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# MOLECULAR BIOLOGY AND PHYSIOLOGY

## Isolation and Characterization of an ADP-Glucose Pyrophosphorylase Gene from *Gossypium hirsutum* L.

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### ABSTRACT

**Roots and stems of cotton (*Gossypium hirsutum* L.) plants store photoassimilate as starch. Partitioning of fixed carbon between starch in vegetative storage tissues and seed is likely to impact cotton development and yield. The enzyme ADP-glucose pyrophosphorylase (ADPGp) plays a rate-limiting role in starch production, and its temporal and spatial expression plays a critical role in determining patterns of starch deposition in plants. The objective of this study was to identify, sequence, and analyze a cotton ADPGp (small subunit) gene involved in starch production in stems and roots. A genomic sequence with extensive similarity to an mRNA encoding the small subunit of ADPGp that is expressed in starchy stems was identified and sequenced. The gene was composed of nine exons and eight introns. The introns were bound by typical splice sites. The open reading frame (ORF) encoded a peptide of 518 amino acids with many catalytic and regulatory features common to plant ADPGp. This gene was abundantly expressed in starchy roots and in leaves. Expression of this gene was low in developing fiber. *In-silico* analysis of the promoter sequence identified regulatory motifs associated with light response and expression in the seed. Motifs associated with response to abscisic acid (ABA), salicylic acid, giberellic acid, and methyl-jasmonate were also identified.**

Cotton plants store substantial amounts of photoassimilate as starch in stems and roots prior to flowering (Wells, 2002). Analysis of greenhouse-grown plants indicated that starch levels peak in domesticated cotton plants at about the time the first flower opens and then starch levels decline (De Souza and Viera da Silva, 1987). Starch is mobilized from starchy tissues as sucrose after the plants begin to flower and supplies up to 28% of the metabolic needs of some developing bolls (Constable and Rawson, 1980). Therefore, starch stored in cotton vegetative tissues early in the growing season supports subsequent reproduction later in the season. However, starch that persists in vegetative tissues during and after seed development is not available to support seed and fiber development. In addition to the normal demand for photoassimilate to support seed development, cotton seed have differentiated ovular trichomes that produce thick secondary cell walls (Graves and Stewart, 1988). The synthesis of secondary cell walls in fiber places an extra demand on photoassimilate to provide the glucose subunits for cellulose biosynthesis.

The rate-limiting step in starch biosynthesis is catalyzed by ADP-glucose pyrophosphorylase (ADPGp) (EC 2.7.7.27). ADPGp catalyzes the conversion of glucose(GLC)-1-phosphate and ATP to GLC-ADP and inorganic phosphate (Pi). GLC-ADP is used to synthesize starch (Martin and Smith, 1995). ADPGp is a heterotetrameric protein composed of two large subunits and two small subunits (Okita et al., 1990). The catalytic site resides in the small subunits. Enzyme activity is regulated by allosteric activation and inhibition by phosphoglyceraldehyde (PGA) and Pi, respectively (Sowokinos and Preiss, 1982). In eudicots, the enzyme is also under redox-dependent post-translational control (Hendriks et al., 2003; Tiessen et al., 2002). ADPGp is inactivated by cross-linking active monomers at a conserved cysteine. Inactivation is reversed by reducing agents such as thioredoxin.

The goal of this research was to identify an ADPGp gene that plays a role in regulating starch levels in cotton stems and roots. Additionally we wanted to identify gene promoters that could target gene expression in starch storing tissues with the aim of developing tools to utilize carbohydrate reserves unavailable to support seed development. This report describes the isolation, sequencing, and characterization of a cotton gene encoding the ADPGp small subunit that increased in expression in starch accumulating tissues.

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## MATERIALS AND METHODS

Expressed sequence tags (ESTs) representing ADPGp were identified from a library representing cDNA derived from cotton stems ( Taliercio and Boykin, 2007; Taliercio et al., 2006). An oligonucleotide (5′-AGTGTTTTGGGGATTA-3′) was used to amplify the 3′-end of an ADPGp cDNA from cotton stems using the SMART RACE (rapid amplification of cDNA ends) cDNA Amplification Kit from Clontech (Mountain View, CA). The clone was sequenced to confirm it encoded ADPGp. A radioactive ($^{32}$P) probe was made of the ADPGp clone using the RadPrime DNA Labeling System (Invitrogen, Carlsbad, CA). The probe was hybridized to blots of bacterial artificial chromosome (BAC) DNAs containing random inserts of EcoRI fragments of TM1 cotton genomic DNA from Texas A&M University (GENEfinder Genomic Resources, http://hbz7.tamu.edu/). Up to 5 blots were prehybridized at 65 °C in 20 ml of 50 mM Pipes, pH 6.5, 100 mM NaCl, 50 mM sodium phosphate, pH 6.5, 1 mM EDTA, and 5% SDS. The probe ($3\times10^6$ cpm ml$^{-1}$) was added after 4 h of prehybridization and incubated overnight at 65 °C in a Model 1000 hybridization oven (Robbins Scientific, Sunnyvale, CA) with moderate rotation. The hybridization solution was replaced with 2XSSC (20XSSC = 3 M NaCl, 0.3 M NaCitrate, pH 7.0) and 5% SDS and the blots rinsed for 30 min at 65 °C, this step was repeated twice. The blots were rinsed with 0.2XSSC and 1% SDS at 65 °C two times for 30 min. Finally, the SDS was removed with two rinses of 0.2XSSC at room temperature. Probe hybridization was visualized on a Molecular Imager (BioRad, Hercules, CA). Hybridizing BACs were ordered from GENEfinder Genomic Resources. BAC DNA isolated using a BACMAX DNA Purification Kit (Epicenter, Madison, WI) was sequenced on an ABI3730 DNA Sequencer (Foster City, CA) using standard methods by the USDA/ARS MidSouth Area Genomics Laboratory. Initially, primer sequences derived from ADPGp-ESTs were used to sequence the BAC clone. Subsequently, primers derived from genomic sequences were used in iterative rounds of sequencing until sufficient coverage was achieved. The sequences were assembled and analyzed in Vector NTI ContigExpress (Invitrogen). The clone was sequenced at least three times and 80% of the sequences covered five times in both directions. The sequence was deposited in GenBank as accession EU268018.

Total polyribosomal RNA was isolated from mature leaves, immature stems, immature roots, roots rapidly accumulating starch, and 13- and 19-days post anthesis (DPA) fibers. Polyribosomal RNA was also isolated from 1-DPA fiber (Taliercio and Boykin, 2007). The cotton genotype ST4793R was the source of all RNA. The RNA isolation included an on-column DNase1 digestion to remove contaminating genomic DNA (Qiagen, Valencia, CA). The RNA quality was confirmed by analysis on a bioanalyzer (Agilent, Palo Alto, CA). Primers for the 3′-end of the ADPGp gene (F: 5′-GACAAGAATGCTCGAATTGGAG-3′ and R: 5′- CCACTAGGAATCAAGGCATCC-3′), and the rRNA gene (F:5′-CGTCCCTGCCCTTTGTACA-3′ and R:5′-AACACTTCACCGGACCATTCA-3′) were designed by Beacon Designer (BioRad, Carlsbad, CA). All primers were obtained from Integrated DNA Technologies (Coralville, IA). The product of the ADPGp specific primers spanned the last intron. The amplification efficiency of the ADPGp primers was determined to be 1.96 on six 0.25-dilutions of a mixture of cDNAs derived from the RNAs of the analyzed tissues. The efficiency of the 18S rRNA primers was 2.01 determined on five 10-fold dilutions (Taliercio et al., 2005). All cDNA were made with 0.5 µg of RNA using the iSCRIPT cDNA Synthesis Kit from BioRad. Real-time (or quantitative) polymerase chain reaction (QPCR) was performed at 55 °C using the iQ SYBR Green Supermix Kit (BioRad) following the manufacturer's standard instructions. All QPCR were performed in triplicate and confirmed on at least two different cDNA preparations. Melting temperature analyses were consistent with a single amplicon produced in these PCR reactions. Relative quantification was calculated by the method of Pfaffl (2001).

Promoter analyses were performed on sequences 5′of the transcriptional start site (TSS) using the Plant-CARE (cis-acting regulatory elements) search function (Lescot et al., 2002). Putative regulatory elements that were in the wrong position (e.g., the "TATA" box), incomplete (e.g., an "A" box), overlapped better characterized regulatory elements (e.g., "G" boxes), or of unknown function were not included in the schematic of the cotton ADPGp gene (Fig. 1). Detailed information about the regulatory elements listed in Fig. 1 is available at the Plant-CARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

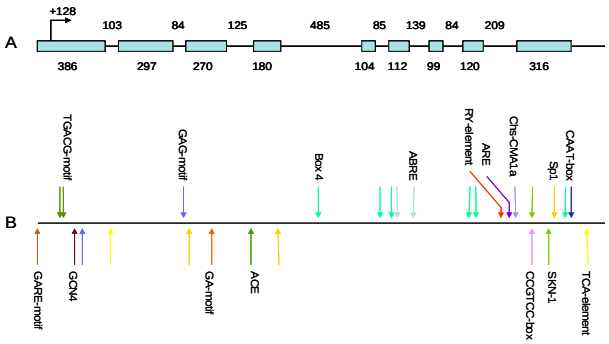


**Figure 1. Structure of the ADPGp gene. Panel A represents 3420 bp of the coding region of the ADPGp. The shaded boxes represent the exons and the numbers below the box indicates the size of the exon in bp. The lines between the boxes represent the introns and the numbers directly above these lines indicate the size of the introns. The right facing arrow indicates the translational start site. The first exon begins at the TSS (+1 bp). Panel B represents 1440 bp of the promoter region ending at the TSS. Arrows below the line indicate the position of transcription factors in the reverse orientation from the orientation listed in Plant-CARE. Arrows above the line indicate the position of transcription factors in the same orientation as the orientation listed in Plant-CARE. Distances from TSS of these putative regulatory elements are: TCA element (-50 bp and -1255 bp), CAAT box (-82 bp), Box 4 (-107 bp, -322 bp, -342 bp, -561 bp, -567 bp, -715 bp), Sp1 (-117 bp, -826 bp, -1053 bp), Skn-1 (-124 bp, -194 bp), CCGTCC box (-184 bp), chs-CMA1a (-216 bp), ARE (-223 bp), RY element (-259 bp), ABRE (-482 bp, -521 bp), ACE (-884 bp, -1386 bp), GA-motif (-984 bp), GAG-motif (-1084 bp, -1314 bp), GCN4 (-1349 bp), TGACG-motif (-1388 bp), and GARE-motif (-1440 bp).**

## RESULTS AND DISCUSSION

**Isolation of an ADPGp gene from cotton.** ESTs representing the small subunit of ADPGp were identified in a cDNA library representing starchy cotton stems (Taliercio et al., 2006). Oligonucleotides designed based on this clone were used to isolate the 3′-end of an ADPGp cDNA from starchy cotton stems. Two nearly identical clones 1737 base pairs (bp) long were isolated. These clones were determined to represent ADPGp transcripts by using them to query GenBank (E = 0.0). One of these clones was used to screen a BAC cotton genomic library. Three positive clones were identified. DNA from a single BAC clone was isolated and the region encoding the ADPGp gene sequenced. The original cDNA aligned nearly perfectly with the exonic regions of the genomic clone. A total of 82 *Gossypium* ESTs were identified from GenBank using BLASTn that were 98% or greater identical to the genomic clone. Only two ESTs, one derived from cotton stems (DN760126.1), the second derived from libraries that included cotton stems (DW504347.1) were 99% identical to these genomic sequences. The remaining ESTs primarily represented young fiber or developing ovules. Thirteen of these ESTs were derived from *Gossypium raimondii* Ulbr. and only one was derived from *Gossypium arboreum* L. Search of the global cotton assembly (http://www.agcol.arizona.edu/pave/old_platform/cotton/) for ADPGp small subunit assemblies identified a *G. raimondii* sequence (Cotton12_00001_244) as the most similar to the genomic sequence under investigation (Udall et al., 2006). The overall identity between these two ADPGp genes was 94.8%. There was a 26 bp insertion in the 3′ untranslated region of the *G. hirsutum* gene relative to the *G. raimondii* sequences and the 5′ untranslated regions were nearly identical. These data suggest that this genomic clone belongs to the D genome with the caveat that starch-producing tissues (e.g., *G. arboreum* leaves) are under-represented in the *Gossypium* EST database at GenBank.

The ESTs similar to the genomic clone were used to identify its complete coding sequence. RACE analysis confirmed that the most 5′ EST contained the transcriptional start site of the gene. Figure 1 shows the schematic structure of the ADPGp gene. The gene is composed of nine exons and eight introns. All of the introns are bound by standard splice sites (Simpson and Filipowicz, 1996). For example, all of the introns are flanked by the dinucleotide GT at the 5′-end and AG at the 3′-end. A potato ADPGp gene also had nine exons (Nakata et al., 1994), whereas an *Arabidopsis* ADPGp gene (locus At5g48300 found at http://arabidopsis.org) is missing intron 2 and therefore contains eight exons. Otherwise, the *Arabidopsis* introns were located in positions similar to the cotton introns. The untranslated 5′ and 3′ regions of the cotton gene were 128 bp and 199 bp, respectively.

Two other BAC clones were also identified with substantial sequence similarity to the cotton ADPGp cDNA. One of the BAC clones contained sequences starting in intron 3 spanning to intron 8 that were greater than 94% identical to the first BAC clone: however, the flanking regions were not similar to other plant ADPG genes or to other known genomic sequences. This result suggested that the second BAC clone may be a pseudogene. Partial DNA sequence of the 3′-end of the third BAC clone indicated it did not match the cDNA used to screen the genomic library and was not relevant to this study.

**Analysis of the open reading frame (ORF).** The ADPGp cotton gene contained an ORF encoding a polypeptide of 518 amino acids aligned with the well characterized potato ADPGp protein (Fig. 2) (Nakata et al., 1994). The amino ends of these proteins are poorly conserved (24 identical amino acids out of 72) and contain a transit sequence (amino acid 1-72 for the potato enzyme) that targets the protein to the amyloplast. Often the amino end of the ADPGp small subunits is not conserved (Choi et al., 2001; Hannah et al., 2001). The remainder of these two genes was highly conserved including motifs involved in catalysis, allosteric regulation by PGA, and thermal stability (Hendriks et al., 2003; Iglesias et al., 2006; Okita et al., 1990; Sowokinos and Preiss, 1982). Conservation of two lysines at positions 471 and 508 of the cotton protein indicated that the cotton enzyme is probably activated by PGA and this activation can be inhibited by Pi. The motif associated with thermal stability (amino acids 77-80) substantially increases the half-life of the enzyme at higher temperatures (Linebarger et al., 2005). Cross-linking at the cysteine of this motif (amino acid 79) is responsible for the redox-dependent posttranscriptional control of ADPGp enzyme activity (Hendriks et al., 2003; Iglesias et al., 2006), a motif characteristic of the ADPGp small subunit of most eudicots. Analysis of the crystal structure of a potato ADPGp identified conserved sites that bind ADP-GLC and ATP all of which are also conserved in the cotton ADPGp (Fig. 2) (Jin et al., 2005).

**Expression of the ADPGp gene and analysis of the promoter**. QPCR analysis was used to identify the sites and levels of expression of this gene. Alignment of the gene with other cotton ADPGp sequences (e.g., other ESTs) identified a unique region at the 3′-end. Primers made to this region amplified a QPCR product from mature leaves, immature root, mature roots, immature stems, meristems, and fiber mRNA (Fig. 3). Leaf and mature roots both had a greater than two-fold increase in the level of expression of this ADPGp gene relative to immature stem. This pattern of expression was consistent with a rate-limiting role for this protein in starch biosynthesis. The other tissues investigated had lower levels of mRNA encoded by this gene. The lowest levels occurred in developing fibers. Query of the cotton EST database in GenBank with the genomic sequence indicated ESTs present in "young fiber" were nearly identical to the coding region of the genomic clone. Although the expected PCR product was amplified in 1-DPA fiber, it was no more abundant than in older fiber and much less abundant than in immature stems (Fig. 3).

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cotton    MVSMAAIGDLR-LPSTASFNASSVCSSRKSSAPWSLSFSASALSGDKLVF
potato    --MAASIGALKSSPSSNNCINERRNDSTRAVSSRNLSFSSSHLAGDKLMP
                                                1         5
cotton    KIATGCS----RTERTASIVSPKAVSDSKNSQTCLDPDASRSVLGIILGG
potato    VSSLRSQGVRFNVRRSPMIVSPKAVSDSQNSQTCLDPDASRSVLGIILGG
                     4 5
cotton    GAGTRLYPLTKKRAKPAVPLGANYRLIDIPVSNCLNSNISKIYVLTQFNS
potato    GAGTRLYPLTKKRAKPAVPLGANYRLIDIPVSNCLNSNISKIYVLTQFNS
                  2                                   5    5
cotton    ASLNRHLSRAYASNMGGYKNEGFVEVLAAQQSPENPNWFQGTADAVRQYL
potato    ASLNRHLSRAYASNMGGYKNEGFVEVLAAQQSPENPDWFQGTADAVRQYL
                     5
cotton    WLFEEHNVLEFLVLAGDHLYRMDYERFIQAHRETDADITVAALPMDEKRA
potato    WLFEEHTVLEYLILAGDHLYRMDYEKFIQAHRETDADITVAALPMDEKRA
            4              4
cotton    TAFGLMKIDEEGRIIEFAEKPKGDQLKAMQVDTTILGLDDERAKEMPFIA
potato    TAFGLMKIDEEGRIIEFAEKPQGEQLQAMKVDTTILGLDDKRAKEMPFIA
          4                       6
cotton    SMGIYVVSKNVMLNLLRDQFPGANDFGSEIIPGATSIGMRVQAYLYDGYW
potato    SMGIYVISKDVMLNLLRDKFPGANDFGSEVIPGATSLGMRVQAYLYDGYW
            4
cotton    EDIGTIEAFYNANLGITKKPVPDFSFYDRSSPIYTQPRYLPPSKMLDADV
potato    EDIGTIEAFYNANLGITKKPVPDFSFYDRSAPIYTQPRYLPPSKMLDADV

cotton    TDSVIGEGCVIKNCKIHHSVVGLRSCISEGAIIEDTLLMGADYYETDADR
potato    TDSVIGEGCVIKNCKIHHSVVGLRSCISEGAIIEDSLLMGADYYETDADR
                                        3
cotton    RFLSAKGSVPIGIGKSSHIKRAIIDKNARIGDNVKIINSENVQEAARETD
potato    KLLAAKGSVPIGIGKNCHIKRAIIDKNARIGDNVKIINKDNVQEAARETD
                     3
cotton    GYFIKSGIVTVIKDALIPSGTVI
potato    GYFIKSGIVTVIKDALIPSGIII
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**Figure 2. Alignment of the conceptual translation of the cotton ADPGp gene with the potato ADPGp (P23509). Amino acids 1-72 constitute the transit peptide of the potato enzyme. Underlined positions marked 1, 2, and 3 indicate the heat stability motif, a conserved amino acid in the catalytic site and conserved amino acids in regulatory sites, respectively. Underlined positions 4, 5, and 6 indicate sites that interact with ADP-GLC, both ADP-GLC and ATP, or ATP only, respectively. Identical amino acids are shaded gray.**

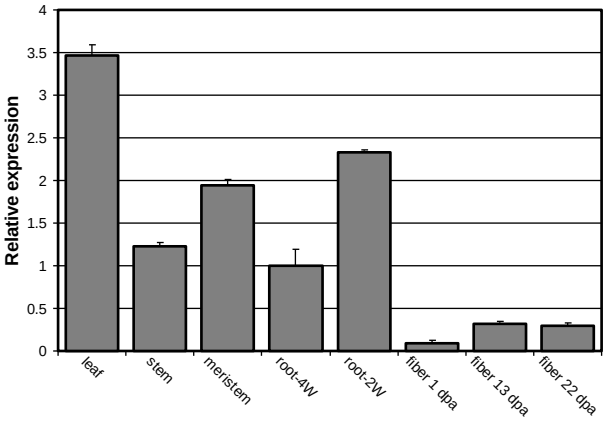


**Figure 3. Expression of the ADPGp gene in various tissues. QPCR was used to quantify expression of ADPGp in RNA from mature leaves (leaf), immature stems (stems), meristematic region (meristem), nonstarchy root 4 wk before plants flower (root -4wk), starchy root 2 wk before plants flower (roots -2W), and fiber 1, 13, and 22 DPA. Expression is relative to root-4W. Bars represent the standard error.**

The genomic sequence of ADPGp included 1516 bp of the putative promoter region an area that often contains elements controlling gene expression (Shahmuradov et al., 2003). *In-silico* analysis of this region identified several motifs such as the CAAT

box associated with gene regulation (Fig.1B). The CAAT boxes are core promoter sequences important in binding RNA Polymerase II (Bucher, 1990; Butler and Kadonaga, 2002; Kusnetsov et al., 1999; Lee and Young, 2000; Lewis and Reinberg, 2003; Smale, 2001; Wenkel et al., 2006). The first TATA box in a forward orientation was 86 bp upstream from the TSS and a few bp upstream from the putative CAAT box. TATA boxes are approximately -30 bp from the TSS and downstream of the CAAT box, indicating this gene may use other motifs to bind RNA polymerase II. Non-TATA RNA polymerase II binding promoters that use other sequences to bind the basal promoter have been reported in plants (Achard et al., 2003). Six regulatory elements (Sp1, chs-CMA1a, box4, ACE, GA-motif, and GAG-motif) associated with light responsiveness are present in the cotton ADPGp promoter consistent with the expression of this gene in leaves (Fig. 1 B). The CCGTCC box was reported to play a role in gene expression in meristems consistent with the observed expression of the ADPGp gene in cotton meristems.

Three regulatory elements associated with seed/endosperm expression (Skn-1, Ry-element, and the GCN4-motif) are present in the cotton ADPGp promoter. The Skn-1 motif cooperates with the GCN4-motif, an AACA-motif, and an ACGT-motif to control endosperm expression (Washida et al., 1999). In addition to the presence of the GCN4-motif, we note that AACA is part of the GARE-motif and ACGT is part of the ABRE-motif. These data suggest that the ADPGp gene might be expressed in the developing seed. Developing endosperm/embryos of cotton do not accumulate starch, but starch has been reported in cotton seed coats (Ruan et al., 1997). The ABRE-motif also has been reported to play a role in abscisic acid (ABA) signaling. The ABA signaling motif could indicate that the expression of this ADPGp gene is influenced by ABA (Himmelbach et al., 2002). ABA induced ADPGp expression and starch accumulation in the presence of sucrose in cultured rice cells and *Arabidopsis* (Akihiro et al., 2005; Rook et al., 2001). The GARE-motif also was reported to play a role in giberellic acid regulation of gene expression, the TCA-motif was reported to play a role in salicylic acid response, the TGACG-motif was reported to play a role in methyl-jasmonate response, and the ARE was reported to play a role in anaerobic induction of gene expression (Lescot et al., 2002).

One goal of this research was to identify an ADPGp gene expressed in starch-accumulating cotton stems and roots that allow targeted expression of transgenes in these tissues. The ADPGp gene characterized in these studies was expressed in the appropriate tissues, however other potential sites of expression based on promoter analysis of this ADPGp gene need to be investigated experimentally. Further studies will include analysis of gene expression in carefully dissected developing cotton seeds to determine if this ADPGp gene is expressed in seed coats and if treatment of cotton tissues with relevant phytohormones affects expression of this gene. More detailed and comprehensive expression analysis can be achieved by using this promoter to drive expression of a reporter gene such as the green fluorescent protein or luciferase, allowing for a direct assessment of sites of activity of this promoter independent of posttranscriptional regulation of ADPGp mRNA accumulation. Use of a reporter gene also allows investigations of how individual regulatory elements coordinate ADPGp gene expression and influence starch levels in cotton tissues.

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