Molecular characterization of an *Arabidopsis thaliana* cDNA encoding a novel putative adenylate translocator of higher plants

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Abstract **We have isolated an *Arabidopsis thaliana* cDNA encoding a highly hydrophobic membrane protein of 589 amino acids which contains 12 potential transmembrane helices and shows a high degree of similarity (43.5% identity, 66.2% similarity) to the ATP/ADP translocase of the Gram-negative bacterium *Rickettsia prowazekii*, an obligate intracellular parasite responsible for the epidemic typhus. This rickettsial translocator resides in the cytoplasmic membrane and allows the bacterium to exploit the host cytoplasmic ATP pool. We hypothesize that the *A. thaliana* homolog of the *R. prowazekii* ATP/ADP translocase is the functional eukaryotic equivalent and resides in the plastid inner envelope membrane where it functions as an ATP importer.**

Key words: *Arabidopsis thaliana*; ATP/ADP translocator; cDNA; Plastid

1. Introduction

Higher plants possess two adenylate translocators in different subcellular compartments. The mitochondrial ADP/ATP translocator resides in the inner membrane and participates in oxidative phosphorylation via exporting matrix ATP from the mitochondria in exchange for cytosolic ADP. The ADP/ATP carriers from mitochondria of higher eukaryotes are remarkably strongly conserved (identity > 50%) over the long evolutionary distance when compared with the yeast transporter [1].

A second adenylate translocator is present in the inner envelope membrane of plastids seemingly having the opposite transport direction under physiological conditions, that is the uptake of cytosolic ATP for stroma ADP [2]. Whereas the metabolic function of the chloroplast ATP/ADP translocator is not yet clear, non-green plastids strongly depend on a supply of cytosolic ATP via this translocator for biosynthesis of starch [3] and fatty acids [4]. The structure of the plastidic ATP/ADP translocator has not yet been elucidated.

In this paper, we report on the structural characterization of an *Arabidopsis thaliana* cDNA that codes for a highly hydrophobic membrane protein showing a high degree of sequence similarity to the ATP/ADP translocator of the bacterium *Rickettsia prowazekii*. We discuss our data in favor of the hypothesis that the *A. thaliana* homolog, designated AATP1 (for ATP/ADP Translocator Protein 1), of the *R. prowazekii* transporter is the functional eukaryotic equivalent and resides in the inner envelope membrane of plastids.

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The nucleotide sequence reported here has been submitted to the EMBL databank and is available under the accession number Z49227.

2. Materials and methods

2.1. Plant material

For DNA and RNA extractions, plants of *Arabidopsis thaliana* (Heyhn.) ecotypes Columbia and Landsberg *erecta* were grown in soil at 23°C with a photoperiod of 16-h light/8-h dark.

2.2. Library screening and DNA sequencing

Via complementation of a copper transporter mutant from yeast, an *A. thaliana* cDNA has been identified which encodes a copper transporter (K. Kampfenkel, unpubl. data). This cDNA was fused via the poly(A) tail to the poly(A) tail of a second, only partial cDNA. The deduced amino acid sequence of this cDNA revealed similarity to only one protein when compared with sequences in the EMBL and Swiss-Prot databases, the ATP/ADP translocase of the bacterium *R. prowazekii*. To isolate a full-length cDNA, an *A. thaliana* cDNA library constructed in the λYES yeast/*Escherichia coli* shuttle vector [5] was screened by hybridization with this partial cDNA. 220,000 plaques were grown in *E. coli* XL1-Blue (Stratagene) and transferred to Hybond-N membranes (Amersham). The membranes were prehybridized for 30 min at 65°C in 0.25 m sodium-phosphate buffer (pH 7.2), 1% (w/v) BSA, 1 mM EDTA, 7% (w/v) SDS. Hybridization was performed for 16 h at 65°C in the same solution plus the cDNA fragment labeled by the random primer method with [α-^{32}P]dCTP using the ^{T7}Quick Prime Kit (Pharmacia). Afterwards, the membranes were washed 2–3× at 58°C for 15 min in 0.05 M sodium-phosphate buffer (pH 7.2), 0.5% (w/v) SDS and autoradiographed. Conversion into the plasmid form was performed as described [5]. The *AATP1* cDNA was sequenced on both strands by using *AATP1*-specific primers which were synthesized with an oligonucleotide synthesizer from Applied Biosystems.

2.3. Southern and Northern blot analysis

Genomic DNA was extracted according to [6] and total RNA was isolated as in [7]. Standard procedures were used for electrophoretic separation and transfer to nylon membranes. Radiolabeled *Hin*dIII digested λDNA served as length standard. Radiolabeling of the cDNA, prehybridization, hybridization and washing was as outlined above.

2.4. Hydrophobicity analysis

The hydropathy profile of the AATP1 protein was obtained according to [8] with the program SOAP in PC/GENE (IntelliGenetics, Mountain View, CA). Potential transmembrane helices were predicted employing the method of Rost et al. [9] which is available by electronic mailing via Internet to 'PredictProtein@EMBL-Heidelberg.DE'.

3. Results

3.1. Sequence analysis of the A. thaliana AATP1 cDNA

The nucleotide sequence of an *A. thaliana* cDNA encoding

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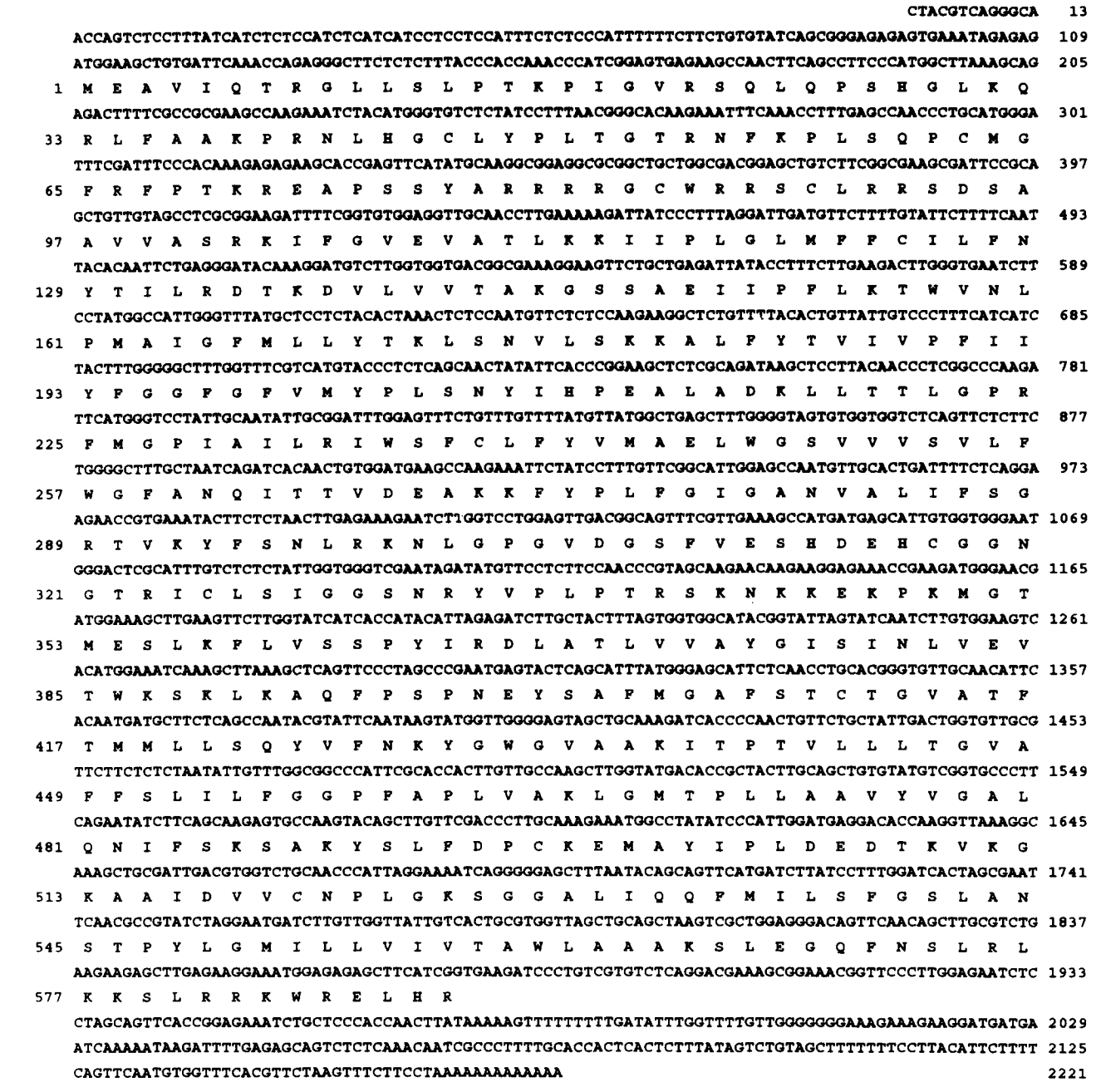

Fig. 1. Nucleotide and predicted amino acid sequence of the *A. thaliana AATP* cDNA. The sequence presented corresponds to the longest *AATP1* cDNA of 2174 bp characterized in this study. The EMBL sequence data bank accession number of the *AATP1* cDNA sequence is Z49227.

a protein of 589 amino acid residues which displays a significant similarity to the ATP/ADP translocator of the bacterium *R. prowazekii* [10] is shown in Fig. 1. The same but only partially sequenced cDNA has been reported recently as an anonymous clone (named EST 20853) among other cDNAs that have been randomly sequenced in conjunction with the *Arabidopsis* genome project [11]. The 5′-proximal ATG triplet has been assumed to be used as the start codon since, according to the 'first AUG rule', it serves as the initiator codon to be used in the translation of ~95% of the eukaryotic mRNAs [12]. This assignment is supported by the nucleotide context around the start codon; GAGAGATG has a purine at position −3 from the initiator codon which is another conserved feature of eukaryotic mRNAs [12]. In front of the start codon, an in-frame stop codon is present at position −12 to −10 supporting the conclusion that the open reading frame is of full length (Fig. 1).

A sequence alignment of the predicted AATP1 protein of *A. thaliana* with the rickettsial transporter is shown in Fig. 2. Within an overlap of 479 amino acid residues, these two proteins are 43.5% identical and 66.2% similar. Analysis of the hydropathy profile of the AATP1 protein sequence indicates that the *A. thaliana* protein is highly hydrophobic except for the first 100 N-terminal amino acid residues (Fig. 3). Thus, the AATP1 protein is most likely an integral membrane protein.

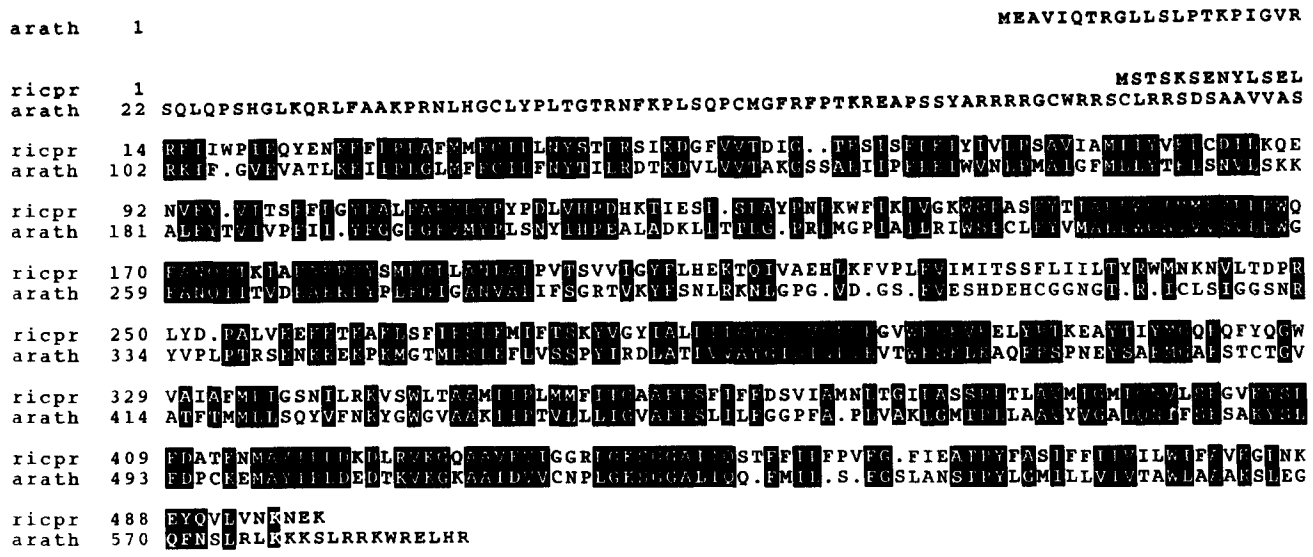

Fig. 2. Alignment of the predicted amino acid sequence of *A. thaliana* AATP1 protein with the *R. prowazekii* ATP/ADP translocase. Arath, *A. thaliana*; ricpr, *R. prowazekii*. Numbers indicate amino acid positions. The dots mark artificial sequence gaps introduced to improve the homology between the proteins.

The only known adenylate translocator activities of higher plants reside in the inner membranes of mitochondria and plastids. Since the AATP1 protein extends beyond the N-terminal region that still displays homology to the rickettsial transporter by ~100 amino acid residues (Fig. 2), we assume that this represents a transit peptide containing targeting information for directing this putative ATP/ADP translocator either to the plastids or the mitochondria. It is tentatively possible to discriminate between mitochondrial and plastidic transit peptides on basis of their gross amino acid content, since mitochondrial targeting sequences are mainly enriched in Arg (14%), Ser (11%) and Ala (14%) whereas plastidic transit peptides are characterized by a high percentage of hydroxylated amino acids, especially Ser (19%) [13]. Based solely on this criterium, the AATP1 transit peptide resembles those of mitochondrial preproteins because of the content of Arg, Ser and Ala (15, 10 and 8%, respectively) in the first 100 N-terminal residues (Fig. 1). The amino acid sequence of the AATP1 protein (Fig. 1) shows no detectable homology to the known plant mitochondrial ADP/ATP translocators, among these, most noteworthy, also those from *A. thaliana* [14] which are otherwise exhibiting a high degree of identity at the amino acid sequence level (data not shown).

Analysis of the AATP1 amino acid sequence using the method of Rost et al. [9] under the assumption that a stretch of 16 amino acid residues is sufficiently long to span the lipid bilayer [15], revealed the possible presence of 12 putative transmembrane helices in the putative mature part of the protein (Fig. 3).

3.2. Northern and Southern blot analysis

An *AATP1* mRNA species of approximately 2.3 kb was found in total RNA extracted from different organs of *A. thaliana* (Fig. 4A). The transcript was of equal size in flowers, stems, leaves and roots. This analysis suggests that the *AATP1* gene or genes (see below) are expressed in all organs of *A. thaliana*.

A Southern blot of genomic DNA from the *A. thaliana* ecotypes Columbia and Landsberg *erecta* which was digested with *Dra*I, *Sac*I, *Eco*RV, *Pst*I and *Hin*dIII and probed with an *AATP1* cDNA fragment is shown in Fig. 4B. The results reveal one or two major hybridizing bands in each of the tracks (Fig. 5B). Currently, we do not know, whether these fragments result from two different *AATP* genes or if they represent separate exons of a single gene.

4. Discussion

We describe the nucleotide sequence of an *A. thaliana* cDNA coding for a membrane protein (Figs. 1,3) which shows striking similarity to the ATP/ADP translocator of the bacterium *R. prowazekii*. Within an overlap of 479 amino acid residues which covers nearly the complete rickettsial translocator sequence of 497 amino acids, these two proteins share 43.5% identity and 66.2% similarity (Fig. 2). Like the rickettsial

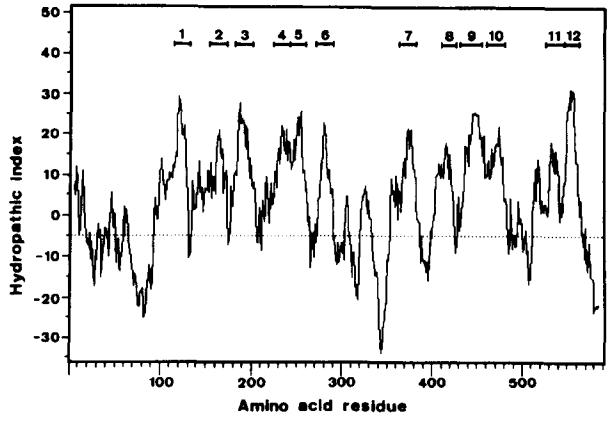

Fig. 3. Hydropathy plot of the deduced amino acid sequence of the AATP1 protein from *A. thaliana*. The analysis was performed according to [13] with a window size of 11 residues (hydrophilic, negative values; hydrophobic, positive values). Tentative membrane spanning α-helices are indicated by cross-bars (1–12).

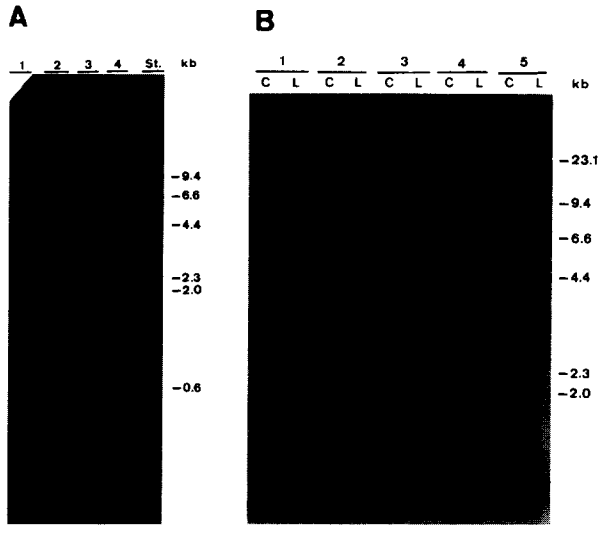

Fig. 4. Expression of *AATP1* in different organs of *Arabidopsis* and Southern blot analysis of *AATP1*. (A) Northern blot analysis of total RNA (10 μg/track) extracted from *A. thaliana* flowers (lane 1), stems (lane 2), leaves (lane 3) and roots (lane 4). The same *AATP1* probe as in (B) has been used. (B) *Arabidopsis* genomic DNA (2 μg/track) extracted from ecotypes Columbia (C) and Landsberg *erecta* (L) was digested with *Dra*I (lane 1), *Sac*I (lane 2), *Eco*RV (lane 3), *Pst*I (lane 4) and *Hin*dIII (lane 5). The blot was probed with a ^{32}P-labeled *AATP1* cDNA fragment corresponding to nucleotides 701–1742 (Fig. 1). Less strong hybridizing bands are indicated by arrow heads. Note this *AATP1* cDNA fragment does not contain *Dra*I, *Sac*I, *Eco*RV or *Pst*I sites, but three *Hin*dIII sites in positions 1173, 1275 and 1502 are present (Fig. 1). St., length standard (kb) in (A) and (B) was *Hin*dIII digested and ^{33}P-labeled λDNA.

translocase [10], the *A. thaliana* homolog contains 12 putative transmembrane helices too (Fig. 3). Regarding the evolutionary distance between *A. thaliana* and the obligate intracellular parasite *R. prowazekii*, this high degree of similarity strongly suggests that the *A. thaliana* protein is also an ATP/ADP translocase. Therefore, we have designated this plant protein as AATP1 for ATP/ADP Translocator Protein 1. We will discuss our observations in favor of the hypothesis that AATP1 resides in the plastid inner envelope membrane where it might function as an ATP importer.

The most striking argument is the close resemblance between the plastidic ATP/ADP translocator and the rickettsial translocator regarding their transport physiology. Upon invading host cells, the Gram-negative rickettsiae escape the phagocytic vacuole and grow directly within the cytoplasm. For exploitation of the host cytoplasmic ATP pool, they possess an ATP/ADP translocator in their cytoplasmic membrane that permits the exchange of ADP from the rickettsia for host cytoplasmic ATP [16]. Exactly this is the physiological transport direction of the plastidic ATP/ADP translocator which is in fact opposite that found in mitochondria. In addition, the plant mitochondrial adenylate translocator is highly sensitive to specific inhibitors like carboxyatractyloside and bongkrekic acid [17], none of which have been shown to be inhibitory for the rickettsial ATP/ADP translocator [18]. Most noteworthy, the plastidic ATP/ADP translocator is only slightly sensitive to these inhibitors [19,20]. Therefore, we believe that the *A. thaliana* homolog AATP1 of the *R. prowazekii* adenylate translocator is a likely candidate for the plastidic ATP/ADP translocator which remained so far unidentified. If so, AATP1 should possess a transit peptide to allow targeting of the preprotein to the inner envelope membrane of plastids. Indeed, the N-terminus of AATP1 extends by ~100 amino acid residues beyond the region that still displays homology to the rickettsial transporter (Fig. 2). This N-terminal extension is presumably a transit peptide which, due to its higher Arg than Ser content [13], seems to be more closely related to mitochondrial transit peptides. However, this does not necessarily contradict our hypothesis that the AATP1 protein resides in the plastid inner envelope membrane. In fact, transit sequences of nearly all of the inner envelope membrane proteins from chloroplasts identified so far (phosphate-triose phosphate-3-phosphoglycerate translocator [21–24]; 37-kDa protein [25]; Bt1 protein [26]; Ca^{2+}-ATPase [27]) do resemble mitochondrial transit peptides more than chloroplast targeting sequences since they all have a comparatively higher Arg than Ser content in their transit peptides. Currently, there is only one exception known, that is the recently identified chloroplast 2-oxoglutarate/malate translocator which has a N-terminal targeting sequence that displays typical features of a chloroplast transit peptide [28]. Interestingly, the residue next to the initiator Met of AATP1 is a Glu which is also the case in the transit peptides of the four sequenced phosphate-triose phosphate-3-phosphoglycerate translocators from spinach [21], pea [22], potato [23] and tobacco [24] as well as the Ca^{2+}-ATPase [27]. In mitochondrial transit peptides, the residue next to the initiator Met is almost never Glu [13].

It has been postulated that the adenylate translocators from plastids and mitochondria are very similar proteins since an antibody directed against the ADP/ATP translocator from *Neurospora crassa* mitochondria cross-reacted with a 32-kDa polypeptide from envelope membranes of spinach chloroplasts and of sycamore amyloplasts [29,30]. However, Schünemann et al. [20] failed to show any cross-reactivity of the same antibody with proteins in the envelope membrane of pea root plastids and could not reproduce the results obtained by Pozueta-Romero et al. [29] with spinach chloroplast envelope membranes. Moreover, the same group did not find a cross-reaction with proteins from sycamore chloroplast envelope membranes [31]. Thus, at present it seems more than questionable that the adenylate translocators in plastids and mitochondria are homologous membrane proteins. Schünemann et al. [20] concluded that they may well derive from different ancestors. The observation that the AATP1 amino acid sequence revealed no detectable homology to the known *A. thaliana* [14] and other plant mitochondrial ADP/ATP translocator sequences which are otherwise highly identical with each other (data not shown) is another argument in favor of our hypothesis that AATP1 is identical with the plastidic ATP/ADP translocator.

We are currently testing our hypothesis and perform transport studies by heterologous expression of the *AATP1* cDNA in yeast cells. With the in vitro translated precursor protein, we can address the question whether the AATP1 protein will be inserted in the inner envelope membrane of isolated plastids.

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