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Occurrence of Plant-Uncoupling Mitochondrial Protein (PUMP) in Diverse Organs and Tissues of Several Plants

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The presence of plant-uncoupling mitochondrial protein (PUMP), previously described by Vercesi *et al.* (1995), was screened in mitochondria of various organs or tissues of several plant species. This was done functionally, by monitoring purine nucleotide-sensitive linoleic acid-induced uncoupling, or by Western blots. The following findings were established: (1) PUMP was found in most of the higher plants tested; (2) since ATP inhibition of linoleic acid-induced membrane potential decrease varied, PUMP content might differ in different plant tissues, as observed with mitochondria from maize roots, maize seeds, spinach leaves, wheat shoots, carrot roots, cauliflower, broccoli, maize shoots, turnip root, and potato calli. Western blots also indicated PUMP presence in oat shoots, carnation petals, onion bulbs, red beet root, green cabbage, and *Sedum* leaves. (3) PUMP was not detected in mushrooms. We conclude that PUMP is likely present in the mitochondria of organs and tissues of all higher plants.

KEY WORDS: Plant-uncoupling mitochondrial protein (PUMP); plant mitochondria; fatty acid-induced uncoupling; mitochondrial respiration; mitochondrial membrane potential; anti-PUMP antibodies.

INTRODUCTION

Plant mitochondria are essential for plant's growth and functional activities. Mitochondria in photosynthetic cells fulfill important roles, even during daylight, and oxidative phosphorylation is the main source of ATP, either in light or darkness, for extrachloroplastic processes (Gardeström and Lernmark, 1995). During photosynthesis, mitochondria regulate the redox balance of the cell by participating in the glycolate cycle. Moreover, plant mitochondria actively oxidize NADH and NADPH, involving external and matrix rotenone-insensitive NADH (and separate NADPH) dehydrogenases (Soole and Menz, 1995) and also contain a cyanide-insensitive alternative oxidase (AOx) (Moore *et al.*, 1995; Vanlerberghe and McIntosh, 1997). With AOx, active oxidation of succinate fails to promote proton pumping and, therefore, no proton-motive force (pmf) is built up. Consequently, AOx presence and operation contribute to the regulation of the electrochemical proton gradient across the inner membrane of plant mitochondria, in addition to the processes acting in animal mitochondria. Therefore, it could be apparently redundant to consider the presence of uncoupling proteins (UCPs) involved in the regulation of energy coupling in plant mitochondria. However, Nature devised an elaborated regulation of mitochondrial coupling in plants, since an uncoupling protein known by the name PUMP (*p*lant-*u*ncoupling *m*itochondrial *p*rotein, Vercesi *et al.*, 1995) was also found in plants.

PUMP was first identified functionally on the basis of the purine nucleotide-sensitive fatty acid-

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induced uncoupling promoted by a 33-kD protein in potato tuber mitochondria (Vercesi *et al.*, 1995, 1998; Ježek *et al.*, 1996, 1997). Later, cDNA coding for proteins, homologous to mammalian UCPs, was cloned and sequenced from potato (*St*UCP, 44% homologous to mammalian UCP1; Laloi *et al.*, 1997) and *Arabidopsis thaliana* flower gene library (81% identical with *St*UCP, Maia *et al.*, 1998). We have shown that PUMP is a product of the *St*UCP gene, since MALDI-mass spectroscopy analysis of peptides obtained by the trypsin cleavage of isolated potato PUMP yields a 35% coverage of the identical sequence (Růžička *et al.*, 1999).

In contrast, four recently revealed mammalian UCPs were identified only on the basis of sequence homology. They form together with PUMP and brown fat UCP1 (Ježek and Garlid, 1997; Ježek *et al.* 1998; Ježek, 1999), a distinct subfamily of the mitochondrial carrier gene family (El Moualij *et al.*, 1997; Ježek and Urbánková, 2000). However, their uncoupling function has not been definitively proved and no specific ligand-gated regulation has been characterized for them. This is valid for all novel UCPs, *i.e.*, for UCP2, ubiquitous in mammalian tissues (Fleury *et al.*, 1997), UCP3 of striated muscle (Boss *et al.*, 1997), and brain-specific BMCP1 (Sanchis *et al.*, 1998) and UCP4 (Mao *et al.*, 1999). For, example, no particular portion of fatty acid-induced uncoupling in brain mitochondria can be ascribed to UCPs existing in the brain, etc.

Physiological roles of novel UCPs are also still rather speculative, in spite of indications of their participation in body weight regulation, fever, etc. Similarly, possible physiological roles of PUMP are subject to speculation as well. For example, PUMP has been found in the pericarp of a variety of fruits of temperate or tropical climates (Ježek *et al.*, 1998; Costa *et al.*, 1999), most of which exhibit a climacteric respiratory rise, *i.e.*, transient respiratory increase during ripening. This finding was a necessary prerequisite for the hypothesis that PUMP-mediated uncoupling could be responsible for such a climacteric respiratory rise (Ježek *et al.*, 1996). The potential of PUMP to uncouple mitochondria of a typical climacteric fruit, tomato, even in state 3, has been clearly established (Jarmuszkiewicz *et al.*, 1998; Costa *et al.*, 1999; Almeida *et al.*, 1999). We have also speculated that PUMP may be important for all physiological events when a sudden cut-off in the ATP synthesis is required (seed formation, transitions to senescence, thermogenesis) or, it needs to be shut off, when an initiation of ATP synthesis is required, as during turn-off from dormancy (Ježek *et al.*,1996, 1997; Vercesi *et al.*, 1998; Nantes *et al.*, 1999).

However, to evaluate the significance of any physiological role of PUMP in detail, its occurrence within the plant in various tissues or organs and its phylogenic distribution need to be determined. It is still uncertain whether PUMP is a ubiquitous protein in all plant structures (as UCP2 is in human tissues; Fleury *et al.*, 1997) or specific for particular organs and tissues (*e.g.*, for fruits or storage organs in which it has been mostly found). Hence, we carried out a screening study of PUMP presence in several plant organs or tissues and among selected plant species. In contrast to mammalian mitochondria (with exception of brown fat), where no nucleotide-sensitive fatty acid-induced uncoupling has yet been found (Matthias *et al.*, 1999), we observed this phenomenon quite frequently in plant mitochondria and ascribed it to PUMP. Thus, PUMP has been functionally identitified in most of the tissues and species tested, which was further confirmed by immunological detection using Western blots.Therefore, we suggest that PUMP might be nearly ubiquitous in plants.

MATERIALS AND METHODS

Chemicals

Nucleotides, bovine serum albumin (BSA), valinomycin, linoleic acid (LA), oligomycin, atractyloside, carbonyl cyanide trifluoromethoxyphenylhydrazone (FCCP), and Percoll were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were commercial products of the purest grade available.

Biological Material

Red beet (*Beta vulgaris* L. var. *rapa*, forma *rubra*) roots, turnip (*Brassica napus* L.) roots, onion (*Allium cepa* L.) bulbs, carrot (*Daucus carota* L.) roots, cauliflower (*Brassica oleracea* L.), broccoli (*Brassica oleracea* L., var. *cauliflora*), green cabbage (*Brassica oleracea* L., var. *sabanda*), dry bean seeds (*Phaseolus multiflorus* L.), mushrooms [*Agaricus bisporus* (J.Lange) Imbach], spinach leaves (*Spinacea oleracea* L.), and white carnations (*Diantus caryophyllus* L.) were purchased in the market. Etiolated shoots or their roots were obtained from maize seeds (*Zea mays* L., cv. Hybrid-3352/Palma-Pioneer), which were surface-

sterilized in a 7% (w/v) solution of commercial bleach, rinsed with tap water, and soaked in running tap water for about 24 h. The seeds were then germinated on damp paper towels in plastic trays in a darkened growth cabinet for 4–5 days at 25°C (Vicente and Vale, 1991) and the shoots or roots used. Oat (*Avena sativa* L.) and wheat (*Triticum aestivum* L.) shoots were grown similarly, but in sand. The field-grown maize plants were harvested to collect the seeds. Immature peas from the pod were collected from 40-day-old plants (*Pisum sativum* L.); immature bean seeds from the pod were collected from 60-day-old plants (*Phaseolus multiflorus* L.). Stems of cacti (*Opuntia* sp.) or *Sedum* sp., young green cones (fruits) of *Platiclatus orientalis* (formerly *Thuja*) were collected in The Botanical Garden of Coimbra University (Department of Botany) Potato calli were obtained from segments of adventitious roots, developed from sprouts of potato (*Solanum tuberosum* L.), excised and cultured on Murashige and Skoog solid medium containing 2 mg/L 2,4-D(2,4-dichlorophenoxyacetic acid).

Mitochondrial Isolation and Protein Determination

Procedures for mitochondrial isolations were optimized for each species or organ tested, namely, in the separation phase on a Percoll gradient. The preceding procedure, leading to crude mitochondria, was carried out as follows: First, a rapid tissue desintegration was performed in 500 ml of a homogenization buffer with 500 g of turnip, 850 g of red beet, 130 g of maize or oat shoots, 45 g of wheat shoots, 1 kg of onions, 700 g of carrots, 460 g of maize seeds, 180 g of maize roots, 210 g of peas, 600 g of cauliflower, 340 g of broccoli, 250 g of carnation petals, 25 g of potato calli; 140 g of *Sedum* leaves; or in 1000 mL for mushrooms (600 g), bean seeds (60 g of dry and 300 g of soft green), green cabbage (500 g), *Thuja* (540 g), *Opuntia* (510 g), and spinach (300 g). The homogenization buffer contained 250 mM sucrose, 40 mM Tris–HEPES, 2 mM Tris–EDTA, pH 7.9, supplemented with 4 mM cysteine, 0.1% BSA and 0.5 mM phenazine methosulfate. For red beet, carnations, onion, spinach leaves, *Thuja*, and *Sedum*, it also contained 0.5% polyvinylpyrrolidone. The plant material was chopped with a knife and then blended, either with a commercial mixer (Moulinex, for bulky parts) or with a Polytron homogenizer, and the homogenate filtered through four layers of cheesecloth. Some materials (maize seeds, broccoli, cauliflower, green cabbage, beans, *Opuntia* stems, and *Thuja*, fruits) were ground initially in a Waring Blendor. Materials containing fibers or gelatinous compounds were pressed over the cheesecloth during filtration. Except for the leaves, a standard centrifugation procedure was carried out (variations for some materials are described in Table I). First, two low-speed centrifugations (3500 $\times$ *g*, for 10 and 15 min, respectively) were performed in four (or six) 250-ml centrifuge tubes. The supernatants, free of floating particles, were used for further centrifugations. The third centrifugation was at 10,000 $\times$ *g* for 20 min. This spins down crude "washed mitochondria," usually contaminated, as reported elsewhere (Neuburger *et al.*, 1982; Edwards and Gardeström, 1987; Moore and Prodlove, 1987; Morré and Anderson, 1994). These "washed mitochondrial fractions" were resuspended in 3–6 mL of the washing medium (250 mM sucrose, 10 mM Na–HEPES, 0.1 mM Na–EDTA, containing 0.05% BSA) and overlaid on the top of a solution containing 28% Percoll, 300 mM mannitol, 10 mM Na–HEPES, 0.1 mM Na–EDTA, pH 7.2, in a 50-ml centrifuge tube Centrifugation on a Percoll self-generated gradient was performed at 30,000 $\times$ *g* for 45 to 60 min. The mitochondrial fraction separates as a brownish cloudly layer at two thirds from the top along the Percoll gradient. As described previously (Neuburger *et al.* 1982; Edwards and Gardeström, 1987; Moore and Proudlove, 1987; Morré and Andersson, 1994), peroxisomes form a small translucent pellet. Pigments and other materials float close to the top. In the case of onion, broccoli, and all chlorophyllous tissues, 28% Percoli gradient did not separate mitochondria from other materials (broken chloroplasts, in the case of green tissues). In some of these cases, a two-component system was successfully used: 10 ml of 60% Percoll in 300 mM mannitol, 10 mM Na–HEPES, 0.1 mM Na–EDTA, pH 7.2, overlaid with 20 ml of 28% Percoll. Mitochondrial layers were carefully removed and resuspended in about 40 ml of washing medium. At least two washing centrifugations were performed at 20 000 $\times$ *g*, for 10 min. The final pellets were resuspended in 0.5–1 ml of washing medium. Protein concentration was determined by the biuret method calibrated with standards of BSA.

Mitochondrial Oxygen Consumption and Membrane Potential Monitoring

Oxygen consumption of mitochondria was measured by a Clark oxygen probe (Yellow Springs Instru-

Table I. Respiration Properties of Tested Mitochondria[a]

Mitochondria[b] (isolation procedure)	Respiration with												
	Succinate					NADH				Pyruvate/malate			
	ADP: O	RCR	Rate III	Rate IV	Rate FCCP	ADP: O	RCR	Rate III	Rate IV	ADP: O	RCR	Rate III	Rate IV
Maize shoots[1,2]	1.7 ± 0.1	2.4 ± 0.2	195 ± 13	82 ± 2	233								
Oat shoots	0	1	37 ± 0.2	37 ± 0.2									
Wheat shoots[1,2]	0	1.4	65	45	65	0	1	45	45				
Turnip root[1]	2.0 ± 0.1	5.1 ± 0.3	160 ± 3	31 ± 2	203	1.6 ± 0.2	3.6 ± 0.3	115 ± 6	32 ± 4	3.1 ± 0.2	4.0 ± 0.8	65 ± 4	19 ± 1
Red beet root	1.9 ± 0.2	3.7 ± 0.2	219 ± 9	61 ± 0.1	260								
Carrot root	1.5 ± 0.1	2.1 ± 0.1	156 ± 11	73 ± 4	168								
Maize roots[1]	1.7 ± 0.1	2.2 ± 0.2	100 ± 4	45 ± 6	79	2.0 ± 0.2	2.5 ± 0.1	80 ± 3	32 ± 2	1.7 ± 0.1	2.4 ± 0.3	58 ± 0.3	22 ± 1
Maize seeds[3]	1.7 ± 0.2	3.1 ± 0.2	51 ± 4	16 ± 0.7	44								
Pea seeds	1.5 ± 0.1	5.6 ± 1	93 ± 1	19 ± 3	101	1.7 ± 0.1	6.5 ± 0.1	89 ± 2	13.7 ± .1	2.5 ± 0.1	6.7 ± 0.5	55 ± 0.5	8 ± 0.5
Dry beans[3]	0	1.03	36 ± 3	34 ± 2	35	1.1 ± 0.05	2.2 ± 0.05	102 ± 0.3	41 ± 0.2	1.5 ± 0.1	2.0 ± 0.1	30 ± 2	15 ± 0.4
Green beans[3]	1.5	1.7 ± 0.1	11.2 ± 0.2	6.5 ± 0.2	10	0	1	6	6	1.34 ± 0.1	2.0 ± 0.2	10 ± 1	4.8 ± 0.1
Broccoli	1.3 ± 0.1	2.6 ± 0.1	100 ± 0.5	38 ± 2	95	1.4 ± 0.2	3.5 ± 0.2	98 ± 2	28 ± 2	2.2 ± 0.1	3.7 ± 0.2	54	15 ± 0.6
Cauliflower	1.5 ± 0.1	6.5 ± 1	193 ± 5	30 ± 6	182	1.5 ± 0.1	5.0 ± 0.8	56 ± 3	11 ± 1	2.8	8.8	134	15.6
Carnation petals					36[c]				65[c]				53[c]
Onions[1]	0	1.06 ± .04	6.5 ± 0.5	6.1 ± 0.3	12								
Potato calli[1,2]	1.6 ± 0.1	1.6 ± 0.1	89 ± 2	57 ± 2[d]	100	1.5 ± 0.1	1.9 ± 0.2	69 ± 8	37 ± 0.2				
Cabbage leaves[4]	2.0 ± 0.1	3.2 ± 0.1	108 ± 5	33 ± 0.3	114	0	1.1	28	26	1.3	1.3 ± 0.1	23 ± 1	12 ± 0.1
Spinach leaves[4]	1.8 ± 0.2	1.7 ± 0.1	33 ± 2	19 ± 1[c]	32								
Mushrooms[1]	1.9 ± 0.1	3.3 ± 0.3	191 ± 2	57 ± 7	202								

[a] Average data taken from three experiments (two in some cases) and their standard deviations are shown: ADP/O- and respiratory control ratios (RCR) were calculated from individual traces and then averaged. Rates for state III (100 μM ADP), state IV, and upon uncoupler (FCCP) addition are expressed in nmol O_2 min^{-1}/mg/$protein^{-1}$ for various plant mitochondria respiring either with succinate, NADH, or pyruvate/malate.

[b] Isolation procedures are described: (1) no Percoll treatment; (2) second low-speed centrifugation omitted; (3) 2000 RPM centrifugation precedes the whole procedure; (4) 60% Percoll/28% Percoll (see Methods).

[c] Describes the cytochrome oxidase assay.

[d] In some cases, cyanide-insensitive rates were observed as 23 nmol O_2 min^{-1}/mg/protein.

[e] Cyanide-insensitive rates of 1.7 nmol O_2 min^{-1}/mg/$protein^{-1}$.

ments Co., Ohio) in a thermostated glass chamber equipped with magnetic stirring and a Teflon stopper, with a capillary hole for adding reactants using Hamilton syringes. The signal of the probe was directed via a control unit to a recorder. The basic assay medium was composed of 250 mM sucrose, 10 mM Na–HEPES, 5 mM KP_1, 20 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, pH 7.2, containing 0.05% BSA. This medium, fully saturated with air, contains 260 nmol of O_2 per ml. In some cases, the Mg^{2+} content was reduced to 1 mM. As substrates, 10 mM succinate, 1 mM NADH, or 30 mM malate/pyruvate (plus 0.5 mM NAD^+ and 0.5 mM thiamin pyrophosphate) were used. For state III–IV transition, 0.1 mM ADP (or 50 μM) was used and, at the end of each run, 1 mM KCN was added. Evaluation of cytochrome oxidase activity was achieved after addition of 20 μM cytochrome and *c* and 10 mM ascorbate.

The mitochondrial membrane potential was monitored by a laboratory-made tetraphenyl phosphonium (TPP^+) electrode (J.A.F. Vicente). Its signal was fed to a pH meter connected via a control unit to a recorder. TPP^+ (3 μM) was used during the measurement and the standard medium was identical, as described for the oxygen probe, except that it contained 1 mM Mg^{2+}. Estimations of mitochondrial membrane potential values in mV were done according to Kamo *et al.* (1979) and Kesseler *et al.* (1992).

Polyacrylamide Gel Electrophoresis and Western Blotting

Mitochondrial samples usually containing 200 μg protein were mixed with a sample–lysis buffer and left at 100°C for 5 min. Prolonged exposure was required to cleave the PUMP dimer. Laemli SDS–PAGE was performed in 12% slab gels. Western blotting (Timmons and Dunbar, 1990) was performed with primary antibodies raised against potato PUMP and secondary alkaline phosphatase-conjugated anti-IgG antibodies, developed with nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) tablets, (Sigma) and photographed. Potato PUMP was isolated on hydroxylapatite, as described previously (Ježek *et al.* 1997), and verified as PUMP by MALDI-mass spectroscopy analysis of peptides that resulted from trypsin cleavage (Růžička *et al.*, 1999).

RESULTS

Quality of Plant Mitochondria Isolated from Various Species

Functional screening of PUMP in plant tissues or organs and among several diverse species requires the isolation of purified mitochondria, capable of at least some energization due to the function of respiratory chain. This aim was achieved with the majority of plant materials tested and most of mitochondrial samples were sufficiently pure, exhibiting tightly coupled respiration with succinate, NADH, or malate–pyruvate (plus NAD^+–thiamin pyrophosphate), as well as phosphorylation (Fig. 1a–g; Table I). It is documented by average ADP/O- and respiratory control ratios, listed in Table I. For example, we succeeded in isolation of phosphorylating mitochondria from dry maize seeds and soft peas taken from the pod (Fig. 1, traces d and e) (cf. Sato and Asahi, 1975). The presence of BSA in the assay medium was essential, since, in its absence, most of the mitochondrial fractions lose their respiratory control and respiration rates were inhibited by unknown factors. Also, the initial addition of a small amount of ATP (0.2 mM) was required for optimal phosphorylation, as indicated by improved ADP/O and respiratory control ratios. Such preconditioning or activation of succinate dehydrogenase has already been described for plant mitochondria (Laties, 1973).

Preparations of onion bulb, wheat shoot, and oat shoot mitochondria exhibited a cyanide-sensitive respiration, but almost no sign of phosphorylation. For example, FCCP accelerated the respiration of onion mitochondria and all above-mentioned mitochondria were energized, as monitored by TPP^+ electrode (see below). Properties of mitochondria from bean seeds were peculiar, as described below. Carnation petal mitochondria exhibited only succinate-supported respiration when external cytochrome *c* was added (Fig. 1h). An assay with the added cytochrome *c* and ascorbate permitted the estimation of cytochrome oxidase activity (Drooillard and Paulin, 1990). We were not successful in isolating mitochondria from young 2-month-old maize leaves, *Opuntia* stems, and young green *Thuja* cones. Our preparation of crude *Sedum* mitochondrial fraction also did not respire.

Uncoupling Induced by Linoleic Acid (LA) in Plant Mitochondria

Since fatty acids are required cofactors in inducing PUMP-mediated uncoupling of mitochondria, we

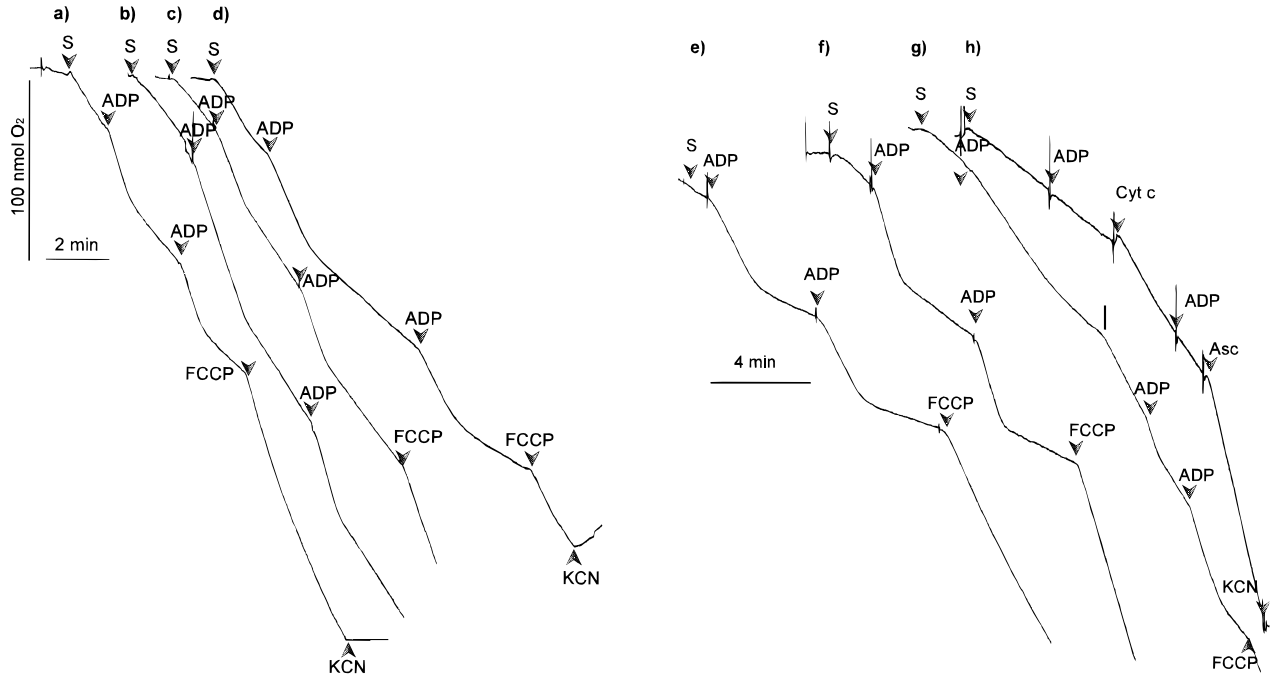

Fig. 1. Respiratory control in isolated mitochondria from selected examples of tested samples. (a) mushrooms [(*Agaricus bisporus* (J.Lange) Imbach]; (b) germinating maize shoots (*Zea mays* L., cv. Hybrid-3352/Palma-Pioneer); (c) carrot roots (*Daucus carota* L.); (d) mature maize seeds (as above); (e) young pea seeds (*Pisum sativum* L.); (f) cauliflower (*Brassica oleracea* L.); (g) germinating maize roots (the same plants as in b); (h) white carnation petals (*Diantus caryophyllus* L.). Samples (c)(e), and (h) were purified using Percoll gradient (0–28%) centrifugation (see Methods). Clark oxygen electrode was used to monitor O_2 consumption in suspensions of respiring mitochondria (0.3 mg protein). ATP (2 mM) was added to the assay medium containing 250 mM sucrose, 10 mM Na–HEPES, 5 mM KP_1, 20 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, pH 7.2, and 0.05% BSA. When indicated, a substrate, 10 mM succinate "S", 0.1 mM ADP (except for second addition in grams), where 50 μM was used), 1 μM FCCP, and 1mM KCN were added. Note, the time scale is different for (a) to (d) and for (e) to (h); for (g) the indicated line is doubled. Evaluation of cytochrome oxidase activity for (h) was achieved after addition of 20 μM cytochrome *c* ("Cyt c") and 10 mM ascorbate ("Asc").

tested sensitivity of isolated plant mitochondria to LA, either by monitoring of the accelerated respiration (Table II) or mitochondrial transmembrane potential decrease, $\Delta\Psi$ (Table III). For the latter, we choose a TPP^+ electrode. Most mitochondria were significantly uncoupled by 50 to 75 μM LA in the presence of 7.5 μM BSA (Fig. 2; Tables II and III). The concentration of LA at which the respiration rate with succinate doubled was minimum for maize shoot mitochondria (40 and 7.5 μM BSA). Mitochondria from mushrooms [*Agaricus bisphorus* (J.Lange) Imbach] exhibited a doubled respiration rate at high concentrations of LA (100 and 7.5 μM BSA). With most of mitochondria, LA at higher concentrations became inhibitory in the sense that FCCP did not further accelerate respiration. TPP^+ electrode monitoring of $\Delta\Psi$ decrease was routinely measured with atractylate in the assays without ATP preconditioning (Table III), to rule out the ADP/ATP carrier participation. Indeed, in some cases (bean seeds, potato calli, wheat shoots, and broccoli), the

Table II. Uncoupling of Plant Mitochondria by Linoleic Acid (LA) as Monitored from Respiration with Succinate[a]

Mitochondria (isolation procedure)[b]	20 μM LA	50 μM LA	50 μM LA & ATP	75 μM LA	90 μM LA
Maize shoots[1,2]	1.39	2.45	1.75		
Turnip root[1]	1.23	3.52	2.62	3.9	
Red beet root		1.9	1.7	2.7	
Maize roots[1]	1.1			1.4	
Pea seeds	1.02	1.82		3.35	3.41
Cauliflower	1.07			1.6	
Cabbage leaves[4]		1.17		2.57	2.4
Mushrooms[1]	1.03	1.22	1.22		2.1

[a] Ratios of respiration rates in the presence of LA vs state IV rates are listed for several LA concentrations (at 50 μ*M*; rates measured with 3 mM ATP are also indicated) and selected samples of plant mitochondria. For other conditions see legend.

[b] See Table I, footnote b for explanation of symbols.

Table III. ATP-Sensitive Uncoupling by Linoleic Acid (LA) as Monitored by TPP^+ Electrode in Various Plant Mitochondria[a]

Mitochondria[b] (isolation procedure)	[LA]: (μM)	ΔΨ decr (mV)	ΔΨ decr and ATP (MV)	% Inhibition by ATP	ΔΨ before LA (mV)	ΔΨ after LA (MV)	ΔΨ after LA and ATP (mV)	ΔΨ and ATP before LA (mV)	ΔΨ and ATP after LA (mV)
Maize shoots[1,2]	22	13	10	23	223	210	216	223	213
Wheat shoots[1,2]	50	15	8	47	145	130	131	156	148
Turnip root[1]	75	26	23	12	216	190	197	220	197
	50	8	7	12					
Carrot root	75	32	19.5	39	218	186	203	214.5	195
		31	19.5	37					
Maize roots[1]	75	27	1	96	182	155	176	177	176
		17	1	94					
Maize seeds[3]	50	21	8	62	199	178	193	204	196
	25	5	1	80					
Pea seeds	75	8	15	0	216	208	209	221	206
Broccoli	75	15	10	33	207	192	197	217	207
Cauliflower	75	23	14	39	203	180	193	214	200
Potato calli[1,2]	50	9.2	5	44	198	190	193	202	197
Cabbage leaves[4]	75	29	30	0	207	178	188	214	184
Spinach leaves[4]	50	43	16	63	176	133	151	164	148
Mushrooms[1]	75	9	9	0	202	193	194	204	195

[a] Table shows typical data for selected samples of plant mitochondria: namely, the magnitudes of LA-induced membrane potential (ΔΨ) decrease ("decr") in the absence or presence of 3 mM ATP, the corresponding % inhibition by ATP, and maximum magnitudes of ΔΨ values from typical pairs of experiments, such as depicted in Fig. 2, conducted without preconditioning and in the presence of 20 μM atractyloside (except for spinach leaves). For other conditions, see Fig. 2 legend.

[b] See Table I, footnote b, for explanation of symbols.

ΔΨ decrease induced by LA was maximized in the absence of atractylate, suggesting participation of the ADP/ATP carrier. In preconditioned spinach leaf mitochondria 50 μM LA (7.5 μM BSA) induced a ΔΨ decrease of 43 mV. The typical experiments with atractylate and 75 μM LA (plus 7.5 μM BSA), yielded a maximum ΔΨ decrease for carrot, (green) cabbage leaves, and maize root mitochondria (32, 29, and 27 mV, respectively; Table III). Other tested mitochondria exhibited a ΔΨ decrease between 10 and 20 mV and a minimum ΔΨ decrease was observed for mitochondria isolated from mushrooms and soft immature pea seeds taken from the pod (Table III).

The observed fatty acid-induced uncoupling might still result from the function of other carriers, besides the ADP/ATP carrier. However, in plant mitochondria only PUMP should be sensitive to ATP, GTP, or GDP (Ježek *et al.*, 1998). ATP (3mM), added after LA, either restored high ΔΨ to the values close to those achieved prior to the LA addition or, at least, substantially increased ΔΨ, in some cases (Fig.2; Table III). Magnitudes of ΔΨ were usually higher with 3 mM ATP (Table III). These results already indicate a possible existence of PUMP function. Nevertheless, for the definitive demonstration of PUMP function, we compared LA-induced uncoupling in the absence and in the presence of ATP, as described below.

Demonstration of PUMP Function in Plant Mitochondria

The best indication of PUMP function is the nucleotide-sensitive fatty acid-induced uncoupling (Vercesi *et al.*, 1995, 1998; Ježek *et al.*, 1996, 1997). To assess the net effect of ATP on LA-induced uncoupling, we tested how ATP prevents the ΔΨ decrease caused by LA. Most of the tested mitochondria behaved in accordance to the assumed function of PUMP, *i.e.*, showed ATP inhibition of the LA-induced uncoupling. Mitochondria exhibited a lower ΔΨ decrease upon the addition of LA with 3 mM ATP with regard to the ΔΨ decrease in the absence of ATP (Fig. 2; Table III). Mitochondria from maize roots that were completely protected from LA-induced uncoupling by 3 and 0.5 mM ATP still inhibited it by 80% (Fig. 2b). ATP (3mM) strongly prevented a ΔΨ decrease induced by 50 μM LA in maize seed (Fig.

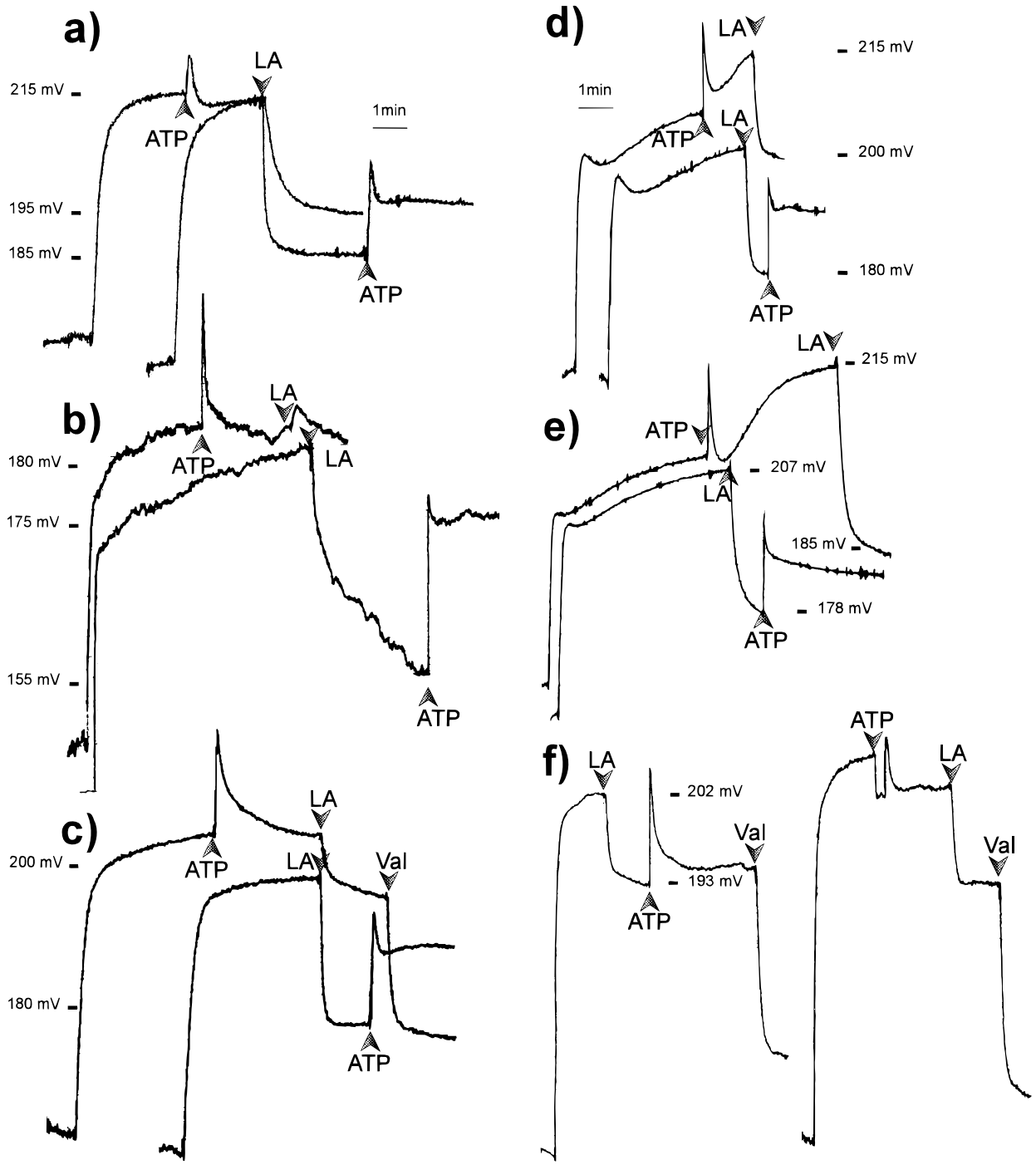

Fig. 2. Fatty acid-induced uncoupling in several types of plant mitochondria—TPP^+ electrode monitoring of membrane potential. Mitochondria were isolated from: (a) carrot roots (*Daucus carota* L.), 0.23 mg mitochondrial protein; (b) germinating maize roots (*Zea mays* L., cv. Hybrid-3352/Palma-Pioneer), 0.47 mg protein; (c) mature dry maize seeds [as in (b)], 0.8 mg protein; (d) cauliflower (*Brassica oleracea* L.); 0.28 mg protein; (e) green cabbage (*Brassica oleracea* L., var. *sabanda*), 0.27 mg protein (f) mushrooms [*Agaricus bisporus* (J. Lange)Imbach], 0.43 mg protein; and (g) spinach leaves (*Spinacea oleracea* L.) 0.43 mg protein. Sample (a) was purified using Percoll gradient (0–2.8%) centrifugation; for sample (e) and (g), a two-component Percoll system (28–/60%) was used (see Methods). Trace pairs are shown for each sample, illustrating the different order of additions of LA and ATP. Thus, ATP prevention of LA-induced uncoupling in the presence of 20 μM atractyloside [except for (g)] was evaluated as a measure of functional PUMP. Usually, 75 μM LA was added [except (c) and (g) where 50 μM was used] and 3 mM ATP (Tris salt). In some traces, 1 μM valinomycin (Val) was added at the end of the run. No ATP was used for preconditioning [except for (g)]. The assay medium contained 250 mM sucrose, 10 mM Na–HEPES, 5 mM KP_i, 20 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, pH 7.2, 0.05% BSA (7.5 μM), 20 μM atractyloside, and 10 mM succinate.

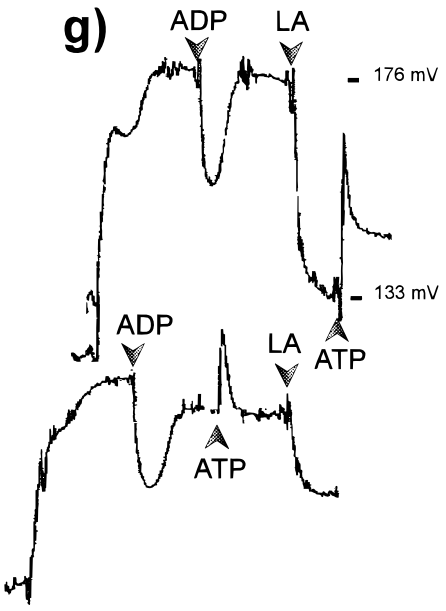

Fig. 2. Continued

2c) and spinach leaf mitochondria (Fig. 2g). It also prevented about one half of the $\Delta\Psi$ decrease in mitochondria from wheat shoots, carrot root (Fig. 2a), cauliflower (Fig. 2d), and potato calli. Partial prevention was found in mitochondria of broccoli, maize shoots, and turnip root (Table III). The assays without atractyloside and with preconditioning by 0.2 mM ATP showed similar results, but ATP inhibitory ability seemed to be lower. No prevention by ATP was observed in the case of mushrooms (Fig. 2f), young pea seed (Table III), and green cabbage mitochondria (Fig. 2e). Accordingly, these mitochondria were relatively insensitive to LA-induced uncoupling. Apparently, mitochondria exhibiting the ATP-sensitive fatty acid-induced uncoupling putatively contain PUMP.

Peculiar Properties of Bean Seed Mitochondria

Mitochondria isolated from bean seeds (*Phaseolus multiflorus* L.), from either dry mature seeds of a colorful pink/purple variety (Fig. 3) or from young soft seeds of a green variety showed some respiratory control, namely, when respiring with pyruvate/ malate (Table I) and exhibited ATP-induced uncoupling with succinate or NADH as a substrate. This uncoupling induced by ADP-free ATP was not observable after a previous uncoupling cycle induced by 75 μM LA (Fig. 3a). Also, LA added after the cycle of ATP-induced uncoupling did not further depress $\Delta\Psi$. However, oligomycin added to bean seed mitochondria respiring with succinate, preconditioned with 0.2 mM ATP, which were capable of oxidative phosphorylation, completely reversed the ATP-induced uncoupling, and LA uncoupled normally after this reversal (Fig. 3b). Oligomycin acted only transiently with NADH. Further study of these effects is beyond the scope of this study. However, note that a similar phenomenon has previously been observed in yeast mitochondria (Prieto *et al.*, 1992).

Immunodetection of PUMP in Mitochondrial Fractions of Various Plant Organs, Tissues, and in Selected Species

Assuming the presence of PUMP in those mitochondrial samples that exhibit ATP inhibition of LA-induced uncoupling as the working hypothesis, based on functional parameters, we confirmed this hypothesis by immunoblotting (Fig. 4). The only exception were the samples of green cabbage mitochondria, for which the functional test failed, but the immunoblotting revealed the positive presence of a possible PUMP dimer and a possible monomer showed a retarded migration on SDS– PAGE. A sufficient PUMP content

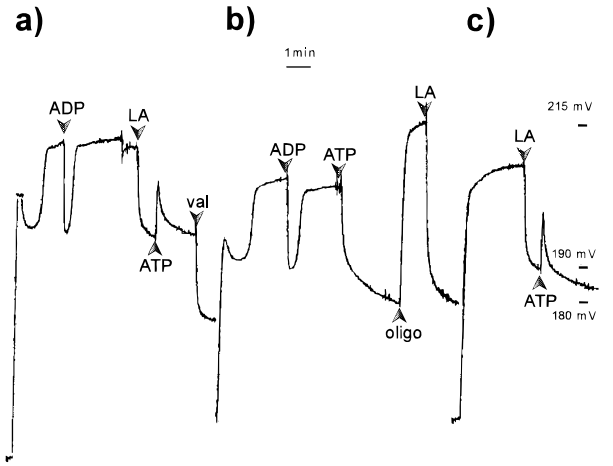

Fig. 3. ATP-induced uncoupling in mitochondria isolated from bean seeds—TPP^+ electrode monitoring of membrane potential. Three experiments (a,b,c) were performed as described in the Fig. 2 legend, but with mitochondria isolated from dry bean seeds (*Phaseolus multiflorus* L., a purple variety) respiring with succinate and with preconditioning by 0.2 mM ATP (a,b) or without preconditioning in the presence of 20 μM atractyloside (c). In experiment (b), 3 mM ATP caused potential decrease from 205 to 180 mV, whereas subsequent oligomycin addition (1 μg/ml) resulted in the recovery of energization to 216 mV; LA addition (80 and 7.5 μM BSA) again caused a potential decrease to 180 mV. In parallel experiments (a) and (c), ATP added after LA did not significantly change the established lower potential value. The phosphorylation ability of bean seed mitochondria is indicated by transient potential changes upon ADP addition (50 μM).

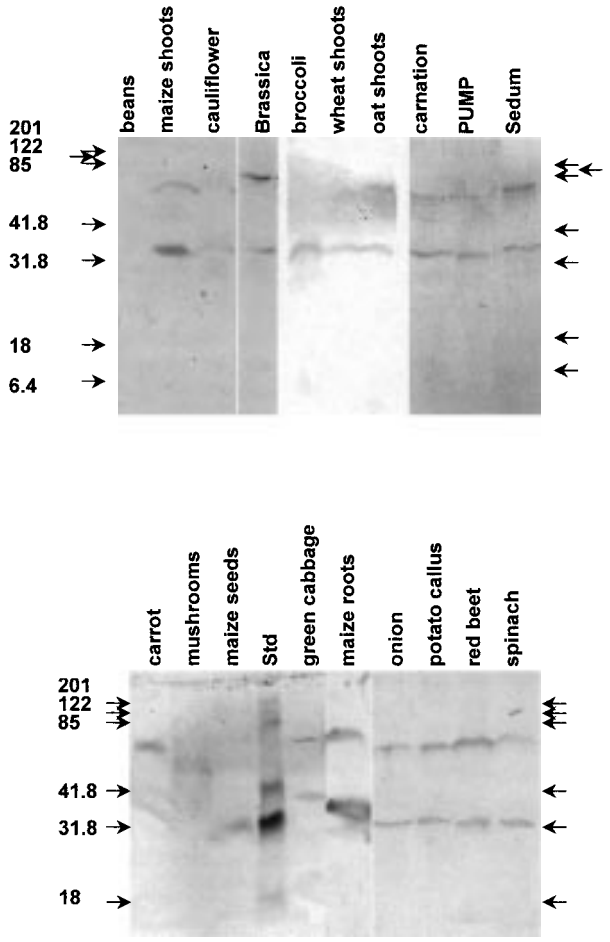

Fig. 4. Western blots of mitochondrial samples isolated from various species or plant parts or organs. Photographs of Western blots for mitochondrial samples indicated by the legend are shown ("PUMP" is the isolated potato PUMP protein, approx. 3 μg), when the primary antibodies raised to the isolated potato PUMP (Ježek *et al.*, 1998; Costa *et. al.*, 1999) and secondary alkaline phosphatase-conjugated anti-lgG antibodies were used. Development of blots proceeded with NBT/BCIP tablets (Sigma). Numbers and arrows indicate positions (in kD) of pre-stained M_w standards ("Std") (Bio-Rad).

giving detectable staining on Western blots using alkaline phosphatase and NBT/BCIP was observed in almost all types of tested mitochondria, except for mushrooms, pea seeds (not shown), and beans. A strong immunoreaction with rabbit anti-potato-PUMP antibodies was observed in mitochondria isolated from maize shoots, maize seeds and roots, onion, potato calli, red beet, spinach, broccoli, wheat shoots, oat shoots, carnation petals, and *Sedum* leaves, whereas a weaker reaction was observed with samples of turnip (*Brassica*) and even weaker with cauliflower. Carrot mitochondria showed only a PUMP dimer of 65 kDa.

DISCUSSION

PUMP Might Be Ubiquitous in Plants

This study was devoted to screening PUMP in mitochondria isolated from several tissues and organs of several plant species. Such screening is particularly relevant to our understanding of its biochemical and physiological role, determining if its presence can be assigned to any particular tissue or organ, or if its distribution is selective to particular lines of evolution in the Plant Kingdom. If the presence of PUMP proves ubiquitous, its role may be rather general and not related to any particular or special function. Nevertheless, it must account for all possible physiological situations when oxidative phosphorylation is regulated. In our screening, we have indicated PUMP either functionally or immunologically in most of the tested species and in all tested tissues or organs including seeds

(excluding the leguminous seeds), roots, stems, leaves, and flowers. We have also demonstrated that PUMP is a widespread protein among a variety of angiosperms (*Magnoliophyta*, with ovules and seeds enclosed in vessel-like carpels and fruits)—either monocots or dicots (Fig. 5). Concerning monocots (or monocotyledons, having a single cotyledon on their embryos), we added to the existing list of PUMP-containing species, *e.g.*, banana (*Musa* sp.) and pineapple (Ježek *et al.*, 1998), maize, wheat, oat, and onion. PUMP was found in maize shoots, roots, and mature seeds. The existence of PUMP in maize is also confirmed by the finding of an incomplete homologous sequence in the plant EST database (Genebank accession Al677128). It is reasonable to suggest that PUMP is also putatively present in species with specific metabolisms, as we have shown in one example of C4-type plant (maize) and one of CAM (crassulacean acid metabolism) plants (*Sedum*). Other tested species included dicots. Finally, we demonstrated that mitochondria isolated from mushrooms and pea or bean seeds exhibited neither functional nor immunological indications of the presence of PUMP. For mushrooms it correlates with their minimum sensitivity to FAs; for beans, it fits with their peculiar properties in having ATP-induced uncoupling. We should also note that negative results in Western blots could originate from an insufficient cross reactivity of anti-potato-PUMP antibodies with PUMP of some species. Nevertheless, this fact does not contradict the idea of the widespread existence of PUMP. In conclusion, we extend our observations to the hypothesis that PUMP is a rather of ubiquitous protein in higher plants and is contained in the mitochondria of most plant tissues and organs. Moreover, there are no expected differences of its phenotype in different tissues. However, the different biochemical (ligand-

Kingdoms:

Monera					
Protista					
Animalia	Invertebrate animals				
	Vertebrate animals	....		**mammals**	UCP1, UCP2,UCP3, UCP4, BMCP
Mycetae	*Zygomycetes*	....		***Saccharomyces***	UCPs were not found[1]
	Ascomycetes				
	Basidiomycetes	....		**mushrooms**	PUMP not found
Plantae	Nonvascular plants				
	Ferns				
	Higher plants	....	*Monocots*	this paper	other papers
				Onion	**Banana [2]**
				Wheat	**Pineapple [2]**
				Oat	**Sugar cane [3]**
				Maize (C4)	
		....	*Dicots*	***Sedum* (CAM)**	**Potato [4]**
				Red beet	***Arabidopsis* [5]**
				Turnip	**Tomato [6]**
				Carrot	**Orange [2]**
				Cauliflower	**Papaya [2]**
				Cabbage	**Musk melon [2]**
				Broccoli	**Peach [2]**
				Spinach	**Apple [2]**
				Carnations	**Pear [7]**
					Avocado [7]
					Mango [7]
					Strawberry [7]
					Tobacco [7]

Fig. 5. Current indications of presence of UCPs or PUMP. Diagram shows a list of species in which PUMP has been found either in this work or in the following papers (as indicated by numbers in superscripts): 1. El Moualij *et al.*, 1997; 2. Ježek *et al.*, 1998; 3. Arruda, P., CBEMG, University of Campinas, Brazil, personal communication; 4. Ježek *et al.*, 1996, 1997; Vercesi *et al.*, 1995; Laloi *et al.*, 1997; 5. Maia *et al.*, 1998; 6. Jarmuszkiewicz *et al.*, 1998; Costa *et al.*, 1999; Almeida *et al.*, 1999; 7. Ježek *et al.*, 2000.

gated or covalent modifications) or transcriptional regulations could be manifested as prerequisites for specific PUMP functions.

Basic Physiological Roles of PUMP

We might consider the basic physiological roles of PUMP to be identical to those valid for UCP2 and other UCPs of mammalian mitochondria. The two of these functions are derived from the thermodynamic considerations for oxidative phosphorylation, when the coupling factor is slightly less than 1 (Ježek *et al.*, 1998). This means that the basic roles of PUMP are manifested during situations of mild uncoupling. It is reasonable to expect that in plants the existing biochemical and transcriptional regulations allow these basic functions to be in effect most of the lifetime. The nonequilibrium thermodynamic treatment proved that the efficiency of the whole organism is optimum at slight uncoupling. Hence, the first basic role of PUMP lies in allowing an accelerated metabolic rate because of the accelerated respiration resulting from mild uncoupling. The second basic role of PUMP that is inherent to the first lies in the concomitant mild thermogenesis. The third role of PUMP, UCP2, and other novel mammalian UCPs, includes the decreased formation of reactive oxygen species as a consequence of mild uncoupling (Kowaltowski *et al.*, 1998). It has been demonstrated that the decrease in membrane potential induced by PUMP in potato mitochondria inhibits mitochondrial generation of reactive oxygen species at the level of the semiquinone forms of coenzyme Q. Increased respiration rates decrease the lifetime of the semiquinone forms of coenzyme Q and reduce the probability of superoxide anion formation through the electron transfer from coenzyme Q to molecular oxygen. Another basic role of PUMP is an autodefense role against oxidative stress, avoiding injury to organelles and tissues. Namely, mitochondria of germinating tissues must be conserved intact during long dormancy periods, as well as tissues of flowers during flowering, or photosynthetic tissues. Some other enzymes, such as an alternative oxidase can exert the same role.

Possible Specific Physiological Roles of PUMP

The presence of PUMP in the mitochondria of fruit pericarp suggests a putative physiological role of mitochondria in fruit development (Ježek *et al.*, 1998; Almeida *et al.*, 1999; Costa *et al.*, 1999). It has been postulated that a regulated uncoupling is useful during fruit ripening and senescence, which terminates all synthetic processes and favors the initiation of seed dormancy (Ježek *et al.*, 1996, 1998). The presence of PUMP in maize seeds found in this work analogously suggests a useful role of active PUMP during seed dormancy and its switch off during germination. PUMP might also provide thermogenesis to promote effective germination. Seeds are complex organs, being cotydelonous, where energetic reserves are accumulated. It is expected that seed mitochondria originate mostly from cotydelons. The presence of PUMP in germinating seeds may be related to a fine control of oxidation flow. We hypothesize that germination, on energetic grounds, might proceed in two sequential steps: (1) a stage with a slightly increased uncoupled respiration (PUMP) that serves the purpose of temperature increase to accelerate metabolism and cell activity; (2) a stage when regulatory mechanisms downregulate PUMP; its activity decreases, resulting in the increased efficiency of oxidative phosphorylation, which provides extra ATP for tissue building during germination.

The presence of PUMP in shoots may serve a similar purpose as described for seeds. PUMP in root mitochondria might fulfill all basic roles inherent to situations of mild uncoupling. Some, yet unknown, specific role of PUMP in roots is suggested by our finding that maize root mitochondria exhibited the strongest ATP sensitivity of LA-induced uncoupling. In storage tap roots, *e.g.*, carrot, turnip, and red beet, another unknown regulation might activate PUMP to provide an intermediate or complete uncoupling that would result in more pronounced thermogenesis, similar to that suggested for potato tubers (Nantes *et al.*, 1999).

Functional tests, as well as Western blots, also indicated the presence of PUMP in spinach leaves. This is one of the first indications of PUMP presence in main photosynthetic tissue (besides PUMP in tobacco leaf mitochondria; P. Arruda, personal communication). Potato PUMP mRNA was also indicated in potato leaves (Laloi *et al.*, 1997). The role of PUMP in leaf mitochondria is more difficult to determine. The presence of PUMP in leaf mitochondria may well serve as an O_2 detoxifying process, because of the massive O_2 production from photosynthesis. PUMP function in thermogenesis may be relevant during the night to maintain metabolism at the required rate. In addition, the role of PUMP in preventing freezing is

an attractive hypothesis. More physiological or genetic studies, such as PUMP overexpression or construction of transgenic plants with a removed PUMP gene, will be required to assess the role of PUMP in leaves.

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