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 absence 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 necessities for starch synthesis in a given tissue.

ADP-glucose pyrophosphorylase (ADP-Glc PPase, EC 2.7.7.27)[1] is the first enzyme of the starch biosynthesis pathway (1–3). It catalyzes the synthesis of ADP-glucose (ADP-Glc) and PP$_i$ 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 P$_i$ (3, 7). 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 P$_i$. 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 P$_i$ (3, 11–14). In addition to the regulation by 3-PGA and P$_i$, 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 (α_4) (18, 19). ADP-Glc PPase from higher plants is a heterotetramer ($\alpha_2\beta_2$) 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 (3, 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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[1] The abbreviations used are: ADP-Glc, adenosine diphosphate glucose; ADP-Glc PPase, ADP-glucose pyrophosphorylase; Glc-1-P, glu-

cose-1-phosphate; 3-PGA, 3-phosphoglycerate; LS, ADP-Glc pyrophosphorylase large subunit(s); SS, ADP-Glc pyrophosphorylase small subunit(s).

to modulate the activity of the heterotetramer in response to the levels of allosteric effectors (31). This fact together with the tissue-specific pattern of expression of LS genes (9, 32, 33) have led us to propose that the LS isoforms could confer different allosteric sensitivities to ADP-Glc PPase related to the specific metabolic conditions of each tissue and organ. Nevertheless, there is no comprehensive study on the possible role played by all the different isoforms of ADP-Glc PPase present in a single species. The identification of all ADP-Glc PPase encoding genes in *Arabidopsis thaliana* opens the possibility of performing studies on subunit interactions paying special attention to the role of the different large subunits.

In this study, all six *Arabidopsis* ADP-Glc PPase cDNAs were cloned and co-expressed in *Escherichia coli*. The activity of the recombinant enzymes was studied and analyzed kinetically. No catalytic activity was detected for APS2, suggesting that APS2 may not be an active ADP-Glc PPase small subunit. The co-expression of APS1 with the different *Arabidopsis* LS (APL1, APL2, APL3, and APL4) resulted in heterotetramers showing distinct affinity for the substrates as well as different regulatory properties. This is the first experimental study on the kinetic and regulatory properties of all the ADP-Glc PPase isoforms from a single species. Our results strongly indicate that ADP-Glc PPases heterotetramers can be formed in different tissues and organs of *Arabidopsis* based on the presence of a specific LS isoform. The heterotetramers formed in a specific tissue or organ would respond to substrates and allosteric effectors attending to its sink source nature, thus controlling starch synthesis and carbon partition in a tissue-specific manner.

EXPERIMENTAL PROCEDURES
Chemicals

The oligonucleotides were purchased from Genset™ Oligos, Taq-Plus™ Precision polymerase was from Stratagene Ltd., and pGEM™-T Easy vector was from Promega Corporation. [^{32}P]PP$_i$ was purchase from PerkinElmer Life Sciences, and [^{14}C]glucose-1-phosphate was from ICN Pharmaceuticals Inc. The MonoQ HR 10/10 column was acquired from Amersham Biosciences. 3-PGA, NaPP$_i$, Glc-1-P, ATP, ADP-Glc, and inorganic pyrophosphatase were obtained from Sigma. All other reagents were purchased at the highest quality available.

Plant Material and Bacterial Strains

A. thaliana plants were ecotype Columbia. *E. coli* DH5α strain was used to clone the cDNAs, and an *E. coli B* mutant deficient in ADP-Glc PPase activity (AC70RI-540; 14) was used in the expression and purification of the *Arabidopsis* ADP-Glc PPases.

Cloning of cDNA and Construction of Plasmids

cDNA fragments encoding the mature sequence of the *Arabidopsis* ADP-Glc PPase proteins were obtained by reverse transcription-PCR from total RNA isolated from leaves and influorescences and cloned into the pGEM™-T Easy vector. The following oligonucleotides were used to clone the *Arabidopsis* ADP-Glc PPase cDNAs: SA239 (ccccatatggtctcagattctcaaaactct) and SA240 (gtggaattcagatgacagtgccggttggga) for *ApS1*; SA241 (aaccatatggtttcaaactctcagcacctt) and SA242 (aaagaattcagaggatggagtcgttcggga) for *ApS2*; SA265 (gaaccatggcgtctctaaatagtgtagctg) and SA244 (gttggtaccattagatcttaaaagtatcat) for *ApL1*; SA266 (caccatggccgttcttactccatttgttg) and SA246 (agaggtaccaaagcttaaatatgcagacca) for *ApL2*; SA267 (ttgccatggctattgctacctcaaagaatg) and SA248 (ggcggtaccgagatcgtttgttcatatca) for *ApL3*; and SA268 (gttgccatggctgttgccacttcagat) and SA250 (ggaggtaccttcaagctctccattcatatt) for *ApL4*. A methionine in the SS or a methionine and an alanine in the LS were engineered by PCR-based mutagenesis, and restriction sites for cloning in the expression vectors were also introduced. *Nde*I-*Eco*RI fragments containing the SS cDNAs were subcloned into pMAB5, a modified version of the expression vector pMON17335 (14) with a *Nde*I restriction site in the N-terminal coding region, to generate plasmids pSAT403 (APS1) and pSAT465 (APS2). The *Nco*I-*Kpn*I fragments containing the LS cDNAs were cloned into the pMON17336 (14) to generate the plasmids pSAT516 (APL1), pSAT524 (APL2), pSAT532 (APL3), and pSAT544 (APL4).

Expression and Purification of Recombinant Enzymes

The ADP-Glc PPase SS alone or together with the LS cDNAs were expressed in *E. coli* strain AC70R1–504, which is deficient in endogenous ADP-Glc PPase 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 A_{600} reached 1.1–1.3. Induction of the small subunits was initiated by adding 400 μM of isopropyl-β-D-thiogalactopyranoside, and 5 μg/ml of nalidixic acid was added to induce the expression of the large subunits. After 16 h of induction at room temperature, the cells were chilled on ice and harvested by centrifugation. Cell pastes were resuspended in 3 ml of buffer A containing 50 mM HEPES (pH 7.9), 5 mM MgCl$_2$, 0.1 mM EDTA, and 10% sucrose and disrupted by sonication. The sonicated suspensions were centrifuged for 20 min at 10,000 $\times$ *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 pyrophosphorolysis 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 pyrophosphorolysis assay was used to monitor the ADP-Glc PPase 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 PPase (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 PPase activity were pooled, and an ammonium sulfate fractionation (30–60%) was performed, with centrifugation for 20 min at 12,000 $\times$ *g*. The ammonium sulfate pellets were resuspended in 6 ml of buffer A and desalted on Econo-Pac™ 10DG 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 PPase 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 PPase 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 bincichoninic acid reagent (34) from Pierce, using bovine serum albumin as the standard.

Assay of ADP-Glc PPase Activity

Pyrophosphorolysis Direction—[^{32}P]ATP formed in the pyrophosphorolysis of ADP-Glc was determined according to Morell *et al.* (20). The reaction mixture consisted of 50 mM HEPES (pH 7.9), 7 mM MgCl$_2$, 2 mM dithiothreitol, 2 mM ADP-Glc, 10 mM NaF, 0.2 mg/ml bovine serum albumin, 1 mM [^{32}P]PP$_i$ (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 NaPP$_i$. 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 MgCl$_2$, 2 mM dithiothreitol, 0.0015 unit/μl pyrophosphatase, 0.2 mg/ml bovine serum albumin, 1.5 mM ATP, 0.5 mM [^{14}C]Glc-1-P (1000 cpm/nmol), 3-PGA, and the enzyme in final volume of 0.2 ml. 0.2 or 2 mM sodium phosphate was included in the mixture to determine sensitivity to phosphate inhibition. The Glc-1-P $S_{0.5}$ was determined measuring the ADP-Glc PPase activity, and in this case, the reaction mixture included [^{14}C]Glc-1-P (4000 cpm/nmol). To determine the $S_{0.5}$ of the substrates, we used 3-PGA at concentrations 5-fold the 3-PGA $A_{0.5}$ 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 pyrophosphorolysis and synthesis direction assays was defined as 1 μmol of product formed per minute.

Kinetic Characterization

The $A_{0.5}$ and $S_{0.5}$ values, which correspond to the concentration of activator and substrate giving 50% of the maximal velocity respectively,

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amino acid position	ATP Site		Catalytic Site		Glc-1-P Site		Activator Site	
	107	115	133	143	183	192	418	435
Consensus	**WFQGTADAV**		**LAGDHLYRMDY**		**IIEF EKP GE**		**SGIV VIK A IP GTVI**	
E. coli	*YR******		*******KQ**		****V***.ANP			
Anabaena	*********		***********		*TD*S***Q**A		N***V***NVT*AD****	
Synechocystis	*********		***********		V*D*S***K**A		****V*L*N*V*TD**I*	
Potato SS	*********		***********		****A***Q**Q		****T***D*L**S*II*	
Tomato SS	*********		***********		****A***Q**Q		****T***D*L**S*I**	
Barley SS	*********		***********		****A***K**Q		****T***D*LL*S****	
Wheat SS	*********		***********		****A***K**Q		****T***D*LL*S****	
Arabidopsis APS1	*********		***********		****A***K**H		****T***D*L**T****	
Arabidopsis APS2	********I		*P*H***K***		VTR*TI*..*QQ		E**IIILRN*V**NDSIL	

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 Asp[142] (37, 38) is *underlined* in the figure. The accession numbers of the aligned sequences, from *top* to *bottom* in the figure, are: P00584, P30521, P52415, 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 data base (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 N-terminal region was determined for the six *Arabidopsis* ADP-Glc PPase genes by aligning and comparing the N-terminal sequences of different ADP-Glc PPase (20, 31), as described by Kavakli *et al.* (36) for APL1. Specific oligonucleotides were designed, and the cDNA fragments encoding for the mature *Arabidopsis* ADP-Glc PPase subunits were cloned in compatible expression vectors (see "Experimental Procedures").

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 pyrophosphorolysis 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 APL subunits expressed alone in accordance with previous data obtained for the potato ADP-Glc PPase recombinant enzyme (14, 31). On the other hand, no significant 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 $A_{0.5}$ 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 recombinant ADP-Glc PPase, and it should be considered in further purifications.

The specific activities in the pyrophosphorolysis 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 inmunoblot 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 Pyrophosphorolysis Direction—The kinetics for the response to 3-PGA conferred by the different *Arabidopsis* LS were studied in detail. The $A_{0.5}$ for 3-PGA values in pyrophosphorolysis 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 $A_{0.5}$ for the homotetrameric APS1 enzyme was 1.2 mM, whereas the $A_{0.5}$ of the heterotetrameric

TABLE I

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

ADP-Glc PPase activity was determined in the pyrophosphorolysis direction as described under "Experimental Procedures." The kinetic parameters (see "Experimental Procedures") were calculated without inhibitor (P_i) and in the presence of inhibitor at 0.2 or 2 mM. The deviation in the 3-PGA $A_{0.5}$ data is the difference between duplicate experiments. ND indicates not determined.

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

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 pyrophosphorolysis 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 280-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 pyrophosphorolysis 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 pyrophosphorolysis 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). Based on the values in Tables I and II, we can divide the *Arabidopsis* ADPG PPase heterotetramers attending to the response to the 3-PGA and P_i. The APS1/APL1 heterotetramer showed the higher response and sensitivity to the allosteric effectors, whereas the heterotetramer composed of APS1 and APL3 or APL4 has an intermediate response, and the APS1/APL2 heterotetramer has the lower one. The possible physiological implications of these results will be further discussed.

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-1P 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 heterotet-
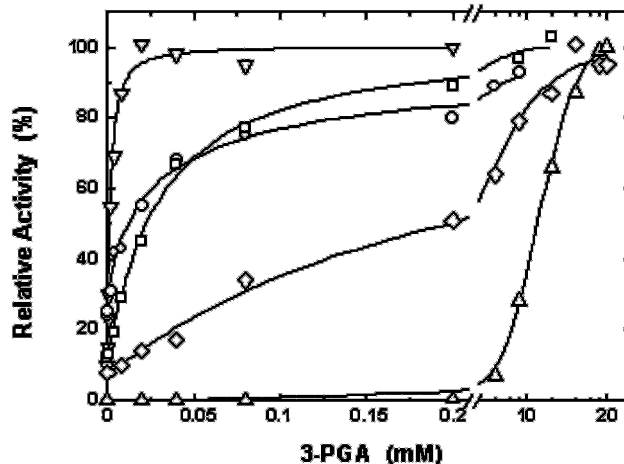

FIG. 2. **Activation by 3-PGA of the recombinant *Arabidopsis* ADP-Glc PPases.** The pyrophosphorolysis assay (see "Experimental Procedures") was used to monitor the activity of the recombinant *Arabidopsis* ADP-Glc PPases. △, APS1; ▽, APS1/APL1; ◇, APS1/APL2; ○, APS1/APL3; □, APS1/APL4. The activity was assayed with 3-PGA at the indicated concentrations in the figure, and the average of two independent experiments for each enzyme was plotted.

ramer 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 P_i. The deviation in the 3-PGA $A_{0.5}$ data is the difference between duplicate experiments. ND indicates not determined.

	Control		0.2 mM P_i		2 mM P_i	
	3-PGA $A_{0.5}$	n_H	3-PGA $A_{0.5}$	n_H	3-PGA $A_{0.5}$	n_H
	mM		*mM*		*mM*	
APS1	5.7 ± 0.5	1.6	13.9 ± 1.8	2.3	ND	
APS1/APL1	0.018 ± 0.004	0.8	0.094 ± 0.009	1.4	1.6 ± 0.1	2.9
APS1/APL2	0.87 ± 0.11	0.9	1.54 ± 0.27	1.3	10.5 ± 1.6	1.5
APS1/APL3	0.34 ± 0.09	0.8	0.70 ± 0.1	1.4	2.7 ± 0.1	2.7
APS1/APL4	0.16 ± 0.03	0.8	0.46 ± 0.1	1.2	1.7 ± 0.1	1.5

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 $S_{0.5}$ (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 $A_{0.5}$ of each enzyme, respectively. The kinetics parameters were calculated at the specified concentrations of 3-PGA without inhibitor (P_i). The deviation in the $S_{0.5}$ data is the difference between duplicate experiments.

	ATP		Glc-1-P	
	$S_{0.5}$	n_H	$S_{0.5}$	n_H
	mM		*mM*	
APS1	0.402 ± 0.04	1.5	0.076 ± 0.018	0.9
APS1/APL1	0.067 ± 0.008	1.0	0.019 ± 0.001	1.0
APS1/APL2	0.575 ± 0.03	1.6	0.085 ± 0.014	0.9
APS1/APL3	0.094 ± 0.008	1.4	0.052 ± 0.007	1.0
APS1/APL4	0.118 ± 0.01	1.2	0.060 ± 0.008	0.9

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 subunit that is expressed in sink and source leaves (27) and is included in the sink group of LS. *Arabidopsis* APL1 is considered to be the main isoform expressed in leaves (36). Correspondingly, APL1 is grouped with other LS expressed in leaf. We can also distinguish three subgroups in the sink LS group: one group corresponding to monocot endosperm LS isoforms; a second group that includes the *Arabidopsis* APL2 and LS present in young leaves, fruits, and roots; and a third group comprising LS from storage organs like tubers or expressed in the stem. *Arabidopsis* APL3 and APL4 share a very high level of identity at the amino acid sequence (88%) and are included in the latter group. It has been shown that *Arabidopsis* APL3 is restricted to the starch sheath layer below the major veins in leaves and petioles and that its expression is strongly induced by sucrose or trehalose in the mesophyl of the leaf (33, 47). Induction by sucrose has also been reported for the tomato and potato LS1 genes (48, 49) that are closely related to *Arabidopsis* APL3 (Fig. 3). Therefore, it seems that for several plant species, ADP-Glc PPase large subunits can be grouped according to their sequences and their pattern of expression. This fact could be related to the specific regulatory and substrate affinity properties that we have demonstrated for different *A. thaliana* APLs and opens the possibility that combinations of SS with different

LS could represent a mechanism for the controlling carbon partitioning among different plants species.

DISCUSSION

ADP-Glc PPase catalyzes the first step in the synthesis of starch. The allosteric control of the activity of this enzyme in photosynthetic and in many nonphotosynthetic tissues is exerted by the relative ratio between the activator (3-PGA) and the inhibitor (P_i). In some plants, ADP-Glc PPase from developing seeds do not respond to these regulators (8–10), although in other cases, like in rice, the response to the effectors is similar to that observed for the leaf enzymes (13). These observations have raised the idea that specific tissues should present different ADP-Glc PPases with distinct regulatory properties 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 in expression vectors 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.[2] So, it is possible that APS2 is a noncatalytic subunit. Nevertheless, further studies are required to confirm this hypothesis and discard other possible roles of the protein.

When analyzed in the pyrophosphorolysis direction, APL1 conferred to the ADP-Glc PPase heterotetramer a very high

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[2] P. Crevillén, unpublished results.

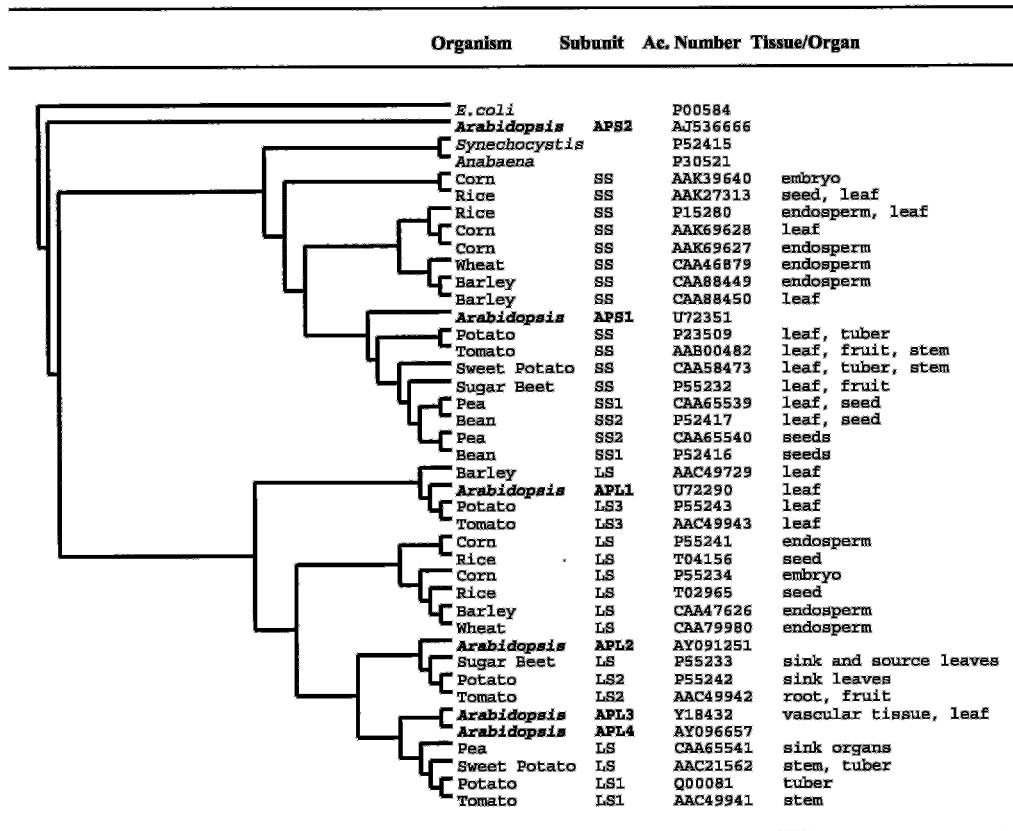| Organism	Subunit	Ac. Number	Tissue/Organ
E.coli		P00584	
Arabidopsis	**APS2**	AJ536666	
Synechocystis		P52415	
Anabaena		P30521	
Corn	SS	AAK39640	embryo
Rice	SS	AAK27313	seed, leaf
Rice	SS	P15280	endosperm, leaf
Corn	SS	AAK69628	leaf
Corn	SS	AAK69627	endosperm
Wheat	SS	CAA46879	endosperm
Barley	SS	CAA88449	endosperm
Barley	SS	CAA88450	leaf
Arabidopsis	**APS1**	U72351	
Potato	SS	P23509	leaf, tuber
Tomato	SS	AAB00482	leaf, fruit, stem
Sweet Potato	SS	CAA58473	leaf, tuber, stem
Sugar Beet	SS	P55232	leaf, fruit
Pea	SS1	CAA65539	leaf, seed
Bean	SS2	P52417	leaf, seed
Pea	SS2	CAA65540	seeds
Bean	SS1	P52416	seeds
Barley	LS	AAC49729	leaf
Arabidopsis	**APL1**	U72290	leaf
Potato	LS3	P55243	leaf
Tomato	LS3	AAC49943	leaf
Corn	LS	P55241	endosperm
Rice	LS	T04156	seed
Corn	LS	P55234	embryo
Rice	LS	T02965	seed
Barley	LS	CAA47626	endosperm
Wheat	LS	CAA79980	endosperm
Arabidopsis	**APL2**	AY091251	
Sugar Beet	LS	P55233	sink and source leaves
Potato	LS2	P55242	sink leaves
Tomato	LS2	AAC49942	root, fruit
Arabidopsis	**APL3**	Y18432	vascular tissue, leaf
Arabidopsis	**APL4**	AY096657	
Pea	LS	CAA65541	sink organs
Sweet Potato	LS	AAC21562	stem, tuber
Potato	LS1	Q00081	tuber
Tomato	LS1	AAC49941	stem

FIG. 3. **Comparison of ADP-Glc PPase sequences present in the EMBL/GenBank/DDBJ data bases.** The amino acid sequences without the putative transit peptide were aligned using the ClustalX program. The phylogenetic tree was constructed with the ClustalX program using the Neighbor Joining method. The tree was rooted to the *E. coli* ADP-Glc PPase and represented using the Treeview program. In the figure are shown the source organism, the name or type of subunit, the accession number for the sequence, and the organ or tissue from which the transcript or the protein was isolated.
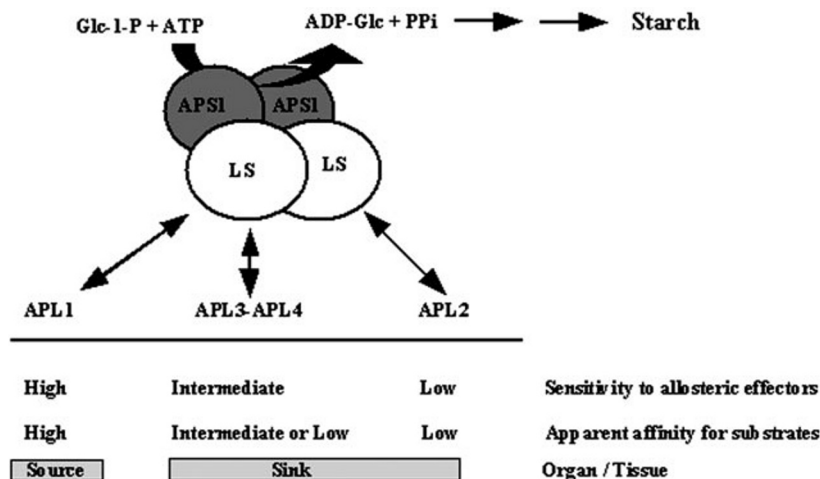

FIG. 4. **Model for the regulation of ADP-Glc PPase activity by the different large subunit isoforms in *A. thaliana* according to the sink/source nature of the organ or tissue.**

sensitivity to both 3-PGA and P_i (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 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 P_i 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 P_i 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 P_i. The regulatory properties of the different ADP-Glc PPase heterotetramers were corroborated by studying the effect of 3-PGA and P_i 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

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_i 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_i. 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_i, 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 ($A_{0.5}$ for ATP 0.067 mM; $A_{0.5}$ for Glc-1-P 0.019 mM), and APL2 confers the lowest affinity ($A_{0.5}$ for ATP 0.575 mM; $A_{0.5}$ 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_i, ATP, and Glc-1-P. In photosynthetic tissues ADP-Glc PPase would be finely regulated by the ratio 3-PGA/P_i, 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 mesophyl 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 APL2, APL3, 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_i 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_i, 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 by 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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