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Differential Pattern of Expression and Sugar Regulation of *Arabidopsis thaliana* ADP-glucose Pyrophosphorylase-encoding Genes*

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ADP-glucose pyrophoshorylase (ADP-Glc PPase) catalyzes the first and limiting step in starch biosynthesis. In plants, the enzyme is composed of two types of subunits (small and large) and is allosterically regulated by 3-phosphoglycerate and phosphate. The pattern of expression and sugar regulation of the six *Arabidopsis thaliana* ADP-Glc PPase-encoding genes (two small subunits, *ApS1* and *ApS2*; and four large subunits, *ApL1–ApL4*) has been studied. Based on mRNA expression, *ApS1* is the main small subunit or catalytic isoform responsible for ADP-Glc PPase activity in all tissues of the plant. Large subunits play a regulatory role, and the data presented define a clear functional distinction among them. *ApL1* is the main large subunit in source tissues, whereas *ApL3* and, to a lesser extent, *ApL4* are the main isoforms present in sink tissues. Thus, in source tissues, ADP-Glc PPase would be finely regulated by the 3-phosphoglycerate/phosphate ratio, whereas in sink tissues, the enzyme would be dependent on the availability of substrates for starch synthesis. Sugar regulation of ADP-Glc PPase genes is restricted to *ApL3* and *ApL4* in leaves. Sugar induction of *ApL3* and *ApL4* transcription in leaves allows the establishment of heterotetramers less sensitive to the allosteric effectors, resembling the situation in sink tissues. The results presented on the expression pattern and sugar regulation allow us to propose a gene evolution model for the *Arabidopsis* ADP-Glc PPase gene family.

Starch is the major storage polysaccharide in plants and is accumulated as granules in many different organs such as leaves, roots, shoots, fruits, or grains, where it is used as a carbon and energy source. The regulatory and rate-limiting step of starch biosynthesis is the synthesis of the glucosyl donor, ADP-glucose, by ADP-glucose pyrophosphorylase (ADP-Glc PPase)[1] (EC 2.7.7.27) (1). ADP-Glc PPase is a heterotetramer ($\alpha_2\beta 2$) composed of two different types of subunits (1, 2). The small subunit (SS) is considered the fully catalytic one, whereas the large subunit (LS) plays a modulatory function regulating the activity of the enzyme (1, 3, 4). Nevertheless, different studies on ADP-Glc PPase from maize and potato have suggested that both subunits may have a regulatory role (5, 6). The activity of most plant ADP-Glc PPases is finely regulated by an allosteric mechanism (7). The enzyme is activated by 3-phosphoglycerate (3-PGA) and inhibited by P_i. This allosteric modulation is considered to represent the main control of the starch biosynthesis rate in photosynthetic tissues and in many non-photosynthetic tissues (1), and it is exerted by the relative ratio between 3-PGA and P_i. However, in some plants, the enzyme from developing seeds does not respond to these regulators (8–10). Besides the allosteric regulation of ADP-Glc PPase activity, a post-translational redox control that involves the action of thioredoxins has been shown (11) and acts in response to sugars and light (12, 13).

Different isoforms of ADP-Glc PPase have been described, and many cDNAs and genomic DNAs have been isolated from both monocot and dicot plants. In most cases, a single SS gene and several LS genes are present in a given species (1, 4). It has been postulated that specific tissues should present different ADP-Glc PPases with distinct regulatory properties that would be dependent on the specific LS present in a given tissue (3, 14). However, there is no comprehensive study of the expression of all the different ADP-Glc PPase isoforms in a single plant system. Six genes encode for proteins with homology to ADP-Glc PPase in the *Arabidopsis* genome. Two of these genes encode for SS, *ApS1* (At5g48300) and *ApS2* (At1g05610); and four of these genes encode for LS, *ApL1 to ApL4* (At15g19220, At1g27680, At4g39210, and At2g21590). We have shown recently that the *Arabidopsis* large subunits confer different regulatory properties to ADP-Glc PPase *in vitro* and would modulate the activity of the ADP-Glc PPase enzyme according to the biosynthetic starch necessities in a tissue-specific manner. Thus, according to their kinetic and regulatory properties, the large subunits were classified as sink and source isoforms (4). However, the distinction between sink LS and source LS needs further confirmation because in *A. thaliana* only partial information concerning some genes has been reported (15–18). To address this question, a complete study of the expression pattern of all ADP-Glc PPase-encoding genes is needed.

Sugars play a basic role as substrates in carbon and energy metabolism as well as precursors of starch biosynthesis. They are also important signaling molecules controlling the growth and development of plants (19). With regard to the starch synthesis pathway, it has been shown that sugars mediate the induction of several genes of the pathway (20–23), including some genes encoding for ADP-Glc PPase (24–26), and also trigger the redox regulation of ADP-Glc PPase activity (12, 13). Induction of *ApL3* mRNA levels in response to sucrose (15, 27)

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[1] The abbreviations used are: ADP-Glc PPase, ADP-glucose pyrophosphorylase; SS, small subunit; LS, large subunit; 3-PGA, 3-phosphoglycerate; RT-PCR, reverse transcription-PCR.

and trehalose (28) has been described in *Arabidopsis* leaves and seedlings. However, information concerning the effect of sugars on the expression of the other ADP-Glc PPase genes from *Arabidopsis* is scarce.

The aim of this work is to understand the role of the different ADP-Glc PPase isoforms in *Arabidopsis thaliana*, a model system to study starch biosynthesis (29). We present novel data about the expression pattern of the six genes encoding for ADP-Glc PPase isoforms in different organ and tissues, and we characterize the effect of sugar supply on the expression of those genes under physiological conditions. The data presented provide solid evidence that confirms a working model proposed previously by us assigning specific roles to the different ADP-Glc PPase subunits (4).

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—*A. thaliana* (Columbia ecotype) was grown in growth cabinets under a 16-h light/8-h dark photo-regime at 23 °C (day)/20 °C (night), 70% humidity, and a light intensity at the plant level of 100 microeinsteins m^{-2} s^{-1}. Plants were sown in soil for mRNA expression studies assays or in perlite for sugar inductions assays. Sugar induction treatments consisted of irrigation of the mature plants with MS medium (30) supplemented with 100 mM sorbitol, 100 mM glucose, 100 mM sucrose, or 25 mM trehalose. We chose sugar concentrations according to previous reports (15, 17). During sugar treatments, plants were maintained under the same growing conditions, and samples were harvested at the middle point of the photoperiod (8 h light) during 4 consecutive days, frozen in liquid nitrogen, and stored at −80 °C until use.

RNA Extraction and Reverse Transcription—Total RNA was isolated as described by Prescott and Martin (31). Before cDNA synthesis, in order to remove contaminating genomic DNA, the RNA preparations were incubated with 10 units of DNase I FPLC Pure for 10 min at 37 °C, extracted with phenol and chloroform, precipitated, and dissolved in nuclease-free MilliQ-water. First-strand cDNA was synthesized from 10 μg of total RNA using Moloney murine leukemia virus reverse transcriptase and oligo$(dT)_{12-18}$ primer, according to the manufacturer's instructions (Amersham Biosciences). The reaction was incubated at 37 °C for 2 h and stopped by adding 1 ml of nuclease-free MilliQ-water. All reagents were from Amersham Biosciences.

Real-time Quantitative RT-PCR Analysis—Real-time quantitative RT-PCR assays were achieved using an iCycler instrument (Bio-Rad). The PCR mixture contained (in a total volume of 25 μl) 5 μl of cDNA, 0.2 mM deoxynucleotide triphosphates, 2.5 mM $MgCl_2$, a 1:100,000 dilution of SYBR® Green I nucleic gel stain (Molecular Probes)/fluorescein calibration dye (Bio-Rad), 0.3 unit of *Taq* polymerase, 2.5 μl of 10× *Taq* polymerase buffer, and 0.2 μM of each primer. Specific oligonucleotides used were as follows: SA253 (5′-GAAAAACCAAAAGGGGAGCA-3′) and SA254 (5′-CCTCAGTCCAAGGGAAGTGG-3′) for *ApS1*; SA255 (5′-CTCGGTGTTTGCCTCCAAG-3′) and SA256 (5′-TCTCCTTCCCTTTTCTTCTCACA-3′) for *ApS2*; SA257 (5′-TCCCCACAGCAAACGACTT-3′) and SA258 (5′-GGTGGCAGGTTTCTCCTTGA-3′) for *ApL1*; SA259 (5′-TCGGAAAAACCAAAGGGAGA-3′) and SA260 (5′-GGCCAACGGGATAATTTCAG-3′) for *ApL2*; SA261 (5′-AGATCGGGAAAAACGTGGTG-3′) and SA262 (5′-CTCTTTTTAACTTCCGGCCAAAC-3′) for *ApL3*; SA263 (5′-AGCAGATAGGCCAGAGGAAGG-3′) and SA264 (5′-GCGGGAAAGAAAAGATCGAAG-3′) for *ApL4*; and UBQF (5′-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3′) and UBQR (5′-CGACTTGTCATTAGAAAGAAAGAGATAACAG-3′) for *UBQ10*. Thermal cycling consisted of 94 °C for 3 min, followed by 40 cycles of 10 s at 94 °C, 15 s at 61 °C, and 15 s at 72 °C. After that, a melting curve was generated to check the specificity of the amplified fragment. In the case of *ApL4* (primers SA263 and SA264), the annealing temperature was 64 °C. The efficiency of all the primers at the conditions described above was between 75% and 110% in all the tested samples, and product identity was confirmed by sequence analysis. *Arabidopsis Ubiquitin 10* (32) was used as a housekeeping gene in the expression analysis. Absolute Quantification (33) was performed by cloning the amplified products in pGEM®-T vector (Promega) and using them as external calibration standards.

In Situ Hybridization Analysis—For mRNA *in situ* hybridizations, sense and antisense RNA probes were obtained by transcription of the 3′-untranslated region of the *Arabidopsis* ADP-Glc PPase-encoding genes cloned into pBluescript SK(−) vector (Clontech) from the T7 promoter. The digoxigenin-labeled riboprobes were prepared following the manufacturer's instructions (Roche Applied Science). *In situ* hybridizations were performed as described by Mérida *et al.* (34), at 50 °C in a solution containing 2× SSC and 50% formamide; signal was detected, and the sections were mounted in Entellan mounting medium (Merck). The sections were observed through an Olympus BX60 microscope by Normarski microscopy and photographed using a JVC GC-X3E digital camera.

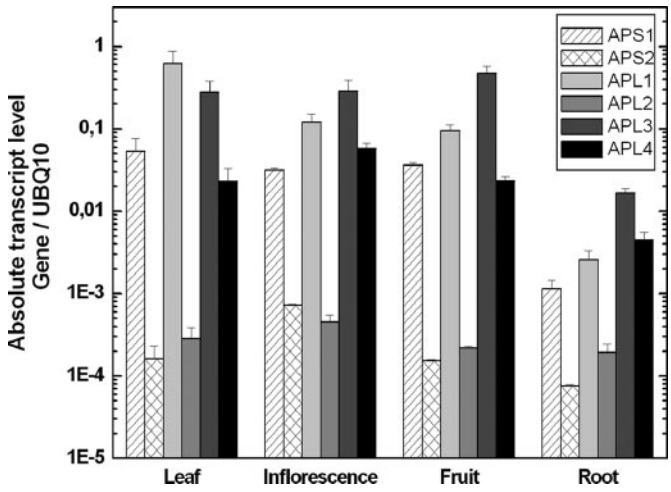

FIG. 1. **Expression profile of the *Arabidopsis* ADP-Glc PPase-encoding genes.** The absolute mRNA levels of all the *Arabidopsis* ADP-Glc PPase-encoding genes were determined by real-time quantitative RT-PCR as described under "Experimental Procedures." In the figure, quantities are represented in a logarithmic plot in order to compare the data among the different genes. The values are the average of the quantification of three cDNA preparations from different experiments. The *error bars* in the plot represent the S.D.

RESULTS

The Arabidopsis ADP-Glc PPase Genes Show Differential mRNA Expression Levels—We have determined the absolute steady-state mRNA levels of the six *Arabidopsis* ADP-Glc PPase-encoding genes by real-time quantitative RT-PCR. Specific primers for the six ADP-Glc PPase genes and for the housekeeping gene, *Ubiquitin 10* of *Arabidopsis* (32), were designed, and their efficiency and reliability were checked. The fragments amplified by these primers were cloned and sequenced, and the resulting plasmids were used as external calibration standards (33).

All six mRNAs were detected in leaves, inflorescences, fruits, and roots of mature plants, but at very different levels (Fig. 1). *ApS1*, *ApL1*, *ApL3*, and *ApL4* showed elevated mRNA steady-state levels, whereas *ApS2* and *ApL2* mRNA were accumulated at very low levels. These data are in accord with previous observations in our laboratory, in which *ApS2* and *ApL2* could not be detected by Northern blot.[2] *ApS1* was the major SS isoform in all organs of the plant. A more complex situation was observed for *ApL* genes. *ApL1* was the most abundant LS in leaves, with levels of mRNA up to three times higher than that of *ApL3* and more than 1 order of magnitude higher levels of transcript than *ApL4*. In inflorescences and fruits, *ApL3* was the most abundant ADP-Glc PPase large subunit transcript. In roots, the level of expression of all ADP-Glc PPase genes was very low compared with leaves, and *ApL3* was the predominant LS with up to 3-fold and 1 order of magnitude higher levels of transcript than *ApL4* and *ApL1*, respectively. These data show that there is a tissue-specific level of expression of the different ADP-Glc PPase subunits in *A. thaliana*.

Expression Pattern at the Cellular Level—The mRNA expression pattern of *Arabidopsis* ADP-Glc PPase genes was studied by *in situ* hybridization in leaves, inflorescences, and fruits.

[2] P. Crevillén, unpublished observations.

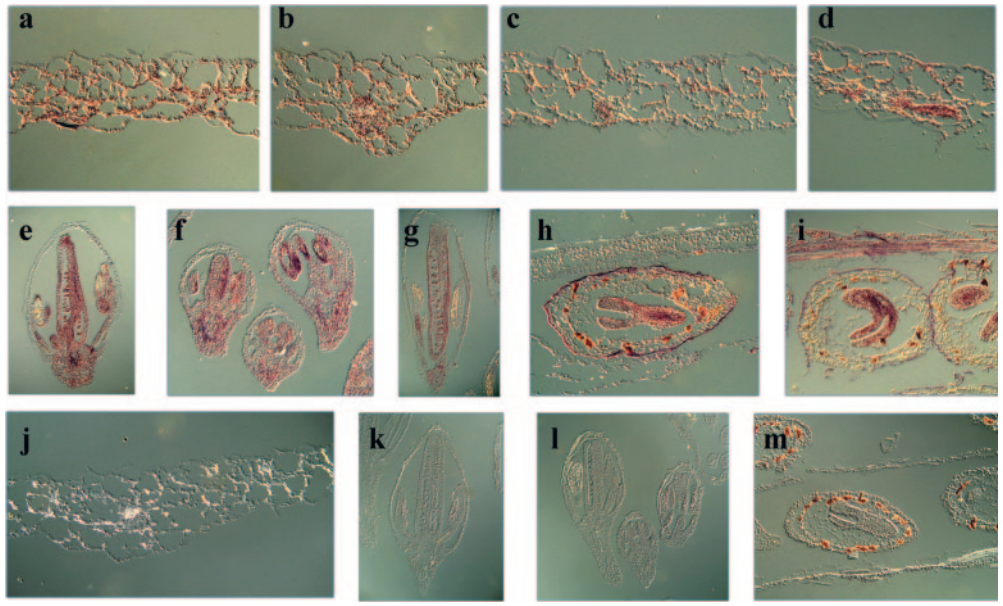

FIG. 2. **Pattern of expression of the *Arabidopsis* ADP-Glc PPase small subunits.** *a–d*, leaf sections hybridized with the antisense probe against *ApS1* (*a* and *b*) and *ApS2* (*c* and *d*); *e–g*, flowers at different developmental stages hybridized with the antisense probe against *ApS1* (*e* and *f*) and *ApS2* (*g*); *h* and *i*, longitudinal sections of fruits showing the seed with the embryo hybridized with the antisense probe against *ApS1* (*h*) and *ApS2* (*i*). *j–m*, control samples hybridized with the *ApS1* sense probe; hybridizations with the *ApS2* sense probe are not shown. The mRNA *in situ* hybridization experiments were performed as described under "Experimental Procedures" using specific digoxigenin-labeled riboprobes against each SS gene.

Fig. 2 shows the expression pattern of the SSs, *ApS1* and *ApS2*. Both genes are detected in the main starch-producing tissues of leaves, the mesophyll and the vascular companions cells (Fig. 2, *a–d*). In flowers, a strong signal is detected in the stamens and pistil, as well as in the receptacle. This pattern was observed in both young (Fig. 2*f* for *ApS1*; *ApS2* data not shown) and mature flowers (Fig. 2, *e* and *g*). *ApS1* and *ApS2* are also clearly expressed in the embryo (Fig. 2, *h* and *i*). A different situation was observed for the ADP-Glc PPase LSs. In leaves, *ApL1, ApL2*, and *ApL3* transcripts are localized in the mesophyll and vascular companions cells (Fig. 3, *a–e*), but *ApL4* appeared to be restricted only to the surrounding cells of the vascular veins (Fig. 3, *f* and *g*). Similar to the SS mRNAs, all the LS mRNAs were detected in all tissues of flowers at different developmental stages (Fig. 3, *h–k*). In fruits, *ApL1* is expressed uniformly in the embryo (Fig. 3, *l* and *m)*, whereas *ApL2*, *ApL3*, and *ApL4* expression was restricted to the provascular cells of the embryo (Fig. 3, *n–r*). An unspecific signal in the seed coat (testa) was observed in most of the hybridizations with fruit sections. No signal was detected with the control sense riboprobes for all the genes (Fig. 2, *j–m* for *ApS1*; *ApS2* and *LS* control sense hybridizations not shown).

The results shown indicate that there is not a differential pattern of expression between the two SSs. On the contrary, a clear differential expression pattern of the LS genes was observed in leaves and fruits. This fact, together with the different mRNA expression levels (Fig. 1), suggests a tissue specificity that could be related to the role of each LS isoform in regulating the activity of the heterotetrameric ADP-Glc PPase enzyme.

ApL3 and ApL4 Are Induced by Sugars in Leaves—Most studies on sugar-regulated transcription of genes involved in the synthesis of starch have been performed with seedlings or detached leaves (15, 24, 27). This type of assay, although very informative, may not reflect the real physiological response of the plant, could magnify the differences in the mRNA levels, and is restricted to specific tissues. To overcome this problem, we have designed a sugar induction assay in which all the different organs of the plant could be harvested under the same experimental conditions (see "Experimental Procedures"). *Arabidopsis* plants irrigated with MS medium were grown to maturity. Then, plants were irrigated with MS medium supplemented with different sugars for several days. Samples were harvested at the middle point of the photoperiod, and the mRNA levels were determined by real-time quantitative RT-PCR. This experimental design reflects the carbon status of mature plants in a better way than in detached leaves or seedlings and at the same time allows global analysis of different organs.

Fig. 4 shows the steady-state ADP-Glc PPase mRNA levels in *Arabidopsis* leaves after irrigation with MS medium supplemented with 100 mM sucrose. A time-course analysis showed that only *ApL3* and *ApL4* mRNAs were sucrose-induced and that, after 72 h of treatment, a significant induction of both genes could be observed (a 10-fold and an 18-fold increase in *ApL3* and *ApL4* mRNA levels, respectively), and induction of both genes was even higher at 96 h. No variation in the transcript levels was observed for *ApS1*, *ApS2*, *ApL1*, and *ApL2* transcripts. A similar behavior was observed when *Arabidopsis* plants were supplemented with glucose (100 mM) or trehalose (25 mM) (data not shown). Results in Fig. 5 show the induction of *ApL3* and *ApL4* after 72 h of sugar treatment. The mRNA levels of *ApL3* were 4-fold higher in the presence of glucose (100 mM), 10-fold higher in the presence of sucrose (100 mM), and 6-fold higher in the presence of trehalose (25 mM) compared with control plants in the absence of added sugars. Induction of *ApL4* was even higher, reaching 20- and 30-fold the control levels in the presence of sucrose and trehalose, respectively. No significant effect was observed when plants were supplemented with sorbitol, thus suggesting that sugar induction of *ApL3* and *ApL4* is not due to osmotic stress (Fig. 5). We also studied the effect of sugar treatment in other organs of mature *Arabidopsis* plants, and no sugar induction of ADP-Glc PPase-encoding genes was observed in fruits, inflorescences, or roots (data not shown). In summary, our data clearly show that only two genes encoding for *Arabidopsis* ADP-Glc PPase, *ApL3* and *ApL4*, are sugar-regulated in source tissues (leaves), whereas sugar regulation in sink tissues does not control the level of any ADP-Glc PPase-encoding gene.

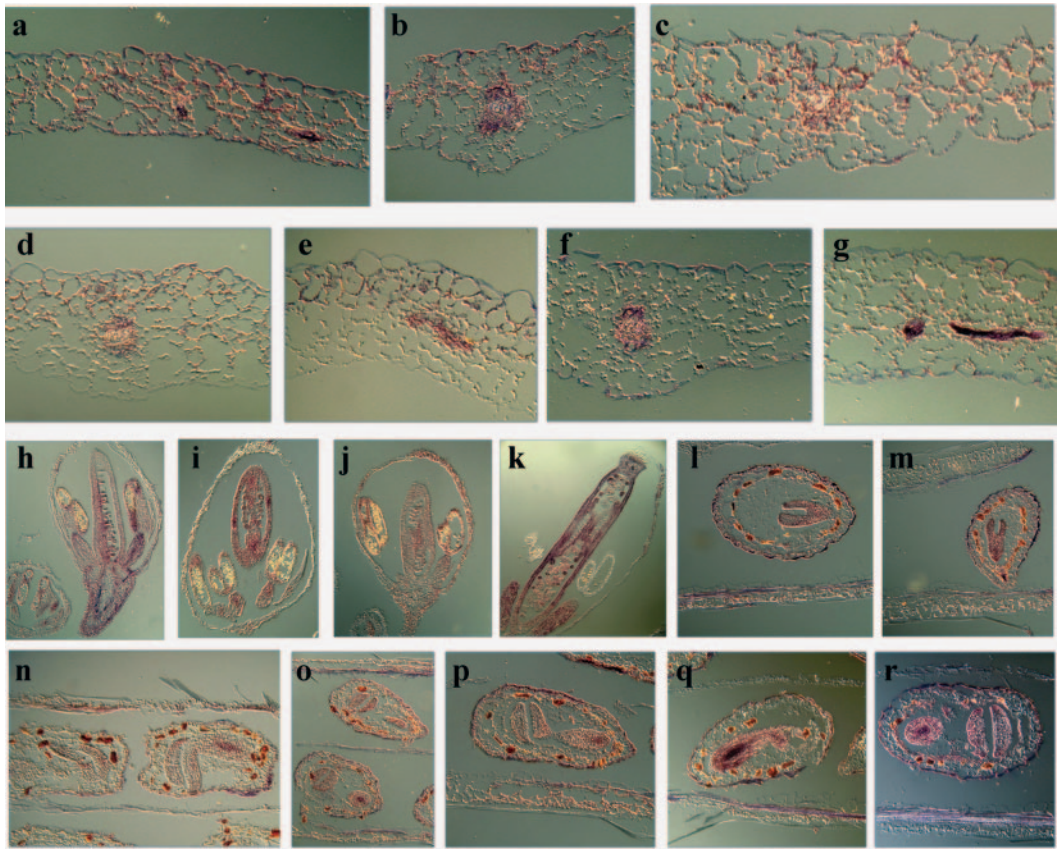

FIG. 3. **Pattern of expression of the *Arabidopsis* ADP-Glc PPase large subunits.** *a–g*, leaf sections hybridized with the antisense probe against *ApL1* (*a* and *b*), *ApL2* (*c*), *ApL3* (*d* and *e*), and *ApL4* (*f* and *g*); *h–k*, flowers at different developmental stages hybridized with the antisense probe against *ApL1* (*h*), *ApL2* (*i*), *ApL3* (*j*), and *ApL4* (*k*); *l–r*, magnifications of longitudinal sections of fruits showing the seed and the embryo hybridized with the antisense probe against *ApL1* (*l* and *m*), *ApL2* (*n*), *ApL3* (*o* and *p*), and *ApL4* (*q* and *r*). Control samples hybridized against the sense probes are not shown. The mRNA *in situ* hybridization experiments were performed as described under "Experimental Procedures" using specific digoxigenin-labeled riboprobes against each LS gene.

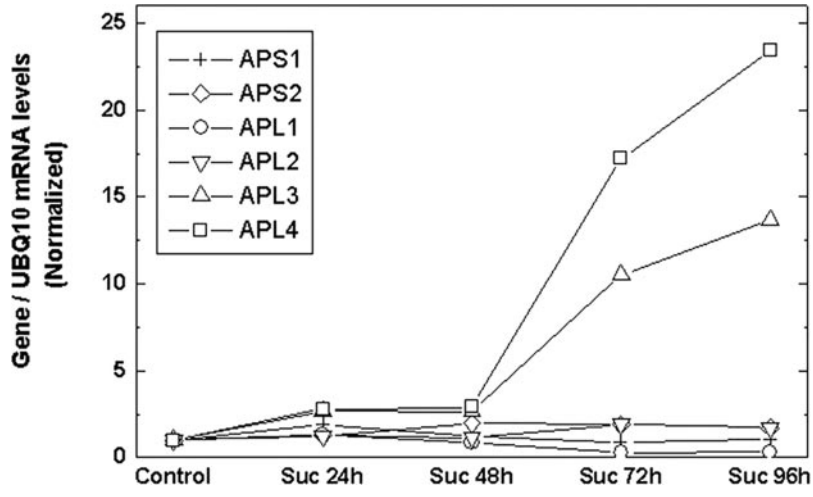

FIG. 4. **Effect of sucrose on the mRNA level of ADP-Glc PPase-encoding genes.** The figure shows a time-course mRNA expression analysis of the six ADP-Glc PPase-encoding genes in *Arabidopsis* leaves of plants induced with 100 mM sucrose. The plants were treated with sucrose, and the mRNA levels were determined by real-time quantitative RT-PCR as described under "Experimental Procedures." The data in the plot are normalized to the value in the control sample without induction. The values are the average of three determinations of two cDNA preparations. The S.D. was less than ±10% for each data point.

DISCUSSION

We have characterized the expression of the *Arabidopsis* ADP-Glc PPase-encoding genes in different organs and tissues, determining their absolute transcript levels by real-time quantitative RT-PCR and their expression pattern by *in situ* hybridizations. Although the six transcripts were detected in all the studied organs, our results clearly show that the different ADP-Glc PPase subunits are expressed at different levels and have specific expression profiles among the different organs of the plant. Both SS-encoding genes are expressed in all the starch-producing tissues of the plant. However, *ApS1* expression level is up to 2 orders of magnitude higher than that of *ApS2*. It has been shown previously that *ApS1* plays a critical role in starch biosynthesis because *adg1-1* mutant, affected in APS1, shows very low levels of starch accumulation (35). Besides, *in vitro* studies with the wild type (4) and a mutated APS2 H137D protein[3] have suggested that *ApS2* is a non-active isoform, with *ApS1* being the only catalytic ADP-Glc PPase subunit in *Arabidopsis*. Our results on the expression data

[3] T. Ventriglia, unpublished observations.

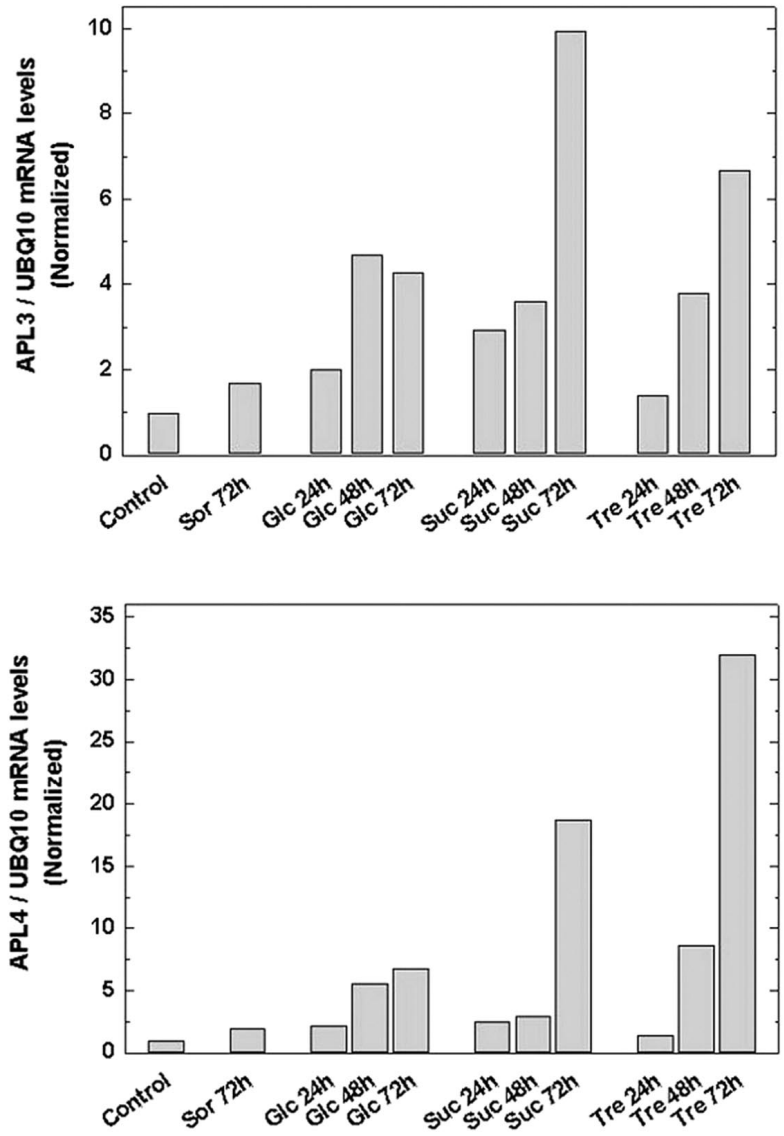

FIG. 5. **Sugar induction of *ApL3* and *ApL4* mRNA expression level.** *Arabidopsis* plants were fed with 100 mM glucose, 100 mM sucrose, 25 mM trehalose, or 100 mM sorbitol, and the ADP-Glc PPase-encoding gene mRNA levels in leaves were determined by real-time quantitative RT-PCR as described under "Experimental Procedures." The figure shows the results for the *ApL3* and *ApL4* genes. The data are normalized to the value of the control without induction. The values are the average of three determinations of two cDNA preparations. The S.D. was less than ±10% for each data point.

reported in this work confirm the role of *ApS1* as the main, if not the sole, SS isoform responsible for ADP-Glc PPase activity in *A. thaliana*.

The ADP-Glc PPase large subunits are considered to play a regulatory role, modulating the activity in response to allosteric effectors (4). The mRNA levels of the four genes encoding for LS show significant differences. Whereas *ApL1* is the most highly expressed ADP-Glc PPase LS in leaves (Fig. 1), *ApL3* reaches the highest levels of expression in sink organs (inflorescences, fruits, and roots). Differentiation is also observed at the cellular level (Fig. 3). In seeds, *ApL1* appeared to be expressed in all the embryo cells, whereas the others LSs are preferentially localized in the pro-vascular cells. In mature leaves, we clearly detect the *ApL1*, *ApL2*, and *ApL3* mRNAs in the mesophyll cells as well as in the bundle sheet, whereas *ApL4* was restricted to the companion cells of the vascular veins. We could not determine which subunit is expressed in the guard cells of the stomata, cells that accumulate significant amounts of starch. However, microarray analysis has shown that *ApL4* is the most abundant LS in guard cells, reasserting the sink nature of this gene (36). Thus, a clear distinction among the LSs can be established. APL1 is the large subunit showing the highest sensitivity to 3-PGA and P_i (4) and, by its level of expression, would be the main isoform in source tissues, thus providing a fine modulation of ADP-Glc PPase by the 3-PGA/P_i ratio to adjust starch synthesis to carbon fixation. In sink tissues, APL3 and, to a lesser extent, APL4 would be the main LS isoforms participating in the establishment of the ADP-Glc PPase heterotetramer. These two LSs have been shown to confer less sensitivity to 3-PGA and P_i to the enzyme (4), and thus starch synthesis would be more dependent on the supply of substrates from source tissues than on the allosteric effectors. The results support our previous model in which a functional distinction among the different LSs was proposed on the basis of the kinetic and regulatory properties that they confer to the ADP-Glc PPase heterotetramer (4).

The specialized function of the different ADP-Glc PPase isoforms is also reflected in their response to sugars. Coordinated sugar transcriptional regulation of several starch biosynthetic genes has been suggested by numerous works (19). Induction of several ADP-Glc PPase-encoding genes has been reported in different species including *Arabidopsis* (16, 25–28). We show that, under physiological conditions, only two ADP-Glc PPase-encoding genes, *ApL3* and *ApL4*, are controlled by sugars (Figs. 4 and 5). Sugar induction of *ApL3* and *ApL4* genes is leaf-specific and does not take place in sink tissue. Induction was stronger in response to disaccharides than to glucose (Fig. 5), suggesting that different signaling pathways may be involved

in the transcriptional control of *ApL3* and *ApL4* by sugars. To date, trehalose-regulated expression of *ApL3* has been shown (28), but no data regarding *ApL4* regulation have been published. Trehalose feeding rescues the phenotype of the *adg2-1* mutant lacking *ApL1* (17), and it was proposed that under these conditions, APL3 would substitute for APL1. Nevertheless, our results demonstrate that trehalose would alter the subunit composition of ADP-Glc PPase by inducing both APL3 and APL4.

The low-starch mutant *adg2-1* confirms our hypothesis that in normal situations, APL1 is the main LS isoform in the leaf mesophyll. In this tissue, there is also a significant amount of APL3 transcript (Figs. 1 and 3). However, it is very likely that in the metabolic environment of the leaf, most of the starch synthesis was driven by the APS1+APL1 heterotetramer. In plants fed with sugars, the intracellular level of hexoses increases (27, 37). In this situation, the change of the metabolic conditions in the mesophyll and the increased *ApL3* and *ApL4* expression would allow the establishment of heterotetramers less sensitive to the allosteric effectors (4), so that the enzyme would respond to or be controlled by the supply of substrates. This would lead to a higher rate of starch synthesis in response to the higher carbohydrate availability, resembling the situation in sink organs. In fact, sugar induction treatments increase starch levels in our plants (data not shown).

Several lines of evidence are in apparent contradiction with the results that we present on sugar regulation of ADP-Glc PPase isoforms. In previous studies, it was reported that the *ApL3* promoter fused to the GUS gene was only expressed in the bundle sheet and that its expression was induced in the leaf mesophyll cells in response to sucrose (15). However, according to the data presented in Fig. 2, *ApL3* can be detected in the mesophyll cells of plants grown in the absence of sugars. The simplest explanation for this discrepancy is that the *ApL3* promoter used for the GUS construct lacked element(s) needed for its normal expression. Induction with sucrose would raise the mRNA levels in the mesophyll cells, making it possible to detect GUS. On the other hand, results have also been published reporting that in *Arabidopsis* detached leaves, *ApS1*, *ApL3*, and *ApL2* genes were induced by sucrose and glucose, whereas *ApL1* was repressed (*ApS2* and *ApL4* were not known at that time) (27). This discrepancy is explained by the probes and assays used in that work. Sokolov *et al.* (27) used probes based on the available sequences at that time, which corresponded to a conserved region of the LS genes (38). Thus, they detected *ApL2*, whereas our observations using specific probes indicate that this gene is undetectable by Northern blot. Besides, they used detached leaves maintained in the dark for the induction assay. In that case, the effect of sugar treatment could be more severe or unspecific due to higher water evaporation rates or dehydration stress (15). We have analyzed the effect of the sugars under normal physiological conditions, and the only ADP-Glc PPase-encoding genes regulated by sugars at the transcriptional level are *ApL3* and *ApL4*.

The characterization of the expression pattern and sugar regulation of the ADP-Glc PPase gene family allows us to propose a model of gene evolution of this family in *Arabidopsis*. APS1 is essential for starch biosynthesis and is the fully catalytic subunit of the enzyme (4, 35), whereas APS2 has several amino acid substitutions and is not active *in vitro* (4, 39). Thus, our hypothesis is that the lone functional *Arabidopsis* SS is APS1. APS2, although it is still expressed at very low levels in the plant, is a nonfunctional ADP-Glc PPase subunit that could be accumulating mutations through a process of pseudogenization (40).

It is accepted that many genes appear by gene duplication (40, 41), and it has been proposed that the different ADP-Glc PPase LSs of a plant specie would result by divergence and specialization (7). It is also accepted that the *Arabidopsis* genome underwent several ancient rounds of duplication (42). Indeed, *ApL3* and *ApL4* genes are located in sister genomic regions that have been described in different genomic analysis (www.tigr.org/tdb/e2k1/ath1/Arabidopsis_genome_duplication.shtml). Our biochemical studies (4) showed that both LSs confer similar regulatory properties to the enzyme, and in the present report, we show that both *ApL3* and *ApL4* genes are similarly induced by sugars (Figs. 4 and 5). On the other hand, the expression pattern of the two genes is different (Figs. 1 and 3). These facts support the hypothesis that they are related genes under a process of functional divergence. Because it is not usual to find twin genes so well characterized, we think this is a great example of subfunctionalization that could be used in further genomic analysis (41).

In summary, we have determined the expression pattern and sugar regulation of the six *Arabidopsis* ADP-Glc PPase-encoding genes. A clear functional distinction can be made between the different large subunit isoforms that could be considered as sink (*ApL2*, *ApL3*, and *ApL4*)- or source (*ApL1*)-specific. Besides, a shift in the ADP-Glc PPase LS composition in response to sugar feeding allows the enzyme to be efficiently regulated according to the metabolic situation and the starch necessities of a given tissue. This hypothesis is in accordance with our previous data concerning the kinetic properties of the enzyme (4).

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