

A new simple fluorimetric method to assay cytosolic ATP content: application to durum wheat seedlings to assess modulation of mitochondrial potassium channel and uncoupling protein activity under hyperosmotic stress

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Abstract: The assays commonly used to determine ATP content in biological samples generally measure total cellular ATP content, but not the different subcellular pools. In this study a new simple method for measuring ATP content in a cytosol-enriched fraction (CEF) was developed, based on a rapid cytosolic ATP extraction (by an isotonic grinding medium that preserves organelle integrity) and its detection monitoring the NADPH fluorescence generated via hexokinase/glucose-6-phosphate dehydrogenase coupled reactions. Four protocols, differing for timing of NADPH generation and for either the presence or absence of some inhibitors of ATP and NADPH metabolism, were compared by determining CEF-ATP, as well as total ATP, in durum wheat (*Triticum durum* Desf.) etiolated seedlings. The best protocol was the one adopting both simultaneous NADPH generation and use of inhibitors during tissue homogenization. This protocol also showed higher performance than the classical trichloroacetic acid extraction. Using the new method, CEF-ATP content was assessed in control, salt- and osmotic-stressed seedlings, resulting 2.68 ± 0.04 , 1.69 ± 0.12 (–40%) and 1.35 ± 0.16 (–50%) $\mu\text{mol/g}$ dry weight, respectively. Finally, the effects of this stress-dependent decrease of cytosolic ATP were evaluated with respect to a possible modulation of two mitochondrial energy-dissipating systems, the uncoupling protein (PUCP) and the K^+ channel (PmitoK_{ATP}), both inhibited by cytosolic ATP. Experiments carried out at different physiological ATP concentrations suggest that the decreased cytosolic ATP content occurring under hyperosmotic stress may contribute to attenuate inhibition of PmitoK_{ATP}, thus promoting its activity (up to about 90%), but not of PUCP, that appears to lose ATP sensitivity under stress condition.

Key words: cytosolic ATP assay; durum wheat; osmotic stress; plant mitochondrial potassium channel; plant uncoupling protein; salt stress.

Abbreviations: ADK, adenylate kinase; ANT, adenine nucleotide translocator; Ap5A, P¹,P⁵-di(adenosine-5')pentaphosphate; ATP D.S., ATP detection system; ADP-Glc PPase, ADP-glucose pyrophosphorylase; BSA, bovine serum albumin; CEF, cytosol-enriched fraction; COX, cytochrome *c* oxidase; DWM, durum wheat mitochondria; EDTA, ethylenediaminetetraacetic acid; E.U., enzymatic units; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; FFAs, free fatty acids; G6PDH, glucose-6-phosphate dehydrogenase; HK, hexokinase; NADH-HPR, NADH-dependent hydroxypyruvate reductase; NTP, nucleotide triphosphate; PEPC, phosphoenolpyruvate carboxylase; PIMAC, plant inner membrane anion channel; PmitoK_{ATP}, plant mitochondria K^+ channel; PUCP, plant uncoupling protein; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)-aminomethane; UCP, uncoupling protein; $\Delta\Psi$, electrical membrane potential.

Introduction

ATP, the “energy currency” of the cell, plays a central role in bioenergetics and represents an important physiological modulator of various cellular activities. Most of the ATP in a plant cell is formed in the mitochondria and chloroplasts, and most of it stays within the borders of the plasma membrane (Roux & Steinebrunner 2007).

Several methods for extracting and assaying ATP

have been described in literature. The extraction of ATP from cells is not a trivial problem, especially in plants with very tough cell walls. Moreover, in a cell the ATP pool has a very high turnover rate. Therefore, extractants should open up cell walls and simultaneously, and within fractions of a second, completely inhibit or ideally inactivate all ATP-degrading enzymes; otherwise a variable fraction of the intracellular ATP could be degraded during extraction or storage of the extract. For this reason, the reagents commonly used

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for this purpose are chaotropic agents, in particular trichloroacetic acid (TCA) and perchloric acid (Lundin & Thore 1975; Velazquez & Feirtag 1997; Ishida et al. 2002; Yang et al. 2002), organic solvents (Brovko et al. 1994; Velazquez & Feirtag 1997) and surfactants (Kamitate et al. 1996; Velazquez & Feirtag 1997; Ishida et al. 2002). Nevertheless, all extractants can adversely affect the subsequent reaction of ATP detection, thus decreasing the sensitivity of the assay (Khlyntseva et al. 2009 and references therein). For the same reason, the use for ATP extraction of boiling deionized water, which efficiently inhibits ATPase, has been already proposed (Yang et al. 2002).

Unfortunately, the above-mentioned methods do not discriminate cytosolic ATP from the total one. Nevertheless, the assessment of cytosolic ATP is of a remarkable interest as it plays a crucial role, in particular when cellular activities modulated by this ATP pool have to be studied.

This is the case of bioenergetic studies on durum wheat (*Triticum durum* Desf.) mitochondria that regard the role of mitochondrial metabolism in cell adaptation to abiotic stress. In fact, the well-established resistance of durum wheat to water stress may depend not only on morphological (Rascio et al. 1988 and refs therein) or photosynthetic adaptations (Flagella et al. 1994, 1995, 1996 and refs therein), but also on adaptation of mitochondrial oxidative metabolism. On this latter point, durum wheat mitochondria (DWM) possess two energy-dissipating systems, namely the plant uncoupling protein (PUCP) (Pastore et al. 2000) and the K^+ channel (PmitoK_{ATP}) (Pastore et al. 1999), both inhibited by ATP from cytosolic side of the inner mitochondrial membrane and both involved in plant defence mechanism against environmental/oxidative stresses (Pastore et al. 2007).

The existence of an uncoupling protein (UCP) in plant mitochondria was described for the first time in potato (Vercesi et al. 1995) and subsequently demonstrated in a variety of organs and tissues of higher plant species including monocots and dicots, C3, C4 and CAM (Ježek et al. 2000). In the presence of free fatty acids (FFAs), PUCP, like animal UCPs (Skulachev 1998), catalyzes H^+ re-entry in the course of substrate oxidation (for a review see, Sluse & Jarmuszkiewicz 2002), so dissipating the electrical membrane potential ($\Delta\Psi$). PUCP is physiologically inhibited by ATP and other purine nucleotides and by bovine serum albumin (BSA), which removes FFAs (Vercesi 2001); interestingly, DWM-PUCP is activated by reactive oxygen species (ROS) (Pastore et al. 2000). Biochemical properties of PUCP, as well as distribution, regulation of gene expression, gene family and evolutionary aspects, have been reviewed by Vercesi et al. (2006). Moreover, the role of the UCP gene families in the plant response to stress has been reported recently (Figueira & Arruda 2011).

The existence of PmitoK_{ATP}, the ATP-sensitive K^+ channel, was first shown in DWM (Pastore et al. 1999) and subsequently in mitochondria from pea

seedlings (Petrussa et al. 2001; Chiandussi et al. 2002; Casolo et al. 2003; Petrussa et al. 2004), soybean cell cultures (Casolo et al. 2005), embryogenic cultures of *Picea abies* (L.) Karst., *Abies cephalonica* Loud (Petrussa et al. 2008a), *Abies alba* Mill. (Petrussa et al. 2009), and *Arum* spadix and tubers (Petrussa et al. 2008b). Moreover, a mitochondrial K^+ uniport has been reported in many other plant species, including bread wheat, barley, spelt, rye, spinach (Pastore et al. 1999), potato (Pastore et al. 1999; Fratianni et al. 2001), and triticale, topinambur and lentil (Laus et al. 2011). Recently, the first patch clamp experiments on DWM have identified an ATP-inhibited K^+ channel also at the level of single channel activity (De Marchi et al. 2010). At the same time, a channel with similar single channel and pharmacological properties was observed in bilayer experiments using plant mitochondria inner membrane vesicles (Matkovic et al. 2011). It should be also reported that an ATP-insensitive K^+ import pathway has been described in potato, maize and tomato (Ruy et al. 2004) and that a large-conductance Ca^{2+} -activated K^+ channel (Koszela-Piotrowska et al. 2009) and a large-conductance Ca^{2+} -insensitive iberitoxin-sensitive K^+ channel have been described in potato tuber mitochondria (Matkovic et al. 2011). Physiology of K^+ channels in the inner mitochondrial membrane has recently been reviewed by Szabò et al. (2012).

In DWM, PmitoK_{ATP} is activated by superoxide anion (Pastore et al. 1999, 2007) and by FFAs and acyl-CoA derivatives (Laus et al. 2011). PmitoK_{ATP} catalyzes the electrophoretic uniport of K^+ across the inner membrane into the matrix and, working together the K^+/H^+ antiporter (Diolez & Moreau 1985), allows the operation of a K^+ cycle that may induce H^+ re-entry in the matrix.

H^+ re-entry generated by the K^+ cycle due to PmitoK_{ATP}- K^+/H^+ antiporter combined function and the FFA-induced H^+ transport by the PUCP have been demonstrated to dampen ROS formation in mitochondria isolated *in vitro* from both control (Pastore et al. 1999) and salt- and osmotic-stressed seedlings (Trono et al. 2004). Under the same hyperosmotic stress conditions, both energy-dissipating systems are activated due to increased mitochondrial FFA content (Laus et al. 2011) and ROS level (Trono et al. 2004). They may thus in turn control large-scale mitochondrial ROS production according to a feed-back mechanism (Trono et al. 2004, 2011; Laus et al. 2011). On the other hand, the activities of both PmitoK_{ATP} and PUCP may be counteracted under stress by ATP inhibition. Therefore to fully understand modulation of PmitoK_{ATP} and PUCP under stress conditions, the possible decrease of ATP content and release of inhibition should be assessed. Indeed, in hyperosmotic-stressed seedlings a decrease in the rate of mitochondrial ATP synthesis has been already reported (Flagella et al. 2006; Trono et al. 2011), but it should be considered that ATP production and consumption occur simultaneously in cell. This means that a decrease in ATP synthesis rate does not necessarily imply an ATP content decrease. In the light of this,

a direct measurement of ATP content is worthwhile.

For this reason, in this study a new simple method was developed, in which the ATP of a cytosol-enriched fraction (CEF) was quickly extracted from durum wheat seedlings and immediately assayed by monitoring the NADPH fluorescence generated via hexokinase (HK)/glucose-6-phosphate dehydrogenase (G6PDH) coupled reactions. Moreover, mitochondria were purified from the same plant material and sensitivity of PUCP and PmitoK_{ATP} to ATP was checked. This to validate the hypothesis that a decrease of cytosolic ATP content may occur under salt and osmotic stress conditions, thus contributing, together with other physiological modulators, to PUCP and PmitoK_{ATP} activation.

Material and methods

Chemicals

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Substrates were used as tris(hydroxymethyl)-aminomethane (Tris) salts at pH 7.20. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), oligomycin and valinomycin were dissolved in ethanol.

Plant material

Control seedlings

Certified durum wheat (*Triticum durum* Desf.) seeds (300 g), cv Ofanto, were dark-grown in deionized water at 25°C and 80–85% relative humidity for 48 h as described in Trono et al. (2011), and early seedlings (shoot length of about 0.3 cm) were used to obtain CEF-ATP and isolate mitochondria.

Salt- and osmotic-stressed seedlings

Salt- and osmotic-stressed seedlings were germinated as described in Trono et al. (2011), by replacing water with 0.21 M NaCl solution (conductivity 20 dS/m; $\Psi\pi = -1.04$ MPa), that induces severe stress on the basis of durum wheat salt tolerance (Francois & Maas 1994), or with 0.42 M mannitol solution, that has the same osmolarity as the NaCl solution, respectively.

Stress lasted 96 h so as to reach the same shoot length as the control, after which the seedlings were harvested. Both stress conditions delayed germination and the percentage of seed germination decreased from 95% in water to 81% under stress conditions (see also Flagella et al. 2006).

Assay of marker enzyme activities and intactness

To assay marker enzyme activities, seedlings homogenization was performed using the grinding buffer adopted for the protocol I (see below). The following enzymes were assayed spectrophotometrically at 25°C in an isotonic reaction medium containing 0.5 M sucrose, buffered at pH 7.75 with Tris-HCl 30 mM: phosphoenolpyruvate carboxylase (PEPC; Trono et al. 2013), NADH-dependent hydroxypyruvate reductase (NADH-HPR; Schwitzguebel & Siegenthal 1984), ADP-glucose pyrophosphorylase (ADP-Glc PPase; Sowokinos 1975; Batz et al. 1992), cytochrome *c* oxidase (COX; Trono et al. 2013), as marker enzymes of cytosol, peroxisomes, plastids and mitochondria, respectively. PEPC activity was measured by monitoring the absorbance decrease at 340 nm due to NADH ($\epsilon_{340} = 6.22$ per mM per cm) oxidation in the course of the PEPC/malate dehydrogenase coupled assay. NADH-HPR activity was measured by following the NADH oxidation in the course of hydroxypyruvate

reduction to glycerate. ADP-Glc PPase activity was determined by measuring the NADPH generation in the course of the ADP-Glc PPase/phosphoglucumutase/G6PDH assay. COX activity was assayed by following the absorbance decrease at 550 nm due to cytochrome *c* ($\epsilon_{550} = 27.6$ per mM per cm) oxidation.

The activities of each marker enzyme were measured before (V_1) and after (V_2) the addition of 0.1% (v/v) Triton X-100. The intactness of cell and organelles was calculated by the formula: $100 \times [1 - (V_1/V_2)]$.

CEF-ATP and total ATP content in durum wheat seedlings Determination of CEF-ATP content

About 300–500 mg of fresh etiolated early seedlings (about 40–70 mg dry weight) were removed from seeds and immediately gently homogenized in ice-water bath by means of a Potter-Elvehjem homogenizer and a teflon pestle (Sigma Chemical Co., St. Louis, MO, USA) using an extraction medium at a (w/v) ratio equal to 1 g/5 mL. The extraction medium was an isotonic grinding buffer capable of preserving the integrity of subcellular organelles, consisting of 0.5 M sucrose, 4 mM cysteine, 30 mM Tris-HCl (pH 7.75), 0.1% (w/v) defatted BSA, 5 mM MgCl₂, 0.6% (w/v) polyvinylpyrrolidone (PVP)-360.

Four extraction/assay protocols were adopted that differed for the presence/absence in the grinding medium of some inhibitors of the main pathways related to ATP and NAD(P)H metabolism and/or of the ATP detection system (ATP D.S.) (see also Table 2). The inhibitors included 30 μ M P¹,P⁵-di(adenosine-5')pentaphosphate (Ap5A), 1 μ g/mL oligomycin, 10 μ M atractyloside, 100 μ M iodoacetic acid, 1 mM KCN, 1 mM salicylhydroxamic acid (SHAM), able to inhibit adenylate kinase (ADK), ATPase, adenine nucleotide translocator (ANT) (Fratianne et al. 2001; Trono et al. 2011), glyceraldehyde-3-phosphate dehydrogenase (Lehner et al. 2009), COX and alternative oxidase (Pastore et al. 2001; Soccio et al. 2010), respectively. The ATP D.S. consisted of 5 mM glucose, 200 μ M NADP⁺, 0.15 enzymatic units (E.U.) per mL HK, 0.15 E.U. per mL G6PDH; it allowed the fluorimetric ATP detection by monitoring NADPH fluorescence ($\lambda_{ex} = 340$ nm, $\lambda_{em} = 456$ nm) generated via HK/G6PDH coupled reactions.

Measurements were carried out at 25°C with a Perkin-Elmer LS-55 spectrofluorimeter (Perkin Elmer, Wellesley, MA, USA). To convert the fluorescence values into ATP concentrations, a titration was carried out with sequential additions of 2 μ M ATP at the end of each experimental trace.

The ATP concentration in the extract was calculated as reported in (a):

$$\text{ATP}(\mu\text{M}) = [(F_2 - F_1)/\Delta F_{\text{ATP}}] \times 100 \quad (\text{a})$$

When the extraction was carried out in the absence of the ATP D.S. according to the protocols I and III, F_1 and F_2 were the fluorescence values measured immediately before and after NADP⁺ addition, respectively (see Figs 1A and 1C). When the protocols II and IV were adopted, F_2 was the fluorescence value obtained after extract addition; as for F_1 , in order to subtract the contribute of endogenous NAD(P)H, a parallel extraction was carried out in the absence of the ATP D.S., so to obtain the true F_1 value (*corrected* F_1) (see Figs 1B and 1D). ΔF_{ATP} was the unitary increment of fluorescence due to addition of 1 μ M ATP and 100 was the dilution factor (20 μ L of extract were diluted in 2 mL of reaction mixture).

Table 1. Activity of COX, ADP-Glc PPase, NADH-HPR and PEPC, and intactness of organelles and cells in the crude extract from control durum wheat seedlings.^a

Compartment	Marker enzyme	Activity (nmol/min per mg of protein)		Intactness (%)
		– Triton X-100	+ Triton X-100	
Mitochondria	COX	1.06 ± 0.07 ^b	32.21 ± 2.21	97
Plastids	ADP-Glc PPase	6.66 ± 0.31	36.62 ± 1.21	82
Peroxisomes	NADH-HPR	5.32 ± 0.42	13.35 ± 0.93	60
Cells	PEPC	107.8 ± 5.9	134.6 ± 7.4	20

^a Enzymatic activities and intactness were calculated as reported in Material and methods.

^b Mean value ± S.E. (n = 4 independent experiments).

ATP content was expressed as $\mu\text{mol/g}$ dry weight as reported in (b)

$$\text{ATP } (\mu\text{mol/g dry weight}) = [\text{ATP}] \times V / (W \times 1000) \quad (\text{b})$$

where [ATP] is the concentration (μM) obtained as reported in (a), V is the volume of extract in milliliters, $[\text{ATP}] \times V$ parameter represents the nmol of ATP, 1000 is the conversion factor from nmol to μmol and W is the dry weight of seedlings in grams.

Finally, the result obtained using the equation (b) was corrected for the contribution of cells not broken during seedling homogenization (see also Table 1 and Result and discussion).

Determination of total ATP content

Total ATP was extracted under both denaturing and non-denaturing conditions.

In the first case, seedling homogenization was carried out by using an extraction medium consisting of 10% (w/v) TCA and 17 mM ethylenediaminetetraacetic acid (EDTA) (Lundin & Thore 1975). TCA was removed by extraction with diethylether (3:4, v/v). After ether extraction, nitrogen gas was bubbled through the water phase for 5–10 min to remove the solubilised ether. Finally, ATP was assayed by monitoring the NADPH fluorescence formed via HK/G6PDH coupled reactions as reported above (protocol I).

To assay total ATP under non-denaturing conditions, seedling homogenization was carried out by using an isotonic grinding medium consisting of 30 mM Tris-HCl (pH 7.75), 0.1% (w/v) defatted BSA, 0.1% (v/v) Triton X-100 and 5 mM MgCl_2 . This medium allows the complete lysis of all subcellular organelles and the overall recovery of ATP. Also in this case, the grinding medium contained the inhibitors of the main pathways related to ATP and NAD(P)H metabolism. Finally, ATP was assayed by monitoring the NADPH fluorescence formed via HK/G6PDH coupled reactions as reported above (protocol IV).

DWM isolation and measurements of P_{mitoKATP} and P_{UCP} activity by means of $\Delta\Psi$ and swelling experiments

About 50–60 g of etiolated early seedlings were removed from seeds and mitochondria were isolated as reported in Pastore et al. (1999). The grinding and washing media were: (i) 0.5 M sucrose, 4 mM cysteine, 1 mM EDTA, 0.1% (w/v) defatted BSA, 0.6% (w/v) PVP-360 and 30 mM Tris-HCl (pH 7.50); and (ii) 0.5 M sucrose, 0.1% (w/v) defatted BSA and 10 mM Tris-HCl (pH 7.40), respectively. Washed mitochondria were purified by isopycnic centrifugation on a Percoll gradient (Moore & Proudlove 1987). The final mitochondrial suspension was diluted with an appropriate volume of sucrose-free washing medium, in order to obtain a

0.3 M sucrose concentration. Mitochondrial protein content was determined by the method of Lowry modified according to Harris (1987) using BSA as a standard.

$\Delta\Psi$ measurements

$\Delta\Psi$ changes were monitored at 25 °C as reported in Pastore et al. (2002), by measuring safranin O fluorescence changes ($\lambda_{\text{ex}} = 520 \text{ nm}$, $\lambda_{\text{em}} = 570 \text{ nm}$). Measurements were carried out in a reaction medium (2 mL) containing 0.3 M mannitol, 5 mM MgCl_2 , 20 mM Tris-HCl (pH 7.20), 2.5 μM safranin O and 0.1 mg/mL DWM protein [(safranin O)/(DWM protein) ratio value of 25]. To avoid ATP entry via ANT and/or its hydrolysis by ATPase, 10 μM atractyloside and 2 μg oligomycin were also present in the reaction mixture. The presence of atractyloside also excludes the uncoupling of mitochondria via ANT mediated by FFAs (Trono et al. 2006).

Calibration of $\Delta\Psi$ as a function of safranin O fluorescence decrease in DWM was carried out by using safranin O response as a function of K^+ diffusion potential in rat liver mitochondria as reported by Zottini et al. (1993). Rat liver mitochondria were isolated according to Pastore et al. (1994); the K^+ diffusion potential in rat liver mitochondria was induced by the addition of 0.05 $\mu\text{g/mL}$ valinomycin (Åkerman & Wikström 1976).

Swelling experiments

Swelling experiments were performed as described in Pastore et al. (1999) in 2 mL of 0.18 M KCl or 0.36 M sucrose isotonic solutions buffered with 20 mM Tris-HCl (pH 7.20). Absorbance changes at 546 nm of a DWM suspension (0.05 mg/mL) were monitored at 25 °C as a function of time by means of a Perkin Elmer $\lambda 18$ UV/VIS spectrometer.

Also in this case, the effect of ATP was evaluated in the presence of 2 μg oligomycin and 10 μM atractyloside. Control was made in order to check that the oligomycin and atractyloside had no effect on sucrose swelling.

Statistical analysis

Statistical analysis was performed by using the StatSoft STATISTICA (data analysis software system), version 7. Data distribution was evaluated by using the Shapiro-Wilk test. Homogeneity of variances was verified by the Bartlett's test. Data of Fig. 2 was submitted to a "one-factor" analysis of variance (ANOVA) model, using a completely randomized block design and the mean separation was tested by the Duncan's test at 0.01 P level of significance.

Results and discussion

Evaluation of cell and organelle intactness in the crude extract from durum wheat etiolated seedlings

The first goal of this study was to set up a new simple method for rapid extraction and measurement of ATP

Table 2. Different protocols adopted to assay CEF-ATP in the extracts from durum wheat seedlings.^a

Protocol	Description
Protocol I	Extraction → ATP consumption by ATP D.S. in the course of NADPH fluorescence measurement (no inhibitors of ATP and NAD(P)H metabolism added).
Protocol II	Extraction and simultaneous ATP consumption by ATP D.S. → NADPH fluorescence measurement (no inhibitors of ATP and NAD(P)H metabolism added).
Protocol III	As protocol I, but in the presence of inhibitors of ATP and NAD(P)H metabolism in the extraction medium.
Protocol IV	As protocol II, but in the presence of inhibitors of ATP and NAD(P)H metabolism in the extraction medium.

^a Measurements were carried out as reported in Material and methods by using extraction media isotonic with intracellular organelles in order to minimize their rupture and allow the assay of the cytosolic ATP.

content in the cytosolic fraction. For this purpose, durum wheat etiolated seedlings were homogenized very gently using an isotonic grinding buffer able to minimize the rupture of subcellular organelles with consequent ATP release and contamination of the cytosolic fraction. Nevertheless, the rupture of vacuole, endoplasmic reticulum and Golgi membranes cannot be avoided; on the contrary, intactness of nuclei, plastids, peroxisomes and mitochondria is expected to be highly preserved. To check this last point, the intactness of mitochondria, plastids and peroxisomes was evaluated in the crude extract by studying the latency of marker enzymes specific for each compartment. Attention was focused on these organelles in the light of the presence of specialized adenine nucleotide carrier proteins (Haferkamp et al. 2011); on the contrary, nuclei do not possess ATP transporters (Haferkamp et al. 2011), suggesting thus a free ATP passive flow and an organelle ATP concentration in equilibrium with the cytosolic one.

In Table 1 specific activities of COX, ADP-Glc PPase and NADH-HPR, marker enzymes of mitochondria, plastids and peroxisomes, respectively, evaluated before and after membrane lysis with Triton X-100, are reported, as well as the deriving organelle intactness. Moreover, the residual integrity of cells after homogenization procedure, evaluated using PEPC latency as a marker of cytosolic compartment, is also reported. COX activity was found to strongly increase after Triton X-100 addition, thus indicating a very high intactness of mitochondria, which are double membrane surrounded organelles. Contrarily, peroxisomes, that are single membrane surrounded organelles, resulted more susceptible to rupture, whereas plastids showed intermediate intactness having larger dimensions than mitochondria, although protected by a double membrane. As for PEPC latency assay, the results shown in Table 1 indicate the presence in the crude extract of an unexpectedly high percentage of intact cells, thus pointing out the gentleness of homogenization procedure. This percentage has been considered to correct the calculation of cytosolic ATP content (see below).

In the whole, these results confirmed that, although a contamination of cytosolic fraction necessarily occurs due to the burst of some organelles, our extraction conditions are able to strongly limit ruptures, in particular of mitochondria. In the light of this, this extract may be defined as a cytosol-enriched fraction (CEF), essentially free of mitochondrial contamination.

This is of main interest since in our experimental systems (etiolated seedlings) mitochondria represent the main cellular site of ATP production; while all other organelles may be considered only as “ATP consumers” and their rupture may presumably release little ATP amount.

Determination of CEF-ATP content

To set up the best experimental conditions to extract and assay the ATP content in the CEF, four different protocols were tested (see also Table 2). As for the protocol I, CEF-ATP was preliminarily extracted in a medium which preserves subcellular organelle integrity and subsequently consumed in course of fluorescence measurements via ATP D.S. consisting of HK, G6PDH, glucose and NADP⁺ (see Material and methods). On the contrary, in the protocol II, the ATP D.S. was already present in the extraction medium, so CEF-ATP extraction and consumption via ATP D.S. occurred simultaneously. In order to avoid ATP degradation or production, as well as ATP transport across inner mitochondrial membrane in the course of extraction, in the protocols III and IV, cytosolic ATP was extracted and assayed as in the protocols I and II, respectively, but in the presence of powerful inhibitors of ATP and NAD(P)H metabolism. In particular, the extraction medium contained: Ap5A to prevent adenylates interconversion via ADK; atractyloside and oligomycin, inhibitors of ANT and ATPase, respectively, in order to avoid ATP/ADP exchange across the mitochondrial membranes, as well as ATP consumption via hydrolysis by ATPase; KCN and SHAM to inhibit NADPH oxidation by respiratory chain; iodoacetic acid, inhibitor of glyceraldehyde 3-phosphate dehydrogenase, to block glycolysis.

In Figure 1, experimental fluorimetric traces relative to ATP determination according to the four protocols described in Table 2 are reported; in this case extracts were obtained from control seedlings.

In Figure 1A CEF-ATP was assayed according to the protocol I. The addition of 20 µL of extract to 2 mL of the reaction mixture caused a slight increase of fluorescence at 456 nm probably due to the presence of endogenous NAD(P)H. After the addition of glucose, HK and G6PDH, the fluorescence remained almost constant, whereas the addition of 200 µM NADP⁺ caused a rapid increase of fluorescence from F_1 to F_2 value due to its conversion to NADPH, which is 1:1 stoichiomet-

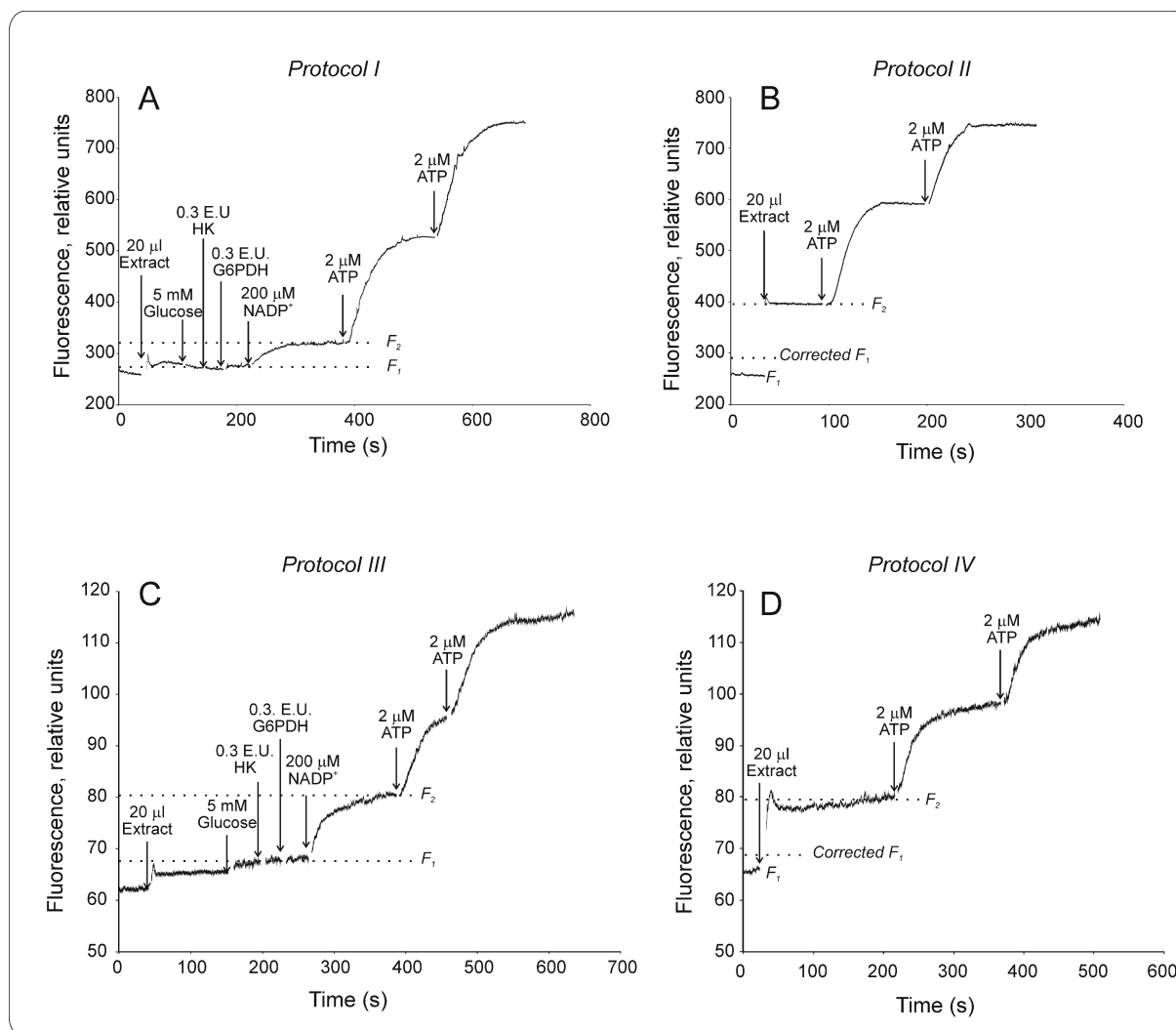


Fig. 1. Measurement of ATP in a CEF from control seedlings. In (A), (B), (C), and (D) CEF-ATP was extracted and assayed as reported in Material and methods by evaluating the fluorescence emission ($\lambda_{em} = 456$ nm, $\lambda_{ex} = 340$ nm) of the NADPH produced by the ATP D.S., according to the protocol I, II, III, and IV from Table 2, respectively. At the time indicated by the arrows, extract, glucose, HK, G6PDH, NADP⁺ and ATP were added at the reported concentration. In (A) and (C), F_1 and F_2 represent the fluorescence values measured before and after NADP⁺ addition, respectively. In (B) and (D), F_1 and F_2 are the values measured before and after the extract addition, respectively; corrected F_1 is the fluorescence value of the extract corrected for endogenous NAD(P)H (see Material and methods).

ric to ATP. Finally, to convert the fluorescence values into ATP concentrations, a titration was carried out with sequential ATP additions at known concentration (2 μ M) at the end of the experimental trace.

In Figure 1B CEF-ATP extraction was performed according to the protocol II, in a medium already containing the ATP D.S. In this case, the enzymes HK and G6PDH immediately used ATP to convert NADP⁺ in NADPH. Therefore, when the extract was added to the reaction medium, a rapid increase of fluorescence from F_1 to F_2 value, higher than in Figure 1A, was observed. To subtract the contribution due to the presence of endogenous NAD(P)H, a parallel assay was carried out without the ATP D.S. to determine the true F_1 value (see corrected F_1 in Figure 1B).

In Figures 1C and 1D, CEF-ATP was extracted and assayed as in Figures 1A and 1B, respectively, but in the presence of powerful inhibitors of ATP and NAD(P)H metabolism, according to protocols III and

IV, respectively. The presence of inhibitors caused a general fluorescence quenching of NADPH, whose values resulted 4–6 times lower than those measured in the absence of inhibitors.

In Figure 2A the different extractions and assays procedures were compared with respect to efficiency of CEF-ATP recovery. Data of ATP content were properly corrected, by considering a CEF-ATP recovery equal to 80%, as indicated by the percentage of unruptured cells (see Table 1) in the crude extract, evaluated by the PEPC latency assay.

The protocols II, III and IV provided an ATP recovery resulting 2.7–3.3 fold statistically significantly higher than that obtained by the protocol I; this confirms that, in the absence of a rapid conversion via ATP D.S. and/or inhibitors of ATP and NAD(P)H metabolism, ATP in the extract is quickly hydrolyzed. On the other hand, no significant differences were obtained when the ATP extraction was performed accord-

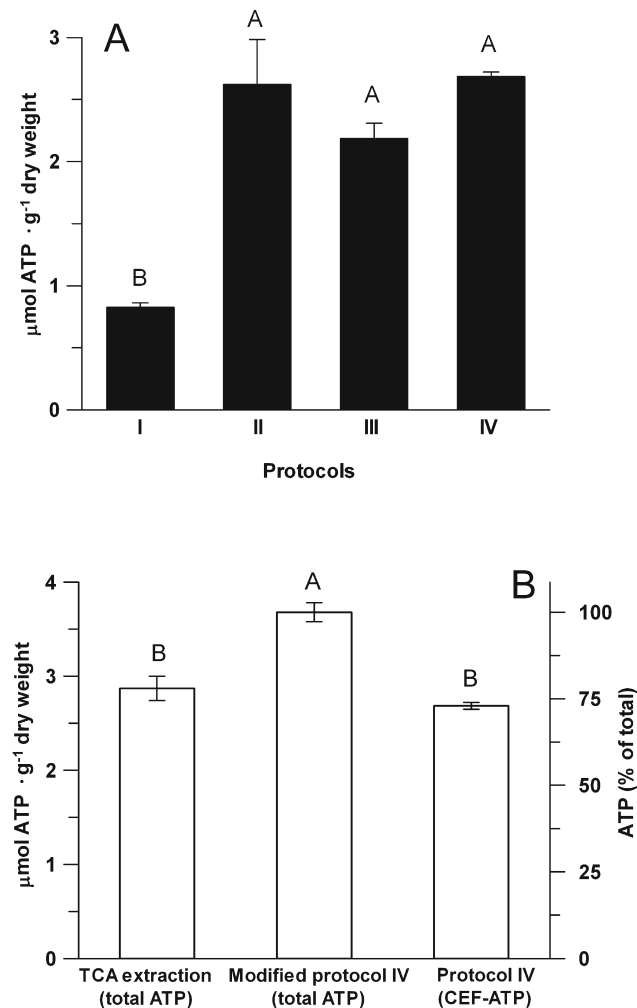


Fig. 2. Performance of various extraction and assay procedures with respect to the efficiency of CEF-ATP recovery (A) and comparison of CEF-ATP with respect to the total pool (B). In (A), CEF-ATP was extracted and assayed from control seedlings as reported in Material and methods by evaluating the fluorescence emission ($\lambda_{em} = 456$ nm, $\lambda_{ex} = 340$ nm) of the NADPH produced by the ATP D.S., according to the protocols I, II, III, and IV, from Table 2 and Fig. 1, respectively. In (B), total cellular ATP was assayed from control seedlings as reported in Material and methods by using the classical TCA extraction procedure and the protocol IV modified by the employment of an ipotonic medium. CEF-ATP value reported in (B) is the same that in (A). Data are expressed as mean value \pm S.E. ($n = 4$ independent experiments). Different letters indicate significant differences at 0.01 P level, according to the Duncan's test.

ing to the protocols II, III and IV. However, since protocol IV assures higher repeatability of measures, it was chosen to evaluate routinely CEF-ATP content in durum wheat seedlings.

In Figure 2B CEF-ATP fraction was compared with respect to the total pool (cellular ATP). Cellular ATP was assayed by modifying the protocol IV using an ipotonic extraction medium containing 0.1% Triton X-100, which lyses the subcellular organelles and cells, allowing thus the recovery of total ATP. As in the protocol IV, also in this case the ATP D.S. and inhibitors of ATP and NAD(P)H metabolism were added to the extraction medium. Under these experimental

conditions, CEF-ATP resulted about 75% of the total one.

Moreover, in Figure 2B the modified protocol IV and the classical denaturing TCA extraction were compared with respect to efficiency of total ATP recovery. Although the TCA extraction represents the reference method for ATP assay in biological materials (see Introduction), it measured only 80% of the ATP recovered using the protocol IV. This is probably due to a partial acid hydrolysis of ATP (Guinn & Eidenbock 1972) and to more manipulations necessary to extract ATP before assaying it. Both the protocol IV and the modified protocol IV provided results statistically different from the classical TCA extraction.

These results confirm that our extraction and assay protocol can be used to evaluate cellular ATP content better than classical TCA extraction.

On the basis of the protocol IV, CEF-ATP content was evaluated also in salt- and osmotic-stressed durum wheat seedlings. It should be outlined that under stress the status of the cell may differ from the control condition and may contribute differently to the ATP recovery during sample homogenization. Therefore preliminarily, in order to evaluate organelle contamination under these stress conditions, the intactness of both cell and organelle membranes was evaluated, as already described for control seedlings. In particular, the percentages of plasma membrane intactness were 4.9 ± 0.3 (S.E., $n = 4$) and 4.8 ± 0.4 under salt and osmotic stress, respectively. Therefore, under these stress conditions, our extraction procedure allows to obtain a higher percentage (about 95%) of ruptured cells in the crude extract with respect to the control. Therefore in this case, a different correction of data of ATP content is to be used, considering a CEF-ATP recovery equal to about 95%. As for mitochondria, a high intactness (89.3 ± 2.1 and 88.0 ± 1.8) was observed in extracts from salt- and osmotic-stressed seedlings, respectively. As already observed in the extract from control seedlings (Table 1), plastids and peroxisomes showed lower intactness than mitochondria both in salt (69.5 ± 2.2 and 50.9 ± 2.9 , respectively) and osmotic (68.6 ± 1.9 and 48.4 ± 3.8 , respectively) stress. In the whole, these results show that, as a result of stress imposition, the intactness of organelles and cells is about 10–25% lower than that observed under control conditions, thus showing greater membrane frailty. This finding is in line with our previous results on mitochondrial intactness under stress conditions (Trono et al. 2004; Soccio et al. 2010).

A decrease in ATP content was observed under both stress conditions. In particular, CEF-ATP was 2.68 ± 0.04 $\mu\text{mol/g}$ dry weight (S.E., $n = 4$) under control condition (Fig. 2A, protocol IV), 1.69 ± 0.12 and 1.35 ± 0.16 $\mu\text{mol/g}$ dry weight under salt and osmotic stress, about 40% and 50% statistically significantly lower than control condition, respectively. It is difficult to translate data of decrease of ATP content in terms of decrease of ATP concentration, but a rough conversion may be carried out on the basis of some

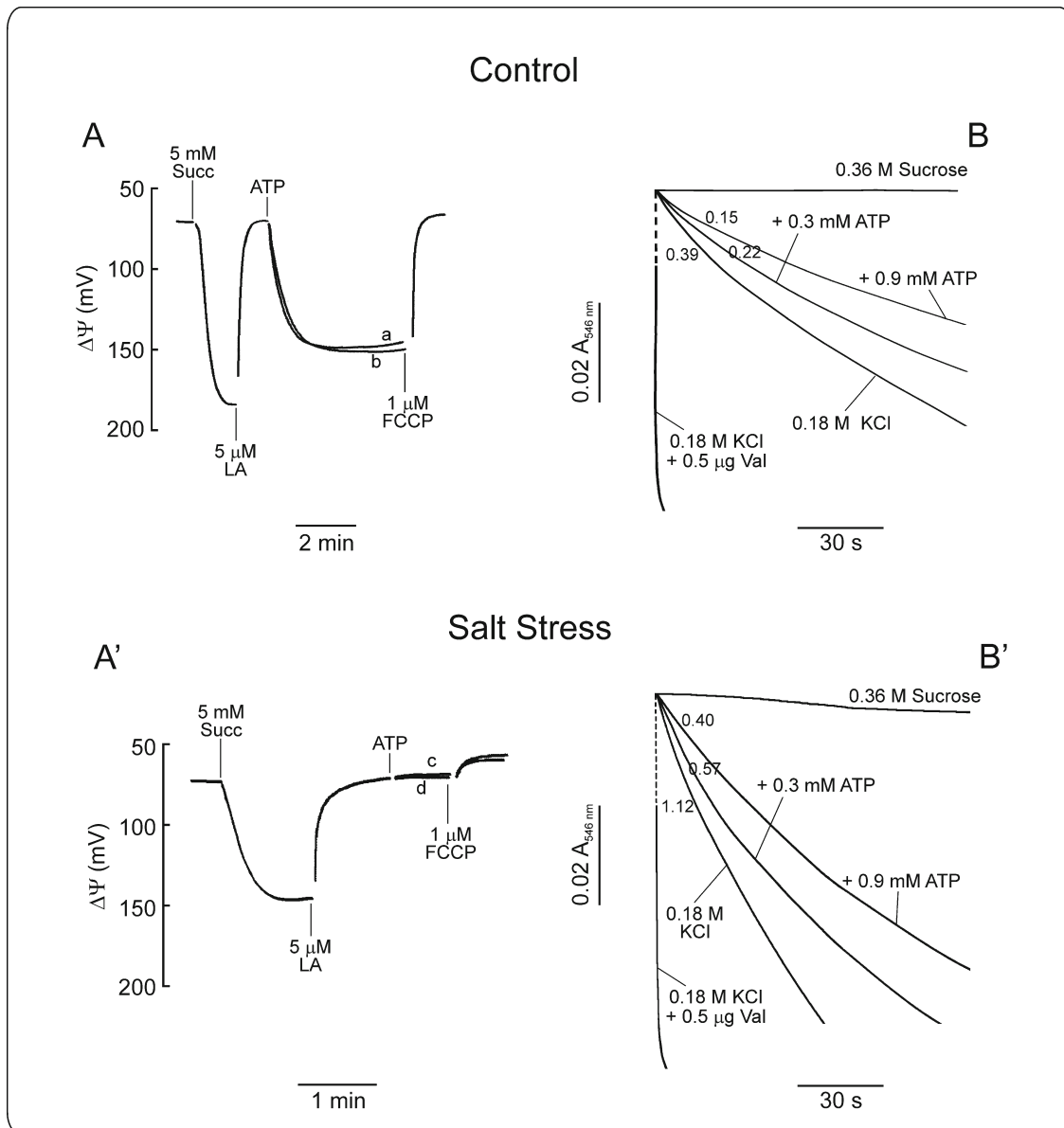


Fig. 3. Effect of ATP on linoleate-induced uncoupling (A and A') and on K^+ uptake (B and B') in DWM from control and salt-stressed seedlings. $\Delta\Psi$ measurements were carried out as reported in Material and methods by using 0.2 mg DWM protein from control (A) and salt-stressed (A') seedlings in the presence of 0.9 mM (traces a and c) and 0.3 mM (traces b and d) ATP. At the time indicated by the arrows, succinate (Succ), linoleate (LA) and FCCP were added at the reported concentrations. K^+ uptake was evaluated by using 0.1 mg DWM protein from control (B) and salt-stressed (B') seedlings by means of swelling experiments in isotonic KCl solutions, as reported in Material and methods, in the absence or presence of 0.3 mM ATP, 0.9 mM ATP, 0.5 μ g valinomicyn (Val). The numbers on the traces refer to the rate of K^+ uptake expressed as $\Delta A_{546\text{nm}}/\text{min}$ per mg of protein. The absence of swelling in isotonic sucrose solution is also shown.

literature data. On the basis of NMR analysis, Gout et al. (1992) reported that the nucleotide triphosphate (NTP) concentration in plant cell is about 0.9–1.2 mM. Moreover, as shown from perchloric acid extract, ATP represents about 70% of NTP (Roby et al. 1987). In the light of these findings, we may hypothesize that, in control condition, cytosolic ATP concentration should be about 0.6–0.8 mM, whereas under our salt and osmotic stress conditions it should decrease up to about 0.35–0.50 mM and 0.30–0.40 mM, respectively.

Whether this drop is only dependent on the decrease of ATP synthesis already reported under the same stress conditions (Flagella et al. 2006; Trono et al. 2011) or it is also due to an increase of ATP de-

mand that occurs under stress to feed defence mechanisms (Davies 1980), remains to be established. In any case, these results are in fairly good agreement to those obtained by Trono et al. (2011) using the same ATP assay method, but here the data on ATP content have the added dimension that, by using the same plant material, a parallel study has been carried out regarding the ATP sensitivity of PUCP and PmitoK_{ATP}. This to validate the hypothesis that the decrease in cytosolic ATP content, observed under salt and osmotic stress, may really modulate their activity.

At this purpose, a set of experiments was carried out to evaluate the effect of proper ATP concentrations on PUCP-dependent $\Delta\Psi$ changes and on PmitoK_{ATP}-

dependent swelling in KCl medium, both in control and salt-stressed DWM (Fig. 3).

As reported in Figure 3A, the addition of succinate to the reaction medium containing DWM from control seedlings (0.2 mg), in the presence of oligomycin (2 µg) and atractyloside (10 µM) (see Material and methods), caused a rapid $\Delta\Psi$ increase, as monitored by safranin O fluorescence decrease, up to a value of about 185 mV; the following addition of linoleate, which activates PUCP (Pastore et al. 2000), resulted in a complete $\Delta\Psi$ collapse. When either 0.9 mM (trace a) or 0.3 mM (trace b) ATP was added after linoleate, a similar $\Delta\Psi$ recovery of about 80 mV was observed, thus showing that the lower ATP concentration was all the same able to accomplish a full inhibition. This result is consistent with that described by Pastore et al. (2000) who reported that inhibition of PUCP in DWM may occur in an ATP micromolar range. Finally, the addition of the non-physiological uncoupler FCCP (1 µM) caused a $\Delta\Psi$ collapse.

In another experiment carried out by using DWM from salt-stressed seedlings, succinate addition generated a lower $\Delta\Psi$ value at a slower rate compared to the control (Fig. 3A'). This result is in accordance with Trono et al. (2006) and it is probably due to the presence of endogenous FFAs which increase under this stress condition (Laus et al. 2011). Interestingly, after ATP addition, safranin O fluorescence was found to remain constant during the time (Fig. 3A'), thus suggesting that, under this experimental condition, PUCP may be insensitive to ATP in DWM from stressed seedlings (see also Trono et al. 2006). At this regard, lower UCP sensitivity to nucleotides as a result of stress has been already observed in mitochondria from cold-stressed rat skeletal muscle (Simonyan et al. 2001) and dehydrated slices of Jerusalem artichoke tubers (Paventi et al. 2006). In the light of this, it appears unlikely that any change of ATP concentration occurring under stress conditions may modulate PUCP, whereas one can assume that the loss of sensitivity to ATP is the key factor for PUCP activation. Moreover, the increase in FFAs due to activation of a mitochondrial phospholipase A₂ (Trono et al. 2013) may strongly contribute to PUCP activation (Trono et al. 2004); 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).

The effect of ATP on PmitoK_{ATP} was assessed on the basis of inhibition of K⁺ uptake evaluated by means of swelling experiments in isotonic KCl solutions, as already stated by Pastore et al. (1999). As reported in Figure 3B, when DWM from control seedlings were suspended in 0.36 M sucrose solution, the absorbance was found to remain constant during the time, thus demonstrating the intactness of mitochondrial membranes. On the other hand, DWM exhibited a fast and clearly evident swelling in isotonic KCl solution containing a physiological (0.9 mM) ATP concentration, depending on K⁺ and Cl⁻ uptake mediated by PmitoK_{ATP} (Pastore et al. 1999) and plant inner membrane anion channel

(PIMAC) (Laus et al. 2008), respectively. To avoid ATP hydrolysis as well as its entry into mitochondria, 2 µg oligomycin and 10 µM atractyloside were also added to the reaction medium. When ATP concentration in KCl medium was lowered from 0.9 mM to 0.3 mM or was absent, a significant increase in the rate of K⁺ uptake was observed. It should be outlined that in all cases the swelling rate in KCl was strongly increased when the K⁺ ionophore valinomycin was present in the reaction medium, thus indicating that, under all tested experimental conditions, the swelling rate in KCl solution is a measure of the rate of K⁺ uptake by PmitoK_{ATP}, while the swelling rate in KCl plus valinomycin reflects the rate of Cl⁻ uptake.

In different mitochondrial preparations, PmitoK_{ATP} activation with respect to 0.9 mM ATP condition resulted: $25.1 \pm 4.1\%$, $35.4 \pm 5.6\%$, $50.5 \pm 6.7\%$ (S.E., $n = 4$), in the presence of 0.7, 0.5 and 0.3 mM ATP and $172.6 \pm 11.4\%$ in the absence of ATP, respectively.

The swelling rate in KCl observed under salt stress condition was found to be faster compared to the control. In particular, in the presence of 0.9 mM ATP, an activation of about 170% was observed with respect to the control condition (compare Figs 3B and 3B'). This is in accordance with previous results obtained under the same stress condition (Trono et al. 2004, 2011; Pastore et al. 2007) and it is probably due to the presence of endogenous FFAs, which increase under this stress condition (Laus et al. 2011). Interestingly, also in this case, PmitoK_{ATP} was activated when in the reaction mixture ATP was lowered to 0.3 mM or was absent (Fig. 3B'). In different experiments, activation was $25.3 \pm 4.3\%$, $37.2 \pm 3.2\%$, $44.7 \pm 7.4\%$ (S.E., $n = 4$), in the presence of 0.7, 0.5 and 0.3 mM ATP and $184.7 \pm 13.2\%$ in absence of ATP, respectively.

These results are in agreement with the starting hypothesis that changes of ATP content measured under stress may induce a change of PmitoK_{ATP} activity in DWM and with theoretical calculation. In fact, in experiments measuring $\Delta\Psi$ changes due to K⁺ uptake, ATP inhibited the channel in a non-competitive manner with a K_i of about 290 µM (Pastore et al. 1999); moreover, a $K_{0.5}$ equal to 0.5 mM ATP was measured in patch clamp experiments (De Marchi et al. 2010). Since K_m for K⁺ is 2.2 mM (Pastore et al. 1999), and considering that mitochondria live in an ionic cytoplasm containing about 100 mM K⁺ and 0.6–0.8 mM ATP (see above), the degree of channel activity, according to the Michaelis-Menten equation, should be about 26–44% of V_{max} in control condition, whereas the measured drop of ATP under our stress conditions may increase channel activity up to about 90%.

It should be noted that, in this respect, the behaviour of PmitoK_{ATP} is rather different from that of its mammalian counterpart, where ATP is about 10–20-fold more effective inhibitor: the mammalian channel is strongly inhibited by very low ATP concentration ($K_{0.5} = 22\text{--}40\text{ }\mu\text{M}$; Garlid 1996; Garlid & Paucek 2003); since physiological ATP concentration falls in a millimolar

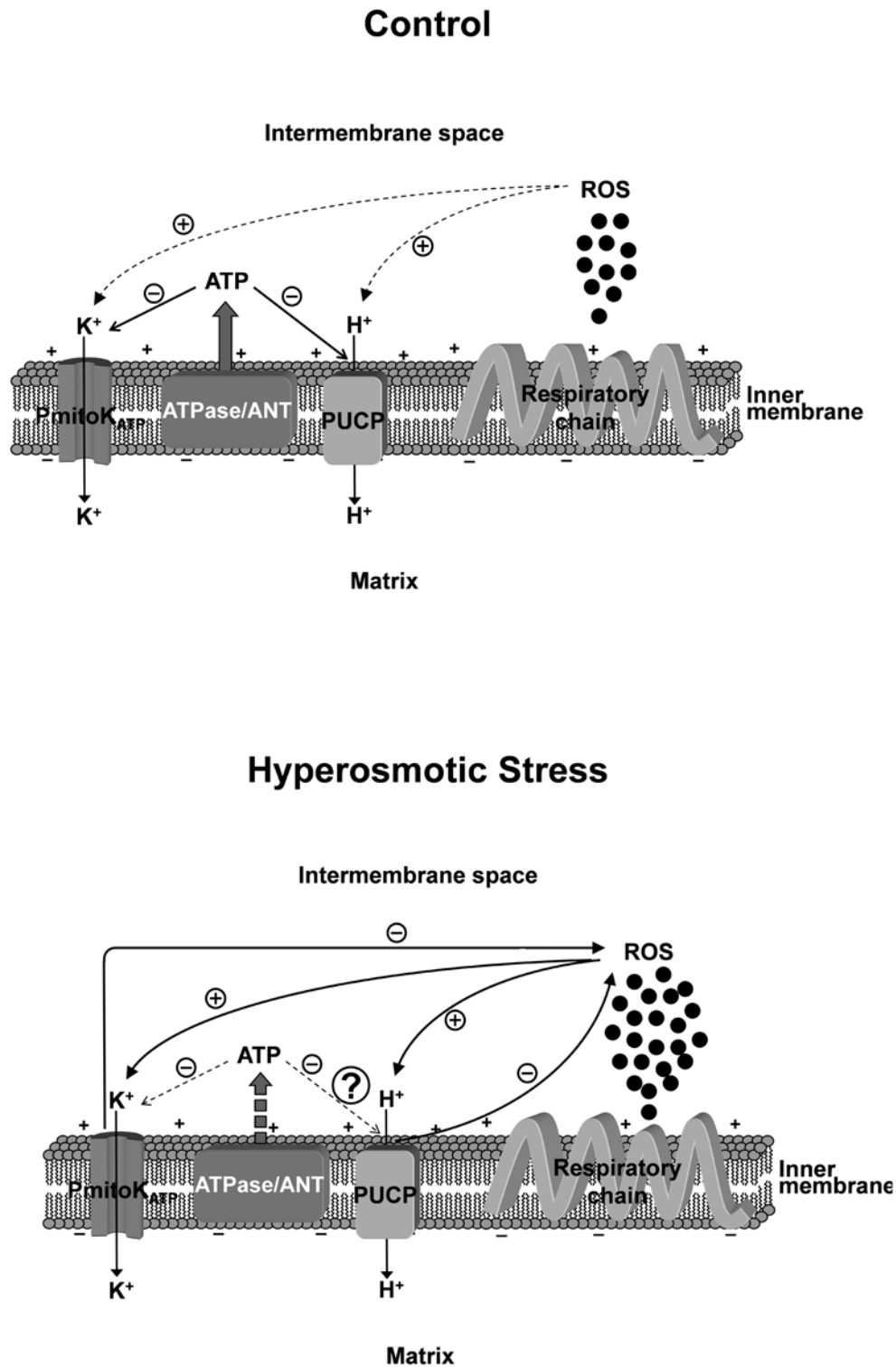


Fig. 4. Hypothetical mechanism of PmitoK_{ATP} and PUCP modulation under hyperosmotic stress conditions due to ATP decrease in DWM from stressed seedlings. For details, see text. The topology of ROS interaction with PmitoK_{ATP} and PUCP is not considered.

range, in mammals the channel should be always inhibited also when some decrease of ATP concentration occurs.

It should be underlined that ATP is only one of the possible modulators of PUCP and PmitoK_{ATP} *in vivo*. A possible mechanism of PUCP and PmitoK_{ATP} modulation under control and hyperosmotic stress conditions

regarding both ATP and ROS is shown in Figure 4. Under control condition, when cytosolic ATP concentration is high and the respiratory chain produces ROS at a basal level, ATP inhibition prevails over ROS activation and both PUCP and PmitoK_{ATP} result inhibited. On the contrary, under hyperosmotic stress conditions, the drop in cytosolic ATP concentration activates

PmitoK_{ATP}, while PUCP appears to lose ATP brake. Concomitantly, under this condition, the increase of mitochondrial ROS generation (Trono et al. 2004; Pastore et al. 2007) activates PmitoK_{ATP} and PUCP, that dissipate $\Delta\Psi$, thus damping further harmful ROS production.

In the whole, the results of this study are consistent with the hypothesis that in durum wheat the decrease in cytosolic ATP concentration under salt and osmotic stress may modulate PmitoK_{ATP}, but not PUCP, thus extending the picture emerged in the recent years about how these mitochondria may act against hyperosmotic stress.

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