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 homotramers. 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: α-glucose-1-phosphate adenylyl 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 α-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 shrunken2 (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...
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 nytran 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.

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 Clal 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 F2 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 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 (XbaI), H (HindIII), B (BamHI), R (EcoRI), S (SstI). 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.
Analysis of Arabidopsis mutant adg2-1

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 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., 1989a). 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.

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 I2–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. (1988), 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 parallel XbaI-Sal\[+\]XbaI-Sa\[+\] fragment of the ADGase large subunit genomic clone was cloned into XbaI–Sal\[+\] vector, supplied by ABRC) was screened with the APL1A cDNA insert. An 8-kb XbaI–Sal\[+\] fragment of the large subunit genomic lambda clone was further subcloned into pBluescript SK\+[+].

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 Agrobacterial transformation

An 8 kb XbaI–Sal\[+\] fragment of the ADGase large subunit genomic clone was cloned into XbaI–Sal\[+\] 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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References


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