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ZmPUMP encodes a fully functional monocot plant uncoupling mitochondrial protein whose affinity to fatty acid is increased with the introduction of a His pair at the second matrix loop

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Abstract

Uncoupling proteins (UCPs) are specialized mitochondrial transporter proteins that uncouple respiration from ATP synthesis. In this study, cDNA encoding maize uncoupling protein (ZmPUMP) was expressed in *Escherichia coli* and recombinant ZmPUMP reconstituted in liposomes. ZmPUMP activity was associated with a linoleic acid (LA)-mediated H^+ efflux with K_m of 56.36 ± 0.27 μM and V_{max} of 66.9 μmol H^+ min^{-1} (mg prot)$^{-1}$. LA-mediated H^+ fluxes were sensitive to ATP inhibition with K_i of 2.61 ± 0.36 mM (at pH 7.2), a value similar to those for dicot UCPs. ZmPUMP was also used to investigate the importance of a histidine pair present in the second matrix loop of mammalian UCP1 and absent in plant UCPs. ZmPUMP with introduced His pair (Lys155His and Ala157His) displayed a 1.55-fold increase in LA-affinity while its activity remained unchanged. Our data indicate conserved properties of plant UCPs and suggest an enhancing but not essential role of the histidine pair in proton transport mechanism.

Keywords: Uncoupling mitochondrial protein; UCP; Proton transport; Functional reconstitution; Mitochondrial carriers; Plant mitochondria; Maize

Mitochondrial inner membrane uncoupling proteins (UCP) are specialized free fatty acid (FFA)-linked H^+ recycling proteins, returning protons that have been expelled from matrix by the respiratory chain, thus promoting a non-phosphorylating bypass [1,2]. The first characterized uncoupling protein from brown adipose tissues, UCP1, promotes heat production through regulated mitochondrial uncoupling [3,4].

The finding of a UCP-like protein in potato [5] denoted that all UCPs probably evolved from a common ancestral protein preceding the divergence of animals and plants [6]. Despite several years of biochemical and physiological experimentation, the precise role of the uncoupling proteins in plants still remains unclear. It has been proposed that UCP-mediated mild uncoupling of mitochondrial respiration could regulate reactive oxygen species production [7,8]. Indeed, over-expression of an uncoupling protein from *Arabidopsis thaliana* (AtPUMP1) in transgenic plants led to a higher tolerance to oxidative stress [9]. Further support to this antioxidant role was recently provided in mammals and plants [10,11]. Energy-dissipating systems such as the one related to uncoupling proteins could also have important implications in general energy metabolism [1,6].

Several cDNAs encoding UCP-like proteins have been identified in a variety of plant species, in both dicots (*AtPUMP1* and *2* in *Arabidopsis* [12,13], *StUCP* in potato [14], *SfUCP* in skunk cabbage [15]) and monocots (*WhUCP* in wheat [16], *OsUCP* in rice [17], and *ZmPUMP* in maize [18]). Although the genes are widely present in various plant species, only dicot proteins have been biochemically and functionally characterized as uncoupling proteins at present [14,19–22]. In fact, a survey of the literature reveals only one report demonstrating an activity reminiscent of UCP in a monocot species [23]. Preliminary biochemical data were derived from reconstitution

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experiments using UCP-enriched fractions purified from potato and tomato [19,20]. Subsequently, the uncoupling activity was investigated using isolated cDNAs and heterologous expression systems. The uncoupling protein from potato (StUCP), for example, was expressed in yeast and shown to promote proton transport [14]. A more precise biochemical characterization was achieved using recombinant AtPUMP1 expressed in *Escherichia coli* and reconstituted into liposomes [21]. In this regard, the major properties of the uncoupling activity of plant UCPs matched those described for their mammalian counterpart, i.e., stimulation by FFA and specific inhibition by purine nucleotides. However, more recent data suggest that superoxide is involved in the activation of StUCP [11].

Whereas the dicot proteins are functionally well characterized, for instance there is no experimental evidence showing whether UCPs from monocots keep the same regulatory properties. A common feature of all known plant UCPs is the absence of a histidine pair in the matrix loop of the second repeat domain of the protein. These residues have been shown, by mutagenesis studies, to be crucial for the FFA-activated proton transport of mammalian UCP1 [24,25]. A Lys at position 155 and an Ala at position 157 replace both histidines in the primary amino acid sequence of ZmPUMP.

We have recently isolated a cDNA (termed *ZmPUMP*) encoding an uncoupling protein from maize (ZmPUMP) [18]. *ZmPUMP* expression is induced by oxidative stress and is cold insensitive [18]. This representative monocot uncoupling protein was expressed in *E. coli* inclusion bodies in order to evaluate its biochemical activity and functional relationships with proteins from dicot plants. Furthermore, we sought to investigate whether the introduction of a histidine pair in the second matrix loop segment of ZmPUMP, as originally found in UCP1, would affect the kinetic properties of the protein. For that, a ZmPUMP substitution mutant carrying a His pair at the corresponding segment was biochemically characterized.

Materials and methods

Materials. Linoleic acid (LA), nucleotides, and other chemicals were from Sigma, undecanesulfonate from Lancaster (UK), and Bio-Beads SM2 from Bio-Rad. Fluorescent probe 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ) was from Molecular Probes. Hybond N membranes and Hyperfilms MP were from Amersham and the CPSD (disodium 3-(4-methoxyspiro{1.2-dioethane-3.2′-(5′-chloro)tricyclo [3.3.1.1$^{3.7}$] decane} -4-yl)phenyl phosphate) was from Applied Biosystems (Tropix).

Construction of the ZmPUMP expression vector. A plasmid carrying the ZmPUMP cDNA [18] was used as template for polymerase chain reaction (PCR) amplification. The ZmPUMP coding region was amplified in a standard PCR using the gene-specific primers ZmNde (5′-GGAA TTCCATATGCCAGGGGACCACGGC-3′) and ZmBam (5′-CGGGA TCCTCAGCTTGTCGCTTTCC-3′). The forward and reverse primers contained *Nde*I and *Bam*HI restriction sites (underlined), respectively, for direct cloning of the PCR product. The amplified DNA fragment was digested with *Nde*I/*Bam*HI, gel purified, and cloned into identically digested pET-28b (Novagen) for protein expression. The resulting construct (pET:ZmPUMP) was sequenced in an ABI-PRISM 3100 automatic sequencer (Perkin-Elmer).

Expression of ZmPUMP, isolation of inclusion bodies, and analysis of the recombinant protein. *Escherichia coli* BL21(DE3) pLysS cells were transformed with the vector pET:ZmPUMP. Transformed cells were grown in Circle Grow medium containing kanamycin (20 μg/ml) and chloramphenicol (25 μg/ml) at 37 °C and induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. The cells were harvested by low-speed centrifugation, resuspended in lysis buffer [100 mM Tris–HCl, pH 7, 0.5 mM EDTA, and 5 mM dithiothreitol (DTT)], and submitted to six freezer-thaw cycles (liquid nitrogen/37 °C). Further lysis of the cells and break of genomic DNA were achieved by sonication (6× 30 s). The suspension was centrifuged (20,000*g* for 30 min), the pellets of inclusion bodies were collected and washed three times with 1 M urea containing 1% Triton X-100. The denaturants were washed out in final wash buffer (50 mM Tris–HCl, pH 7.0, 1 mM EDTA, and 2 mM DTT).

The expressed protein was analyzed by SDS–PAGE (0.1% SDS, 12% polyacrylamide) stained with Coomassie blue. Immunoblot assays were performed according to standard protocols using anti-AtPUMP1 polyclonal antibodies [21] diluted 1:1000. The bands were detected by autoradiography using CSPD (1:2000).

Reconstitution of ZmPUMP and fluorescent monitoring of H^+ fluxes. Reconstitution of the recombinant protein was performed essentially as described by Borecký et al. [21] with minor modifications. Four milligrams of inclusion bodies was washed twice in 1 ml of internal medium [IM; 28.8 mM tetraethylammonium *N*-tris [hydroxymethyl]methyl-2-aminoethanesulfonate (TEA–TES; $[TES^-]_{free} = 9.2$ mM), pH 7.2, 84.4 mM TEA_2SO_4, and 0.6 mM TEA–EGTA]. The pellet was solubilized in 1 ml of IM containing 1.67% sodium lauroylsarcosinate and 1% decylpolyoxyethylene (decylPOE) at 4 °C for 2.5 h. The insoluble fraction was removed by centrifugation. This procedure yielded a crude preparation (with a negligible contamination by inclusion body proteins) used for reconstitutions.

Lipid film was prepared from ethyl ether solution of 39 mg egg yolk lecithin, 1.66 mg cardiolipin, and 0.66 mg phosphatidic acid by stream of nitrogen. The film was hydrated in 875 μl of IM by vortexing at 50 °C under nitrogen and solubilized by adding 56 mg of decylPOE. The micelles were cooled down on ice, mixed with 2 mM SPQ probe plus solubilized ZmPUMP (lipid/protein ratio of 410:1), and then incubated in a column of Bio-Beads SM2 at 4 °C for 2.5 h. Proteoliposomes were recovered by centrifugation. Traces of detergent were removed by reincubation of proteoliposomes with fresh Bio-Beads for 10 min and recentrifugation. External SPQ was removed by gel-filtration through Sephadex G25-300 spin column. All columns were pre-washed with IM. The resulting proteoliposomes contained ~100 μg of incorporated protein.

H^+ flux assays were based on the quenching of SPQ fluorescence by TES^- [26]. Vesicles (30 μl) were added to 1.5 ml of external medium (EM; 28.8 mM TEA–TES [9.2 mM TES^-], pH 7.2, 84.4 mM K_2SO_4, and 0.6 mM TEA–EGTA). Linoleic acid was added after 20 s and, 20 s later, an H^+ efflux was initiated by 1.3 μM valinomycin. The fluorescence was calibrated to $[H^+]$ by the addition of 10 aliquots of 6 μmol of KOH in the presence of 1.5 μM nigericin to proteoliposomes suspended in IM. Fluorescent data were converted into "H^+ traces" by fitting with modified Stern–Volmer equation

$$[H^+] = (1/K_q) \cdot (F_0 - F)/(F - L), \quad (1)$$

where F is the experimental and F_0, the unquenched fluorescence. Parameters K_q (quenching constant) and L (background, mostly light scattering) were obtained by linear regression of F vs. $(F_0 - F)$/[KOH] plot.

Rates of H^+ efflux were derived from "H^+ traces", multiplied by the internal proteoliposome volume (V, estimated from volume distribution of the SPQ [2]), and corrected for protein content to yield final rates in μmol H^+ min^{-1} (mg prot)$^{-1}$. When expressed per μmol of PUMP dimer, rates represented the minimum values for the turnover number, since not all of the protein was inserted into the vesicles. The inhibition kinetic of ATP was determined by Lineweaver–Burk analysis varying both the LA (13.31, 19.95, 33.23, and 39.87 μM) and ATP (1.0, 1.33, 2.00, and 4.00 mM) concentrations.

Mutagenesis, bacterial expression, and reconstitution of mutant ZmPUMP. A ZmPUMP double substitution mutant (K155H/A157H)

was prepared using the QuickChange site-directed mutagenesis kit (Strat agene) with pET:ZmPUMP as a template. A specific primer (5′CAGG CCGAGGGCCACCTTCACCCTGGTGTGCCA3′) was designed to mutate the ZmPUMP codons Lys155 and Ala157 to a His codon. The presence of the mutations was confirmed by DNA sequencing. Expression in *E. coli* and reconstitution of the mutant protein were accomplished as described for wild type ZmPUMP.

Results

Expression of wild type ZmPUMP in E. coli

The entire reading frame of *ZmPUMP* was cloned into the expression vector pET-28b under the control of an IPTG-inducible promoter and *E. coli* BL21(DE3) cells were transformed with this construct in order to generate a recombinant protein. The induction of the cells at 37 °C for 3 h produced a polypeptide bearing a His tag with an apparent molecular mass of 36 kDa (Fig. 1A, compare lanes 1 and 2) that was present in the inclusion body fraction (Fig. 1A, compare lanes 3 and 4). Inclusion bodies were solubilized to obtain an enriched fraction of the recombinant ZmPUMP (Fig. 1A, lane 5). The identity of the recombinant protein was confirmed by immunoblot analysis employing anti-AtPUMP1 polyclonal antibodies [21]. A reactive band with the expected mobility (36 kDa) was observed (Fig. 1B, compare lanes 3 and 4). The small amounts of protein detected in the non-induced bacterial extract were attributed to the basal level of expression normally observed.

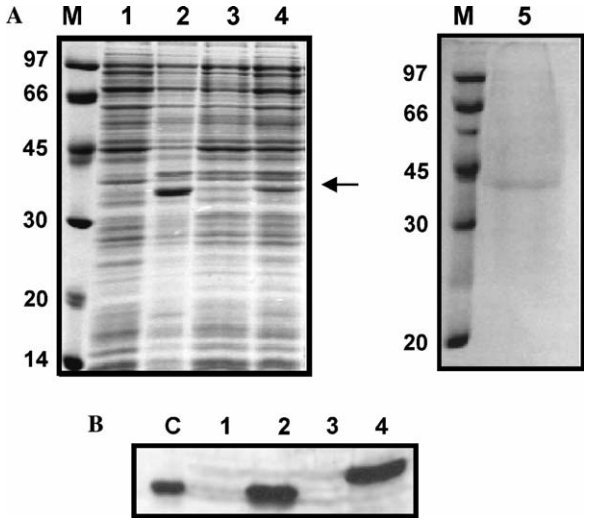

Fig. 1. Analysis of the expression of ZmPUMP by SDS–PAGE. (A) Coomassie blue staining. Lane M, protein markers (sizes in kDa); lanes 1 and 2, total proteins of non-induced and IPTG-induced *E. coli* cells, respectively; lanes 3 and 4, total proteins of soluble and inclusion body fractions, respectively; lane 5, ZmPUMP solubilized from inclusion bodies. The position of ZmPUMP is indicated (arrow). (B) Immunoblot analysis using anti-AtPUMP1 polyclonal antibodies. C, solubilized AtPUMP1 [21] used as positive control; lanes 1 and 2, total proteins of non-induced and IPTG-induced *E. coli* cells expressing AtPUMP1; lanes 3 and 4, total proteins of non-induced and IPTG-induced *E. coli* cells expressing ZmPUMP as shown in (A).

Kinetics of linoleic acid-induced ZmPUMP-mediated H^+ efflux in proteoliposomes

Fig. 2 illustrates the H^+ fluxes in proteoliposomes containing reconstituted wild type ZmPUMP. The addition of 33.23 μM LA caused vesicle acidification, indicating the redistribution of LA molecules in both leaflets of the lipid bilayer leading to internal H^+ release. H^+ efflux that represented FA cycling [2,22,27–30] was initiated by addition of 1.3 μM valinomycin.

The kinetics of LA cycling mediated by ZmPUMP was evaluated by varying the total LA concentration. Protein-independent basal H^+ fluxes were simulated in protein-free liposomes, under identical LA and valinomycin concentrations and membrane potential. Data, corrected for the different (lower) volume of proteoliposomes, were used for a theoretical estimation of the basal H^+ flux in proteoliposomes. Evaluation of the kinetics of LA cycling mediated by ZmPUMP produced a typical dose response (Fig. 3). The corresponding Eadie–Hofstee plot yielded a K_m of 56.36 ± 0.27 μM when using an iterative procedure for evaluating the proteoliposomal basal H^+ flux (Fig. 3; inset) giving a V_{max} of 66.9 μmol H^+ min^{-1} (mg prot)$^{-1}$ that corresponded to minimum turnover numbers of 12.6 s^{-1} for dimeric ZmPUMP.

Inhibition of fatty acid-induced ZmPUMP-mediated H^+ efflux by ATP

The inhibition kinetics for ATP in proteoliposomes containing wild type ZmPUMP were determined in the presence of various concentrations of LA and ATP. Lineweaver–Burk analysis revealed a special mixed

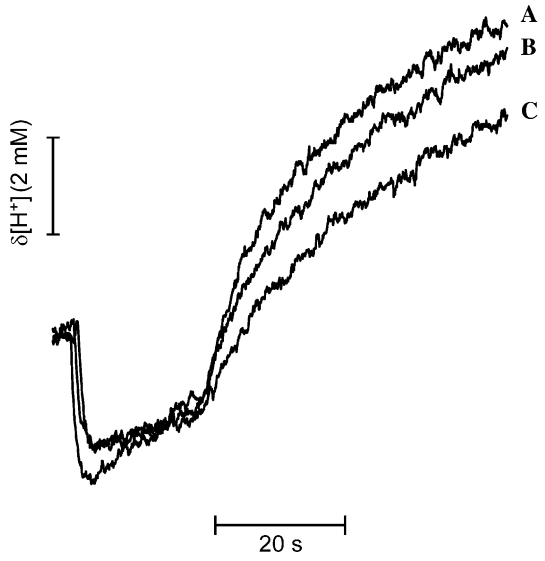

Fig. 2. Typical H^+ fluxes in proteoliposomes reconstituted with wild type ZmPUMP. The H^+ traces are depicted for a H^+ efflux in proteoliposomes containing ZmPUMP induced by 53.12 μM linoleic acid (A), 33.23 μM linoleic acid (B), and 33.23 μM linoleic acid plus 4 mM ATP (C), in the presence of 1.3 μM valinomycin at approximately 179.5 mV.

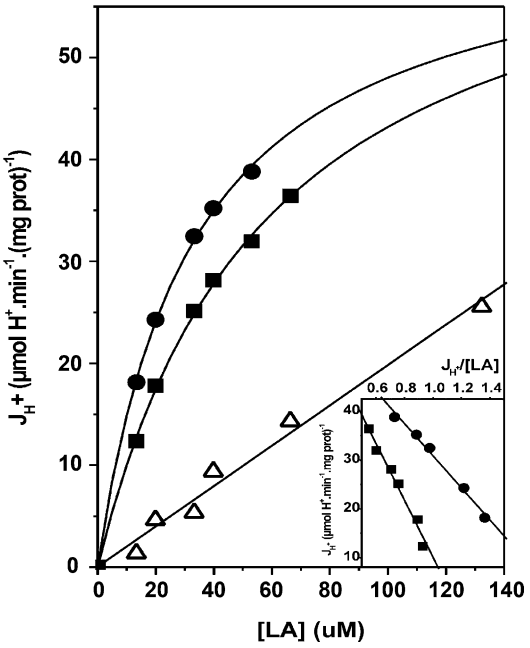

Fig. 3. Kinetics of LA-induced H^+ fluxes in protein-free liposomes (△) and proteoliposomes with reconstituted wild type (■) and mutant ZmPUMP (●). Measurements were conducted in the presence of 1.3 μM valinomycin. The fluxes were calculated per μg of protein to yield final rates in μmol H^+ min^{-1} (mg prot)$^{-1}$. Inset shows Eadie–Hofstee plot for the kinetics of LA cycling with the kinetic data for H^+ fluxes induced by LA in proteoliposomes with wild type (■) and mutant (●) ZmPUMP.

inhibition known as noncompetitive inhibition where α is equal to α', since the K_i values were virtually unchanged in the range of LA concentrations used. Therefore, the inhibitory effect of ATP yielded a dynamic K_i of 2.61 ± 0.36 mM at pH 7.2 (Fig. 4), when considered the even or random orientation of ZmPUMP molecules [2,19,27,30].

Effect of the K155H/A157H mutation on ZmPUMP activity

The K155H/A157H double mutant was over-expressed in inclusion bodies in *E. coli*, purified and reconstituted in proteoliposomes as described for wild type ZmPUMP. Kinetic analyses (Fig. 3) showing that the doubly mutated protein exhibited 1.55-fold higher LA-affinity ($K_m = 36.32 \pm 0.51$ μM) than wild type ZmPUMP ($K_m = 56.36 \pm 0.27$ μM) while its corresponding V_{max} value was not altered (Table 1). Thus, a clear increase in the relative efficiency of H^+ transport of the mutant protein as compared to wild type ZmPUMP was observed (Table 1). The K155H/A157H mutant also displayed a modest increase in sensitivity to ATP inhibition (about 1.3-fold; Table 1).

Discussion

In a previous study, we identified a gene coding for a maize uncoupling protein [18]. In contrast to genes

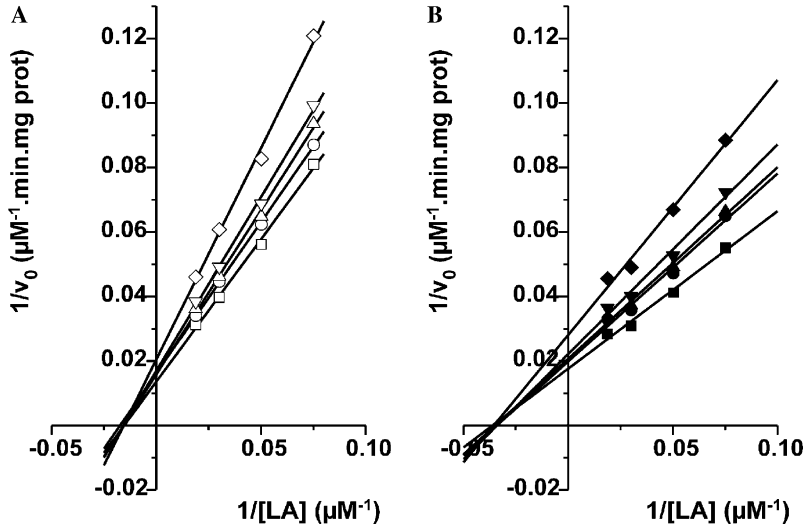

Fig. 4. Lineweaver–Burk plots for the mixed ATP inhibition of FA-induced H^+ fluxes mediated by wild type ZmPUMP (A) or the K155H/A157H mutant (B) at pH 7.2. The employed ATP concentrations were: 0 (□,■), 1.0 (○,●), 1.33 (△,▲), 1.99 (▽,▼), and 3.99 mM (◇,◆). Inhibition kinetic analysis of wild type ZmPUMP yielded a derived K_i of 2.61 ± 0.36 mM. Inhibition kinetic analysis of the K155H/A157H double mutant yielded a derived K_i of 2.03 ± 0.19 mM.

Table 1
Kinetic parameters for H^+ flux in proteoliposomes containing wild type and mutant ZmPUMP

Protein	K_m (μM)	V_{max} μmol H^+ min^{-1} (mg prot)$^{-1}$	K_i (mM)	Relative efficiency μmol H^+ min^{-1} (mg prot)$^{-1}$ μM^{-1}
Wild type	56.36 ± 0.27	66.9	2.61 ± 0.36	1.19
K155H/A157H	36.32 ± 0.51	66.83	2.03 ± 0.19	1.84

encoding two isoforms of PUMP in rice [17], *ZmPUMP* expression resulted in full-length protein product, ZmPUMP, as detected immunogenetically [18]. In the present study, we biochemically characterized ZmPUMP as a recombinant protein expressed in *E. coli*. ZmPUMP was solubilized from inclusion bodies and the authenticity of the recombinant protein confirmed using polyclonal antibodies directed against AtPUMP1 [21]. Solubilized ZmPUMP was reconstituted into liposomes and shown to be fully active; it induced a saturable FFA-dependent proton flux across the liposomal membrane that exceeded the non-specific and unsaturable H^+ flux observed in protein-free liposomes under the same conditions (Fig. 3). Kinetic analysis of LA-dependent H^+ fluxes yielded a $K_m = 56.36 \pm 0.27$ μM, a value that is close to those found for dicot PUMP from *Arabidopsis* and potato [20,21]. Moreover, the proton transport capacity of ZmPUMP was estimated as 66.9 μmol H^+ min^{-1} (mg prot)$^{-1}$, which is similar to those reported for mammalian and plant UCPs [20,21,28,31].

The LA-dependent ZmPUMP-mediated H^+ flux was sensitive to inhibition by purine nucleotides. Kinetic analysis revealed inhibition constant $K_i = 2.61 \pm 0.36$ mM for ATP, a value that falls into the physiological range of concentrations of intracellular ATP (2–15 mM) [32]. The inhibition constant observed is slightly higher than those estimated for recombinant AtPUMP1 (0.85 mM) [21] and native potato UCP (0.78 mM) [20] reconstituted in liposomes. Furthermore, the kinetic analysis of ZmPUMP inhibition by ATP revealed a special type mixed inhibition (noncompetitive, where $\alpha = \alpha'$ hence inhibitor binds either to free or substrate-occupied ZmPUMP with the same rate) that contrasts the competitive inhibition of the LA-dependent potato PUMP-mediated H^+ flux by undecanesulfonate [20]. Altogether, these data confirmed that ZmPUMP behaves as a typical uncoupling protein.

A close analysis of the deduced amino acid sequences of the maize and *Arabidopsis* [12] uncoupling proteins revealed only 83 non-identical residues (over 310), including a monocot-specific alanine motif [18]. Nonetheless, the dicot and monocot uncoupling proteins exhibit almost the same biochemical properties. In this regard, our data suggest that the uncoupling activity catalyzed by PUMPs from monocot and dicot plants shares common regulatory mechanisms.

Contradictory results have been published concerning the role of a His pair located in the second matrix loop in modulating UCP activity. Replacement of these histidines with neutral groups (H145Q/H147N) in UCP1 reconstituted in proteoliposomes had a knockout effect on FA-dependent H^+ transport, whereas the single mutants H145Q and H147N still retained some activity (about 10%), indicating a key role of these residues in proton transport by UCP1 [24]. The results from Urbánková et al. [25] corroborated this observation demonstrating halved FA-affinity and a 70% reduction of V_{max} for an equivalent double mutant (H145L plus H147L). Discordant results were obtained when the mentioned H145Q/H147N mutant of UCP1 was expressed in yeast mitochondria. Under these conditions, replacement of this His pair had no effect on uncoupling activity [33]. Moreover, introduction of the His pair and adjacent amino acids found in UCP1 into UCP3 (that lacks the second His) was not sufficient to promote FA activation [34], indicating that these residues are not required for FA stimulation.

The existing differences prompted us to investigate the effect of these residues on the catalytic activity of a plant uncoupling mitochondrial protein. It is worth to note that all plant UCPs lack both histidines, having lysine and apolar or serine residues at positions of the first and second histidine, respectively. The presence of a strong base (Lys) in PUMPs that substitutes two weak bases (histidines) in UCP1 could be a reason why plant UCPs are able to mediate FA-induced uncoupling. We assumed that if the His pair is functionally important, it would improve proton transport when placed in a plant UCP context. The characterization of the K155H/A157H double mutant of ZmPUMP has shown a 1.55-fold increase in the affinity of the mutant to LA, while its activity remained unchanged. From a mechanistic standpoint, the observed increase in LA-affinity could be a consequence of the better positive charge distribution provided by two weak histidine bases than by one strong lysine base and supports the hypothesis that the His pair is important but not essential for UCP activity. Increased affinity to LA could also be a result of a local conformational change in the second matrix loop caused by the introduced histidines.

To our knowledge, this is the first study reporting the direct functional characterization of a PUMP from a monocot species describing the kinetic of its activation by one of FAs, i.e., linoleic acid, and the kinetic of its sensitivity to inhibition by a classic UCP inhibitor, ATP. Furthermore, this study reports a first mutational analysis of plant uncoupling proteins, describing biochemical kinetic analysis of a protein with introduced His pair. Up to now, the only occurrence of PUMP activity was reported indirectly in isolated durum wheat mitochondria [23]. The authors attributed the LA-induced purine nucleotide-sensitive $\Delta\Psi$ decrease to PUMP, although they did not exclude a possible involvement of ADP/ATP antiporter in this process. Our study prevents such fact, using recombinant PUMP isolated from bacteria and characterized in liposomes, therefore the experimental set-up free of any mitochondrial protein contamination. Hence, our results suggest that the uncoupling activity in monocots can be attributed to PUMP.

More-in-depth molecular and biochemical analyses of uncoupling proteins from different plant species will not only improve our knowledge of their functional properties but also give new insights of their physiological role in the plant kingdom.

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