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Possible plant mitochondria involvement in cell adaptation to drought stress

A case study: durum wheat mitochondria

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Abstract

Although plant cell bioenergetics is strongly affected by abiotic stresses, mitochondrial metabolism under stress is still largely unknown. Interestingly, plant mitochondria may control reactive oxygen species (ROS) generation by means of energy-dissipating systems. Therefore, mitochondria may play a central role in cell adaptation to abiotic stresses, which are known to induce oxidative stress at cellular level. With this in mind, in recent years, studies have been focused on mitochondria from durum wheat, a species well adapted to drought stress. Durum wheat mitochondria possess three energy-dissipating systems: the ATP-sensitive plant mitochondrial potassium channel (PmitoK$_{ATP}$); the plant uncoupling protein (PUCP); and the alternative oxidase (AOX). It has been shown that these systems are able to dampen mitochondrial ROS production; surprisingly, PmitoK$_{ATP}$ and PUCP (but not AOX) are activated by ROS. This was found to occur in mitochondria from both control and hyperosmotic-stressed seedlings. Therefore, the hypothesis of a 'feed-back' mechanism operating under hyperosmotic/oxidative stress conditions was validated: stress conditions induce an increase in mitochondrial ROS production; ROS activate PmitoK$_{ATP}$ and PUCP that, in turn, dissipate the mitochondrial membrane potential, thus inhibiting further large-scale ROS production. Another important aspect is the chloroplast/cytosol/mitochondrion co-operation in green tissues under stress conditions aimed at modulating cell redox homeostasis. Durum wheat mitochondria may act against chloroplast/cytosol over-reduction: the malate/oxaloacetate antiporter and the rotenone-insensitive external NAD(P)H dehydrogenases allow cytosolic NAD(P)H oxidation; under stress this may occur without high ROS production due to co-operation with AOX, which is activated by intermediates of the photorespiratory cycle.

Key words: Alternative oxidase, drought stress, durum wheat, oxidative stress, plant mitochondria, potassium channel, salt stress, uncoupling protein.

Introduction

It is known that plant mitochondria are involved in some metabolic processes implicated in cell adaptation to abiotic stresses. Mitochondria interact with chloroplasts and peroxisomes in the photorespiratory cycle; this allows excess

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Abbreviations: $\Delta\Psi$, electrical membrane potential; AOX, alternative oxidase; DWM, durum wheat mitochondria; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FFAs, free fatty acids; MAL/OAA, malate/oxaloacetate; MDH, malate dehydrogenase; ME, malic enzyme; NAD(P)H DHext, rotenone-insensitive external NAD(P)H dehydrogenases; NAD(P)H DHin, rotenone-insensitive internal NAD(P)H dehydrogenases; O_2^-, superoxide anion; PmitoK$_{ATP}$, ATP-sensitive plant mitochondrial potassium channel; PUCP, plant uncoupling protein; ROS, reactive oxygen species; SHAM, salicylhydroxamate.

reducing equivalents produced during photosynthesis under conditions of restricted Calvin cycle to be eliminated, thus preventing an over-reduction of the carriers of photosynthetic electron transport (Krömer, 1995). Mitochondria are implicated in the metabolism of proline, which accumulates under hyperosmotic stress conditions as an osmoprotectant, being the first step of its oxidation catalysed by proline dehydrogenase, an inner mitochondrial membrane enzyme (Kiyosue *et al*., 1996, and references therein). Moreover, respiration controls synthesis of ascorbate, a powerful antioxidant playing a key role in reactive oxygen species (ROS) detoxification; this occurs by regulating the activity of L-galactono-γ-lactone dehydrogenase, a mitochondrial enzyme which catalyses the last step of ascorbate synthesis (Millar *et al*., 2003). It has also been reported recently that leaf mitochondria may play a central role in counteracting environmental stresses by modulating cell redox homeostasis and by setting antioxidant capacity (Dutilleul *et al*., 2003). Plant mitochondria may also sense cellular stress early and regulate programmed cell death (Jones, 2000; Rhoads *et al*., 2006). Furthermore, mitochondria may play a major role in inter-organelle cross-talk under environmental/oxidative stresses by signalling with chloroplasts (Millar *et al*., 2001) and by inducing altered nuclear gene expression through mitochondria-to-nucleus signalling, which is referred to as mitochondrial retrograde regulation (Rhoads *et al*., 2006).

A key role of mitochondria, that is receiving increasing interest, is their ability to defend themselves (and the cell) from an excess of ROS. In fact, in the plant cell, mitochondria represent a major source of ROS production and consequent oxidative damage, as indicated by proteomic studies (Sweetlove *et al*., 2002; Bartoli *et al*., 2004; Taylor *et al*., 2005). This occurs under abiotic stresses, drought and salinity in particular (Alscher *et al*., 1997), thus suggesting that high tolerance of plants to environmental stresses may be associated with efficient defence against oxidative stress at cellular and subcellular levels. To do this, plant mitochondria display three different strategies (Møller, 2001): the first line of defence is the avoidance of ROS production, achieved by keeping the electron transport chain adequately oxidized, while the second and the third ones are ROS detoxification and the repair of ROS-mediated damages, respectively. Since the last two lines of defence require continuous consumption and regeneration of small antioxidant molecules, such as ascorbate, glutathione, and NADPH, the avoidance of ROS production may appear more advantageous for mitochondria.

These topics have been investigated by many research groups through the classical biochemical techniques using isolated mitochondria, as well as by more sophisticated approaches (gene expression, proteomics, transgenic plants, isotope fractionation, '*in vivo*' microscopy) applied to organelles and '*in vivo*' systems from cultured cells to whole plants. Commonly studied species are *Arabidopsis*, a useful genetic model, together with tobacco, pea, potato, tomato, and cereals. In the last few years, studies have been focused on the first line of defence against oxidative stress, the avoidance of ROS production, which is still largely unknown. In particular, bioenergetics of mitochondria purified from durum wheat, a species widely cultivated in semi-arid regions of the Mediterranean area and well adapted to drought and thus representing a useful model system, have been studied (Flagella *et al*., 1998, and references therein). Durum wheat mitochondria (DWM) possess three active energy-dissipating systems, namely the ATP-sensitive plant mitochondrial potassium channel ($PmitoK_{ATP}$) (Pastore *et al*., 1999*a*), the plant uncoupling protein (PUCP) (Pastore *et al*., 2000), and the alternative oxidase (AOX) (Pastore *et al*., 2001), all three able to control ROS production. To date nothing is known about the possible existence in DWM of the cold shock protein, CSP 310, acting as a non-phosphorylating bypass of the respiratory chain in some cereals (Kolesnichenko *et al*., 2005, and references therein).

DWM also possess the rotenone-insensitive external NAD(P)H dehydrogenases [NAD(P)H DHext] and a very active malate/oxaloacetate (MAL/OAA) shuttle, both allowing cytosolic NAD(P)H oxidation (Pastore *et al*., 2003). They may co-operate with the AOX thus protecting the chloroplast/cytosol from over-reduction by oxidizing excess NAD(P)H without high ROS production (Pastore *et al*., 2001). The effect of oxidative/abiotic stresses on the activity of the three dissipating systems and on the electrical membrane potential ($\Delta\Psi$) generation by DWM was checked. This was done by using '*in vitro*' isolated mitochondria treated with ROS (Pastore *et al*., 1999*a*, 2000, 2001, 2002) and mitochondria purified from seedlings subjected to mannitol or NaCl stress (Trono *et al*., 2004; Flagella *et al*., 2006).

Here, recent papers concerning mitochondria from durum wheat and the related literature are reviewed to infer the possible involvement of plant mitochondria in adaptation to drought-induced oxidative stress. Among the three energy-dissipating systems, the focus is particularly on the potassium channel; in fact, while the AOX and, more recently, the PUCP have been heavily studied by several groups using different plant systems, to date fewer than 10 papers in the literature deal with the potassium channel in plant mitochondria. So, this channel is certainly not well-known to many scientists in this field and, so far, it has very often been forgotten in the papers about plant mitochondria and stress.

Dissipating systems in DWM and their effect on $\Delta\Psi$ and ROS production

$PmitoK_{ATP}$

The existence of a potassium channel inhibited by ATP in plant mitochondria was first shown by using DWM

(Pastore *et al*., 1999*a*). This channel was named PmitoK$_{ATP}$ in analogy with the animal counterpart (mitoK$_{ATP}$; Paucek *et al*., 1992). The main characteristics of this channel are summarized in Table 1. Notably, ATP inhibits this channel with at least 10-fold lower efficiency than the mammalian counterpart (Garlid, 1996); moreover Mg^{2+}, which inhibits the mammalian channel, is ineffective in DWM. Some variability of data reported in Table 1 may be observed depending on the stock of seeds and the conditions of seedling growth.

The plant species in which the existence of a potassium uniport has been reported to date are listed in Table 2. Only the channel of pea is well characterized and shows some analogies with durum wheat (DW)-mitoK$_{ATP}$, as, for example, the inhibition by ATP (Petrussa *et al*., 2001; Chiandussi *et al*., 2002; Casolo *et al*., 2003); on the contrary, potato, tomato, and maize mitochondria were found to show an ATP-insensitive potassium import pathway (Ruy *et al*., 2004).

In DWM, PmitoK$_{ATP}$ catalyses the electrophoretic uniport of potassium through the inner mitochondrial membrane; the co-operation between PmitoK$_{ATP}$ and the K^+/H^+ antiporter, very active in plant mitochondria (Diolez and Moreau, 1985), allows the operation of a potassium cycle. In the course of substrate oxidation, protons are ejected in the intermembrane space by complexes I, III, and IV of the respiratory chain, thus generating the protonmotive force, which drives ATP synthesis; the potassium cycle causes re-entry of protons in the mitochondrial matrix, thus potentially uncoupling mitochondria (Fig. 1A). While in mammalian mitochondria this uncoupling is negligible due to the low activity of the cycle (Garlid and Paucek, 2003), the capacity of the potassium cycle in DWM is so high as to completely collapse $\Delta\Psi$ (Pastore *et al*., 1999*a*), the main component of protonmotive force in plant mitochondria (Douce *et al*., 1987). This is clearly shown in Fig. 2A, in which $\Delta\Psi$ of DWM was measured by using the fluorimetric probe safranin 'O' (Pastore *et al*., 1996). When succinate (5 mM), a respiratory substrate, was added to DWM, a rapid and large $\Delta\Psi$ was generated; this was largely decreased by 25 mM KCl plus 10 μM diazoxide, a potassium channel opener (Table 1), while 0.2 mM ATP restored $\Delta\Psi$. Finally, the artificial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) completely collapsed $\Delta\Psi$. In a previous set of experiments, the depolarization induced by KCl was even faster, although obtained without diazoxide (Pastore *et al*., 1999*a*), thus showing that the channel is able to depolarize DWM at different rates in different mitochondrial preparations. Here is shown an experiment where the channel is less active in order to highlight the sensitivity to diazoxide and

Table 1. *Characteristics of PmitoK$_{ATP}$ in DWM*

Activators and inhibitors of durum wheat (DW)-mitoK$_{ATP}$ are listed. Activation and inhibition are reported as increase or decrease with respect to the control activity, taken equal to 100. Some ineffective compounds and other properties of this channel are also reported. Data are from Pastore *et al*. (1999*a*).

Compound	Effect
ATP (1 mM)[a]	Inhibition (–79±7.5%)[c], K_i=290 μM
ADP (1 mM)[a]	Inhibition (–38±2.9%)
ADP plus Pi (1+1 mM)[a]	Inhibition (–48±5.8%)
NADH (1 mM)[a]	Inhibition (–74±8.2%), K_i=390 μM
Zn^{2+} (400 μM)[b]	Inhibition (–46±12.2%)
Diazoxide (10 μM)[a,b]	Activation (counteracts ATP inhibition)
GTP (1 mM)[a,b]	Activation (counteracts ATP inhibition)
PalmitoylCoA (1 μM)[b]	Activation (+140±29.8%)
Coenzyme A (100 μM)[a,b]	Activation (+102±28.1%)
Mersalyl (1 mM)[b]	Activation (+132±16.4%)
N-Ethylmaleimide (30 nmol mg^{-1})[b]	Activation (+86±19.1%)
Compounds having an effect on other potassium channels but not on DW-mitoK$_{ATP}$	
Mg^{2+} (0.1 and 5 mM)[a,b,d,e], Al^{3+} (50 μM)[a], Ba^{2+} (1 mM)[a], TEA^+ (10 mM)[a,e], Ca^{2+} (1–3 μM)[a], glyburide (5–10 μM)[a,b,f], 4-aminopyridine (5 mM)[a,b], AMP (1 mM)[a], phosphate (1 mM)[a], EGTA (10 mM)[a,e], NAD^+ (1 mM)[a]	
Other properties	
Specificity: $Cs^+>K^+=Rb^+>Na^+=Li^+$ [b]	
ΔΨ dependence: independent in the 90–140 mV and 175–185 mV ranges; linearly dependent in the 140–175 mV range	
V_{max}=12.5±1.96 mV s^{-1}[g]	
K_m=2.2±0.78 mM[g]	

[a] Evaluated by ΔΨ experiments.
[b] Evaluated by swelling experiments.
[c] Mean value ±SE (n=3 experiments).
[d] Mg^{2+} was tested in both the absence and presence of 1 mM ATP.
[e] Osmolarity of the medium was kept constant by acting on mannitol concentration.
[f] Glyburide was tested in both the absence and presence of Mg^{2+} (0.1 and 0.5 mM) plus ATP (1 mM) plus either diazoxide (10 μM) or GTP (1 mM).
[g] Represent V_{max} and K_m values relative to the hyperbolic dependence of the rate of K^+-induced ΔΨ decrease on K^+ concentration.

Table 2. *Plant species showing a protein-mediated mitochondrial K^+ uniport*

Plant species	References
Durum wheat (*Triticum durum* Desf.)[a,b]	Pastore *et al*. (1999*a*)
Bread wheat (*Triticum aestivum* L.)[a,b]	Pastore *et al*. (1999*a*)
Spelt (*Triticum dicoccum* Schübler)[a,b]	Pastore *et al*. (1999*a*)
Rye (*Secale cereale* L.)[a,b]	Pastore *et al*. (1999*a*)
Barley (*Hordeum vulgare* L.)[a,b]	Pastore *et al*. (1999*a*)
Spinach (*Spinacea oleracea* L.)[a,b]	Pastore *et al*. (1999*a*)
Potato (*Solanum tuberosum* L.)[a,b]	Pastore *et al*. (1999*a*), Ruy *et al*. (2004)
Pea (*Pisum sativum* L.)[a,b]	Petrussa *et al*. (2001, 2004), Chiandussi *et al*. (2002), Casolo *et al*. (2003), D Pastore, D Trono, MN Laus (unpublished data)
Soybean (*Glycine max* L.)[a,b]	Casolo *et al*. (2005)
Maize (*Zea mays* L.)[b]	Ruy *et al*. (2004), D Pastore, D Trono, MN Laus (unpublished data)
Tomato (*Lycopersicon esculentum* Mill.)[b]	Ruy *et al*. (2004)
Topinambur (*Helianthus tuberosus* L.)[b]	D Pastore, D Trono, MN Laus (unpublished data)
Triticale (*Triticum*×*Secale*)[b]	D Pastore, D Trono, MN Laus (unpublished data)
Lentil (*Lens esculenta* Moench.)[b]	D Pastore, D Trono, MN Laus (unpublished data)

[a] Activity evaluated by $\Delta\Psi$ experiments.
[b] Activity evaluated by swelling experiments.

ATP better and unambiguously to identify the operation of the PmitoK$_{ATP}$ as uncoupling mode. It should be reported that ΔpH, the other component of protonmotive force, is negligible in succinate-respiring DWM (about 0.03 units), as expected in the light of the high activity of the K^+/H^+ antiporter (D Pastore, D Trono, MN Laus, unpublished data). Therefore, PmitoK$_{ATP}$ is able to completely collapse $\Delta\Psi$ (and protonmotive force) in '*in vitro*' isolated DWM, whereas the degree of $\Delta\Psi$ decrease '*in vivo*' should be dependent on the balance of modulators under different physiological conditions, as proposed later.

PUCP

The existence of an uncoupling protein in plant mitochondria was described for the first time in potato (Vercesi *et al*., 1995) and it is now demonstrated in a variety of organs and tissues of higher plant species including monocots and dicots, and C_3, C_4, and CAM plants (Ježek *et al*., 2000). cDNAs for uncoupling proteins were obtained from potato, *Arabidopsis*, bread wheat, and skunk cabbage (references given in Hanák and Ježek, 2001), and from rice (Watanabe and Hirai, 2002). Current knowledge of PUCPs including biochemical properties, distribution, and regulation of gene expression, as well as gene family and evolutionary aspects, have been reviewed recently by Vercesi *et al*. (2006).

It has been shown that DWM possess a very active PUCP (Pastore *et al*., 2000). With the notable exception of UCP1 in brown adipose tissue (Klingenberg and Echtay, 2001), the PUCP in DWM is probably the most active uncoupling protein so far described among animal and plant mitochondria. In Table 3 the main characteristics of DW-UCP are summarized. ATP is the main inhibitor, while GDP and GTP are less effective.

In the presence of free fatty acids (FFAs), PUCP catalyses re-entry of protons in the course of substrate oxidation, so allowing $\Delta\Psi$ to decrease in plant mitochondria (Fig. 1B). The mechanism of activation of UCPs by FFAs has been one of the most debated issues over the years, but it is beyond the scope of this paper (for further elucidation see the following reviews: Skulachev, 1998; Klingenberg and Echtay, 2001; Vercesi *et al*., 2006). In particular, as shown in Fig. 2B, DW-UCP is so active it quickly and completely collapses $\Delta\Psi$ of succinate-respiring mitochondria, even at low FFA concentration (8 μM linoleic acid in this experiment). ATP (0.5 mM) induced a large recoupling. FCCP (1 μM) completely collapsed $\Delta\Psi$ at high rate. It should be mentioned that the ATP/ADP antiporter may also significantly uncouple mitochondria in the presence of FFAs (Skulachev, 1998, and references therein), but its contribution was inhibited by including atractyloside in the reaction medium (Trono *et al*., 2006). Also in this case, even though PUCP is able to completely collapse $\Delta\Psi$ of DWM '*in vitro*', the activity '*in vivo*' should be dependent on physiological conditions (see below).

AOX

Plant mitochondria possess an AOX showing a quinol-oxidizing activity; it branches from the respiratory chain at the level of ubiquinone and it is known to be insensitive to cyanide and antimycin A, inhibitors of cytochrome *c* oxidase. The AOX bypasses the last two sites of energy conservation associated with the cytochrome pathway (complexes III and IV; compare A and B with C in Fig. 1), showing a non-phosphorylating and, consequently, an energy-dissipating character (for excellent reviews, Moore and Siedow, 1991; Vanlerberghe and McIntosh, 1997). In this case, non-coupled rather than uncoupled respiration takes place (Skulachev, 1998).

At present, the physiological role of the AOX has been well established only in specialized thermogenic plant tissues (Meeuse, 1975). The AOX function in non-thermogenic plants has not been completely understood, but it is commonly accepted that the AOX energy-dissipating character can play a protective role under biotic and abiotic stress by lowering mitochondrial ROS generation in isolated mitochondria, cultured cells (Vanlerberghe and McIntosh, 1997; Maxwell *et al*., 1999, and references therein), and the whole plant (Umbach

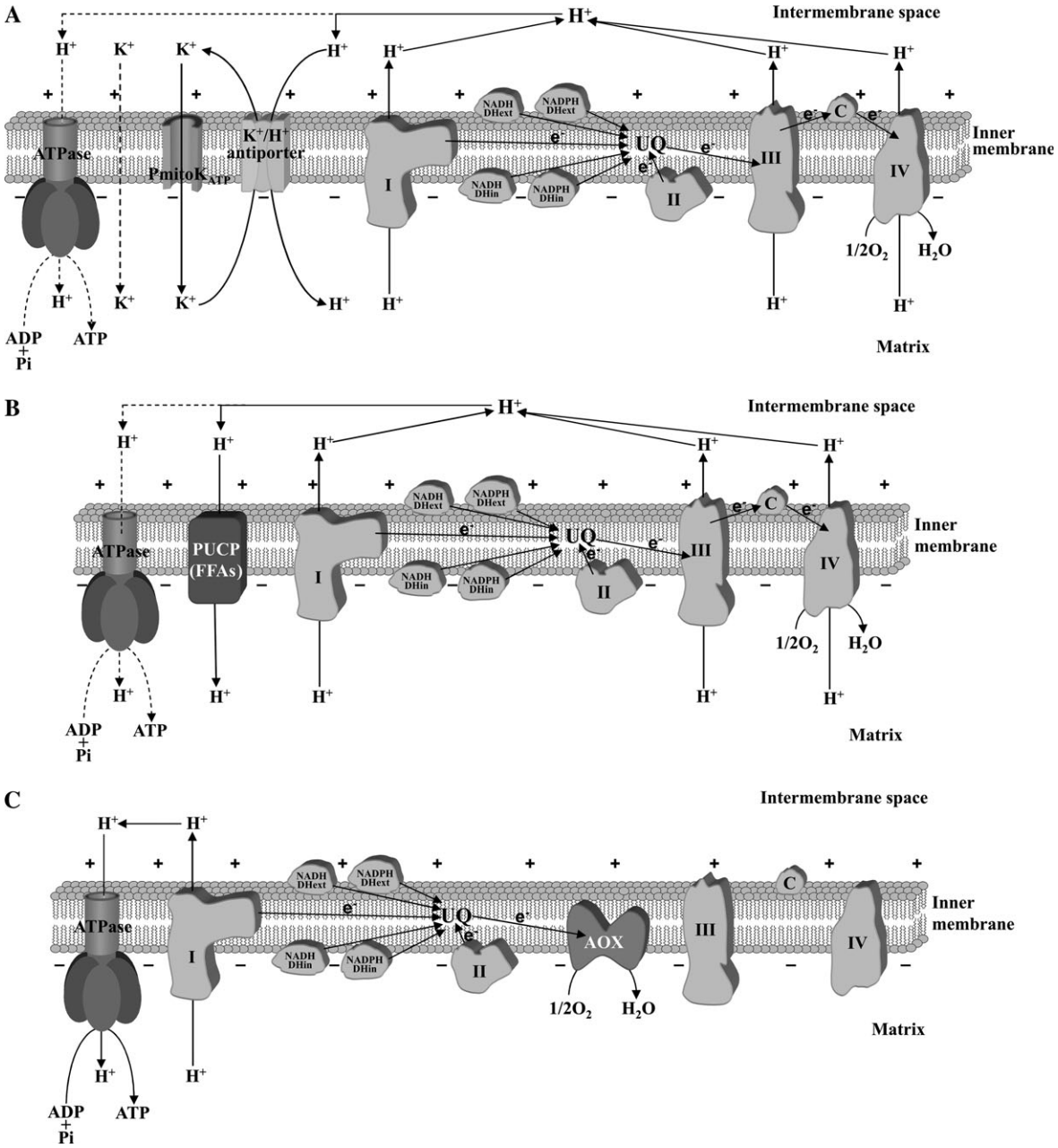

Fig. 1. Mechanisms of DWM uncoupling due to either PmitoK$_{ATP}$ (A) or PUCP (B) and of non-coupling due to AOX (C). Abbreviations: UQ, ubiquinone; C, cytochrome *c*; e^-, electrons or other reducing equivalents. The electron transport chain of plant mitochondria is shown. In addition to respiratory complexes I–IV, four rotenone-insensitive non-proton-pumping NAD(P)H DHs are reported: two of them are localized on the outer side of the inner membrane [NAD(P)H DHext] and two on the matrix side [NAD(P)H DHin], two oxidize specifically NADH and two NADPH. The plant electron transport chain also contains a non-proton-pumping AOX, which catalyses electron transfer from ubiquinol to molecular oxygen. Reducing equivalent flux through the respiratory complexes to molecular oxygen is indicated, together with the proton ejection into the intermembrane space by complexes I, III, and IV, which generates a proton electrochemical gradient. This protonmotive force consists of two components: the electrical membrane potential or $\Delta\Psi$ (+ and − signs in the figure), which is the main component of protonmotive force in plant mitochondria, and the proton gradient or ΔpH, which is even negligible in DWM. Proton re-entry into the matrix via the ATPase drives ATP synthesis. The three energy-dissipating systems may lower $\Delta\Psi$ in three different ways. (A) PmitoK$_{ATP}$ catalyses the electrophoretic K^+ uptake across the inner membrane towards the matrix. When K^+ uptake via PmitoK$_{ATP}$ is associated with a K^+ efflux through the K^+/H^+ antiporter, a very active K^+ cycle, that allows proton re-entry into the matrix, is generated. K^+ influx by means of diffusive leak is also shown. (B) In the presence of FFAs, PUCP mediates proton re-entry into the matrix. (C) The electron transport through AOX bypasses complexes III and IV, two of the three sites of energy conservation, so less ATP is synthesized in the course of substrate oxidation, with consequent 'non-coupling'.

et al., 2005). The analysis of extensive mRNA expression data in *Arabidopsis* indicates that five *Aox* genes are expressed, but organ and development regulation are evident, suggesting regulatory specialization of *Aox* gene members (Clifton *et al.*, 2006). Interestingly, a microarray study using an *Arabidopsis* AtAOX1a anti-sense line

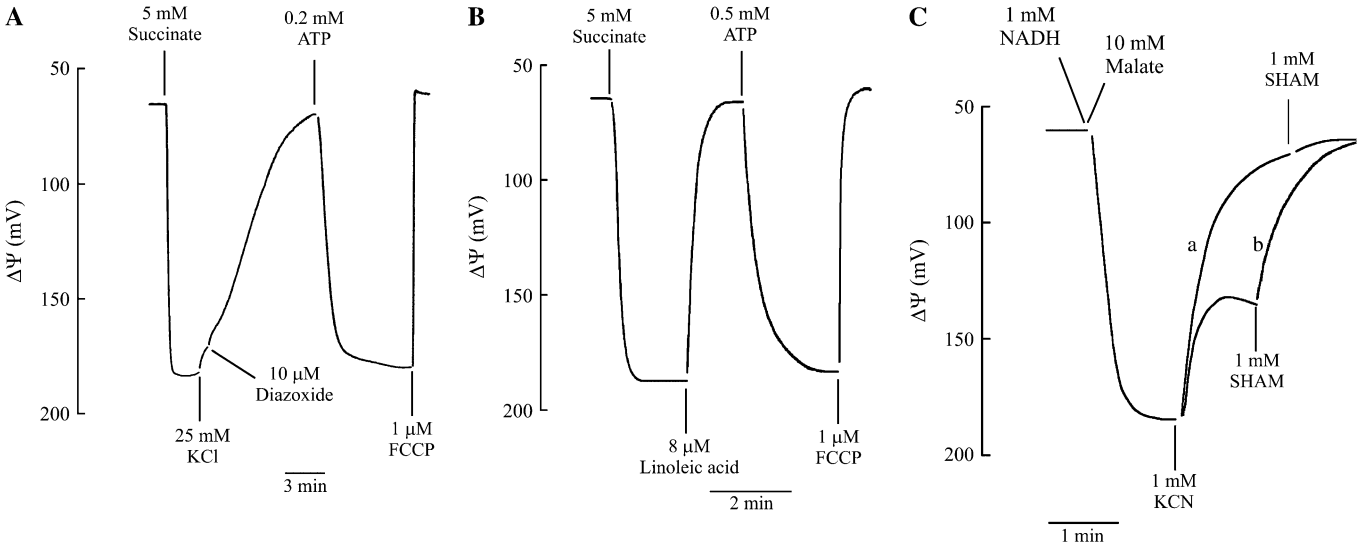

Fig. 2. Effect of PmitoK$_{ATP}$ (A), PUCP (B), and AOX (C) on ΔΨ in DWM. ΔΨ measurements were carried out by monitoring safranin 'O' fluorescence as a function of time; calibration of fluorescence changes versus ΔΨwas carried out as in Pastore *et al*. (1996). (A) DWM (0.2 mg protein) were suspended in 2.0 ml of a medium consisting of 0.3 M mannitol, 20 mM TRIS-HCl pH 7.20, 5 mM $MgCl_2$, 2.5 μM safranin, 2 μg oligomycin, and 30 μM P_1, P_5, di(adenosine-5′)pentaphosphate, inhibitors of ATPase and adenylate kinase, respectively. (B) DWM (0.2 mg protein) were suspended in 2.0 ml of a medium consisting of 0.125 M mannitol, 65 mM NaCl, 2.5 mM Na-phosphate, 0.33 mM EGTA, 10 μM atractyloside (inhibitor of the ADP/ATP carrier), 2 μg oligomycin, 10 mM TRIS-HCl pH 7.20, 2.5 μM safranin. (C) Trace a, DWM (0.2 mg protein) were suspended in 2.0 ml of a medium consisting of 0.27 M mannitol, 20 mM TRIS-HCl pH 7.20, 5 mM $MgCl_2$, 10 mM EGTA, and 10 mM EDTA (in order to inhibit NADH oxidation via the Ca^{2+}-dependent NADH DHext), 0.1% (w/v) defatted BSA, 0.5 EU MDH (in order to allow the MAL/OAA shuttle), 2.5 μM safranin. Trace b, the reaction medium also contained 1 mM pyruvate. All the additions were carried out at the time indicated.

Table 3. *Characteristics of PUCP in DWM*

Inhibitors and other properties of DW-UCP are reported. Data are from Pastore *et al*. (2000).

Inhibitor	Effect
ATP (0.5 mM)[a]	Inhibition (–71%)[b]
Mg^{2+} (5 mM)[a]	Inhibition (–43%)[b]
BSA (0.1–1% w/v)[a]	Complete inhibition
GTP (70 μM)[a]	Inhibition (ΔΨ recovery of 20 mV)[c]
GDP (70 μM)[a]	Inhibition (ΔΨ recovery of 40 mV)[c]
Other properties	
Fatty acid specificity: Palmitoleic>linoleic>γ-linolenic>lauric>palmitic>stearic>phenylvaleric=undecanesulphonic[d] ΔΨ dependence: Slow increase of the linoleic acid-induced ΔΨ decrease rate in 65–125 mV and 135–160 mV ranges; fast increase in 125–135 mV range $K_{0.5}$=16 μM[e,f]	

[a] Evaluated by ΔΨ experiments.
[b] Calculated by inhibition of the ΔΨ decrease rate induced by 12 μM linoleic acid.
[c] Calculated by reversion of ΔΨ decrease induced by 8 μM linoleic acid.
[d] Evaluated on the basis of the ΔΨ decrease rate induced by different fatty acids (12 μM).
[e] Represents $K_{0.5}$ value relative to the sigmoidal dependence of the linoleic acid-induced ΔΨ decrease rate on increasing linoleic acid concentration.
[f] Measurements were carried out in the presence of 0.5 mM ATP in order to lower the rate of ΔΨ decrease.

shows AOX influences outside mitochondria, particularly in chloroplasts and on several carbon metabolism pathways (Umbach *et al*., 2005). Also, analysis of genes co-expressed with *Aox*s from studies of responses to various treatments altering mitochondrial functions and/or from plants with altered AOX levels reveals that this gene set encodes more functions outside the mitochondrion than functions in mitochondria, thus showing a role in re-programming cellular metabolism in response to the ever-changing environment encountered by plants (Clifton *et al*., 2006).

In the past, it was reported that DWM do not show a cyanide-insensitive alternative pathway (Goldstein *et al*., 1980). This point was reinvestigated and it was shown

that AOX activity may be elicited in DWM by using suitable activators (Pastore *et al*., 1999*b*, 2001). In particular, DW-AOX is mainly activated by glyoxylate and hydroxypyruvate, two intermediates of the photorespiratory cycle, while pyruvate, a typical activator of AOXs, is less effective (Table 4). Other intermediates of the photorespiratory cycle, such as glycine, glycolate, phosphoglycolate, and serine, as well as 2-oxoglutarate, activators of other AOXs (Millar *et al*., 1996), are ineffective on DW-AOX. The effect of AOX functioning on $\Delta\Psi$ is shown in Fig. 2C. The reaction was started by treating DWM with 10 mM malate plus 1 mM NADH, thus inducing a high $\Delta\Psi$ value. EGTA and EDTA were also present in the reaction medium to inhibit NADH oxidation via the Ca^{2+}-dependent rotenone-insensitive external NADH dehydrogenase (NADH DHext). Since DWM cannot freely oxidize malate via the mitochondrial NADH-dependent malic enzyme (ME), malate dehydrogenase (MDH) was also added to the reaction medium in order to allow $\Delta\Psi$ generation via the MAL/OAA shuttle (Pastore *et al*., 2001, 2003). The addition of cyanide caused an almost complete depolarization (Fig. 2C, trace a), which was not further enhanced by salicylhydroxamate (SHAM), an AOX inhibitor (Fig. 2C; Table 4). On the other hand, when the reaction medium also contained 1 mM pyruvate, the addition of cyanide depolarized DWM to a smaller extent compared with the control (Fig. 2C, trace b). In this case, SHAM addition induced obvious depolarization. These results are consistent with the oxidation of malate through the AOX pathway, which leads to the maintainance of some $\Delta\Psi$ due to the complex I proton pumping activity (Vianello *et al*., 1997). Therefore, under conditions in which the cytochrome pathway is restricted, DW-AOX is unable to maintain a high $\Delta\Psi$. In the light of this, the non-coupled respiration due to AOX, although differing in its mechanism, is energy dissipating, the same as the uncoupled respiration due to $PmitoK_{ATP}$ and PUCP.

Table 4. *Characteristics of AOX in DWM*

Activators and inhibitors of DW-AOX are listed. Data are from Pastore *et al*. (2001).

Activator	$K_{1/2}$ (μM)	Maximal activity (%)[a]	Concentration (mM)[b]
Pyruvate[c,d]	145	100	0.5
Hydroxypyruvate[c,d]	100	170	1.0
Glyoxylate[c,d]	370	233	2.5
Compound	**Effect**		
Citrate (10 mM)[e]	Activation[f]		
Propylgallate (1 mM)[c]	Complete inhibition		
SHAM (1 mM)[e]	Complete inhibition		

[a] Maximal activity expressed as percentage of that obtained with 0.5 mM pyruvate, taken equal to 100.
[b] Concentration giving maximal activity.
[c] Evaluated by oxygen uptake measurements.
[d] Measurements were carried out in the presence of 10 mM citrate.
[e] Evaluated by $\Delta\Psi$ experiments.
[f] Maintenance of a $\Delta\Psi$ of about 100 mV in cyanide-treated DWM.

Effect of the dissipating systems on mitochondrial ROS production in DWM

One of the major sources of oxygen radicals in cells are mitochondria, which basically convert to ROS about 2–6% of the total oxygen consumed (Boveris and Chance, 1973; Liu, 1997). In fact, some components of the initial (complex I) and middle part (Q-cycle, complex III) of the respiratory chain are the main reductants involved in one-electron reduction of oxygen to superoxide anion (O_2^-); among them, semiquinone (CoQH•) is apparently employed especially often (Skulachev, 1998).

In plants, the oxygen concentration in the photosynthesizing cell is very high (Scandalios, 1993), thus plant mitochondria are potentially exposed to high ROS production causing an even greater oxidative damage than the animal counterpart.

Harmful ROS production is enhanced when $\Delta\Psi$ is high and the intermediates of the respiratory chain are kept in a more reduced state; also Skulachev (1994, 1998) proposed that 'mild' uncoupling, by lowering $\Delta\Psi$, may protect mitochondria from high ROS production. Therefore, the ability of DWM dissipating systems to control mitochondrial production of O_2^- was checked (Table 5). Activation of $PmitoK_{ATP}$ by KCl, of PUCP by linoleic acid, and of AOX by pyruvate, reduced the rate of O_2^- generation by half; moreover, if the dissipating systems were inhibited, the rate came back to control levels. Notably, if the $PmitoK_{ATP}$ was activated by KCl plus mersalyl (Table 1), O_2^- generation was reduced to zero. It was concluded that dissipating systems may strongly control ROS production in purified DWM. This result is not unique (Skulachev, 1997; Braidot *et al*., 1999; Casolo *et al*., 2000, and references therein). In order to compare DWM with other plant mitochondria also see Møller (2001). It should be underlined that ROS production by '*in vitro*' isolated mitochondria may be quite different from the physiological one; nevertheless, '*in vitro*' experiments give useful indications that are confirmed '*in vivo*'. A recent paper confirms that AOX dampens ROS generation by using a whole plant model system (transformed lines of *Arabidopsis*) and an '*in vivo*' microscopy technique (Umbach *et al*., 2005). Also, transgenic plant cells lacking AOX have increased susceptibility to oxidative stress and programmed cell death (Robson and Vanlerberghe, 2002). Moreover, overexpression of PUCP in transgenic tobacco increases tolerance to oxidative stress (Brandalise *et al*., 2003).

Table 5. *Superoxide anion generation by DWM in different experimental conditions*

O_2^- production was determined essentially as described in Pastore *et al.* (1999*a*). The reaction medium contained: 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM TRIS-HCl pH 7.20, 2 EU catalase, and one of the respiratory substrates listed below. The rate of O_2^- generation was monitored spectrophotometrically at 480 nm and 25 °C by measuring the absorbance increase due to epinephrine oxidation to adrenochrome (ϵ_{480}=4.00 mM^{-1} cm^{-1}), in the absence (Control) and presence of the compounds listed below.

Superoxide anion generation rate (nmol min^{-1} mg^{-1} protein)	
DWM respiring 5 mM succinate	
Control	44±5.8[a,b]
0.5 mM KCl[c]	42±8.8[a,d]
100 mM KCl[c]	22±5.6[a,d]
20 μM Linoleic acid	25±3.1[a,b]
20 μM Linoleic acid+1% (w/v) BSA	43[b,e]
DWM respiring 10 mM malate plus 2 mM glutamate	
Control	13±2.9[f,g]
2.5 mM Pyruvate	6±0.9[f,g]
2.5 mM Pyruvate+1 mM propylgallate	12[e,g]
DWM respiring 2.5 mM ascorbate plus 0.25 mM TMPD (*N*, *N*, *N*', *N*'-tetramethyl-*p*-phenylenediamine)	
100 mM KCl[c]	15±3.4[f,h]
100 mM KCl[c]+1 mM ATP	30±7.0[f,h]
100 mM KCl[c]+1 mM mersalyl	0.2±0.08[f,h]

[a] Mean value ±SE (*n*=5 experiments).
[b] Pastore *et al.* (2000).
[c] Osmolarity of the medium was kept constant by acting on mannitol concentration.
[d] Pastore *et al.* (1999*a*).
[e] Single measurement.
[f] Mean value ±SE (*n*=4 experiments).
[g] Pastore *et al.* (2001).
[h] D Pastore, D Trono, MN Laus (unpublished data).

Effects of oxidative and abiotic stresses on dissipating systems in DWM

Effect of ROS on PmitoK$_{ATP}$, PUCP, and on the mechanism of ΔΨ generation

The demonstration that the DWM dissipating systems reduce ROS production is not surprising, since most modes of uncoupling have this effect (Wagner and Krab, 1995; Zoratti and Szabò, 1995; Skulachev, 1996*a*, *b*, 1997; Negré-Salvayre *et al.*, 1997; Kowaltowski *et al.*, 1998). Of potentially greater interest is the novel finding, reported in Table 6, showing that ROS can quickly stimulate PmitoK$_{ATP}$ (Pastore *et al.*, 1999*a*) and PUCP in DWM, the latter being evaluated as ATP-sensitive FFA-mediated uncoupling (Pastore *et al.*, 2000). Activation of the mitochondrial potassium channel by ROS in cardiomyocytes (Lebuffe *et al.*, 2003), as well as by NO in ventricular myocytes (Sasaki *et al.*, 2000) and pea seedlings (Chiandussi *et al.*, 2002), was successively reported.

Sensitivity of UCPs to ROS also seems to be a general property: besides DW-UCP, it was successively reported that animal UCP1, UCP2, and UCP3 are also activated by O_2^- (Echtay *et al.*, 2002*b*), with UCP2 activation occurring from the matrix side (Echtay *et al.*, 2002*a*), probably through a mechanism involving lipid peroxidation breakdown products such as hydroxyl-nonenal (Murphy *et al.*, 2003). As for plant mitochondria, PUCP of potato tubers (Considine *et al.*, 2003) and topinambur tubers (Paventi *et al.*, 2006) were also shown to be activated by ROS; in potato, this has been shown to occur via hydroxyl-nonenal (Smith *et al.*, 2004).

Interestingly, AOX activity was insensitive to quick regulation by ROS and little inhibited by H_2O_2 in minutes. On the other hand, it is known that in tobacco suspension cells, H_2O_2 increased *Aox1* mRNA within 2 h (Vanlerberghe and McIntosh, 1996) and that, in *Arabidopsis*, *Aox1a* and *Aox1d* are amongst the most stress-responsive genes amongst the hundreds of genes known to encode mitochondrial proteins (Clifton *et al.*, 2006).

In another set of experiments the effect of O_2^- on ΔΨ generation in DWM respiring either succinate or externally added NADH was studied (Table 6; Pastore *et al.*, 2002). In a few minutes O_2^- inhibited succinate oxidation as a result of a mixed (competitive/non-competitive) inhibition exerted on succinate transport across the inner mitochondrial membrane. In addition, no effect was observed on the oxidation of NADH, which is oxidized by the NADH DHext without crossing the membrane; this also shows that, in a few minutes of treatment, no effect occurs on the common electron carriers of the respiratory chain. After prolonged treatment (tens of minutes), gross damage occurred with complete impairment of DWM capability to generate ΔΨ (for oxidative damages to plant mitochondria, Taylor *et al.*, 2004).

From the above results, the following picture emerges: during oxidative stress ROS may very rapidly elicit PmitoK$_{ATP}$ and PUCP activity, which in turn may control ROS production. This suggests a possible feed back

Table 6. *Effect of ROS on some components of the inner mitochondrial membrane*

The effect of ROS on the functioning of some components of the inner membrane was studied as reported in the text. Activation, inhibition or absence of effect, as well as the time response, are reported. Abbreviations: COX, cytochrome *c* oxidase; SDH, succinate dehydrogenase.

Component of the inner membrane	Effect of ROS	Time response	References
$PmitoK_{ATP}$	Activation[a,b,c]	Seconds	Pastore *et al*. (1999*a*)
PUCP	Activation[a,c,d]	Seconds	Pastore *et al*. (2000)
AOX	No effect (O_2^-)[a,c]	Minutes	Pastore *et al*. (2001)
AOX	Little inhibition (H_2O_2)[a,e]	Minutes	Pastore *et al*. (2001)
Succinate carrier	Inhibition[a,c]	Minutes	Pastore *et al*. (2002)
Respiratory chain (NADH DHext→COX span)	No effect[a,c]	Minutes	Pastore *et al*. (2002)
Respiratory chain (SDH→COX span)	No effect[a,c]	Minutes	Pastore *et al*. (2002)
Respiratory chain (SDH→COX span)	Inhibition[a,c,f]	Tens of minutes	Pastore *et al*. (2002)

[a] Evaluated by $\Delta\Psi$ experiments.
[b] Evaluated by swelling experiments.
[c] ROS was O_2^- generated by a system consisting of 0.1 mM xanthine plus the amount of xanthine oxidase (xanthine: oxygen oxidoreductase, EC 1.1.3.22) required to generate O_2^- at an initial rate of 20 nmol min^{-1}, i.e. about 5-fold the physiological one measured in control DWM.
[d] ROS was 10 μM hydrogen peroxide (H_2O_2).
[e] ROS was 1 mM H_2O_2.
[f] Complete impairment in 30 min.

mechanism to protect mitochondria against ROS (see below). On the contrary, AOX regulation by ROS probably involves new synthesis under oxidative stress (Vanlerberghe and McIntosh, 1996), chilling stress (Vanlerberghe and McIntosh, 1997), and pathogen attack (Simons *et al*., 1999), but probably not under water stress (Ribas-Carbo *et al*., 2005). As for damage to membrane proteins, substrate carriers may be a sensitive early target (Atlante *et al*., 1992; Yang and Yang, 1997; Yan and Sohal, 1998; Valenti *et al*., 2002), thus possibly preventing further substrate oxidation and ROS production, while the respiratory chain is more resistant to ROS.

The time-course and the opposite effects induced by ROS confirm that they should be considered not only highly harmful reactive species, but also key factors for regulation and signalling (for recent reviews, Laloi *et al*., 2004; Rhoads *et al*., 2006).

Effect of hyperosmotic stress on $PmitoK_{ATP}$ and PUCP in DWM

Modulation by ROS and ATP: It is well known that cellular ROS production can be increased as a result of plant exposure to various environmental stresses, thus inducing oxidative stress (Scandalios, 1993; Foyer *et al*., 1994; Møller, 2001). Mitochondria, which are a major source of ROS in the plant cell, were reported to increase ROS production, in particular under drought and salt stress (Alscher *et al*., 1997). Therefore, it is feasible that $PmitoK_{ATP}$ and PUCP, which are quickly activated by ROS, serve as defence mechanisms under these conditions. Also, both dissipating systems were found to be activated in potato cells adapted to hyperosmotic stress (Fratianni *et al*., 2001) and in early etiolated seedlings of durum wheat submitted to moderate and severe osmotic and salt stress (Trono *et al*., 2004). In the latter case, durum wheat was chosen because it is a crop well adapted to semi-arid regions, where it undergoes water and salt stress, and etiolated early seedlings were used because of the central role mitochondria play in cellular bioenergetics at this stage. Main results regarding DWM are reported in Table 7. Both dissipating systems were strongly activated under all stress conditions; in particular, $PmitoK_{ATP}$ showed a higher response to osmotic stress, and PUCP to the salt stress. The remarkable activation even under moderate stress is very interesting. In fact, the moderate stress imposed is unable to affect mitochondrial integrity or oxygen uptake rate and efficiency of phosphorylation of succinate-respiring mitochondria (Trono *et al*., 2004; Flagella *et al*., 2006). So, activation of $PmitoK_{ATP}$ and PUCP precedes the occurrence of frank mitochondrial damage and may be considered an early response to stress. Moreover, under the severe stress imposed, which significantly damages mitochondrial integrity and functionality (Trono *et al*., 2004; Flagella *et al*., 2006), $PmitoK_{ATP}$ and PUCP were further activated rather than inhibited, thus suggesting that both proteins can withstand high-stress conditions and that they are involved in a prolonged response to stress. As for the mechanism of $PmitoK_{ATP}$ and PUCP activation, an increase of O_2^- production by DWM purified from stressed seedlings was measured; in the case of severe salt stress, this was even double with respect to the control. It was also observed that inclusion in the reaction medium of superoxide dismutase plus catalase, which scavenge ROS, resulted in a strong reversion of $PmitoK_{ATP}$ and PUCP activation. These results show that both systems are activated by ROS in DWM from hyperosmotic-stressed seedlings, the same as in control DWM (Trono *et al*., 2004). Finally, a strong inhibition of ATP synthesis under severe stress was measured (Flagella *et al*., 2006). These data are consistent with the hypothetical mechanism of $PmitoK_{ATP}$ and PUCP activation reported in Fig. 3. Under control

Table 7. *PmitoK$_{ATP}$ and PUCP activity in DWM from control and hyperosmotic-stressed seedlings*

The experimental conditions are detailed below the table (see also Trono *et al*., 2004).

Control[b]	Moderate stress[c]				Severe stress[d]			
	Osmotic	%[e]	Salt	%	Osmotic	%	Salt	%
PmitoK$_{ATP}$ activity[a] ($V_{KCl}/V_{KCl+Val}$)								
0.034±0.002[f]	0.120±0.008***	353	0.062±0.004**	182	0.141±0.009***	415	0.108±0.007***	318
PUCP activity[g] (nmol H^+ min^{-1} mg^{-1} protein mV^{-1})								
0.37±0.061	1.91±0.639**	516	3.14±0.940***	849	2.62±0.828***	708	5.82±1.058***	1573

[a] Channel activity was expressed as the ratio between the swelling rate in 180 mM KCl and that in 180 mM KCl plus 0.1 μg valinomycin used as internal reference.
[b] Control DWM were purified from 2-d-old seedlings germinated in distilled water.
[c] Moderate stress: DWM were purified from 3-d-old seedlings germinated in either 0.25 M mannitol solution (osmotic stress) or 0.125 M NaCl solution (conductivity 12 dS m^{-1}; $\Psi\pi$= –0.62 MPa).
[d] Severe stress: DWM were purified from 4-d-old seedlings germinated in either 0.42 M mannitol solution (osmotic stress) or 0.21 M NaCl solution (conductivity 20 dS m^{-1}; $\Psi\pi$= –1.04 MPa).
[e] % of the control.
[f] Mean value ±SE (n=4 experiments). ** P<0.01, *** P<0.001, P represents the probability level according to Student's t-test relative to the comparison between each value with the corresponding control.
[g] PUCP activity was expressed as ATP-sensitive proton conductance calculated as proton leak rate/$\Delta\Psi$ (at 100 mV). Proton leak rate curves were obtained by simultaneous measurements of oxygen uptake and $\Delta\Psi$ of succinate-respiring DWM by titrating $\Delta\Psi$ with sequential pulses of cyanide to progressively inhibit the respiratory chain (Trono *et al*., 2004).

conditions, ATP inhibition prevails over ROS activation. Under stress, as ROS production is increased and ATP synthesis is inhibited, the balance between modulators changes; therefore, ROS activate PmitoK$_{ATP}$ and PUCP that, in turn, dissipate $\Delta\Psi$, thus inhibiting further large-scale ROS production according to a feedback mechanism.

As for PUCP, a possible increase in FFAs may contribute to activation; in line with this hypothesis, PUCP-dependent uncoupling in tomatoes at different stages of ripening was found to change as a result of the availability of endogenous FFAs (Costa *et al*., 1999). Further studies are necessary to check this possibility.

Are new syntheses involved?: To date, new synthesis of PmitoK$_{ATP}$ and PUCP cannot be excluded. Anyway, the observation that ROS may largely account for the activation, strongly suggests that this mechanism may function to quickly switch on the decrease of $\Delta\Psi$ and, as a consequence, of ROS production without waiting for protein expression. Also, it was observed that the transcript levels of two mitochondrial uncoupling protein-related genes (*WhUCP1* and *TC265881*) are not affected under the experimental conditions of the study (Trono *et al*., 2006). This hypothesis is in agreement with results from other plant systems: *Arabidopsis* suspension cells subjected to a broad range of abiotic stresses showed no changes in the transcript levels of the two UCP genes under the majority of treatments (Clifton, 2005). Moreover, in dehydrated slices of topinambur tubers, PUCP was found to acquire ROS sensitivity without increasing protein content, as shown by western blot analysis (Paventi *et al*., 2006).

Results are quite different when using other plant/stress systems, probably due to a different plant model and kind of stress as well as its intensity, duration, and mode of imposition. Analysis of tobacco leaves acclimated to oxidative stress shows an increase in the expression of a gene encoding for a putative PUCP (Vranová *et al*., 2002); on the other hand, differential impact of environmental stresses on the pea mitochondrial proteome has been observed recently, with an increase of the PUCP as a result of drought, but not chilling or oxidative stress imposition (Taylor *et al*., 2005). Contrasting results were also obtained when the expression of PUCP was investigated under cold stress, in view of its possible thermogenic role: expression was enhanced in potato (Laloi *et al*., 1997; Nantes *et al*., 1999; Calegario *et al*., 2003) and in the case of *AtUCP1* of *Arabidopsis* (Maia *et al*., 1998), but was unaffected in the case of *AtUCP2* (Watanabe *et al*., 1999) and *WhUCP1* of bread wheat (Murayama and Handa, 2000).

The cold-induced increase of PUCP expression is consistent both with the protein's thermogenic effect (Jarmuszkiewicz *et al*., 2001, and references therein) and with the control of ROS production under abiotic stress proposed herein, whereas the lack of increased expression appears to be in contrast to these effects. The hypothesis reported in Fig. 3 may help to reconcile these apparently conflicting findings. It is suggested that, as a result of ROS activation (and possibly of the increased availability of endogenous FFAs), PUCP-dependent uncoupling can be enhanced, even though protein content is unchanged.

Possible effect of drought stress on the oxidation of cytosolic NADH and the AOX activity in DWM

In green tissues, an important aspect is the chloroplast/cytosol/mitochondrion co-operation under stress to modulate

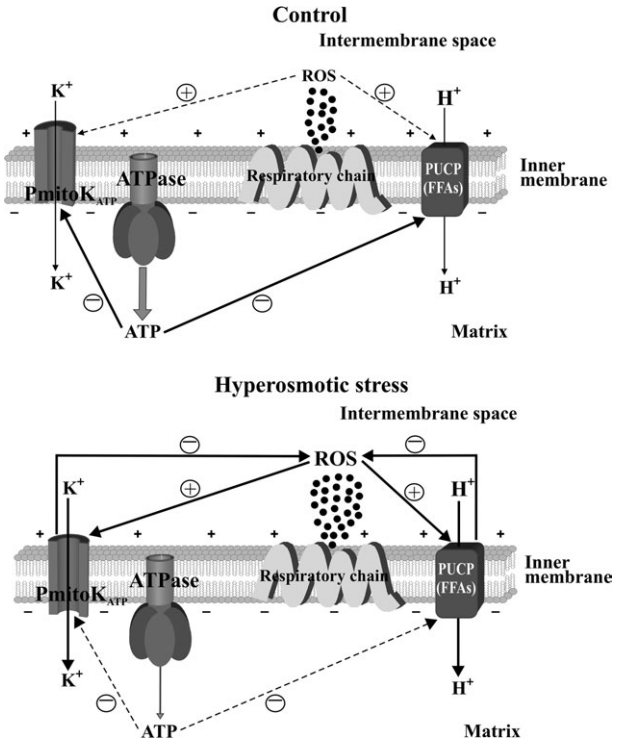

Fig. 3. Hypothetical mechanism of $PmitoK_{ATP}$ and PUCP activation in DWM based on the modulation by ROS and ATP. Under control conditions, the respiratory chain produces ROS at a basal level; on the other hand, ATP is synthesized at high rate, with consequent inhibition of $PmitoK_{ATP}$ and PUCP. Under hyperosmotic stress, ROS generation is increased, while ATP synthesis is inhibited; therefore, ROS activation prevails on ATP inhibition. Functioning of $PmitoK_{ATP}$ and PUCP is able to collapse $\Delta\Psi$, so inducing an inhibition of ROS production, according to a feed back mechanism. The topology of ROS and ATP interactions with the proteins is not considered. In DWM, under severe salt stress, 3-fold and 15-fold activation was measured by ROS of $PmitoK_{ATP}$ and PUCP, respectively, and an about 60% and 70% inhibition of ROS production due to $PmitoK_{ATP}$ and PUCP functioning, respectively.

cell redox homeostasis (Krömer, 1995; Raghavendra and Padmasree, 2003, and references therein). Under restriction of the Calvin cycle due to suboptimal CO_2 availability (for example, under drought), chloroplasts may defend themselves from over-reduction and photoinhibition by releasing excess reducing power and energy via photorespiration, triose phosphate export, and the so-called 'malate valve' (Backhausen *et al*., 1998; Backhausen and Scheibe, 1999).

As for mitochondrial help, excess chloroplastic NAD(P)H exported in the cytosol is thought to be directly oxidized by mitochondria via NAD(P)H DHext. On the contrary, the MAL/OAA shuttle was reported to export reducing equivalents from the matrix towards the cytosol (Raghavendra and Padmasree, 2003, and references therein); in fact, plant cells are generally thought to be more aligned energetically to export NADH from mitochondria than to import it.

Surprisingly, in DWM and potato cell mitochondria, results quite different from the expected ones were observed (Pastore *et al*., 2003); they are summarized in Fig. 4. The systems involved in cytosolic NAD(P)H oxidation are shown and kinetic parameters of the enzymes implicated are reported in the legend. Cytosolic NADH can be oxidized by the MAL/OAA shuttle, which is highly active in DWM. In particular, a novel MAL/OAA antiporter representing the rate-limiting step of the shuttle has been described, thus determining the rate of NADH oxidation. On the basis of the kinetic analysis, at low physiological NADH concentrations (about 1 μM; Heineke *et al*., 1991), oxidation via the MAL/OAA shuttle occurs in DWM with about 100-fold higher efficiency than that due to the NADH DHext (10-fold in potato mitochondria). On the other hand, the contribution of the NADH DHext to NADH oxidation is expected to increase under stress conditions, when an increase of NADH cytosolic concentration and, possibly, in the NADH DHext content (Clifton *et al*., 2005) and of the activator Ca^{2+} (Krömer, 1995, and references therein) may occur.

As for NADPH, it is oxidized by the NADPH DHext, but not by the MAL/OAA shuttle. In Fig. 4 the possible link between NAD(P)H oxidation and the AOX pathway is also shown. In plant mitochondria, malate metabolism and AOX activity are generally linked to each other; in fact, malate or malate-generating substrates produce pyruvate, an AOX activator, via ME (Millar *et al*., 1993, 1996). Anyway, this does not occur in DWM (Pastore *et al*., 1999*b*, 2001). Really, pyruvate can activate DW-AOX (Table 4), but it cannot be generated from malate inside DWM because of the very low activity of ME and very high activity of mitochondrial MDH (see the legend to Fig. 4). Also, in the course of malate oxidation, DWM from both etiolated shoots and green leaves release oxaloacetate but not pyruvate via the MAL/OAA antiporter reported above (Pastore *et al*., 2001). On the other hand, as stated above, DW-AOX is efficiently activated by hydroxypyruvate and glyoxylate, thus showing a metabolic link between the AOX pathway and the photorespiratory cycle in durum wheat.

This result suggests that under environmental stress, for example, drought, when photorespiration is really active, AOX is activated to oppose oxidative stress. In addition, Ribas-Carbo *et al*. (2005) reported recently that in soybean leaves severe water stress caused a significant shift of electrons from the cytochrome to the alternative pathway, due to a biochemical regulation other than protein synthesis. So, although it is clear that photorespiration increases relative to net CO_2 uptake during drought, it is less evident whether flux through the photorespiratory pathway increases in absolute terms or not (Noctor *et al*., 2002, and references therein). On the other hand, studies of several barley mutants with decreased amounts of enzymes involved in the photorespiratory pathway suggest that flux does increase to some extent during drought stress (Wingler *et al*., 1999).

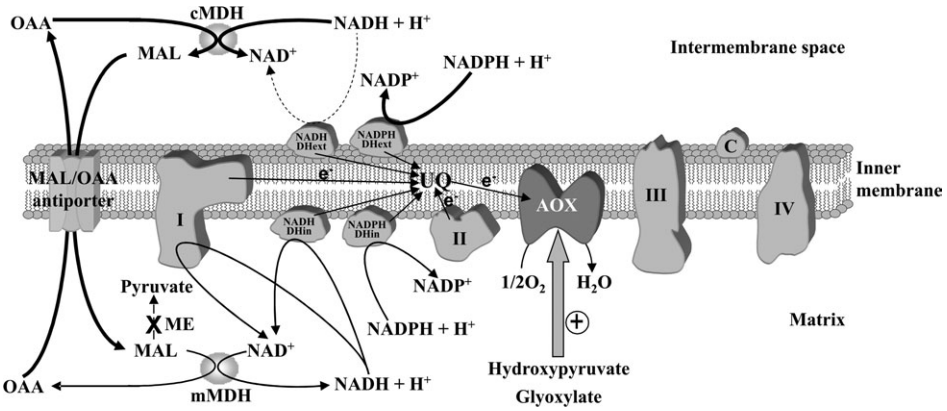

Fig. 4. Possible mechanism of cytosolic NAD(P)H oxidation and AOX activity in DWM under drought. The mitochondrial electron transport chain is described in Fig. 1. The systems involved in cytosolic NAD(P)H oxidation, i.e. the MAL/OAA shuttle and the NAD(P)H DHext, are shown. As for NADH oxidation via the MAL/OAA shuttle, MAL enters mitochondria, where it is oxidized to OAA with NAD^+ reduction by the highly active mitochondrial MDH (mMDH) ($50\times10^3\pm5.2\times10^3$ nmol min^{-1} mg^{-1} protein), but it is not converted into pyruvate due to the very low activity of ME in DWM (0.98 ± 0.16 nmol min^{-1} mg^{-1} protein). NADH produced by mMDH is then re-oxidized to NAD^+ by complex I and/or NADH DHin. OAA is released in the extramitochondrial phase in exchange with entering MAL via the MAL/OAA antiporter (V_{max} and MAL K_m calculated in DWM were 120 ± 5.1 nmol min^{-1} g^{-1} tissue and $1.0\times10^3\pm0.1\times10^3$μM, respectively). Outside mitochondria, OAA is converted into MAL with NADH consumption by cytosolic MDH (cMDH) (V_{max} value was $90\times10^3\pm9.3\times10^3$ nmol min^{-1} g^{-1} tissue and NADH and OAA K_m were 30 ± 5.0 and 40 ± 4.1 μM, respectively). Cytosolic NADH should also be oxidized by the NADH DHext (V_{max} and K_m were 370 ± 24 nmol min^{-1} g^{-1} tissue and 115 ± 25 μM, respectively). On the basis of kinetic parameters and on the assumption that the physiological cytosolic NADH concentration is about 1 μM, the rate of NADH oxidation by NADH DHext is expected to be 100-fold lower than that of the shuttle-dependent oxidation. As for cytosolic NADPH, it is oxidized by the NADPH DHext (V_{max} and K_m were 260 ± 15 nmol min^{-1} g^{-1} tissue and 290 ± 59 μM, respectively), but not by the MAL/OAA shuttle, since DW-cMDH shows no specificity towards NADPH. The kinetic data reported above were from Pastore *et al.* (2001, 2003). A possible metabolic link between the AOX pathway and cytosolic NAD(P)H oxidation in green tissues under drought is also shown. Excess NAD(P)H released in the cytosol from chloroplasts may be oxidized by mitochondria as described above. Under these conditions, glyoxylate and hydroxypyruvate, photorespiratory cycle intermediates, may activate AOX, so electron transport through the alternative pathway increases. Therefore, AOX may work as a dissipating system with a consequent decrease in ROS production. When endogenous NADH is oxidized via the NADH DHin, minimal ROS production is to be expected.

As a whole, it is proposed that, in green tissues under drought stress, the mitochondrial oxidation of cytosolic NADH may occur via the MAL/OAA shuttle and, probably, to a less extent via the NAD(P)H DHext. Concurrently, the photorespiratory cycle intermediates, glyoxylate and hydroxypyruvate, activate AOX, which works as an energy-dissipating system. Therefore, DWM may prevent chloroplast/cytosol over-reduction by discharging reducing equivalents without high ROS production by the respiratory chain. It should be noted that endogenous NADH produced via the shuttle may be oxidized via the rotenone-insensitive internal NADH dehydrogenase (NADH DHin) (Fig. 4). This would provide the mitochondria with a pathway for re-oxidation via ubiquinone to the AOX and oxygen, where no protons are pumped because all three sites of proton pumping are bypassed (Fig. 1). In this way, over-reduction and the accompanying production of ROS at complexes I and III is minimized (Møller, 2002). The hypothesis that there is co-operation among the MAL/OAA shuttle, NADH DHin, and AOX in green tissues is also in agreement with the diurnal regulation of the *NDA1* gene and with the co-ordinated up-regulation of *NDA2* and *Aox1a* genes under various treatments in *Arabidopsis* (Elhafez *et al.*, 2006).

Conclusions

It is well known that plant mitochondria participate in some metabolic processes involved in response to drought stress (photorespiration, proline oxidation, and ascorbate synthesis). Here it is proposed that mitochondria may also play a key role in adaptation to drought by acting against drought-induced oxidative stress. In addition, durum wheat that is used as a model species is well adapted to drought and even possesses three mitochondrial dissipating systems acting against oxidative stress. $PmitoK_{ATP}$ and PUCP are very active and are modulated by ROS in such a way to warrant a quick control of mitochondrial ROS production. On the other hand, AOX is ROS-insensitive, while it appears to be regulated by photorespiratory metabolism.

This meets the expectation that the three systems should be subjected to cross-regulation under different metabolic conditions and/or developmental stages. In line with this hypothesis, an opposite regulation of PUCP and AOX by FFAs was reported (Sluse *et al.*, 1998); moreover, hydroxyl-nonenal, a lipid peroxidation breakdown product, which activates PUCP (Smith *et al.*, 2004), was reported recently to inhibit AOX (Winger *et al.*, 2005). Interestingly, it has been suggested that, while both AOX

and PUCP may act to forestall mitochondrial ROS production, only PUCP may be able to operate when ROS levels become increased (Rhoads *et al*., 2006).

Differences also exist in gene expression profiles. AOX and PUCP were differently expressed and regulated during off-vine ripening in tomato (Almeida *et al*., 1999), but not in mango (Considine *et al*., 2001) or during on-vine ripening in tomato (Holtzapffel *et al*., 2002). Very recently, the expression profiles of the gene families of PUCP and AOX in a monocot (sugarcane) and a dicot (*Arabidopsis*) have been studied (Borecký *et al*., 2006). Uncoupling protein genes were expressed more ubiquitously than the AOX genes. Distinct expression patterns among gene family members were observed between species and during chilling stress, thus showing a variation in cell or tissue/organ transcriptional regulation (Borecký *et al*., 2006).

In DWM, the picture emerging from the results is that the AOX may act as an antioxidant defence system when green tissues are exposed to drought stress by working in combination with the photorespiratory cycle, while, in etiolated tissues, this role is questionable in the light of the high $PmitoK_{ATP}$ and PUCP functioning under stress.

Further investigations will be necessary to understand the significance and regulation of the three energy-dissipating systems under different stress conditions and plant species.

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