# *AtPUMP*: an *Arabidopsis* gene encoding a plant uncoupling mitochondrial protein

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**Abstract A cDNA clone (AtPUMP) encoding a plant uncoupling mitochondrial protein was isolated from *Arabidopsis thaliana*. The cDNA contains an open reading frame of 921 nucleotides encoding 306 amino acids (predicted molecular weight 32 708). The predicted polypeptide is 81% identical and 89% similar to the potato UCP-like protein, and includes an energy transfer protein motif common to mitochondrial transporters. The AtPUMP gene exists as a single copy in the *Arabidopsis* genome. The corresponding transcript was expressed in all tissues and was strongly induced by cold treatment. We suggest that the putative AtPUMP protein may play a role in heat-requiring physiological events in *Arabidopsis*.**



*Key words:* Uncoupling protein; Plant uncoupling mitochondrial protein; Cold acclimation; *Arabidopsis thaliana*

## 1. Introduction

Uncoupling proteins (UCP) found in the mammalian inner mitochondrial membrane allow for transmembrane $H^+$ flux which uncouples respiration from ATP synthesis permitting the dissipation of chemical energy into metabolic heat [1,2]. Three UCPs have been described in mammals: UCP1 is located in brown adipose tissue (BAT) and may be involved in the regulation of body temperature [1,3]; UCP2 is ubiquitously found in many tissues and thought to play a role in diseases like diabetes, obesity and in infection responses [4]; and UCP3 is highly specific for skeletal muscle and probably involved in the modulation of respiration in this tissue [5,6]. UCP1 has been shown to be induced by cold acclimation [7–10] whereas UCP2 and UCP3 were unaffected by this treatment [5].

Among the three mammalian UCPs, UCP1 has been studied in detail (for a review, see [10]). This protein is active in freshly isolated mitochondria [2], it is allosterically inhibited by purine nucleotides and dependent on the presence of free fatty acids (FFA). The apparently exclusive localization of UCP1 in BAT mitochondria led to the proposition that this protein is a late evolutionary acquisition of mammals [1]. However, a detailed analysis of the respiratory control of plant mitochondria, together with the coupling effect of BSA and ATP, provided new insights into the possible existence of an UCP-like protein in plants [11]. Thereafter, it was demonstrated that in potato mitochondria a fully coupled state can be reached only in the simultaneous presence of purine nucleotides, such as ATP, and absence of FFA [12]. At this time, a hydrophobic 32-kDa protein was isolated from potato mitochondria and reconstituted into liposomes which was in all aspects similar to the UCP of BAT mitochondria [12,13]. Hence, this protein was termed PUMP (plant uncoupling mitochondrial protein).

In addition to potato, PUMP was also isolated from tomato [14] and immunologically detected in several climacteric fruits (P. Jezek, M. Zackova, A.D.T. Costa, P. Arruda and A.E. Vercesi, unpublished), suggesting a possible role during fruit ripening and senescence. Further support for this hypothesis comes from the observation that PUMP activity in mitochondria incubated in the absence of BSA was much more pronounced in red (ripe) than in green tomatoes (A.E. Vercesi, I.L. Nantes, A.D.T. Costa, P. Jezek, A. Leite and P. Arruda, unpublished). In this case, the higher contents of FFA observed in ripened tomatoes may contribute to this activation.

Although much is known about the PUMP-mediated uncoupling mechanisms [12–14], the characterization and expression patterns of UCP-like genes in plants has only recently being addressed. For instance, a cDNA encoding an UCP-like protein from potato (namely StUCP) has been identified and its gene product shown to possess uncoupling activities in yeast mitochondria [15]. In this report we describe the cloning and initial characterization of an *Arabidopsis thaliana* cDNA encoding a putative cold-inducible protein homologous to StUCP. This is the first evidence of the presence of an UCP-like gene in a non-climacteric plant, which may suggest other physiological roles for UCP-like proteins.

## 2. Materials and methods

### 2.1. Plant material, growth and treatments

Wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0) was used in all experiments. Seedlings were grown in Petri dishes containing MS medium [16], and transferred to fresh MS every week. The seedlings were maintained in a growth chamber at 22°C under a 16-h light/8-h dark photoperiod. For the cold induction treatment, 21-day-old seedlings were incubated either at 4°C or at room temperature (22°C) for 24, 48 and 72 h, respectively.

### 2.2. Cloning of the AtPUMP cDNA

The expressed sequence tag (EST) clone 304H12T7 was kindly provided by the *Arabidopsis* Biological Resource Center (Columbus, OH) and the λZAP II cDNA library of *Arabidopsis* (ecotype Landsberg erecta) flowers was kindly donated by Dr. Elliot M. Meyerowitz (California Institute of Technology, Pasadena, CA). In order to clone the full-length AtPUMP cDNA, the library was screened following the

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manufacturer's protocols using the above EST clone as a probe. The positive clones were excised from the λZAP II as a recombinant pBluescript SK plasmid (Stratagene, La Jolla, CA). The nucleotide sequence of the clones containing the largest inserts was determined on both strands in an ABI 310 automatic sequencer (Perkin-Elmer, Foster City, CA).

*2.3. DNA extraction and Southern blot analysis*

Genomic DNA was extracted as described [17]. DNA samples (10 μg) were electrophoresed in a 1% agarose gel and transferred onto nylon membrane (Hybond $N^+$; Amersham, Buckinghamshire, UK). The blots were hybridized for 16 h at 42°C with a $^{32}$P-labeled probe containing the DNA insert of the EST clone 304H12T7 or a *Xho*I/*Xho*I restriction fragment spanning 1197 nucleotides of the AtPUMP cDNA. Hybridization was performed in 5×SSPE, 50% formamide, 10×Denhardt's solution and 1% SDS. Membranes were washed twice with 2×SSC containing 0.1% SDS for 15 min at 42°C and twice with 0.2×SSC containing 0.1% SDS for 15 min at 42°C. Bands were detected by autoradiography.

*2.4. RNA extraction and Northern blot analysis*

Total RNA from various *Arabidopsis* tissues was extracted as previously described [18]. RNA samples (10 μg) were electrophoresed on 1% formaldehyde-agarose gels [17], transferred onto nylon membranes (Hybond $N^+$) by capillary blot and fixed by UV cross-linking according to the manufacturer's instructions. The blots were hybridized for 16 h at 42°C using either the EST clone 304H12T7 or the *Xho*I/*Xho*I restriction fragment of the AtPUMP cDNA as a probe and washed as described for the Southern blots. Blots were stripped off the radioactive probes in a boiling 0.1% SDS solution and sequentially reprobed. Bands were detected by autoradiography.

## 3. Results

*3.1. Sequence analysis of the AtPUMP cDNA and predicted polypeptide*

We have identified in the *Arabidopsis* database an EST clone (304H12T7) sharing significant sequence homology with the mammalian UCPs, including a mitochondrial carrier gene family signature. The EST clone was found to contain a ∼0.5-kb insert which was used to screen a cDNA library of *Arabidopsis* flowers and resulted in the cloning of a cDNA of 1225 nucleotides, designated AtPUMP. The nucleotide sequence of the 3′ end of the cloned AtPUMP cDNA was identical to the EST clone used as a probe and probably represented a full-length copy of the mRNA. An open reading frame (ORF) of 921 nucleotides flanked by untranslated regions of 150 and 154 nucleotides at its 5′ and 3′ ends, respectively, could be identified in the AtPUMP cDNA sequence (Fig. 1). This ORF encodes a predicted polypeptide of 306 amino acids (estimated molecular weight 32.7 kDa), which upon alignment with known UCP proteins showed 81% (89%), 40% (57%) and 45% (62%) amino acid identity (similarity) with StUCP, human UCP1 and human UCP2, respectively (Fig. 2). The predicted AtPUMP protein possesses the tripartite structure characteristic of all known mitochondrial carriers (Fig. 2A–C). These repeats, made up of related sequences of about 100 amino acids in length [19], are characterized by the presence of two predicted transmembrane domains as well as a typical mitochondrial energy transfer protein signature domain [19] (Fig. 2).

*3.2. The AtPUMP gene exists as a single copy*

To investigate the number of AtPUMP genes present in the *Arabidopsis* genome, genomic DNA was digested with three different restriction enzymes and hybridized with either the EST clone 304H12T7 or the *Xho*I/*Xho*I restriction fragment

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   1  GACGATCTTTTCTATAACTGAAACACTACTCGAGGCCAAGTTGCTTTA   48
  49  GCCGTAATCGTCGTCGTCCCTCTTCCCGAAATTATCTCTTCTCTGTTC   96
  97  TTCGATTTCGAAACCCTAACCTCCTCTCTTTAATTCGCGTTTTCTGGA  144
 145  TCGAAGATGGTGGCGGCTGGTAAATCCGACCTTTCCTTGCCCAAAACT  192
   1        M  V  A  A  G  K  S  D  L  S  L  P  K  T    14
 193  TTCGCCTGCAGTGCCTTCGCTGCTTGCGTCGGCGAGGTATGCACAATT  240
  15   F  A  C  S  A  F  A  A  C  V  G  E  V  C  T  I   30
 241  CCATTGGACACTGCTAAAGTTAGGCTTCAGCTCCAAAAGTCTGCTCTT  288
  31   P  L  D  T  A  K  V  R  L  Q  L  Q  K  S  A  L   46
 289  GCTGGTGATGTTACTCTGCCTAAATATCGAGGATTGTTGGGAACTGTT  336
  47   A  G  D  V  T  L  P  K  Y  R  G  L  L  G  T  V   62
 337  GGTACCATAGCAAGGGAAGAAGGGTTACGTTCACTATGGAAAGGTGTT  384
  63   G  T  I  A  R  E  E  G  L  R  S  L  W  K  G  V   78
 385  GTACCTGGATTGCATCGTCAATGCCTATTTGGAGGTCTTAGGATTGGA  432
  79   V  P  G  L  H  R  Q  C  L  F  G  G  L  R  I  G   94
 433  ATGTATGAGCCGGTGAAAAACTTGTATGTTGGAAAAGACTTTGTAGGT  480
  95   M  Y  E  P  V  K  N  L  Y  V  G  K  D  F  V  G  110
 481  GATGTTCCATTGAGCAAGAAAATTCTTGCTGGTTTGACAACAGGTGCA  528
 111   D  V  P  L  S  K  K  I  L  A  G  L  T  T  G  A  126
 529  CTGGGTATCATGGTAGCAAATCCCACTGATCTTGTGAAAGTTAGGCTT  576
 127   L  G  I  M  V  A  N  P  T  D  L  V  K  V  R  L  142
 577  CAGGCGGAAGGAAAATTAGCTGCAGGTGCGCCAAGACGGTACTCTGGA  624
 143   Q  A  E  G  K  L  A  A  G  A  P  R  R  Y  S  G  158
 625  GCGCTGAATGCGTATTCAACAATTGTGAGACAGGAAGGAGTCCGAGCT  672
 159   A  L  N  A  Y  S  T  I  V  R  Q  E  G  V  R  A  174
 673  CTTTGGACTGTTCTTGGACCTAACGTAGCAAGAAACGCTATTATCAAT  720
 175   L  W  T  V  L  G  P  N  V  A  R  N  A  I  I  N  190
 721  GCTGCTGAATTAGCGAGTTACGATCAAGTGAAAGAGACTATCTTGAAG  768
 191   A  A  E  L  A  S  Y  D  Q  V  K  E  T  I  L  K  206
 769  ATTCCAGGGTTCACTGACAACGTTGTCACACATATTCTATCTGGACTG  816
 207   I  P  G  F  T  D  N  V  V  T  H  I  L  S  G  L  222
 817  GGGGCAGGATTCTTTGCTGTTTGCATCGGTTCTCCTGTTGACGTGGTT  864
 223   G  A  G  F  F  A  V  C  I  G  S  P  V  D  V  V  238
 865  AAGTCAAGAATGATGGGAGATTCTGGTGCTTACAAGGGCACCATTGAT  912
 239   K  S  R  M  M  G  D  S  G  A  Y  K  G  T  I  D  254
 913  TGCTTCGTCAAAACTCTGAAGAGCGACGGTCCTATGGCATTTTACAAG  960
 255   C  F  V  K  T  L  K  S  D  G  P  M  A  F  Y  K  270
 961  GGTTTCATCCCCAACTTTGGACGCCTTGGCTCATGGAACGTAATCATG 1008
 271   G  F  I  P  N  F  G  R  L  G  S  W  N  V  I  M  286
1009  TTTTTGACCCTCGAACAGGCAAAGAAGTATGTCCGGGAACTCGATGCG 1056
 287   F  L  T  L  E  Q  A  K  K  Y  V  R  E  L  D  A  302
1057  TCCAAAAGAAACTGAGACACAAAGTTTTAAGCAGAGGGAATGAGAGCA 1104
 303   S  K  R  N  *                                    307
1105  ACATTGTTTTCTTCTTCATTTTCGGTGATTGAGAGAGGCCAGAACTTG 1152
1153  GTCGAATATTGTTTTCGGAATAGAGATTCAGTTTTCGAGTAAAACTGT 1200
1201  GAAATAAAATTTCTGTGGATTGCTC                         1225
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Fig. 1. Nucleotide and deduced amino acid sequence of AtPUMP (GenBank accession number AJ223983). The deduced amino acid sequence is shown below the nucleotide sequence. A putative polyadenylation signal is underlined.

of the AtPUMP cDNA. The simple banding pattern observed in Fig. 3 suggests that the AtPUMP gene exist as a single copy in the *Arabidopsis* genome.

*3.3. Tissue specificity and cold induction of AtPUMP gene expression*

To determine the tissue specificity of AtPUMP expression, the EST clone 304H12T7 containing the 3′ end of the AtPUMP cDNA sequence was used to probe blots of total RNA extracted from different *Arabidopsis* organs. As shown in Fig. 4, hybridization to a single band of ∼1.5 kb was observed. The AtPUMP gene was expressed in all tissues examined, showing higher levels of transcript accumulation in roots and flowers. The same results were obtained when using the *Xho*I/*Xho*I restriction fragment of the AtPUMP cDNA (not shown).

In order to test whether the expression of AtPUMP is affected by cold acclimation, the transcript accumulation in seedlings exposed to low temperature (4°C) or maintained at 22°C (RT) was monitored by Northern blot. As was observed for StUCP [15] and the mammalian UCP1 [8,9], AtPUMP gene expression is significantly induced by cold, with transcripts accumulating to peak amounts after 48 h, as compared to control seedlings (Fig. 5).

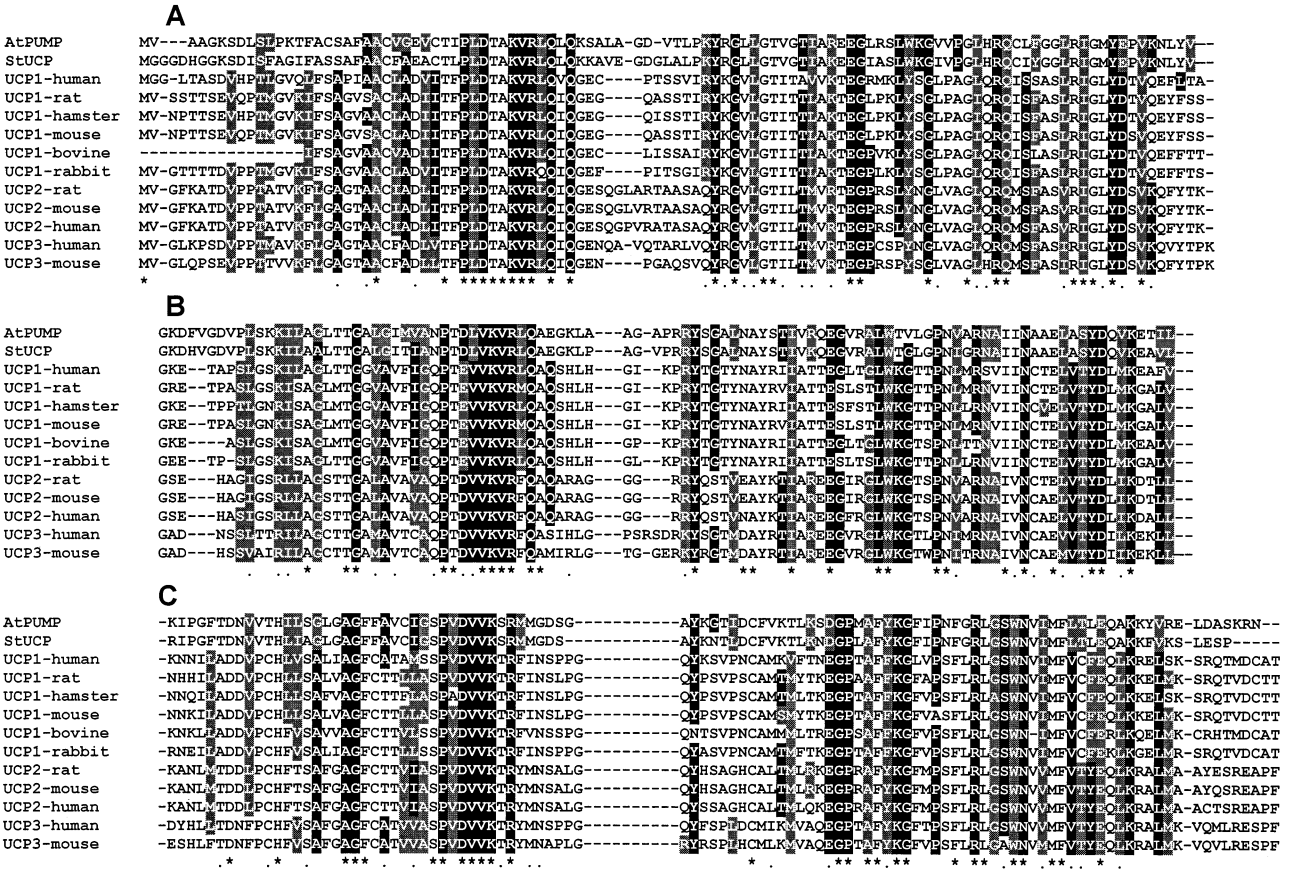


Fig. 2. Alignment of the deduced amino acid sequence of the putative AtPUMP protein with the potato (StUCP) and several mammalian UCPs. Black and dark gray boxes indicate residues that are identical or similar in at least 50% of the sequences within the conserved repeats (A, B, C). Asterisks indicate perfect matches and dots represent conservative changes within all sequences. The gaps introduced to optimize the sequence alignment are represented by dashes. The alignment was obtained with CLUSTAL W applying the Dayhoff PAM 250 matrix [20]. Shading was performed by the BOXSHADE program (ISRC Bioinformatics Group). Accession numbers are: StUCP (Y11220); UCP1-human (P25874); UCP1-rat (P04633); UCP1-golden hamster (P04575); UCP1-mouse (P12242); UCP1-bovine (P10861); UCP1-rabbit (P14271); UCP2-rat (AF039033); UCP2-mouse (P70406); UCP2-human (P55851); UCP3-human (P55916) and UCP3-mouse (AF032902).

## 4. Discussion

In this study, we describe the isolation and characterization of a novel cDNA clone encoding a mitochondrial uncoupling protein from *Arabidopsis*. Two lines of evidence supports the hypothesis that the putative AtPUMP protein possesses uncoupling properties. First, the predicted polypeptide shares high sequence identity and similarity (81% and 89%, respectively) with potato StUCP, for which an uncoupling activity has been shown to occur in yeast mitochondria [15]. Second, as deduced from the amino acid sequence, the putative AtPUMP protein has structural features similar to those of other members of the UCP family, including the highly conserved motifs [19]. Thus, the high levels of sequence identity and similarity reported here strongly suggest that the AtPUMP gene product is involved in regulation of mitochondrial uncoupling.

The data presented in this study show that AtPUMP, like StUCP [15] and the mammalian UCP2 [5], is ubiquitously expressed. A great similarity as compared to StUCP is its high level of expression in roots and flowers, indicating mainly biochemical functions and common roles in these tissues. Moreover, AtPUMP and StUCP transcript accumulation was found to be strongly induced following exposure to low temperature, suggesting that both genes probably share a common mechanism of regulation. An analogous effect on the expression of the mammalian UCP1 in BAT was also observed [8–10]. The differential expression of the AtPUMP

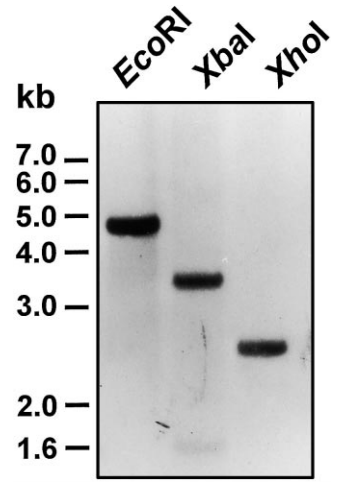


Fig. 3. Southern blot analysis of *Arabidopsis* genomic DNA. Genomic DNA was digested with *Eco*RI, *Xba*I and *Xho*I and probed with the EST clone 304H12T7 containing the partial AtPUMP cDNA. Numbers on the left represent the sizes in kb of the molecular weight markers.

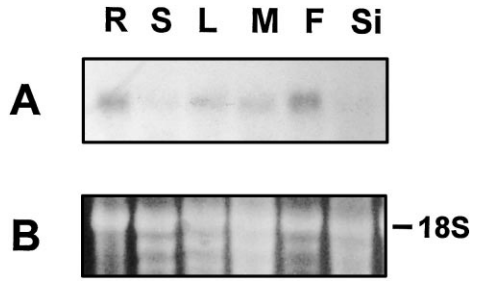


Fig. 4. Northern blot analysis of AtPUMP gene expression in various *Arabidopsis* tissues. A: Total RNA from roots (R), seedlings (S), young (L) and mature (M) leaves, flowers (F) and siliques (S) was hybridized with the EST clone 304H12T7. A ~1.5-kb transcript is detected in the different plant tissues analyzed. B: 18S rRNA stained with ethidium bromide prior to blotting.

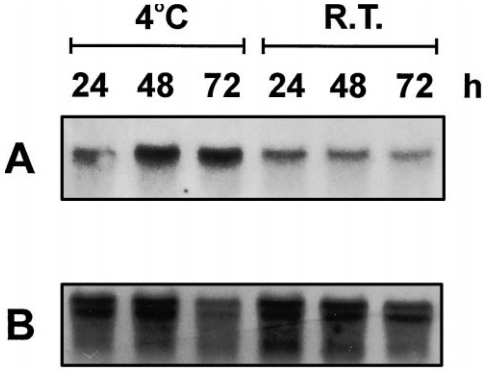


Fig. 5. Cold-inducible effect on AtPUMP gene expression analyzed by Northern blot. A: Total RNA extracted from *Arabidopsis* seedlings incubated either at 4°C or at 22°C (R.T.) and collected after 24, 48 and 72 h, was hybridized with the EST clone 304H12T7. B: After autoradiography, the blot was stripped and reprobed with a tobacco ubiquitin probe to give a loading control.

gene in response to cold acclimation is consistent with a possible role of its gene product in heat requiring events during the *Arabidopsis* life cycle.

In addition to the potato and *Arabidopsis* isoforms, the presence of PUMP was immunologically shown to be widespread in many climacteric fruits and fruits in which an increase in respiration has been observed fruits (P. Jezek, M. Zackova, A.D.T. Costa, P. Arruda and A.E. Vercesi, unpublished) [21]. Although there has been considerable progress in the elucidation of the physiological role of PUMP during the ripening process (A.E. Vercesi, I.L. Nantes, A.D.T. Costa, P. Jezek, A. Leite and P. Arruda, unpublished), little is known about its possible role in a non-climacteric plant such as *Arabidopsis*. Since PUMP has been shown to efficiently reduce the generation of mitochondrial reactive oxygen species [22], it may be important to prevent oxidative mitochondrial damage. The availability of this new gene provides a good tool to further elucidate other PUMP-mediated physiological events in plants.

The presence of uncoupling proteins in plant and mammalian mitochondria shows that the ancestral genes probably evolved at the initial point of the phylogenetic tree of eukaryotes prior to the divergence into protozoa, animals, fungi and plants. The molecular cloning and characterization of similarly functional uncoupling proteins able to regulate the mitochondrial transmembrane potential in different organisms will facilitate the further elucidation of their evolution and regulatory functions and help to explain many physiological events in plants.

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