

SHORT COMMUNICATION

## ***adg2-1* represents a missense mutation in the ADPG pyrophosphorylase large subunit gene of *Arabidopsis thaliana***

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### **Summary**

*Arabidopsis* mutants affecting ADPG pyrophosphorylase (ADGase) activity can be divided into two complementation groups, *adg1* and *adg2*. Previous biochemical studies of *adg2-1* mutant indicated that mutant plants do not accumulate ADGase large subunit protein and that ADGase small subunits assemble as homotetramers. This suggested that the *ADG2* gene may encode the large subunit of ADGase. In this paper, it is shown that *adg2-1* mutant plants accumulate near wild-type levels of transcripts encoding both the large and small subunits of ADGase. However, by RFLP analysis and complementation of *adg2-1* with the ADGase large subunit gene, we show that the *adg2-1* mutant does represent a mutation of the ADGase large subunit gene. Sequence analysis of the *adg2-1* allele revealed a missense mutation. The results therefore suggest either that the missense mutation affects the stability of the ADGase large subunit protein or that it prevents assembly of the large subunit into holoenzyme.

### **Introduction**

ADP-glucose pyrophosphorylase (ATP:  $\alpha$ -glucose-1-phosphate adenyl transferase, EC 2.7.7.27; ADGase) plays an important role in the synthesis of starch in both photosynthetic and non-photosynthetic plant tissues (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  $\alpha$ -glucan synthesis by various starch synthases (Preiss, 1991). Allosteric regulation of this

enzyme by metabolites such as inorganic phosphate and 3-P-glycerate in plants and 5'-adenylate and fructose-1,6-bisphosphate in bacteria plays a vital role in controlling the rate of starch synthesis (Preiss, 1988).

In bacteria and plants, the size of the native ADP-glucose pyrophosphorylase is similar, ranging from about 200 to 240 kDa (Copeland and Preiss, 1981). The *Escherichia coli* enzyme is a homotetramer with a subunit size of about 50 kDa (Haugen *et al.*, 1976). By contrast, two different polypeptides of 51 and 54 kDa copurified with the activity from spinach leaves (Copeland and Preiss, 1981; Morell *et al.*, 1987). Other species such as *Arabidopsis* (Lin *et al.*, 1988a,b), *Chlamydomonas* (Iglesias *et al.*, 1994), wheat, rice, maize (Krishnan *et al.*, 1986), and pea (Hylton and Smith, 1992) also contain immunologically related peptides of similar size. It was suggested that the native ADGase of plants was formed as heterotetramer with two large and two small subunits (Iglesias *et al.*, 1995; Smith-White and Preiss, 1992). However, the assembly process has not been well characterized.

Mutants with low ADGase activity exhibit decreased starch accumulation. Such decreases are observed in leaves of the *Arabidopsis* mutants *adg1* (Lin *et al.*, 1988a) and *adg2* (Lin *et al.*, 1988b), in kernels of the maize mutants *shrunk2* (*sh2*) and *brittle2* (*bt2*) (Weaver *et al.*, 1972), in seeds of the pea mutant *rb* (Smith *et al.*, 1989), and in tubers of potato plants expressing an antisense construct of an ADGase small subunit gene (Muller-Rober *et al.*, 1992).

*Arabidopsis* mutants affecting ADGase activity can be divided into two complementation groups, *adg1* and *adg2*. The *adg2* mutant accumulates no ADGase large subunits and as such exhibits 5% of wild-type ADGase activity and 40% of wild-type starch levels in leaves. This mutant therefore demonstrates that the 54 kDa large subunit protein is required for efficient ADGase function (Lin *et al.*, 1988b). However, since *adg2* mutant plants accumulate no ADGase large subunit protein yet have some ADGase activity, it appears that the homotetramer of small subunits is at least partially functional (Li and Preiss, 1992).

The *adg1-1* mutant is conditioned by a monogenic recessive mutation. Mutant plants accumulate less than 2% of wild-type starch levels and have no detectable ADGase activity in the leaves (Lin *et al.*, 1988a). By Western blot analysis, the *adg1-1* mutant was shown to lack both the large and small subunit proteins of ADGase. Based on

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these results, a hypothesis was proposed that the *adg1-1* is a regulatory gene affecting both large and small subunit gene expression (Lin *et al.*, 1988a).

In this paper, we show that *adg2* represents a missense mutation in the large subunit gene of ADGase. Our results suggest that the large subunit protein is not stable in the chloroplast unless it is assembled as a heterotetramer.

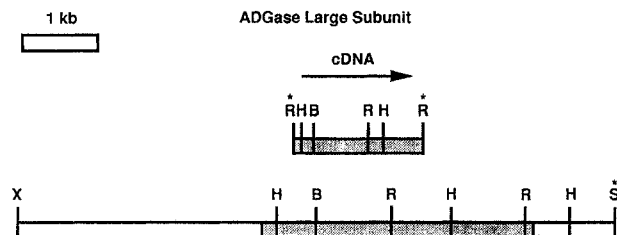
## Results

### Characterization of cDNA and genomic clones of ADGase large subunit

To examine ADGase small and large subunit gene expression in *adg2* mutant plants, we characterized previously isolated ADGase cDNA clones (Villand *et al.*, 1993) and isolated the ADGase genomic clones. 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 and RNA isolated from whole plants (Villand *et al.*, 1993). Two cDNA clones corresponding to the small (APS1A) and one of the large subunits (APL1A) were isolated from a leaf (*Columbia* ecotype) cDNA library using the PCR isolated cDNAs (APS and APL1) as probes (Villand *et al.*, 1993). We sequenced the APL1A cDNA clone and found that it contained an insert of 1.67 kb with an open reading frame encoding the entire ADGase large subunit protein (Figure 1). To isolate genomic clones of the ADGase large subunit, we screened a genomic library (*Landsberg erecta* ecotype) with the APL1A cDNA probe. By restriction enzyme mapping and partial sequence analysis of the genomic clones, we showed that one of the genomic clones (APL14B) included the complete ADGase large subunit transcription unit plus 3.2 kb of 5' upstream and 1 kb of 3' downstream sequence. The restriction map and orientation of the transcription unit are shown in Figure 1.

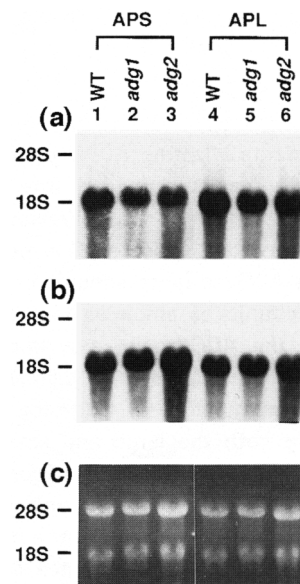
### Northern blot analysis

We examined ADGase transcript accumulation in leaves of wild-type and ADGase mutant plants by Northern blot analysis. Total RNA was isolated from leaves of plants grown in continuous light, electrophoresed on formaldehyde-agarose gels, transferred to a nylon membrane, and then probed with the small subunit and large subunit cDNAs of ADGase. As shown in Figure 2, transcripts encoding the ADGase large and small subunits were detected at similar levels in wild-type, *adg1-1* and *adg2-1* mutants. Since *adg2-1* mutants do not accumulate ADGase large subunit protein but do accumulate large subunit transcripts, we reasoned that the *adg2-1* mutation must perturb either the translation or post-translational processing of ADGase large subunit protein.



**Figure 1.** Restriction maps of *Arabidopsis* large subunit cDNA and genomic clones.

The transcription orientation of *Arabidopsis* ADGase large subunit is indicated with an arrow along with the restriction enzyme maps of large subunit cDNA and genomic clones: X (*Xba*I), H (*Hind*III), B (*Bam*HI), R (*Eco*RI), S (*Sst*I). Sites labeled with an asterisk are present in the vectors; shaded boxes represent transcribed regions.



**Figure 2.** Northern blot analysis of small subunit and large subunit transcripts of ADGase.

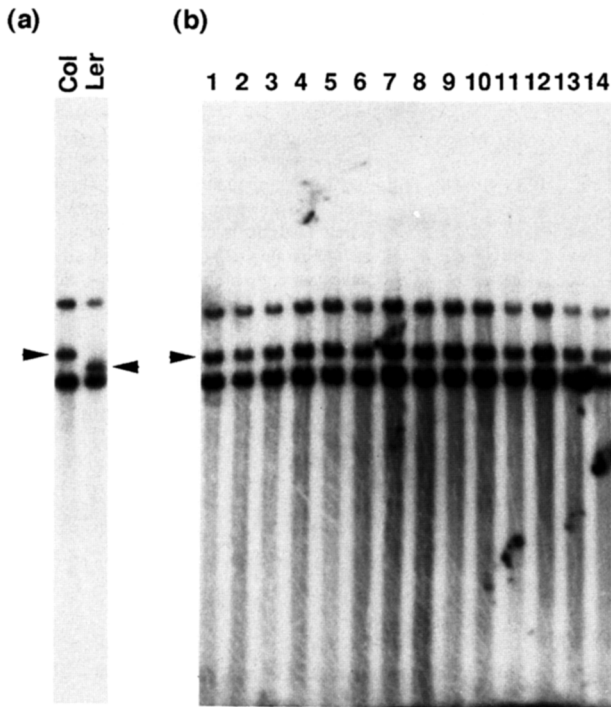
(a) Northern blots of total leaf RNA (20 µg) isolated from wild-type, *adg1-1* and *adg2-1* plants probed with radioactively labeled small subunit cDNA (APS) and large subunit cDNA (APL).

(b) After removal of the hybridization signals of small and large subunit probes, the same RNA blots as shown in (Figure 2a) were hybridized with a tubulin cDNA probe.

(c) The RNA gels were stained with ethidium bromide, showing that a similar amount of RNA was loaded.

### RFLP and DNA sequence analyses of *adg2-1*

We mapped the ADGase large subunit gene by RFLP analysis. RFLP was identified by digesting genomic DNA of recombinant inbred lines (Lister and Dean, 1993) with *Cl*aI and probing with the APL1A cDNA. The data were analyzed using the JOINMAP computer program (Stam, 1993), indicating that the gene is located at chromosome 5–18.9 cM. To test whether the *adg2* mutation is linked to the ADGase large subunit, we carried out RFLP analysis with selfed F<sub>2</sub> progenies of *adg2-1* (Col) crossed with the wild-type (Ler). In a population of 60 *adg2-1* homozygous plants, which were identified by assaying for ADGase



**Figure 3.** RFLP analysis of  $F_2$  plants of *adg2-1* (Col)  $\times$  *ADG2* (Ler). (a) Genomic DNAs (2  $\mu$ g) isolated from wild-type *Columbia* and *Landsberg* ecotypes were digested with *Cla*I, Southern-blotted and probed with labeled *ADGase* large subunit cDNA. A polymorphism was detected. (b) Southern blot analysis of genomic DNA isolated from  $F_2$  progenies of *adg2-1* (Col)  $\times$  *ADG2* (Ler). DNA samples derived from plants with the low leaf starch phenotype of *adg2-1* (lanes 1–13) and from wild-type (*Columbia*) plants (lane 14) were digested with *Cla*I, Southern-blotted, and probed with *ADGase* large subunit cDNA.

activity and leaf starch content, the APL1A Col band cosegregated with the low starch phenotype (Figure 3). This result strongly suggested that *adg2-1* is a mutation in the *ADGase* large subunit gene.

We validated this suggestion by cloning and sequencing the *adg2-1* cDNA. Primers were designed to the coding region of the gene. Reverse-transcribed cDNAs were synthesized from mRNA isolated from wild-type (*col*) and *adg2-1* (*col*) plants, amplified by PCR, cloned and sequenced. Multiple clones were sequenced from independent PCR reactions. The sequence data indicated that, in the *adg2-1* mutant, there was a single nucleotide mutation at position 366 (G to A) that caused a glycine to glutamic acid (G118E) transition (Figure 4). The sequence around position 366, which is downstream of the predicted transit peptide, is conserved in all other known *ADGase* large subunit genes (Smith-White and Preiss, 1992). To confirm that the mutant protein can be imported into chloroplasts, we synthesized radiolabeled proteins of the wild-type and the mutant *ADGase* large subunits through *in vitro* transcription and translation. Import assay with isolated pea chloroplasts indicated that the mutant *ADGase*

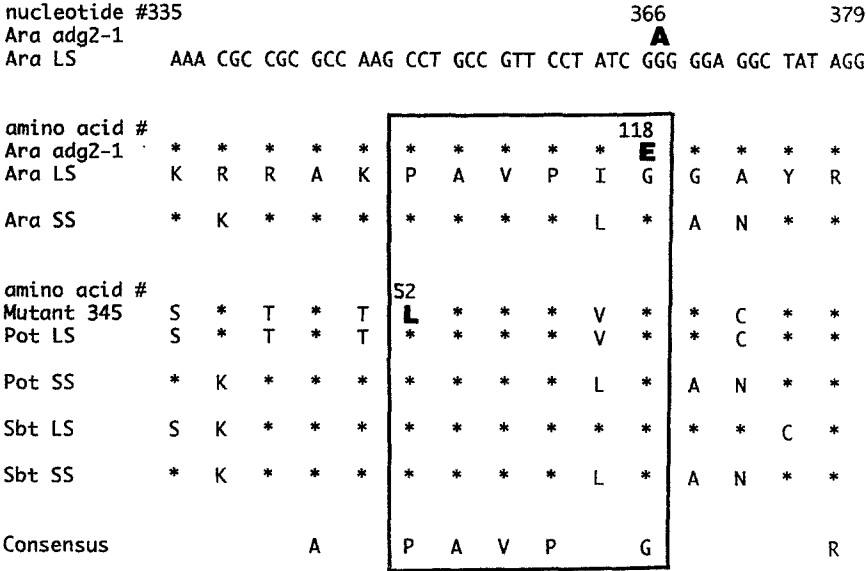
large subunit protein was imported into chloroplasts and processed to the mature size (data not shown).

#### *Complementation of adg2-1 mutant by transforming with the wild-type gene*

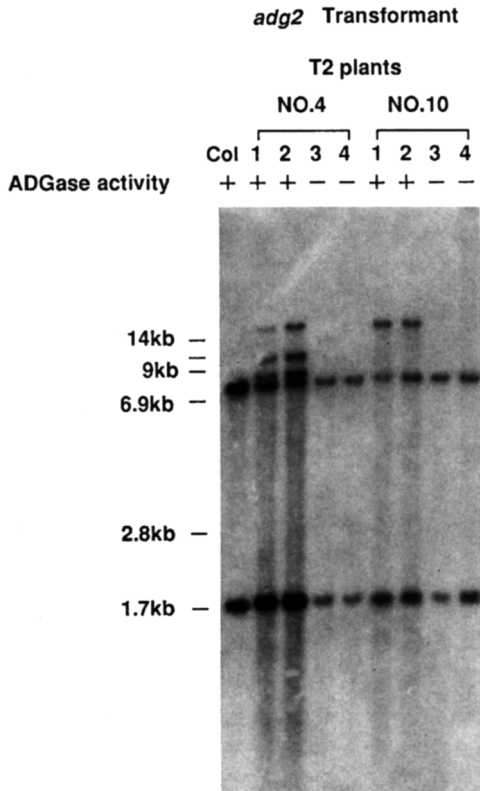
To prove further that *adg2-1* is an *ADGase* large subunit mutation, we transformed *adg2-1* mutant plants with the wild-type genomic clone of the *ADGase* large subunit gene. The wild-type APL1 gene (an 8-kb *Xba*I-*Sal*I fragment) was inserted into pBin19 and transformed into *Agrobacterium tumefaciens* C58 rif. *adg2-1* mutants were transformed by vacuum infiltration, and 10 independent kanamycin resistant transformants were selected from the  $T_1$  seeds. The kanamycin resistant transformants accumulated wild-type levels of starch. The  $T_1$  plants were selfed, and the  $T_2$  plants were scored for kanamycin resistance and assayed for *ADGase* activity and leaf starch content. Southern blot analysis indicated that transformants that exhibited kanamycin resistance, wild-type levels of *ADGase* activity and leaf starch, carried a single copy or multiple copies of the wild-type *ADGase* large subunit gene (Figure 5). In contrast, kanamycin sensitive plants showed the original *adg2-1* phenotype. This result confirms that the *adg2-1* mutant phenotype is caused by a mutation in the *ADGase* large subunit gene.

#### Discussion

Previous studies have shown that leaves of *adg2-1* mutant plants accumulate no detectable *ADGase* large subunit protein (Lin *et al.*, 1988b). By genetic and molecular analyses, we have shown that *adg2-1* represents a missense mutation in the *ADGase* large subunit gene. A point mutation (G to A) at position 366 results in a glycine to glutamic acid (G118E) transition in the peptide sequence. Because the mutation site is not in the predicted transit peptide, the mutated nascent protein is transported into the chloroplast. However, since no large subunit protein is detected in *adg2-1* mutant plants, the mutated protein must not be stable. It is possible that the mutated protein shows increased sensitivity to proteases and is thus degraded before it is assembled into the holoenzyme. Alternatively, the *adg2-1* mutation may prevent the large subunit from being assembled into the holoenzyme, and unassembled large subunits may be rapidly degraded. Support for the latter hypothesis comes from analysis of the *adg1* mutant. *adg1* represents a mutation in the *ADGase* small subunit gene (Chen, unpublished results). In *adg1* mutant plants, neither the small nor large subunit proteins accumulate, despite the fact that *adg1* mutants have a normal large subunit gene (Lin *et al.*, 1988a). This suggests that the assembly of large subunits into the holoenzyme is essential for stability.



**Figure 4.** Sequence alignment around the missense mutation site of *adg2-1*. The DNA and protein sequence of ADGase around the missense mutation site (nucleotide 366, G to A and amino acid 118 glycine to glutamic acid) of *adg2-1* were aligned with the small and large subunit sequences of potato (Pot) and sugar beet (Sbt), and the large subunit mutant 345 of potato (with the missense mutation at amino acid 52 proline to leucine). The boxed region indicates the proposed domain for the interaction of small and large subunits of ADGase.



**Figure 5.** Southern blot analysis of two transgenic lines of *adg2-1* carrying the transformed wild-type *APL* gene. T<sub>2</sub> progenies of two representative transgenic lines (Nos 4 and 10) that co-segregated for kanamycin resistance, wild-type level leaf starch, and wild-type level leaf ADGase activity (+) (lanes 1–2); and for kanamycin sensitivity, low leaf starch, and low leaf ADGase activity phenotypes (–) (lanes 3–4). Genomic DNA was isolated from the leaves of wild-type (*Columbia* ecotype) and transgenic plants, digested with *Eco*RI, Southern blotted, and probed with ADGase large subunit cDNA. The results showed that transgenic plants with a wild-type phenotype carried the transformed wild-type ADGase large subunit gene.

While the *adg2-1* allele appears to disrupt the assembly of large and small subunit ADGase proteins, other alleles may exist that affect the catalytic or allosteric regulatory domains of the enzyme. Such mutations permit the large subunit to be incorporated into the holoenzyme; however, holoenzyme activity is perturbed. This type of mutant has been identified by *in vitro* mutagenesis of the potato large subunit gene expressed in *E. coli* (Greene *et al.*, 1996). One of these mutants (345) is deficient in glycogen synthesis but possesses ADGase enzyme activity comparable to wild-type as assayed *in vitro*. Like *adg2-1*, mutant 345 is a missense mutation of the ADGase large subunit gene. In mutant 345, leucine exists at position 52 rather than proline (P52L). Position 52 is five amino acids upstream of the altered amino acid in *adg2-1* (Figure 4). Significantly, the missense mutation of mutant 345 affects the interaction of the ADGase enzyme and its activator but does not affect the assembly of ADGase. Two suppressor mutants of mutant 345 that restore normal allosteric properties of ADGase have been isolated (Greene *et al.*, 1996). Both mutants have the same P52L missense mutation within the ADGase small subunit, suggesting that the allosteric domain of the heterotetramer holoenzyme is formed by the interaction of both the large and small subunits. Because the *adg2-1* mutation alters the large subunit protein sequence only five amino acids downstream of that affected in mutant 345, it is likely that the domain shown in Figure 4 is involved in the interaction of large and small subunits. This suggestion is further supported by the fact that the glycine at position 57 is conserved in all known ADGase sequences (Smith-White and Preiss, 1992). As such, the domain shown in Figure 4 is probably important for both allosteric regulation and holoenzyme assembly.

In *Arabidopsis*, ADGase large subunit genes exist as a small gene family with three members (Villand *et al.*, 1993).

Our complementation test suggested that the ADGase large subunit mRNA that accumulates in leaves is primarily transcribed from the *ADG2* gene. The expression profiles of the other two copies of the ADGase large subunit genes remain to be determined. It would be interesting to ascertain whether overexpression of the other two genes could functionally complement the *adg2* mutation.

## Experimental procedures

### Plant materials and growth conditions

*adg1-1*, *adg2-1* and recombinant inbred lines (Lister and Dean, 1993) were obtained from the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State and Nottingham *Arabidopsis* Stock Center (NASC). Plants were grown in soil at 23°C under c. 5000 Lx continuous fluorescent light.

### Mutant screening

Mutant screening was carried out as described by Caspar *et al.* (1985). Plants were screened first after they developed four to six true leaves. Leaf pieces were cut out, depigmented in 95% ethanol or methanol and stained with I<sub>2</sub>-KI (5.7 mM iodine and 43.4 mM potassium iodide in 0.2 N HCl).

### 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).

### Native gel assay

The ADGase gel assays were carried out according to Lin *et al.* (1988a) using 7.5% polyacrylamide.

### Isolation of genomic and cDNA clones

*Arabidopsis* ADGase small subunit cDNA clone ALPC10 (APS1A) and large subunit cDNA clone ALPA4 (APL1A), both clones 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 large subunit, a genomic library of *Arabidopsis* Ler ecotype (constructed as partially digested *Mbo*I genomic DNA fragments cloned into the *Xho*I site of lambda Fix vector, supplied by ABRC) was screened with the APL1A cDNA insert. An 8-kb *Xba*I-*Sal*I fragment of the large subunit genomic lambda clone was further subcloned into pBluescript SK<sup>+</sup>.

### 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).

### Complementation of ADGase mutants with wild-type genes by *Agrobacterium* transformation

An 8 kb *Xba*I-*Sal*I fragment of the ADGase large subunit genomic clone was cloned into *Xba*I-*Sal*I sites of pBin19 and denoted pBin19-APL. The resulting T-DNA binary constructs were transformed into *Agrobacterium tumefaciens* pGV2260/C58 rif strain. *adg2-1* plants were transformed with pBin19-APL 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 analysis.

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The GenBank Data Library accession number U72290 (ADPG pyrophosphorylase large subunit cDNA sequence).