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Isolation and expression analysis of two tomato ADP-glucose pyrophosphorylase *S* (large) subunit gene promoters

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Abstract

ADP-glucose pyrophosphorylase (AGPase, EC2.7.7.27) is a key enzyme in starch synthesis. The enzyme is a heterotetramer with two S (large) and two B (small) subunits. In tomato (*Lycopersicon esculentum* Mill), there are three *S* subunit genes. The Agp *S1* and *Agp S3* genes and their promoters were isolated from a tomato genomic library. The *Agp S1* promoter region has a TATAA box at −57 bp and a CCAAT box at the −8 bp position. Tomato *Agp S1* promoter is active in starch storage tissues, and in organs that are carbohydrate sinks. Its activity is localized in the guard cells and veins of the leaf, in the root cap and root vascular tissues, in the starch sheath cells of the stem and in the ovary and stamens of the flower. It is also active in pollen, seeds and in the inner pericarp wall and placental tissue of developing tomato fruits. No activity was observed in mesophyll cells, or in the ovaries at anthesis. The *Agp S3* promoter has a CCAAT box at the −115 bp position and multiple GC rich regions around the CCAAT box, but it does not contain a TATAA box. Tomato *Agp S3* promoter is expressed in the mesophyll cells, guard cells and veins of the leaf. In the flower, *Agp S3* is strongly expressed in the sepals, ovary and anthers at anthesis. Deletions in the distil 5′ upstream region of *Agp S3* indicated that only a 0.5 kb fragment of the *Agp S3* promoter is required for complete expression in transgenic plants. *Agp S3* contains a minimal promoter with little evidence of functional *cis*-acting regulatory elements. For AGPase, the different requirements for regulation of enzyme activity between source leaves and sink organs are met in part by the evolution of multiple *S* subunit genes with very different promoters.
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1. Introduction

ADP-glucose pyrophosphorylase (AGPase, EC2.7.7.27) is the critical enzyme in starch synthesis, both in photosynthetic and non-photosynthetic tissues [1]. It catalyzes the reaction of glucose-1-phosphate and ATP to ADP-glucose, which is the substrate for starch synthesis. Several lines of evidence indicate that alternative pathways to provide activated glucose for starch synthesis do not exist. Maize mutants with only 5–10% AGPase activity have reduced starch levels, with only 20–30% of the starch of wild type [2]. In *Arabidopsis*, a mutant lacking leaf starch was identified that had no extractable AGPase activity, but other enzymes such as starch synthase and UDP-glucose pyrophosphorylase were unaffected [3]. Transgenic potato plants with reduced levels of AGPase activity had lower starch content in the tubers [4], whereas expression of an AGPase variant from *E. coli* in potato tubers increased the starch content by an average of 35% [5].

The AGPase enzyme in plants is a heterotetramer of 200–240 kDa consisting of two S (large) and two B (small) subunits with molecular weights of 54 and 51 kDa, respectively [6]. In a number of plants there are multiple forms of the genes for both subunits. In tomato, there are three *S* subunit genes (*Agp S*1, *Agp S*2, and *Agp S*3) and one gene for the B subunit (*Agp B*) [7–9]. All of the cDNAs from these genes are unique and do not hybridize with each other. Multiple cDNAs for each of the subunit genes have been isolated from other plants such as potato, wheat, rice and *Arabidopsis*.

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AGPase activity may be regulated allosterically, transcriptionally, and/or post-transcriptionally. AGPase is activated by 3-PGA and inhibited by Pi with the ratio of the two effectors playing a major role in modulating AGPase activity [10,11]. In source tissues, during photosynthetic carbon fixation, which produces 3-PGA and consumes Pi during ATP synthesis, the ratio of 3-PGA/Pi is high, activating AGPase and increasing starch biosynthesis. While in sink tissues, allosteric control may not be the main regulatory mechanism, since the precursor for starch synthesis is imported sucrose. In this case, the steady supply of sucrose to the sink may induce a sustainable increase in the transcription of the AGPase subunit genes and/or an increase in mRNA stability. For example, in rice endosperm, AGPase B subunit mRNA was detected 3 days after pollination (dap) and reached its maximum at 14 dap. These changes were closely correlated to the AGPase protein level, suggesting that AGPase gene expression is regulated at the transcription level in sink tissues [12]. Similar results were found in other plants like potato, maize, and bean. A third level of regulation of AGPase activity is the light or sucrose induced formation of an interchain disulfide bond between the small subunits, which leads to activation of the enzyme [13]. There is also evidence that sugar may be an important regulatory factor for AGPase gene expression [14–16]. For example, AGPase subunit genes in *Arabidopsis* were found to be significantly and differentially regulated by sugar and light/dark exposures [17].

Our previous research [18] and the research of [19] also indicated that sucrose is an important regulatory factor for AGPase, and that the transcription of *Agp S1* was directly correlated to the sucrose content of sink tissue.

In source tissues, such as potato leaves, the AGPase B subunit protein, detected by using western blots, was not well correlated to its mRNA level. This suggests that the expression of *Agp B* subunit gene in source tissue was regulated post-transcriptionally [20]. The research of [16] suggested that in source tissues the primary mode of regulation of AGPase is allosteric and post-transcriptional, whereas in sink tissue, the predominant level of AGPase regulation is transcriptional. In *Arabidopsis* three cDNAs for the large subunit (designated *ApL1*, *ApL2* and *ApL3*) were obtained and their kinetic and regulatory properties studied by expressing the *ApL* genes with the small subunit gene in *E. coli*. They found that *ApL1* is regulated allosterically [21], whereas [17] found that *ApL3* is regulated at the transcriptional level.

In tomato, the AGPase activity in mature leaves is very high, in young leaves and fruits moderate and in roots low [7]. In fruit, *Agp S1* and *Agp B* are expressed very highly compared to *Agp S2* and *Agp S3*, whereas in leaves, *Agp S3* and *Agp B* are highly expressed compared to *Agp S1* and *Agp S2* [8]. In roots, only *Agp S1* and *Agp S2* were expressed.

There is less information concerning the spatial expression of the *S* subunit genes within leaves, roots and fruit. *Agp S1* from potato is active in the guard cells of the leaf, the starch sheath of the stem and in the flower anthers and ovary. No activity was detected in the root or in the mesophyll of the leaves [22]. Given our finding that the tomato *S1* gene was expressed in roots [8] in contrast to the results for the potato gene, and that there are no reports on the spatial expression of *Agp S3*, we isolated the tomato *Agp S1* promoter, and we report for the first time the expression pattern in roots and a fleshy fruit. We also isolated the tomato *Agp S3* gene (equivalent to the *Arabidopsis ApL1*) and determined the expression pattern of the promoter and truncated versions in transgenic tobacco plants.

2. Materials and methods

2.1. Screening of the tomato genomic library

A tomato genomic library was constructed in λ-phage. Plaques were transferred to nitrocellulose filters, and the DNA denatured and neutralized. Tomato *Agp S1* and *Agp S3* cDNA were labeled with [α-32P]dCTP and used as probes. Hybridization was performed at 65 °C in 5 × SSC, 0.1% SDS, 0.2 mg/ml bovine serum albumin, 0.2 mg/ml Ficoll, and 0.2 mg/ml denatured herring sperm DNA. Filters were washed in 2 × SSC, 0.1% SDS at room temperature once for 20 min, and in 0.1 × SSC, 0.1% SDS at 65 °C twice for 20 min. The filters were exposed between intensifying screens at −70 °C for 24 h to Kodak XOMAT films. Several hybridizing phage clones were obtained. An 8.1-kb long insert clone for *Agp S1* and a 6.4 kb long insert clone for *Agp S3* were selected and purified by three rounds of hybridizations.

2.2. Sub-cloning

λ-DNA was extracted according to the technical bulletin of Promega (Purification of Lambda DNA with Wizard™ Lambda Preps DNA Purification System). Selected λ-phages were picked up from fresh plates and put in phage buffer (150 mM NaCl, 40 mM Tris–HCl, 10 mM $MgSO_4$, pH 7.4) at 4 °C overnight. *E. coli* was cultured in 1 ml LB (10 g Bacto Tryptone, 5 g Bacto Yeast Extract, 10 g NaCl in 1 l) medium supplemented with 10 μl of 20% maltose and 10 μl of 1 M $MgSO_4$ at 37 °C overnight and then mixed with 2 μl phage buffer containing λ-phage and cultured at 37 °C for 20 min. LB (10 ml) was added and the cultures kept at 37 °C for 5 h until lysis occurred. The lysate was centrifuged at 8000 × *g* for 10 min and the supernatant was collected. Nuclease mixture (40 μl) (containing 0.25 mg/ml RNAase A, 0.25 mg/ml DNAase I, 150 mM NaCl, 50% glycerol) was added to the supernatant and incubated at 37 °C for 15 min followed by the addition of 4 ml phage precipitant (33% polyethylene glycol, 3.3 M NaCl) with incubation on ice for 30 min. The mixture was centrifuged at 10,000 × *g* for 10 min, and the supernatant was discarded. The pellet was resuspended with 500 μl phage buffer. Purification resin (1 ml) was added and transferred to a minicolumn. The

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