Journal of Experimental Botany, Vol. 56, No. 415, pp. 1269–1276, May 2005
doi:10.1093/jxb/eri111 Advance Access publication 21 March, 2005

Journal of Experimental Botany

RESEARCH PAPER

Up-regulation of the mitochondrial alternative oxidase pathway enhances photosynthetic electron transport under drought conditions

Carlos G. Bartoli[1]*, Facundo Gomez[1], Gustavo Gergoff[1], Juan J. Guiamet[1] and Susana Puntarulo[2]

[1] *Plant Physiology Institute (INFIVE), Schools of Agronomy and of Natural Sciences, National University of La Plata, cc 327 1900 La Plata, Argentina*

[2] *Physical Chemistry-PRALIB, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina*

Received 13 August 2004; Accepted 22 December 2004

Abstract

The aim of this study was to explore the role of the mitochondrial alternative oxidase (AOX) in the protection of photosynthesis during drought in wheat leaves. The relative water contents of water-replete and drought-exposed wheat plants were 97.2±0.3 and 75±2, respectively. Drought increased the amount of leaf AOX protein and also enhanced the rate of AOX-dependent O_2 uptake by the respiratory electron transport chain. The amount of the reduced, active form of the AOX protein was specifically increased by drought. The AOX inhibitor salicylhydroxamic acid (1 mM; SHAM) inhibited 70% of AOX activity *in vivo* in both water-replete and drought-exposed plants. Plants treated with SHAM were then exposed to low (100), high (350), or excess light (800 μmol photons m^{-2} s^{-1}) for 90 min. SHAM did not modify chlorophyll *a* fluorescence quenching parameters in water-replete controls after any of these treatments. However, while the maximal quantum yield of photosystem II (PSII) electron transport (F_v/F_m) was not affected by SHAM, the immediate quantum yield of PSII electron transport (Φ_{PSII}) and photochemical quenching (*qP*) were gradually reduced by increasing irradiance in SHAM-treated drought-exposed plants, the decrease being most pronounced at the highest irradiance. Non-photochemical quenching (*NPQ*) reached near maximum levels in plants subjected to drought at high irradiance. However, a combination of drought and low light caused an intermediate increase in *NPQ*, which attained higher values when AOX was inhibited. Taken together, these results show that up-regulation of the respiratory AOX pathway protects the photosynthetic electron transport chain from the harmful effects of excess light.

Key words: Alternative oxidase, chlorophyll fluorescence, drought, photoprotection, respiration, wheat.

Introduction

Respiration is a central metabolic process that contributes in many different ways to cell function. It produces ATP, ascorbic acid, and other metabolites that are used in many synthetic processes and are essential for growth and maintenance of the cell (McCabe *et al.*, 2000; Bartoli *et al.*, 2000). In addition, key steps of the photorespiration pathway are localized in mitochondria (Douce and Heldt, 2000). Mitochondria are implicated in the control of developmental processes particularly programmed cell death (Tiwari *et al.*, 2002).

In the last decade there has been an increase in interest in the interactions between respiration and photosynthesis. Reducing power generated in the chloroplasts through photosynthesis may be exported as malate to the cytosol, and used in other cell compartments such as mitochondria (Atkin *et al.*, 2000; Krömer, 1995). Several important metabolic pathways such as photorespiration and nitrogen assimilation require reactions occurring in both mitochondria and chloroplasts (Foyer and Noctor, 2002). Evidence of the connection between these organelles comes from the observation that chloroplast development is altered in the *albostrians* barley mutant which has increased mitochondrial

* To whom correspondence should be addressed. Fax: +54 221 4233698. E-mail: carlos.bartoli@ceres.agro.unlp.edu.ar

gene copies per cell (Hedtke *et al.*, 1999). Furthermore, the light-dependent expression of mitochondrial genes also suggests that there is a close association between photosynthesis and mitochondrial metabolism (Svensson and Rasmusson, 2001).

There is a branch point in the respiratory electron transport chain after ubiquinone such that electrons can either flow through the cytochrome *c* pathway that generates the proton-motive force used for ATP synthesis, or through an alternative, non-phosphorylating pathway that channels the electrons directly from ubiquinone to O_2 through the alternative oxidase (AOX). Both pathways appear to have important functions in the regulation of photosynthesis (Foyer and Noctor, 2002). Evidence of this interaction comes from experiments using low concentrations of oligomycin, a mitochondrial phosphorylation inhibitor which causes a decrease of photosynthesis even though it does not inhibit chloroplast phosphorylation (Krömer *et al.*, 1993). Furthermore, the addition of the AOX inhibitor salicylhydroxamic acid (SHAM) lowers photosynthetic O_2 evolution rates in isolated protoplasts (Padmasree and Raghavendra, 1999). Moreover, loss of mitochondrial complex I function in the CMSII *Nicotiana sylvestris* mutant slowed the induction of photosynthesis after a period of darkness and decreased optimal rates of photosynthesis under photorespiratory conditions (Dutilleul *et al.*, 2003).

Photosynthetic light absorption and energy utilization must be kept in balance to prevent the formation of reactive oxygen species in the chloroplasts. Drought causes stomatal closure, which limits the diffusion of CO_2 to chloroplasts and thereby causes a decrease in CO_2 assimilation in favour of photorespiration that produces large amounts of hydrogen peroxide (Noctor *et al.*, 2002). Under these conditions the probability of singlet oxygen production at PSII and superoxide production at PSI is increased (Niyogi, 1999; Foyer *et al.*, 2005). These may cause direct oxidative damage or induce a cell suicide programme (Tambussi *et al.*, 2000). The probability of generating singlet oxygen and superoxide is decreased by decreasing light absorption, by increasing thermal energy dissipation or consuming excess reducing power in compartments other than the chloroplast (Niyogi, 2000).

The aim of this study was to test the hypothesis that under adverse environmental conditions, such as drought, the mitochondrial AOX pathway is up-regulated and that this acts as a sink for reducing power, preventing the accumulation of excess reducing equivalents in the chloroplasts and thereby decreasing the probability of loss of photosynthetic function.

Materials and methods

Plant material

Wheat (*Triticum aestivum* L. cv. Buck Chambergo) seeds were sown in 0.5 l pots containing a loam soil, and grown at a *PPFD* of 350 μmol m^{-2} s^{-1} and 22±3 °C with a photoperiod of 12 h. Drought was imposed on 3-week-old plants by withholding watering until the soil water content had reached 14% (about −1 MPa, as measured with a Wescor HR33T psychrometer and PST55 soil probes). Soil water content was maintained at 14% for the following 3 d by replacing the amount of water lost each day (as estimated by weighing the pots). At this time water-replete and water-deficient plants were used for the experiments described below. Relative water content (*RWC*) was measured to monitor leaf water status and was calculated as: $(FW–DW)/(TW–DW)$, where *FW* is the fresh weight, *DW* is the dry weight, and *TW* represents the fresh weight at full turgor. Leaf segments were floated in distilled water for 3 h in order to achieve full turgor.

Respiration measurements

Respiration was measured as O_2 uptake using an air-tight chamber fitted with a Clark type electrode (Hansatech LD2/3 leaf disc electrode unit). To assess the maximum capacity of the alternative oxidase (AOX) pathway, the cytochrome *c* pathway was inhibited with 1 mM KCN, whereas the AOX pathway was inhibited with 0.1–10 mM salicylhydroxamic acid (SHAM). Both respiratory inhibitors were supplied for 4 h through the cut tips of leaves attached to the plant and maintained at a very low light intensity (*PPFD*s of around 10–20 μmol m^{-2} s^{-1}). Thereafter, leaves were cut into 3 cm long segments, placed in the oxygen electrode chamber, and respiration measured as described above. For plants subjected to drought, solutions of respiratory inhibitors were supplemented with 440 mM mannitol to maintain a similar water potential to that in droughted leaf tissues (leaves supplied with SHAM+440 mM mannitol maintained a similar *RWC* as water-stressed leaves without respiratory inhibitor solutions, data not shown). Total respiration was measured in leaves without any inhibitor treatment.

Chlorophyll fluorescence measurements

After treatment with respiratory inhibitors as described above, leaves were exposed to *PPFD*s of 100, 350, and 800 μmol m^{-2} s^{-1} for 90 min. Chlorophyll fluorescence parameters were then measured with a Fluorescence Modulated System (FMS2, Hansatech Instruments, Ltd., Norfolk, UK). Steady-state fluorescence parameters were measured at each light level. F_v/F_m was measured after 30 min darkness. Fluorescence parameters were calculated as described by Genty *et al.* (1989). *NPQ* was calculated as $(F_m–F'_m)/F'_m$.

Mitochondria isolation

Mitochondria were isolated as described by Moore and Whitehouse (1997) with minor modifications. Briefly, 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 ascorbic acid, and 4 mM cysteine. The homogenate was centrifuged at 3000 *g* for 5 min and then the supernatant was centrifuged at 17 400 *g* for 20 min. The pellet was resuspended in wash medium [20 mM MOPS pH 7.5, 300 mM sucrose, 2 mM EDTA, and 0.1% (w/v) BSA] and centrifuged at 17 400 *g* for 20 min. The last step was repeated again and mitochondria were finally suspended in a small volume of wash medium.

Chloroplast isolation

Leaves were ground in buffer containing 50 mM HEPES pH 7.6, 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 5 mM ascorbic acid, and 0.05% (w/v) BSA using a hand-held homogenizer. The homogenate was filtered through a 20 μm pore size nylon mesh and centrifuged at 3000 *g* for 5 min. The pellet was suspended in wash medium (50 mM HEPES pH 7.6 and 330 mM sorbitol), loaded in a solution consisting of 35% (v/v) Percoll in wash medium, and centrifuged at 2500 *g* for 5 min. The pellet containing intact chloroplasts was used to measure O_2 evolution and fluorescence in the absence or presence of 1 mM SHAM.

Content of AOX protein

Proteins isolated from mitochondria were separated by SDS-PAGE as previously described (Tambussi *et al.*, 2000). The AOX protein was detected according to Elthon *et al.* (1989) using a chemiluminescent detection kit.

Chloroplast O_2 evolution

Chloroplast O_2 evolution was measured in a reaction mixture containing 50 mM HEPES pH 7.6, 330 mM sorbitol, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM ATP, 0.5 mM K_2HPO_4, 10 mM $NaHCO_3$, and chloroplasts (10 μg of chlorophyll). The reaction was started by switching on the light (800 μmol photons m^{-2} s^{-1}) and O_2 evolution was measured with a Clark type O_2 electrode in liquid phase.

Statistical analyses

Data are expressed as mean ±SD from 4–6 independent experiments. The effects of treatments were tested for significance using a single-factor analysis of variance (ANOVA). Significantly different means were separated using the Fisher PLSD test, Statview SE, v 1.03 (Abacus Concepts Inc, Berkeley, CA).

Results

Effect of water stress on respiration

Water stress was imposed on wheat plants by discontinuing watering until the soil reached a water potential of −1 MPa. The soil water content was then maintained at −1 MPa for the next 3 d. The intensity of drought experienced by the leaves was monitored by measuring leaf relative water content (*RWC*). Well-irrigated plants showed a *RWC* of 97.2±0.3% while water deficit significantly decreased *RWC* to values of 75±2%.

The respiration rates of wheat leaves were increased by 41% when water availability was restricted (Table 1). This increase in respiration was due mostly to an increase in the capacity of the AOX pathway. While the maximum capacity of the cytochrome *c* oxidase pathway (as estimated in leaves treated with 10 mM SHAM) was not affected by drought, oxygen uptake by the AOX doubled in water-stressed leaves (Table 1). Surprisingly, both the cytochrome *c* and AOX pathways appeared to operate at maximum rates under these conditions as determined by their maximum rates relative to total leaf respiration under either adequate water supply or drought conditions. Drought increased the amount of the AOX protein resulting in an increase in the capacity of the AOX pathway (Fig. 1). Specific antisera identified two AOX bands corresponding to the reduced, active monomer (of about 36 kDa) and the oxidized, less active dimer forms of AOX, respectively (Vanlerberghe and McIntosh, 1997). The levels of both the reduced and oxidized forms of AOX increased under drought-stress conditions, but the increase in the reduced active form was more dramatic than that observed in the oxidized form.

Table 1. *Effect of drought on total respiration, maximum cytochrome* c*-dependent respiration (O_2 uptake in the presence of 10 mM SHAM) and maximum AOX-dependent respiration rates (O_2 uptake in the presence of 1 mM KCN) in wheat leaves*

Treatment	Total respiration (μmol O_2 uptake h^{-1} g^{-1} DW)	Cyt *c* pathway (μmol O_2 uptake h^{-1} g^{-1} DW)	AOX pathway (μmol O_2 uptake h^{-1} g^{-1} DW)
Well irrigated	59±5	44±5	15±2
Droughted	83±7[a]	53±8	30±4[a]

[a] Significantly different from well-irrigated plants (ANOVA $P \leq 0.05$). The values represent the mean ±SD for five independent experiments.

AOX inhibition

To determine the function of AOX in the protection of photosynthetic electron transport in plants experiencing drought, AOX activity was inhibited by feeding SHAM to intact leaves. *In vivo* AOX activity (i.e. as measured by cyanide-insensitive respiration) was reduced by 30–90%

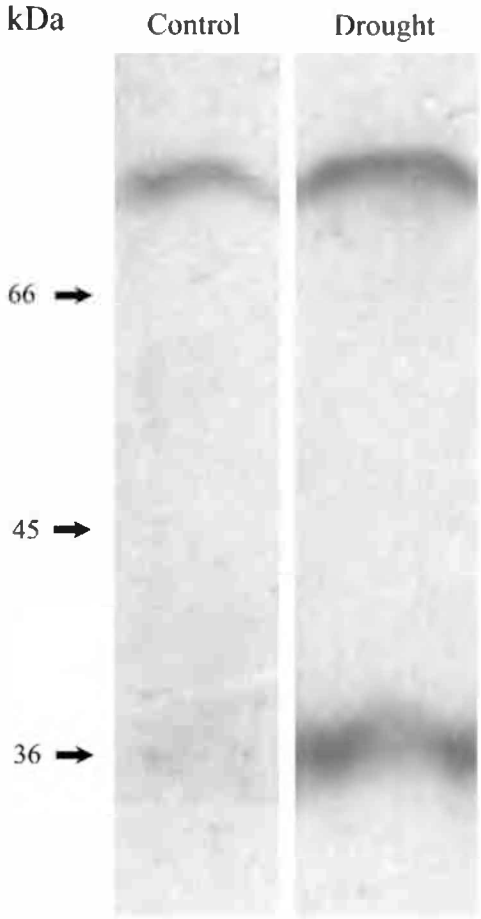

Fig. 1. Western blot analysis of the content of the alternative oxidase (AOX) protein in mitochondria isolated from leaves of well-watered wheat plants compared with plants experiencing drought. Equivalent amounts of mitochondrial proteins were separated by SDS–PAGE in each sample. AOX was detected in immunoblots developed with anti-AOX monoclonal antibodies. Similar results were obtained in three independent experiments. The position of the molecular mass standards is shown on the left side of the figure.

when leaves were fed with SHAM concentrations of 0.1–10 mM (Fig. 2). Since treatment with 1 mM SHAM inhibited cyanide-insensitive oxygen uptake by about 70% in leaves from both well-irrigated and water-stressed plants, this concentration was used in all the subsequent experiments. This concentration is also sufficiently low to avoid the possible side-effects that are observed with higher levels of this AOX inhibitor (Moller *et al.*, 1988, and references therein).

CO_2-dependent O_2 evolution and PSII quantum yield (Φ_{PSII}) were measured simultaneously in isolated intact chloroplasts in the presence of 1 mM SHAM to determine whether this concentration of the inhibitor had any direct effects on photosynthesis (i.e. independent of AOX inhibition). There was no effect of 1 mM SHAM on either Φ_{PSII} (Fig. 3) or CO_2-dependent O_2 evolution (29±7 and 32±5 μmol O_2 mg^{-1} chlorophyll h^{-1}, in the absence or presence of SHAM, respectively), indicating that, at this concentration, SHAM has no direct effects on photosynthesis. Likewise, 1 mM SHAM had no effect on the F_v/F_m ratio of leaves from well-watered plants at any of the light regimes tested (Fig. 4), showing that SHAM did not inhibit PSII activity at the concentrations used in these experiments.

Effect of AOX inhibition on chlorophyll fluorescence under drought conditions

The quantum yield of PSII, and the chlorophyll fluorescence quenching parameters (photochemical and non-photochemical quenching, *qP* and *NPQ*, respectively) were measured in leaves following SHAM treatment. Values for Φ_{PSII} in well-watered plants under low, high, and excess light were 0.68±0.001, 0.52±0.001, and 0.23±0.03, respectively. While SHAM had no statistically significant effect on either Φ_{PSII}, *qP*, or *NPQ* in well-watered plants (data not shown), Φ_{PSII} decreased following the addition of the AOX inhibitor (Fig. 5) in plants subjected to drought. In plants experiencing low light and drought, SHAM caused a decrease in Φ_{PSII} of 21.5%. The effect of SHAM on Φ_{PSII} was even more severe in plants exposed to higher light levels with a 57% and 69% decrease, respectively, at high and excess light (Fig. 5). However, the F_v/F_m ratio was not modified by drought either in the presence or absence of 1 mM SHAM under any of the conditions examined here (data not shown), demonstrating that the observed SHAM-induced decrease in Φ_{PSII} is not due to irreversible inactivation.

Photochemical quenching provides an estimation of the relative proportion of open and oxidized PSII reaction centres (Maxwell and Johnson, 2000). In water-replete plants, *qP* decreased as light intensity increased having values of 0.9±0.006, 0.8±0.01, and 0.66±0.025, respectively, for low, high, and excess irradiance and these levels were unchanged in the presence of SHAM (data not shown). Drought significantly lowered *qP* only at the highest level of irradiance. Moreover, the addition of 1 mM SHAM under these conditions caused further decreases in *qP* values of 12%, 30%, and 67%, respectively, under low, high, and excess light (Fig. 6).

Increasing irradiance enhanced *NPQ* obtained in leaves of well-irrigated plants with values of 0.10±0.03,

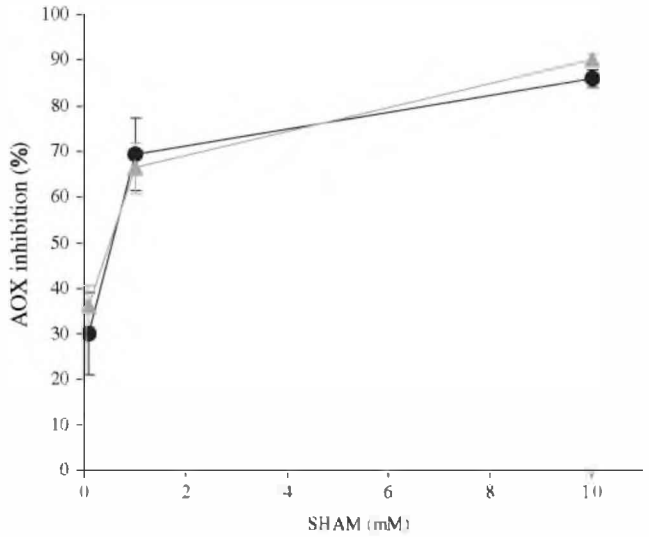

Fig. 2. *In vivo* inhibition of AOX activity in wheat leaves treated with 1 mM KCN and either 0.1, 1.0, or 10 mM SHAM. Circles and triangles correspond to leaves from well-irrigated plants and plants experiencing drought, respectively. Oxygen uptake by leaves treated with 1 mM KCN in the absence of SHAM was denoted as the 100% value for maximal AOX capacity. Each value represents the mean ±SD (vertical bars) of three independent experiments.

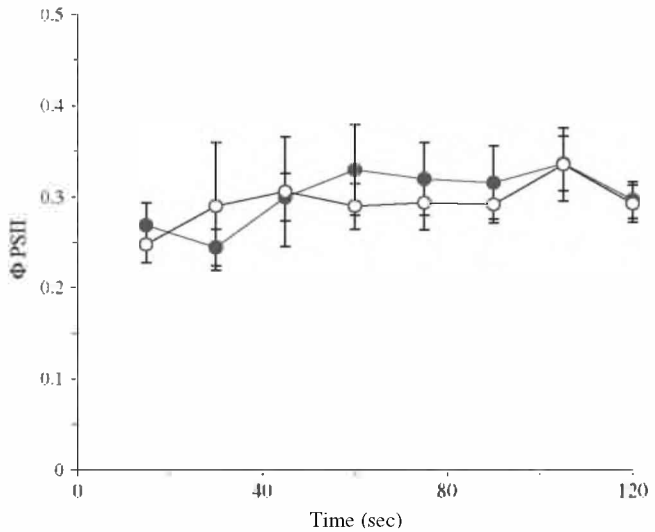

Fig. 3. Effect of SHAM on Φ_{PSII} in chloroplasts isolated from wheat leaves. Closed and open circles represent controls and chloroplasts treated with 1 mM SHAM, respectively. Isolated chloroplasts were resuspended in buffer containing $NaHCO_3$ as a source of CO_2, illuminated at 800 μmol photons m^{-2} s^{-1}. and Φ_{PSII} was recorded over time until it reached a steady state. Vertical bars show the SD.

0.57±0.07, and 0.94±0.09, respectively, obtained at low, high, and excess light. However, *NPQ* was not affected by treatment with 1 mM SHAM. Drought increased thermal energy dissipation under low (4.8-fold), high (3.4-fold), and excess irradiances (100%) relative to well-irrigated plants (Fig. 7). The addition of 1 mM SHAM to plants experiencing drought had no significant effect on *NPQ*, except under low irradiance where it caused a significant increase (50%) of *NPQ* (Fig. 7).

Discussion

Chloroplast/mitochondria interactions

Photosynthesis requires the interaction of chloroplasts with the cytosol and other organelles such as mitochondria to attain optimal rates (Hoefnagel *et al.*, 1998; Padmasree *et al.*, 2002). It is now well established that mitochondrial electron transport is important to optimize photosynthesis (Padmasree and Raghavendra, 1999; Dutilleul *et al.*, 2003; Noctor *et al.*, 2004). In the present study it is shown that, under stressful conditions such as those imposed by drought, the mitochondrial AOX pathway is up-regulated, and that this represents an important response that protects the photosynthetic machinery against the harmful effects of excess energy.

Up-regulation of the alternative oxidase pathway in plants experiencing drought

There are many reports in the literature showing that the AOX pathway is up-regulated in plants subjected to adverse environmental conditions (Purvis and Shewfelt, 1993; Wagner and Krab, 1995; Yip and Vanlerberghe, 2001; Rizhsky *et al.*, 2002). For example, the level of AOX proteins was increased in pea plants subjected to drought (Taylor *et al.*, 2002). A major function of the AOX is to balance the requirements of carbon metabolism and mitochondrial electron transport (Vanlerberghe and McIntosh, 1997). Under stress conditions, the activity of the AOX pathway allows the TCA cycle to continue providing carbon skeletons for metabolism and this is particularly important in the synthesis of compatible solutes (Mckenzie and McIntosh, 1999). It also prevents overreduction of mitochondrial transporters that might otherwise lead to an increase in the production of reactive oxygen species (Millar and Day, 1997; Maxwell *et al.*, 1999). In the experiments reported here respiration was enhanced by drought, the drought-induced increase in oxygen uptake being due mostly to a 2-fold increase in the capacity of the AOX pathway. AOX activity is subject to complex controls (Vanlerberghe and McIntosh, 1997) such that an increase in AOX activity can result from either increased amounts of the protein and/or increased proportions of the enzyme in

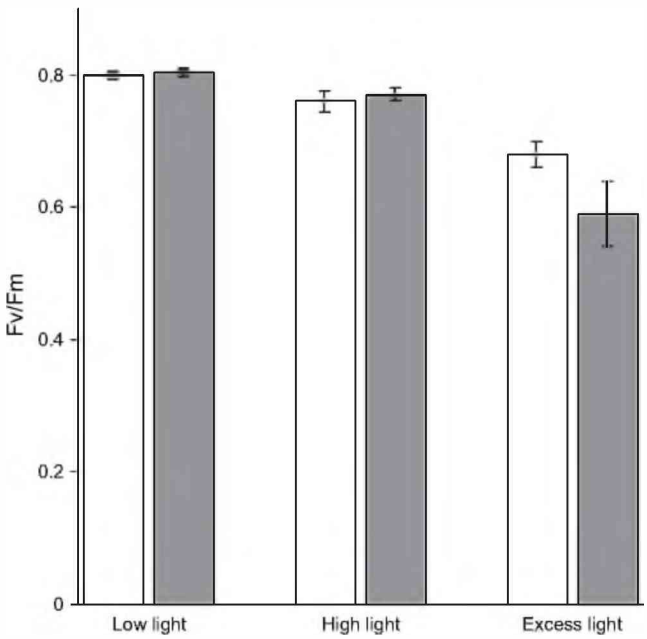

Fig. 4. Effect of SHAM on F_v/F_m in leaves of well-irrigated plants. F_v/F_m was measured in leaves that had been exposed to low, high, and excess light regimes (100, 350, and 800 μmol photons m^{-2} s^{-1}, respectively) for 90 min in the absence (white bars) and presence (grey bars) of 1 mM SHAM. Each value represents the mean ±SD (vertical bars) of four independent experiments.

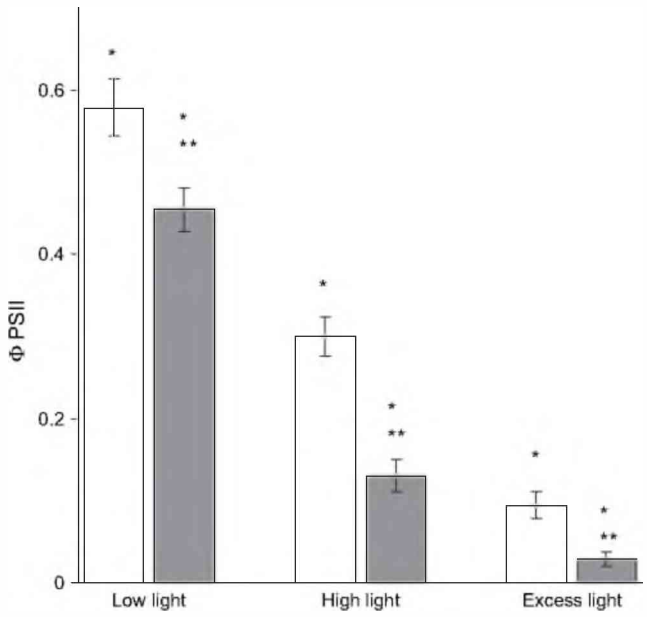

Fig. 5. Changes in the actual quantum yield (Φ_{PSII}) of leaves of wheat plants subjected to drought and treated with SHAM (grey bars). White bars represent leaves experiencing drought incubated without SHAM. One asterisk indicates significant difference from well-watered plants at the same irradiance (ANOVA, $P \leq 0.05$). Two asterisks indicate significant difference from drought-exposed plants in the absence of SHAM, at an identical light regime (ANOVA, $P \leq 0.05$). Each value represents the mean ±SD (vertical bars) of four independent experiments.

the reduced, active form. The results presented here show that both mechanisms are involved in the increase of AOX activity in wheat leaves experiencing drought. Wheat leaves subjected to drought accumulated higher amounts of AOX proteins and contained a higher proportion of the reduced active form than leaves of well-irrigated plants.

AOX activity prevents overreduction of PSII under water deficit

While it is known that the AOX pathway is stress-inducible, its importance in the protection of the photosynthetic apparatus under drought has not been extensively studied to date. The rationale for the experiments reported here was that, if AOX activity contributes to excess energy dissipation, then the inhibition of AOX activity by SHAM would result in an increased proportion of reduced PSII centres resulting in a decrease in Φ_{PSII} and *qP*.

There was no change in the Φ_{PSII}, which is an estimate of the proportion of photons used in photochemistry (Maxwell and Johnson, 2000) or *qP* or *NPQ* when AOX activity was inhibited by SHAM under well-irrigated conditions, indicating that AOX function was not crucial for photosynthetic efficiency under these conditions. However, in plants experiencing drought, the inhibition of AOX activity caused a decrease in Φ_{PSII}, compared with leaves on well-irrigated plants. The SHAM-induced effect on Φ_{PSII} was proportional to the degree of stress applied (i.e. it was higher when drought stress was accompanied by high irradiance). The SHAM-induced decrease of Φ_{PSII} in leaves experiencing drought correlated closely with a decline in *qP*. Since *qP* is an estimate of the proportion of oxidized PSII centres, the observed decrease of *qP* in SHAM-treated leaves reflects an increase in the proportion of reduced PSII centres. These results indicate that AOX activity is important in avoiding overreduction of PSII in the chloroplasts of plants experiencing drought. Thermal energy dissipation mechanisms seem to be operating at near maximum rates in plants exposed to a combination of drought and SHAM, particularly at the higher irradiances used here. However, an intermediate increase in *NPQ* was detected in leaves at low light levels and exposed to drought, while the addition of SHAM further increased thermal dissipation in these circumstances. This suggests that the decrease in AOX activity could be compensated, at least in part, by an increase in *NPQ* under mild stress conditions.

Possible mechanisms involving AOX in protection of photosynthesis under drought

Several mechanisms operate in chloroplasts to maintain a high proportion of oxidized PSII centres and, thereby limit the generation of reactive oxygen species (Niyogi, 1999). However, extra-chloroplastic mechanisms that

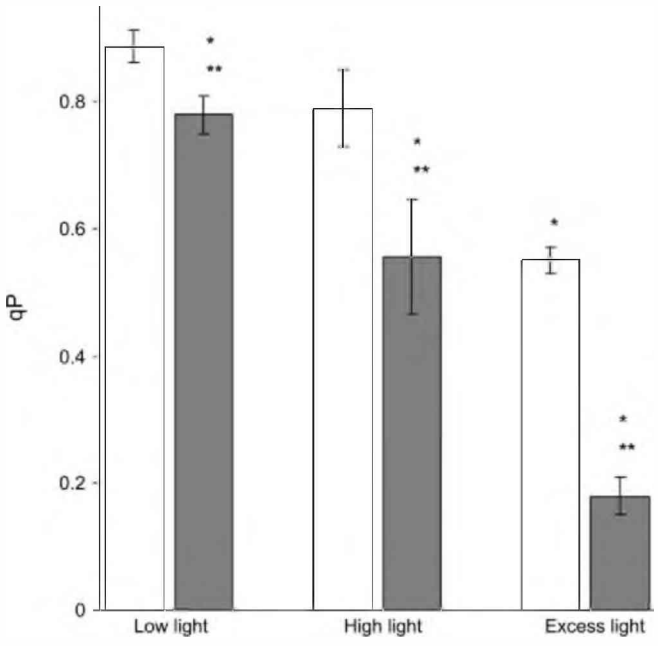

Fig. 6. Changes in the photochemical quenching parameter (*qP*) in leaves of wheat plants subjected to drought and treated with SHAM (grey bars). White bars represent droughted leaves incubated without SHAM. One asterisk indicates significant differences between drought-exposed and well-irrigated plants at the same irradiance (ANOVA. $P \leq 0.05$). Two asterisks indicate significant differences from droughted plants in the absence of SHAM, at an identical light regime (ANOVA. $P \leq 0.05$). Each value represents the mean ±SD (vertical bars) of four independent experiments.

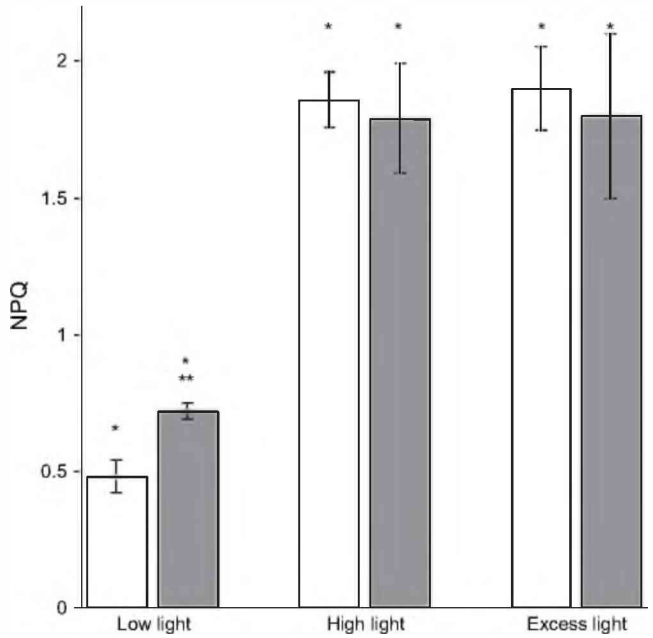

Fig. 7. Changes in non-photochemical quenching (*NPQ*) in leaves of wheat plants subjected to drought and treated with SHAM (grey bars). White bars represent drought-exposed leaves incubated without SHAM. One asterisk indicates significant difference from well-irrigated plants at an identical light regime (ANOVA. $P \leq 0.05$). Two asterisks indicate significant differences from droughted plants in the absence of SHAM, at an identical light regime (ANOVA. $P \leq 0.05$). Each value represents the mean ±SD (vertical bars) of four independent experiments.

remove excess NADPH formed by photosynthesis may be important in preventing photodamage (Hoefnagel *et al.*, 1998, and the results presented here). Dissipation of reducing equivalents produced in the chloroplasts by other organelles requires transport via the malate/oxalacetate shuttle. It is noteworthy that the activity of $NADP^+$-malate dehydrogenase was increased in wheat leaves exposed to drought, suggesting that the malate valve is activated to transport excess reducing equivalents out of chloroplasts in these circumstances (Biehler *et al.*, 1996).

According to the data shown in Fig. 5, the inhibition of AOX activity by 1 mM SHAM produced a decrease in the photosynthetic electron flux of about 19.5 μmol m^{-2} s^{-1}, as calculated by the product of $\Phi_{PSII} \times PAR \times 0.5 \times 0.8$ (Maxwell and Johnson, 2000). These results demonstrate that only 10% of this photosynthetic electron flux reduction might be linked directly to the AOX inhibition. If respiratory rates are similar in darkness and light, data from Table 1 show that the maximum AOX activity of leaves under drought could consume about 8.3 nmol O_2 g^{-1} DW s^{-1}, i.e. 0.25 μmol O_2 m^{-2} s^{-1}, assuming a specific leaf weight of 30 g m^{-2}, or about 1 μmol electrons m^{-2} s^{-1}. Thus, only 5% of the pool of redox equivalents generated by chloroplasts could be directly oxidized by AOX-dependent respiration. Therefore, protection of the photosynthetic apparatus by AOX activity must involve the recruitment of other processes in addition to the direct consumption of reducing power. Inhibition of AOX would also decrease mitochondrial CO_2 release. Under drought conditions it has been demonstrated that the reassimilation of CO_2 released by respiration and photorespiration increases significantly, allowing continued NADPH utilization in the chloroplasts (Haupt-Herting *et al.*, 2001, and references therein). Although the rate of respiratory CO_2 refixation is difficult to estimate, it constitutes an additional NADPH-utilization mechanism. Considering an AOX-dependent CO_2 production of 0.25 μmol m^{-2} s^{-1} and at least four electrons needed for the reduction of each CO_2 molecule, another 5% of the pool of redox equivalents generated by chloroplasts could be oxidized.

In addition to its direct role in sustaining the mitochondrial production of CO_2, AOX activity seems to be necessary for the operation of the photorespiratory cycle. Igamberdiev *et al.* (1997) showed that oxidation of glycine in mitochondria is coupled to the operation of non-phosphorylating pathways such as that of AOX. Glycine oxidation, involving CO_2 release, is an essential step of photorespiration. Photorespiration is an important process preventing the overreduction of chloroplast electron carriers, especially under stress conditions (Osmond and Grace, 1995; Noctor *et al.*, 2002). Therefore, inhibition of AOX activity might also impact on photorespiration and contribute to an overreduction of chloroplast electron carriers and thus a decrease of PSII quantum yield (Noctor *et al.*, 2004).

The results presented here show that the AOX pathway is up-regulated in leaves of plants experiencing drought, and that this up-regulation involves an increase not only in the overall content of the AOX protein but also an elevation of the reduced, active form in particular. *In vivo* inhibition of the AOX pathway in drought-stressed plants with SHAM results in a significant decrease in PSII quantum yield and in the proportion of oxidized, open PSII centres. This implies that AOX activity is important in maintaining photosynthetic electron transport under stress, either through direct consumption of reducing power, through sustained production of CO_2, or by allowing other power-consuming processes (e.g. photorespiration) to operate unabated. In any case, the data presented here show that AOX activity helps plants to cope with excess energy under drought by avoiding the overreduction of chloroplast electron carriers.

Acknowledgements

We are grateful to the Associate Editor who contributed interesting suggestions for the improvement of our work and to Dr Christine Foyer for her careful revision of the manuscript. The authors thank Dr TE Elthon for the gift of AOX antibodies. This work was supported by Fundación Antorchas, CONICET, FONCYT, and the University of Buenos Aires. SP and CGB are career investigators from the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET). JJG is a career investigator from the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires.

References

Atkin OK, Millar AH, Gardeström P, Day DA. 2000. Photosynthesis, carbohydrate metabolism and respiration in leaves of higher plants. In: Leegood RC, Sharkey TD, von Caemmerer S, eds. *Photosynthesis: physiology and metabolism.* Dordrecht: Kluwer Academic Publishers, 153–175.

Bartoli CG, Pastori GM, Foyer CH. 2000. Ascorbate bioshynthesis in mitochondria is linked to the electron transport chain between complexes III and IV. *Plant Physiology* **123,** 335–343.

Biehler K, Migge A, Fock HP. 1996. Gas exchange, chlorophyll fluorescence and malate dehydrogenase activity of *Triticum aestivum* and *Triticum kotschyi* in relation to water stress. *Photosynthetica* **32,** 431–438.

Douce R, Heldt HW. 2000. Photorespiration. In: Leegood RC, Sharkey TD, von Caemmerer S, eds. *Photosynthesis: physiology and metabolism.* Dordrecht: Kluwer Academic Publishers, 115–136.

Dutilleul C, Driscoll S, Cornic G, De Paepe R, Foyer CH, Noctor G. 2003. Functional mitochondrial complex I is required by tobacco leaves for optimal photosynthetic performance in photorespiratory conditions and during transients. *Plant Physiology* **131,** 264–275.

Elthon TE, Nickels RL, McIntosh L. 1989. Monoclonal antibodies to the alternative oxidase of higher plant mitochondria. *Plant Physiology* **89,** 1311–1317.

Foyer CH, Noctor G. 2002. Photosynthetic nitrogen assimilation: interpathway control and signaling. In: Foyer CH, Noctor G, eds. *Advances in photosynthesis and respiration,* Vol. 12. *Photosynthetic nitrogen assimilation and associated carbon and respiratory metabolism.* Dordrecht: Kluwer Academic Publishers, 1–22.

Foyer CH, Trebst A, Noctor G. 2005. Protective and signaling functions of ascorbate, glutathione and tocopherol in chloroplasts. In: Demmig-Adams B, Adams, WW, eds. *Advances in photosynthesis and respiration,* Vol. 19. *Photoprotection, photoinhibition, gene regulation, and environment*. (in press)

Genty B, Briantais JM, Baker NR. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* **990,** 87–92.

Haupt-Herting S, Klug K, Fock HP. 2001. A new approach to measure gross CO_2 fluxes in leaves. Gross CO_2 assimilation, photorespiration, and mitochondrial respiration in the light in tomato under drought stress. *Plant Physiology* **126,** 388–396.

Hedtke B, Wagner I, Börner T, Hess WR. 1999. Inter-organellar crosstalk in higher plants: impaired chloroplast development affects mitochondrial gene and transcript levels. *The Plant Journal* **19,** 635–643.

Hoefnagel MHN, Atkin OK, Wiskich JT. 1998. Interdependence between chloroplasts and mitochondria in the light and the dark. *Biochimica et Biophysica Acta* **1366,** 235–255.

Igamberdiev AU, Bykova NV, Gardeström P. 1997. Involvement of cyanide-resistant and rotenone-insensitive pathways of mitochondrial electron transport during oxidation of glycine in higher plants. *FEBS Letters* **412,** 265–269.

Krömer S. 1995. Respiration during photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **46,** 45–70.

Krömer S, Malmberg G, Gardeström P. 1993. Mitochondrial contribution to photosynthetic metabolism. *Plant Physiology* **102,** 947–955.

Maxwell K, Johnson GN. 2000. Chlorophyll fluorescence: a practical guide. *Journal of Experimental Botany* **51,** 659–668.

Maxwell DP, Wang Y, McIntosh L. 1999. The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proceedings of the National Academy of Sciences, USA* **96,** 8271–8276.

Mckenzie S, McIntosh L. 1999. Higher plant mitochondria. *The Plant Cell* **11,** 571–585.

McCabe TC, Daley D, Whelan J. 2000. Regulatory, developmental and tissue aspects of mitochondrial biogenesis in plants. *Plant Biology* **2,** 121–135.

Millar AH, Day DA. 1997. Alternative solutions to radical problems. *Trends in Plant Science* **2,** 289–290.

Moller IA, Bérczi A, van der Plas LHW, Lambers H. 1988. Measurement of the activity and capacity of the alternative pathway in intact plant tissues: identification of problems and possible solutions. *Physiologia Plantarum* **72,** 642–649.

Moore AL, Whitehouse DG. 1997. Isolation and purification of functionally intact mitochondria and chloroplasts from plant cells. In: Graham JM, Rickwood D, eds. *Subcellular fractionation: a practical approach*. New York: Oxford University Press Inc, 243–270.

Niyogi KK. 1999. Photoprotection revisited: genetic and molecular approaches. *Annual Review of Plant Physiology and Plant Molecular Biology* **50,** 333–359.

Niyogi KK. 2000. Safety valves for photosynthesis. *Current Opinion in Plant Biology* **3,** 455–460.

Noctor G, Veljovic-Jovanovic S, Driscoll S, Novitskaya L, Foyer CH. 2002. Drought and oxidative load in the leaves of C_3 plants: a predominant role for photorespiration? *Annals of Botany* **89,** 841–850.

Noctor G, Dutilleul C, De Paepe R, Foyer CH. 2004. Use of mitochondrial electron transport mutants to evaluate the effects of redox state on photosynthesis, stress tolerance and the integration of carbon/nitrogen metabolism. *Journal of Experimental Botany* **55,** 49–57.

Osmond CB, Grace SC. 1995. Perspectives on photoinhibition and photorespiration in the field: quintessential inefficiencies of the light and dark reactions of photosynthesis? *Journal of Experimental Botany* **46,** 1351–1362.

Padmasree K, Padmavathi L, Raghavendra AS. 2002. Essentiality of mitochondrial oxidative metabolism for photosynthesis: optimization of carbon assimilation and protection against photoinhibition. *Critical Reviews in Biochemistry and Molecular Biology* **37,** 71–119.

Padmasree K, Raghavendra AS. 1999. Importance of oxidative electron transport over oxidative phosphorylation in optimizing photosynthesis in mesophyll protoplasts of pea (*Pisum sativum* L.). *Physiologia Plantarum* **105,** 546–553.

Purvis AC, Shewfelt RL. 1993. Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? *Physiologia Plantarum* **88,** 712–718.

Rizhsky L, Liang H, Mittler R. 2002. The combined effect of drought stress and heat shock on gene expression in tobacco. *Plant Physiology* **130,** 1143–1151.

Svensson AS, Rasmusson AG. 2001. Light-dependent gene expression for proteins in the respiratory chain of potato leaves. *The Plant Journal* **28,** 73–82.

Tambussi EA, Bartoli CG, Beltrano J, Guiamet JJ, Araus JL. 2000. Oxidative damage to thylakoid proteins in water-stressed leaves of wheat (*Triticum aestivum*). *Physiologia Plantarum* **108,** 398–404.

Taylor NL, Day DA, Millar AH. 2002. Environmental stress causes oxidative damage to plant mitochondria leading to inhibition to glycine decarboxylase. *Journal of Biological Chemistry* **277,** 42663–42668.

Tiwari BS, Belenghi B, Levine A. 2002. Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death. *Plant Physiology* **128,** 1271–1281.

Vanlerberghe GC, McIntosh L. 1997. Alternative oxidase: from gene to function. *Annual Review of Plant Physiology and Plant Molecular Biology* **48,** 703–734.

Wagner AM, Krab K. 1995. The alternative respiration pathway in plants: role and regulation. *Physiologia Plantarum* **95,** 318–325.

Yip JYH, Vanlerberghe GC. 2001. Mitochondrial alternative oxidase acts to dampen the generation of active oxygen species during a period of rapid respiration induced to support a high rate of nutrient uptake. *Physiologia Plantarum* **112,** 327–333.