gas exchange, chlorophyll fluorescence, ribulose-1,5-bisphosphate carboxylase, phosphoenolpyruvate carboxylase, sucrose, resurrection plant.

INTRODUCTION

Desiccation tolerance is most frequently seen as a capability possessed by mature seeds of higher plants. It is rarely seen in other higher plant tissues. However, of the approximately 160,000 species of angiosperms, about 100 so-called "poikilohydric" or "resurrection" plants can dry to ~4–13% relative water content without sustaining damage (Gaff, 1997). Resurrection plants are able to tolerate dehydration and then return as functional units upon rehydration (Ingram and Bartels, 1996). These plants are widespread and found in most taxonomic groups ranging from pteridophytes to dicotyledons (Gaff, 1977; 1987; Oliver, 1996).

In recent years, many studies have found that leaves of resurrection plants possess three key mechanisms by which they survive tissue water loss. First, the plants produce proteins called dehydrins and late embryogenesis abundant proteins called LEAs. It has been suggested that these proteins are involved in stabilizing the membranes and preventing protein denaturation (Hoekstra et al., 2001), but clear evidence for this role is still lacking. Second, plants possess varying levels of protective mechanisms such as antioxidant accumulation to reduce damage to proteins and nucleic acids and to prevent chlorophyll removal (Black et al., 2002). Third, the plants increase the expression of genes associated with carbohydrate metabolism (Hoekstra et al., 2001). This permits the plant to accumulate large amounts of sucrose. The buildup of this carbohydrate in tissue has been suggested to act in two ways. In interactions with proteins and lipids, sucrose replaces water associations in the molecules and maintains structural integrity (Crowe et al., 1998). Thus the functional properties of the membranes and enzymes are preserved. In addition, sucrose assumes a glass-like state during drying. This state is not crystalline, but is an amorphous solid in which molecular movement is severely restricted (Crowe, 2002). This again helps to stabi-

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lize the cell structure. However, the amount of sucrose accumulated in different species of poikilohydric plants varies greatly (Ghasempour et al., 1998; Scott, 2000). In the majority of plants there will inevitably be interaction between the expression of a range of proteins and sugars to achieve the desiccation-tolerant state (Scott, 2000).

While there is some understanding of the mechanisms underlying the ability of tissues to survive desiccation in some resurrection plants (Mundree et al., 2002; Vicre et al., 2004), including physiological and molecular adaptations, it is much more limited in *Reaumuria soongorica* (Pall.) Maxim. *R. soongorica*, a short woody shrub with vegetative organs that can survive desiccation. During the dry season, the plant desiccates and the leaves abscise. Even several weeks later, the stem is still able to reactivate and develop new leaves upon rainfall. Therefore it is qualified as a resurrection plant. (Liu et al., 2007a,b). The objective of this research was to characterize the photosynthesis and metabolite levels in dehydrating leaves of *R. soongorica* in order to offer referenced evidence for an understanding of the mechanism of acclimating desiccation in this poikilohydric plant.

MATERIALS AND METHODS

PLANT MATERIALS AND EXPERIMENTAL DESIGN

Seeds of *R. soongorica* were obtained in the northern foothills of Lanzhou City, Gansu, China (36°17' N, 103°48' E, 1700–1900 m a.s.l.), and planted in pots containing 9 l soil. The pots were placed in the experimental field of the Botanical Garden of Lanzhou University. All the experimental plants were 2 years old. Thirty plants were watered to ensure full hydration prior to the stress experiments. In summer (July), thriving plants were selected and subjected to drought stress by withholding watering; the controls were well watered. During drought treatment the maximum irradiance reached 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ after mid-day (at 1:00 p.m.), and maximum air temperature 40°C. All measurements were made at days 0, 3, 6, 9, 12 and 15 after the beginning of dehydration. At the same intervals, leaves were collected from the plants for all physiological and biochemical measurements and immediately stored in liquid nitrogen.

DETERMINATION OF SOIL WATER CONTENT AND LEAF WATER POTENTIAL

Soil water content was measured by the method of Mao et al. (2004); three samples were taken at predawn and dried at 105°C for 24 h. Plant leaf water potential was determined from freshly cut leaves at 9:00 a.m. using a WP4 Dewpoint Potentiometer (Decagon Devices, Inc, Pullman, Washington).

MEASUREMENTS OF GAS EXCHANGE AND CHLOROPHYLL FLUORESCENCE PARAMETERS

Measurements of net photosynthetic rate (Pn), transpiration rate (Tr) and stomatal conductance (Gs) were made from different shoots at 9:00 a.m. using a portable photosynthetic measurement system (CIRAS-1, PP-system, UK). At the same time, chlorophyll *a* fluorescence measurements were made from 10 shoots by the saturation pulse method (Schreiber et al., 1994) with a pulse-modulated fluorometer (MFMS-2, Hansatech, UK). Maximal photochemical efficiency of photosystem II (PS II), (*Fv/Fm*) quantum efficiency of linear electric transport of PSII (Φ_{PSII}) and non-photochemical quenching of fluorescence (NPQ) were calculated as follows: $Fv/Fm = (Fm-Fo)/Fm$, $\Phi_{PSII} = (Fm'-Fs)/Fm'$ and $NPQ = (Fm-Fm')/Fm'$ (Zhao et al., 2002). The relative photosynthetic electron transport rate (ETR) between PS II and PS I was calculated as $ETR = \Phi_p \sigma_a$ E0 (PAR)(Kolber and Falkowski, 1993; Hofstraat et al., 1994).

DETERMINATION OF CARBOHYDRATE AND PROLINE CONTENT

Sucrose and starch content in leaf tissues was estimated according to the method of Ramachandra Reddy et al. (1996). Total soluble sugar content in 80% ethanolic extract was determined using the anthrone method (Dubois et al., 1956). Free proline from leaves was extracted in aqueous sulfosalicylic acid and estimated using ninhydrin according to the method of Bates et al. (1973).

DETERMINATION OF PHOTOSYNTHETIC PIGMENTS

Chlorophyll *a* and *b*, carotenoids and zeaxanthin were extracted with acetone from leaf segments according to the method of Cooper and Farrant (1996) and quantified by HPLC (Val et al., 1994).

SOLUBLE PROTEINS EXTRACTION AND SDS-PAGE GEL ELECTROPHORESIS

Soluble proteins were extracted at 4°C by homogenizing 0.5 g leaf tissue in 1 ml extraction buffer containing 200 mM Tris-HCl (pH 8.0), 2 mM ethylene diamine tetraacetic acid (EDTA), 1 mM 1,4-Dithiothreitol (DTT), 10 μM phenylmethylsulfonyl fluoride (PMSF), 2% (w/w) insoluble polyvinylpolypyrrolidone (PVPP), and 2% (w/v) polyethylene glycol (PEG) 20000 with 60 mg sodium bicarbonate, and then centrifuging at 13,000 g for 10 min. Total soluble protein content was determined by the Bradford dye method (Bradford, 1976). Polyacrylamide gel elec-

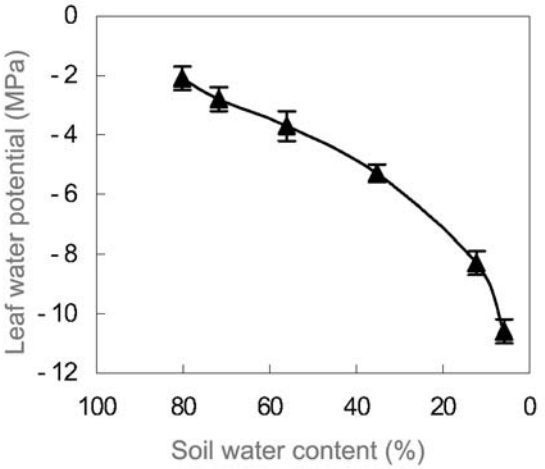

Fig. 1. Changes of leaf water potential in two-year-old *R. soongorica* as the soil water content decreased during dehydration. Data represent means ±SE, n=3.

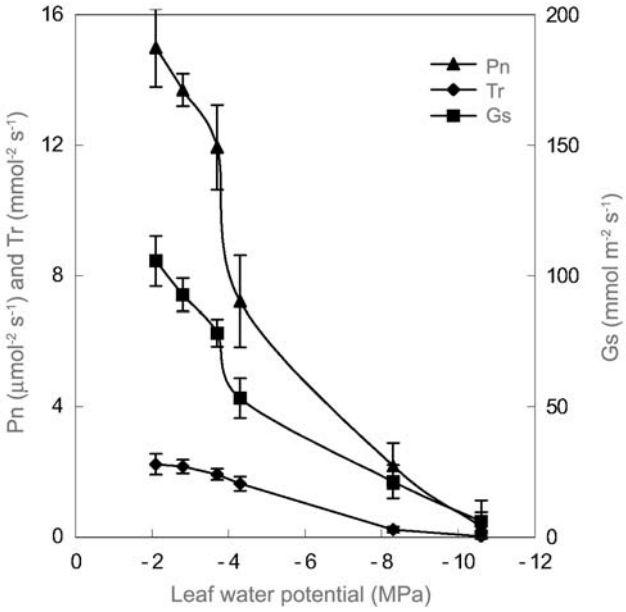

Fig. 2. Effects of different leaf water status on photosynthetic rate (Pn), stomatal conductance (Gs) and transpiration rate (Tr) in two-year-old *R. soongorica* leaves during progressive drought. Data represent means ±SE, n=6.

trophoresis with sodium dodecyl sulfate (SDS-PAGE) on 12% (m/v) polyacrylamide gels was conducted on the same protein extracts according to Laemmli (1970). Leaf extracts were solubilized in 5x-SDS loading buffer containing 250 mM Tris-HCl (pH 6.8), 10%(m/v) SDS, 5% 2-mercaptoethanol, 0.5% (m/v) bromophenol blue and 50% (v/v) glycerin, and maintained in a boiling bath for 5 min. The apparent molecular masses of proteins were estimated by comparison with the mobility of standard proteins (from Sangon, Shanghai, China). Proteins on the gel after SDS-PAGE were visualized with Coomassie brilliant blue following a standard protocol (Sambrook et al., 1989).

ACTIVITY OF THE ENZYMES IN PHOTOSYNTHESIS

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBPCO) was extracted by homogenizing 0.5 g leaf tissue in 1 ml extraction buffer at 4°C containing 400 mM Hepes-KOH (pH 7.5), 5 mM $MgCl_2$, 2% (w/v) PEG 20000, 5 mM EGTA, 14 mM mercaptoethanol and 2% (w/w) insoluble PVPP, and centrifuging at 13,000 g for 5 min. Initial activity of RuBPCO (i.e., not fully activated) was measured by the method of Borland et al. (1998) in a reaction mix containing 100 mM Bicine-KOH (pH 8.0), 25 mM $NaHCO_3$, 20 mM $MgCl_2$, 3.5 mM adenosine triphosphate (ATP), 3.5 mM P-creatine, 0.25 mM nicotinamide adenine dinucleotide, reduced (NADH), 10 units creatine P-kinase, 10 units glyceraldehyde 3-phosphate-dehydrogenase, 10 units 3-phosphoglyceric phosphokinase and 50 μl extract. The reaction was initiated by the addition of ribulose bisphosphate (RuBP; final concentration 0.5 mM) and the change in absorbance at 340 nm was followed for 4 min at 25°C. Enzyme units are $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein. The activity of phosphoenolpyruvate carboxylase (PEPCase) was assayed in crude protein extract in a reaction mix containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.2 mM NADH, 10 mM $NaHCO_3$ and 2 mM phosphoenolpyruvate (PEP). The reaction was initiated by the addition of 50 μl of extract and the change in absorbance at 340 nm was measured for 4 min at 25°C.

STATISTICAL ANALYSIS

The experiments were performed at least in triplicate and the results averaged (means ±SE). The significance of differences between the control and each treatment was analyzed using the SPSS statistical package (version 10.0 for Windows; SPSS, Chicago, IL, U.S.A.).

RESULTS

Figure 1 shows the course of dehydration of leaves after withholding water. After 15 days of drought, the water potential of *R. soongorica* leaves had dropped from an initial value of –2.1 to –10.6 MPa. Soil water content was less than 6% at late stages of the treatment. By withholding watering, Pn declined as the leaf water potential decreased (Fig. 2). Although Pn reached high values in well-watered

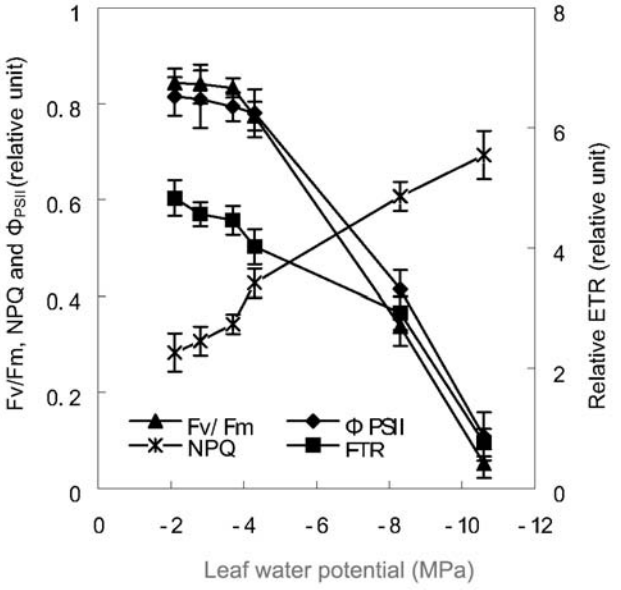

Fig. 3. Effects of different leaf water status on the maximal photochemical efficiency (*Fv/Fm*), the quantum efficiency of linear electric transport (Φ_{PSII}), non-photochemical quenching of fluorescence (NPQ) and photosynthetic electron transport rate (relative ETR) of photosystem II in two-year-old *R. soongorica* leaves during progressive drought. Data represent means ±SE, n=6.

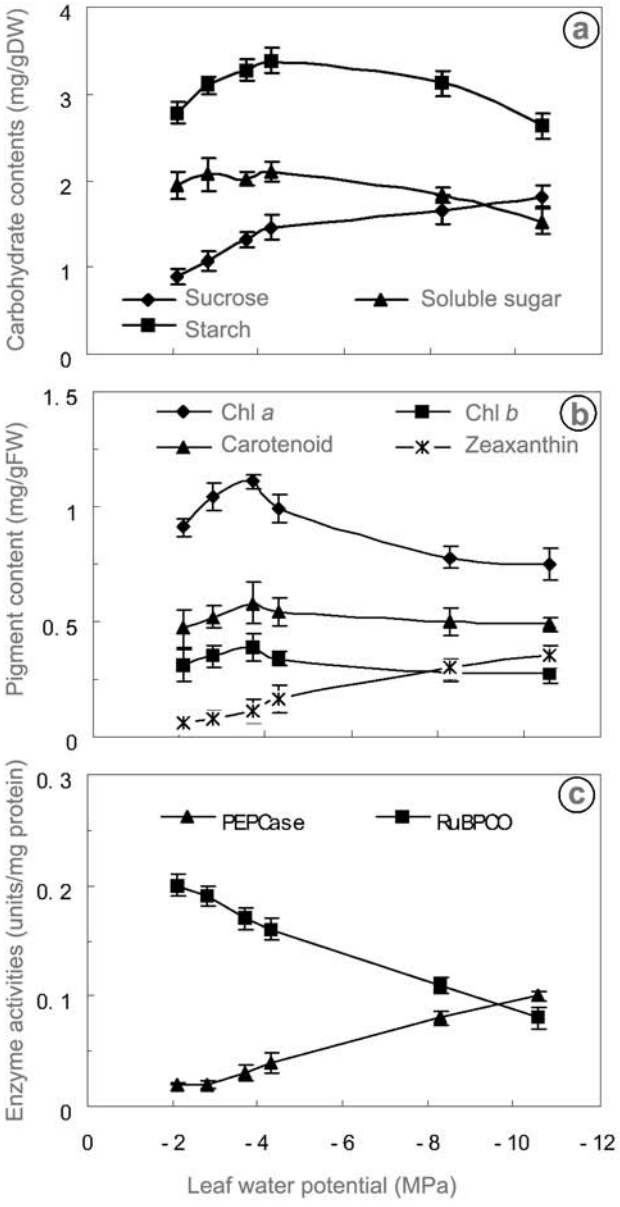

Fig. 4. Effects of different leaf water status on: (**a**) Carbohydrate contents, (**b**) Pigments contents and (**c**) Enzyme activities in 2-year-old *R. soongorica* leaves during progressive drought. Data represent means ±SE, n=3.

leaves, Gs was very low, indicating that little water evaporated from the stomata. The Gs measurements correlated well with those of Pn; Pn stopped at leaf water potential lower than –10 MPa. *Fv/Fm* and *Fv/Fm* and Φ_{PSII} did not decrease significantly when leaf water potential was higher than –4 MPa (Fig. 3), but NPQ rose ~30%. Under extreme arid conditions (leaf water potential lower than –6 MPa), *Fv/Fm*, Φ_{PSII} and relative ETR decreased rapidly.

Drought stress also caused an increase in sucrose content (Fig. 4a), but this increase occurred during the first days, after which the sucrose level remained almost constant. Total soluble sugar decreased only during the late stages of dehydration (below –4.3 MPa leaf water potential). Starch content rose and then fell during treatment.

During dehydration of this plant there was little variation in chlorophyll *b* and carotenoid content (Fig. 4b). Chlorophyll *a* content rose slightly in the early stages of dehydration and then decreased to approximately the initial level. However, there was about a sixfold increase of zeaxanthin, the xanthophyll cycle pigment previously reported as a sensitive indicator of plant stress (Demming-Adams and Adams, 1993; Jeyaramraja et al., 2005).

Drought stress resulted in a substantial decrease in Rubisco activity in leaves (Fig. 4c). Although there was very low PEPCase activity in these leaves, it showed an upward trend. Soluble protein content decreased in leaves during dehydration (Fig. 5), and the SDS-PAGE protein profile showed no induced polypeptides in drought-stressed plants (Fig. 6). Interestingly, an abundant polypeptide with about 52 kD molecular mass disappeared as drought stress progressed. This protein was closely related to water loss, and there has been

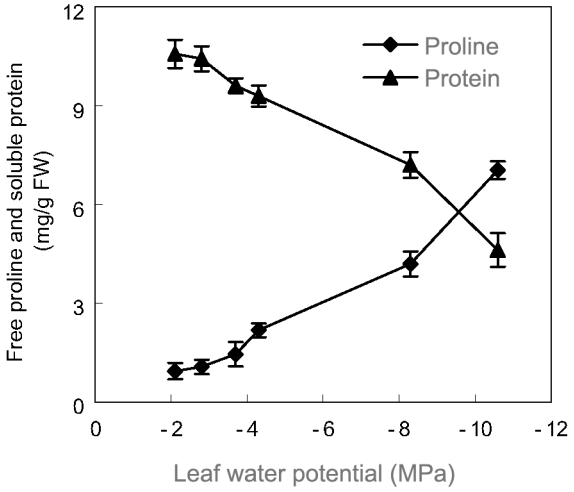

Fig. 5. Changes of free proline and total soluble proteins in two-year-old *R. soongorica* leaves during progressive drought. Data represent mean ±SE, n=3.

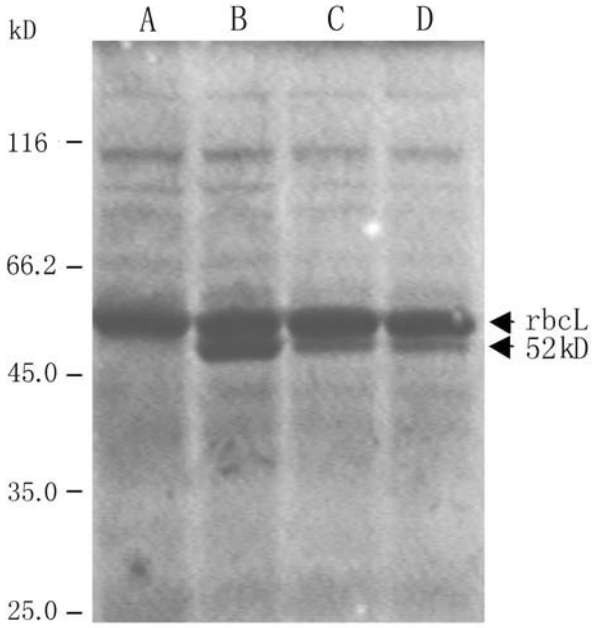

Fig. 6. Effects of different water status on the protein profile in *Reaumuria soongorica* leaves during dehydration. 12% SDS-PAGE analysis was performed to determine the total soluble proteins. From lane A to lane D, leaf water potential was –10.6 MPa, –2.1 MPa (control plants), –3.7 MPa and –4.3 MPa, respectively. The standard proteins (kDa) used to estimate the molecular masses of proteins. rbcL – RuBisCo large subunit.

no report as to its function up to now. Drought stress induced a more than sevenfold increase of proline content (Fig. 5).

DISCUSSION

Although this investigation was not focused on the metabolic networks of dehydration in the resurrection plant *R. soongorica*, it is important to understand how this plant prepares itself for this unusual capability. From these measurements, some significant observations stand out in this report.

In resurrection plants, the leaf water potential can fall below –50 MPa. Low leaf water potential was found in *R. soongorica* as well, both in the controls and dehydrated plants. Leaf water potential can indicate water-stress intensity in plants (Hsiao, 1973). Only resurrections plant can survive with such low leaf water potential. In the control plants, Pn was high and Gs was very low. It is an important point that low Gs did not inhibit natural photosynthesis but protected the leaves from water loss, suggesting that *R. soongorica* is able to hold water under extreme conditions. Low Gs was one of the most important factors behind a decline of Pn under drought stress (Ramachandra Reddy et al., 2004).

Reaumuria soongorica did not accumulate a large amount of sucrose in the leaves during dehydration. Sucrose accumulation varies in different species. In dicotyledonous species, the amount of accumulated sucrose in dehydrated leaf tissues ranges from 150 to 2000 mmol/g dry weight (DW) (Ghasempour et al., 1998). It is clear from the extent of this accumulation in dicotyledons that sucrose must be playing an important role in the tissues. However, in dehydrated monocotyledonous poikilohydric plants, sucrose accumulation is much lower, ranging between 65 and 100 mmol/g DW (Ghasempour et al., 1998; Scott, 2000). Moreover, it is not clear that sucrose is in fact accumulating during dehydration in some leaf tissues (Ghasempour et al., 1998). This may mean that sucrose accumulation plays a less important role in desiccation tolerance in monocotyledons. Sucrose content in *R. soongorica* leaves initially increases 1.8-fold in stressed leaves (at leaf water potential –5.3 MPa). This result suggests that the protection afforded by this molecule was incomplete in the leaves.

According to the $\delta^{13}C$ value of the leaves, *R. soongorica* employs the C_3 photosynthetic pathway (Ma et al., 2005). However, drought stress caused an increase (5-fold) in the PEPCase activity of the leaves during dehydration. This suggested that PEPCase, a C4 enzyme, played an important role in photosynthetic carbohydrate metabolism in late stages of leaf dehydration. Despite an increase in PEPCase activity, Pn decreased by ~98% versus the control value, which correlates with a marked decrease of RuBPCO activity. In general, plants exploit one mode of photosynthesis in their leaves, but some

plants can alter the mode of photosynthesis in response to changes in environmental conditions (Ueno, 1998). In some plants it seems likely that specific environmental stimuli induce new photosynthetic tissues with either C3-like or C4-like traits (Ueno et al., 1988). In some succulent plants, salt stress and drought stress induce the expression of CAM (Winter, 1985). Thus, other evidence is needed to verify a shift of the photosynthetic carbon assimilation pathway in *R. soongorica*.

Zeaxanthin, previously reported as a sensitive indicator of plant stress (Jeyaramraja et al., 2005), accumulated in *R. soongorica* leaves under drought stress, indicating that the conversion of violaxanthin to zeaxanthin far exceeded the reverse reaction that recycles violaxanthin. Similarly, accumulation of zeaxanthin has been reported in desiccating *Craterostigma plantagineum* (Alamillo and Bartels, 2001) and *Myrothamnus flabellifolia* (Krannerl et al., 2002).

Total soluble protein content in the leaves dropped progressively during drought. Stress inhibition of protein synthesis could be the cause to this decline (Barathi et al., 2001). However, a polypeptide with ~52 kD molecular mass was presented. In control plants it occurred in equal amounts with a large subunit of Rubisco (rbcL), and disappeared under desiccation. There are many reports of proteins associated with dehydration in plant tissues, such as late embryogenesis abundant (LEA) (Schneider et al., 1993) and aldehyde dehydrogenase proteins (Alamillo et al., 1995). Such abundance of protein was first reported in response to water loss. Further quantitative and qualitative analyses are needed.

Apart for the above significant changes, decreased *Fv/Fm*, *Fv/Fm*, Φ_{PSII} and relative ETR accompanied by increased NPQ, could protect the photosynthetic apparatus. Reductions in *Fv/Fm*, Φ_{PSII} could represent a photoprotective mechanism, adjusting the rate of photochemistry to match that of ATP and NADPH consumption (Cruz et al., 2003; Havaux et al., 2003). *Fv/Fm*, Φ_{PSII} and relative ETR decreased rapidly under extreme arid conditions, indicating that the protective mechanism in leaf had been damaged. Decreased Pn correlated well with Rubisco activity. Drought stress progressively decreases Pn, indicating that the decreased CO_2 assimilation rate was due to both reduced Gs and the damage to the photosynthetic apparatus. Starch content rose and then fell, suggesting its role as storage carbohydrate of photosynthetic production, mobilized at late stages of dehydration. The minor changes of chlorophyll content and chlorophyll *a/b* ratio in drought-treated plants indicate that damage to the light-harvesting complex was much less or negligible, which in turn could maintain a certain level of photosynthetic efficiency under severe water deficit (Lawlor, 1993). Carotenoids, major components of antenna systems, were only slightly affected by drought stress. Accumulation of proline under stress protects the cell by balancing the osmotic pressure of cytosol with that of vacuoles and the external environment (Gadallah, 1999; Hellebust, 1976). The higher osmolyte concentration in *R. soongorica* presumably maintains the comparatively lower leaf water potential.

We conclude that drought tolerance in *R. soongorica* leaves is achieved through a complex of changes in metabolism. Work is in progress to investigate the metabolic process that *R. soongorica* employs in order to allow tissues to dehydrate and then resurrect.

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