Two Arabidopsis ADP-Glucose Pyrophosphorylase Large Subunits (APL1 and APL2) Are Catalytic[1]

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ADP-glucose (Glc) pyrophosphorylase (ADP-Glc PPase) catalyzes the first committed step in starch biosynthesis. Higher plant ADP-Glc PPase is a heterotetramer ($\alpha_2\beta_2$) consisting of two small and two large subunits. There is increasing evidence that suggests that catalytic and regulatory properties of the enzyme from higher plants result from the synergy of both types of subunits. In Arabidopsis (*Arabidopsis thaliana*), two genes encode small subunits (*APS1* and *APS2*) and four large subunits (*APL1–APL4*). Here, we show that in Arabidopsis, APL1 and APL2, besides their regulatory role, have catalytic activity. Heterotetramers formed by combinations of a noncatalytic APS1 and the four large subunits showed that APL1 and APL2 exhibited ADP-Glc PPase activity with distinctive sensitivities to the allosteric activator (3-phosphoglycerate). Mutation of the Glc-1-P binding site of Arabidopsis and potato (*Solanum tuberosum*) isoforms confirmed these observations. To determine the relevance of these activities in planta, a T-DNA mutant of *APS1* (*aps1*) was characterized. *aps1* is starchless, lacks ADP-Glc PPase activity, *APS1* mRNA, and APS1 protein, and is late flowering in long days. Transgenic lines of the *aps1* mutant, expressing an inactivated form of APS1, recovered the wild-type phenotype, indicating that APL1 and APL2 have catalytic activity and may contribute to ADP-Glc synthesis in planta.

ADP-Glc pyrophosphorylase (ADP-Glc PPase; EC 2.7.7.27) is a heterotetrameric enzyme that catalyzes the synthesis of ADP-Glc and inorganic pyrophosphate from Glc-1-P and ATP (Espada, 1962). ADP-Glc is the donor for glycogen synthesis in bacteria and starch synthesis in plants (Sivak and Preiss, 1998; Slattery et al., 2000). ADP-Glc PPase is an allosteric enzyme regulated by intermediates of the major pathway of carbon assimilation in the organism (Ballicora et al., 2003, 2004). The activity of many higher plant ADP-Glc PPases is allosterically activated by 3-phosphoglycerate (3-PGA) and inhibited by inorganic phosphate (Preiss, 1973; Sivak and Preiss, 1998). Most bacterial enzymes are homotetramers composed of four identical subunits (α_4; Haugen et al., 1976; Ballicora et al., 2003), while higher plant ADP-Glc PPases are heterotetramers composed of two closely related types of subunits (S_2L_2; Morell et al., 1987; Okita et al., 1990; Preiss et al., 1991; Smith-White and Preiss, 1992; Ballicora et al., 2004). In higher plants, a number of studies suggest that the regulatory properties of ADP-Glc PPase are a product of synergistic interactions between the two types of subunits (Ballicora et al., 1998; Slattery et al., 2000; Cross et al., 2004; Hwang et al., 2005; Ventriglia et al., 2007). Besides, recent evidence suggests that some L subunits may have catalytic activity or influence the net catalysis of the enzyme (Burger et al., 2003). It has been reported that potato (*Solanum tuberosum*) tuber L subunit can be turned catalytically active by mutagenesis (Ballicora et al., 2005; Hwang et al., 2008) and that mutation of potato tuber L subunit (PLS) amino acid P44 (numbered as P47 in Fig. 1) caused a decrease of the heterotetrameric enzyme activity of 11- to 36-fold (Hwang et al., 2007). However, no direct evidence that the wild-type L subunit has activity or an active substrate site has been obtained to date.

In Arabidopsis (*Arabidopsis thaliana*), six genes encode proteins with homology to ADP-Glc PPase. Two of these genes encode for S subunits (*APS1* and *APS2*) and four encode L subunits (*APL1–APL4*; Crevillen et al., 2003, 2005). According to enzyme activity and

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A

E. coli	25	LAGGRGT**R**LKDLTNKRA**K**PAVH	46
PSS	26	LGGGAGT**R**LYPLTKKRA**K**PAVP	47
APS1	95	LGGGAGT**R**LYPLTKKRA**K**PAVP	116
APL1	95	LGGGAGT**R**LFPLTKRRA**K**PAVP	116
APL2	90	LGGGAGT**R**LFPLTSKRA**K**PAVP	111
APL3	94	LGGGDGA**K**LFPLTKRAA**T**PAVP	115
APL4	96	LGGGNGA**K**LFPLTMRAA**T**PAVP	117
PLS	37	LGGGEGT**K**LFPLTSRTA**T**PAVP	58

B

PSS	188	EEGRIIEFAE**K**PQGEQLQAMKVDT	211
APS1	257	EEGRIIEFAE**K**PKGEHLKAMKVDT	280
APL1	261	DKGRVISFSE**K**PKGDDLKAMAVDT	284
APL2	257	QSGKIIQFSE**K**PKGDDLKAMQVDT	280

Figure 1. Sequence comparison of ADP-Glc PPase subunits in a conserved region with critical amino acids for catalysis and Glc-1-P binding. Sequence comparison of the catalytic site (A) and Glc-1-P binding region (B) of ADP-Glc PPase isoforms from *E. coli*, Arabidopsis, and potato. Sequences and their accession numbers are as follows: *E. coli*, P00584; PSS, P23509; APS1, P55228; APL1, P55229; APL2, P55230; APL3, P55231; APL4, Q9SIK1; and PLS, Q00081. Conserved Arg (R) and Lys (K) are indicated in bold and underlined in A. The critical K residues involved in Glc-1-P binding are indicated in bold and underlined in B.

mRNA expression pattern studies, it has been proposed that the only functional S subunit in Arabidopsis is APS1, while APS2 may be in a process of pseudogenization (Crevillen et al., 2003, 2005; Zhang, 2003). It has also been proposed that the different L subunits are encoded by related genes that evolved by divergence, specialization, and subfunctionalization from a common ancestor (Simillion et al., 2002; Crevillen et al., 2003, 2005; Ballicora et al., 2004). Recently, site-directed mutagenesis studies have suggested that the PLS derives from a catalytic ancestor (Ballicora et al., 2005). Evolution of nonenzymes from a catalytic precursor is more common than the reverse, and many nonenzymes derived from enzyme ancestors have lost one or more critical catalytic residues (Todd et al., 2002; Pils and Schultz, 2004). Loss of catalytic activity opens new possibilities to adopt new functions, especially regulatory functions (Pils and Schultz, 2004). The genes encoding the S and L subunits are paralogs originated during an ancient duplication. It has been proposed that in Angiosperms, the S subunits have been subjected to more strict evolutionary constraint than the L subunits, as the former is less tissue specific and must interact with different L subunits (Georgelis et al., 2007). Considering that ADP-Glc PPase subunits evolved from a catalytic ancestor, it is of interest to analyze if the Arabidopsis L subunits have catalytic activity. Arabidopsis is a good system to perform this analysis, because the regulatory properties and the expression pattern of the ADP-Glc PPase gene family are well known (Crevillen et al., 2003, 2005).

In this study, we provide evidence that supports the hypothesis that two of the L subunits from Arabidopsis have catalytic activity, while the other two have lost their catalytic capacity during evolution. These catalytic L subunits also display distinct regulatory properties in relation to the allosteric activator 3-PGA.

RESULTS

Catalytic Activity of APL1 and APL2 Expressed in *Escherichia coli*

In multicellular organisms, S and L ADP-Glc PPase subunits form two and five distinct phylogenetic groups, respectively (Ballicora et al., 2005; Georgelis et al., 2007). While the S subunit phylogenetic groups correspond to monocot and dicot plants, the L subunit groups correlate with the reported tissue expression and the conservation of critical amino acid residues (Crevillen et al., 2003, 2005; Ballicora et al., 2005). In Arabidopsis, each L subunit shows a distinct pattern of expression. APL1 is the main L subunit in source tissues, whereas APL2, APL3, and APL4 are mainly present in sink tissues (Crevillen et al., 2005). APL1 belongs to a group of L subunits preferentially expressed in photosynthetic tissues. APL2 is included in an L subunit group showing a broader expression pattern but mainly restricted to sink tissues. On the other hand, APL3 and APL4 are included in a group that comprises the dicot L subunits expressed in sink tissues and is clearly separated from monocot seed-specific L subunits (Crevillen et al., 2003, 2005; Ballicora et al., 2005; Georgelis et al., 2007). Moreover, transcriptional regulation mediated by sugars of ADP-Glc PPase genes in Arabidopsis is restricted to *APL3* and *APL4* (Crevillen et al., 2005).

Amino acid residues R33 and K43 from the potato S subunit (PSS) have been shown to be critical for catalysis (Ballicora et al., 2005). These two amino acids are conserved in all S subunits and L subunits from groups 1 and 2 (Ballicora et al., 2005; Georgelis et al., 2007). As shown in Figure 1, the homologous residues in the active S subunit of Arabidopsis ADP-Glc PPase, APS1, are R102 and K112, while in the L subunits are R102 and K112 for APL1 and R97 and K107 for APL2. In Arabidopsis L subunits belonging to group III, the corresponding residues are K and T (K101 for APL3 and K103 for APL4; T111 for APL3 and T113 for APL4) as in the PLS (K44 and T54). It has been shown that mutation of both K44 to R44 and T54 to K54 in the PLS renders an enzyme with catalytic activity (Ballicora et al., 2005; Hwang et al., 2008). These mutagenesis approaches strongly suggest that the L subunits from groups 1 and 2 (APL1 and APL2, respectively, in the case of Arabidopsis) may have catalytic activity. To determine the putative activity of APL1 and APL2, it is necessary to avoid activity that comes from the S subunit participating in the heterotetrameric enzyme. Studies with *E. coli*-expressed proteins showed that none of the Arabidopsis L subunits was able to form active heterotetramers when coexpressed in the presence of APS2 (Crevillen et al., 2003). However, APS2 is a nonfunctional ADP-Glc PPase S subunit that has been accumulating mutations through a process of pseudogenization and might not be able to properly interact with the large subunits to form heterotetramers (Crevillen et al., 2003). To overcome this problem

and to obtain heterotetramers lacking APS1-dependent activity, an inactivated APS1 subunit was generated. Amino acid D145 of the PSS and the homologous residue in *E. coli* ADP-Glc PPase (D142) are essential for catalysis (Blankenfeldt et al., 2000; Frueauf et al., 2001, 2003). A PSS mutated in D145 (PSS_{D145N}) is four orders of magnitude less active than the wild-type subunits (Frueauf et al., 2003). Therefore, by site-directed mutagenesis, the homologous residue of Arabidopsis APS1 (D214) was changed to H, the corresponding amino acid present in the inactive Arabidopsis S subunit (APS2; Crevillen et al., 2003).

The mutated APS1 cDNA ($APS1_{D214H}$) and the cDNAs coding for the wild-type ADP-Glc PPase isoforms were expressed in an *E. coli* strain deficient in ADP-Glc PPase activity (AC70RI-504; Iglesias et al., 1993), and the activity of the different homo- and heterotetramers was analyzed in partially purified extracts (see "Materials and Methods"). $APS1_{D214H}$ homotetramers did not display detectable activity when assayed in the presence of a saturating concentration of the activator 3-PGA (Table I). Coexpression of $APS1_{D214H}$ with APL1 or APL2 produced active heterotetramers, while heterotetramers formed by $APS1_{D214H}$ and APL3 or APL4 showed no detectable activity (Table I). The activities determined for $APS1_{D214H}$/APL1 and $APS1_{D214H}$/APL2 heterotetramers in partially purified extracts in the pyrophosphorolysis direction were 0.74 and 0.46 units/mg protein, respectively. The response to the allosteric activator (3-PGA) was studied in partially purified $APS1_{D214H}$/APL1 and $APS1_{D214H}$/APL2 heterotetramers. In the pyrophosphorolysis direction, the kinetic for the response to 3-PGA of the $APS1_{D214H}$/APL1 heterotetramer indicated that the $A_{0.5}$ for 3-PGA was 0.62 μM, around 3-fold lower than the $A_{0.5}$ for the wild-type APS1/APL1 heterotetramer. A similar situation was observed for the $APS1_{D214H}$ /APL2 heterotetramer with an $A_{0.5}$ of 41 μM, around 6-fold lower than the wild-type APS1/APL2 heterotetramer (Table I; Fig. 2; Crevillen et al., 2003). The same behavior was observed when the activity was determined in the synthesis direction. The $A_{0.5}$ for $APS1_{D214H}$/APL1 and $APS1_{D214H}$/APL2 were also lower than the corresponding wild-type heterotetramers (Table I; Fig. 2). The results strongly indicate that both APL1 and APL2 have catalytic activity, while APL3 and APL4 are noncatalytic isoforms. Moreover, the APL1- and APL2-dependent activity showed a significantly higher sensitivity to the allosteric activator than the corresponding wild-type heterotetramers (Table I).

Mutation of Glc-1-P Binding Site Affects APL1- and APL2-Dependent Activity

It has been determined that residue K-195 in ADP-Glc PPase from *E. coli* is involved in the binding of the substrate Glc-1-P (Parsons and Preiss, 1978; Hill et al., 1991). This residue is conserved in both the S and the L subunits of higher plant ADP-Glc PPases. Site-directed mutagenesis and x-ray crystallography studies have shown that the corresponding residue of the PSS (K198) is in fact involved in Glc-1-P binding (Fu et al., 1998; Jin et al., 2005). The apparent affinity for Glc-1-P decreases 135-fold when K198 is changed to R in the PSS (Fu et al., 1998). In Arabidopsis, the homologous residues in APS1, APL1, and APL2 are K267, K271, and K267, respectively (Fig. 1).

To further corroborate that APL1 and APL2 are catalytic, the Lys residue involved in Glc-1-P binding was changed to R in $APS1_{D214H}$, APL1, and APL2 to generate $APS1_{D214H,K267R}$, $APL1_{K271R}$, and $APL2_{K267R}$. The mutated cDNAs were expressed in *E. coli* and the affinity for Glc-1-P determined in partially purified heterotetramers.

Coexpression of the wild-type APS1 subunit with mutated $APL1_{K271R}$ or $APL2_{K267R}$ subunits produced enzymes with altered Glc-1-P $S_{0.5}$ (Table II). This suggests

Table I. *Kinetic parameters for 3-PGA of Arabidopsis recombinant ADP-Glc PPase*

ADP-Glc PPase activity was determined in the pyrophosphorolysis direction and in the synthesis direction with partially purified enzymes as described in "Materials and Methods." The specific activity data correspond to the final step of a representative purification. The deviation in the 3-PGA $A_{0.5}$ data is the difference between duplicate experiments.

Subunit	Pyrophosphorolysis Direction			Synthesis Direction	
	Specific Activity	3-PGA $A_{0.5}$	n_H	3-PGA $A_{0.5}$	n_H
	unit/mg protein	μM		μM	
APS1	2.1	1,200 ± 92	2.5	5,300 ± 510	1.7
$APS1_{D214H}$	0[a]	–	–	–	–
APS1/APL1	1.6	1.9 ± 0.6	1.2	15.7 ± 3	1.5
$APS1_{D214H}$ /APL1	0.74	0.62 ± 0.06	1.5	2.3 ± 0.2	0.6
APS1/APL2	0.044	230 ± 14	1.2	821 ± 45	1.1
$APS1_{D214H}$ /APL2	0.46	41 ± 3	1.6	14.6 ± 0.4	1.6
APS1/APL3	0.95	35 ± 2.5	0.7	376 ± 52	0.7
$APS1_{D214H}$ /APL3	0[a]	–	–	–	–
APS1/APL4	15.4	28 ± 1.7	1.3	220 ± 30	0.7
$APS1_{D214H}$ /APL4	0[a]	–	–	–	–

[a]Detection limit of ADP-Glc PPase activity is about 1 nmol min^{-1} mg^{-1} protein.

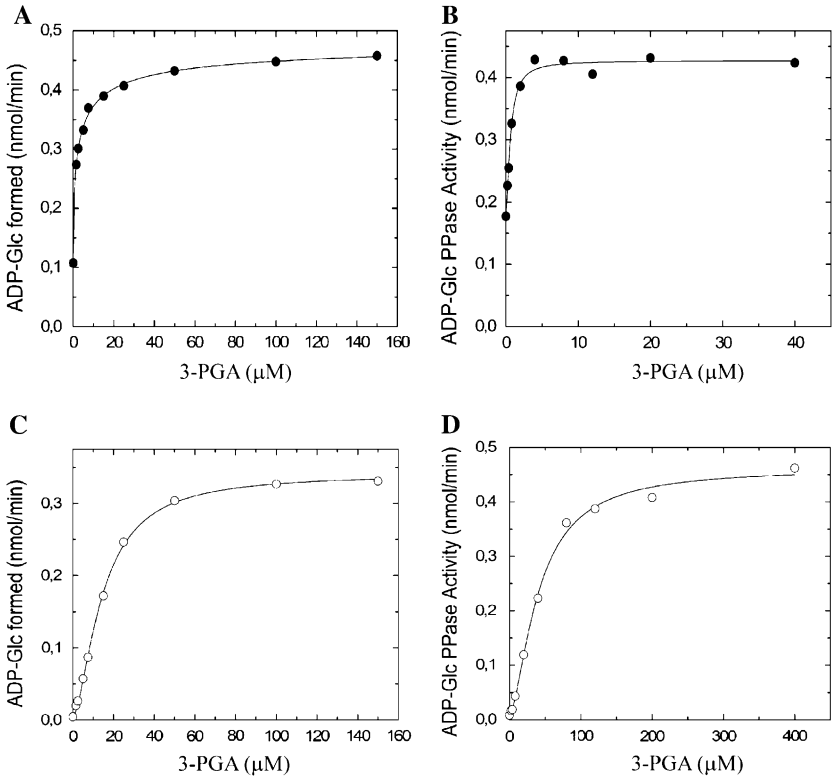

Figure 2. 3-PGA activation of recombinant Arabidopsis ADP-Glc PPases expressed in *E. coli*. Partially purified recombinant $APS1_{D214H}$/APL1 and $APS1_{D214H}$/APL2 heterotetramers were assayed for ADP-Glc PPase activity in the pyrophosphorolysis direction and in the synthesis direction with 3-PGA at the indicated concentrations in the figure. A and B (black circles) are 3-PGA saturation curves of partially purified recombinant $APS1_{D214H}$/APL1 (0.84 μg of enzyme). C and D (white circles) are 3-PGA saturation curves of partially purified recombinant $APS1_{D214H}$/APL2 (0.86 μg of enzyme). Curves in A and C are in the synthesis direction and B and D in the pyrophosphorolysis direction. The average of two experiments is plotted.

that catalysis occurred in the L subunits examined. Previously, a mutation of the homologous Glc-1-P site on an inactive PLS showed no effect (Fu et al., 1998). Here, in all cases, the $S_{0.5}$ increased and the Hill coefficients were clearly below 1.0. Curves that show a deviation from hyperbolic behavior may be the combination of separate active sites with different affinities. Because it was not possible with these constructs to discriminate the contribution from the catalytic S subunit, we proceeded to perform the same experiment with a catalytically deficient S subunit ($APS1_{D214H}$).

As shown in Table II, coexpression of $APS1_{D214H}$ and APL1 or APL2 produced enzymes with an apparent affinity for Glc-1-P ($S_{0.5}$) in the micromolar range. Similar results were obtained for heterotetramers formed by APL1 or APL2 with the APS1 double mutant ($APS1_{D214H,K267R}$). However, heterotetramers formed by $APS1_{D214H}$/$APL1_{K271R}$ or $APL2_{K267R}$ showed drastic increases in $S_{0.5}$ for Glc-1-P with values in the millimolar range (Table II). These results suggest that the activity determined for the different heterotetramers are not dependent on Glc-1-P binding by APS1 but are dependent on APL1 and APL2.

We have recently demonstrated that PSS can form active heterotetramers with the L subunits of Arabidopsis (Ventriglia et al., 2007). To further confirm the above-described data, the inactive PSS_{D145N} (Frueauf et al., 2003) and a generated double mutant, $PSS_{D145N,K198R}$, were coexpressed with the wild-type APL1 or APL2 isoforms (Table II). The calculated $S_{0.5}$ for Glc-1-P was in the micromolar range for these heterotetramers, as in the case of Arabidopsis heterotetramers. However, for heterotetramers formed by PSS_{D145N}/$APL1_{K271R}$ or $APL2_{K267R}$, the $S_{0.5}$ for Glc-1-P changed to the millimolar range, thus corroborating that activity is dependent on Glc-1-P binding by APL1 and APL2.

APL1- and APL2-Dependent Starch Synthesis in Planta

The results presented above strongly suggest that both APL1 and APL2, apart from playing a regulatory role in ADP-Glc PPase heterotetramers (Crevillen et al., 2003), also have catalytic activity and may contribute to starch synthesis in planta. To study the possible activity of the L subunits in planta, we have followed a similar strategy to that used for the *E. coli* protein expression studies. An Arabidopsis T-DNA insertional mutant in the *APS1* gene from the SALK collection (SALK_040155) was selfed and homozygotes for T-DNA insertion (*aps1*) were selected and characterized. Southern blot and PCR analysis show that the *aps1* mutant is homozygous for the T-DNA insertion at the *APS1* locus (Fig. 3, A and B) and presents undetectable levels of *APS1* mRNA and APS1 protein (Fig. 3, C and D). Growth of *aps1* mutant plants under short-day conditions (8 h light/16 h dark) was greatly hampered and flowering severely delayed (data not shown). Under long-day conditions (16 h light/8 h dark), the *aps1* mutant showed a clear delay in growth and flowering in comparison with the wild type, whereas in continuous light, growth and flowering time were equivalent to wild-type plants (Table III;

Table II. *Effect of mutations on the S and L subunits on the apparent affinity for Glc-1-P*

Reactions were performed in the synthesis direction as described in "Materials and Methods." Data were determined at least in duplicates, and the SE was determined by nonlinear regression as indicated in "Materials and Methods." When the n_H were different from 1.0, they are shown in parenthesis. In all the other cases, the coefficients were not significantly different from 1.0 and the curves showed a hyperbolic behavior. In the case of the last mutant, PSS_{D145N} /$APL2_{K267R}$, the activity was negligible until 10 mM Glc-1-P and the $S_{0.5}$ could not be measured with confidence (N/A).

Small Subunit	Large Subunit	Glc-1-P $S_{0.5}$	
		μM (n_H)	*relative (-fold)*
APS1	APL1	19 ± 1[a]	
APS1	APL2	85 ± 14[a]	
APS1	$APL1_{K271R}$	1,170 ± 290 (0.68)	
PSS	$APL1_{K271R}$	1,000 ± 110 (0.47)	
APS1	$APL2_{K267R}$	235 ± 20 (0.62)	
PSS	$APL2_{K267R}$	930 ± 90 (0.53)	
$APS1_{D214H}$	APL1	37 ± 4	1
$APS1_{D214H,K267R}$	APL1	22 ± 2	0.6
$APS1_{D214H}$	$APL1_{K271R}$	8,200 ± 800	221
$APS1_{D214H}$	APL2	131 ± 12	1
$APS1_{D214H, K267R}$	APL2	22 ± 5	0.17
$APS1_{D214H}$	$APL2_{K267R}$	4,700 ± 1,400	36
PSS_{D145N}	APL1	17 ± 3	1
$PSS_{D145N, K198R}$	APL1	20 ± 3	1.2
PSS_{D145N}	$APL1_{K271R}$	9,300 ± 900	547
PSS_{D145N}	APL2	47 ± 8	1
$PSS_{D145N, K198R}$	APL2	56 ± 12	1.2
PSS_{D145N}	$APL2_{K267R}$	N/A	N/A

[a]Data were from literature (Crevillen et al., 2003).

Fig. 4, A, B, and D). No ADP-Glc PPase activity in leaves or starch in leaves and roots could be detected in the *aps1* mutant (Table IV; Figs. 4C and 7). Mutants with low starch content affected in ADP-Glc PPase or other genes have been described bearing similar phenotypes as the *aps1* mutant (Lin et al., 1988a, 1988b; Li and Preiss, 1992; Yu et al., 2000; Dumez et al., 2006). The *aps1* mutant is a T-DNA null mutant completely lacking starch and, to confirm that it is specifically affected in ADP-Glc PPase, it was transformed with the wild-type *APS1* full-length cDNA under the 35S promoter. Three independent transgenic lines [35S:*APS1*(*aps1*)#1; #2; #4] were analyzed and showed similar levels of starch accumulation as the wild-type plant and partially recovered ADP-Glc PPase activity (Table IV). The transgenic lines behaved as wild-type plants in relation to growth rate and flowering time (Table III). Furthermore, transformation of the *aps1* mutant with the wild-type *APS2* full-length cDNA under the 35S promoter [35S:*APS2*(*aps1*)] did not complement the *aps1* mutation, corroborating that APS2 is a nonfunctional S subunit (Table IV; Fig. 4D). These results confirm that the SALK_040155 T-DNA insertion line is specifically affected in the *APS1* gene.

The inability to accumulate starch of the *aps1* mutant is most likely due to the fact that in the absence of APS1 protein, no active ADP-Glc PPase can be formed, because the S subunit is necessary for stability of the L subunits (Wang et al., 1998). Even if APL1 and APL2 had enzymatic activity, *aps1* mutant plants would not synthesize starch, because both ADP-Glc PPase L subunits would not be able to form a stable enzyme.

The full-length cDNA coding for $APS1_{D214H}$, including the chloroplastic signal peptide of *APS1*, was cloned in vector cTapi.289.gw under the 35S promoter and used to transform the *aps1* mutant. Different transgenic lines were selected and characterized. $APS1_{D214H}$ protein was detected by western blot in the transgenic lines [35S:$APS1_{D214H}$(*aps1*)#4 and 35S:$APS1_{D214H}$(*aps1*)#10] as well as APS1 in a control *aps1* mutant plant transformed with the wild-type *APS1* cDNA [35S:*APS1*(*aps1*)#2; Fig. 3D]. The level of $APS1_{D214H}$ mRNA in leaves of different transgenic lines (Fig. 5) correlates with the level of ADP-Glc PPase activity and starch accumulation (Table IV). Lines with a very low level of $APS1_{D214H}$ mRNA expression, as 35S:$APS1_{D214H}$(*aps1*)#2, also showed limited levels of starch accumulation and undetectable ADP-Glc PPase activity. On the contrary, line 35S:$APS1_{D214H}$(*aps1*)#10 showed similar levels of $APS1_{D214H}$ mRNA, equivalent to *APS1* mRNA levels in wild-type plants (Fig. 5), and correspondingly showed significant levels of ADP-Glc PPase activity and similar levels of starch accumulation as the wild type (Table IV). Interestingly, flowering time in 35S:$APS1_{D214H}$(*aps1*) lines correlated with the level of starch accumulation. Lines 35S:$APS1_{D214H}$(*aps1*)#4 and 35S:$APS1_{D214H}$(*aps1*)#10 showed both starch levels and bolting time equivalent to wild-type plants. However, line 35S:$APS1_{D214H}$(*aps1*)#8 reached only 25% of wild-type starch accumulation and was late flowering in

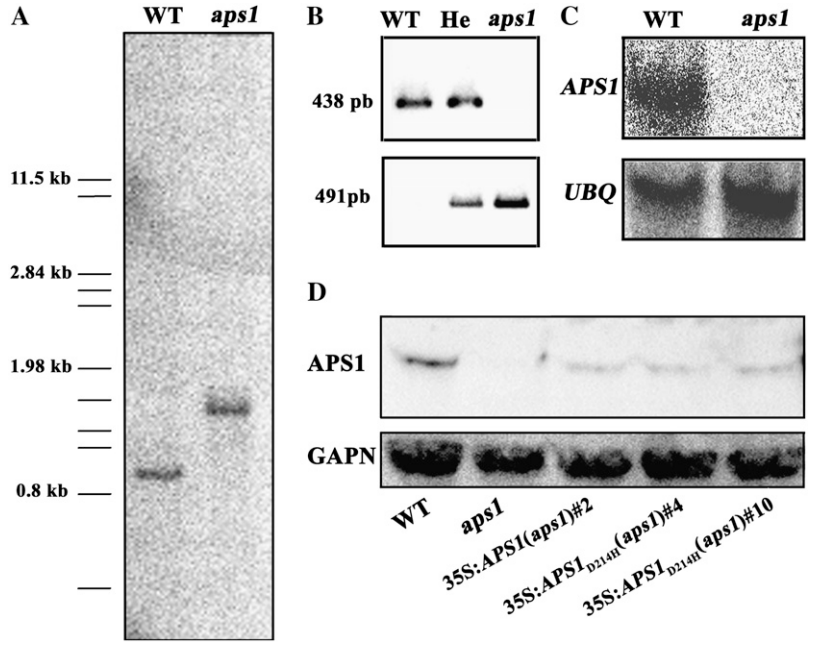

Figure 3. Molecular analysis of *aps1*, a T-DNA insertional mutant of *APS1*. A, Southern-blot analysis of genomic DNA of wild type and SALK_040155 line (*aps1*). A total of 20 μg of genomic DNA was cut with *Eco*RI and analyzed by Southern blot using a 0.4-kb cDNA fragment as probe (see "Materials and Methods"). B, PCR of genomic DNA isolated from wild type and homozygous (*aps1*) and heterozygous lines of SALK_040155. *APS1*-specific primers SA340 and SA341, flanking the T-DNA insertion, produced a band of 438 bp in wild-type plants. Combination of SA340 with LBb1 primer from the left border of the T-DNA produced a band of 491 bp using mutant genomic DNA. C, Northern analysis of *APS1* expression in leaves. Total RNA (30 μg) from leaves was probed with a 0.4-kb *APS1* cDNA fragment (see "Materials and Methods"). *UBIQUITIN10* gene was used as loading control. D, Western blot of leaf extracts from different plants probed with antibodies against the spinach (*Spinacia oleracea*) leaf ADP-Glc PPase. GAPN was used as loading control and detected with anti-GAPN serum.

long days (Fig. 4E; compare Tables III and IV). As shown in Figure 6A, the growth rate of the transgenic lines, estimated as fresh weight, was also correlated with the level of starch content in each line. Starch accumulation in 35S:*APS1*$_{D214H}$(*aps1*) transgenic lines is not due to an altered capacity of starch degradation that may mediate accumulation of the polymer. As shown in Figure 6B, 35S:*APS1*$_{D214H}$(*aps1*)#4 and 35S:*APS1*$_{D214H}$(*aps1*)#10 plants display the same rate of both starch accumulation during the day and starch degradation during the dark period as wild-type plants. These results strongly indicate that the *aps1* mutant is specifically affected in the *APS1* gene and that some Arabidopsis ADP-Glc PPase L subunits have catalytic activity. In the presence of a noncatalytic S subunit, they are most probably able to generate active heterotetramers. According to *E. coli* expression studies, APL1 and APL2 should have catalytic activity and be responsible for starch accumulation and ADP-Glc PPase activity in 35S:*APS1*$_{D214H}$(*aps1*) transgenic lines.

APL3 and *APL4* are the main L subunit isoforms expressed in roots (Crevillen et al., 2005); thus, ADP-Glc PPase heterotetramers would preferentially be formed by APS1/APL3 and/or APL4 in roots. As shown in Figure 7, leaves of wild type and transgenic lines 35S:*APS1*$_{D214H}$(*aps1*)#4 and #10 showed similar starch content. Starch levels in the 35S:*APS1*$_{D214H}$(*aps1*)#8 line were lower than in the wild type but significantly higher than in the *aps1* mutant. However, the starch content in the columella cells was significantly lower in all transgenic 35S:*APS1*$_{D214H}$(*aps1*) lines in relation to leaves, reflecting the low level of *APL1* and *APL2* expression in roots (Fig. 7). These results indicate that the levels of APL1 and APL2 in roots are not sufficient to fully restore the wild-type starch content in the transgenic lines. On the contrary, the level of starch content in leaves, where APL1 is the main L subunit isoform (Crevillen et al., 2005), is recovered.

From the data presented above, we propose that some L subunits (APL1 and APL2 in Arabidopsis) contribute to the synthesis of ADP-Glc, the precursor of starch synthesis.

DISCUSSION

In plants, ADP-Glc PPase is a heterotetrameric enzyme composed of two small and two large subunits. Different studies have suggested that both types of subunits may have catalytic and regulatory roles (Ballicora et al., 1998; Burger et al., 2003; Cross et al., 2004, 2005) and that large subunit can be converted in a catalytic form by site-directed mutagenesis (Ballicora et al., 2005; Hwang et al., 2008). However, no reports have been published showing that any large subunit has catalytic activity per se.

In Arabidopsis, four genes encode L subunits (*APL1– APL4*). Among them, APL1 and APL2 have two amino acids (R102 and K112 in APL1 and R97 and K107 in APL2; see Fig. 1) that are also present in the S subunits of all plants and are known to be critical for catalysis (Ballicora et al., 2005). We wondered whether APL1 and/or APL2 were catalytic subunits. To test this hypothesis, an inactive form of APS1 (APS1$_{D214H}$) was produced and expressed in combination with the four Arabidopsis L subunits in *E. coli*. The analysis of ADP-Glc PPase activity both in the pyrophosphorolysis and synthesis directions indicated that coexpression of APS1$_{D214H}$ with either APL1 or APL2 produced active heterotetramers (Fig. 2; Table I). These results strongly suggest that APL1 and APL2 are catalytic isoforms as well as regulatory. The critical R and K

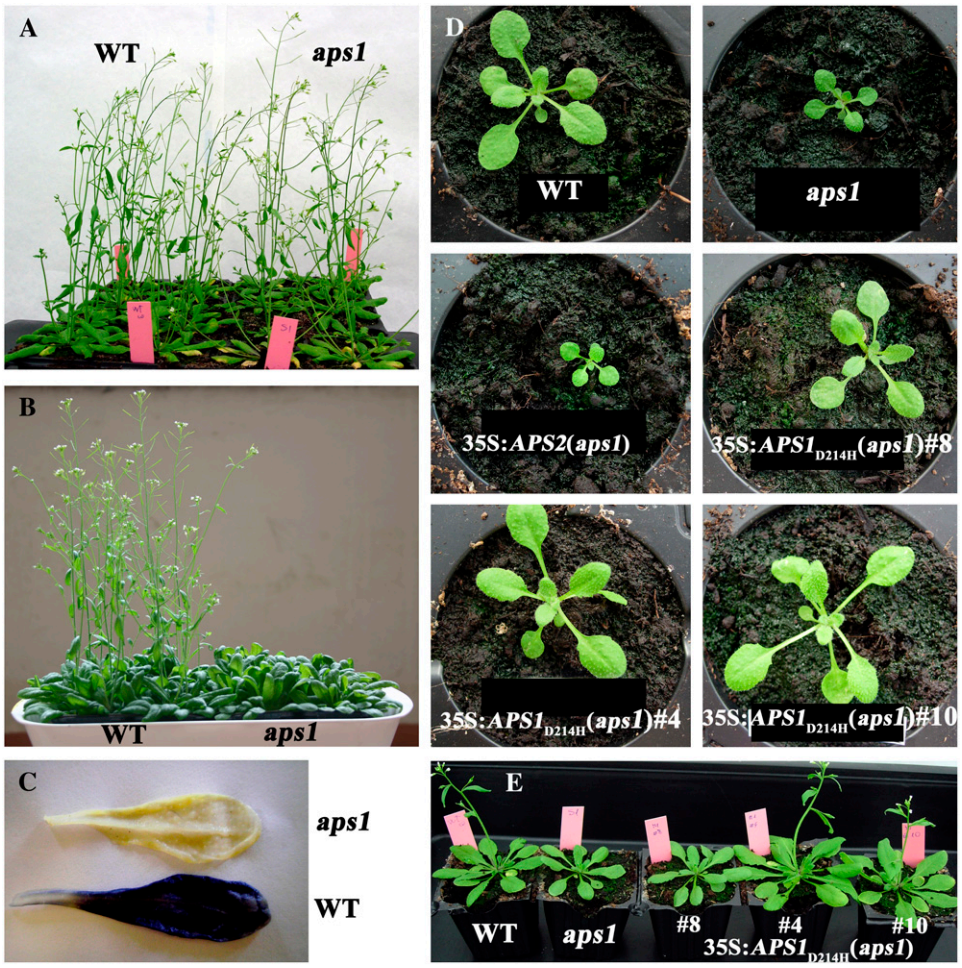

Figure 4. Phenotypic differences between Arabidopsis wild type, *aps1* mutant, and 35S:$APS1_{D214H}$ transgenic lines. A and B, Wild-type and *aps1* plants were grown for 3 weeks under continuous light (A) or long-day conditions for 4 weeks (B). C, Iodine staining of rosette leaves of wild-type and *aps1* mutant plants grown for 3 weeks under long-day conditions. D and E, Phenotypes of wild type, *aps1*, and different 35S:$APS1_{D214H}$ transgenic lines grown for 2 (D) or 4 weeks (E) under long-day conditions.

residues are not conserved in APL3 and APL4 and, as expected, coexpression of $APS1_{D214H}$ with APL3 or APL4 did not produce active heterotetramers. The response to the allosteric activator (3-PGA) of $APS1_{D214H}$/APL1 and $APS1_{D214H}$/APL2 heterotetramers was also analyzed and shown to be more sensitive than the corresponding wild-type APS1/APL1 and APS1/APL2 (Fig. 2; Table I; Crevillen et al., 2003). This higher sensitivity for the allosteric activator of APL1- and APL2-dependent ADP-Glc PPase activity suggests that the regulatory properties of the wild-type heterotetramer result from the synergy between the S and L subunits. Thus, the overall response of the wild-type enzyme to the allosteric effectors would result from the combination of a very sensitive response of the L subunits and a lower sensitivity of the S subunits.

Site-directed mutagenesis of the Glc-1-P binding site in S and L subunits of Arabidopsis confirmed that the activity of heterotetramers containing an inactive APS1 protein is dependent on the L subunits APL1 and APL2 (Table II). This observation is further corroborated in heterotetramers containing an inactive PSS with a mutation in the Glc-1-P binding site.

To determine the catalytic nature of the L subunits in planta, we reconstituted the starch synthesis capacity in a starchless mutant (*aps1*). Previously reported starch-deficient mutants affected in ADP-Glc PPase such as TL46 and TL25 showed decreased ADP-Glc PPase activity and starch contents of 40% and 2% in relation to wild-type plants, respectively (Lin et al., 1988a, 1988b). A T-DNA mutant showing about 20% starch has also been described (Dumez et al., 2006), but the *aps1* mutant is a T-DNA insertional null mutant completely lacking starch. Transformation of the *aps1* line with *APS2*, a nonfunctional ADP-Glc PPase isoform, did not restore the wild-type phenotype, while the wild-type *APS1* gene complemented the mutant phenotype in the transgenic *aps1* lines (Tables III and IV).

The presence of both S and L ADP-Glc PPase subunits is necessary for stability of the heterotetramer (Giroux et al., 1994; Wang et al., 1998). Thus, to detect L subunit-dependent ADP-Glc PPase activity, we generated transgenic lines of the *aps1* mutant transformed with an $APS1_{D214H}$ construct rendering a noncatalytic S subunit. Transgenic *asp1* lines were characterized and showed a recovery of ADP-Glc PPase activity, starch content, and bolting time that correlated with the $APS1_{D214H}$ mRNA level (Tables III and IV; Fig. 5). These results clearly indicate that the L subunits, in the presence of an inactive form of APS1, are able to

Table III. *Bolting time of wild type (Columbia ecotype), aps1 mutant, and several transformant aps1 lines containing the aps1$_{D214H}$ cDNA or the wild-type APS1 cDNA under the 35S promoter*

Plants were grown under long-day conditions (16 h light/8 h dark). Data represent the mean for 20 plants.

Line	Bolting Time	No. of Rosette Leaves	No. of Cauline Leaves
	d		
Wild type	21 ± 1.5	13.8 ± 1.3	4.1 ± 0.5
aps1	32 ± 1.0	23.8 ± 1.2	4.8 ± 0.7
35S:*APS1*(*aps1*)#1	21 ± 0.5	15.0 ± 1.1	3.8 ± 0.8
35S:*APS1*(*aps1*)#2	20.8 ± 0.8	14.8 ± 1.1	3.8 ± 0.4
35S:*APS1*(*aps1*)#4	20.8 ± 0.5	14.3 ± 0.9	4.0 ± 0.0
35S:*aps1*$_{D214H}$(*aps1*)#8	29 ± 2.0	20.9 ± 1.9	4.3 ± 0.5
35S:*aps1*$_{D214H}$(*aps1*)#4	21 ± 2.0	16.2 ± 1.3	4.0 ± 0.0
35S:*aps1*$_{D214H}$(*aps1*)#10	21 ± 2.0	14.8 ± 0.8	4.2 ± 0.4

mediate the synthesis of ADP-Glc in planta. According to the *E. coli* expression studies performed, this activity would be dependent upon APL1 and APL2. Therefore, the role of the L subunits would be just regulatory (APL3 and APL4) or both catalytic and regulatory (APL1 and APL2), modulating enzyme activity but also contributing to ADP-Glc synthesis.

Higher plant ADP-Glc PPases are heterotetramers that show different regulatory properties according to subunit composition in different organs and tissues (Crevillen et al., 2003). The L subunits are differentially expressed in relation to the necessities of a given organ or tissue for starch synthesis. In roots, the prevalent heterotetramers would be formed by APS1/APL3 or APL4 with a lower contribution of APL1 and APL2. Accordingly, in 35S:*APS1*$_{D214H}$(*aps1*) transgenic lines, recovery of starch accumulation in columella cells is significantly lower than in leaves (Fig. 7), as the catalytic L subunits (APL1 and APL2) would contribute to a lesser extent to heterotetramer formation in roots.

During evolution from a common ancestor, divergence leading to new roles (catalytic and/or regulatory), subfunctionalization of regulatory genes, as well as the establishment of specific patterns of expression have been necessary to control starch synthesis in different tissues with distinct physiological necessities. According to our data, in Arabidopsis, one S subunit (APS1) and two L subunits (APL1 and APL2) have catalytic activity, thus indicating the evolution from a common ancestor for both types of subunits as proposed previously (Ballicora et al., 2005). APL3 and APL4 should have reached a higher level of subfunctionalization and specialization, as they are sink subunits (Crevillen et al., 2003) lacking catalytic activity.

Recently, it was shown that the S subunits have more evolutionary constraints than the L subunits (Georgelis et al., 2007). S subunits are less tissue specific in comparison to L subunits and form heterotetramers with different L subunits in different tissues. In Arabidopsis, two genes code for proteins with homology to S subunits, *APS1* and *APS2*. Our data

Table IV. *ADP-Glc PPase activity and starch content in leaves of wild type (Columbia ecotype), aps1 mutant, and several transformant lines containing the full-length cDNA of APS1 or aps1$_{D214H}$ under the 35S promoter*

Plants were grown under long-day conditions (16 h light/8 h dark). In brackets, the percentage of starch content and activity, normalized to wild-type values, is indicated. Data are the mean of three independent experiments.

Line	Starch	ADP-Glc PPase Activity
	mg/g fresh weight	*milliunits/mg protein*
Wild type	6.4 ± 0.4 (100)	354 ± 19.3 (100)
aps1	0[a]	0[b]
35S:*APS1*(*aps1*)#1	5.6 ± 0.52 (86.8)	27.2 ± 0.5 (7.7)
35S:*APS1*(*aps1*)#2	6.3 ± 0.50 (97.5)	42.8 ± 1.0 (12.1)
35S:*APS1*(*aps1*)#4	6.5 ± 0.47 (101.7)	41.8 ± 1.1 (11.8)
35S:*APS2*(*aps1*)	0[a]	0[b]
35S:*aps1*$_{D214H}$(*aps1*)#2	0.7 ± 0.05 (10.8)	0[b]
35S:*aps1*$_{D214H}$(*aps1*)#8	1.6 ± 0.19 (25.9)	4.4 ± 1.3 (1.2)
35S:*aps1*$_{D214H}$(*aps1*)#9	1.7 ± 0.15 (26.1)	10.4 ± 3.1 (2.9)
35S:*aps1*$_{D214H}$(*aps1*)#4	5.8 ± 0.50 (88.9)	35.8 ± 5.6 (10.1)
35S:*aps1*$_{D214H}$(*aps1*)#10	6.5 ± 0.48 (102.1)	73.9 ± 7.2 (20.9)

[a]Limit of detection for starch is about 0.2 μg. [b]Detection limit of ADP-Glc PPase activity is about 1 nmol min^{-1} mg^{-1} protein.

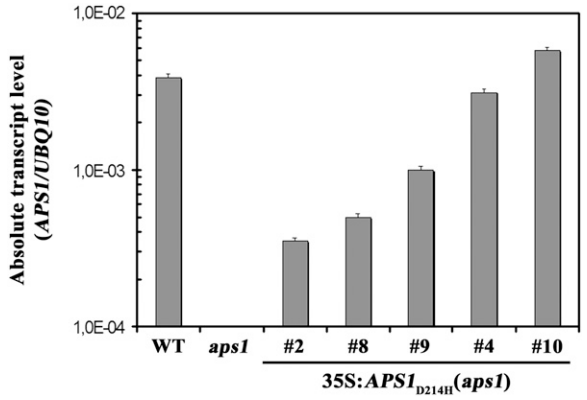

Figure 5. *APS1* mRNA levels in wild type, *aps1* mutant, and different *aps1* transgenic lines. Plants were grown under long-day conditions. Leaf samples were collected at the end of the light period and the level of mRNA determined by real-time quantitative RT-PCR as described under "Materials and Methods." Data are the average of three independent determinations in each of two different cDNA preparations.

confirm that *APS2* is a nonactive S subunit as proposed previously (Crevillen et al., 2003, 2005). Evolutionary changes in *APS1* might then have been more constrained than for the L subunit genes, as the only functional S subunit in Arabidopsis forms heterotetramers with four different L subunits.

APL1 is the main L subunit isoform present in source tissues, conferring a very high sensitivity to 3-PGA and inorganic phosphate to the heterotetrameric enzyme, while APL2 is mainly present in sink tissues and confers a very low sensitivity to the allosteric effectors. This suggests a process in which the L subunits have subfunctionalized from their common ancestor with the S subunit and diverged to have more specific roles (Kavakli et al., 2002; Crevillen et al., 2003, 2005). It is noteworthy that the $A_{0.5}$ for 3-PGA of heterotetramers formed by $APS1_{D214H}$ and APL1 or APL2 is lower than the $A_{0.5}$ displayed by the wild-type heterotetramers (Table I). These data indicate that the S subunit also has regulatory capacity (with lower affinity for 3-PGA than L subunits) and confirm the synergistic interactions between both types of subunits (Ballicora et al., 1998; Cross et al., 2004; Hwang et al., 2005, 2008).

The data presented suggest that synthesis of ADP-Glc in planta is carried out by the combined activity of S and L subunits. Further studies with double and triple L subunit mutants would provide insights on the contribution of APL1 and APL2 to starch synthesis and confirm the lack of APL3 and APL4 activity in planta.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) Columbia ecotype was grown in growth cabinets under long-day conditions (16 h light/8 h dark), short-day conditions (8 h light/16 h dark), or continuous light at 23°C (day)/20°C (night), 70% humidity, and a light intensity of 100 μE m^{-2} s^{-1} at the plant level. The APS1 mutant line of Arabidopsis was obtained from the SALK T-DNA mutant collection (SALK_040155; Alonso et al., 2003). T-DNA insertion was analyzed using the left-border T-DNA oligonucleotide LBb1 (5′-GCGTGGACCGCTTGCTGCAAC-3′) and *APS1*-specific oligonucleotides (SA340, 5′-TGGACTCCGTTCCTGCA-3′ and SA341, 5′-TCAGATGACAGTGCCGGTTGG-3′).

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 (RT)-PCR as described by Crevillen et al. (2003). *Nde*I-*Eco*RI fragments containing the S subunit cDNAs were subcloned into pMAB5, a modified version of the expression vector pMON17335 (Iglesias et al., 1993), with an *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 L subunit cDNAs were cloned into the pMON17336 (Iglesias et al., 1993) to generate the plasmids pSAT516 (APL1), pSAT524 (APL2), pSAT532 (APL3), and pSAT544 (APL4; Crevillen et al., 2003). Change of D214 to H in APS1 was performed by site-directed mutagenesis of pSAT403 using the QuickChangeIIXL kit with oligonucleotides SA415 (5′-CTCATTCTTGCTGGG**C**ATCATTTGTATAGAATGG-3′) and SA416 (5′-CCATTCTATACAAATGAT**G**CCCAGCAAGAATGAG-3′) to generate plasmid pM1 ($APS1_{D214H}$; nucleotide changes are indicated in bold and underlined). We performed site-directed mutagenesis on the Glc-1-P binding site of APS1-K267R, APL1-K271R, and APL2-K267R using PCR and primers 5′-CCGTATTATTGAATTTGCGGAA**CGC**CCGAAAGGCGAACATCTG-3′, 5′-GGAAGAGTTATCTCATTCAGTGAA**CGT**CCTAAAGGAGACGACCTG-3′, 5′-CATTCAATTCTCGGAA**CGT**CCAAAGGGAGATGACTTAAAGGC-3′, respectively. The PCR product was inserted into the Strataclone

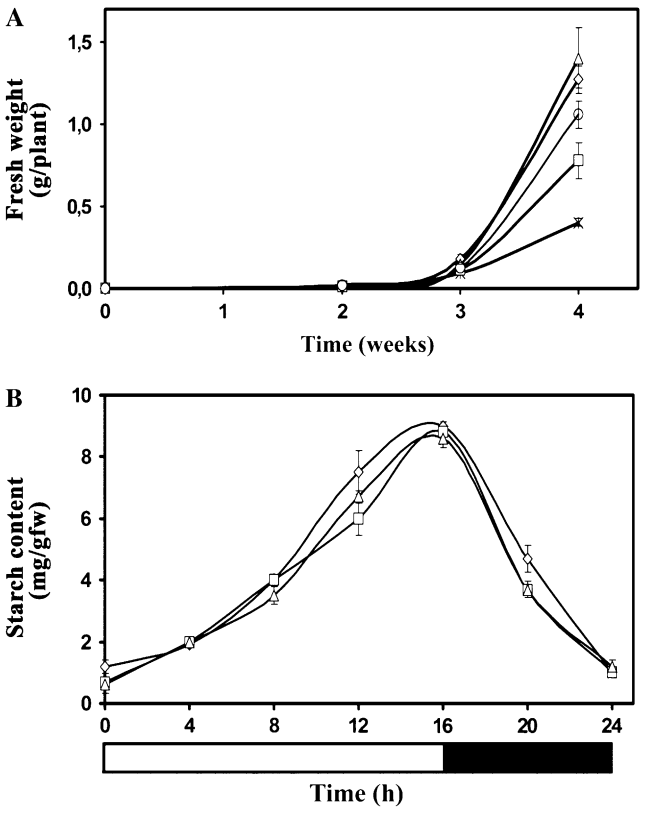

Figure 6. Growth rate (A) and starch content during 24 h (B) in wild type and different *aps1* transgenic lines. Plants were grown as described in "Materials and Methods" under long-day conditions. A, Fresh weight of aerial organs in grams per plant is plotted against time after sowing. B, Starch content in leaves during a day/night cycle. The bar at the bottom of the graph indicates the corresponding day/night periods: day, white box; night, black box. Rhombuses, wild type; asterisk, *aps1* mutant; squares, 35S:$aps1_{D214H}$(*aps1*)#4 line; circles, 35S:$aps1_{D214H}$(*aps1*)#8 line; triangles, 35S:$aps1_{D214H}$(*aps1*)#10 line. Data are the mean of three independent experiments.

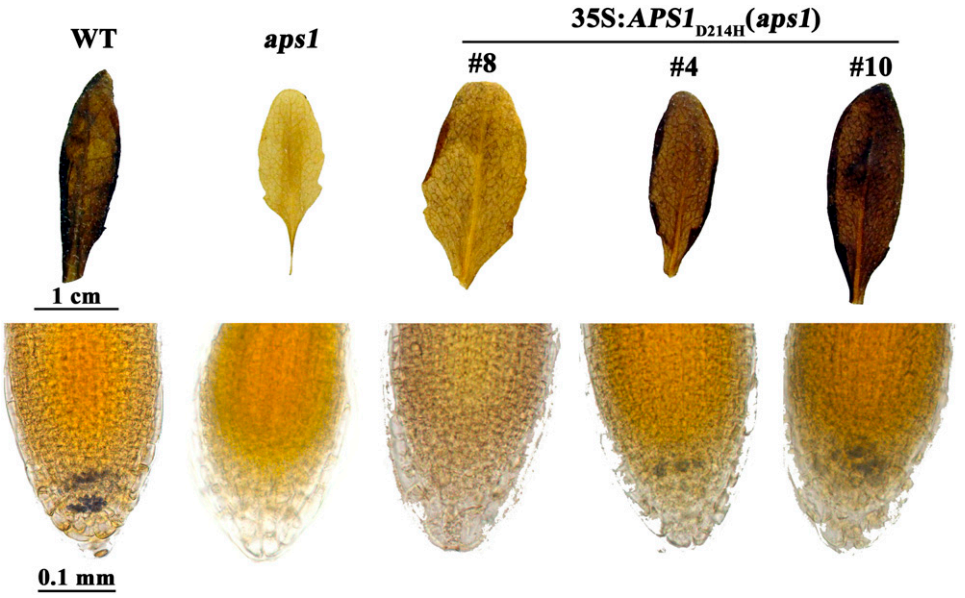

Figure 7. Iodine staining of leaves and roots of wild type, *aps1* mutant, and 35S:$APS1_{D214H}$ transgenic lines. Rosette leaves from plants grown for 3 weeks (top) and roots dissected from seedlings grown for 1 week (bottom) were stained with lugol. Scale bars = 1 cm (top) and 0.1 mm (bottom).

vector system (Stratagene), screened, and sequenced. Once the correct sequence was obtained, the mutated genes were ligated into derivatives of pMON17335 (APS1) and pMON17366 (APL1 and APL2) expression vectors, respectively.

Arabidopsis was transformed using plasmid cTapi.289.gw (Rohila et al., 2004) using the Gateway cloning system (Invitrogen). cDNA fragments encoding the mature sequence of APS1 and $APS1_{D214H}$ were cloned in pGEM-T Easy vector (Promega) as described by Crevillen et al. (2003). The signal peptide was obtained by RT-PCR from total RNA isolated from leaves with oligonucleotides SA467 (5′-ATGGCGTCTGTATCTGCAATTGG-3′) and SA468 (5′-CAAAGACGCGGAATTGAACTGAG-3′) and cloned in pGEM-T Easy vector. The unique *Bgl*II restriction site in *APS1* was used to add the cDNA fragment of pSAT403 and pM1 to the signal peptide and generate pSAT409 (*APS1*) and pSAT412 ($APS1_{D214H}$), respectively. The full-length *APS2* cDNA was obtained by RT-PCR using the oligonucleotides SA103 (5′-CGACCATGGTGATTTCTTCTTCTTTTATTACC-3′) and SA104 (5′-CTGCAGTCAGAGGATGGAGTCGTT-3′) and cloned in pGEM-T Easy vector (pSAT450). The att-B recognition site for BP-recombinase was introduced using oligonucleotides SA511 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTGGGTGGGTGGCCGTGACAGTAGACT-3′) and SA514 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCGTCTGTATCTGCAATTGG-3′) for *APS1* and $APS1_{D214H}$; and SA512 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCAGATTTCTTCTTCTTCTT-3′) and SA513 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTCAGAGGATGGAGTCGTTC-3′) for *APS2*. pDONR221 and cTapi.289.gw were used as donor and destination vectors, respectively, for the Gateway gene transfer system.

Transformation of Arabidopsis was performed by the floral dipping method (Clough and Bent, 1998). All experiments were performed with F3 seeds. The insertions of the transgenes in all transgenic lines were sequenced to check for integrity of the inserted constructs.

Expression and Purification of Recombinant Enzymes

For expression and purification of the Arabidopsis ADP-Glc PPases, an *Escherichia coli B* mutant strain deficient in ADP-Glc PPase activity (AC70RI-504; Iglesias et al., 1993) was used. The ADP-Glc PPase S subunits alone or together with the L subunit cDNAs were expressed in *E. coli* strain AC70R1-504 and purified as described previously (Crevillen et al., 2003). Single colonies were grown in Luria-Bertani medium at 37°C with the suitable antibiotic until the A_{600} reached 1.1 to 1.3. Induction of the small subunits was initiated by adding 400 μM of isopropyl-β-D-thiogalactopyranisode and 5 μg/mL of nalidixic acid to induce the expression of the large subunits. After 16 h of induction, cells were chilled on ice and harvested by centrifugation. Cell pastes were resuspended in buffer A containing 50 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, and 10% Suc, and disrupted by sonication. The sonicated suspensions were centrifuged for 20 min at 10,000*g*, and the supernatants (crude extracts) were retained for activity assays.

Recombinant enzymes were purified for kinetic studies. Crude extracts were subjected to an ammonium sulfate fractionation (30%–60%) with centrifugation for 20 min at 12,000*g*. The ammonium sulfate pellets were resuspended in 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 a 10-kD molecular mass cutoff (Millipore), and stored at −80°C. ADP-Glc PPase activity of the samples was stable for at least 3 months. Integrity of heterotetramers was tested as described previously (Crevillen et al., 2003; Ventriglia et al., 2007).

Protein Assay

Protein concentrations of the fractions obtained during the purification were determined by using bicinchoninic acid reagent (Smith et al., 1985) from Pierce Chemicals using bovine serum albumin (BSA) as the standard.

Western-Blot Analysis

Immunoblot analysis was performed using antibodies against spinach-leaf ADP-Glc PPase (Morell et al., 1987). Proteins (75 μg) from leaf crude extracts were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membrane by electroblotting in a Trans-Blot SD transfer cell (Bio-Rad) according to the manufacturer's instructions. Blots were probed with rabbit antiserum raised against spinach-leaf ADP-Glc PPase followed by horseradish peroxidase-conjugated goat-anti-rabbit serum (SIGMA) and detected using ECL Plus Advance Western Blotting Reagent (Amersham Biosciences). Non-phosphorylating glyceraldehyde-3-P dehydrogenase (GAPN) was used as loading control and probed as reported previously (Valverde et al., 1999).

Assay of ADP-Glc PPase Activity

Pyrophosphorolysis Direction

For *E. coli*-expressed proteins, the [^{32}P]ATP formed in the pyrophosphorolysis of ADP-Glc was determined according to Morell et al. (1987). The reaction mixture consisted of 50 mM HEPES, pH 7.9, 7 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 2 mM ADP-Glc, 10 mM NaF, 0.2 mg/mL BSA, 1 mM sodium [^{32}P]pyrophosphate (1,000 cpm/nmol), variable concentrations of 3-PGA, and the aliquot of the enzyme in a final volume of 0.25 mL.

Synthesis Direction

Synthesis of ADP-Glc was measured as described by Yep et al. (2004) and was used to characterize the response to 3-PGA. The reaction was conducted in the presence of 50 mM HEPES, pH 8.0, 0.2 mg/mL BSA, 7 mM $MgCl_2$, 1.5 mM ATP, 2 mM DTT, 1.5 unit/mL inorganic pyrophosphatase, and 0.5 mM [^{14}C]Glc-1-P (10.7 Bq/nmol), and variable concentrations of 3-PGA in a total volume of 200 μL. The product ADP-[^{14}C]Glc was determined by the glycogen synthesis procedure (Yep et al., 2004). Saturation curves of Glc-1-P were obtained in a similar fashion, but the concentration of 3-PGA was kept constant at 10 mM and the concentration of [^{14}C]Glc-1-P was varied.

Synthesis Direction in Plant Extracts

ADP-Glc PPase activity in plant extracts was performed estimating the amount of ADP-Glc formed by anionic HPLC. Leaves were quickly frozen in liquid nitrogen, homogenized, and kept at −80°C until use. Leaf material (0.2 g) was ground in a mortar at 4°C and resuspended in 1 mL of extraction buffer containing 100 mM MOPS, pH 7.5, 10 mg/mL leupeptin, and 100 mM phenyl methyl sulphonyl fluoride. Crude extracts were centrifuged for 10 min at 12,000 rpm at 4°C, and the supernatant filtered through a Sephadex G-25 column equilibrated with 100 mM MOPS, pH 7.5, and immediately assayed for ADP-Glc PPase activity. The reaction mixture consisted of 100 mM MOPS, pH 7.5; 10 mM 3-PGA, 7 mM $MgCl_2$, 1.5 mM ATP, 0.5 mM Glc-1-P, 2 mM DTT, 0.5 units/mL pyrophosphatase, and 100 μL of crude extract in a final volume of 1 mL. The assays were initiated by Glc-1-P addition, stopped after 10 min by boiling the samples during 4 min, and centrifuged for 10 min at 12,000 rpm. The soluble fractions were filtered through nylon mesh and kept at −20°C. ADP-Glc produced in the reaction was estimated at 254 nm after chromatographic separation by anionic HPLC. Then 20 μL of sample was applied onto a Partisil 10 SAX analytical column (Whatman) and eluted for 20 min at 1 mL/min solvent flow with a linear gradient of 88% buffer A (10 mM $NH_4H_2PO_4$, pH 3.0):12% buffer B (450 mM $NH_4H_2PO_4$, pH 4.3) to 0% buffer A:100% buffer B.

One unit of enzyme activity in the pyrophosphorolysis and synthesis direction assays was defined as 1 μmol/min of product formed.

Kinetic Characterization

$A_{0.5}$ values, which correspond to the concentration of activator giving 50% of the maximal velocity, 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 kinetic parameters are the mean of at least two independent determinations.

Starch Measurement

Leaf starch was quantified as described previously (Lin et al., 1988a). Starch was converted to Glc by incubation with amyloglucosidase (from *Aspergillus niger*; Sigma), and the amount of Glc was analyzed enzymatically using hexokinase and Glc-6-P dehydrogenase (Jones et al., 1977).

Iodine Staining of Starch

Rosette leaves from plants grown in soil for 3 weeks were excised from the plants, incubated in 100% ethanol until the chlorophyll was eliminated, and rinsed with distilled water. The leaves were stained with lugol for 10 min, rinsed with water, and photographed immediately with a digital camera (JVC GC-X3E). Roots were dissected from seedlings grown for 1 week in Murashige and Skoog agar plates, washed with distilled water, stained with lugol for 10 min, rinsed with water, and visualized in a microscope (Olympus BX60) at 40× magnification. Images were captured with a color video camera (model JVC TK-C1381) using the MicroImage image analysis software (Olympus).

Extraction, Gel Analysis of DNA and RNA, and cDNA Synthesis

DNA was extracted from leaves using E.Z.N.A. Plant DNA Miniprep kit (Omega) and digested with *Eco*RI. Southern hybridizations were performed as described previously (Sommer et al., 1985) using an *APS1*-specific probe (438 bp) obtained by PCR using oligonucleotides SA340 and SA341 to amplify leaf cDNA.

Total RNA was isolated according to Prescott and Martin (1987) and was analyzed by electrophoresis through formaldehyde gels and transferred to Gene Screen Plus filters (New England Nuclear). Filter hybridizations were performed according to the manufacturer's instructions using the above-indicated probe.

cDNA was synthesized from total RNA. Prior to cDNA synthesis and 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 total RNA using Moloney murine leukemia virus retrotranscriptase and oligo(dT)$_{12\text{-}18}$ primer according to the manufacturer's instructions. The reaction was incubated at 37°C during 2 h and stopped by adding 1 mL of nuclease-free MilliQ-water. All the reagents were from Amersham Pharmacia Biotech.

Real-Time PCR Analysis

Real-time quantitative PCR assays were achieved using an iCycler instrument (Bio-Rad). The PCR reaction mixture contained (in a total volume of 25 μL): 5 μL of cDNA, 0.2 mM dNTPs, 0.125 μL of Exiqon Universal Probe Library (Roche), 1.25 μL of 50 mM $MgCl_2$, 2.5 μL of Ecotaq buffer 10× [670 mM Tris-HCl, pH 8.8, 166 mM $(NH_4)_2SO_4$, and 0.1% Tween 20], 0.3 units of EcoTaq polymerase (Ecogen), and 0.2 μM of each primer. Specific oligonucleotides and Exiqon probes used were: SA594 (5′-ACGCTAATCTTGGAATCACCA-3′), SA595 (5′-CGGAGCAGAACGGTCATAG-3′), and probe 154 for *APS1*; and SA532 (5′-GAAGTTCAATGTTTCGTTTCATGT-3′), SA533 (5′-GGATTATA-CAAGGCCCCAAAA-3′), and probe 119 for ubiquitin. Thermal cycling consisted of 94°C for 3 min, followed by 60 cycles of 20 s at 94°C and 1 min at 60°C. The efficiency of all the primers at the above conditions was between 90% and 110% in all the tested samples. Arabidopsis *UBIQUITIN10* (Sun and Callis, 1997) was used as a housekeeping gene control in the expression analysis. Absolute quantification (Ginzinger, 2002) was performed by cloning the amplified products in pGEM-T Easy vector (Promega) and using them as external calibration standards.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative data library under accession numbers At5g48300 (*APS1*), At1g05610 (*APS2*), At5g19220 (*APL1*), At1g27680 (*APL2*), At4g39210 (*APL3*), At2g21590 (*APL4*), and SALK_040155 (*aps1*). ADP-Glc PPase proteins and their accession numbers are as follows: *E. coli*, P00584; APS1, P55228; APL1, P55229; APL2, P55230; APL3, P55231; APL4, Q9SIK1; PSS, P23509; and PLS, Q00081.

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