Structure and expression analysis of genes encoding ADP-glucose pyrophosphorylase large subunit in wheat and its relatives

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| Complete List of Authors: | Zhang, Xiao-Wei; Triticeae Research Institute, Sichuan Agricultural University  
Li, Si-Yu; Triticeae Research Institute, Sichuan Agricultural University  
Zhang, Ling-Ling; Triticeae Research Institute, Sichuan Agricultural University  
Yang, Qiang; Triticeae Research Institute, Sichuan Agricultural University  
Jiang, Qian-Tao; Sichuan Agricultural University, Triticeae Research Institute  
Ma, Jian; Triticeae Research Institute, Sichuan Agricultural University  
Qi, Peng-Fei; Triticeae Research Institute, Sichuan Agricultural University  
Li, Wei; Triticeae Research Institute, Sichuan Agricultural University  
Chen, Guo-Yue; Triticeae Research Institute, Sichuan Agricultural University  
Lan, Xiu-Jin; Sichuan Agricultural University, Triticeae Research Institute  
Deng, Mei; Sichuan Agricultural University, Triticeae Research Institute  
Lu, Zhen-Xiang; Agriculture and Agri-Food Canada Lethbridge Research Centre  
Liu, Chunji; CSIRO Plant Industry  
Wei, Yu-Ming; Triticeae Research Institute  
Zheng, You-Liang; Sichuan Agricultural University, |
| Keyword:        | Wheat, AGP-L, Gene structure, Starch accumulation, Expression |
Structure and expression analysis of genes encoding ADP-glucose pyrophosphorylase large subunit in wheat and its relatives

Xiao-Wei Zhang\textsuperscript{1+}, Si-Yu Li\textsuperscript{1+}, Ling-Ling Zhang\textsuperscript{1}, Qiang Yang\textsuperscript{1}, Qian-Tao Jiang\textsuperscript{1*}, Jian Ma\textsuperscript{1}, Peng-Fei Qi\textsuperscript{1}, Wei Li\textsuperscript{1}, Guo-Yue Chen\textsuperscript{1}, Xiu-Jin Lan\textsuperscript{1}, Mei Deng\textsuperscript{1}, Zhen-Xiang Lu\textsuperscript{2}, Chunji Liu\textsuperscript{3}, Yu-Ming Wei\textsuperscript{1*}, You-Liang Zheng\textsuperscript{4}

\textsuperscript{1}Triticaceae Research Institute, Sichuan Agricultural University, Chengdu, Sichuan, 611130, China

\textsuperscript{2}Lethbridge Research Centre, Agriculture and Agri-Food Canada, Lethbridge T1J 4B1, Canada

\textsuperscript{3}CSIRO Agriculture Flagship, 306 Carmody Road, St Lucia, Brisbane, QLD 4067, Australia

\textsuperscript{4}Key Laboratory of Southwestern Crop Germplasm Utilization, Ministry of Agriculture, Sichuan Agricultural University, Ya’an, Sichuan, 625014, China

\textsuperscript{+}The first and second authors contributed equally to this paper.

*Authors for correspondence:

Dr. Qian-Tao Jiang
E-mail: qiantaojiang@sicau.edu.cn
Tel: +86 28 86290958; Fax: +86 28 82650350

Dr. Yu-Ming Wei
E-mail: ymwei@sicau.edu.cn
Tel: +86-28-86290909; Fax: +86-28-82650350
Abstract

ADP-glucose pyrophosphorylase (AGP), which consists of two large subunits (AGP-L) and two small subunits (AGP-S), controls the rate-limiting step in the starch biosynthetic pathway. In this study, a full-length open reading frame (ORF) of AGP-L gene (named as Agp2) in wheat and a series of Agp2 gene sequences in wheat relatives were isolated. The coding region of Agp2 contained 15 exons and 14 introns including a full-length ORF of 1566 nucleotides, and the deduced protein contained 522 amino acids (57.8 kDa). Generally, the phylogenetic tree of Agp2 indicated that sequences from A and D genome donor species were most similar to each other and sequences from B genome donor species contained more variation. Starch accumulation and Agp2 expression in wheat grains reached their peak at 21 and 15 days post anthesis (DPA), respectively.

Keywords AGP-L · Expression · Gene structure · Starch accumulation · Wheat
Introduction

Common wheat (*Triticum aestivum* L.) is one of the most important cereal crops in the world. Starch is the most abundant storage reserve carbohydrate in wheat seed and comprises approximately 70% of wheat grain dry weight (Morell et al. 1995). The starch content influences wheat yield; starch also influences the processing quality of wheat flour (Blazek and Copeland 2008; Lee et al. 2001).

Synthesis of adenosine diphosphate glucose (ADP-glucose), the substrate for starch synthesis, is catalyzed by ADP-glucose pyrophosphorylase (AGP), which controls the rate-limiting step in the starch biosynthetic pathway (Hannah 1997). In plants, AGP is an allosteric heterotetramer, consisting of two large subunits (AGP-L) and two small subunits (AGP-S). In most cases, the small subunit is catalytic, while the large subunit affects interactions between the small subunit and 3-phosphoglyceric acid (3-PGA) (Ballicora et al. 2004). Recently, catalytic AGP-Ls have been discovered (Ventriglia et al. 2008). 3-PGA and orthophosphate (Pi) are positive and negative allosteric effectors for AGP, respectively (Hannah 1997; Preiss 1997).

In *Arabidopsis thaliana* (L.) mutants, both AGP activity and starch synthesis decreased owing to the lack of one of the subunits (Li and Preiss 1992; Lin et al. 1988). Low levels of AGP activity were correlated with low levels of starch in maize mutants (Dickinson and Preiss 1969). Mutations created by site-specific mutagenesis in the maize AGP-L gene (*shrunk*2) increased maize seed weight by 18% without changing starch content (Giroux et al. 1996). An altered maize AGP-L gene (*Sh2r6hs*) that was transformed into and expressed in wheat increased total plant biomass, seed yield including seed number and AGP activity in developing seed endosperm (Meyer et al. 2004; Smidansky et al. 2007).

Subcellular localization has demonstrated that AGP may be plastidial or cytosolic in different species. In maize endosperm, AGP-Ls and AGP-Ss are encoded by two separate genes (Giroux and Hannah 1994). In barley, plastidial and cytosolic AGP-Ss are produced from a single gene (Thorbjørnsen et al. 1996). In wheat, genes encoding AGP-L were designated as *Agp2* (Ainsworth et al. 1995). Up to now, our knowledge on plastidial and cytosolic AGP-L is insufficient. In previous reports, only a cDNA sequence and a gene sequence of *Agp2* from the 1B chromosome of the Chinese Spring cultivar were identified (Ainsworth et al. 1995; Thorneycroft et al. 2003). In addition, histochemical analysis of GUS expression initiated by the *Agp2B* promoter indicated that GUS expression was detected in the endosperm and aleurone but not in leaves (Thorneycroft et al. 2003). Wheat relatives like *Triticum urartu* Thumanjan ex Gandilyan, *Aegilops speltoides* Tausch, and *A. tauschii* Coss. are
highly similar to wheat because they or their close relatives were the donors of the wheat A, B, and D genomes, so they could contribute to research on functional genes. To date, no Agp2 sequences have been reported from relatives of common wheat.

Materials and methods

Plant material

*Triticum aestivum* cv. ‘Bobwhite 7’ (AABBDD, 2n = 42) and 18 accessions of *Triticum urartu* (A^u^A^u^ genome, 2n = 14), *Triticum monococcum* L. (A^m^A^m^, 2n = 14), *Aegilops speltoides* (SS, 2n = 14), *Aegilops bicornis* (Forssk.) Jaub. & Spach (S^b^S^b^, 2n = 14), *Aegilops longissima* Schweinf. & Musch. (S^l^S^l^, 2n = 14), *Aegilops sharonensis* Eig (S^sh^S^sh^, 2n = 14), and *Aegilops tauschii* (DD, 2n = 14) were kindly provided by USDA-ARS (http://www.arsgrin.gov) (Table 1). The accessions were grown in a glasshouse with a daytime temperature of 23°C and a nighttime temperature of 18°C, with a photoperiod of 16 h. Wheat seeds were collected at different developmental stages and stored at −80°C until DNA and RNA were extracted. Three replicates were used for all samples.

Isolation of the Agp2 open reading frame (ORF) and gene sequences

Genomic DNA and total RNA were extracted from endosperm at 20 days post anthesis (DPA), using the modified SDS method described by Aljanabi and Martinez (1997) and Trizol (Tiangen, Beijing, China), respectively. cDNA was obtained from reverse transcription using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). A set of primers (Table 2) was designed from the cDNA sequence of Agp2 in wheat (Ainsworth et al. 1995) to isolate the ORF or gene sequences of Agp2 from wheat and 18 diploid Triticeae accessions. PCRs were performed in 50-µL reactions containing 5 U PrimerStar HS DNA polymerase, 25 µL 2× PrimeSTAR GC Buffer (Mg^2+ Plus), 200 µM of each dNTP, 200 nM of each primer, and 100 ng of DNA. The PCR program included an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 10 min. Target bands were purified and cloned into the pEasy-T1 (Transgen, Beijing, China) vector and then transformed into *E. coli* (DH5α) (Transgen, Beijing, China). For each target band, three positive clones were selected and sequenced by Invitrogen (Shanghai, China).

Structure analysis of gene and protein
Intron-exon structures of Agp2 were analyzed from the cDNA and gene sequences with CLC Genomics Workbench 8.0.2. Predictions of conserved domains in proteins were performed with Conserved Domain Database Tools (CDD) (http://www.ncbi.nlm.nih.gov/cdd). Gene and protein sequences were aligned with DNAMan 7.0 (Lynnon Biosoft, San Ramon, CA, USA). AGP-L protein sequences from A. thaliana (NP_197423.1), Hordeum vulgare L. (CAX51355.1), Oryza sativa L. (ACJ71342.1), Phaseolus vulgaris L. (BAC66692.1), Sorghum bicolor (L.) Moench (XP_002456012.1), and Zea mays L. (AFP90368.1) were obtained from GenBank (http://www.ncbi.nlm.nih.gov/protein/). Amino acid secondary structures were predicted with PredictProtein (http://www.predictprotein.org) (Rost et al. 2004).

**Phylogenetic analysis of Agp2**

The phylogenetic tree was constructed from 18 accessions of Triticum urartu, Triticum monococcum L., Aegilops speltoides, Aegilops bicornis, Aegilops longissima, Aegilops sharonensis and Aegilops tauschii (Table 1). A dendrogram showing the evolutionary relationships of Agp2 genes was generated by CLC Main Workbench 7.6.4 (CLC bio, Aarhus, Denmark) with the maximum likelihood method (Felsenstein 1981).

**Starch accumulation pattern in wheat grains**

The total starch content in grains of Bobwhite 7 was measured at 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 DPA. The grains were ground in liquid nitrogen and assessed with the Total Starch Assay Kit (Megazyme, Bray, Wicklow, Ireland). Three replicates were used for all samples.

**Expression analysis by real-time quantitative PCR (RT-qPCR)**

The expression levels of Agp2 in Bobwhite 7 grains were detected at 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 DPA by RT-qPCR. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as the internal reference. Primers used for RT-qPCR are listed in Table 1; the E values of primers for Agp2 and GAPDH were 1.031 and 1.041, respectively. Total RNA extraction and cDNA synthesis were performed with the same methods outlined previously. The RT-qPCR program included an initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 5 s, 60°C for 15 s, 72°C for 15 s, and a melt curve stage. Relative expression levels of Agp2 were calculated using the Pfaffl method (Pfaffl 2001).

**Results**
Sequence analysis of Agp2 in wheat and related species

A full-length clone of the Agp2 ORF from Bobwhite 7 and sequences of Agp2 from its relatives were successfully isolated (Table 1). Gene structures and ORFs of Agp2 were identified from these sequences with CLC Genomics Workbench 8.0.2. (CLC bio). The coding region of Agp2 contained 15 exons and 14 introns (Fig. 1a). All species had an ORF of 1566 bp and the full gene ranged from 3264 to 3350 bp. The ORFs were 98.7% similar and the full gene was 93% similar across the species (Fig. S1). Most variation in the gene was located in intron 2 and exon 15. Intron 2 ranged from 425 to 508 bp.

Analysis of AGP-L protein structure

Agp2 from hexaploid wheat and its relatives encoded AGP-L consisting of 522 amino acid residues, including a putative transit peptide of 62 amino acids. The predicted secondary structure composition of AGP-L was 13.4% helix, 21.7% strand, and 65.0% loop (Fig. 1b). An alignment indicated that similarity of amino acid sequences between hexaploid wheat and its relatives was 99.4% and the similarity between Triticeae species and others (i.e., rice, maize, and Arabidopsis) was 63.5%. BlastP results against CDD databases indicated that conserved domains (i.e., oligomer interface, dimer interface, N-terminal domain interface, sulfate 1 binding site, ligand binding site) (Jin et al. 2005) were present in AGP-L from members of Triticeae and rice, maize, and Arabidopsis (Fig. 1b, Fig. 2).

Phylogenetic analysis of Agp2

The phylogenetic analysis of Agp2 from 18 Triticeae accessions showed that the evolutionary tree was divergent and contained two clades: one was for all of A, B, D genome donor species and the other was only for B genome donor species (Fig. 3). In the upper clade, PI 428170 (Triticum monococcum) was categorized as an individual branch; A and D genome donor species (except PI 428170) were clustered in a branch and all D genome donor species composed a subgroup; B genome donor species (A. bicornis, A. sharonensis and A. speltoides) composed the rest of branches. In the nether clade, 5 accessions of A. longissima, A. sharonensis and A. speltoides were clustered in different subgroups.

Starch accumulation and Agp2 expression analysis in wheat grain

Total starch content and total expression levels of Agp2 were measured in wheat grains at different developmental stages to analyze the relationship between starch biosynthesis and Agp2 expression. Starch content rose steadily after 6 DPA and reached a peak at 21 DPA before plateauing (Fig. 4a). Total expression levels of Agp2 rose slowly from 3 DPA to 9 DPA before they decreased slightly. The
expression levels rose rapidly from 12 DPA and reached a peak at 15 DPA before slowly declining (Fig. 4a). The starch accumulation curve was similar to the expression curve (Fig. 4b), and there was a high correlation ($R^2=0.89$) between starch accumulation and $Agp2$ expression.

Discussion

AGP controls the rate-limiting step in the starch biosynthetic pathway (Hannah 1997), and AGP-L increases the activity of AGP-S (Ballicora et al. 2004). In this study, a full-length ORF of $Agp2$ encoding AGP-L from common wheat Bobwhite 7 and sequences of $Agp2$ from its relatives were successfully isolated. As previously reported (Thorneycroft et al. 2003), the coding region of $Agp2$ isolated from these species contained 15 exons and 14 introns (an additional intron has been reported from the 3’ untranslated region), including a full-length ORF of 1566 nucleotides. The deduced protein includes 522 amino acids ($\approx 58$ kDa) and includes a putative transit peptide of 62 amino acids (6.5 kDa) (Ainsworth et al. 1995).

The large subunit of AGP in higher plant is less conserved (50%–60% identity) than the small subunit (85%–95% identity) (Smith-White and Preiss 1992), which probably reflects differences in regulation of small subunit sensitivity to allosteric activation and inhibition in different tissues and species (Ballicora et al. 2004). In this study, there was a low similarity (63.5%) between AGP-L protein sequences from $Triticeae$ and other species (rice, maize, and Arabidopsis), but a high similarity (99.4%) within members of $Triticeae$. The sequence conservation suggests that these $Triticeae$ species use AGP-L in a similar manner. On the whole, the phylogenetic analysis of $Agp2$ indicated that sequences from A and D genome donor species (except PI 428170) are most similar to each other and sequences from B genome donor species contain more variation. These sequences could be useful references for future research on AGP in wheat and its relatives.

Starch steadily accumulated from 6 DPA, but the peak starch content in Bobwhite 7 was recorded at 21 DPA (Fig. 4a), two weeks earlier than reported by Laudencia-Chingcuanco et al. (2007). Although the peak expression was observed at a similar time (15 DPA), the relative expression levels of $Agp2$ in Bobwhite 7 changed 8-fold and just 2.6-fold in earlier report (Stamova et al. 2010). These differences might be due to differences between wheat varieties or growth conditions. In addition, both our study and earlier studies (Laudencia-Chingcuanco et al. 2007; Stamova et al. 2010) showed that $Agp2$ expression levels reached their peak earlier than starch content peaked. In this study, $Agp2$ expression reached its peak one week earlier than starch content peaked, indicating that the
accumulation of starch grains is a protracted and complicated process. Despite the time lag, there was a high correlation ($R^2=0.89$) between starch accumulation and Agp2 expression.

Wheat relatives not only contribute to research on wheat functional genes, but also play important roles in wheat breeding. Sequences and expression of Agp2 in Triticaceae species were insufficiently known. Our study provides a better understanding of AGP-L, and should be a useful reference for further studies of AGP in wheat and its relatives.

Acknowledgments

This work was supported by the International Science & Technology Cooperation Program of China (No. 2015DFA30600), and the International Science & Technology Cooperation project of Sichuan province, China (2016HH0057).

References


Table 1 Plant materials used in this study.

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**Fig. 1** Gene and protein structures of AGP-L. **a** Gene structure of Agp2 and location of primers. Black boxes and thick lines represent exons and introns, respectively. Regions that vary within Agp2 from the species examined are indicated by red boxes. The positions and directions of primers are indicated by arrows. **b** Protein structure of AGP-L. Symbols in gray boxes indicate conserved features in AGP-L. TP = transit peptides, LBS = ligand binding site, DI = dimer interface, and NDI = N-terminal domain interface. The blue boxes represent strands and the red boxes represent helixes.

**Fig. 2** Sequence alignment of AGP-L amino acid sequences from 10 species. Amino acids that constitute conserved domains have been labeled. The violet regions indicate a similarity of 100%; the pink regions indicate a similarity ≥ 75%, the blue regions indicate a similarity was ≥ 50%, and the white regions indicate a similarity was < 50%.

**Fig. 3** Phylogenetic relationships from 18 Triticeae accessions based on Agp2 gene sequences. The bootstrap values were calculated based on 1000 replications and were showed as percentages.

**Fig. 4** Analysis of starch accumulation and Agp2 expression pattern. **a** Total starch contents and expression levels of Agp2 in wheat grains at different developmental stages. **b** Correlations between starch accumulation and Agp2 expression in grains of wheat.
Gene and protein structures of AGP-L. a Gene structure of AGP-L and location of primers. Black boxes and thick lines represent exons and introns, respectively. The varied regions in AGP-L from a range of species are indicated by red box. The position and direction of primers are indicated by arrows. b Protein structure of AGP-L. Symbols in gray boxed indicate conserved features in AGP-L. TP represent transit peptides, LBS represent ligand binding site, DI represent dimer interface and NDI represent N-terminal domain interface. The blue boxes represent strand structure and the red boxes represent helix structure.
Sequence alignment of AGP-L amino acid sequences from 10 species. Amino acids that constitute conserved domains have been labeled. The violet regions indicate a similarity of 100%; the pink regions indicate a similarity ≥ 75%, the blue regions indicate a similarity was ≥ 50%, and the white regions indicate a similarity was < 50%.

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Phylogenetic relationships from 18 Triticeae accessions based on Agp2 gene sequences. The bootstrap values were calculated based on 1000 replications and were showed as percentages.

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Analysis of starch accumulation and Agp2 expression pattern. a Total starch contents and expression levels of Agp2 in wheat grains at different developmental stages. b Correlations between starch accumulation and Agp2 expression in grains of wheat.

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Fig. S1 DNA sequence alignment of Agp2 from 18 Triticeae accessions. The violet regions indicate a similarity of 100%; the pink regions indicate a similarity ≥ 75%, the blue regions indicate a similarity was ≥ 50%, and the white regions indicate a similarity was < 50%.