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FOTOSÍNTESIS, FOTOPROTECCIÓN, PRODUCTIVIDAD Y ESTRÉS ABIÓTICO: ALGUNOS CASOS DE ESTUDIO

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5. Oxidative damage to thylakoid proteins in water-stressed leaves of wheat (*Triticum aestivum* L.)

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5.0. Abstract

The production of reactive oxygen species in the chloroplast may increase under water deficit. To determine if this causes oxidative damage to the photosynthetic apparatus, we analyzed the accumulation of oxidatively damaged proteins in thylakoids of water-stressed wheat (*Triticum aestivum* L.) leaves. Water stress was imposed on four week-old plants by withholding watering for 10 days to reach a soil water potential of about - 2.0 MPa. In thylakoids of water-stressed leaves there was an increase in oxidative damage, particularly in polypeptides of 68, 54, 41 and 24 kDa. High molecular weight oxidized (probably cross-linked) proteins accumulated in chloroplasts of droughted leaves. Oxidative damage was associated with a substantial decrease in photosynthetic electron transport activity. L-galactono-1,4-lactone reduced oxidative damage to photosynthetic proteins of droughted plants, and partially reverted the decrease in electron transport activity. Increasing the ascorbic acid content of leaves might be an effective strategy to protect thylakoid membranes from oxidative damage in water-stressed leaves.

Abbreviations used: Chl, chlorophyll; Fm, maximum fluorescence in dark-adapted leaves; F_v, variable fluorescence; F_v/F_m, ratio of variable to maximum fluorescence; *Gal*, L-galactono-1,4-lactone; *LHCII*, light harvesting Chl-protein complex of *PSII*; $O_2^{\cdot -}$, superoxide anion; *RWC*, relative water content; *ROS*, reactive oxygen species.

5.1. Introduction

Growth and primary production of plants are severely reduced by water deficit. Even in habitats with high average rainfall, plants may experience water stress in certain periods of the year, or at times during the day (Cornic and Massacci 1996). Drought is an important limitation to grain yield in many crops (Blum 1996).

Photosynthetic activity is reduced by water stress. One of the earliest responses to drought is stomatal closure, which limits CO_2 diffusion to chloroplasts (Muller and Whitsitt 1996). With short periods of drought, or with relatively mild water stress, stomatal limitations account for most of the decrease in photosynthesis (Cornic and Massacci 1996). However, when drought is prolonged and/or more severe, the breakdown of the photosynthetic apparatus may contribute to the inhibition of CO_2 gain in water-stressed plants. In leaves experiencing a 30% or higher leaf water deficit there was a significant decrease in photosynthetic O_2 evolution at high CO_2 concentration, i.e., where stomatal limitations to CO_2 have a minimal impact (Cornic and Massacci 1996). Photosystem activity and photophosphorylation have been shown to decrease in droughted plants (Hanson and Hitz 1983; He et al. 1995).

Non-stomatal limitations to photosynthesis in droughted plants might be related to oxidative damage to chloroplast lipids, pigments and proteins. In droughted wheat, sunflower and pea leaves (Moran et al. 1994; Menconi et al. 1995; Sgherri and Navari-Izzo 1995; Sgherri et al. 1996) there was an increase in the production of reactive oxygen species (*ROS*) and in lipid peroxidation, probably initiated through photo-reduction of O_2 by the photosynthetic electron transport system (Menconi et al. 1995; Sgherri et al. 1996). *ROS* in plants are removed by a variety of anti-oxidant enzymes (e.g., superoxide dismutase, ascorbate peroxidase) and by small *ROS* scavenging molecules (Foyer et al. 1994). The expression of anti-oxidant enzymes, including superoxide dismutase, increases in droughted leaves (e.g., Smirnoff and Colombe 1988; Zhang and Kirkham 1996), affording some protection against oxidative damage in these conditions. Significantly, over-expression of superoxide dismutase in transgenic plants improves water-stress tolerance (McKersie et al. 1996). Another anti-oxidant defense in chloroplasts is provided by the ascorbate-glutathione cycle that removes H_2O_2 arising from dismutation of $O_2^{\cdot -}$ formed by the Mehler reaction (Foyer et al. 1994). Thus, ascorbic acid is critically involved in the defense against *ROS* produced by the photosynthetic apparatus (Smirnoff 1996).

Oxidative stress may cause protein oxidation, with loss of enzyme activity and the formation of protease-resistant cross-linked aggregates (Roberts et al. 1991; Berlett and Stadtmam 1997). Oxidatively-damaged proteins accumulate in pea leaves subjected to moderate water stress (Moran et al. 1994) and in chilled maize seedlings (Prassad 1996). However, besides these reports of bulk protein oxidation, there is no information on oxidative damage to photosynthetic proteins in stressed leaves, and on the impact of oxidative damage on photosynthetic performance in water-stressed plants. In this work, we analyzed oxidative damage to different proteins of the photosynthetic membranes in water-stressed leaves, and related the changes in protein oxidation to photosynthetic activity of thylakoids. In addition, we determined if application of the ascorbic acid precursor L-galactono-1,4-lactone (Smirnoff 1996) could increase the ascorbic acid content of droughted leaves and protect their photosynthetic apparatus from oxidative damage.

5.2. Material and methods

5.2.1. Plant material, water stress, and L-galactono-1,4-lactone application

Wheat plants (*Triticum aestivum* L. cv. Buck Poncho) were grown from seed in pots (0.5 L) filled with soil under controlled conditions (average maximum/minimum temperature of 26/18°C, 13-h photoperiod and 350 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of photosynthetically active radiation). Four week-old plants were subjected to water stress by withholding watering for 10 days until soil water potential reached about - 2.0 MPa. L-galactono-1,4-lactone (30 mM) or distilled water (control plants) were sprayed daily from the 4th to the 7th day of the water deficit treatment, i.e., *Gal* applications were discontinued three days before sampling on day 10. Preliminary trials showed that the amount of water sprayed on the plants had no effect on leaf or soil water potential. All measurements were made on the third leaf, the youngest fully expanded leaf at the start of the drought treatment. The experiments were repeated twice with at least 3 replicates per treatment in each experiment.

5.2.2. Soil water potential and leaf water status

Leaf and soil water potential were measured with a Wescor HR33T dew point psychrometer (Wescor Inc., Logan, Utah, U.S.A.) and C-52 leaf chambers or PST-55 probes placed in the soil at the start of the experiment. For relative water content (*RWC*) leaf segments were weighed (*wi*), floated on distilled water at 4°C in the dark overnight, weighed again (*wf*) and dried at 80°C for 48 hs for the determination of dry mass (*wd*). Relative water content was calculated as: $RWC = (wi - wd)\ (wf - wd)^{-1} \times 100$.

5.2.3. Photosynthesis and stomatal conductance

Photosynthesis was measured with a Licor LI-6200 Portable Photosynthesis System (Li-Cor Inc., Lincoln, Nebraska, U.S.A.) at 25°C and 1000 $\mu mol.\ m^{-2}\ s^{-1}$ of photosynthetically active radiation. Stomatal conductance was determined under the same conditions with a Licor LI-1600 Steady State Porometer (Li-Cor Inc., Lincoln, Nebraska, U.S.A.).

5.2.4. Determination of ascorbic acid content

For the determination of ascorbic acid (Law et al.1983) leaf segments were ground in 5% (w/v) metaphosphoric acid, centrifuged at 22000 g for 10 min and the supernatant was mixed with the reaction mixture (5 mM EDTA, 1.7% (w/v) TCA, 7.6% (w/v) o-phosphoric

acid 7.6%, 16 mM α-α'-dipyridyl, $FeCl_3$, dissolved in phosphate buffer pH 7.4). The reaction mixture was incubated at 40°C for 40 min and the absorbance at 525 nm was measured with a Shimadzu UV-VIS 160 spectrophotometer (Shimadzu Corporation, Tokyo, Japan).

5.2.5. Thylakoid isolation and carbonyl derivatization

Thylakoid membranes were isolated basically as in Camm and Green (1980). Leaves were homogenized with a blender in chilled buffer (50 mM HEPES, pH 7.5, 0.4 M sucrose, 2 mM $MgCl_2$, 1 mM EDTA, 0.2% (w/v) bovime serum albumin) added with protease inhibitors (0.5 $\mu g.ml^{-1}$ aprotinin, 0.5 $\mu g.ml^{-1}$ leupeptin, 0.7 $\mu g.ml^{-1}$ pepstatin and 40 $\mu g.ml^{-1}$ phenyl methyl sulfonylfluoride). The homogenate was filtered through a 10 µm nylon mesh and centrifuged at 3000 g and 4°C for 5 min. The pellet was resuspended in wash buffer (50 mM HEPES, pH 7.5, 10 mM NaCl) and centrifuged again as above. This second pellet was the thylakoid membrane preparation.

Carbonyl groups in thylakoid proteins were derivatized as described in Levine et al. (1994). Thylakoids were mixed with an equal volume of SDS (12% w/v) and then with 2 volumes of 20 mM dinitrophenylhydrazine dissolved in 10% (v/v) trifluoroacetic acid. This mixture was incubated for 25 min at room temperature, and the reaction was stopped by adding 1.5 sample volumes of 2 M Tris/30% (v/v) glycerol.

5.2.6. SDS-PAGE and Western blotting

Derivatized thylakoid proteins were loaded in 12% (w/v) acrylamide concentration mini-gels and electrophoresed at 15 mAmps for 2 hs. For Western blotting, proteins were electrotransferred to nitrocellulose membranes at 70V for 3 hs, or at 200 mAmps overnight. Blots were blocked with 5% (w/v) non-fat dry milk dissolved in TBS-T (20 mM Tris, pH 7.4, 150 mM NaCl, 0.02% (v/v) Tween-20), incubated for 1 h in primary antibody dissolved in blocking buffer, and washed several times with TBS-T. For carbonyl detection, the primary antibody was rabbit anti-DNP (SIGMA, St. Louis, Missouri, U.S.A.). Blots were then incubated for 1 h in secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase), washed several times with TBS-T and developed with a chemiluminescence detection kit (Renaissance TM, DuPont, Boston, Massachussetts, U.S.A.). Band intensity was determined by scanning films with a DMU-33C densitometer (Advantec Toyo Kaisha Ltd., Tokyo, Japan).

5.2.7. Photosynthetic electron transport

Whole chain electron transport was measured spectrophotometrically as in Holloway et al. (1983). Thylakoids (5 µg Chl) were resuspended in buffer (5 mM Na-Tricine, pH 7.5, 30 mM NH_4Cl, 80 µM K-ferricyanide) and illuminated with a dichroic lamp at 200 $\mu mol. m^{-2}.s^{-1}$ of photosynthetically active radiation. Ferricyanide reduction was calculated from the change in absorbance at 430 nm measured at 15 s intervals with a Shimadzu UV-Visible 160 spectrophotometer (Shimadzu Corporation, Tokyo, Japan). The activity of duplicate samples containing dibromothymoquinone (10 µM) was substracted to calculate the rate of electron transport from H_2O to the donor side of *PSI*. Typically, dibromothymoquinone inhibited this reaction by 80-85%.

5.3. Results

5.3.1. Physiological responses to water stress

Drought reduced leaf water potential to -2.6 MPa and the relative water content of water-stressed leaves to 71%, indicating that these plants were experiencing a stress of moderate to severe intensity at the time of the sampling (Table 1). Midday net photosynthetic rate was non-detectable in droughted plants, mostly because of very low stomatal conductance. Drought caused partial breakdown of the photosynthetic apparatus, i.e. photosynthetic electron transport declined in water-stressed leaves (Table 3).

Table 1 - *Leaf water potential, relative water content, and gas exchange parameters in wheat plants subjected to water stress for 10 days. Means followed by the same letter are not significantly different at 5% level of probability according to Tukey's test. Nd: not detectable.*

	Leaf water potential, *(MPa)*	**RWC** (%)	**Photosynthesis** *($\mu mol\ CO_2.\ m^{-2}.s^{-1}$)*	**Stomatal conductance** *($mmol\ H_2O.\ m^{-2}.s^{-1}$)*
Control	- 0.66 [a]	97.4 [a]	11.2	147 [a]
Water-stressed	- 2.60 [b]	71.0 [b]	Nd	15 [b]

Table 2 *- Ascorbic acid content of wheat leaves subjected to water stress and treated with 30 mol . m^{-3} of L-galactonic acid γ-lactone. For the water stress treatment, watering was withheld for 10 days. L-galactonic acid γ-lactone was sprayed on days 4th. to 7th., and measurements of ascorbic acid content were made on day 10th. For each water supply treatment, means followed by the same letter are not significantly different at 5% level of probability according to Tukey's test.*

	Ascorbic acid content, *μmol . g^{-1} dry weight*	
	Well-watered	**Water stressed**
Control	12.7 [a]	14.0 [a]
L-galactono-1,4-lactone	19.0 [b]	36.4 [b]

Water stress had no effect on leaf chlorophyll content (data not shown) nor on the protein composition of the thylakoid membranes (Fig. 1). Coomassie-stained gels did not reveal differences in the polypeptide pattern of thylakoid membranes nor in the abundance of specific proteins in control and droughted leaves. The levels of representative polypeptides of the *ATP* synthase, *PSII* and *PSI*, determined by Western blotting with specific antibodies, did not change in response to water stress (Fig. 1). Similarly, leaf soluble protein content and the levels of the large and small subunits of ribulose bisphosphate carboxylase/oxygenase (detected by Coomassie staining of denaturing gels) were not affected by the stress treatment (data not shown).

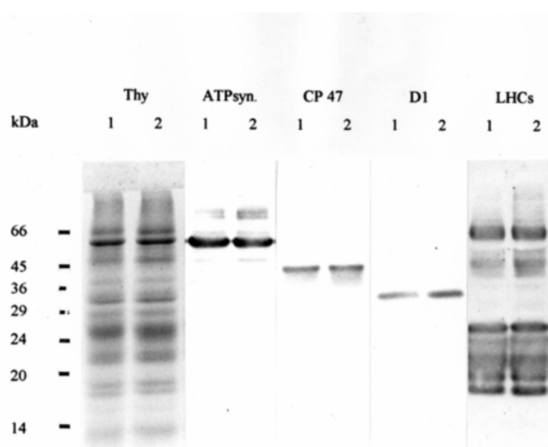

Figure 1 *- Changes in thylakoid protein composition detected by Coomassie-blue staining (lane labeled Thy) and in selected proteins revealed by Western blotting with specific antibodies (ATP synthase, CP47, D1 and LHCs) in well-watered and droughted leaves of wheat. Lanes: 1- well-watered; 2- water stressed. Figures on the left margin show the position of molecular weight standards.*

5.3.2. Oxidative damage to thylakoid proteins

Thylakoid membranes of well-watered leaves of wheat contained carbonylated (oxidized) polypeptides with apparent molecular weights of 68, 54, 45, 41, 36, 32 and 24 kDa (Fig. 2). The carbonylated protein of about 41 kDa represented the most heavily oxidized protein in thylakoids, although in Coomassie-stained gels this 41 kDa polypeptide appeared as a minor component of the thylakoid membranes (Fig. 1). There was also significant carbonylation of a polypeptide of 32 kDa (Fig. 2) located in *PSII*-enriched fractions of thylakoids (not shown). A 32 kDa protein was recognized by an anti-*D1* antibody (Fig. 1). Therefore, based on its location and recognition by an specific antibody, we identify the carbonylated polypeptide of 32 kDa as the *D1* protein of *PSII* reaction center. In Western blots of thylakoids proteins, antibodies specific for *CP47* and the β–subunit of *ATP* synthase recognized bands of 45 and 54 kDa, respectively (Fig. 1). An anti-*CPI* antibody that cross-reacts with *LHCII* and *LHCI* hybridized to bands at 68, 28 and 17 kDa (Fig. 1), corresponding to *CPI*, *LHCII* and *LHCI* (White and Green 1987). However, an unambiguous identification of the oxidized proteins of 68, 54 and 45 kDa was not possible because the carbonylated protein and the polypeptide recognized by each antibody might be different proteins co-migrating in our gels. Interestingly, there was no detectable carbonylation of *LHCII* (28 kDa) in wheat thylakoids, although *LHCII* represented the most abundant protein in these thylakoids (see Coomassie-stained lane 1 in Fig. 1).

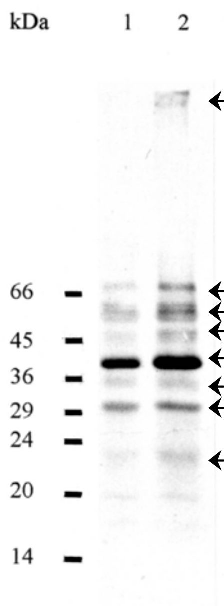

Figure 2 *-Western blot of thylakoid proteins from well-watered (lane 1) and water-stressed (lane 2) leaves of wheat. Blots were developed with an anti-DNP antibody to detect carbonylated proteins. Figures on the left margin of the blot show the position of molecular weight standards. Arrowheads on the right margin mark, from top to bottom: high molecular weight band (probably cross-linked proteins) and polypeptides of 68, 54, 45, 41, 36, 32 and 24 kDa.*

While the steady-state levels of thylakoid proteins were not affected by drought (Fig.1), there was an increase in oxidative damage to photosynthetic proteins in water-stressed leaves (Figs. 2 and 3). Overall, the carbonyl content of thylakoid proteins increased by 50% under drought conditions. Large increases in carbonyl content in response to water-stress occurred in a band of high molecular weight (193% increase over controls), probably representing cross-linked proteins in the interface between the stacking and resolving gels, and in a polypeptide of 24 kDa (170% increase over controls) (Figs.2 and 3). Oxidation of the 41 kDa protein increased by 46% in droughted leaves. The carbonyl content of the 68 and 45 kDa proteins increased by 61 and 56%, respectively, in droughted plants, while oxidation of the *D1* protein of the reaction center of *PSII* increased slightly less (Figs. 2 and 3). As with thylakoids from well-watered plants, samples from droughted leaves had no detectable carbonylation in the region of the blot corresponding to *LHCII* (i.e., at about 28 kDa).

5.3.3. Effects of L-galactono-1,4-lactone

Treatment with L-galactono-1,4-lactone increased the ascorbic acid content of leaves by 50 and 160%, in control and water-stressed plants, respectively (Table 2). The increased ascorbic acid content in plants treated with *Gal* resulted in a significant decrease in thylakoid protein oxidation. Overall, the carbonyl content of thylakoid proteins from treated leaves decreased by about 30 in control and droughted leaves (Fig. 3). In water-stressed leaves treated with *Gal* the carbonyl content of some thylakoid proteins decreased by ca. 20% (e.g., 45 and the 41 kDa polypeptide), whereas larger decreases were seen in the 68 and 24 kDa proteins.

5.3.4. Protection of the photosynthetic apparatus by L-galactono-1,4-lactone

In vitro photosynthetic electron transport from water to the donor side of *PSI* decreased in droughted plants. At day 10, uncoupled electron transport activity of droughted thylakoids represented 34% of the rate in control leaves (Table 3). Treatment with L-galactono-1,4-lactone had little effect in well-watered plants, but in water-stressed thylakoids *Gal* increased the electron transport activity by 33% compared to thylakoids of droughted plants receiving no *Gal*. Likewise, the decrease in the quantum yield (*Fv/Fm*) of *PSII* in droughted leaves was almost completely overcome by *Gal* treatment.

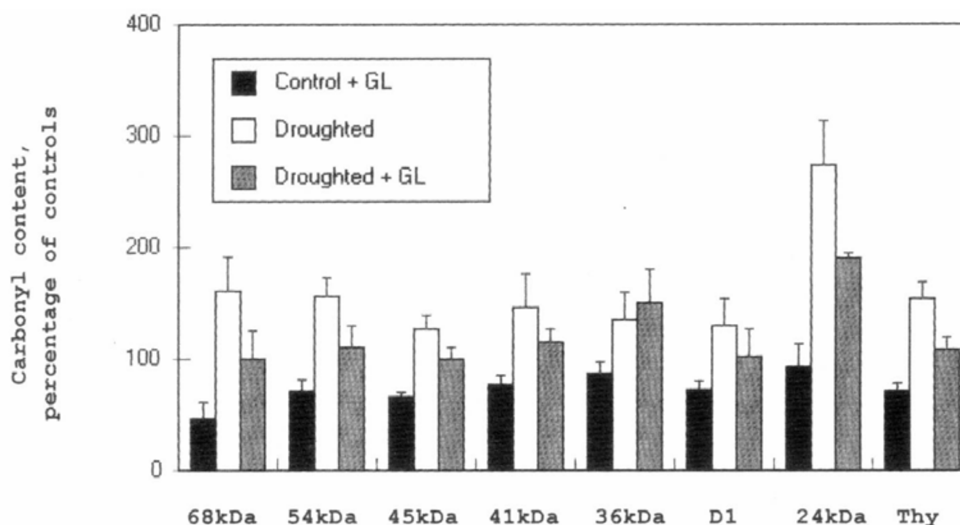

Figure 3. *Changes in carbonyl content in D1, in proteins of 68, 54, 45, 41, 36, and 24 kDa, and overall protein carbonylation in the thylakoid membranes (labeled thy) in response to water-stress and treatment with L-galactonic acid γ-lactone (GL). Carbonyl content was measured in Western blots developed with a chemiluminescent detection system. The exposed films were quantified with a densitometer and the carbonyl content of selected proteins was expressed as a percentage of the corresponding values in control leaves. Vertical bars represent the standard error of the mean.*

Table 3 - *Effects of water-stress and L-galactonic acid γ-lactone (Gal) application on photosynthetic electron transport from H_2O to the donor side of photosystem I. Means followed by the same letter are not significantly different at 5% level of probability according to Tukey's test.*

	Photosynthetic electron transport *µmol.mg Chl^{-1}. h^{-1}*
Control	138.0 [a]
Control + *Gal*	148.0 [a]
Water-stressed	47.0 [b]
Water-stressed + *Gal*	62.5 [c]

5.4. Discussion

5.4.1. Oxidative stress in droughted plants

The generation of *ROS* within the chloroplast may increase under conditions that limit CO_2 supply, such as in droughted leaves. There are several sources of *ROS* in the chloroplast. Photosystem I may donor electrons to O_2 (i.e., the Mehler reaction) to generate $O_2^{\cdot -}$ when the pool of NADP is mostly reduced. Also, excited *Chl* molecules can interact directly with O_2 to generate the very reactive singlet oxygen (Foyer et al. 1994). Photoproduction of superoxide by *PSII* has also been reported (Ananyev et al. 1994). The rate of $O_2^{\cdot -}$ formation increases under water-stress in a number of species, leading to lipid peroxidation, fatty acid saturation, and ultimately, membrane damage (Moran et al. 1994; Menconi et al. 1995; Sgherri and Navari-Izzo 1995; Sgherri et al. 1996). The content of oxidatively damaged proteins, which is a sensitive indicator of oxidative damage (Berlett and Stadtman 1997), increased in thylakoids of droughted leaves. However, in similar experiments with wheat, we did not detect any increase in the steady-state levels of lipid peroxides or fatty acid radicals in droughted plants (Bartoli et al. 1998), which suggests that proteins may be more susceptible to oxidative damage, or that lipid peroxides may be catabolized more rapidly than carbonylated proteins. Protein oxidation appears to be a more sensitive indicator of oxidative stress in chloroplasts than the accumulation of lipid peroxides or carbon centered radicals.

The largest increases in oxidative damage in response to water stress were detected in a 24 kDa protein and in a very high molecular weight band probably composed of cross-linked proteins. Cross-linked protein aggregates are a hallmark of oxidative damage to proteins, and they form in large quantities in thylakoid membranes in response to free-radical attack (Roberts et al. 1991). Interestingly, there was only a modest increase in the pool of oxidatively damaged *D1* protein in droughted leaves, although the *D1* protein of *PSII* is subjected to photo-oxidative damage under a number of stress conditions, including high light intensity, chilling, etc (Vermaas and Ikeuchi 1991). Damaged *D1* polypeptides are rapidly broken down in the stroma-exposed regions of the thylakoids, and damaged *PSII* centers are repaired through synthesis of new *D1* polypeptides (Vermaas and Ikeuchi 1991). The operation of this repair cycle may prevent the accumulation of oxidatively damaged *D1* in droughted leaves.

LHCII, the most abundant protein in thylakoid membranes (Green and Durnford 1996) including wheat thylakoids used here (Fig.1), showed no detectable carbonylation

either in well-watered or drougthed plants. We have not seen much carbonylation of *LHCII* even in leaves treated with 50 µM methyl viologen, a treatment causing heavy oxidation of other thylakoid proteins (Tambussi E, Bartoli CG and Guiamet JJ, unpublished observations). This is quite surprising given that a large part of chlorophyll in leaves is bound to *LHCII*, and, therefore, large amounts of singlet oxygen could be generated in this chlorophyll-protein complex under stress conditions. However, pigment molecules in *LHCII* seem to be separated from O_2 by an oxygen-impermeable barrier (Siefermann-Harms and Angerhofer 1998), which might limit the production of *ROS* by *LHCII*. This barrier might also protect part of the *LHCII* molecule from oxidation by *ROS* formed in other parts of the thylakoid membrane. It is also possible that the lipid environment surrounding *LHCII* limits the access of oxygen and oxygen radicals to this chlorophyll-protein complex (Siefermann-Harms and Angerhofer 1998). In any event, *LHCII* appears to be quite resistant to oxidative damage.

5.4.2. Protection by L-galactono-1,4-lactone

Treatment with L-galactono-1,4-lactone increased the pool of ascorbic acid in wheat leaves and this protected thylakoid proteins of water-stressed leaves from oxidative damage. Similarly, increased anti-oxidant capacity ameliorates the tolerance to water deficit in other species. For example, drought-resistance in maize correlates with heightened induced expression of anti-oxidant enzymes under oxidative stress (Pastori and Trippi 1993) and over-expression of superoxide dismutase confers drought-tolerance in transgenic alfalfa (McKersie et al. 1996).

The synthesis of ascorbic acid in response to the supply of L-galactono-1,4-lactone was significantly higher in droughted plants. L-galactono-1,4-lactone dehydrogenase catalyzes the conversion of L-galactono-1,4-lactone into ascorbic acid in plants, the last step in one the putative pathways leading to ascorbic acid biosynthesis in plants (Smirnoff 1996; Arrigoni et al. 1997). Our data suggest that the *in vivo* activity of L-galactono-1,4-lactone deshydrogenase is enhanced in water-stressed leaves.

The increased pool of ascorbic acid in droughted plants treated with *Gal* protected the photosynthetic membranes from oxidative damage. Several mechanisms could be involved in this protection. First, ascorbic acid can scavenge *ROS* directly, or indirectly serving as an electron donor for ascorbate peroxidase (Smirnoff 1996). Secondly, ascorbic acid contributes to the dissipation of excess energy in thylakoid membranes, thereby

decreasing the probability of *ROS* formation through interactions between excited *Chl* and O_2, or through photo-reduction of O_2. Ascorbic acid stimulates violoxanthin de-epoxidase activity and, thereby, the formation of zeaxanthin, which is involved in non-photochemical quenching of excitation energy (Havir et al. 1997). It is difficult to assess how much of the protection afforded by exogenously applied *Gal* was due to scavenging of *ROS* or to increased non-photochemical quenching, but it is clear that increasing the ascorbic acid content of leaves reduced oxidative damage to thylakoid membranes in water-stressed plants.

5.4.3. Protein oxidation and photosynthetic activity of thylakoid membranes

Increased protein oxidation under stress conditions correlated with reduced photosystem activity, whereas protection from oxidation by *Gal* partially restored the activity of thylakoids from droughted leaves. Thus, oxidative damage to thylakoid membranes may be an important factor lowering the photosynthetic activity of thylakoids in droughted plants. However, other factors in addition to oxidative damage may be involved in the decrease of photosynthetic electron transport activity. For example, treatment of water-stressed leaves with *Gal* reduced overall thylakoid protein oxidation to the levels in well-watered, untreated leaves, yet photosynthetic electron transport activity did not recover fully. Although we did not see important differences in the abundance of various thylakoid proteins, it remains possible that part of the drop in photosystem activity might be due to the loss of a critical component of the thylakoid membranes not detected in our gels and immunoblots. For example, with a stress level similar to that used in this work (i.e., about 70% *RWC*), levels of the *D2* protein of *PSII* started to decline in droughted wheat leaves before any change in the levels of *D1* (He et al. 1995).

Despite protection to thylakoid membranes, net CO_2 fixation during the water-deficit period did not increase in leaves supplied with *Gal*, compared to leaves receiving no *GL* (data not shown), most likely because of extremely low stomatal conductance for CO_2 in stressed leaves. Thus, anti-oxidant treatment may not improve the carbon balance of droughted plants despite the protection to the photosynthetic apparatus. However, protection against damage caused by *ROS* may ameliorate plant survival under stress, and might possibly improve other processes sensitive to oxidative stress, e.g., nitrogen assimilation (Kenis et al. 1994). The importance of protection to processes other than

photosynthesis is highlighted by the observation that survival of transgenic alfalfa plants over-expressing superoxide dismutase (*SOD*) in mitochondria is higher than with *SOD* targeted to the chloroplast (McKersie et al. 1996). Increasing the ascorbic acid pool of water-stressed leaves appears to be an effective strategy to enhance harmless dissipation of excess energy in the photosynthetic membranes, reduce the formation of *ROS*, and scavenge *ROS* produced. This should contribute to increase stress tolerance in crops and plant survival under water deficit.

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