Identification of an Arabidopsis inorganic pyrophosphatase capable of being imported into chloroplasts

Silke Schulze[a,c], Alexandra Mant[b], Jens Kossmann[c], James R. Lloyd[a,c,*]

[a] *Max-Planck-Institute of Molecular Plant Physiology, Am Mühlenberg 1, D-14476 Golm, Germany*
[b] *Department of Plant Biology, Plant Biochemistry Laboratory, The Royal Agricultural & Veterinary University, 40, Thorvaldsensvej, DK-1871 Frederiksberg C, Denmark*
[c] *Plant Research Department, RisøNational Laboratory, DK-4000 Roskilde, Denmark*

Received 20 February 2004; revised 25 March 2004; accepted 29 March 2004

First published online 9 April 2004

Edited by Julian Schröder

Abstract An Arabidopsis cDNA coding for a previously uncharacterized isoform of inorganic pyrophosphatase was isolated. It was used to complement an *E. coli* mutant, demonstrating that it coded for an active enzyme. $MgCl_2$ was necessary for the protein's activity, whilst NaF inhibited it. The K_m for pyrophosphate and the pH optimum of the protein was determined. The gene coding for this protein was expressed in all tissues, and its expression in rosette leaves was induced by incubation on metabolizable sugars. In vitro import experiments demonstrated that the protein could be imported into chloroplasts and localized to the stromal compartment.

Keywords: Inorganic pyrophosphatase; Arabidopsis; Plastid

1. Introduction

Inorganic pyrophosphatase (EC 3.6.1.1, PPase) is an enzyme that cleaves pyrophosphate (PPi) molecules, producing inorganic phosphate (Pi). Its role in metabolism is thought to be to remove PPi, which is a product of many reactions, especially those involved in the synthesis of polymers. Removal of PPi stops such reactions from reaching equilibrium and is, thus, essential for maintaining the direction of the reaction [1].

In plants, many important biosynthetic pathways are localized in plastids. Some of these, such as the synthesis of purines, pyrimidines or starch, contain reactions that generate PPi. It is assumed that if PPi accumulates within the plastid these pathways would be inhibited and there are two theories about how the PPi becomes eliminated. The first of these is that PPase degrades it to Pi and the Pi is exported into the cytosol by Pi transporters. Approximately 80% of the total PPase activity found in plant cells is located in plastids [2,3], indicating that breakdown of PPi by PPase may be an important pathway for eliminating PPi from that compartment. The second theory is that PPi is exported directly by a PPi transporter [4]. Research into understanding this important process has been hampered as genes coding for neither a plastidial isoform of PPase, nor a PPi transporter have been isolated.

The first plant *PPase* gene identified was from Arabidopsis [5] and many cDNAs have since been found from several other plant species that are highly similar to this original sequence [6–9]. Immuno-gold labelling using an antibody that recognizes the protein encoded by a potato cDNA demonstrated that it is localized in the cytosol [7], and it is reasonable to assume that all these similar genes code for cytoplasmic isoforms. In this paper, we report on an Arabidopsis gene revealed by the genome sequencing project coding for a PPase that is clearly different from all other higher plant PPases so far identified. We demonstrate that the protein it codes for has PPase activity and that it is capable of being imported into chloroplasts.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from either Sigma (St. Louis, MO, USA), Roche (Hvidovre, Denmark) or Merck (Darmstadt, Germany).

2.2. Isolation of cDNA and its manipulation

Arabidopsis genes coding for soluble PPase proteins were sought using the BlastP program at the NCBI database. The sequence used for the search was that of the *E. coli* PPase (NCBI# NP_418647). The DNA sequence from one of the genes (At5g09650) identified as coding for a soluble PPase was used to identify Arabidopsis expressed sequence tags coding for the gene. One of these (NCBI# Z34857) was obtained from the Arabidopsis Biological Resource Center and both strands of the insert were sequenced using a commercial service (AGOWA, Berlin, Germany). This demonstrated that although the cDNA was full length, it was in reverse orientation. The cDNA was cut from the vector using the restriction enzymes *Not*I and *Xho*I and, following agarose gel electrophoresis, the fragment was isolated using a commercially available kit (Qiagen, Hilden, Germany). This fragment was ligated into the *Xho*I and *Not*I sites of the vector pBluescript KS using another kit (Stratagene, La Jolla, CA, USA). This yielded the plasmid pAtPPa6.

2.3. Complementation of E. coli

The *E. coli* strain k37 EKTR pE′ [10] was transformed with either the plasmid pBluescript KS or pAtPPa6. Transformed colonies were selected on solid YT media containing appropriate amounts of ampicillin, kanamycin and chloramphenicol. One individual colony from both was isolated and inoculated into liquid media containing the same antibiotics. This was grown overnight at 28 °C and 100 μl of each of these cultures was used to inoculate two separate cultures of 100 ml of

* Corresponding author. Fax: +45-4677-4122.
E-mail address: james.richard.lloyd@risoe.dk (J.R. Lloyd).

Abbreviations: PPase, inorganic pyrophosphatase; PPi, inorganic pyrophosphate; Pi, inorganic phosphate; AGPase, ADP–glucose pyrophosphorylase

0014-5793/$22.00
doi:10.1016/j.febslet.2004.03.080

liquid media containing ampicillin and kanamycin only. These were grown overnight at either 28 or 44 °C. These cultures were then streaked onto solid YT media containing ampicillin and kanamycin and were grown overnight at 28 °C.

2.4. Production of E. coli *extracts containing AtPPa6 protein*

Escherichia coli containing the pAtPPa6 plasmid were grown in liquid YT culture containing ampicillin and kanamycin until it reached an OD_{600} of 0.6. The culture was centrifuged at $3000\times g$ for 15 min at 4 °C and the pellet re-suspended in 50 mM HEPES–KOH (pH 7.5), 2 mM EDTA and 2 mM DTT. *E. coli* cells were disrupted by sonication and the cell debris was removed by centrifuging at $3000\times g$ for 15 min at 4 °C. The extracts were aliquoted, and immediately frozen in liquid nitrogen, before being stored at −80 °C until use.

2.5. Measurement of PPase activity

PPase activity was measured using a variation of a previously published method [11]. Fifteen microliters *E. coli* extract was incubated for 15 min at 30 °C with 285 μl of 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$ and 2 mM NaPPi. The reaction was stopped by adding 400 μl of 12% (w/v) trichloroacetic acid and left on ice for 10 min. The blank was stopped with 400 μl of 12% (w/v) trichloroacetic acid immediately upon initiation of the reaction. The reaction was centrifuged at $20\,000\times g$ for 10 min and 500 μl of the supernatant was placed in a new container. 500 μl of 1% (w/v) ammonium molybdate, 5% (w/v) $FeSO_4\cdot 7H_2O$ and 0.5 M H_2SO_4 was added, and the reaction was left for 10 min at room temperature. The absorbance at 595 nm was determined and compared with that of the blank. The activity at different pH values was determined using the same concentration of Tris–HCl in the incubation buffer, but set at different pH values. The effect of $MgCl_2$ was determined by varying the amount in the incubation buffer, and the effect of NaF was determined by adding it at different concentration to the incubation buffer. The K_m was determined by varying the NaPPi concentration in the incubation buffer. Protein in the extract was measured by the method of Bradford [12].

2.6. Incubation of rosette leaves on sugars

Leaf discs were incubated overnight in darkness on water, and on two different concentrations (100 or 300 mM) of the following sugars: sorbitol, glucose, fructose and sucrose.

2.7. RNA blots

RNA was extracted from plant tissue by a standard method [13]. Forty micrograms total RNA was separated on a 1.5% (w/v) agarose gel containing formaldehyde [14] and blotted onto a nylon membrane (Hybond N+, Amersham, Hillerød, Denmark). Membranes were hybridized at 65 °C in 250 mM sodium phosphate (pH 7.2), 1 mM EDTA, 1% (w/v) BSA and 7% (w/v) SDS. The radioactive probe was made by the random primed method using a commercially available kit (High-Prime, Roche, Hvidovre, Denmark) using the entire *AtPPa6* or *agpS* cDNAs. The filters were washed once with 2× SSC + 0.5% (w/v) SDS, once with 2× SSC + 0.1% (w/v) SDS and once with 1× SSC + 0.1% (w/v) SDS at 65 °C. Radioactivity was detected either by auto-radiography, or by use of a phosphoimager.

2.8. Chloroplast import assays

Plasmid pAtPPa6 was transcribed in vitro using T3 RNA polymerase and the resulting mRNA was used to programme a wheat germ lysate system (Promega) in the presence of L-[^{3}H] leucine (Amersham Biosciences). Intact chloroplasts were isolated from pea seedlings (*Pisum sativum*, var. Kelvedon Wonder), as described in [15]. Import assays were also based on the procedure described by Robinson and Mant [15]; briefly, intact chloroplasts equivalent to 50 μg chlorophyll were mixed with 8 mM MgATP, 5 mM unlabelled L-leucine, 5 mM $MgCl_2$ and 12.5 μl translation mixture, all in HS buffer (330 mM sorbitol, 50 mM HEPES–KOH, pH 8.0) to a final volume of 150 μl. Assay mixtures were incubated for 30 min at 26 °C in an illuminated water bath (150 μmol photon m^{-2} s^{-1}). Incubations were halted by the addition of 1 ml ice-cold HS, followed by centrifugation at $3000\times g$ for 5 min at 4 °C. Chloroplast pellets were resuspended in 120 μl HS and 30 μl removed, representing total chloroplasts. The remaining 90 μl chloroplasts were incubated for 40 min on ice with 0.2 mg ml^{-1} thermolysin (Protease type X, Sigma), 2.5 mM $CaCl_2$ in a final volume of 250 μl HS. Simultaneously, 2 μl in vitro translation mixture was digested with 48 μl thermolysin mixture for 40 min on ice (final concentration 0.2 mg ml^{-1} thermolysin, 2.5 mM $CaCl_2$). The protease digestion was halted by the addition of 10 mM EDTA and one third of the chloroplast sample removed (protease-treated chloroplasts sample). The remaining chloroplasts were centrifuged at $3000\times g$ for 5 min at 4 °C and resuspended in lysis buffer (10 mM HEPES–KOH, pH 8.0, 5 mM $MgCl_2$ and 10 mM EDTA). After 5 min incubation on ice, the chloroplast lysate was centrifuged at $20\,000\times g$ for 5 min at 4 °C. The supernatant represented stromal extract, and the pellet, thylakoid and envelope membranes. All chloroplast fractions were mixed with protein sample buffer and heated to 95 °C for 4 min before analysis by SDS–PAGE and fluorography.

3. Results and discussion

3.1. Identification of the AtPPa6 cDNA

Analysis of the Arabidopsis genome demonstrated the presence of six genes that appeared to code for PPase proteins. The predicted protein sequences of five of these (AtPPa1-5; At1g01050, At2g18230, At2g46860, At3g53620 and At4g01480) were extremely similar, being at least 69% identical to each other. The sixth (AtPPa6; At5g09650), however, was at most only 22% identical with the other five predicted protein sequences.

We used the MacVector 7.0 program to align AtPPa6 with the other plant PPase protein sequences. This alignment was used to produce a dendrogram indicating the phylogeny of the sequences (Fig. 1), revealing that AtPPa6 was most closely related to the predicted protein sequence of a cDNA from *Chlamydomonas rheinhardtii* (NCBI#, CAC42762). Although this is annotated in the NCBI database as being a chloroplastidic isoform, to our knowledge no data have been presented to support that claim.

An alignment of the Arabidopsis sequences, as well as the putative chloroplastidic *Chlamydomonas* isoform is shown in Fig. 2. This demonstrates that both AtPPa6 and the *Chlamydomonas* protein have an N-terminal extension in comparison with the other five Arabidopsis proteins. This extension means that the predicted molecular weight of AtPPa6 is approximately 33.4 kDa in comparison with 24.5–25 kDa for the other Arabidopsis sequences. The comparison also revealed that all the sequences contain a motif (shown in bold in the figure), which is thought to be part of the active site of the enzyme [16,17]. In addition, all plant PPase protein sequences so far studied contain two deletions (D1 and D2) in compar-

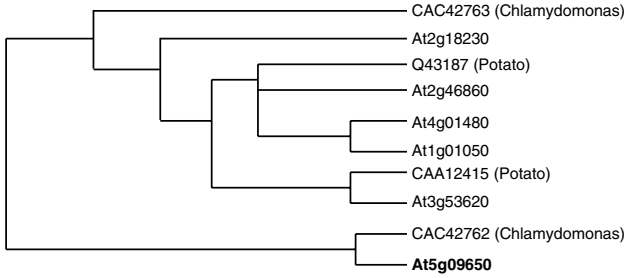

Fig. 1. Dendrogram showing the evolutionary distance of some plant PPases. Amino acid sequences were aligned using the program MacVector 7.0. This alignment was used for the generation of the dendrogram using the UPGMA method and uncorrected distances. Sequences are Arabidopsis, unless otherwise stated. The genomic locus coding for AtPPa6 is in bold.

ison with animal or fungal PPases [18], however, neither AtPPa6 nor the *Chlamydomonas* protein contain these.

Using the At5g09650 DNA sequence we identified an Arabidopsis EST which appeared to correspond to the sequence. We obtained this EST and sequenced both strands of the insert. This revealed that the EST was 100% identical to the At5g09650 sequence.

3.2. AtPPa6 is a functional PPase

To test the function of the AtPPa6 protein we decided to complement an *E. coli* mutant. *E. coli* contains one gene encoding PPase and a mutation within it is lethal. A strain has been manufactured where the gene has been disrupted, but the PPase activity is maintained through the presence of a plasmid (pMAK705::*PPa*) containing the genomic *PPa* sequence [10]. This plasmid contains a thermo-sensitive replicon, meaning that it is possible to replace it by growing the bacteria at temperatures above 40 °C. We transformed this strain with a plasmid allowing expression of the full-length AtPPa6 protein, and also the same plasmid, but not containing the *AtPPa6* cDNA. These were gown in liquid culture overnight at either 28 or 44 °C, before being streaked onto solid media and allowed to grow at 28 °C. It could be demonstrated that pre-incubation at the permissive temperature for replication (28 °C) had no effect on growth (Fig. 3A). This is, of course, expected as the pMAK705::*PPa* plasmid would not become lost. When the cells were grown at 44 °C, however, those containing the empty vector were not able to grow after being plated onto solid media, whilst those containing pAtPPa6 were (Fig. 3B).

3.3. Analysis of AtPPa6 activity

As the *E. coli*'s own *PPase* gene has been replaced by *AtPPa6*, this means that all PPase activity in the *E. coli* comes from the plant protein. As a result it is possible to examine AtPPa6 without encountering problems of background activity of the *E. coli* protein. We made, therefore, extracts from *E. coli* cells containing AtPPa6. Using these crude extracts we examined first whether AtPPa6 was dependant on $MgCl_2$ for its activity, as is normal for PPases [16]. This was indeed the case, with no activity being detected with a concentration of 0 mM $MgCl_2$. The optimal concentration was found to be 5 mM. The pH optimum of the enzyme was also determined. Activities below pH 6.5 were extremely low, but increased to a maximum at pH 7.5. The activity decreased slightly at more basic pHs, but at pH 9.0 was only 10% less than that found at pH 7.5. The K_m for PPi was also determined using a Michaelis–Menten plot and was determined to be approximately 0.6 mM. Finally, the effect of NaF was determined. As has been found with other PPase enzymes [16] NaF was found to be a potent inhibitor of AtPPa6 activity, greatly reducing the activity at concentrations of 1 and 5 mM, and completely eliminating it at 10 mM.

The construct that we used to express the AtPPa6 protein in *E. coli* contained an insert coding for the full-length protein, including any transit peptide. Clearly a transit peptide may affect the kinetic properties of the protein, but it is nevertheless striking how similar the properties are that we measured for AtPPa6 in comparison to those measured for PPa isoforms purified from various other plant species. In studies where PPa was purified from the chloroplast the optimal $MgCl_2$ concentration was determined as between 2 and 7 mM [19–25], as compared to 5 mM for AtPPa6. In addition, all the purified chloroplastidic PPa isoforms have been found to be active at alkaline pHs [19–23], as is AtPPa6. One property that might have been altered either by the presence of a transit peptide, or due to lack of some

```
                1                   20                  40                  60                  80
At4g01480       ..............................................MNGEEVKTSQP.....QKKLQNPTPRLNERILSSLSKRSVA  36
At3g53620       ..............................................MAPPIEVSTKS.....YVEKHVSLPTLNERILSSMSHRSVA  36
At1g01050       ..............................................MSEETKDNQ........RLQRPAPRLNERILSSLSRRSVA  32
At2g18230       ..............................................MAEIKDEGSAKGY...AFPLRNPNVTLNERNFAAFTHRSAA  38
At2g46860       ..............................................MSEEAYEETQE.....SSQSPRPVPKLNERILSTLSRRSVA  36
At5g09650       MAATRVLTAATAVTQTTSCFLAKQAFTLPAKKSCGGFGGLCFSRRALVLKSKRPFSCSAIYNPQVKVQEEGPAESLDYRVFFLDGS.GKK  89
CAC42762        ..................MALAIRSSLRAAAMGRKAFRQAVPVRVAPAQRVRSVTTASAEIT.AYSVEEKGPKDSLEYRMFFKQG..AKE  69

                                                                                D1                  D2
                91                  110                 130                 150                 170
At4g01480       AHPWHDLEIGP.GAPVIFNVVIEISKGSKVKYELDKKTGLIKVDRILYSSVVYPH....NYGFVPRTLCE.............DNDPIDV  108
At3g53620       AHPWHDLEIGP.EAPIIFNCVVEIGKGSKVKYELDKTTGLIKVDRILYSSVVYPH....NYGFIPRTLCE.............DSDPIDV  108
At1g01050       AHPWHDLEIGP.GAPQIFNVVVEITKGSKVKYELDKKTGLIKVDRILYSSVVYPH....NYGFVPRTLCE.............DNDPIDV  104
At2g18230       AHPWHDLEIGP.EAPTVFNCVVEISKGGKVKYELDKNSGLIKVDRVLYSSIVYPH....NYGFIPRTICE.............DSDPMDV  110
At2g46860       AHPWHDLEIGP.EAPLVFNVVVEITKGSKVKYELDKKTGLIKVDRILYSSVVYPH....NYGFIPRTLCE.............DNDPLDV  108
At5g09650       VSPWHDIPLTLGDGVFNFIVEIPKESKAKMEVATDEDFTPIKQDTKKGKLRYYPYNINWNYGLLPQTWEDPSHANSEVEGCFGDNDPVDV  179
CAC42762        VSCWHEIPLYAGDGHLHYICEIPKETSAKMEVATDEPRTPIKQDVKKGKLRFYPYNINWNYGMLPQTWEDPGHTD.ATLGAAGDNDPVDV  158

                181                 200                 220                 240                 260
At4g01480       LVIMQEPVLPGCFLRARAIGLMPMIDQGEKDDKIIAVCVDDPEYKHITNINELPPHR...LSEIRRFFEDYKKNENKEVAVNDFLQPGP.  194
At3g53620       LVIMQEPVIPGCFLRAKAIGLMPMIDQGEKDDKIIAVCADDPEYRHYNDISELPPHR...MAEIRRFFEDYKKNENKEVAVNDFLPATA.  194
At1g01050       LVIMQEPVLPGCFLRARAIGLMPMIDQGEKDDKIIAVCVDDPEYKHYTDIKELPPHR...LSEIRRFFEDYKKNENKEVAVNDFLPSES.  190
At2g18230       LVLMQEPVLTGSFLRARAIGLMPMIDQGEKDDKIIAVCADDPEFRHYRDIKELPPHR...LAEIRRFFEDYKKNENKKVDVEAFLPAQA.  196
At2g46860       LVLMQEPVLPGCFLRARAIGLMPMIDQGEKDDKIIAVCADDPEYKHFTDIKQLAPHR...LQEIRRFFEDYKKNENKKVAVNDFLPSES.  194
At5g09650       VEIGETQRKIGDILKIKPLAALAMIDEGELDWKIVAISLDDPKAHLVNDVEDVEKHFPGTLTAIRDWFRDYKIPDGKPANRFGLGDKPAN  269
CAC42762        VEIGAAAAKRGGVYKVKPVGVLAMIDDGELDWKVIAISADDPKAALCNDVEDVEKHFPGEIQKVLEWFRDYKIPDGKPANKFGYDNKCMN  248

                271                 290
At4g01480       ...AIEAIQYSMDLYAEYILHTLRR.....    216
At3g53620       ...AYDAVQHSMDLYADYVVENLRR.....    216
At1g01050       ...AVEAIQYSMDLYAEYILHTLRR.....    212
At2g18230       ...AIDAIKDSMDLYAAYIKAGLQR.....    218
At2g46860       ...AHEAIQYSMDLYAEYILHTLRR.....    212
At5g09650       KDYALKIIQETNESWAKLVKRS.VDAGDLSLY  300
CAC42762        KEFTLNVIKETHEAYVKLKSGARANSEELSLI  280
```

Fig. 2. Comparison of the deduced primary protein sequences of Arabidopsis PPases and a putative chloroplastidic isoform from *C. rheinhardtii*. Sequences were aligned using the Blosum matrix. Two deletions characteristic of the sequences of most plant PPa proteins are shown as D1 and D2, while a motif thought to be part of the active site of the proteins is shown in bold lettering.

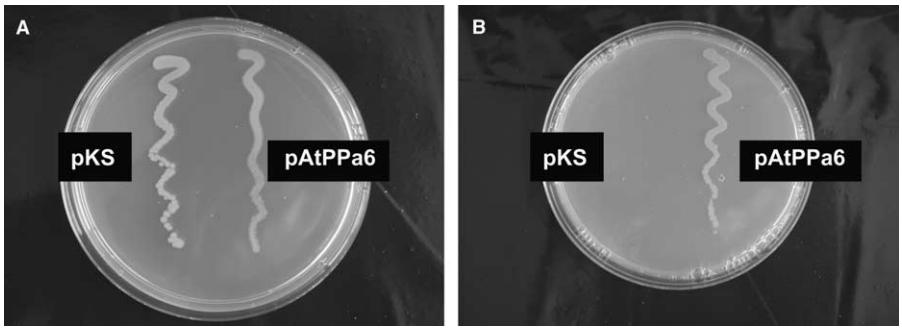

Fig. 3. Complementation of the k37 EKTR pE′ strain with AtPPa6. The *E. coli* were transformed with either the empty vector (pKS), or the same containing an insert coding for AtPPa6 (pAtPPa6). The cells were pre-incubated at either 28 °C (Fig. 1A) or 44 °C (Fig. 1B) before being streaked onto solid media and grown at 28 °C. Cells pre-incubated at 44 °C can only grow when they contain pAtPPa6 due to loss of the plasmid containing the *E. coli* gene coding for PPase.

post-translational modification of the recombinant protein, is the K_m for PPi. In several studies [2,3,19,23,24], the K_m for PPi of PPases isolated from chloroplasts was determined as being approximately 10–50-fold lower than we measured with AtPPa6, however in others [17,21] it was found to be in the same order of magnitude as we determined for AtPPa6.

3.4. *AtPPa6 is targeted to the chloroplast stroma*

To examine the sub-cellular localization of AtPPa6, we performed in vitro import experiments. In vitro-translated, radioactive AtPPa6 protein was incubated with pea chloroplasts and the chloroplasts subsequently fractionated before analysis by SDS–PAGE and fluorography (Fig. 4). The translated full-length precursor of AtPPa6 migrated just under the 37 kDa marker (lane Tr). Following incubation with washed chloroplasts (lane C) the protein migrated with a reduced molecular mass (approximately 28 kDa), indicating processing of the precursor to remove the envelope transit peptide. After digestion of the chloroplasts with thermolysin to remove unimported precursor protein (lane C+), the 28 kDa band is still visible, demonstrating that it is inside the chloroplasts. An important control experiment is to digest the in vitro translation mixture with thermolysin, to ensure that the protease alone does not produce a degradation product with the same size as imported AtPPa6. Lane Tr+ shows the results of such a control: although still visible, the digested protein is clearly slightly smaller than that seen in lane Tr, demonstrating that the imported protein is not an artefact caused by thermolysin. Lysis of the chloroplasts and their separation into stromal (lane S) and thylakoid (lane T) fractions clearly demonstrate that AtPPa6 fractionates with the stromal, but not the membrane fraction. This experiment demonstrates that AtPPa6 is a plastidial isoform and is targeted to the chloroplast stroma.

3.5. *AtPPa6 is expressed in many tissues in Arabidopsis and is induced by metabolizable sugars in leaves*

To examine the extent of expression of the *AtPPa6* gene, an RNA blot containing RNA from various organs was probed. It was found that *AtPPa6* transcript accumulated in all organs examined, but to different extents. It was most highly expressed in flowers, leaves and roots, and to a lesser extent in siliques and stems (Fig. 5A). It would be expected that a plastidial PPase would be present in all tissues, as metabolic pathways producing PPi in plastids will also be present in all tissues. The enzyme that most likely produces the majority of PPi within plastids is ADP–glucose pyrophosphorylase (AGPase), the first enzyme on the committed pathway of starch synthesis [26]. It has been suggested that plastidial PPase activity is necessary to allow starch synthesis, as potato tuber discs incubated on NaF (which inhibits PPase) synthesize less starch than controls [27]. The expression of the genes coding for AGPase is known to be induced by metabolizable sugars [28]. We were interested to see if *AtPPa6* was also induced by

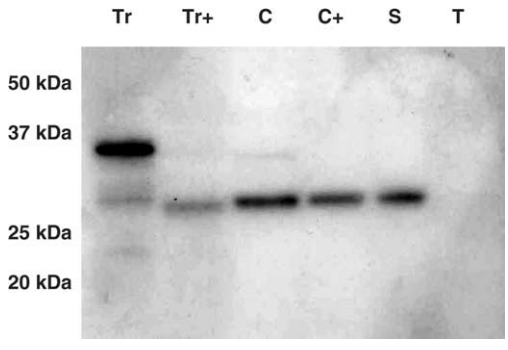

Fig. 4. Import of AtPPa6 into pea chloroplasts. Tr, 0.5 μl in vitro-translated AtPPa6 protein; Tr+, 0.5 μl in vitro-translated AtPPa6 protein treated with thermolysin; C, AtPPa6 following incubation with isolated pea chloroplasts (6 μg chlorophyll); C+, same as lane 3, but post-treated with thermolysin (6 μg chlorophyll); S, stromal extract (6 μg chlorophyll); T, thylakoid membranes (6 μg chlorophyll).

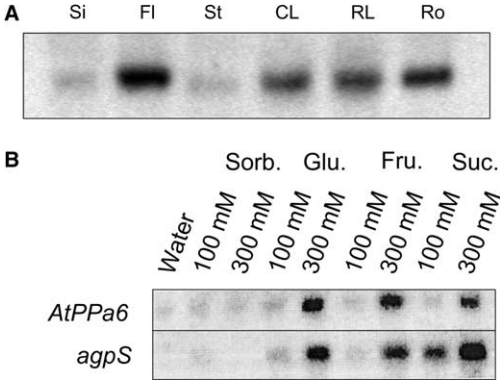

Fig. 5. Transcript accumulation of AtPPa6. (A) Accumulation in different tissues of Arabidopsis. Si, silique; Fl, flower; St, stem; CL, cauline leaf; RL, Rosette leaf; Ro, Root. (B) Sugar induction of *AtPPa6* in comparison with *agpS*. Sorb., sorbitol; Glu., glucose; Fru., fructose; Suc., sucrose.

such sugars. This is because if AtPPa6 plays an important role in catabolizing PPi within the plastid to allow starch synthesis, it will most likely be regulated in a similar way to genes coding for AGPase. Therefore, we incubated rosette leaves on different concentrations of sorbitol, sucrose, glucose and fructose in darkness. We extracted RNA from these leaves and probed this with either the Arabidopsis *agpS* cDNA (coding for the small sub-unit of AGPase) or the *AtPPa6* cDNA. Similar to results reported previously [28], expression of *agpS* is induced by all the metabolizable sugars at 300 mM and by sucrose at 100 mM (Fig. 5B). The expression of *AtPPa6* is also induced at 300 mM by the metabolizable sugars, but not at 100 mM (Fig. 5B). The expression of neither gene was induced by incubation on either 100 or 300 mM sorbitol (Fig. 5B).

In conclusion, we have isolated an Arabidopsis cDNA coding for a plastidial isoform of PPase. RNA blots indicate co-expression of this gene with that coding for the small sub-unit of AGPase, suggesting that it may play a role in starch synthesis. Our future work will concentrate on the isolation and analysis of mutant and transgenic plants lacking this protein.

Acknowledgements: The authors thank Dr. Pierre Plateau (Laboratoire de Biochemie, Ecole Polytechnique, Palaiseau Cedex, France) for the gift of the pMAK705::*PPa E. coli* strain, Dr. Christian Sohlenkamp (Max-Planck-Institute of Molecular Plant Physiology, Golm, Germany) for gift of the Arabidopsis tissue RNA blot, and Prof. Leszek Kleczkowski (University of Umeå, Sweden) for providing the *agpS* cDNA.

References

[1] Kornberg, A. (1957) Adv. Enzymol. 18, 191–240.
[2] Gross, P. and ap-Rees, T. (1986) Planta 167, 140–145.
[3] Weiner, H., Stitt, M. and Heldt, H.W. (1987) Biochim. Biophys. Acta 893, 13–21.
[4] Lunn, J. and Douce, R. (1993) Biochem. J. 290, 375–379.
[5] Kieber, J.J. and Signer, E.R. (1991) Plant Mol. Biol. 16, 345–348.
[6] du Jardin, P., Rojas-Beltran, J., Gebhardt, C. and Brasseur, R. (1995) Plant Physiol. 109, 853–860.
[7] Rojas-Beltrán, J.A., Dubois, F., Mortiaux, F., Portetelle, D., Gebhardt, C., Sangwan, R.S. and du Jardin, P. (1999) Plant Mol. Biol. 39, 449–461.
[8] Sterky, F., Lundeberg, J. and Kleczkowski, L.A. (1999) Plant Physiol. 120, 934.
[9] Visser, K., Heimovaara-Dijkstra, S., Kijne, J.W. and Wang, M. (1998) Plant Mol. Biol. 37, 131–140.
[10] Chen, J., Brevet, A., Fromant, M., Lévêque, Schmitter, J.-M., Blanquet, S. and Plateau, P. (1990) J. Bacteriol. 172, 5686–5689.
[11] Taussky, H.H. and Shorr, E. (1953) J. Biol. Chem. 202, 675–685.
[12] Bradford, M.M. (1976) Anal. Biochem. 72, 248–252.
[13] Logemann, J., Schell, K. and Willmitzer, L. (1987) Anal. Biochem. 163, 16–20.
[14] Lehrach, H., Diamond, D., Wozney, J.M. and Boedtker, H. (1977) Biochemistry 16, 4743–4751.
[15] Robinson, C. and Mant, A. (2002) Import of proteins into isolated chloroplasts and thylakoid membranes. in: Molecular Plant Biology, Vol. 2: A Practical Approach (Gilmartin, P. and Bowler, C., Eds.), pp. 123–146, Oxford University Press, Oxford.
[16] Cooperman, B.S., Baykov, A.A. and Lahti, R. (1992) Trends Biochem. Sci. 17, 262–266.
[17] Lahti, R., Kolakowski, L.F., Heinonen, J., Vihinen, M., Pohjanoksa, K. and Cooperman, B.S. (1990) Biochim. Biophys. Acta 1038, 338–345.
[18] Sivula, T., Salminen, A., Parfenyev, A.N., Pohjanjoki, P., Goldman, A., Cooperman, B.S., Baykov, A.A. and Lahti, R. (1999) FEBS Lett. 454, 75–80.
[19] Simmons, S. and Butler, L.G. (1969) Biochim. Biophys. Acta 172, 150–157.
[20] El-Badry, A.M. and Bassham, J.A. (1970) Biochim. Biophys. Acta 197, 308–316.
[21] Bucke, C. (1970) Phytochemistry 9, 1303–1309.
[22] Schwenn, J.D., Lilley, McC. and Walker, A. (1973) Biochim. Biophys. Acta 325, 586–595.
[23] Klemme, B. and Jacobi, G. (1974) Planta 120, 147–153.
[24] Ananda Krishnan, V.A: and Gnanam, A. (1988) Arch. Biochem. Biophys. 260, 277–284.
[25] Mortain-Bertrand, A., El Amrani, A., Davail, S., Rey, P., Suire, C. and Lamant, A. (1996) Plant Physiol. Biochem. 34, 343–352.
[26] Kossmann, J. and Lloyd, J.R. (2000) Crit. Rev. Plant Sci. 19, 171–226.
[27] Viola, R. and Davies, H.V. (1991) Plant Physiol. 97, 638–643.
[28] Sokolov, L.N., Dejardin, A. and Kleczkowski, L.A. (1998) Biochem. J. 336, 681–687.