Plant Biotechnol Rep (2017) 11:135–146
DOI 10.1007/s11816-017-0438-7

Online ISSN 1863-5474
Print ISSN 1863-5466

ORIGINAL ARTICLE

A soybean plastidic ATP/ADP transporter gene, *GmAATP*, is involved in carbohydrate metabolism in transgenic *Arabidopsis*

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Received: 18 January 2017 / Accepted: 24 April 2017 / Published online: 26 May 2017

Abstract The plastidic ATP/ADP transporter (AATP) imports adenosine triphosphate (ATP) from the cytosol into plastids, resulting in the increase of the ATP supply to facilitate anabolic synthesis in heterotrophic plastids of dicotyledonous plants. The regulatory role of *GmAATP* from soybean in increasing starch accumulation has not been investigated. In this study, a gene encoding the AATP protein, named *GmAATP*, was successfully isolated from soybean. Transient expression of *GmAATP* in *Arabidopsis* protoplasts and *Nicotiana benthamiana* leaf epidermal cells revealed the plastidic localization of *GmAATP*. Its expression was induced by exogenous sucrose treatment in soybean. The coding region of *GmAATP* was cloned into a binary vector under the control of 35S promoter and then transformed into *Arabidopsis* to obtain transgenic plants. Constitutive expression of *GmAATP* significantly increased the sucrose and starch accumulation in the transgenic plants. Real-time quantitative PCR (qRT-PCR) analysis showed that constitutive expression of *GmAATP* up-regulated the expression of phosphoglucomutase (*AtPGM*), ADP-glucose pyrophosphorylase (AGPase) small subunit (*AtAGPase-S1* and *AtAGPase-S2*), AGPase large subunit (*AtAGPase-L1* and *AtAGPase-L2*), granule-bound starch synthase (*AtGBSS I* and *AtGBSS II*), soluble starch synthases (*AtSSS I*, *AtSSS II*, *AtSSS III,* and *AtSSS IV*), and starch branching enzyme (*AtSBE I* and *AtSBE II*) genes involved in starch biosynthesis in the transgenic *Arabidopsis* plants. Meanwhile, enzymatic analyses indicated that the major enzymes (AGPase, GBSS, SSS, and SBE) involved in the starch biosynthesis exhibited higher activities in the transgenic plants compared to the wild type (WT). These findings suggest that *GmAATP* may improve starch content of *Arabidopsis* by up-regulating the expression of the related genes and increasing the activities of the major enzymes involved in starch biosynthesis. All these results suggest that *GmAATP* could be used as a candidate gene for developing high starch-accumulating plants as alternative energy crops.

Keywords *Arabidopsis* · Constitutive expression · *GmAATP* · Sucrose and starch content · Soybean

Introduction

Biofuel, which can decrease environmental damage by reducing the extraction and use of fossil fuels, is more and more important with the society's development. Breeding of non-food energy crops for economically viable production as environmentally friendly biofuels is on the way (Sanz-Barrio et al. 2013; Wang et al. 2016a, b). Due to the conversion of starch into fermentable sugars is relatively easy, starch has been taken as a major feedstock for first-generation biofuel production (Smith 2008; Sanz-Barrio et al. 2013). Therefore, it is vital to know how

Electronic supplementary material The online version of this article (doi:10.1007/s11816-017-0438-7) contains supplementary material, which is available to authorized users.

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carbohydrates are metabolized in plants, which could be of great help in the development of crops by means of enhancing starch synthesis, and in the improvement of biofuel production efficiency (Sanz-Barrio et al. 2013; Wang et al. 2017a).

As a main source of nutrition in the human and animal diet, starch is a major storage of carbohydrates in plants (Blennow et al. 2013), and plays a fundamental role in plant survival and adaptation to various environmental conditions (Skryhan et al. 2015). In plants, starch exists as an insoluble glucan, comprised of two glucose polymers: amylose and amylopectin. Amylose is mainly comprised of linear chains that are linked by α-1,4*O*-glycosidic bonds, whereas amylopectin is highly branched and contains 5–6% α-1,6*O*-glycosidic bonds to generate glucan branches of various lengths (Delvallé et al. 2005). Four major enzymes are involved in starch biosynthesis: ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching enzyme (SBE), and starch debranching enzyme (DBE) (Fujita et al. 2006).

It is the best option to enhance starch biosynthesis by increasing the adenosine triphosphate (ATP) supply to the plastid in below-ground storage organs (Smith 2008). ATP, as the basic energy currency in living cells, is needed for almost every step of biochemical reactions. Meanwhile, ATP is also an indispensable participant in the AGPase reaction, which is a rate-limiting step of starch biosynthesis catalyzing the formation of ADP-glucose (ADPG) (Jeon et al. 2010). ATP exchange between organelles and the cytosol is mediated by adenylate carrier proteins (ACPs). There are two types of ACPs. The mitochondrial ADP/ATP carrier (AAC), one type of ACPs, can export ATP, which is produced previously via oxidative phosphorylation in a one-to-one exchange of cytosolic ADP (Fiore et al. 1998). The mitochondrial AAC is a dimer, and each monomer contains 6 transmembrane helices (Winkler and Neuhaus 1999). Another type of ACPs is the plastidic ATP/ADP transporter protein (AATP) discovered in spinach chloroplasts (Winkler and Neuhaus 1999; Wang et al. 2016d, e). AATP, an important energy transporter, is generally found in the heterotrophic plastids (amyloplasts, chromoplasts, and leucoplasts) of higher and lower land plants (Heldt 1969; Schünemann et al. 1993; Emes and Neuhaus 1997; Möhlmann et al. 1998; Linka et al. 2003; Meng et al. 2005; Yuen et al. 2009). The further studies found that its main function is implicated in providing the plastid stroma with cytosolic ATP, to further participate in anabolic processes, such as starch and fatty acid synthesis (Möhlmann et al. 1998; Wang et al. 2016d, e).

There are a few studies about the function of *AATP* in starch biosynthesis in plants. Constitutive expression of the *Arabidopsis AtAATP1* increased ADPG level up to two-folds and starch content by 16–36% in potato tubers (Tjaden et al. 1998). In contrast, the antisense inhibition of the potato *StAATP* decreased ADPG level by 25–70% and starch content by 19–51% in potato tubers (Geigenberger et al. 2001). Ectopic expression of *SlAATP/StAATP* from tomato/potato increased the starch accumulation in the leaves of transgenic *Arabidopsis* plants (Wang et al. 2016d, 2017a). Overexpression of *IbAATP* increased starch and amylose content in transgenic sweetpotato (Wang et al. 2016e). In another report, down-regulation of the plastidic adenylate kinase (*StAK*) gene, interconverting ATP and AMP into ADP, caused a substantial effect on the adenylate pool size and the ADP-glucose level in potato tubers, further increasing the starch content (Regierer et al. 2002). These results indicate that the manipulation of the enzymes that modulate the ATP supply in plastids is an effective way to enhance starch biosynthesis in plants.

Although the function of *AATP* from other plant species has been well studied, the regulatory role of *GmAATP* (Genbank accession No. XP_003543665) from soybean in increasing starch accumulation still remained unknown. In this study, we isolated *GmAATP* from soybean and estimated its roles in transgenic *Arabidopsis*. Heterologous expression of *GmAATP* was found to significantly increase the starch accumulation in the leaves of transgenic plants, indicating a potential of *GmAATP* in the development of high starch-accumulating plants.

Materials and methods

Plant materials

Soybean (*Glycine max*) cultivar Williams 82 was employed for *GmAATP* gene cloning in this study. *Arabidopsis* [ecotype Columbia-0, wild type (WT)] was used as a model plant to investigate the functions of *GmAATP*.

Cloning of the soybean *GmAATP* gene

Total RNA was extracted from the leaves of Williams 82 with the RNAprep Pure Kit (Tiangen Biotech, Beijing, China). RNA samples were reverse-transcribed according to the instructions of the Quantscript Reverse Transcriptase Kit (Tiangen Biotech, Beijing, China). Based on the sequence of *GmAATP* (Genbank accession No. XP_003543665), we designed one gene-specific primers (GC-F/R) of reverse transcription PCR (RT-PCR) (Table S1) to obtain its full-length cDNA sequence. The genomic sequence of *GmAATP* was amplified with primers GA-F/R (Table S1) using genomic DNA extracted from the leaves of Williams 82 as a template.

Sequence analysis of the *GmAATP* gene

The open-reading frame (ORF) of the cloned *GmAATP* gene was predicted with ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The homology of GmAATP protein was identified using protein BLAST in the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The conserved domain of GmAATP protein was scanned by the InterProScan program (http://www.ebi.ac.uk/Tools/ pfa/iprscan/). The theoretical molecular weight and isoelectronic point (*p*I) were calculated using ProtParam tool (http://web.expasy.org/protparam/). The genomic structure of *GmAATP* was analyzed using the Spidey Program (http://www.ncbi.nlm.nih.gov/spidey/). The transmembrane helices in the GmAATP protein were detected using TMHMM Server (v. 2.0, http://www.cbs.dtu.dk/services/TMHMM/). For multiple sequence alignment analysis, the amino acid sequences of GmAATP and other AATP homologs from different plant species retrieved from NCBI were aligned using the DNAMAN software (Lynnon Biosoft, Quebec, Canada). Phylogenetic analysis was conducted with the MEGA4 software (http://www.megasoftware.net/).

Subcellular localization of GmAATP

The subcellular localization of GmAATP was predicted using TargetP (version 1.1) and ChloroP (version 1.1) (http://www.cbs.dtu.dk/services/). The ORF of *GmAATP* was cloned and then inserted into the pMDC83 expressing vector containing the green fluorescent protein gene (GFP) at the *Spe I* and *Asc I* restriction sites, under the control of the CaMV35S promoter and NOS (nopaline synthase) terminator (Table S1). *Arabidopsis* mesophyll protoplasts were used for the subcellular localization of GmAATP as described previously (Wang et al. 2016c). Meanwhile, the positive bacterial strains were used for transient expression in *Nicotiana benthamiana* leaf epidermal cells according to the method of Strasser et al. (2007). After co-cultivation, the transfected *Arabidopsis* protoplasts and agroinfiltrated tobacco leaves were visualized with a laser scanning confocal microscope (Nikon Inc., Melville, NY, USA).

Expression analysis of *GmAATP* in soybean

The response of *GmAATP* to exogenous sucrose was investigated based on the method of Wang et al. (2001) with some modifications. Leaves from Williams 82 were supplied with water (control) or 175 mM sucrose in darkness at 28 °C after being cultured in water in the dark for 1 day. Real-time quantitative PCR (qRT-PCR) was conducted to determine the transcript levels of *GmAATP* in the leaves at different timepoints (0, 3, 6, 12, 24, and 48 h) after treatment using SYBR Green PCR Master Mix (Tiangen Biotech, Beijing, China) and ABI PRISM 7500 (Software for 7500 and 7500 fast real-time PCR systems, V2.0.1, USA). Total RNA was isolated using the RNAprep Pure Plant Kit (Tiangen Biotech, Beijing, China) and first-strand cDNA was prepared by the Quantscript Reverse Transcriptase Kit (Tiangen Biotech, Beijing, China). The primers used to amplify *GmAATP* were listed in Table S1. The soybean *Gmβ-tubulin* gene (Genbank accession No. M21297) was used as an internal control (Rao et al. 2014) (Table S1).

Generation of transgenic *Arabidopsis* plants

The coding region of *GmAATP* was amplified using a pair of specific primers with terminal *Bam*H I and *Sac* I restriction sites, and then inserted into the same enzyme sites in pCAMBIA1301 to create the plant expression vector pCAMBIA1301-*GmAATP*, under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (NOS) terminator. This vector also contains β-glucuronidase (*gusA*) and hygromycin resistance (*hptII*) genes driven by the CaMV 35S promoter. Both pCAMBIA1301-*GmAATP* and the control vector (VC) pCAMBIA1301 were transformed into the *Agrobacterium tumefaciens* strain LBA4404 cells by the electroporation method for *Arabidopsis* transformation (Lou et al. 2007). Transgenic plants were produced according to methods described previously (Zhang et al. 2006). Transformants were selected based on their resistance to hygromycin (Hyg). Putative transformant seeds were germinated on agar-solidified MS (Murashige and Skoog 1962) medium containing 25 mg/L Hyg. Positive transgenic seedlings were grown in pots containing a mixture of soil, vermiculite, and humus (1:1:1, v/v/v) for T_2 and T_3 seed selection. The incubation and growth conditions of *Arabidopsis* were the same as described previously (Zhang et al. 2006).

Molecular confirmation of transgenic plants

The presence of *GmAATP* in hygromycin-resistant plants was assessed by PCR analysis using specific primers (Table S1) to amplify fragments of the *hptII* coding sequence. DNA was first extracted from *Arabidopsis* leaves according to the instructions of the EasyPure Plant Genomic DNA Kit (Transgen, Beijing, China). PCR amplifications were performed with an initial denaturation 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and final extension 72 °C for 10 min. PCR products were separated by electrophoresis on a 1.0% (w/v) agarose gel.

Assays of sucrose, ADPG, and starch content

The content of sucrose was measured using the anthrone method according to the method of Zhang (1977). Starch extraction and quantification were performed as described previously (Smith and Zeeman 2006). ADP-glucose (ADPG) content was determined exactly as described in Geigenberger et al. (1998). Seeds were grown on MS medium for 2 weeks and transferred to pots containing a mixture of soil, vermiculite, and humus (1:1:1, v/v/v). Plants were grown in growth chamber for 4 weeks at 22 °C under the standard long day conditions (14 h light and 10 h dark). Leaves of 4-week-old plants were harvested to determine starch content in light at 10–11 a.m. All treatments were performed in triplicate.

Measurement of photosynthesis

Photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate of the transgenic *Arabidopsis* plants were measured according to the methods of Jiang et al. (2013). The experiments were conducted at 10–11 a.m. of sunny days. All treatments were performed in triplicate.

Expression analysis of the related genes

The expression of *GmAATP* and starch biosynthesis-related genes was analyzed by qRT-PCR. Transgenic, VC, and WT plants were grown in pots for 4 weeks under normal condition. Total RNA was extracted from the leaves of these plants, respectively, using the RNAprep Pure Plant Kit (Tiangen Biotech, Beijing, China). RNA samples were reverse-transcribed using the Quantscript Reverse Transcriptase Kit (Tiangen Biotech, Beijing, China). The cDNA solution was used as templates for PCR amplification with gene-specific primers (Table S1). *Arabidopsis Atactin* gene (Genbank accession No. NM112764) was used as an internal control (Li et al. 2013) (Table S1).

AGPase, GBSS, SSS, and SBE activity analyses

The activity of four starch biosynthetic enzymes (AGPase, GBSS, SSS, and SBE) in the leaves of 4-week-old transgenic, VC, and WT plants was performed according to the method described by Nakamura et al. (1989). One unit of enzyme activity (AGPase, GBSS, and SSS) was defined as the formation of 1 nmol ADP per min at 30 °C and 1 unit of SBE activity was defined as the amount of enzyme required to increase the spectrophotometric absorbance by 1 unit in 1 min.

Fig. 1 Analyses of soybean plastidic ATP/ADP transporter protein GmAATP. **a** Structure of *GmAATP* and GmAATP. The GmAATP protein contained an ADP/ATP carrier protein domain. Exon/intron organization of *GmAATP* gene with location of exons (*brown boxes*) and introns (*blue lines*). **b** Multiple sequence alignment of the GmAATP protein with its homologous proteins from other plant species. The proteins are as follows: *Arabidopsis thaliana* (AtAATP1, NP_173003; AtAATP2, NP_178146), *Arachis ipaensis* (AiAATP, XP_016170350), *Cajanus cajan* (CcAATP, KYP59581), *Cicer arietinum* (CaAATP, XP_004506888), *Oryza sativa* (OsAATP1, AAX58120; OsAATP2, BAD24996), *Solanum lycopersicum* (SlAATP, XP_004235723), *Solanum tuberosum* (StAATP, Y10821), *Theobroma cacao* (TcAATP, XP_007023973), *Vigna radiata* (VrAATP, XP_014516633), and *Zea mays* (ZmAATP, NM_001154379). The ten transmembrane helices of GmAATP predicted by TMHMM are outlined. Five highly conserved motifs [FLKT, AELWG, FANQIT, AYG(I/V)S(I/V)NLVE, and (L/I)GKSGGA(L/I)IQ] present in the plant ATP/ADP transporter proteins are indicated in *yellow boxes*. **c** Phylogenetic tree of the GmAATP protein with its homologous proteins from other plant species. The branch lengths are proportional to distance

Statistical analysis

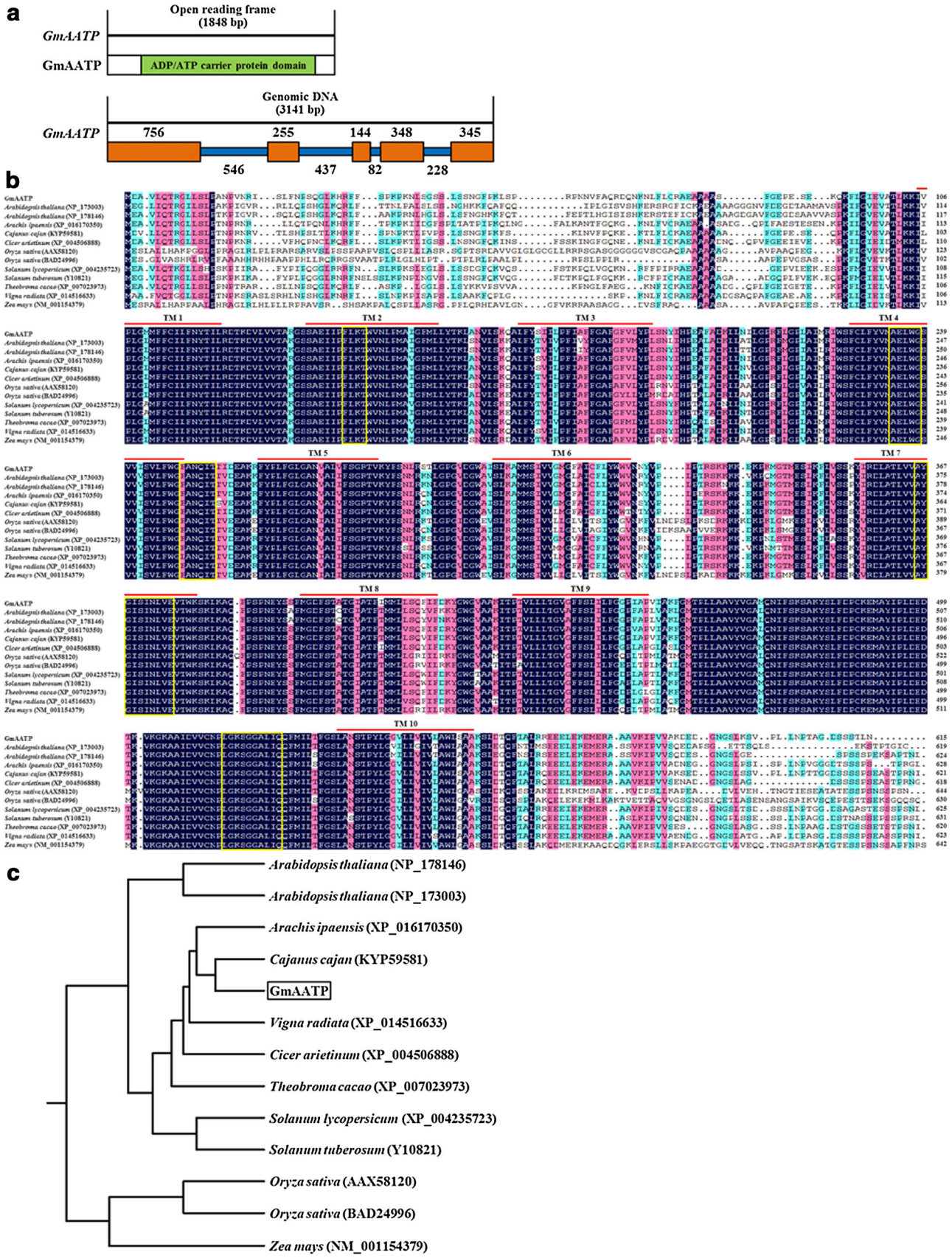
All experiments were repeated three times and the data presented as the mean ± standard error (SE). Where applicable, data were analyzed by Student's *t* test in a two-tailed analysis. Values of $P < 0.05$ or <0.01 were considered to be statistically significant difference.

Results

Cloning and sequence analysis of *GmAATP*

The *GmAATP* gene was cloned by RT-PCR. The *GmAATP* gene contained a 1848 bp ORF, encoding a polypeptide of 615 amino acids, with a molecular weight of 67.59 kDa and a theoretical isoelectric point (*p*I) of 9.53. Sequence analysis via the InterProScan program (http://www.ebi.ac.uk/Tools/pfa/iprscan/) showed that the GmAATP protein contained an ADP/ATP carrier protein domain (Fig. 1a). The genomic sequence of *GmAATP* was 3141 bp long and contained 5 exons and 4 introns (Fig. 1a). According to the online software TMHMM (v. 2.0), GmAATP contained 10 transmembrane (TM) helices (Fig. 1b), which is one of the main structural characteristics of the AATP family (Winkler and Neuhaus 1999; Yuen et al. 2009).

The deduced amino acid sequence alignment showed that GmAATP was highly conserved and homologous to the AATPs from *Arabidopsis thaliana* (AtAATP1, NP_173003; AtAATP2, NP_178146), *Arachis ipaensis* (AiAATP, XP_016170350), *Cajanus cajan* (CcAATP, KYP59581), *Cicer arietinum* (CaAATP, XP_004506888), *Oryza sativa* (OsAATP1, AAX58120; OsAATP2, BAD24996), *Solanum lycopersicum* (SlAATP, XP_004235723), *Solanum*


a
Open reading frame
(1848 bp)
GmAATP
ADP/ATP carrier protein domain
Genomic DNA
(3141 bp)
GmAATP
756
255
144
348
345
546
437
82
228
b
TM 1
TM 2
TM 3
TM 4
TM 5
TM 6
TM 7
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TM 9
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c
Arabidopsis thaliana (NP_178146)
Arabidopsis thaliana (NP_173003)
Arachis ipaensis (XP_016170350)
Cajanus cajan (KYP59581)
GmAATP
Vigna radiata (XP_014516633)
Cicer arietinum (XP_004506888)
Theobroma cacao (XP_007023973)
Solanum lycopersicum (XP_004235723)
Solanum tuberosum (Y10821)
Oryza sativa (AAX58120)
Oryza sativa (BAD24996)
Zea mays (NM_001154379)

tuberosum (StAATP, Y10821), *Theobroma cacao* (TcAATP, XP_007023973), *Vigna radiata* (VrAATP, XP_014516633), and *Zea mays* (ZmAATP, NM_001154379), especially in the TM regions (Fig. 1b). Further homology analyses using DNAMAN showed that GmAATP had 66.05–90.24% amino acid identity to OsAATP1, OsAATP2, ZmAATP, AtAATP1, AtAATP2, StAATP, SlAATP, TcAATP, VrAATP, CaAATP, AiAATP, and CcAATP (Fig. 1b). Five highly conserved motifs [FLKT, AELWG, FANQIT, AYG(I/V)S(I/V)NLVE, and (L/I)GKSGGA(L/I)IQ] present in the plant and bacterial ATP/ADP transporter proteins were also identified (Fig. 1b), indicating the importance of these motifs in determining AATP function (Möhlmann et al. 1998; Meng et al. 2005). Phylogenetic analyses found that GmAATP had a close relationship with the predicted protein products of *Cicer arietinum* (Fig. 1c).

Plastid localization of GmAATP

Computer programs, such as TargetP and ChloroP, suggested a chloroplastic localization of GmAATP. The subcellular localization of GmAATP protein was examined in *Arabidopsis* mesophyll protoplasts and *Nicotiana benthamiana* leaf epidermal cells. An GmAATP-green fluorescent protein (GFP) construct (Fig. 2a) was, therefore, transiently expressed in *Arabidopsis* mesophyll protoplasts and tobacco (*Nicotiana benthamiana*) epidermal cells and visualized using a laser scanning confocal microscope (Nikon Inc., Melville, NY) (Fig. 2b). In the *Arabidopsis* protoplasts, GmAATP was observed as scattered patches and co-localized with the autofluorescence of chloroplasts (Fig. 2b). In the tobaccoepidermal cells, signals associated with the GmAATP were also localized to the chloroplasts (Fig. 2b). These results are in good agreement with the plastidic localization of GmAATP.

Expression analysis of *GmAATP* in soybean

To examine whether the expression of *GmAATP* responded to exogenous sucrose, we floated young leaves in water (control) or 175 mM sucrose for up to 48 h. The results showed that no induction of the *GmAATP* transcript occurred when the leaves were treated with water as control (Fig. 3). In contrast, the presence of exogenous sucrose could significantly enhance the accumulation of the *GmAATP* transcript, reaching the highest level (2.21-folds) at 12 h and declining thereafter (Fig. 3).

Increased sucrose and starch content in *Arabidopsis* expressing *GmAATP*

The ORF of *GmAATP* was heterologously expressed in *Arabidopsis* (Col-0, WT) using the binary vector pCAMBIA1301-*GmAATP* (Fig. 4a). Nine independent transgenic lines constitutively expressing *GmAATP* (T_1 generation) were obtained by Hyg resistance selection, named #1–#9,

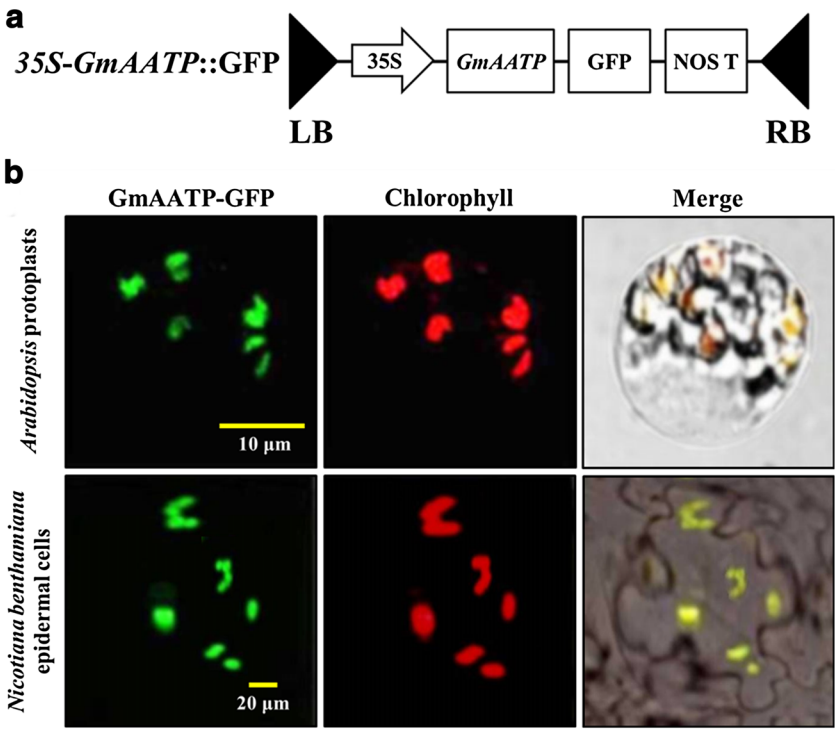

Fig. 2 Subcellular localization of GmAATP protein. **a** Schematic diagram of the 35S-GmAATP::GFP vector construct. **b** *Upper row* co-localization of GmAATP-GFP signals with autofluorescence of chloroplasts in *Arabidopsis* mesophyll protoplast. *Scale bar* 10 μm. *Lower row* confocal images of green fluorescence of GmAATP-GFP fusion protein are shown in *Nicotiana benthamiana* leaf hypodermal cells, which co-localized with the autofluorescence of chloroplasts. *Scale bar* 20 μm

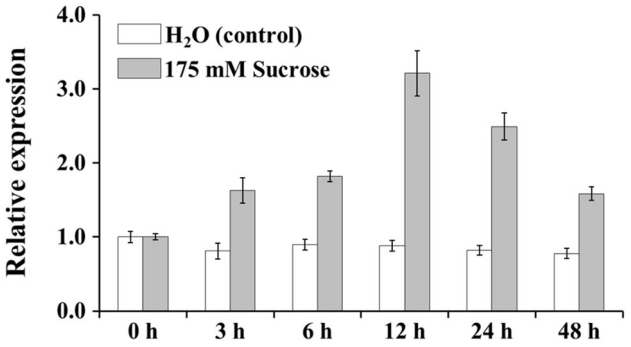

Fig. 3 Effect of exogenous sucrose treatment on *GmAATP* transcript accumulation in leaves. The results were expressed as relative values with respect to 0 h, which were set to 1.0. The soybean *Gmβ-tubulin* gene was used as an internal control. Data are presented as the mean ± SE ($n = 3$)

respectively, and their progenies (T_3 generation) were generated. PCR analyses showed that these plants were transgenic plants (Fig. 4b). qRT-PCR analyses showed that the highest expression levels of *GmAATP* were observed in transgenic lines #1, #4, #5, and #7, while no transgene expression was observed in VC and WT (Fig. 4c). Therefore, transgenic lines #1, #4, #5, and #7 were selected for further analyses.

2-week-old WT, VC, and transgenic plants (lines #1, #4, #5, and #7) were grown in pots under normal condition for 4 weeks. Expression of *GmAATP* did not change the growth of transgenic plants, since no morphological variations were observed between WT, VC, and the transgenic plants under normal conditions. The sucrose, ADPG, and starch content in *GmAATP* expressing plants were increased by 61–128, 42–106, and 37–82%, respectively, compared to that in WT (Figs. 5, 6, 7), whereas no significant difference was observed between VC and WT plants (Figs. 5, 6, 7).

Enhanced photosynthesis in *Arabidopsis* expressing *GmAATP*

Photosynthesis in the leaves of the transgenic plants was measured. The results showed that photosynthetic rate, stomata conductance, intercellular CO_2 concentration, and transpiration rate were significantly higher in the transgenic plants than in WT and were increased by 19–36, 41–76,

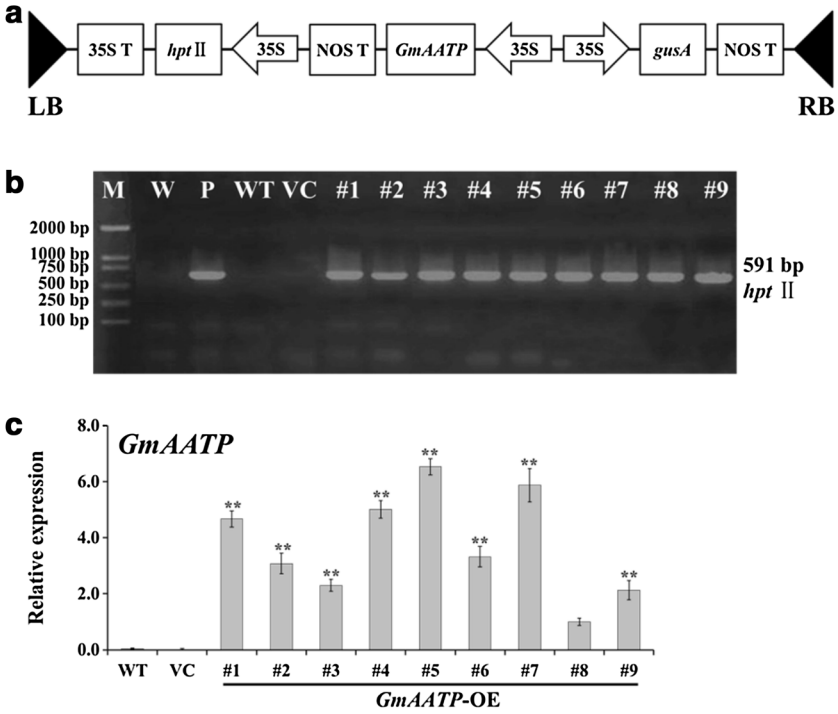

Fig. 4 Molecular confirmation of transgenic plants. **a** Schematic diagram of the T-DNA region of binary plasmid pCAMBIA1301-*GmAATP*. LB, left border; RB, right border; *hptII*, hygromycin phosphotransferase II gene; *GmAATP*, soybean plastidic ATP/ADP transporter protein gene; *gusA*, β-glucuronidase gene; 35S, cauliflower mosaic virus (CaMV) 35S promoter; 35S T, CaMV 35S terminator; NOS T, nopaline synthase terminator. **b** PCR analysis of *GmAATP* expressing *Arabidopsis* plants. Lane M, DL2000 DNA marker; Lane W, water as negative control; Lane P, plasmid pCAMBIA1301-*GmAATP* as positive control; Lane WT, wild type; VC, control vector; Lanes #1–#9, different transgenic *lines*. **c** Expression levels of *GmAATP* in different transgenic *lines*. The *Arabidopsis actin* gene was used as an internal control. Data are presented as mean ± SE ($n = 3$). * and ** indicate a significant difference from that of WT at $P < 0.05$ and < 0.01, respectively, by Student's *t* test

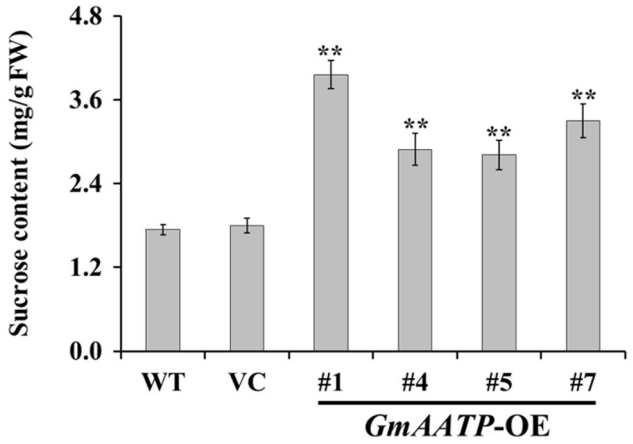

Fig. 5 Sucrose content assay in the leaves of WT and transgenic plants. 4-week-old WT and transgenic plants grown under normal condition were used. Data are presented as mean ± SE ($n = 3$). * and ** indicate a significant difference from that of WT at $P < 0.05$ and < 0.01, respectively, by Student's t test

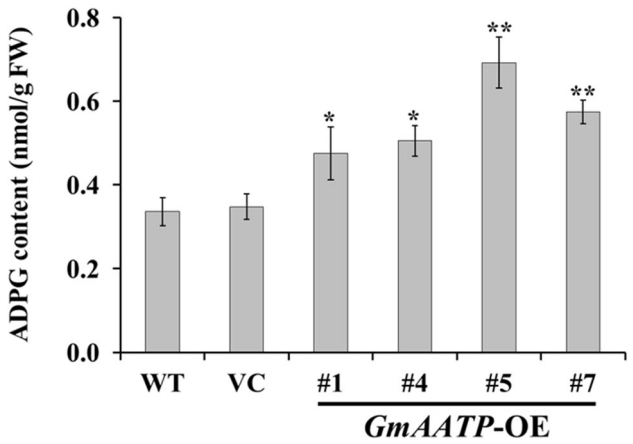

Fig. 6 ADPG content assay in the leaves of WT and transgenic plants. 4-week-old WT and transgenic plants grown under normal condition were used. Data are presented as mean ± SE ($n = 3$). * and ** indicate a significant difference from that of WT at $P < 0.05$ and < 0.01, respectively, by Student's t test

25–48, and 66–101%, respectively (Fig. 8), whereas no significant difference was observed between VC and WT plants (Fig. 8).

Up-regulation of starch biosynthetic genes in *Arabidopsis* expressing *GmAATP*

To dissect how expression of *GmAATP* increased starch content in transgenic plants, the transcript levels of 13 starch biosynthetic genes in WT, VC, and transgenic plants (lines #1, #4, #5, and #7) were examined by qRT-PCR (Fig. 9). Expression of the genes related to starch biosynthesis pathway, such as phosphoglucomutase (*AtPGM*),

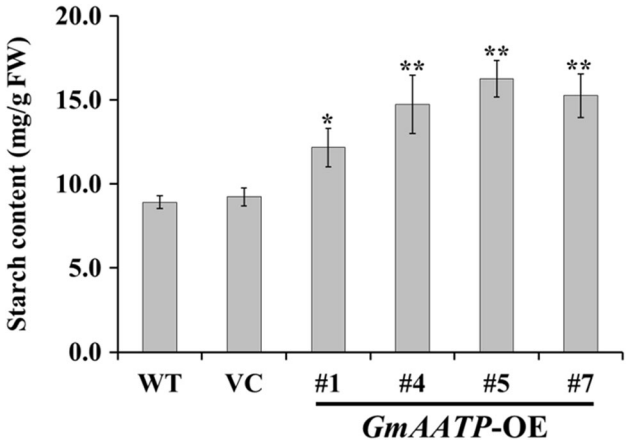

Fig. 7 Starch content assay in the leaves of WT and transgenic plants. 4-week-old WT and transgenic plants grown under normal condition after different times of light were used. Data are presented as mean ± SE ($n = 3$). * and ** indicate a significant difference from that of WT at $P < 0.05$ and < 0.01, respectively, by Student's t test

AGPase small subunit (*AtAGPase-S1* and *AtAGPase-S2*), AGPase large subunit (*AtAGPase-L1* and *AtAGPase-L2*), granule-bound starch synthase (*AtGBSS I* and *AtGBSS II*), soluble starch synthases (*AtSSS I*, *AtSSS II*, *AtSSS III,* and *AtSSS IV*), and starch branching enzyme (*AtSBE I* and *AtSBE II*) was up-regulated in transgenic plants (Fig. 9). These results indicate that *GmAATP* might be involved in the regulation of starch biosynthetic processes.

Enhanced enzyme activities in *Arabidopsis* expressing *GmAATP*

The activity of major enzymes (AGPase, GBSS, SSS, and SBE) involved in starch biosynthesis was also investigated in the leaves of WT, VC, and transgenic plants (lines #1, #4, #5, and #7) (Fig. 10). These results showed that the activity of these enzymes was significantly enhanced in transgenic plants compared to that in the WT (Fig. 10). While it was not possible to resolve the biochemical activities of individual isoforms, the increases in enzyme activity in transgenic plants were consistent with the increased transcription levels of their corresponding genes. All these results demonstrate that *GmAATP* has significant effects on the activities of AGPase, GBSS, SSS, and SBE in transgenic *Arabidopsis* plants.

Discussion

Starch is the major dietary source of carbohydrates and the most abundant storage of polysaccharide in higher plants. The *AATP* gene has been shown to be involved in starch

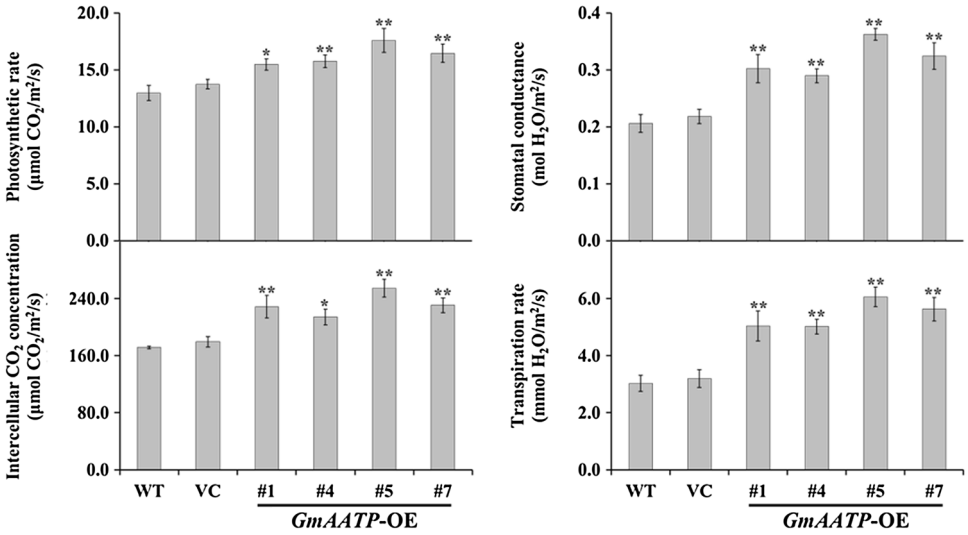

Fig. 8 Photosynthetic rate, stomata conductance, intercellular CO_2 concentration, and transpiration rate assays in the leaves of WT and transgenic plants. 4-week-old WT and transgenic plants grown under normal condition were used. Data are presented as mean ± SE ($n = 3$). * and ** indicate a significant difference from that of WT at $P < 0.05$ and < 0.01, respectively, by Student's t test

accumulation in plants. In potato tubers, heterologous expression of the *Arabidopsis AtAATP1* increased the level of ADP-glucose (ADPG) up to twofold and the starch content by 16–36% (Tjaden et al. 1998). In contrast, the antisense inhibition of the potato *StAATP1* reduced the level of ADP-glucose by 25–70% and the starch content by 19–51% (Geigenberger et al. 2001). The transgenic *Arabidopsis*/sweetpotato plants expressing *SlAATP*/*StAATP*/*IbAATP* exhibited the increased starch content (Wang et al. 2016d, e, 2017a).

In the current study, we isolated the *GmAATP* gene from soybean, and sequence analysis indicated that *GmAATP* was a member of the plastidic ATP/ADP transporter gene family (Fig. 1). We also found that the transcript level of *GmAATP* was induced by exogenous sucrose treatment in soybean (Fig. 3), indicating that *GmAATP* may be tightly associated with starch biosynthesis in soybean and may serve as a means to reprogram chloroplasts into starch-accumulating ATP-importing storage plastids (Koch 1996; Reiser et al. 2004). Once the sugar level in the cytosol is higher than actually needed, *GmAATP* will be strongly induced, which couples this high sugar level by importing more ATP into the plastids, thus triggering the starch biosynthesis. Our work indicated that constitutive expression of *GmAATP* significantly increased the sucrose and starch accumulation in the transgenic *Arabidopsis* plants (Figs. 5, 7).

In heterotrophic organs, the carbon precursors and ATP needed for anabolic processes are mainly imported from the cytoplasm (Emes and Neuhaus 1997; Winkler and Neuhaus 1999). The levels of ATP or ADP-glucose (ADPG) are key targets for genetic manipulation when attempting to accelerate starch biosynthesis in amyloplasts. ATP uptake by amyloplasts is mediated by a plastidic ATP/ADP transporter (AATP) (Schünemann et al. 1993). Constitutive expression of *AtAATP1* in potato resulted in the more imported ATP levels from the cytosol into the stroma, which facilitated the synthesis of ADPG, leading to an increase in starch accumulation in tubers (Tjaden et al. 1998). In this study, the ADPG and starch content in the transgenic plants were significantly enhanced (Figs. 6, 7). Constitutive expression of *GmAATP* could also increase ATP import levels into amyloplasts, energizing the pivotal AGPase reaction in starch biosynthesis (Fig. 7). We found that the expression of *AtAGPase-S1* and *AtAGPase-S2*, encoding AGPase small subunit, and *AtAGPase-L1* and *AtAGPase-L2*, encoding AGPase large subunit, was up-regulated in transgenic plants (Fig. 9). Consistently, enzymatic activity of AGPase was also significantly increased in the *GmAATP* expressing *Arabidopsis* plants (Fig. 10). These results showed that ADPG, as a large amount of the ultimate precursor for starch synthesis, could be more accumulated (Fig. 6). Meanwhile, the consumption of glucose-1-phosphate (G-1-P) in the AGPase reaction required the accelerated conversion of glucose-6-phosphate (G-6-P) to G-1-P, which was catalyzed by plastidic phosphoglucomutase (PGM) (Harrison et al. 2000), and consequently, the transcription of *AtPGM* was up-regulated (Fig. 9). These results suggested that the expression of *GmAATP* enhanced the starch biosynthesis due to the increased ATP supply into amyloplasts, which further increased the production of precursors (ADPG and G-1-P) and the expression of starch biosynthesis-related genes (Fig. 11).

The higher level of starch content is related to the increased expression of starch biosynthesis genes (Delvallé et al. 2005; Jiang et al. 2013; Wang et al. 2016a, b, 2017a, b). Starch synthase can be grouped into

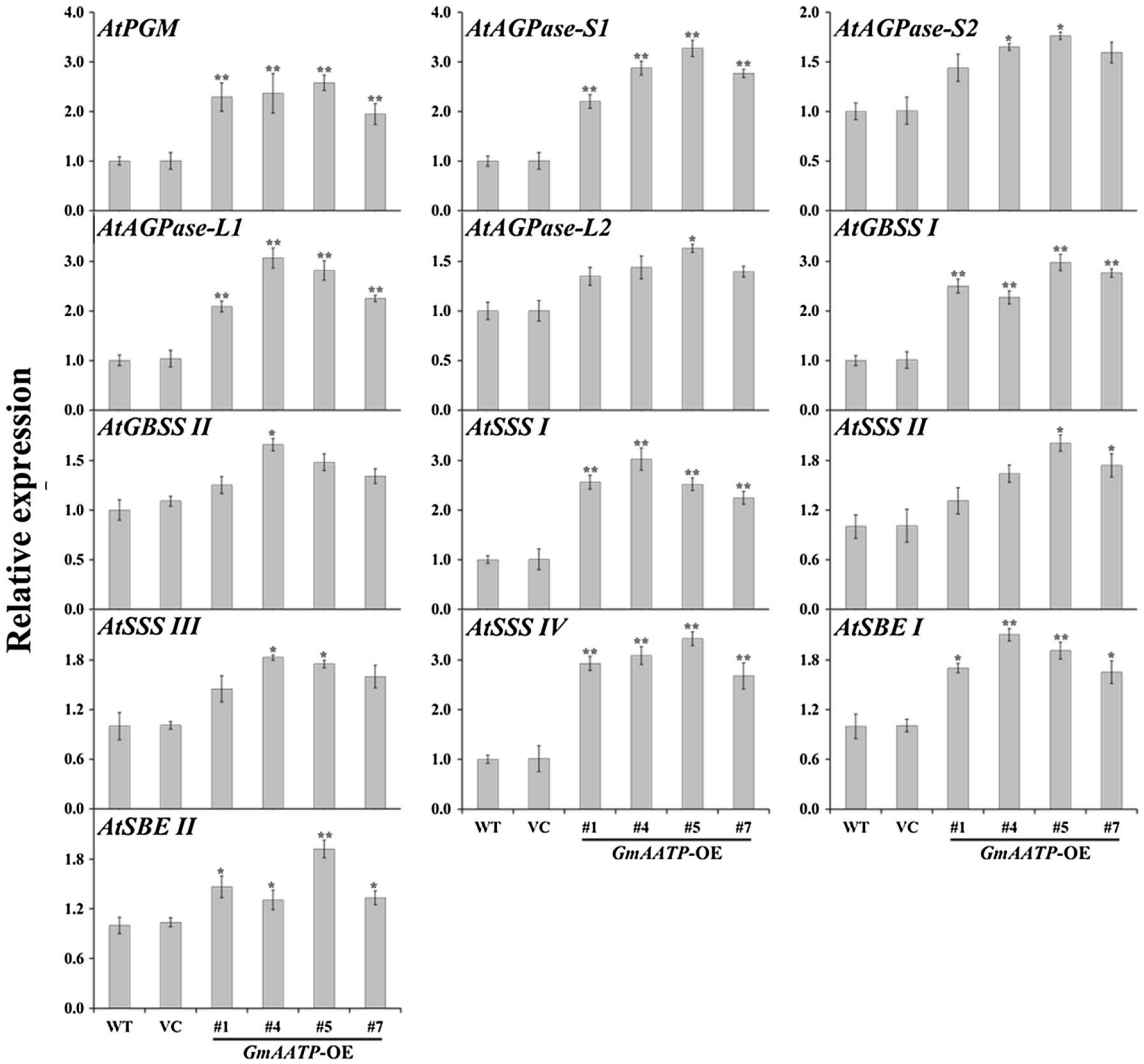

Fig. 9 Transcript levels of starch biosynthesis genes in the leaves of WT and transgenic plants. 4-week-old WT and transgenic plants grown under normal condition were used. The *Arabidopsis actin* gene was used as an internal control. Results are expressed as relative values with respect to WT, which was set to 1.0. Data are presented as mean ± SE ($n = 3$). * and ** indicate a significant difference from that of WT at $P < 0.05$ and < 0.01, respectively, by Student's *t* test

five types of granule-bound starch synthases (GBSS) and four types of soluble starch synthases (SSS): SSS I, SSS II, SSS III, and SSS IV (Delvallé et al. 2005; Fujita et al. 2006; Szydlowski et al. 2009). A large body of evidence has illustrated that up-regulation of these genes could increase starch accumulation in plants (Burton et al. 2002; Bustos et al. 2004; Roldan et al. 2007; Szydlowski et al. 2009; Jiang et al. 2013; Wang et al. 2016a, b, d, e, Wang et al. 2017a, b). In our study, the activities of the major enzymes (GBSS, SSS, and SBE) involved in starch biosynthesis were increased in transgenic plants (Fig. 10). Consistent with this phenomenon, systematic up-regulation of these genes (*AtGBSS I*, *AtGBSS II*, *AtSSS I*, *AtSSS II*, *AtSSS III*, *AtSSS IV*, *AtSBE I,* and *AtSBE II*) involved in starch biosynthesis pathway was also observed in transgenic plants (Fig. 9). Therefore, it is thought that constitutive expression of *GmAATP* increases the expression of the genes and the activity of the major enzymes involved in starch biosynthesis, resulting in the improved starch accumulation in transgenic plants (Fig. 11).

In conclusion, the *GmAATP* gene was successfully isolated from soybean. Constitutive expression of *GmAATP* was found to significantly increase starch content in transgenic *Arabidopsis* plants by the up-regulation of starch biosynthetic-related genes. Our results suggest that *GmAATP* plays a crucial role in starch metabolism, and has great potential in the engineering of alternative energy crop plants with improved starch accumulation.

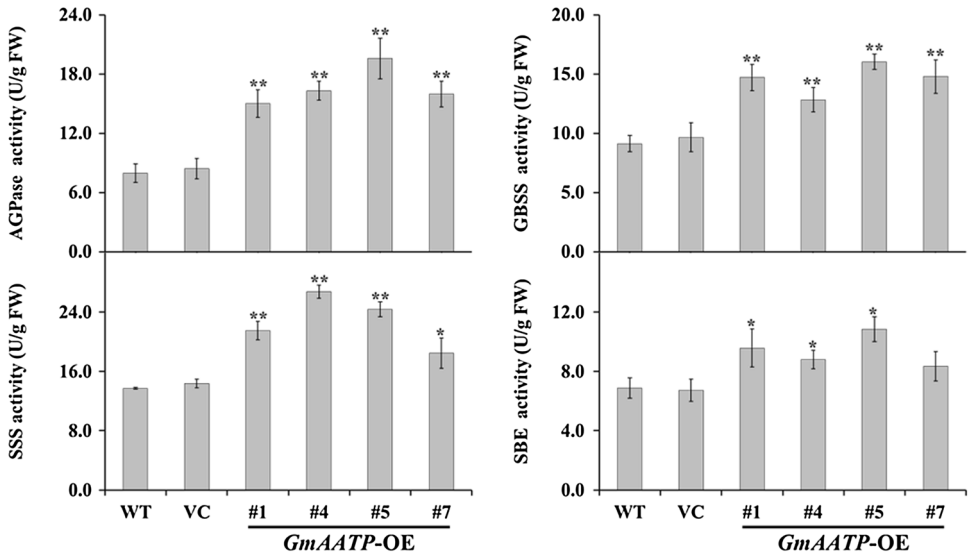

Fig. 10 AGPase, GBSS, SSS, and SBE enzyme activity assays in the leaves of WT and transgenic plants. 4-week-old WT and transgenic plants grown under normal condition were used. Data are presented as mean ± SE ($n = 3$). * and ** indicate a significant difference from that of WT at $P < 0.05$ and < 0.01, respectively, by Student's t test

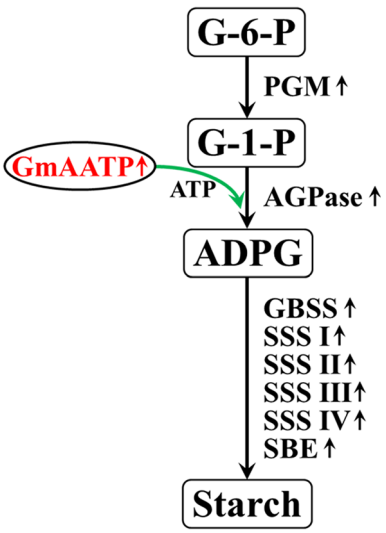

Fig. 11 Proposed model of the regulatory network of *GmAATP* in starch accumulation. Biosynthesis pathway is shown with solid arrows and regulatory interactions with broken arrows. *Upward arrow* indicates up-regulation of the relative enzyme encoding genes

Acknowledgements This work was supported by the Research Project of Talent Introduction of Huaiyin Institute of Technology (Z301B16534), the Natural Science Foundation of Jiangsu Province of China (BK2013256), and the National Spark Plan Project of China (2014GA69002).

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