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RESEARCH PAPER

Mitochondria are the main target for oxidative damage in leaves of wheat (*Triticum aestivum* L.)

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Abstract

Photosynthesis, respiration, and other processes produce reactive oxygen species (ROS) that can cause oxidative modifications to proteins, lipids, and DNA. The production of ROS increases under stress conditions, causing oxidative damage and impairment of normal metabolism. In this work, oxidative damage to various subcellular compartments (i.e. chloroplasts, mitochondria, and peroxisomes) was studied in two cultivars of wheat differing in ascorbic acid content, and growing under good irrigation or drought. In well-watered plants, mitochondria contained 9–28-fold higher concentrations of oxidatively modified proteins than chloroplasts or peroxisomes. In general, oxidative damage to proteins was more intense in the cultivar with the lower content of ascorbic acid, particularly in the chloroplast stroma. Water stress caused a marked increase in oxidative damage to proteins, particularly in mitochondria and peroxisomes. These results indicate that mitochondria are the main target for oxidative damage to proteins under well-irrigated and drought conditions.

Key words: Ascorbic acid, chloroplasts, drought, mitochondria, oxidative damage, peroxisomes, *Triticum aestivum* L., wheat.

Introduction

Plant cells produce harmful reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), the superoxide anion (O_2^-), and the hydroxyl radical ($HO^\bullet$), as by-products of their normal metabolism (Mittler, 2002). ROS may react with proteins, lipids, and DNA causing oxidative damage and, thereby, impairing the normal functions of cells. The deleterious effects of many environmental stresses are due to an increase in ROS production and, therefore, to oxidative damage to macromolecules (Foyer and Fletcher, 2001).

Many cell compartments produce ROS. Chloroplasts are a potentially important source of ROS because excited pigments in thylakoid membranes may interact with O_2 to form strong oxidants such as O_2^- or $^1O_2$ (Niyogi, 1999). Further downstream reactions produce other dangerous ROS like H_2O_2 and $HO^\bullet$. The interaction of O_2 with reduced components of the electron transport chain in mitochondria can lead to ROS formation (Møller, 2001), and peroxisomes produce H_2O_2 when glycolate is oxidized to glyoxylic acid during photorespiration (Douce and Heldt, 2000).

Plant cells synthesize a variety of antioxidants to cope with ROS produced under normal and stress conditions. Among non-enzymatic antioxidants, ascorbic acid (AA) plays a central role in plant defence by reacting directly with $HO^\bullet$, O_2^-, and $^1O_2$, and by recovering α-tocopherol from its oxidized form (Noctor and Foyer, 1998). An increased content of AA protects proteins and lipids against oxidative damage in plants subjected to water and salt stress (Tambussi *et al.*, 2000; Shalata and Neumann, 2001). In addition to their effects as oxidants, increased production of H_2O_2 and other ROS may constitute an alarm signal that regulates gene expression for the development of a co-ordinated protection against harmful environments (Pastori and Foyer, 2002). While plant cells may use this increase in ROS production to monitor the extent of environmental stress, the higher steady-state levels of ROS may lead to oxidative damage and trigger programmed cell death (Fath *et al.*, 2001; Tiwari *et al.*, 2002).

Although chloroplasts, mitochondria and peroxisomes are important sources of ROS, the relative extent of oxidative damage to these organelles has not been studied. Finding out the organelle(s) most vulnerable to oxidative damage under stress conditions might help to design stress-tolerant crops through targeted overexpression of antioxidant

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enzymes. The main goal of this work was to determine the intracellular site(s) most susceptible to oxidative damage in plants growing under normal or water stress conditions. The extent of oxidative damage to different organelles was also correlated with cultivar-specific differences in leaf AA contents.

Materials and methods

Plant material

The experiments were carried out using two wheat cultivars with either high (cv. Buck Chambergo, BCH) or low (cv. Cooperativa Maipún, CM) leaf ascorbic acid (AA) content. Plants were grown in 5.0 l pots containing an enriched soil mixture in a growth chamber with a photosynthetic photon flux density of 450 μmol m^{-2} s^{-1}, 25 °C and a 14 h photoperiod. Drought was imposed 3 weeks after seed germination by withholding watering. The soil water content decreased slowly to 15% w/w (about −1.5 MPa) and thereafter plants were kept at that level of stress for another 5 d by replacing the amount of water lost every day. Control plants were watered daily to keep the soil at field capacity (soil water content around 30% w/w). Relative water content (*RWC*) was calculated as in Tambussi *et al.* (2000).

Subcellular fractionation

The procedures for the isolation of organelles were carried out at 4 °C, and grinding mediums were added with a mixture of protease inhibitors (40 μg ml^{-1} phenylmethylsulphonyl fluoride, 0.5 μg ml^{-1} leupeptin, and 0.5 μg ml^{-1} aprotinin).

For chloroplast isolation, leaves were homogenized in 50 mM HEPES pH 7.6 containing 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 5 mM AA, and 0.05% (w/v) BSA, and filtered through a 20 μm pore mesh. The homogenate was loaded on a Percoll cushion (50 mM HEPES pH 8.0, 330 mM sorbitol, 35% (w/v) Percoll) and centrifuged at 2500 *g* for 5 min. The pellet containing intact chloroplasts was washed in 50 mM HEPES pH 8.0, 330 mM sorbitol and centrifuged again at 2500 *g* for 5 min. This final pellet was resuspended in a similar medium lacking sorbitol, and incubated in an ice bath for 10 min for chloroplast lysis. Stroma and thylakoid fractions were separated by a final centrifugation at 3000 *g* for 5 min.

Mitochondrial fractions were prepared by two different methods. Mitochondria were isolated by centrifugation in a PVP-40 gradient (Moore and Whitehouse, 1997) in method 1. Leaves were ground in a medium consisting of 40 mM MOPS pH 7.5, 300 mM sucrose, 25 mM $Na_4P_2O_7$, 2 mM EDTA. 1% (w/v) PVP-40, 20 mM AA, 4 mM cysteine, and 0.1% (w/v) BSA. The homogenate was centrifuged first at 3000 *g* for 5 min and then the supernatant was centrifuged at 17 400 *g* for 20 min. The resulting pellet, containing mitochondria and peroxisomes, was resuspended in wash medium (20 mM MOPS pH 7.5, 300 mM sucrose, 2 mM EDTA, 0.1% (w/v) BSA) and centrifuged at 17 400 *g* for 20 min. The pellet was resuspended again in wash medium and loaded on a 0–10% (w/v) PVP-40 gradient (PVP-40 dissolved in 20 mM phosphate buffer pH 7.5, 300 mM sucrose, 0.1% (w/v) BSA, 28% (v/v) Percoll). This gradient was centrifuged at 39 000 *g* for 40 min and the loose pellet containing mitochondria was washed twice as described above. Finally, mitochondria were resuspended in a small volume of wash medium without BSA.

Mitochondria isolated by method 1 were partly contaminated by peroxisomes and so mitochondria were also isolated by centrifugation in a Percoll gradient (Purvis, 1997) in method 2 to yield a preparation devoid of peroxisomal contamination. Leaves were ground in a medium consisting of 75 mM MOPS pH 7.5, 600 mM sucrose, 4 mM EDTA, 0.2% (w/v) PVP-40, 8 mM cysteine, and 0.2% (w/v) BSA. The homogenate was centrifuged at 3000 *g* for 10 min and the supernatant was centrifuged again at 16 000 *g* for 10 min. The resulting pellet was resuspended in wash buffer (10 mM MOPS pH 7.2, 300 mM sucrose), layered on top of a Percoll gradient (7.5 ml of 20% Percoll underlaid with 2.5 ml of 45% Percoll dissolved in wash buffer). The Percoll gradient was centrifuged at 26 000 *g* for 15 min and mitochondria were recovered in the interface between 20% and 45% Percoll. Finally, mitochondria were washed twice with wash buffer before carbonyl derivatization or subcellular marker determinations.

Peroxisomes were isolated as in Jimenez *et al.* (1997). Leaves were ground in 40 mM MOPS pH 7.5, 300 mM sucrose, 25 mM $Na_4P_2O_7$, 2 mM EDTA, 1% (w/v) PVP-40, 20 mM AA, 4 mM cysteine, and 0.1% (w/v) BSA. The homogenate was centrifuged first at 3000 *g* for 5 min and then the supernatant was centrifuged at 17 400 *g* for 20 min. The 17 400 *g* pellet, containing mitochondria and peroxisomes, was loaded on a gradient consisting of a bottom layer of 15 ml of 28% (v/v) Percoll in 10 mM MOPS, pH 7.2, 300 mM sucrose, and 0.1% (w/v) BSA, and a top layer of 21 ml of the same solution with 300 mM mannitol instead of sucrose. After centrifugation, peroxisomes were recovered close to the bottom of the gradient and washed twice as described for mitochondria.

Organelle integrity

The intactness of the organelles was estimated by measuring the latency of subcellular specific activities. Latency was calculated as in Burgess *et al.* (1985):

$$\text{latency} = [(\text{activity of broken organelle}) - (\text{activity of intact organelle})]/(\text{activity of broken organelle}) \times 100.$$

Immediately after isolation the integrity of the chloroplasts was assayed by measuring ferricyanide-dependent O_2 evolution in intact or osmotically broken organelles. The integrity of mitochondria and peroxisomes was assayed measuring the activities of marker enzymes in the presence (broken organelles) or absence (intact organelles) of Triton X-100, and calculated as above. Mitochondrial outer membrane intactness was estimated by measuring the activity of cytochrome *c* oxidase (CCO, EC 1.9.3.1), which is localized in the inner membrane facing the intermembrane space. Mitochondrial inner membrane integrity was assayed measuring the activity of the matrix soluble enzyme fumarase (EC 4.2.1.2). Integrity of peroxisomes was estimated by measuring the activity of hydroxypyruvate reductase (HPR, EC 1.1.1.29), which is located in the matrix of peroxisomes.

Oxygen evolution

Chloroplast O_2 evolution was determined with a Clark type electrode (Hansatech LD2/3 leaf disc electrode unit). The reaction mixture consisted of 1 vol. of reaction buffer (660 mM sorbitol, 4 mM EDTA, 2 mM $MgCl_2$, 2 mM $MnCl_2$, and 100 mM HEPES pH 7.6), 1 vol. of water, 1 mM $K_3Fe(CN)_6$, 5 mM NH_4Cl, and the chloroplast sample (about 50 μg chlorophyll ml^{-1}). O_2 evolution was measured at 800 μmol m^{-2} s^{-1} of photosynthetically active radiation.

Rubisco, chlorophyll, AA, and enzyme assays

Rubisco content was determined with specific antibodies in blots developed using a chemiluminiscence detection kit (Reinessance TM, DuPont, Boston, MA, USA), chlorophyll was determined after extraction with dimethylformamide (Inskeep and Bloom,1985) and AA as previously described (Bartoli *et al.*, 2000).

Fumarase activity was determined as fumarate formation following the increase in the absorbance at 240 nm (ε=2.4 mM^{-1} cm^{-1}) with a Shimadzu UV-Visible 160 spectrophotometer (Shimadzu Corporation, Tokyo, Japan). The reaction mixture consisted of 100 mM Tricine pH 7.5, 1 mM L-malate, and 50 μg of protein, in the presence

or absence of 0.02% (v/v) Triton X-100. HPR and CCO activities were measured as described previously (Bartoli *et al.*, 2000).

Carbonyl derivatization, SDS-PAGE, and western blotting

Derivatization and analysis of carbonylated proteins by western blotting was performed as described in Levine *et al.* (1994). Carbonyl content was expressed as a relative value on the basis of the protein content of each subcellular fraction.

Protein content

The protein content of each fraction was estimated by running aliquots in SDS–PAGE gels and scanning the gels after Coomassie blue staining. The scanned gels were quantified with the SigmaGel software (v. 1.0, Jandel Scientific), and the signals corresponding to all the proteins in each fraction (e.g. mitochondria) were totalled to estimate the concentration of protein in each fraction.

Results

The extent of oxidative damage to chloroplasts, mitochondria, and peroxisomes was determined in two cultivars of wheat, Buck Chambergo (BCH) and Cooperativa Maipún (CM), that differ in their leaf contents of AA (Table 1). AA content was twice as high in leaves of BCH as in CM, and it did not change significantly in response to water stress.

Organelle purity and intactness

Chloroplasts, mitochondria, and peroxisomes were isolated, and the contents of Rubisco and chlorophyll, and the activities of fumarase and HPR were used as specific markers for chloroplast stroma, thylakoids, mitochondria, and peroxisomes, respectively. Table 2 shows that preparations of chloroplast stroma, thylakoids, and peroxisomes obtained from BCH leaves were largely pure, with negligible contamination by other fractions. Similar results were obtained for CM (data not shown). The activity of fumarase, an enzyme of the Krebs cycle, was only detected in the mitochondria-enriched fraction, indicating that mitochondria did not contaminate any other fraction. HPR activity, a marker for peroxisomes, was also detected in the mitochondria-enriched fraction prepared by method 1, albeit at a lower specific activity, indicating some peroxisomal contamination of this mitochondrial preparation. Therefore, mitochondria from leaves of BCH were also isolated by a second procedure (method 2) that produced mitochondrial fractions where HPR activity was not detectable (well-watered plants) or extremely low (droughted plants).

Organelles retained a high degree of intactness after the isolation procedure (Table 3). Latency of ferricyanide-dependent O_2 evolution showed that chloroplasts were isolated with a high degree of intactness. CCO activity showed a latency of 90% or 86% for mitochondria isolated from control or stressed leaves, indicating that the mitochondrial outer membrane was mostly intact. Fumarase is a soluble enzyme localized in the mitochondrial matrix and its activity could only be detected after organelle disruption after adding Triton X-100. Similar results were obtained with HPR, which is located in the peroxisome matrix. The high degree of intactness and the recovery of soluble proteins (e.g. fumarase and HPR) demonstrate that these organelles were isolated retaining their internal contents.

Table 1. *Ascorbic acid content in leaves of wheat plants growing under well-watered (control) or drought conditions*

Cultivar	Ascorbic acid content (μmol g^{-1} dry weight)[a]	
	Control	Stressed
Buck Chambergo	16.2±1.3	13.6±2.0
Cooperativa Maipún	7.2±1.6 b	6.0±1.8 b

[a] Values are the means ±SE of five independent experiments. b indicates means statistically different between cultivars with the same treatment (ANOVA, $P \leq 0{,}05$).

High levels of oxidative damage to mitochondrial proteins

The highest contents of oxidatively modified proteins were found in the mitochondrial-enriched fractions (Table 4). Compared with mitochondria, the protein carbonyl concentrations of other fractions were much lower, ranging from 9-fold (cf. chloroplast stroma and mitochondria of

Table 2. *Distribution of subcellular markers among the organelle fractions isolated from leaves of wheat (cv. Buck Chambergo) grown under well-watered (control) or drought conditions*

Subcellular fraction	Rubisco content[a] (AU mg^{-1} protein)[b]		Chlorophyll content[a] (μg mg^{-1} protein)		Fumarase activity[a] (nmol substrate mg^{-1} protein min^{-1})		HPR activity[a] (nmol substrate mg^{-1} protein min^{-1})	
	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed
Stroma	9.6±3.0	10.4±3.0	3.2±0.7	1.9±1	nd	nd	9.5±3.5	3±1
Thylakoids	nd[c]	nd	405±86	460±90	nd	nd	19±10	3±2
Peroxisomes	nd	nd	0.55±0.2	1.8±1.0	nd	nd	600±80	220±18
Mitochondria, method 1	nd	nd	20±4	22±9	60±16	46±52	379±4	158±50
Mitochondria, method 2	nd	nd	19±15	18±12	185±52	36±4	nd	7±7

[a] Values are the means ±SE of 3–5 independent experiments.
[b] AU: arbitrary units.
[c] nd: non detectable.

CM) to 28-fold (cf. chloroplast stroma and mitochondria of BCH) lower. Although mitochondria prepared with method 1 were contaminated by peroxisomes, levels of carbonylation in peroxisomes (which were devoid of mitochondrial contamination) were about 10-fold lower than in mitochondria, which clearly implies that peroxisomal contamination could not contribute significantly to the carbonylation signal in the mitochondrial fraction from this study. Furthermore, a second mitochondrial preparation (method 2), with non-detectable or extremely low peroxisome contamination, showed the same high level of protein oxidation, confirming that mitochondria contain the highest levels of protein carbonylation of the organelles tested. In general, CM, the cultivar with the lower content of AA, showed a higher level of carbonylated proteins in every subcellular fraction, except for thylakoids where both cultivars had similar protein carbonylation levels. The concentrations of carbonylated proteins in chloroplast stroma, mitochondria, and peroxisomes were 5-, 1.8- and 1.8-fold higher, respectively, in CM than in BCH.

Oxidative damage in water-stressed leaves

Oxidative stress and oxidative damage to macromolecules may be partly responsible for the deleterious effects of water deficit in some species (Sgherri *et al.*, 1996; Tambussi *et al.*, 2000). In this experiment, the extent of oxidative damage to proteins was determined in chloroplasts, mitochondria, and peroxisomes of plants subjected to an episode of moderate water deficit. Drought reduced the relative water content (*RWC*) of leaves to similar values in both cultivars, i.e. from about 97% in well-watered plants to 77–79% in droughted plants of cv. CM or BCH.

The overall protein carbonyl content increased significantly in all subcellular fractions from droughted plants, reaching the highest amounts in mitochondria of both cultivars (Table 4). The largest increase in carbonylation was detected in mitochondrial proteins of BCH (4.6-fold increase), followed by peroxisomes of both cultivars (2.9-fold and 3.5-fold) and mitochondria of CM (2.1-fold). Protein carbonylation increased less in thylakoids of both cultivars, and in the chloroplast stroma of BCH, but no significant increase was detected in the chloroplast stroma of water-stressed plants of CM. However, carbonylation was 3-fold higher in stroma proteins of droughted plants of CM than BCH.

Table 3. *Intactness of isolated organelles determined as the latency of specific activities of subcellular markers in the fractions isolated from leaves of wheat (cv. Buck Chambergo) grown under well-watered (control) or drought conditions*

Latency is expressed as % using the formula described in the Materials and methods. Oxygen evolution rates by chloroplasts were 132±6 and 71±10 μmol O_2 h^{-1} mg^{-1} Chl for control and stressed leaves, respectively. The activities of CCO showed by mitochondria were 560±10 and 360±22 nmol cyt *c* min^{-1} mg^{-1} protein for fractions obtained from control and stressed leaves, respectively. The activities of fumarase and HPR are shown in Table 2.

Subcellular fraction	Intactness (latency, %)	
	Control	Stressed
Chloroplasts (O_2 evolution)	75±8	78±12
Mitochondria		
CCO	90±2	86±1
Fumarase	100	100
Peroxisomes		
HPR	97±4	100

Discussion

The main objective of this work was to assess the relative extent of oxidative damage to proteins in chloroplasts, mitochondria, and peroxisomes isolated from leaves of wheat plants grown under good irrigation and drought. In this work, evidence is presented that, among the subcellular fractions studied, mitochondria are the organelles with the highest concentrations of oxidatively damaged proteins in plant cells.

Oxidative damage in different subcellular fractions

Many plant cell compartments are capable of producing ROS. Thylakoid membranes constitute an important site of ROS production in leaves exposed to light, partly because the triplet excited state of chlorophyll interacts with ground state O_2 forming the dangerous $^1O_2$ (Foyer and Fletcher, 2001). Once formed, $^1O_2$ rapidly reacts with nearby molecules causing oxidative damage to proteins, lipids, and DNA. Peroxisomes are another important source of ROS, especially in C_3 plants where photorespiration contributes

Table 4. *Protein carbonyl content in different subcellular fractions of wheat leaves*

Plants were grown under well-watered (control) or water-stress conditions. Values are expressed as arbitrary units $unit^{-1}$ protein.

Cultivar	Chloroplast stroma		Thylakoids		Mitochondria 1		Mitochondria 2		Peroxisomes	
	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed
Buck Chambergo	1.28±0.4[a]	2.78±0.3 a	2.1±0.3	3.5±0.2 a	36±1	167±47 a	36.5±0.2	110±5 a	3.5±0.17	10±1.5 a
Cooperativa Maipún	7.0±0.4 b	8.4±0.5 b	2.4±0.15	3.7±0.3 a	66±9 b	140±23 a	nd	nd	6.5±0.7 b	23±13 a

[a] Values are the means±SE of 3–5 independent experiments. a indicates means statistically different between stressed and control plants. b indicates means statistically different between cultivars with the same treatment (ANOVA, $P \leq 0{,}05$); nd: not determined.

large amounts of H_2O_2 (Corpas *et al.*, 2001; Noctor *et al.*, 2002). However, in plants growing under normal, non-stressful conditions, mitochondria contained the highest concentrations of oxidatively modified proteins. The concentrations of protein carbonyl groups in mitochondria were at least 9 times higher than in the other compartments tested (i.e. chloroplast stroma, thylakoids, and peroxisomes). Mitochondria are an important source of free radicals (Salin, 1991), and the two main sites of ROS formation in plant mitochondria are complexes I and III, where interactions with O_2 can lead to the production of $O_2^{\cdot-}$ (Møller, 2001). Whether the high level of carbonylated proteins in mitochondria is due to a high ROS production, or to comparatively lower levels of antioxidant defences remains to be determined.

The effect of differences in AA content

AA has multiple functions in photosynthesis and photoprotection, and it plays a central role in the antioxidant defence system in plants (Foyer and Harbinson, 1994; Noctor and Foyer, 1998; Smirnoff and Wheeler, 2000). Cultivars BCH and CM differ in their leaf AA content, with BCH having around twice as much AA as CM, under both well-watered and drought conditions. This natural variation in AA content established a test to discover if higher levels of AA might protect different organelles from oxidative damage. In well-watered plants, the higher AA content of BCH correlated with lower levels of oxidatively modified proteins. The large difference (5.5-fold) between BCH and CM in oxidative damage to stromal proteins suggests that AA afforded protection mostly in the water-soluble environment of the chloroplast where AA occurs in high concentrations (Noctor and Foyer, 1998). The higher AA content of BCH also resulted in significant, albeit not as striking, antioxidant protection to mitochondrial and peroxisomal proteins.

It was previously reported that thylakoid protein carbonylation increases in wheat leaves under drought, and that increasing the levels of ascorbic acid by exogenous supplementation with L-galactono-1,4-lactone may protect thylakoid membranes from oxidative stress (Tambussi *et al.*, 2000). By contrast, in this work the higher AA content of BCH was reflected in lower carbonylation levels of stromal proteins, but thylakoid proteins were apparently not protected by the higher levels of AA in BCH. However, the stress level in this work was milder (*RWC*=77–79%) than in previous experiments (*RWC*=71%, Tambussi *et al.*, 2000) and the increase of AA content induced by L-galactono-1,4-lactone was slightly higher than the constitutive difference between BCH and CM reported here.

Effects of drought on oxidative damage to subcellular fractions

Many environmental factors, including chilling, salinity, and drought, cause an increase in oxidative stress in plants (Hernández *et al.*, 1993; Hippeli and Elstner, 1996; Bartoli *et al.*, 1999; Kingston-Smith and Foyer, 2000). Among the subcellular fractions studied in this work, the largest increases in protein oxidation brought about by drought occurred in mitochondria and peroxisomes.

The over-reduction of mitochondrial electron transporters can lead to an increase in the generation of ROS, and risk of oxidative damage to cell molecules (Millar and Day, 1997; Møller, 2001). Oxidative damage to mitochondria may be crucial in determining the survival of cells exposed to different kinds of stress. For example, pre-acclimation treatments at moderately low temperatures improved survival after a chilling period in maize, probably by triggering the induction of mitochondrially-targeted antioxidant enzymes (Prasad *et al.*, 1994). Survival after a chilling episode correlated with enhanced recovery of mitochondrial function, and particularly of the CCO pathway (Prasad *et al.*, 1994). Likewise, exposure to ozone triggered oxidative stress in birch leaves, with H_2O_2 accumulation in mitochondria (Pellinen *et al.*, 1999). Hydrogen peroxide accumulation in mitochondria coincided with disintegration of the mitochondrial matrix and the appearance of the first symptoms of leaf damage (Pellinen *et al.*, 1999). An increase in H_2O_2 content in chilled maize leaves, independently of photosynthetic activity, also suggests that cell compartments other than the chloroplast are major sources of ROS (Kingston-Smith *et al.*, 1999). This work shows that mitochondria contain higher levels of oxidatively damaged proteins than chloroplasts or peroxisomes, and that protein carbonylation increases significantly in mitochondria of water-stressed plants. The increase in the production of ROS, for example, H_2O_2, by mitochondria might be an important alarm signal up-regulating antioxidant defence systems (Prassad *et al.*, 1994) or triggering programmed cell death if oxidative stress becomes rampant (Tiwari *et al.*, 2002).

Peroxisomes proliferate in plants subjected to pathogen attack, wounding, and, probably under other stresses (Lopez-Huertas *et al.*, 2000). It has been postulated that H_2O_2 generation through the glycolate oxidase reaction increases when leaf CO_2 concentration declines, as in plants growing under water stress, and that, under such conditions, the rate of H_2O_2 production in peroxisomes is higher than H_2O_2 production by the Mehler reaction in chloroplasts (Noctor *et al.*, 2002). The results obtained here are in agreement with this observation since drought-associated oxidative damage was more intense in peroxisomal than in chloroplast proteins.

Conclusions

The identification of intracellular sites that are susceptible to oxidative damage may contribute to the design of transgenic plants with improved performance under a variety of abiotic stresses (Millar *et al.*, 2001). The results of

this work suggest that protecting mitochondria against ROS might improve plant performance under non-stress and water-stress conditions. While most of the attention has so far focused on chloroplasts, damage to mitochondria may be equally important since they play many important functions in plant metabolism, for example, supply of ATP and carbon skeletons for the biosynthesis of several compounds, participation in photorespiration and programmed cell death (McCabe *et al.*, 2000, and references therein), optimization of photosynthesis (Padmasree *et al.*, 2002), and synthesis of AA (Bartoli *et al.*, 2000; Millar *et al.*, 2003). Future studies might be aimed at understanding antioxidant protection and ROS production in mitochondria, and the possible role of mitochondrial oxidative damage as an alarm signal for the development of plant responses under stress conditions.

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