The Different Large Subunit Isoforms of Arabidopsis thaliana ADP-glucose Pyrophosphorylase Confer Distinct Kinetic and Regulatory Properties to the Heterotetrameric Enzyme*

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ADP-glucose pyrophosphorylase catalyzes the first and limiting step in starch biosynthesis and is allosterically regulated by the levels of 3-phosphoglycerate and phosphate in plants. ADP-glucose pyrophosphorylases from plants are heterotetramers composed of two types of subunits (small and large). In this study, the six Arabidopsis thaliana genes coding for ADP-glucose pyrophosphorylase isoforms (two small and four large subunits) have been cloned and expressed in an Escherichia coli mutant deficient in ADP-glucose pyrophosphorylase activity. The co-expression of the small subunit APS1 with the different Arabidopsis large subunits (APL1, APL2, APL3, and APL4) resulted in heterotetramers with different regulatory and kinetic properties. Heterotetramers composed of APS1 and APL1 showed the highest sensitivity to the allosteric effectors as well as the highest apparent affinity for the substrates (glucose-1-phosphate and ATP), whereas heterotetramers formed by APS1 and APL2 showed the lower response to allosteric effectors and the lower affinity for the substrates. No activity was detected for the second gene coding for a small subunit isoform (APS2) annotated in the Arabidopsis genome. This lack of activity is possibly due to the presence of essential amino acids involved in catalysis and/or in the binding of glucose-1-phosphate and 3-phosphoglycerate. Kinetic and regulatory properties of the different heterotetramers, together with sequence analysis, has allowed us to make a distinction between sink and source enzymes, because the combination of different large subunits would provide a high plasticity to ADP-glucose pyrophosphorylase activity and regulation. This is the first experimental data concerning the role that all the ADP-glucose pyrophosphorylase isoforms play in a single plant species. This phenomenon could have an important role in vivo, because different large subunits would confer distinct regulatory properties to ADP-glucose pyrophosphorylase according to the necessary for starch synthesis in a given tissue.

ADP-glucose pyrophosphorylase (ADP-Glc PPase, EC 2.7.7.27) is the first enzyme of the starch biosynthesis pathway (1–3). It catalyzes the synthesis of ADP-glucose (ADP-Glc) and PP from glucose 1-phosphate (Glc-1-P) and ATP (4). ADP-Glc serves as the glucosyl donor for starch biosynthesis mediated by starch synthase and starch branching enzyme (3, 5). Most of the ADP-Glc PPase enzymes studied are allosterically regulated by intermediates of the major pathway of carbon assimilation in the organism, and it has been proposed that this allosterical modulation of ADP-Glc PPase represents the main control of the starch synthesis rate (6). In cyanobacteria, green algae and photosynthetic cells, and tissues of plants, ADP-Glc PPase is allosterically activated by 3-phosphoglycerate (3-PGA) and inhibited by Pi (7, 8). However, in nonphotosynthetic tissues of higher plants, a less uniform situation is observed. ADP-Glc PPases from pea embryos (8), bean cotyledons (9), and barley endosperm (10) have been described to be insensitive to 3-PGA and Pi. On the contrary, ADP-Glc PPases from wheat, maize, and rice endosperm, as well as from potato tuber and cassava root, are regulated by 3-PGA and Pi (11, 14). In addition to the regulation by 3-PGA and Pi, a redox control has also been proposed for potato tuber ADP-Glc PPase (15–17).

ADP-Glc PPases are tetrameric enzymes. However, whereas the bacterial enzyme is composed of four identical subunits (α4) (18, 19), ADP-Glc PPase from higher plants is a heterotetramer (αβ2) composed of two closely related but different types of subunits (20–23). The small subunit (SS) is responsible for the catalytic activity, whereas the large subunit (LS) plays a regulatory function (3). Different isoforms for ADP-Glc PPase subunits have been described, and many cDNAs and genomic DNAs encoding for them have been isolated from both monocot and dicot plants. The most frequent situation is the existence of one SS gene and several LS genes that are differentially expressed (9, 23–29). In Arabidopsis one SS gene (ApS1) and three LS genes (ApL1, ApL2, and ApL3) have been previously identified (30). Recently, the Arabidopsis Genome Initiative provided evidence for the existence of two new putative ADP-Glc PPase encoding genes: one with homology to SS (At1g05610) and another with homology to LS (At2g21590) that we designated ApS2 and ApL4, respectively.

Many attempts have been made to determine the function of the higher plants ADP-Glc PPase subunits. Studies with the potato tuber recombinant ADP-Glc PPase shows that the large subunit regulates the activity of the small subunit, increasing its apparent affinity for the activator and decreasing the affinity for the inhibitor. The function of the large subunit would be
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Expression and Purification of Recombinant Enzymes

The ADP-Glc P-Pase SS alone or together with the LS cDNAs were expressed in E. coli strain AC70RI–504, which is deficient in endogenous ADP-Glc P-Pase activity. In small scale expression assays, single colonies were grown in 50 ml of LB medium at 37 °C with the suitable antibiotic until the A600 reached 1.1–1.3. The induction of the small subunits was initiated by adding 400 μM of isopropyl-β-D-thiogalactopyranoside, and 5 μg/ml of naldixic acid was added to induce the expression of the large subunits. After 16 h of induction at room temperature, the cells were harvested by centrifugation. Cell paste were resuspended in 3 ml of buffer A containing 50 mM HEPES (pH 7.9), 5 mM MgCl2, 0.1 mM EDTA, and 10% sucrose and disrupted by sonication. The sonicated suspensions were centrifuged for 20 min at 10,000 × g, and the supernatants (crude extracts) were retained. The activity of the constructions was tested using 10 μl of 10- or 5-fold diluted crude extracts in the pyrophosphorylases assay that is described below.

Recombinant enzymes were purified for kinetic and regulatory studies. To purify the recombinant APS1 homotetramer and the APS1 plus the four large subunits, the cells were grown in 10 liters of LB medium. The induction and sonication was followed as described above, but sonicated cells were resuspended in 100 ml of buffer A. All subsequent protein purification steps were performed at 0–4 °C. The pyrophosphorylases assay was used to monitor the ADP-Glc P-Pase activity during the purification steps. Crude extracts were subject to heat treatment at 65 °C (or 70 °C for the APS1 homotetramer) as described for purification of the potato recombinant ADP-Glc P-Pase (31). The supernatants obtained after the heat treatment were absorbed onto a DEAE-Fractogel column of 40–ml bed volume equilibrated with buffer A. The enzymes were eluted with a NaCl linear gradient (10 bed volumes, 0–0.5 M) in buffer A, and fractions of 24 ml were collected. Fractions containing ADP-Glc P-Pase activity were pooled, and an ammonium sulfate fractionation (30–60%) was performed, with centrifugation for 20 min at 12,000 × g. The ammonium sulfate pellets were resuspended in 6 ml of buffer A and desalted on Econo-Pac™ 100G columns (Bio-Rad) equilibrated in buffer A. The desalted samples were applied to a MonoQ HR 10/10 column equilibrated with buffer A. To elute the enzymes, a NaCl linear gradient (20 bed volumes, 0–0.5 M) was applied, and fractions of 4 ml were collected. Fractions containing ADP-Glc P-Pase activity were pooled, concentrated by Ultra Free-4™ centrifugal filters with 10-kDa molecular mass cut-off (Millipore) and stored at ~80 °C. ADP-Glc P-Pase activity of the samples was stable for at least 3 months.

Protein Assay

Protein concentrations of the fractions obtained during the purification were determined by using bichinchonic acid reagent (34) from Pierce, using bovine serum albumin as the standard.

Assay of ADP-Glc P-Pase Activity

Pyrophosphorylization Direction—[32P]ATP formed in the pyrophosphorylases assay of ADP-Glc was determined according to Morell et al. (120). The reaction mixture consisted of 50 mM HEPES (pH 7.9), 7 mM MgCl2, 2 mM dithiothreitol, 2 mM ADP-Glc, 10 mM NaF, 0.2 mg/ml bovine serum albumin, 1 mM [γ-32P]ATP (1000 cpm/nmol), 3-PGA, and the aliquot of the enzyme in a final volume of 0.25 ml. For kinetic characterization the reaction mixture also contained 0.2 or 2 mM NaF, and the concentration of 3-PGA was 10 mM in the small scale induction experiments and was the indicated range in the kinetic characterizations.

Synthesis Direction—Synthesis of ADP-Glc was measured as described by Ghosh and Preiss (35) and was used to characterize the response to effectors and the apparent affinity for the substrates. The reaction mixture contained 50 mM HEPES (pH 7.9), 7 mM MgCl2, 2 mM dithiothreitol, 2 mM ADP-Glc, 10 mM ATP, and the enzyme in final volume of 0.2 ml. 0.5 mM of isopropyl-β-D-thiogalactopyranoside was included in the mixture to determine sensitivity to phosphophosphate inhibition. The Glc-1-P S0.5 was determined measuring the ADP-Glc P-Pase activity, and in this case, the reaction mixture included [14C]Glc-1-P (1000 cpm/nmol). To determine the S0.5 of the activators, we used 3-PGA at concentrations 5-fold the 3-PGA A determined for each enzyme. This approach compensated for the differences in sensitivity to the activator of the different recombinant tetramers and allowed us to compare the values obtained. One unit of enzyme activity in the pyrophosphorylases and synthesis direction assays was defined as 1 μmol of product formed per minute.

Kinetic Characterization

The A0.5 and S0.5 values, which correspond to the concentration of activator and substrate giving 50% of the maximal velocity respectively,
**Properties of ADP-Glc Pyrophosphorylase**

<table>
<thead>
<tr>
<th></th>
<th>ATP Site</th>
<th>Catalytic Site</th>
<th>Glc-1-P Site</th>
<th>Activator Site</th>
</tr>
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<tbody>
<tr>
<td><strong>a</strong></td>
<td>107</td>
<td>115</td>
<td>133</td>
<td>143</td>
</tr>
<tr>
<td>Consensus</td>
<td>WPQQTADAV</td>
<td>LAGQHLYRMDY</td>
<td>IIEF HRP GR</td>
<td>SIVG VIK A IP OTV</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>**********</td>
<td>**********</td>
<td>*<strong>V</strong></td>
<td>** smelling **</td>
</tr>
<tr>
<td>Anabaena</td>
<td>**********</td>
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<td>**********</td>
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</tr>
<tr>
<td><em>Synecocystis</em></td>
<td>**********</td>
<td>**********</td>
<td><strong>Q</strong></td>
<td>** smelling **</td>
</tr>
<tr>
<td>Potato SS</td>
<td>**********</td>
<td>**********</td>
<td><strong>Q</strong></td>
<td>** smelling **</td>
</tr>
<tr>
<td>Tomato SS</td>
<td>**********</td>
<td>**********</td>
<td><strong>Q</strong></td>
<td>** smelling **</td>
</tr>
<tr>
<td>Barley SS</td>
<td>**********</td>
<td>**********</td>
<td><strong>Q</strong></td>
<td>** smelling **</td>
</tr>
<tr>
<td>Wheat SS</td>
<td>**********</td>
<td>**********</td>
<td><strong>Q</strong></td>
<td>** smelling **</td>
</tr>
<tr>
<td>Arabidopsis APS1</td>
<td>******* <strong>I</strong></td>
<td><strong>Q</strong></td>
<td>** smelling **</td>
<td></td>
</tr>
<tr>
<td>Arabidopsis APS2</td>
<td>**********</td>
<td><strong>Q</strong></td>
<td>** smelling **</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1. Comparison of motifs in the Arabidopsis APS2 amino acid sequence.** The sequence of the APS2 protein was aligned with different plant and prokaryotic ADP-glucose pyrophosphorylases, and the catalytic (37, 38), ATP, Glc-1-P, and Activator sites (3) are showed in the figure. A consensus of the alignment and the amino acid position corresponding to the APS2 sequence are also displayed. The *E. coli* residue Apn (36) (37, 38) is underlined in the figure. The accession numbers of the aligned sequences, from top to bottom in the figure, are: P00584, P30521, P24215, P23509, AAB00482, CAA88449, CAA46879, U72351, and AJ536666.

and Hill coefficients (n_H) were calculated by fitting the data with a nonlinear least square formula and the Hill equation using the software Origin™ 6.0. All of the kinetic parameters are the means of at least two determinations. The standard deviation was between 20% and 20% of the average value.

**RESULTS**

**Cloning and Expression in E. coli of the Arabidopsis ADP-Glc PPase cDNAs—** The APS1 and the four LS cDNAs sequences were obtained from the Arabidopsis Information Resource database (www.arabidopsis.org). The putative cDNA sequence of the *ApS2* gene was predicted by the GENSCAN program (genes.mit.edu/GENESCAN.html), and the corresponding full-length cDNA was cloned and sequenced (EMBL accession number AJ536666). All ADP-Glc PPases from dicotyledonous plants studied so far are plastidial proteins. Thus, a putative mature and prokaryotic ADP-glucose pyrophosphorylases, and the catalytic (37, 38), ATP, Glc-1-P, and Activator sites (3) are showed in the figure.

**Expression of the different constructs was carried out in E. coli strain AC70R1–504, mutated in the structural gene of the bacterial ADP-Glc PPase.** Crude extracts of *E. coli* AC70R1–504 cells transformed with the different plasmids were analyzed for ADP-Glc PPase activity in the pyrophosphorylation direction at saturating concentrations of 3-PGA. When the APS1 subunit was expressed alone, an activity of ~0.13 units mg⁻¹ protein was determined. Similar results were obtained in extracts of *E. coli* AC70R1–504 cells co-expressing APS1 with any of the LS. No ADP-Glc PPase activity was detected for APS2 subunit expressed alone (<0.003 units mg⁻¹ protein) or co-expressed with any of the LS.

The absence of activity of APS2 could be explained by analyzing the sequence of the protein. Aspartate residues 142 of *E. coli* and 145 in potato ADP-Glc PPase have been demonstrated to be essential for catalysis (37, 38). It is one of the highly conserved residues among the superfamily of pyrophosphorylases (39, 40) and is present in all plant ADP-Glc PPases. In the case of APS2 protein, the aspartate residue is substituted by a histidine residue (amino acid 136) (Fig. 1). A sequence comparison between APS2 and other SS of the region comprising the ATP, Glc-1-P, and the activator sites, as defined by Sivak and Preiss (3), is also shown in Fig. 1. Although the ATP site in APS2 is well conserved when compared with other SS, the Glc-1-P site shows significant sequence variations in relation to both plant and bacterial ADPG PPases. In addition, the activator site, which is conserved among cyanobacteria, plant SS, and plant LS ADP-Glc PPases (3), shows several amino acid substitutions in the APS2 protein (Fig. 1). These sequence divergences suggest that Arabidopsis APS2 is a non-functional ADP-Glc PPase subunit, although further studies will be necessary to discern the role of the APS2 gene.

**Purification of the Arabidopsis Recombinant ADP-Glc PPases—** Arabidopsis APS1 homotetramer or heterotetramers formed by APS1 with any of the LS were partially purified to study the subunit interactions between the four Arabidopsis LS and the fully active Arabidopsis SS. Purification was carried out through heat treatment, followed by two anion-exchange chromatography steps on DEAE-Fractogel and MonoQ HR columns, as described under “Experimental Procedures.” We detected the presence of SS homotetramers during the purification of heterotetramers, probably because of the imbalanced expression of the small and the large subunit genes in *E. coli*. It has been described that the activity of plant ADP-Glc PPase heterotetramers has a lower 3-PGA A0.5 than the homotetramers (31). To select those fractions containing the heterotetrameric forms, the activity was monitored during the purification at two different concentrations of the activator 3-PGA (0.5 mM and 10 mM). Thus, only fractions that showed activity in both conditions were pooled and considered in further steps. It is the first time that this situation has been reported for the purification of a plant recombiant ADP-Glc PPase, and it should be considered in further purifications.

The specific activities in the pyrophosphorylation direction obtained for the partially purified Arabidopsis recombinant ADP-Glc PPases in the presence of 10 mM 3-PGA were 16.0 units mg⁻¹ protein for the APS1 homotetramer and 3.7, 5.0, 2.5, and 17.0 units mg⁻¹ protein for the APS1 plus APL1, APL2, APL3, and APL4 heterotetramers, respectively. The enzymes were estimated to be ~4–25% pure, considering a specific activity for the pure enzyme of ~60 units mg⁻¹ protein (14). It was of importance that no degrading reactions for either ADP-Glc or Glc-1-P were noted during the kinetic assays. The recombinant enzymes were subjected to immunoblot analysis using antibodies against spinach-leaf ADP-Glc PPase (20) and showed no apparent degradation during the purification process (data not shown).

**Kinetic Parameters of the Arabidopsis Recombinant ADP-Glc PPase in the Pyrophosphorylase Direction—** The kinetics for the response to 3-PGA conferred by the different Arabidopsis LS were studied in detail. The A0.5 for 3-PGA values in pyrophosphorylase direction are shown in Table I. The data indicate that, as previously reported for other plant recombinant enzymes (14, 31), the APS1 homotetramer has a much lower apparent affinity for the activator, 3-PGA, than the heterotetrameric enzymes. The A0.5 for the homotetrameric APS1 enzyme was 1.2 mM, whereas the A0.5 of the heterotetrameric
enzyme composed of APS1 and APL1 is 3 orders of magnitude lower (0.0017 mM). The $A_{0.5}$ values for APS1/APL3 or APS1/APL4 are 40-fold lower, and the corresponding $A_{0.5}$ for the APS1/APL2 is 5-fold lower (Table I). The different responses of the Arabidopsis recombinant ADP-Glc PPases are plotted in Fig. 2. These results indicated that the different Arabidopsis LS confer distinct regulatory properties to ADP-Glc PPase, with the heterotetramer APS1/APL1 being the most sensitive to 3-PGA.

The effect of the inhibitor was also studied in the pyrophosphorylation direction. The magnitude of $P_i$ inhibition is dependent on the concentration of the activator, 3-PGA, and to compare the effect of $P_i$ on the different enzymes, we studied the shift in the 3-PGA $A_{0.5}$ caused by the presence of $P_i$. This strategy allowed us to avoid the differences in affinity for 3-PGA. The data in Table I show that the sensitivity to $P_i$ was also different between the Arabidopsis recombinant enzymes. The presence of 0.2 mM $P_i$ induced a 4.6-fold increase on the 3-PGA $A_{0.5}$ for the APS1 homotetramer. However, $A_{0.5}$ for 3-PGA of the APS1/APL1 heterotetramer was 11-fold higher in the presence of $P_i$, 0.2 mM and 28-fold higher with 2.0 mM $P_i$. This shift was less pronounced for the other heterotetramers (Table I). This results indicate that the APS1/APL1 heterotetramer is more sensitive to both allosteric effectors in the pyrophosphorylation direction than the other heterotetramers.

### Kinetic Parameters of the Arabidopsis Recombinant ADP-Glc PPase in the Synthesis Direction—The characterization of the Arabidopsis recombinant ADP-Glc PPases was completed studying the activity in the physiological direction. The data of the response to the allosteric effectors (Table II) confirm the results obtained in the pyrophosphorylation direction. Moreover, our 3-PGA $A_{0.5}$ data are in accordance with a previously reported work on the recombinant APS1/APL1 enzyme (36) and the native Arabidopsis leaf enzyme (41).

*Table I.* Kinetic parameters for the 3-PGA of A. thaliana recombinant ADP-Glc PPase in the pyrophosphorylation direction

<table>
<thead>
<tr>
<th></th>
<th>Control 3-PGA $A_{0.5}$</th>
<th>$n_H$</th>
<th>0.2 mM $P_i$ 3-PGA $A_{0.5}$</th>
<th>$n_H$</th>
<th>2 mM $P_i$ 3-PGA $A_{0.5}$</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS1</td>
<td>1.2 ± 0.09</td>
<td>1.8</td>
<td>5.6 ± 0.29</td>
<td>2.5</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>APS1/APL1</td>
<td>0.0017 ± 0.0005</td>
<td>1.0</td>
<td>0.019 ± 0.0019</td>
<td>1.9</td>
<td>0.48 ± 0.005</td>
<td>2.7</td>
</tr>
<tr>
<td>APS1/APL2</td>
<td>0.219 ± 0.024</td>
<td>0.8</td>
<td>0.820 ± 0.12</td>
<td>0.9</td>
<td>6.95 ± 1.59</td>
<td>1.5</td>
</tr>
<tr>
<td>APS1/APL3</td>
<td>0.029 ± 0.009</td>
<td>0.6</td>
<td>0.105 ± 0.025</td>
<td>0.8</td>
<td>0.29 ± 0.048</td>
<td>1.0</td>
</tr>
<tr>
<td>APS1/APL4</td>
<td>0.030 ± 0.003</td>
<td>0.9</td>
<td>0.110 ± 0.008</td>
<td>1.0</td>
<td>0.80 ± 0.167</td>
<td>1.2</td>
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</table>

The catalytic activity of the ADP-Glc PPase is considered to be mainly due to the action of the SS (37, 38, 42, 43). LS appeared to interact with the substrate but does not play a direct role in catalysis (44). We investigated whether the different large subunits affect the affinity of ADP-Glc PPases for its substrates, Glc-1-P and ATP. As shown in Table III, APL1 induced a 4-fold increase in the apparent affinity for the Glc-1-P compared with the APS1 homotetramer, whereas the other APLs did not significantly affect the $S_{0.5}$ for Glc-1-P. In contrast, the ATP $S_{0.5}$ was affected for Arabidopsis APL1, APL4, and APL3 (Table III). The ATP $S_{0.5}$ APS1/APL1 heterotetramer was 6-fold lower than the APS1 homotetramer, whereas the APS1/APL3 or APS1/APL4 heterotetramers were 4-fold lower. On the other hand, the APS1/APL2 heterotetramer showed a similar $S_{0.5}$ for ATP in relation to the APS1 homotetramer. In summary, the Arabidopsis APL1, APL3, and APL4 confer higher affinity for ATP, whereas APL2 confers an affinity for ATP similar to the APS1 homotetramer. These are novel data supporting the idea that the role of large subunits is not only to modulate ADP-Glc PPase in response to the allosteric effectors, but they can also change the apparent affinity for the substrates.

### Sequence Analysis of ADP-Glc PPase Subunits—The predicted amino acid sequences of several plant ADP-Glc PPases can be divided in two groups corresponding to APS and APL subunits (23, 24). To analyze the putative function of the different Arabidopsis ADP-Glc PPase subunits, we compare a set of ADP-Glc PPase amino acid sequences analyzing the tissue where the proteins are present. A phylogenetic tree built with protein sequences deposited in the EMBL/GenBank/DDBJ data bases including the six Arabidopsis ADP-Glc PPase subunits is shown in Fig. 3. As previously reported, sequences can be divided in two main groups corresponding to small and large subunits. The higher plant SS group can be divided in two subgroups: one corresponding to monocots and a second one corresponding to dicots (including APS1 from Arabidopsis). APS1 from Arabidopsis is essential to starch synthesis in vivo as revealed the study of starch-lacking mutant plants (45). Small subunits are considered to be ubiquitous, although in the case of rice, Vicia faba, and pea, for which two genes coding for
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Table II

Kinetic parameters for the 3-PGA of A. thaliana recombinant ADP-Glc PPase in the synthesis direction

ADP-Glc PPase activity was determined in the synthesis direction as described under “Experimental Procedures.” The kinetics parameters (see “Experimental Procedures”) were calculated at the specified concentrations of Pᵢ. The deviation in the 3-PGA data is the difference between duplicate experiments. ND indicates not determined.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.2 mM Pᵢ</th>
<th>2 mM Pᵢ</th>
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<tbody>
<tr>
<td></td>
<td>3-PGA Aᵢ₅, nᵢ</td>
<td>3-PGA Aᵢ₅, nᵢ</td>
<td>3-PGA Aᵢ₅, nᵢ</td>
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<tr>
<td>APS1</td>
<td>5.7 ± 0.5</td>
<td>13.9 ± 1.8</td>
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<td>APS1/APL1</td>
<td>0.018 ± 0.004</td>
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<tr>
<td>APS1/APL2</td>
<td>0.87 ± 0.11</td>
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<tr>
<td>APS1/APL3</td>
<td>0.34 ± 0.09</td>
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<td>APS1/APL4</td>
<td>0.16 ± 0.03</td>
<td>0.48 ± 0.1</td>
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</tr>
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</table>

Table III

Kinetic parameters for the substrates in the synthesis direction

The ADP-Glc PPase activity was determined in the synthesis direction as described under “Experimental Procedures” except that the concentration of 3-PGA was different for each enzyme. To avoid the different apparent affinity for the activator, we determined the Sₒ.₅ (see “Experimental Procedures”) for the ATP and the Glc-1-P at 20 mM of 3-PGA for the APS1, 0.1 mM for the APS1/APL1, 4 mM for the APS1/APL2, 2 mM for the APS1/APL3, and 1 mM for the APS1/APL4. These concentrations were five times the 3-PGA Aᵢ₅ of each enzyme, respectively. The kinetics parameters were calculated at the specified concentrations of 3-PGA without inhibitor (Pᵢ). The deviation in the Sₒ.₅ data is the difference between duplicate experiments.

<table>
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<th>ATP</th>
<th>Glc-1-P</th>
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<td>Sₒ.₅, nᵢ</td>
<td>Sₒ.₅, nᵢ</td>
</tr>
<tr>
<td>APS1</td>
<td>0.402 ± 0.04</td>
<td>0.076 ± 0.018</td>
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<td>APS1/APL1</td>
<td>0.067 ± 0.008</td>
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<td>0.052 ± 0.007</td>
</tr>
<tr>
<td>APS1/APL4</td>
<td>0.118 ± 0.01</td>
<td>0.060 ± 0.008</td>
</tr>
</tbody>
</table>

different APS subunits have been described, distinct patterns of expression have been reported (9, 13, 46). The Arabidopsis APS2 protein appears in a separate branch of the tree. Although APS2 is related to ADP-Glc PPase SS, its sequence is clearly different from any other ADP-Glc PPase sequence present in the data bases. This is reflected in Fig. 3, supporting the idea of the nonfunctionality of the APS2 protein, with APS1 being the only functional SS in Arabidopsis.

The LS group can be divided into two subgroups corresponding to sink (sites of net sugar import like endosperm, roots, and young leaves) and source (sites of net sugar export like mature leaves) tissues, with the exception of the sugar beet large subunits that would be dependent on the specific large subunits present in a given tissue (31, 46).

In the present study, we have cloned the six A. thaliana mature cDNAs coding for ADP-Glc PPase isoforms and characterized the regulatory properties of the different ADP-Glc PPase heterotetramers. Recombinant enzymes expressed in E. coli were partially purified, and the regulatory and kinetic properties were studied.

We could not detect activity for the APS2 homotetramer or for heterotetramer composed of APS2 and any of the LS. The lack of activity of APS2 subunit is probably due to the absence of essential amino acids for catalysis and/or amino acids implicated in the binding of Glc-1-P and the allosteric activator (Fig. 1). Besides, ApS2 mRNA can only be detected by reverse transcription-PCR, indicating that its level of expression is very low. So, it is possible that APS2 is a noncatalytic subunit.

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When analyzed in the pyrophosphorylation direction, APL1 conferred to the ADP-Glc PPase heterotetramer a very high...
sensitivity to both 3-PGA and Pi (Table I). The $A_{0.5}$ for 3-PGA was estimated to be 0.0017 mM for APS1/APL1 heterotetramer. The corresponding values for were 100-fold higher for APS1/APL2 and 10-fold higher for APS1/APL3 or APL4. The response of the LS isoforms to 3-PGA are graphically represented in Fig. 2, indicating the great differences in the response to the effector 3-PGA. The inhibition by Pi is more drastic for the APS1/APL1 heterotetramer, inducing a 10-fold increase of the $A_{0.5}$ for 3-PGA at 0.2 mM and an approximately 280-fold increase at 2.0 mM. The effect of Pi was less pronounced for heterotetramers formed by APS1 and any of the other three APLs and showed an increase in the range of 3-4-fold at 0.2 mM and 10-30-fold at 2.0 mM Pi. The regulatory properties of the different ADP-Glc PPase heterotetramers were corroborated by studying the effect of 3-PGA and Pi in the synthesis direction (Table II). Our results are consistent with the idea that ADP-Glc PPase regulatory properties in a given tissue would be dependent on the expression of specific isoforms that could generate different heterotetramers. APS1/APL1 heterotetramers, the main enzyme in leaves, should respond in a

![Fig. 3. Comparison of ADP-Glc PPase sequences present in the EMBL/GenBank/DDBJ data bases.](image)

![Fig. 4. Model for the regulation of ADP-Glc PPase activity by the different large subunit isoforms in A. thaliana according to the sink/source nature of the organ or tissue.](image)
more sensitive manner to changes in 3-PGA concentration than source enzymes. It has been shown that tomato leaf ADP-Glc PPase is more sensitive to 3-PGA than the fruit enzyme (25, 26). In photosynthetic tissues, the levels of 3-PGA and P, will fluctuate according to the photosynthetic process and is considered to be one of the mechanisms controlling carbon partitioning between sucrose and starch. Changes in these metabolites are involved in the physiological regulation of starch synthesis in leaves (50). The high sensitivity of APS1/APL1 ADP-Glc PPase heterotetramer to the allosteric effectors strongly suggests that starch synthesis in Arabidopsis leaves would be finely tuned by the ratio 3-PGA/P,. In contrast, heterotetramers of APS1 with APL2, APL3, or APL4 would only respond to more drastic changes in the levels of the allosteric effectors. A distinction can also be made between the regulatory properties conferred by APL2 and APL3/APL4 to ADP-Glc PPase. The enzyme formed by APS1/APL2 is the less sensitive to both 3-PGA and P,, than the APS1/APL1 enzyme, whereas APL3 and APL4 conferred an intermediate response to the effectors. The high level of identity between APL3 and APL4 (88%) would explain the similar response of both isoforms to the effectors.

Our data strongly suggest that different LS confer distinct regulatory properties to ADP-Glc PPase that may be related to the metabolic profile of different tissues. It would, however, be necessary to determine the pattern of expression of the different genes by in situ hybridization to have a clear view on the tissue and the type of cells expressing each isoform.

The apparent affinity of ADP-Glc PPase for the substrates ATP and Glc-1-P was also dependent on the large subunit co-expressed with APS1. APL1 confers the highest affinity for the substrates to ADP-Glc PPase (A, for ATP 0.067 mM; A, for Glc-1-P 0.019 mM), and APL2 confers the lowest affinity (A, for ATP 0.575 mM; A, for Glc-1-P 0.085 mM), whereas APL3 and APL4 confer an intermediate affinity (Table III). These results indicate that APL subunits affect not only the response of ADP-Glc PPase to the allosteric regulator but also the binding of the substrates, with the tetramer formed by APS1 and APL1 being the one showing the most sensitive response to the regulators and at the same time the highest affinity for the substrates. Studies in tomato have also shown differences in the apparent affinity for the substrates between ADP-Glc PPase enzymes from fruit and leaves (25, 26). Our results strongly indicate that the distinct properties of the different APL subunits may control the synthesis of starch in sink and source tissues attending to the levels of 3-PGA, P,, ATP, and Glc-1-P. In photosynthetic tissues ADP-Glc PPase would be finely regulated by the ratio 3-PGA/P,, whereas in nonphotosynthetic tissues the rate of starch synthesis would be controlled by the supply of the substrates.

As inferred from Fig. 3, SS genes are expressed in both photosynthetic and nonphotosynthetic tissues, whereas LS genes are expressed preferentially in a tissue-specific manner (9, 23, 27). Arabidopsis APL1 is mainly expressed in leaves (36) and, correspondingly, is included in the leaf group of APLs (Fig. 3). The other three APL from Arabidopsis are included in the sink group of dicot plants. This division between sink and source APL isoforms is consistent with the kinetic properties of the different heterotetramers discussed above. Expression studies using the Arabidopsis APL3 promoter indicates that it is expressed in sink tissues (33) and that sucrose feeding induced APL3 expression in mesophyll cells, in a tissue-specific pattern similar to that reported in potato (32). Induction by sucrose has also been described for the Arabidopsis APL2 and APL3 genes in leaves, whereas expression of APL1 gene is repressed by sucrose (51). This suggests that APL3 and/or APL2 could participate in starch synthesis in photosynthetic tissues in the presence of an excess of sugars. It has been shown in Arabidopsis that sucrose feeding in the dark induces a 2-fold increase in starch levels without a significant effect on the level of ADP-Glc PPase activity and protein (51). So, it is possible that changes in the allosteric properties of ADP-Glc PPase account for starch synthesis when the Apl1 gene is repressed by sucrose. In this situation, expression of Apl3 gene (Apl2 and/or Apl4) will permit the formation of heterotetramers with different regulatory properties, thus allowing starch synthesis to proceed. In sink tissues the allosteric effectors are unlikely to fluctuate; thus APL3, APL2, and APL4 would participate in the synthesis of starch attending to the supply of the substrates Glc-1-P and ATP. However, to be able to ascertain the role of the different APL isoforms, it will be necessary to determine the level of 3-PGA and P, in the plastids of the different tissues where they are expressed.

In conclusion, we have studied the kinetic and regulatory interactions between all of the ADP-Glc PPase subunits present in A. thaliana. We have found differences on the regulatory properties conferred by the Arabidopsis large subunits to ADP-Glc PPase in vitro. The distinction between sink and source APL proteins points to the possibility that starch synthesis is modulated in response to 3-PGA and P,, as well as to the level of substrates, in a tissue-specific manner. As depicted in Fig. 4, ADP-Glc PPase heterotetramers formed by APS1 and APL1 are very sensitive to the effectors, display high apparent affinity for the substrates, and would be finely regulated in source tissues. On the other hand, in sink tissues heterotetramers could be formed by APS1 and APL2, APL3, and APL4, with lower sensitivity to the effectors and lower affinity for substrates. However, combination of the different LS would provide even more plasticity to ADP-Glc PPase because in different situations more than one LS could be expressed in a given tissue. This situation could take place in other plants having different isoforms of ADP-Glc PPase and may be a general system by which starch synthesis is differentially controlled in sink and source tissues and organs.

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The Different Large Subunit Isoforms of Arabidopsis thaliana ADP-glucose Pyrophosphorylase Confer Distinct Kinetic and Regulatory Properties to the Heterotetrameric Enzyme
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