

Characterization of *ADG1*, an *Arabidopsis* locus encoding for ADPG pyrophosphorylase small subunit, demonstrates that the presence of the small subunit is required for large subunit stability

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Summary

Two mutants of *Arabidopsis* have been isolated that affect ADPG pyrophosphorylase (ADGase) activity. Previously, it has been shown that *ADG2* encodes the large subunit of ADGase. This study characterizes the *adg1* mutant phenotype and *ADG1* gene structure. RNA blot analyses indicate that the *adg1-1* mutant accumulates transcripts encoding both the large and small subunits of ADGase, while the *adg1-2* mutant accumulates only large subunit transcripts. RFLP analysis and complementation of *adg1* mutants with the ADGase small subunit gene demonstrate that *ADG1* encodes the small subunit. Sequence analysis indicates that *adg1-1* represents a missense mutation within the gene. Western blot analysis confirms that *adg1* mutants contain neither the large nor the small subunit proteins, suggesting that the presence of functional small subunits is required for large subunit stability.

Introduction

ADP-glucose pyrophosphorylase (ATP:α-glucose-1-phosphate adenyl transferase, EC 2.7.7.27; ADGase) is a key regulatory enzyme in the starch biosynthetic pathway of plants (for review, see Preiss, 1991). Within chloroplasts and amyloplasts, the enzyme catalyzes the synthesis of ADP-glucose and pyrophosphate from glucose-1-phosphate and ATP. ADP-glucose functions as the glucosyl donor for α-glucan synthesis by various starch synthases (Preiss, 1991). ADGase from almost all higher plant sources is activated by 3-phosphoglyceric acid (3PGA) and inhibited by orthophosphate (Plaxton and Preiss, 1987).

ADGase from all sources is found to be a tetrameric

structure (Smith-White and Preiss, 1992) with a size ranging from about 200–240 kDa (Copeland and Preiss, 1981; Preiss *et al.*, 1987). However, the bacterial enzyme is homo-tetrameric in structure (Haugen *et al.*, 1976), whereas the plant enzyme comprises two subunits (Morell *et al.*, 1987; Okita *et al.*, 1990; Preiss, 1991). Immunological studies of *Arabidopsis* (Lin *et al.*, 1988a, b), wheat, rice and maize (Krishnan *et al.*, 1986; Preiss *et al.*, 1990), *Chlamydomonas* (Iglesias *et al.*, 1994) and pea (Hylton and Smith, 1992) demonstrated that the two subunits differed in size. It was suggested that the native ADGase of plants formed as heterotetramer with two large and two small subunits (Preiss, 1988). Expression of both potato ADGase subunits in the same *Escherichia coli* cell leads to assembly *in vivo* into active homo-tetramers, while the large subunit expressed on its own leads to very little enzyme activity (Iglesias *et al.*, 1993). However, the detailed assembly process of ADGase has not been well characterized.

Starch accumulation is severely affected in plants that are deficient in ADGase activity. This has been shown in *Arabidopsis* leaves (Lin *et al.*, 1988a, b), maize endosperm (Weaver *et al.*, 1972), pea cotyledons (Smith *et al.*, 1989), *Chlamydomonas* (Van den Koornhuyse *et al.*, 1996), and in tubers of potato plants expressing antisense constructs of an ADGase gene (Muller-Rober *et al.*, 1992). The *Arabidopsis adg2* mutant has only ~5% ADGase activity but accumulates ~40% of wild-type starch levels (Lin *et al.*, 1988b). Western blot analysis has shown that the *adg2* mutant is deficient in the large subunit protein (Lin *et al.*, 1988b), and this suggests that the functional ADGase that is present in the *adg2* mutant is a homo-tetramer of small subunits. This suggestion was confirmed by Li and Preiss (1992). By molecular analysis and complementation of the mutant with the wild-type large subunit gene, we have shown that *ADG2* encodes the ADGase large subunit (Wang *et al.*, 1997). DNA sequence analysis indicated that *adg2-1* is a missense mutation (glycine 118 changed to glutamic acid, G118E) in a domain (PAVPXG) proposed to be involved in the interaction of ADGase small and large subunits. This change is thought to be important both for allosteric regulation and for enzyme assembly (Wang *et al.*, 1997).

The *adg1-1* mutant is a monogenic recessive mutation that has no measurable ADGase activity in leaf extracts (Lin *et al.*, 1988a). The absence of starch in these mutants demonstrates that starch synthesis in the chloroplast is entirely dependent on a pathway involving ADGase.

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Western blot analysis indicated that both the large and small subunits of ADGase are absent in the *adg1-1* mutant but the specific activity of ADGase in *adg1-1/ADG1* heterozygous plants is indistinguishable from wild-type (Lin *et al.*, 1988a). Based on these results, it was hypothesized that *adg1* is a regulatory mutant affecting both the ADGase small and large subunit gene expression (Lin *et al.*, 1988a).

Here we show that *adg1* is not a regulatory mutation. Rather, it is a mutation in the structural gene encoding the ADGase small subunit. The absence of large subunit protein in *adg1* mutants suggests that stability of the large subunit protein is dependent on the presence of the small subunit.

Results

Isolation and characterization of *Arabidopsis* starch metabolic mutants

We have isolated *Arabidopsis* mutants affecting starch metabolism (Eimert *et al.*, 1995). Two of them were isolated on the basis that leaves grown under continuous light did not stain blue with iodine. We measured the starch content in these two mutants (ke122 and ke478 of RLD ecotype) and confirmed that the level of starch accumulation in mutant leaves (0.3 mg starch per g leaf tissue) is significantly lower than that of the wild-type (5 mg starch per g leaf tissue).

So far, studies of *Arabidopsis* starch-deficient mutants have identified two types of defect. Biochemical analysis showed that one class of mutant is deficient in chloroplastic phosphoglucomutase (PGM) activity, e.g. *pgm1-1* mutant (Caspar *et al.*, 1985), and the other class perturbs ADGase activity, e.g. *adg1* and *adg2* mutants. The *adg1* mutant has no ADGase activity (Lin *et al.*, 1988a), and the *adg2* mutant has decreased ADGase activity (Lin *et al.*, 1988b). We analyzed the PGM and ADGase activity of ke122 and ke478 mutants. As shown in Figure 1, ke478 has the same level of ADGase activity as the wild-type but, similar to *pgm1-1*, has lost the chloroplastic PGM activity. In contrast, the ke122 mutant has normal levels of PGM activity but has lost the ADGase activity (Figure 1). In order to examine the basis for the deficiency of ADGase activity, the presence of ADGase proteins in leaf extracts was detected by Western blot analysis. We found that both the small and large subunit proteins were present in the wild-type and the *pgm* mutants (Figure 2). In the mutants carrying either *adg1-1* or *adg1-2* allele, both the small subunit (Figure 2a, lanes 3 and 4) and large subunit proteins (Figure 2b, lanes 3 and 4) were below the detectable level. The amount of small subunit protein (Figure 2a, lane 5) and large subunit protein (Figure 2b, lane 5) in the *adg2* mutant was significantly reduced compared with that of the wild-type. Cross-reactive bands were detected by the antibodies. Because these proteins were not affected by the mutations,

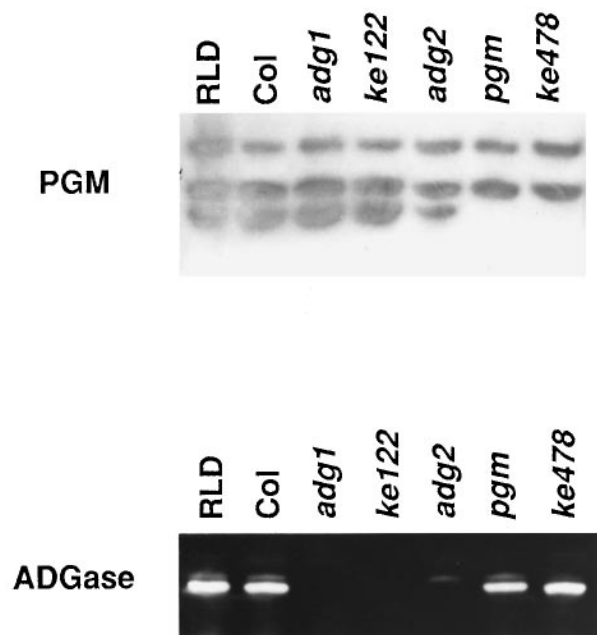


Figure 1. Activity gel assay for phosphoglucomutase and ADPG pyrophosphorylase in wild-type and mutant leaf extracts. Leaf extracts (20 µl) from the wild-type (RLD ecotype and *Columbia* (Col) ecotype), *adg1-1* (*adg1*), *adg1-2* (ke122), *adg2*, *pgm1-1* (*pgm*), and *pgm1-2* (ke478) were separated in a 7% native polyacrylamide gel and assayed for phosphoglucomutase (PGM) and ADPG pyrophosphorylase (ADGase) activity.

they may be related proteins that share antigenic sites with the ADGase proteins or *E. coli*-derived proteins.

Notably, two bands were detected in the ADGase activity assay in both the wild-type and *pgm1* mutants (Figure 1). Only the upper band was found in the *adg2-1* mutant. Western blot analysis demonstrated that the main activity (lower) band of the wild-type contains both the small and large subunits whereas the minor (upper) band seen in *adg2-1* and wild-type samples contains only the small subunits. The amount of protein detected by Western blot analysis is roughly correlated to enzyme activity assayed *in vitro*, and as such this result is consistent with the fact that ADGase in *adg2-1* is a homo-tetramer of small subunits (Li and Preiss, 1992).

The F_1 progeny of test crosses between ke478 and *pgm1-1* have no chloroplast PGM activity, indicating that ke478 is allelic to *pgm1-1*. ke478 was therefore designated *pgm1-2*. Test crosses of ke122 and *adg1-1* yielded F_1 progeny plant with no starch and no ADGase activity, indicating that ke122 is allelic to *adg1-1*. ke122 was therefore designated *adg1-2*.

The *adg1-1* mutant is a monogenic recessive mutation that has no ADGase activity in leaf extracts. Both the ADGase small and large subunits are absent in the *adg1-1* mutant as shown by Western blot analysis (Lin *et al.*, 1988a). Similarly, we have found that both subunits are absent in *adg1-2* mutants. Previous reports have suggested

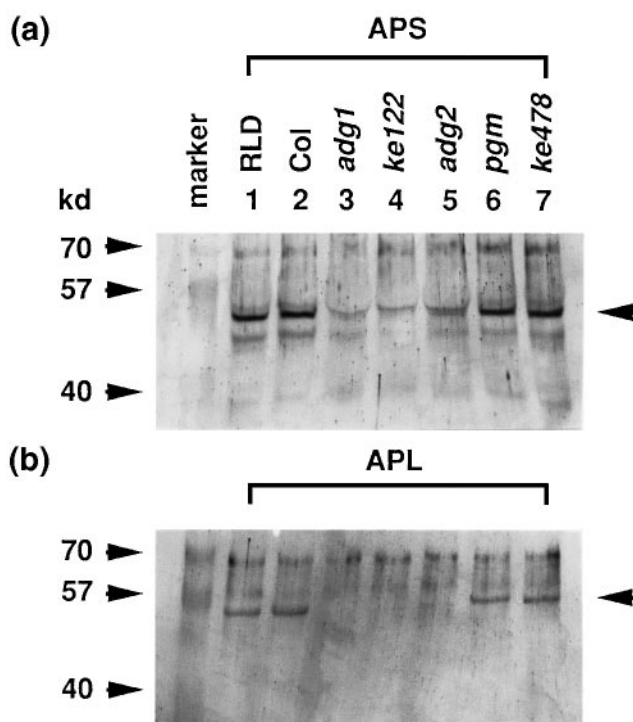


Figure 2. Western blot analysis for the ADGase proteins in the wild-type and mutant leaf extracts.

(a) Leaf extracts containing 20 µg proteins from the wild-types (RLD ecotype and *Columbia* (Col) ecotype), *pgm1-1* (*pgm*) and *pgm1-2* (*ke478*) and 40 µg proteins from *adg1-1* (*adg1*), *adg1-2* (*ke122*) and *adg2* were separated by 10% SDS-polyacrylamide gel and probed with the antiserum against ADGase small subunit protein (APS).

(b) Leaf extracts containing 20 µg proteins for all the samples were probed with the antiserum against the ADGase large subunit protein (APL).

The predicted size of ADGase small subunit protein is 49 kDa and the size of the large subunit protein is 52 kDa. Bands of expected size were detected as indicated by the arrows. Cross-reactive bands were detected by the antibodies. They may be related proteins sharing antigenic sites with the ADGase proteins or *E. coli*-derived proteins.

that this finding demonstrates that *ADG1* is a regulatory gene affecting small and large subunit gene expression (Lin *et al.*, 1988a).

Characterization of cDNA and genomic clones of the ADGase small subunit

In order to characterize expression patterns of ADGase small and large subunit transcripts in *adg* mutants, we isolated and characterized cDNA and genomic clones of *Arabidopsis* ADGase small subunit. Previously, four cDNA clones of ADGase, including one small subunit (APS) and three large subunits (APL1, APL2 and APL3), were isolated by PCR with two primers corresponding to conserved domains (Villand *et al.*, 1993). Using APS and APL1 as probes, two cDNA clones corresponding to the small (APS1A) and one of the large subunits (APL1A) were isolated from a cDNA library prepared with leaf poly(A)+ RNA (*Columbia* ecotype, Villand *et al.*, 1993). Since mRNA

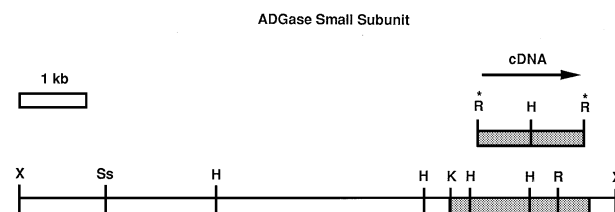


Figure 3. Restriction maps of *Arabidopsis* small subunit cDNA and genomic clones.

The transcription orientation of the small subunit of ADGase is indicated with an arrow: X (*Xba*I), Ss (*Sst*I), H (*Hind*III), K (*Kpn*I), R (*Eco*RI). Sites labeled with an asterisk are derived from the vector; shaded boxes represent transcribed regions.

used for the cDNA library construction was isolated from leaves, both large and small subunit transcripts must be expressed in leaf tissue. Sequence analysis showed that the insert of the small subunit cDNA clone (APS1A) contains 1470 bp of coding region and 202 bp of 3' untranslated region. To clone the complete sequence of the small subunit coding region, we sequenced the small subunit genomic clone (see below). Using the sequence of the 5' genomic region, primers were designed for RT-PCR and full-length cDNA clones (*Columbia* ecotype) were isolated. cDNA sequence showed that the ADGase small subunit is composed of 520 amino acids (GenBank accession number U72351). Genomic clones of the ADGase small subunit were isolated by screening a genomic library (*Landsberg erecta* ecotype) with the cDNA probe. Restriction enzyme mapping and sequence analysis indicated that the genomic clone contains the complete small subunit transcription unit plus 6.4 kb of 5' upstream sequence and 0.4 kb of 3' downstream sequence. The restriction enzyme map and transcription orientation are shown in Figure 3.

Northern blot analysis of ADGase expression

We examined the expression pattern of ADGase transcripts in leaves of wild-type, *pgm* mutants and the ADGase mutants *adg1-1*, *adg1-2* and *adg2-1*. Total RNA was isolated from the leaves of plants grown in continuous light and hybridized with the small and large subunit cDNAs of ADGase. As shown in Figure 4, the ADGase large subunit transcript was present in all samples. ADGase small subunit transcripts could be detected in the wild-type, *adg1-1* and *adg2-1* samples, but not in the *adg1-2* mutant sample. The absence of small subunit transcripts in *adg1-2* leads us to suggest that *adg1* mutants may represent a structural mutation in the small subunit gene as opposed to the previously proposed regulatory mutation (Lin *et al.*, 1988a).

RFLP and DNA sequence analyses of the *adg1* mutant

We mapped the ADGase small subunit gene using recombinant inbred lines and the APS cDNA as probe. The

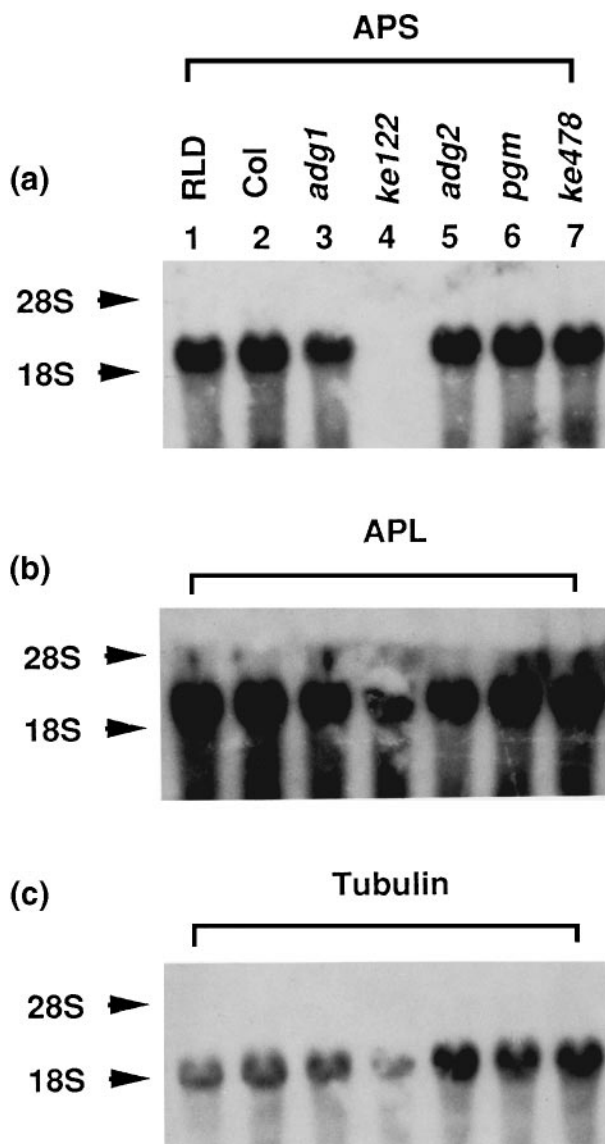


Figure 4. Northern blot analysis of small subunit and large subunit transcripts of ADGase.

(a) Northern blot of total leaf RNA (20 µg) isolated from the wild-type (RLD) ecotype and *Columbia* (Col) ecotype, *adg1-1* (*adg1*), *adg1-2* (*ke122*), *adg2*, *pgm1-1* (*pgm*) and *pgm1-2* (*ke478*) plants probed with radioactively labeled small subunit cDNA (APS).

(b) The same blot as shown in (a) hybridized with the large subunit cDNA probe (APL) after recycling the membrane.

(c) The same blot as shown in (a) reprobed with a tubulin cDNA probe.

mapping data indicated that the ADGase small subunit gene is located on chromosome 5 at 69.1 cM. If *adg1* is an ADGase small subunit gene mutation, we should be able to show linkage of *adg1* to the small subunit cDNA probe. To test this hypothesis, we examined RFLPs in selfed F_2 progenies of *adg1* (Col) \times *ADG1* (*Ler*). As shown in Figure 5, we found that in a population of 60 plants that failed to accumulate starch, the APS Col band co-segregated with

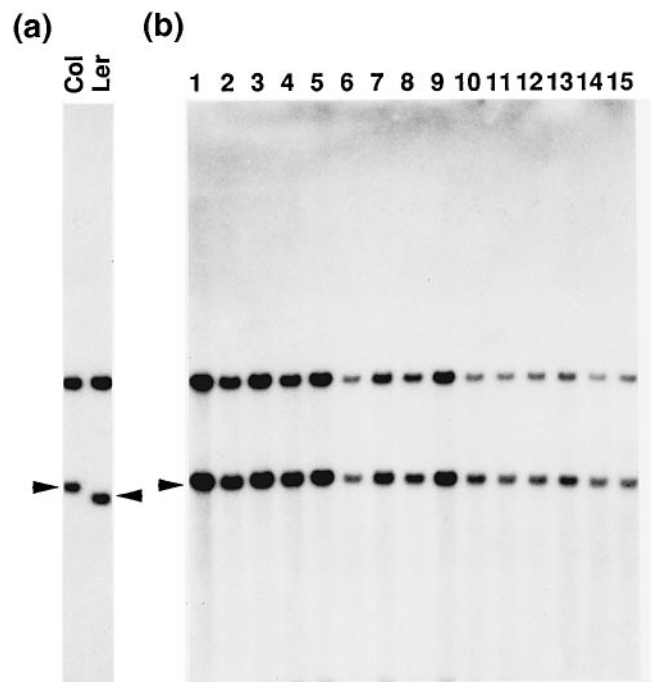


Figure 5. RFLP analysis of F_2 plants of *adg1-1* (Col) \times *ADG1* (*Ler*).

(a) Genomic DNAs (2 µg) isolated from wild-type *Columbia* (Col) and *Landsberg erecta* (*Ler*) ecotypes were digested with *Bcl*I, Southern blotted and probed with labeled APS cDNA. A polymorphism was detected.

(b) Southern blot analysis of genomic DNA isolated from the F_2 progenies of *adg1-1* (Col) \times *ADG1* (*Ler*). DNA samples derived from plants that failed to accumulate starch (lanes 1–14) and from wild-type (*Col*) plants (lane 15) were digested with *Bcl*I, Southern blotted and probed with the APS cDNA.

Potato	1	MAASIGALKS	SPSSNNCINE	RRNDSTRAVS	SRNLSFSSSH	LAGDKIMFVS	50
Spinach		
Arabidopsis		.MASVSAIGV	LKVPPASTSN	STGKATEAVP	TRILSFSSSV	TSSDDKISLK	
Potato	51	SLRSQGVREN	VRRSPMIVSP	KAVSQSNSQ	TCLDPDASRS	VLGIILGGGA	100
Spinach		
Arabidopsis		STVSRICKSV	VRRNPIIVSP	KAVSQSNSQ	TCLDPDASSS	VLGIILGGGA	
<i>adg1-1</i>						R	
Potato	101	GTRLYPLTKK	RAKPAVELGA	NYRLIDIPVS	NCLNSNISKI	YVLTQFNSSAS	150
Spinach		GTRLYPLTKK	RAKPAVELGA	NYRLIDIPVS	NCLNSNISKI	YVLTQFNSSAS	
Arabidopsis		GTRLYPLTKK	RAKPAVELGA	NYRLIDIPVS	NCLNSNISKI	YVLTQFNSSAS	

Figure 6. Protein sequence alignment around the missense mutation site of the *adg1-1* allele.

The N-terminal portions of ADGase small subunit sequences of potato, spinach and *Arabidopsis* were aligned. The sequence of spinach ADGase was determined by protein sequencing and cDNA sequencing. The missense mutation of *adg1-1* allele causes the amino acid glycine (92) change to arginine. The PAVPXG domain is shown in bold.

the *adg1-1* phenotype. This result suggested that *adg1-1* is a mutation in the ADGase small subunit gene.

We confirmed this suggestion by cloning and sequencing both *adg1-1* and *ADG1* (wild-type) alleles. Sequence comparison indicated that, in *adg1-1*, there is a single nucleotide change from G (nucleotide 309) to A causing a missense mutation (glycine 92 mutated to arginine, G92R) in the ADGase small subunit gene. As shown in Figure 6, comparison with the N-terminal peptide sequence of mature spinach leaf ADGase small subunit (Morell *et al.*, 1987),

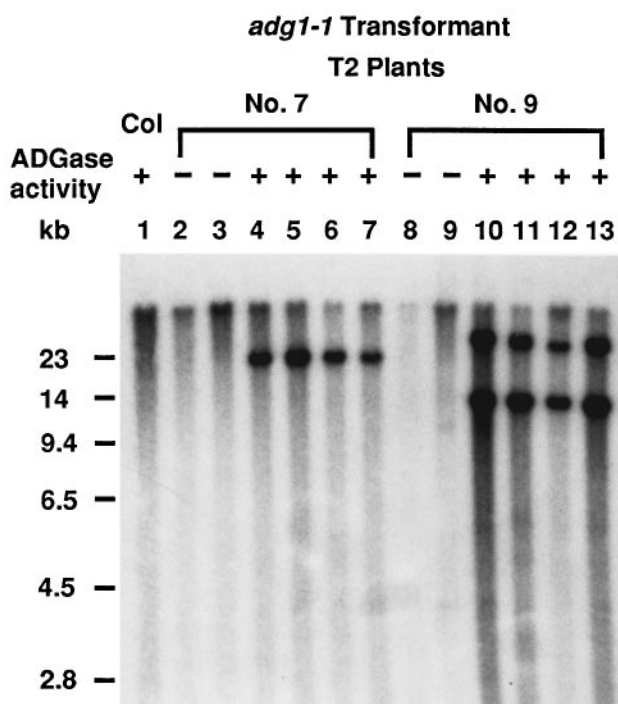


Figure 7. Southern blot analysis of two transgenic lines of *adg1-1* carrying the transformed wild-type APS gene.

T₂ progenies of two representative transgenic lines (No. 7 and No. 9) that co-segregated for kanamycin resistance, wild-type level leaf starch, and wild-type level leaf ADGase activity (labeled as +, lanes 4–7 and 10–13) and kanamycin sensitivity, no leaf starch and no leaf ADGase activity (labeled as –, lanes 2, 3, 8 and 9) were analyzed. Genomic DNA was isolated from leaves of wild-type (*Columbia* ecotype, lane 1) and transgenic plants, digested with *Kpn*I, Southern blotted, and probed with the APS cDNA. The results showed that transgenic plants with a wild-type phenotype carried the transformed wild-type APS gene.

indicated that this missense mutation is at position 21 of the mature protein.

Complementation of *adg1* mutants with the wild-type ADGase small subunit gene

To further prove that *adg1* is an ADGase small subunit mutation, we transformed the *adg1* mutants with the wild-type ADGase small subunit gene. The wild-type APS gene (a 7.6 kb *Sst*I–*Xba*I fragment) was inserted into pBin19 and transformed into *Agrobacterium*. *adg1-1* and *adg1-2* plants were transformed by vacuum infiltration and independent kanamycin-resistant transformants (15 for *adg1-1* and four for *adg1-2*) were selected from the T₁ seeds. The resulting transformants reversed the starch-deficient phenotype of *adg1* to wild-type. The kanamycin-resistant T₁ plants were selfed and T₂ plants were scored for their ADGase activity. As shown in Figure 7, T₂ transformants with wild-type level of ADGase activity and leaf starch contain the transformed small subunit gene. This result confirms that the *adg1* mutant phenotype is caused by a mutation in the small subunit gene.

Discussion

Both the *adg1-1* and *adg1-2* mutants have no detectable ADGase activity (Figure 1). By RFLP analysis and complementation of the mutant phenotype with the wild-type ADGase small subunit gene, we have shown that *ADG1* encodes the ADGase small subunit. The *adg1-2* mutant has no detectable ADGase small subunit RNA, thus no detectable small subunit protein, suggesting that this mutation perturbs gene expression at the transcriptional level, either affecting the promoter activity or mRNA stability. The presence of ADGase large subunit RNA in the *adg1-2* mutant which has no detectable small subunit RNA indicated that the transcription of ADGase large subunit gene is independent of that of the small subunit gene. By Northern blot analysis and ADGase activity gel assays, we showed that *pgm1-1* and *pgm1-2* mutants accumulate ADGase small and large subunit transcripts (Figure 4) and exhibit ADGase activity (Figure 1) similar to that of the wild-type. In the *pgm* mutants, both the chloroplastic phosphoglucomutase activity and leaf starch accumulation are defective, suggesting a deficiency of glucose 1-P necessary for starch synthesis in the chloroplast. Our results suggest that the expression of ADGase genes is not modulated by the glucose-1-P concentration.

We have previously shown that *ADG2* encodes the large subunit of ADGase. Complementation analysis suggests that the ADGase large subunit mRNA that accumulates in leaves is primarily transcribed from the *ADG2* gene (Wang *et al.*, 1997). The expression profiles and functions of the other two copies of the ADGase large subunit genes remain to be determined. Molecular analysis of the *adg2-1* allele indicated that it is a missense mutation (G118E) adjacent to a conserved domain (PAV,113–115) involved in the allosteric property of the holoenzyme (Greene *et al.*, 1996). From biochemical/genetic analyses of potato ADGase mutants, Greene *et al.* (1996) proposed that the PAV allosteric domain of the hetero-tetramer enzyme is formed by interaction of both ADGase small and large subunits. Western blot analysis of ADGase in the *adg2-1* mutant indicated that it is composed of a homo-tetramer of small subunits (Li and Preiss, 1992), suggesting that the mutated large subunit protein is not assembled into holoenzyme and is not stably maintained in chloroplasts. Thus, the domain PAVPXG (113–118) is thought to be important both for allosteric regulation and for enzyme assembly (Wang *et al.*, 1997).

Sequence analysis indicated that the *adg1-1* mutant contains a missense mutation (G92R) located 21 amino acids upstream of the PAVPXG domain. Although the N-terminal sequence of the mature small subunit protein of *Arabidopsis* ADGase has not yet been determined, protein sequence comparisons with the spinach small subunit mature protein (Figure 6) indicated that the first 20 amino acids differ only by two amino acids (Morell *et al.*, 1987).

This similarity suggests that the missense mutation of *adg1-1* is not within the transit peptide and probably would not affect the transport of nascent protein into chloroplasts. It is likely that the missense mutation may either directly affect the stability of the protein in the chloroplast or may affect the assembly process and then indirectly affect the stability of unassembled proteins. If the latter proposal is true, the mutation may prevent the small subunit from being folded in a proper conformation for subsequent assembly into an oligomeric structure with the large subunits. Alternatively, the N-terminal portion of the small subunit may be a part of the domain involved in the interactions of the subunits. The missense mutation G92R may interfere with the interactions between subunits. Since the N-terminal domain is highly conserved among all known ADGase small subunits (Smith-White and Preiss, 1992), this suggestion has credence.

Western blot analysis indicated that both the small and large subunits of ADGase are absent in *adg1-1* (Lin *et al.*, 1988a) and *adg1-2* mutants (Figure 2). These monogenic recessive *adg1* mutants are mutants of the ADGase small subunit and have wild-type large subunit genes. Thus the large subunit genes of *adg1* mutants would be transcribed (Figure 4) and translated normally in mutant plants. These results suggest that the presence of functional small subunits is required for the stability and/or assembly of large subunits and furthermore that large subunit proteins cannot assemble into a functional homo-tetramer in the *adg1-1* and *adg1-2* mutants.

In the *adg2* mutant, we observed a reduced amount of both small subunit and large subunit proteins (Figure 2). The presence of small amounts of large subunit proteins that was not observed by Lin *et al.* (1988b) may reflect the experimental variations of plants affecting the stability of mutated large subunit protein. However, this result did support the suggestion that the stability of small subunit protein in the chloroplast is also affected by the amount of large subunit protein. A similar effect was observed in maize in which the rate of SH2 or BT2 protein turnover is dependent on the presence of BT2 or SH2 protein, respectively (Giroux *et al.*, 1994). It has been suggested that SH2 and BT2 proteins are more susceptible to degradation when the formation of a SH2/BT2 polymer does not occur (Giroux *et al.*, 1994). Similar to *Arabidopsis*, two groups of maize mutants that affect ADGase activity have been isolated (*bt2* and *sh2*). *Bt2* encodes the small subunit protein and *SH2* encodes the large subunit protein (Preiss *et al.*, 1990). Both genes are expressed specifically in the endosperm. Whereas *Arabidopsis adg1* mutants have no detectable small nor large subunits, large subunit protein is detected in maize *bt2* mutants in the absence of small subunit proteins (Giroux and Hannah, 1994). It has been suggested that there are other genes expressed in maize endosperm that are homologous to *SH2/BT2*, i.e. *AGP1*

and *AGP2* (Giroux and Hannah, 1994). Biochemical analysis indicated that the *bt2* mutation specifically eliminates the extra-plastidial ADGase activity (Denyer *et al.*, 1996), suggesting that the plastidial small subunit is not encoded by *BT2* (Denyer *et al.*, 1996). It seems likely that the plastidial small subunit is encoded by *AGP2*. It is reasonable to suppose that the ADGase proteins detected in the *bt2* mutants correspond to the *AGP1/AGP2* gene products. Alternatively, large subunit proteins of maize may be able to interact with each other and become stabilized. Differences in the proteolytic activity of endosperm and leaves could also explain why the large subunit protein can accumulate even in the absence of small subunit proteins in *bt2* mutants. The underlying mechanism for the presence of ADGase large subunit in maize *bt2* mutant requires further analysis.

Existing data from studies of *Arabidopsis* ADGase showed that wild-type ADGase is mainly composed of two small and two large subunits (Li and Preiss, 1992). In the absence of the large subunit, small subunit proteins can assemble into a homo-tetrameric quaternary structure (Li and Preiss, 1992) but in the absence of small subunit proteins, large subunit proteins become unstable in the chloroplast (Lin *et al.*, 1988a; Wang *et al.*, 1997). Based on these data, we propose that the ADGase assembly process may be initiated by homodimer formation of small subunits. The small subunit homodimer then interacts further with large subunit or small subunit proteins. In the absence of the large subunit, however, the homodimers of small subunits would assemble into a homo-tetramer form of ADGase as observed in the *adg2-1* mutant. However, this homo-tetramer form of ADGase, has a different allosteric regulatory response to the effectors, in as much as it requires a higher concentration of 3PGA for maximal activation and is more sensitive to inhibition by P_i than the wild-type hetero-tetramer form (Li and Preiss, 1992). Future analysis of the ADGase structure would elucidate the interactions among subunits and its catalytic and regulatory domains.

Experimental procedures

Plant materials and growth conditions

The M_2 seeds of ethyl methanesulfonate-treated seeds of *Arabidopsis thaliana* (RLD ecotype) were obtained from Lehle Seeds (Round Rock, TX). *adg1-1*, *adg2-1* and recombinant inbred lines (Lister and Dean, 1993) were obtained from the *Arabidopsis* Biological Resource Center at Ohio State University (ABRC, Columbus, OH) and Nottingham *Arabidopsis* Stock Center (NASC, Nottingham, UK). Plants were grown in soil at 23°C under ca. 100 $\mu\text{mol quanta m}^{-2} \text{sec}^{-1}$ continuous fluorescent light.

Mutant screening

Mutant screening was carried out as described by Caspar *et al.* (1985). Plants were screened after they developed 4–6 true leaves.

Leaf pieces were cut out, de-pigmented in ethanol or methanol and stained with I_2 -KI (5.7 mM iodine and 43.4 mM potassium iodide in 0.2 N HCl). Quantitative starch assays were conducted according to Eimert *et al.* (1995).

Native gel assay

The ADGase (Lin *et al.*, 1988a) and PGM (Caspar *et al.*, 1985) gel assays were carried out according to previously described methods using 7% polyacrylamide gels. Leaf samples were extracted with extraction buffer (100 mM Tris-HCl pH 7.0, 40 mM β -mercaptoethanol, 10 mM $MgCl_2$, 100 mM KCl, 15% glycerol, 2 ml per g⁻¹ fresh tissue). The crude extract was centrifuged at 14 000×g for 5 min and the activity present in the supernatant fraction was assayed. Quantitative assay of ADPG pyrophosphorylase activity was conducted as described by Sowokinos (1976) in the presence of 2 mM 3-phosphoglyceric acid (3PGA).

General molecular analysis

Standard cloning, Southern blot and Northern blot techniques were used as described by Sambrook *et al.* (1989). DNA sequencing was performed with double-stranded plasmids, using Sequenase (United States Biochemical).

Isolation of genomic and cDNA clones

Arabidopsis ADGase small subunit cDNA clone ALPC10 (APS1A) and large subunit cDNA clone ALPA4 (APL1A), both containing 1.7 kb inserts in pBluescript vector (Stratagene), were isolated from a leaf cDNA library by screening with APS and APL1 probes, respectively (Villand *et al.*, 1993). APS, APL1, APS1A and APL1A plasmids were kindly supplied by Dr Villand. To isolate the corresponding genomic clones of ADGase small subunit, a genomic library of *Arabidopsis* *Ler* ecotype (constructed as partially digested *MobI* genomic DNA fragments cloned into the *XhoI* site of λ Fix vector, supplied by ABRC) was screened with the APS1A cDNA insert. A 9 kb *XbaI* fragment of the small subunit genomic λ clone (APS3B) was further subcloned into pBluescript SK+.

Western blot analysis

Leaf extracts were electrophoresed by 10% SDS-polyacrylamide gels and electroblotted to nitrocellulose membranes. Rabbit antiserum was prepared against the *E. coli*-expressed ADGase small subunit and large subunit proteins and used as probes for the Western blot analyses. The primary antibody was detected with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The ADGase small subunit protein (Ala340-Ile520) was isolated from an *E. coli* strain carrying a plasmid with the *HindIII*-*XhoI* 0.7 kb fragment of the small subunit cDNA clone (APS1A) inserted into pET30A (Novagen). The ADGase large subunit protein (Glu37-Ile522) was prepared from an *E. coli* strain carrying a plasmid with an *SstI*-*XhoI* 1.6 kb fragment of the large subunit cDNA clone (APL1A) inserted into pET30A.

RFLP mapping

RFLP mapping was performed according to Chang *et al.* (1988) and Nam *et al.* (1989) and the data were analyzed using the JoinMap computer program (Stam, 1993).

Complementation of adg1 mutants with wild-type genes

A 7.6 kb *XbaI*-*SstI* fragment of the ADGase small subunit genomic clone was cloned into *XbaI*-*SstI* sites of pBin19 and denoted pBin19-APS. The resulting T-DNA binary constructs were transformed into the *Agrobacterium tumefaciens* strain pGV2260/C58 rif. *adg1-1* and *adg1-2* plants were transformed with pBin19-APS by vacuum infiltration (Bechtold *et al.*, 1993). Kanamycin-resistant transformants were selected by germinating seeds on selective medium. The resistant plants were transferred to soil and self-pollinated. Seeds were harvested for subsequent genetic, biochemical and molecular analyses.

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GenBank accession number U72351 (ADPG pyrophosphorylase small subunit cDNA).