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Effects of fatty acids, nucleotides and reactive oxygen species on durum wheat mitochondria

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Abstract Linoleic acid (LA) and other fatty acids added to respiring durum wheat mitochondria (DWM) were found to cause a remarkable membrane potential ($\Delta\Psi$) decrease, as monitored by measuring safranin fluorescence. The rate of $\Delta\Psi$ decrease showed (i) saturation dependence on LA concentration; (ii) fatty acid specificity; (iii) inhibition by externally added ATP, GDP, GTP and Mg^{2+} and (iv) sigmoid dependence upon initial $\Delta\Psi$, thus suggesting the existence of an active plant mitochondrial uncoupling protein (PUMP) in mitochondria from monocotyledonous species (durum wheat, *Triticum durum* Desf.). Surprisingly, the rate of the linoleate dependent $\Delta\Psi$ decrease was found to be activated by reactive oxygen species (ROS) (hydrogen peroxide and superoxide anion) and, moreover, linoleate proved to lower the mitochondrial generation of superoxide anion. These results suggest that ROS can activate PUMP, thus protecting the cell against mitochondrial ROS production.

Key words: Plant mitochondrion; Uncoupling protein; Durum wheat; Reactive oxygen species

1. Introduction

In plants, the oxygen concentration in the cell is very high [1], thus, plant mitochondria are potentially exposed to high reactive oxygen species (ROS) production and ensuing oxidative damage, which can be increased as a result of plant exposure to various environmental stresses [1–3]. Therefore, plant mitochondria must possess efficient defence systems. Consistently, besides antioxidants and enzymatic ROS scavengers ([4] and references therein), plant mitochondria possess energy dissipating systems, namely the alternative oxidase [5–7], the plant mitochondrial ATP-sensitive potassium channel ($PmitoK_{ATP}$) [8] and the plant mitochondrial uncoupling protein (PUMP) [9–12]. In particular, PUMP, which like the animal uncoupling proteins [13–15] can dissipate the mitochondrial transmembrane proton gradient in the presence of free fatty acids, was recently discovered in potato [9,10], isolated from potato and tomato [9,11] and immunologically detected in corn, avocado and other fruit mitochondria ([12] and references therein). However, at present, the occurrence of PUMP activity in monocotyledonous species has not yet been described.

All the above reported energy dissipating systems were found to prevent mitochondrial ROS generation [8,16–20]. In particular, in potato tuber mitochondria, the hydrogen peroxide production was found to increase as a result of PUMP inhibition and to decrease following the addition of linoleic acid (LA) [18].

Thus, in the light of the possible role of PUMP in the plant defence against ROS, an investigation, dealing with the occurrence of the PUMP in a monocotyledonous plant species resistant to environmental stresses, such as durum wheat, was carried out. In this paper, we show that in durum wheat the PUMP activity exists. Moreover, we show that PUMP is activated by ROS, and that it may work as a defence system against ROS.

2. Materials and methods

2.1. Chemicals and plant material

All reagents were purchased from Sigma. They were of the purest grade available and they were used without a further purification. Substrates were used as Tris salts at pH 7.20. Solution pH was adjusted with either Tris or HCl. Oligomycin and FCCP were dissolved in ethanol. Fatty acids were dissolved in dimethyl sulfoxide.

Certified seeds of durum wheat (*Triticum durum* Desf.), cv. Ofanto, were kindly supplied from Dr N. Di Fonzo (Italian Cereal Crop Institute, Foggia, Italy).

2.2. Isolation of durum wheat mitochondria (DWM)

Mitochondria were isolated as in [21,22] from 72 h old aetiolated shoots of durum wheat seedlings. The grinding and washing buffers were 0.5 M sucrose, 4 mM cysteine, 1 mM EDTA, 30 mM Tris–HCl, pH 7.50, 0.1% (w/v) defatted bovine serum albumin (BSA), 0.6% (w/v) PVP and 0.5 M sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.40, 0.1% (w/v) defatted BSA, respectively. Purification of washed mitochondria was performed by isopycnic centrifugation in a self-generating 28% (v/v) Percoll gradient. After purification, mitochondria were suspended in a washing medium containing 0.3 M sucrose. Mitochondrial protein content was determined by the Lowry method modified as in [23], using BSA as a standard. The purified mitochondria showed a 95% and a 90% intactness of inner and outer membrane, respectively, determined as in [24].

2.3. Oxygen uptake measurements

Oxygen uptake was measured at 25°C, by means of a Gilson Oxygraph (model 5/6 servo channel pH 5) equipped with a Clark-type electrode, in a medium (1.5 ml) containing 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 0.1% (w/v) defatted BSA, 10 mM K-phosphate buffer, pH 7.20.

2.4. Fluorimetric measurements of $\Delta\Psi$ changes and other assays

Mitochondrial $\Delta\Psi$ changes were monitored at 25°C essentially as in [25], using a Perkin-Elmer LS50B spectrofluorimeter to measure safranin fluorescence changes (λ_{ex} 520 nm, λ_{em} 570 nm). The standard medium (2 ml) contained DWM (0.2 mg), 0.125 M mannitol, 65 mM

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Abbreviations: DWM, durum wheat mitochondria; LA, linoleic acid; $PmitoK_{ATP}$, plant mitochondrial ATP-sensitive potassium channel; PUMP, plant uncoupling mitochondrial protein; ROS, reactive oxygen species

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NaCl, 2.5 mM Na-phosphate, 0.33 mM Tris–EGTA, 0.01 mM atractyloside, 2 μg of oligomycin, 10 mM Tris–HCl pH 7.20, 2.5 μM safranin O. Absolute $\Delta\Psi$ values reported in Fig. 2b(abscissa) were obtained by calibrating the safranin fluorescence response as a function of K^+ diffusion potential according to [26], by using rat liver mitochondria isolated as in [27]. The K^+ diffusion potential in rat liver mitochondria was induced by adding 0.05 μg/ml valinomycin [8]. LA concentration was assayed by using lipoxygenase reaction as reported in [28]. Production of superoxide anion was determined essentially as in [29], by monitoring photometrically (480 nm, $\varepsilon_{480} = 4.00$ mM^{-1} cm^{-1}) the rate of epinephrine oxidation to adrenochrome. The assay was carried out at 25°C in 2 ml of a medium consisting of 310 mM mannitol, 0.5 mM KCl, 5 mM $MgCl_2$, 5 mM succinate, 2 EU catalase, 10 mM Tris–HCl, pH 7.20. Superoxide anion was generated at a rate of about 20 nmol/min, by using the superoxide anion producing system already used in [8], consisting of 100 μM xanthine plus 65 μg xanthine oxidase (from buttermilk, Sigma-X 4376).

3. Results

In order to check the PUMP occurrence in DWM, the effect of externally added LA on the mitochondrial $\Delta\Psi$ was studied by means of safranin fluorescence. DWM were incubated in a medium containing atractyloside (see [13]) and then added with 5 mM succinate, thus increasing $\Delta\Psi$ rapidly, as monitored by safranin fluorescence decrease (Fig. 1a). Externally added LA (12 μM) was found to collapse $\Delta\Psi$ with a rate about 60% lower with respect to that measured after FCCP (1 μM) addition (Fig. 1b). The LA-induced $\Delta\Psi$ decrease rate was found to be inhibited by either $MgCl_2$ (5 mM) or ATP (0.5 mM), which have been reported to inhibit PUMP [9,10], and completely prevented by BSA (1%), which binds free fatty acids (Fig. 1b). Consistently, when either BSA (0.1%) or ATP (0.5 mM) were added about 90 s after LA, $\Delta\Psi$ was largely recovered (Fig. 1c). ATP and other PUMP inhibitors, i.e. GDP and GTP [9–11], were found to reverse LA-induced $\Delta\Psi$ decrease in DWM with a different effectiveness: ATP > GDP > GTP; in particular about 50% reversal was found as a result of 30 μM ATP addition to DWM (Fig. 1d).

In the same experiment, in order to test the DWM coupling, measurements of oxygen uptake by purified organelles in the presence of some respiratory substrates were carried out. The respiratory profiles clearly showed that DWM can oxidise efficiently succinate, 2-oxoglutarate and malate plus glutamate (for other substrates, see [22]), with high oxygen uptake rates. The respiratory control ratio was found to range between 2 and 2.4 (succinate), 4 and 8 (malate plus glutamate), and 8 and 12 (2-oxoglutarate); the ADP/O ratio was about 1.7 (succinate), 2.5–3.5 (2-oxoglutarate) and about 1.8 (malate plus glutamate). In all cases, cyanide was found to block the oxygen uptake (see also [22]). Interestingly, LA (40 μM) was found to stimulate 3–6-fold the oxygen uptake rate by DWM added with different respiratory substrates (not shown).

The LA-induced $\Delta\Psi$ decrease rate was measured as a function of LA concentration (Fig. 2a). A sigmoidal saturation dependence was found.

In order to ascertain whether and how the LA-induced $\Delta\Psi$ decrease rate is $\Delta\Psi$ dependent, DWM, previously energised by succinate, were treated with FCCP in a nM concentration range, designed to partially collapse $\Delta\Psi$. Then the dependence of the $\Delta\Psi$ decrease rate on the actual mitochondrial $\Delta\Psi$ was investigated, using 40 μM LA (Fig. 2b). The LA-induced $\Delta\Psi$ decrease rate was largely enhanced between 120 mV and 130 mV, whereas a lower activation was observed below 120 mV and over 130 mV.

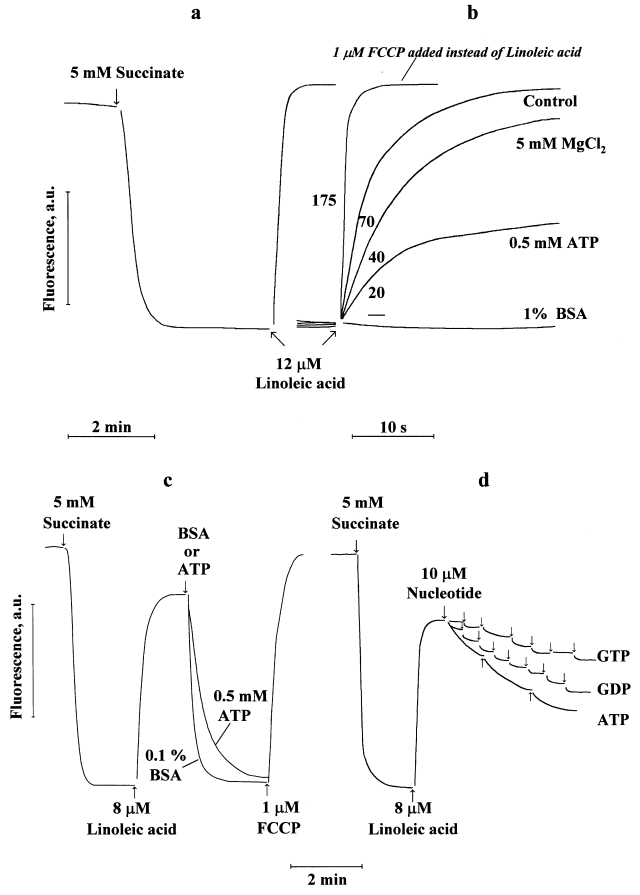

Fig. 1. Mitochondrial uncoupling caused by externally added LA. DWM (0.2 mg protein) were incubated in the standard medium for about 1 min with continuous measurement of the safranin fluorescence as a function of time. At the arrows, succinate and LA were added (a and b), followed (c and d) by either BSA or a nucleotide (ATP, GDP or GTP) and by FCCP (c). In (d), the arrows without indications refer to each addition of 10 μM nucleotide. In (b), the fluorescence decrease due to succinate addition was not shown; the experiments were carried out either as in (a) (control trace) or in the presence of the indicated compounds. It should be noted that, in the presence of either ATP or BSA, DWM showed a higher state 4 $\Delta\Psi$, nonetheless, in the figure, the different fluorescence levels were equalised in order to better compare the rates of fluorescence decrease in the different conditions. Numbers refer to the $\Delta\Psi$ decrease rate calculated as a tangent to the linear part of the progress curve and expressed as Δarbitrary units (a.u.) of fluorescence/min. The experiment showing the faster $\Delta\Psi$ decrease was carried out by adding FCCP (1 μM) instead of LA.

The $\Delta\Psi$ decrease rate showed fatty acid specificity (Fig. 2c,d), being the unsaturated fatty acids the most effective substrates and lauric acid a good substrate among the tested saturated fatty acids (see also [30]). Interestingly, phenylvaleric and undecanesulfonic acids fail to cause DWM $\Delta\Psi$ decrease; consistently, these compounds were unable to induce proton movement in liposomes incorporating reconstituted PUMP [11]. In Fig. 2d, a typical experiment is reported, in which both the uncoupling due to lauric acid (with recoupling by either BSA or ATP) and the ineffectiveness of phenylvaleric acid can be observed.

Taken together, the results reported in Figs. 1 and 2 show the occurrence of PUMP activity in DWM.

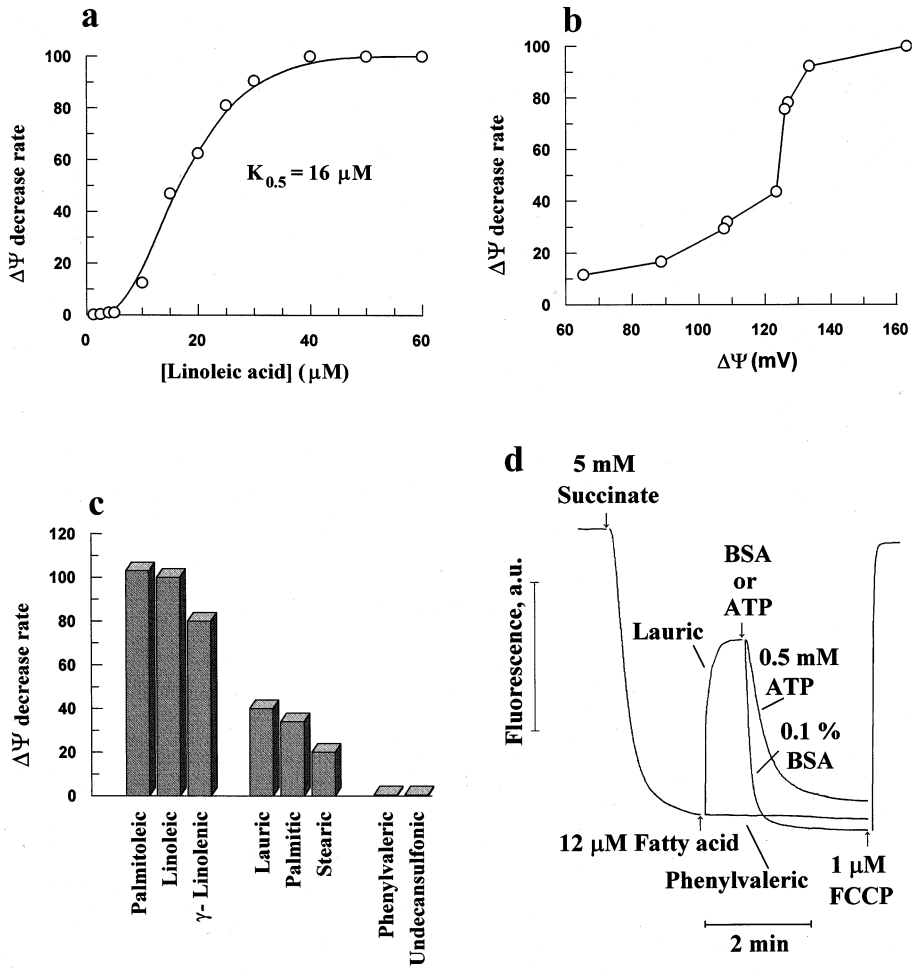

Fig. 2. Certain features of the LA-induced mitochondrial uncoupling. The experiments and $\Delta\Psi$ change measurements were carried out as in Fig. 1 except that 0.5 mM ATP was present in the reaction medium in order to lower the rate of $\Delta\Psi$ decrease. (a) The dependence of the $\Delta\Psi$ decrease rate on increasing LA concentration. (b) The $\Delta\Psi$ dependence of the LA-induced $\Delta\Psi$ decrease; in this case, mitochondria were energised with 5 mM succinate; then, FCCP was added at nM concentrations (1–10 nM); when a constant $\Delta\Psi$ was reached, 40 μM LA was added and the obtained $\Delta\Psi$ decrease rate was reported as a function of the imposed absolute $\Delta\Psi$, calculated as reported in Section 2. (c) The effectiveness of different fatty acids (12 μM) to cause $\Delta\Psi$ decrease; in (d), the effect of lauric and phenylvaleric acids was shown in detail; the experiment was carried out as reported in Fig. 1c with the only modification that either lauric or phenylvaleric acids were added instead of LA; at the arrows, either BSA or ATP and FCCP were added at the reported concentration. In (a) and (b), the $\Delta\Psi$ decrease rate was expressed as % of the higher one, in (c), it was expressed as % of the $\Delta\Psi$ decrease rate induced by LA.

In the light of [18], an investigation was made in order to establish whether DWM PUMP reaction and superoxide anion generation are somehow related. In particular, we wanted to ascertain whether hydrogen peroxide and superoxide anion could modulate PUMP activity in DWM. Mitochondria were energised with succinate (Fig. 3a) and partially uncoupled with 4 μM LA; when $\Delta\Psi$ remained almost constant, 10 μM hydrogen peroxide was added, that was found to cause a further $\Delta\Psi$ decrease. In another experiment, the dose effect of hydrogen peroxide was investigated at a 0.1 μM–10 mM range. Maximal hydrogen peroxide-induced $\Delta\Psi$ decrease was observed at 10 μM, with half maximal $\Delta\Psi$ change at about 1 μM. $\Delta\Psi$ decrease was also found when the superoxide anion generating system xanthine plus xanthine oxidase was added to DWM respiring succinate in the presence of LA (Fig. 3b). It should be noted that in this case, xanthine per se causes about 50% of the observed depolarisation (not shown). $\Delta\Psi$, in control DWM lacking LA addition, was not affected (Fig. 3a) or slightly affected (Fig. 3b) by hydrogen peroxide or xanthine plus xanthine oxidase, respectively.

PUMP activation by ROS was confirmed also in a different way: either hydrogen peroxide or xanthine plus xanthine oxidase were added before 8 μM LA; in this case, the LA-induced $\Delta\Psi$ decrease was faster (about 40%) and was found to occur to a higher extent (not shown).

In another set of experiments, mitochondria were incubated for 2 min in the assay medium in either the absence or presence of different compounds (Fig. 3c), then 1 mM epinephrine was added with the superoxide anion formation monitored as in [29]. A high superoxide anion generation rate (45 nmol/min/mg of protein) was observed in a control experiment. A 45% reduction of superoxide anion generation rate was observed in the presence of externally added LA; LA plus BSA proved to have no effect. In a series of five experiments with different mitochondrial preparations, the rate of superoxide anion generation was found to be 44 ± 12.9 (S.D.) nmol/min/mg protein

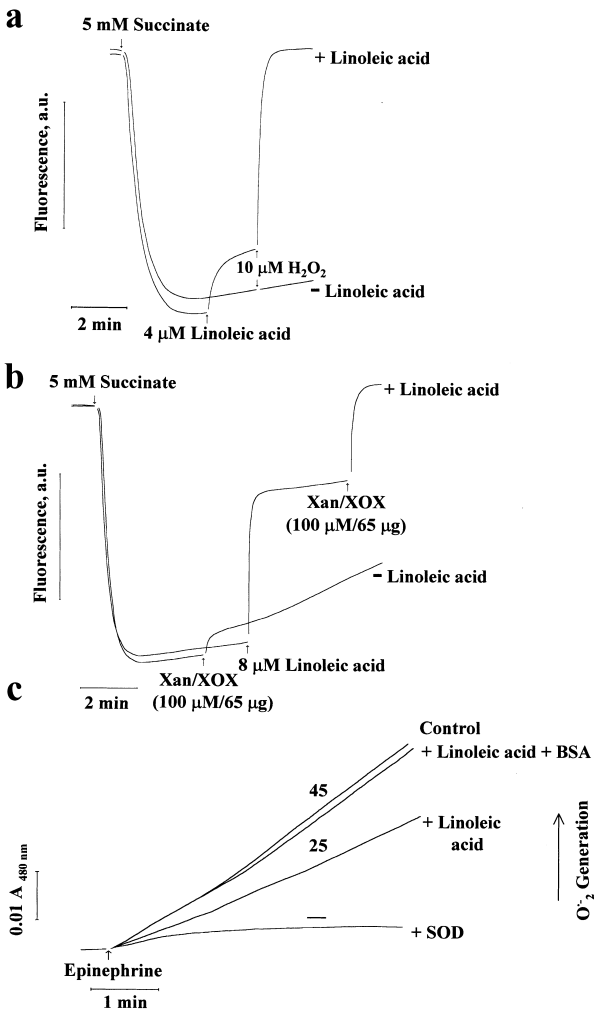

Fig. 3. Effect of ROS on LA-induced mitochondrial uncoupling and effect of LA on superoxide anion generation by DWM. The experiments were carried out as in Fig. 1. At the arrows, succinate, LA and either hydrogen peroxide (a) or the superoxide anion generating system xanthine plus xanthine oxidase (Xan/XOX) (b) were added. In the control experiments, LA addition was omitted. (c) Measurements of superoxide anion generation by succinate respiring DWM were carried out as described in Section 2 (Control) or in the presence of 20 μM LA plus 1% BSA, 20 μM LA or 20 μg SOD. Numbers alongside the trace represent the rate of superoxide anion generation expressed in nmol/min/mg protein.

with about 43% decrease in the presence of LA (25 ± 6.9 nmol/min/mg protein). As expected, externally added superoxide dismutase was found to prevent the epinephrine response (Fig. 3c), thus confirming that absorbance increase was dependent on superoxide anion formation. Control was made in order to check that the LA had no effect on epinephrine response (not shown).

4. Discussion

In this paper, we show the occurrence of the PUMP activity in durum wheat, i.e. in a monocotyledonous plant. Such a conclusion derives from the successful application of criteria used to assess the occurrence of a protein-mediated transport process; in fact, we show that the rate of LA dependent $\Delta\Psi$ decrease, which is a measurement of the PUMP reaction, exhibits saturation kinetics, substrate specificity, inhibitors sensitivity and $\Delta\Psi$ dependence. The features of DWM PUMP, including ATP, GDP, GTP and Mg^{2+} sensitivity and $\Delta\Psi$ dependence, are rather similar to those reported for other PUMPs [9–11]. Since uncoupling by fatty acids occurs in mammalian mitochondria due to the activity of the ATP/ADP antiporter (ANT) with partial inhibition found caused by atractyloside [31], the possible involvement of the ANT in the reported results cannot be completely excluded.

Here, we show a novel PUMP reaction feature, i.e. the PUMP modulation by ROS. As recently reported for $PmitoK_{ATP}$ [8], durum wheat PUMP reaction is activated by exogenous ROS production. Such a conclusion derives from the results reported in Fig. 3a,b in which both hydrogen peroxide and superoxide anion, produced in situ by the xanthine/xanthine oxidase system, were found to increase the extent and the rate of $\Delta\Psi$ decrease stimulated by LA. Indeed, these findings could be a result of other processes different from PUMP stimulation, including mitochondrial damage, spontaneous LA independent uncoupling and formation of LA hydroperoxide derivative, which could be PUMP substrates more efficient than LA. All these possibilities can be excluded in the light of control experiments showing that: (i) DWM incubated for 1 min with either hydrogen peroxide (up to 10 mM) or with xanthine/xanthine oxidase system, then added with succinate, show both a $\Delta\Psi$ generation rate and final $\Delta\Psi$ value similar to those measured in the control; (ii) in the absence of LA, no ROS dependent $\Delta\Psi$ decrease was found even though mitochondria were partially uncoupled with a low FCCP concentration; (iii) the ROS dependent $\Delta\Psi$ decrease was observed as due to saturated fatty acids such as lauric and stearic acids, thus excluding that the ROS effect may be due to the possible generation of hydroperoxides of the polyunsaturated LA. Finally, the direct PUMP involvement in the ROS dependent $\Delta\Psi$ decrease is confirmed by the observation that PUMP inhibitors, such as ATP (0.5 mM) or BSA (0.1%), prevent any ROS effect (not shown).

In addition to the PUMP activation by ROS, the PUMP capability to partially prevent ROS production in DWM is also shown. Although in vitro and in vivo situations are assumed to differ from each other, ROS-mediated PUMP activation could induce mitochondrial uncoupling in the cell, with reduction of the mitochondrial ROS generation as proposed by Skulachev [32,33]. Thus, the picture emerging from [18] and this paper is that cell ROS can stimulate the PUMP activity and that PUMP activity, according to a feed-back mechanism, can decrease ROS mitochondrial production. Consistently, a number of other energy dissipating systems in plant and animal mitochondria have been proposed to decrease the mitochondrial ROS generation [8,16,17,19,20,32–36].

The physiological role of PUMP has been discussed in several papers. It has been suggested that PUMP plays a major role in thermogenesis [10,37–39], in seed development and dormancy and in fruit ripening and senescence [10,11]; in particular, PUMP was reported to be responsible for the fruit climateric respiratory burst [40]. In the light of the novel PUMP feature reported in this paper (see also [10]), another physiological role of PUMP is the cell defence against mitochondrial oxidative stress. In particular, since ROS generation is enhanced when plants are subjected to unfavourable environmental stimuli including drought, salinity, chilling, high

light, UV light, herbicides, pathogens and various other stresses [1–3], the ROS dependent PUMP stimulation could be suggested to play a major role in plant defence against environmental stresses.

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