Silencing *GhAGPL1* Reduces the Quality and Quantity of Corms and Cormels in Gladiolus

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ABSTRACT. Starch accumulation is important during corm development. ADP-glucose pyrophosphorylase (AGPase) is the rate-limiting enzyme in starch synthesis. AGPL is the large subunit of AGPase. Here, we isolated and characterized the large subunit of AGPase gene *GhAGPL1* in gladiolus (*Gladiolus hybridus*). *GhAGPL1* was highly expressed in sink organs (corms and cormels). The expression of *GhAGPL1* was induced by glucose, sucrose, and mannitol, and it was repressed by abscisic acid (ABA). Overexpression of *GhAGPL1* in the arabidopsis (*Arabidopsis thaliana*) *apl1* mutant resulted in complementation of AGPase activity and thus starch synthesis. Silencing *GhAGPL1* in gladiolus decreased the transcript level of *GhAGPL1* and *GhSus*, and resulted in the reduction of AGPase activity and starch content in gladiolus corm and cormel. Meanwhile, sucrose content was higher in *GhAGPL1*-silenced corm. Surprisingly, silencing *GhAGPL1* in gladiolus produced smaller corms and fewer number of cormels. Overall, *GhAGPL1* contributed to the quality and quantity of gladiolus corms and cormels.

Worldwide, gladiolus is a commercially sold cut flower, whose sales rank the second highest among bulb flowers (Wu et al., 2015). Corms and cormels are the only materials used for commercial propagation. Yearly, gladiolus produces new corms and cormels, whereas the mother corm undergoes atrophy. The new corm forms at the shoot base, just above the old corm (mother corm); then, after 11 weeks, stolons develop from the axillary buds situated between the mother and new corms; finally, the cormels are formed at the tips of branched stolons in weeks 14 to 17 (Chen and Yi, 2004). The new corm (diameter >2.4 cm) is used for the production of cut flowers. The quality of corms is correlated with flowering and corm yield (Sharma and Gupta, 2003). The cormel is used for new corm reproduction. Therefore, the quality of the cut flower is determined by the quality of the corm, and the production of cut flower is determined by the quantity of corms and cormels. However, corm degeneration is a major problem in gladiolus commercial cultivation. Thus, the production of cut flowers benefits from improving the yield of corms and cormels.

Studies have shown that phytohormones like ABA, gibberellin, cytokinins, and auxins are involved in corm development (Dantu and Bhojwani, 1995). Macronutrients, such as nitrogen and phosphorus, also contribute to corm and cormel yields (Butt, 2005). In addition, starch accounts for half of dry weight in corms (Seng et al., 2016). Starch is a major reserve carbohydrate, and also functions to balance source and sink metabolites (Yu et al., 2015). Sucrose produced in leaves is transported into underground organs (new corm and cormels) where it is used

for starch synthesis by AGPase. Starch synthesis and accumulation affect the growth of underground organs, including corm enlargement, stolon swelling, and the production of cormels.

AGPase is a rate-limiting enzyme in starch synthesis, which catalyzes the conversion of glucose-1-phosphate (Glc-1-P) and ATP to pyrophosphate and ADP-glucose (Kang et al., 2013; Seng et al., 2016). In higher plants, AGPase functions as a heterotetramer, with two different subunits (large subunits AGPL and small subunits AGPS). AGPS is proposed to play a dominant role in catalysis. Many studies have shown that both AGPL and AGPS influence catalytic and allosteric regulatory properties of the enzyme, suggesting diverse catalytic properties exist in higher plants. AGPLs are involved in seed development (Tuncel et al., 2014a), thermotolerance (Saripalli and Gupta, 2015), redox (Tuncel et al., 2014b), and tuberization (Müller-Röber et al., 1992). However, AGPL function in corm development is not yet well characterized.

The large subunits of AGPase, AGPLs, are proposed to have evolved by divergence, specialization, and subfunctionalization from a common ancestor (Ventriglia et al., 2008). Different large subunits confer distinct regulatory properties to AGPase, according to the necessities for starch synthesis in a given tissue (Crevillén et al., 2003). In arabidopsis, AGPL1 is the main large subunit in source tissues, whereas AGPL2 and AGPL3 are the main isoforms in sink tissues (Crevillén et al., 2005). In addition, different large subunit shows different response to sugar, light, and osmotic agents (Crevillén et al., 2005; Sokolov et al., 1998).

In this study, we isolated *GhAGPL1* gene from gladiolus corm. We explored whether *GhAGPL1* can function in arabidopsis, and how it influences gladiolus corm development. Our results provide definitive evidence that starch biosynthesis during corm development is regulated by *GhAGPL1*. Moreover, silencing *GhAGPL1* reduced the quality and quantity of gladiolus corms and cormels.

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Materials and Methods

PLANT MATERIALS AND GROWTH CONDITIONS. *Gladiolus* (cv. Rose Supreme) was planted in the Science Research Garden at China Agricultural University. Organs were taken from 25-week-old plants to detect *GhAGPL1* expression. Cormels (0.6–1.0 cm diameter) were cultured at 22 °C in growth chamber with 16/8 h light/dark photoperiod, with light intensity of 54 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. For abiotic stress, cormels were placed in a petri plate in sugar-free medium containing 200 mM mannitol, 200 mM glucose, 200 mM sucrose, or 100 μM ABA in the dark for 36 h.

Arabidopsis seeds were sterilized with 1% (v/v) NaClO for 20 min, washed five times with sterile water, and placed on Murashige and Skoog (MS) medium. Following stratification for 3 d at 4 °C in the dark, the seeds were transferred into a growth chamber and were germinated at 22 °C with 16/8 h light/dark photoperiod, under 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity. Seedlings were transferred to forest soil and vermiculite (1:1 v/v) in the growth chamber after 7 d. *Nicotiana benthamiana* seeds were planted into pots and cultured under the same conditions as the arabidopsis.

ISOLATION AND SEQUENCE ANALYSIS OF *GhAGPL1*. RNA extraction kit (Tiangen, Beijing, China) was used to extract total RNA from gladiolus cormels. Subsequently, cDNA was synthesized by M-MLV reverse transcriptase (TaKaRa, Dalian, China) with a 3' adaptor [5'-CCAGTGAGCAGAGTGAC-GAGGACTCGAGCTCAAGC(T)₁₇-3']. Degenerate primers were designed for *GhAGPL1* cloning based on conserved regions of homologous *AGPL1* from other species. The amplified product was used to clone the full-length *GhAGPL1* by the rapid amplification of cDNA ends (RACE) according to the manufacturer's protocol (Clontech, Mountain View, CA). All primers are listed in Supplemental Table 1.

ClustalX1.8 (Thompson et al., 1997) and BioEdit7.0 (Hall, 1999) were used to perform multiple amino acid alignments, and phylogenetic tree was constructed by the neighbor-joining method using the MEGA5.0 software (Tamura et al., 2011). Accession numbers for public sequences used in the phylogenetic analysis are listed in Supplemental Table 2.

SUBCELLULAR LOCATION OF *GhAGPL1*-GFP FUSION PROTEINS. The full length of *GhAGPL1* coding region was digested by *Sall/KpnI* and subcloned into pCambia1300-GFP vector to generate pCambia1300-*GhAGPL1*-GFP expression plasmid. The *GhAGPL1*-GFP plasmid was transformed into onion (*Allium cepa*) epidermal cells by previously described particle bombardment method (Wang and Fang, 2002). The *GhAGPL1*-GFP fusion protein transformed into onion cells was microscopically detected with a light microscope (Eclipse TE2000-Econfocal; Nikon, Melville, NY). Then, images were analyzed using EZ-C1 software (Nikon).

EXPRESSION ANALYSIS OF *GhAGPL1* BY QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION. Primers for quantitative real-time polymerase chain reaction (qRT-PCR) system (StepOne; Applied Biosystems, Foster City, CA) are listed in Supplemental Table 1. *GhActin* and *AtActin2* were used as internal control for gladiolus and arabidopsis, respectively. Relative expression was calculated by the $2^{-\Delta\Delta\text{CT}}$. The detailed procedures were described previously (Livak and Schmittgen, 2001). Each qRT-PCR assay was run using three biological replicates.

DETERMINING AGPASE ACTIVITY, STARCH, AND SUCROSE CONTENT. For AGPase activity, samples were flash frozen using liquid nitrogen. About 0.05 g of frozen powder was resuspended

on ice with 500 μL extraction buffer [1 mM ethylenediaminetetraacetic acid, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 5 mM dithiothreitol, 5 mM MgCl_2 , 20% sucrose, and freshly added 1 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g}\cdot\text{mL}^{-1}$ pepstatin, and 1 $\mu\text{g}\cdot\text{mL}^{-1}$ leupeptin]. The slurry was centrifuged at 13,400 g_n for 15 min at 4 °C, and the supernatant was immediately used for AGPase enzyme assay (Cross et al., 2004). Three biological replicates were used in each experiments.

A previously described method was used for determining sucrose and starch content (Hendriks et al., 2003). Pulverized material (0.02 g) was extracted with 250 μL ethanol (80% ethanol twice, followed by 50% ethanol once). After that, the samples were heated at 90 °C for 30 min. The supernatants were collected in another tube. The pellets were reextracted twice more, using the same procedure. The combined supernatants were used for the determining sucrose content as described previously (Hendriks et al., 2003). For starch quantification, the pellets from the ethanol extraction were incubated at 95 °C in 0.1 M NaOH for 30 min and neutralized with HCl/sodium acetate (0.5 M HCl + 0.1 M acetate/NaOH, pH 4.9). Suspensions (50 μL) were digested overnight with amyloglucosidase and α -amylase.

IODINE STAINING OF STARCH. Leaves from arabidopsis or gladiolus were incubated in 75% ethanol at 80 °C until chlorophyll was totally eliminated. Then, treated leaves were rinsed with distilled water and were stained with Lugol's solution (0.06% I_2 , 0.1% KI, and 4 mM HCl) for 10 min. Finally, the stained leaves were rinsed with distilled water for 15 min (Caspar et al., 1985).

COMPLEMENTATION OF *APL1* MUTANT WITH *GhAGPL1*. Arabidopsis *apl1* mutant plants (Salk_139228) were transformed using the floral dip method (Clough and Bent, 1998) with *Agrobacterium tumefaciens* GV3101 harboring 35S:GFP-*GhAGPL1*/pCambia1301. Infected seeds were screened on MS medium containing 50 $\mu\text{g}\cdot\text{mL}^{-1}$ hygromycin. Lines with homozygous seeds were used for related assays.

SILENCING OF *GhAGPL1* IN GLADIOLUS. Silencing of *GhAGPL1* by virus-induced gene silencing (VIGS) was performed as described previously (Zhong et al., 2014). Cormels with 6 to 10 mm in diameter were used for VIGS assays. First, a 313-base pair (bp) specific fragment of *GhAGPL1* were generated and cloned into pTRV2 vector. TRV1, TRV2, and TRV2-*GhAGPL1* were transformed individually into agrobacterium GV3101. The transformed agrobacterium colonies were cultured overnight in Luria-Bertani medium supplemented with 50 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin and 100 $\mu\text{g}\cdot\text{mL}^{-1}$ rifampicin. After overnight culture, the agrobacterium lines were collected and resuspended in infiltration buffer [10 mM MgCl_2 , 200 mM acetosyringone, and 10 mM 2-(N-morpholino) ethanesulfonic acid (pH 5.6)] to a final OD₆₀₀ of 1.8. Equal volumes of TRV1 and TRV2 (used as the control), as well as TRV1 and TRV2-*GhAGPL1* were mixed together and placed at room temperature for 3 h in the dark before vacuum infiltration. Young plants were submerged in infiltration buffer and were subjected to vacuum infiltration under 0.7 MPa for 20 min. After infiltration, the plants were transferred into pots containing a mixture of 1:1 (v/v) soil:sand mixture and grown in a greenhouse (22 °C, relative humidity 60% to 70%, 16/8 h light/dark cycle).

TRANSMISSION ELECTRON MICROSCOPY. The leaves and corms from plants cultured in a 16/8 h light/dark photoperiod were collected at the midpoint of the light period. Small pieces (2 mm²) were immediately fixed by submersion in a cacodylate

buffer [0.05 M sodium cacodylate and 3% (v/v) glutaraldehyde, pH 7.4] for 3 h at 4 °C, under vacuum. After that, the specimens were washed three times in a solution of 1% sucrose in 0.05 M sodium cacodylate buffer, for 30 min each at 4 °C, and postfixed with a solution of 1% osmium tetroxide in 0.05 M sodium cacodylate buffer, then kept overnight for complete removal of fixative at 4 °C. After two washes of 30 min each at 4 °C with the same cacodylate buffer, the samples were transferred into 30%, 50%, and 70% ethanol for 10 min each at 4 °C to dehydrate. Then they were dehydrated with 1% uranyl acetate in 70% ethanol for 16 h, 90% ethanol for 10 min, 96% ethanol for 30 min, and 100% ethanol for 2 h, with one change after the first hour at 4 °C. London Resin White (London Resin, Reading, UK) infiltration and polymerization at 60 °C were performed as previously described (Gonzalez-Melendi et al., 2008). Ultra-thin (70 to 90 nm) sections were stained as described previously by Roldan et al. (2007).

Results

ISOLATION AND SEQUENCE ANALYSIS OF GhAGPL1. *GhAGPL1* was isolated using the RACE method. The length of *GhAGPL1* gene was 1919 bp with a 1554-bp open reading frame (ORF). *GhAGPL1* encodes a 517 amino acid residues protein, with a calculated molecular mass of 56.5 kDa and an isoelectric point of 7.47.

To further investigate the *GhAGPL1* protein, we used MEGA5.0 for phylogenetic analysis. The results (Fig. 1A) suggested that *GhAGPL1* is more similar to homologs in monocotyledons such as *Zea mays*, *Oryza sativa*, *Triticum aestivum*, and *Hordeum vulgare* rather than dicotyledons. Sequence analysis (Fig. 1B) indicated that *GhAGPL1* shares four conserved domains [motif (a) to (d): ATP-binding motif, catalytic motif, Glc-1-P binding motif, and activator site] with other homologs.

SUBCELLULAR LOCALIZATION OF GhAGPL1 IN ONION EPIDERMAL CELLS. To examine the subcellular localization of *GhAGPL1*, the ORF

Fig. 1. Phylogenetic analysis and sequence alignment of gladiolus *GhAGPL1* protein. (A) Phylogenetic analysis of *GhAGPL1* and AGPLs in other species. (B) Sequence alignment of *GhAGPL1* with its homologs in *Arabidopsis thaliana*, *Citrullus lanatus*, *Solanum lycopersicum*, *Vitis vinifera*, and *Zea mays*. The conserved (a) ATP-binding motif, (b) catalytic motif, (c) Glc-1-phosphate motif, and (d) activator site are represented by red boxes, respectively.

of *GhAGPL1* was fused to the C-terminal of GFP, driven by CaMV35S promoter. Both recombinant and unrecombined vectors were transferred into onion epidermal cells by particle bombardment. The results (Fig. 2) showed that GFP signals of the control were observed throughout the cell, whereas the signals of GFP-*GhAGPL1* were observed in the cytoplasm.

EXPRESSION PROFILE OF *GhAGPL1*. To better understand *GhAGPL1*, we analyzed its expression profile in different organs and under abiotic stress conditions using qRT-PCR. The data indicates that *GhAGPL1* was expressed constitutively in different organs, and was largely expressed in sink organs (corms and cormels) especially cormels (Fig. 3A). When treated with sucrose, mannitol, or glucose, the transcription level of *GhAGPL1* increased dramatically. However, ABA had a negative effect on *GhAGPL1* expression, inhibiting its transcription (Fig. 3B).

***GhAGPL1* CONTRIBUTES TO STARCH SYNTHESIS IN ARABIDOPSIS.** To detect whether *GhAGPL1* plays a role in starch synthesis, we complemented the *apl1* arabidopsis mutant (Salk_139228) with the overexpression of *GhAGPL1*. Three different transgenic complementary lines (2, 6, and 12)

were chosen for further study (Fig. 4A). As *apl1* is a transfer DNA null mutant, it had low starch content resulting from low AGPase activity (Supplemental Fig. 1). However, AGPase activity was dramatically higher in the complementary lines. The level of AGPase activity was correlated with the transcript level of *GhAGPL1*, although its activity was not as high as wild type [WT (Fig. 4B)].

Next, we investigated the effect of *GhAGPL1* on starch synthesis. Starch accumulation in WT and complementary lines followed a similar pattern (Fig. 4C): starch content increased throughout the day, and reached its peak at the end of the day, after which the starch content decreased quickly throughout the night.

Furthermore, we explored the relationship between *GhAGPL1* and fresh weight. In the first 3 weeks, the fresh weight of all arabidopsis lines was similar. The growth and development of all arabidopsis lines was slow. After 4 weeks, there was a great difference between rescued lines and *apl1* mutant. The fresh weight of the *apl1* mutant was only 0.99 g, whereas the fresh weight of the rescued lines was similar to WT (Fig. 4D). These results showed *GhAGPL1* could rescue the slower growth rate of arabidopsis *apl1* mutant.

Taken together, these results indicate that *GhAGPL1* shows conserved function to *AtAGPL1* and its overexpression promotes AGPase activity, starch synthesis, and fresh weight in arabidopsis.

SILENCING OF *GhAGPL1* REDUCES AGPASE ACTIVITY AND STARCH SYNTHESIS IN GLADIOLUS. Intensive studies focused on function of *AGPL* in perennial and herbaceous plants, but there is little information about *AGPL* function in corm organs. Therefore, we used VIGS to eliminate *GhAGPL1* gene expression in

Fig. 2. Subcellular localization of gladiolus *GhAGPL1*. 35S:GFP-*GhAGPL1* (left panels) and 35S:GFP (right panels) were transiently expressed in onion epidermal cells by the particle bombardment.

Fig. 3. Quantitative real-time polymerase chain reaction (PCR) analysis of *GhAGPL1* expression levels in various gladiolus organs and different treatments. (A) *GhAGPL1* expression levels in various gladiolus organs (root, corm, cormel, stolon, leaf, flower). (B) *GhAGPL1* expression levels under different abiotic stress. For abiotic stress, cormels were placed in a petri plate in the sugar-free medium containing 200 mM mannitol, 200 mM glucose, 200 mM sucrose, or 100 μ M abscisic acid (ABA), respectively. Different letters indicate significant differences (Duncan's multiple range test at $P < 0.05$); identical letters indicate no statistical difference. Actin was used as the internal control to normalize PCR efficiency (Larionov et al., 2005).

Fig. 4. *GhAGPL1* complemented starch synthesis defect in *apl1* arabidopsis mutant. (A) Transcriptional analysis of *GhAGPL1* in *apl1* and complementary lines (35S:*GhAGPL1/apl1*-2, 6, 8, 9, 12), (B) AGPase activity, (C) starch content, and (D) fresh weight in wild type (WT), *apl1* mutant, and complementary lines (35S:*GhAGPL1/apl1*-2, 6, 12). WT was arabidopsis Columbia (Col) ecotype. (C) Starch content in leaves during a 16/8 h light/dark photoperiod. The bar at the bottom of the graph represents day (white) and night (black). (D) Fresh weight per plant plotted against time after germination. Leaves were sampled at the end of the light period. The error bars represent $\pm$ SD of data from three biological replicates. Different letters indicate significant differences (Duncan's multiple range test at $P < 0.05$); identical letters indicate no statistical difference.

gladiolus. First, we detected the existence of tobacco (*Nicotiana tabacum*) rattle virus by RT-PCR after agroinfiltration of sprouted cormels. Compared with the control, a larger PCR product was observed in TRV-*GhAGPL1*-treated groups (Fig. 5A), indicating successful infection of gladiolus.

Then we performed qRT-PCR to select silenced plants. The expression of *GhAGPL1* was found to be significantly lower in several silenced plants (Fig. 5B). In addition, we also tracked the expression of *GhSus*. *GhSus* encodes sucrose synthase (*Sus*), an enzyme which plays an important role in conversion of sucrose to UDP-glucose in sink organs, a necessary step for starch synthesis. In *GhAGPL1*-silenced plants, *GhSus* expression was 18% lower compared with control (Fig. 5B), suggesting *GhAGPL1* accumulation may play a role in the upstream feedback regulation of *GhSus* transcription.

To investigate the effect of silencing *GhAGPL1* in gladiolus starch synthesis, we measured AGPase activity, starch content, and sucrose content. AGPase activity in *GhAGPL1*-silenced corm was reduced by around 25%, and it further decreased in

silenced cormels (Fig. 5C). Starch accumulation in silenced corms was around a half, compared with the control (Fig. 5D). Using transmission electron microscopy, we found fewer and smaller starch bodies in silenced corm than the control (Fig. 5F). A similar situation was observed in silenced leaves (Fig. 5E and F). In contrast, sucrose content was 22% higher in silenced corms. Thus, silencing *GhAGPL1* has a negative effect on starch accumulation.

SILENCING *GhAGPL1* DECREASES THE QUALITY AND QUANTITY OF GLADIOLUS CORMS AND CORMELS. The onset of corm formation is accompanied with starch accumulation. Therefore, we investigated whether inhibition of *GhAGPL1* expression would result in any change with respect to corm formation and development.

Plants from the control and silenced lines were harvested at the end of cultivation (when leaves were totally withered). We analyzed their corm weight, corm size, cormel number, and cormel weight. Although there was no visual difference in corm diameter, fresh weight and dry weight were reduced by 16% and 24% in silenced corms, respectively (Fig. 6A). For cormels, there was a significant reduction in fresh weight and dry weight (Fig. 6B). In addition, the number of cormels was only 14% of the control (Fig. 6B and C). These results indicate that a reduction in *GhAGPL1* is paralleled by a decrease in the fresh weight and dry weight of corm or cormels, and is correlated with a lower number of cormels.

Fig. 5. Downregulation of *GhAGPL1* decreases starch biosynthesis and increases sucrose content in gladiolus. (A) Detection of TRV1 and TRV2 in untreated, TRV and TRV-*GhAGPL1* plants (TRV-*GhAGPL1*-1, TRV-*GhAGPL1*-2, TRV-*GhAGPL1*-3, TRV-*GhAGPL1*-4). TRV1 is partial TRV, containing its movement protein, whereas TRV2 contains the coat protein. TRV control refers to empty TRV2. TRV-*GhAGPL1* plant: *GhAGPL1*-silenced plant. (B) Transcription analysis of *GhAGPL1* and *GhSus* in TRV and four TRV-*GhAGPL1* lines. (C) AGPase activity in TRV and silenced corm and cormels. (D) Starch and sucrose content in TRV and four TRV-*GhAGPL1* lines. (E) The phenotype of gladiolus leaves in TRV and TRV-*GhAGPL1* lines via Lugol's iodine stain. (F) Transmission electron microscopy of leaf and corm section from TRV and four TRV-*GhAGPL1* lines. Different letters indicate significant differences (Duncan's multiple range test at $P < 0.05$); identical letters indicate no statistical difference.

Discussion

Corm degeneration is a severe problem in the production of gladiolus cut flowers. Degenerated corms produce slim and branched inflorescences, and fewer cormels, which do not meet the standards of commercial sales and propagation (Seng et al., 2016). In addition, degeneration also confers weak disease resistance which leads to great economic loss. Degeneration also occurs in sweetpotato [*Ipomoea batatas* (Adikini et al., 2015)]. Hence, the quality and quantity of corms and cormels predominantly influence the commercial cut flower industry.

Over decades, *AGPL* genes were studied intensively in annual plants such as arabidopsis, *O. sativa*, *T. aestivum*, and *Z. mays* (Huang et al., 2011; Meyer et al., 2004; Tuncel et al., 2014; Ventriglia et al., 2008). However, since they do not have modified organs, we still do not know much about the function of *AGPL* in corm. In our study, we silenced *GhAGPL1* in gladiolus,

Fig. 6. Silencing *GhAGPL1* decreases the quality and quantity of gladiolus corms and cormels. (A) Fresh weight, dry weight of corm, and corm diameter in TRV and four TRV-*GhAGPL1* lines. (B) Fresh weight, dry weight of cormel, and cormel number in TRV and four TRV-*GhAGPL1* lines. (C) Silencing *GhAGPL1* in gladiolus leads to fewer number of cormels. Different letters indicate significant differences (Duncan's multiple range test at $P < 0.05$); identical letters indicate no statistical difference.

and found a reduction in corm weight and cormel number (Fig. 6). Previously, studies have shown that an increase in the amount of sucrose within an appropriate range, promotes gladiolus corm formation, diameter, and fresh weight (Dantu and Bhojwani, 1995). The weight and the number of cormels were closely correlated with mother corm size in *brodiaea* [*Brodiaea laxa* (Han et al., 1991)]. Our results are in agreement with their research. We provide molecular evidence that *GhAGPL1* plays a vital role in corm development. In addition, we find a distinct role of *AGPL* in gladiolus and potato (*Solanum tuberosum*). In potato, silencing *AGPase B* reduced tuber size but resulted in an increase in both total tuber number and the number of tubers formed per stolon (Müller-Röber et al., 1992). We hypothesize that this difference may result from different mechanisms of tuberization and cormel initiation. However, they do share similarities, such as weak sink ability in silenced tubers or corms.

Downregulating *GhAGPL1* via VIGS leads to low expression of *GhSus*, but resulted in higher sucrose accumulation in gladiolus (Fig. 5). Silencing *GhAGPL1* reduced AGPase activity, resulting in low level of sucrose availability and reduction of starch content. Furthermore, silencing *GhAGPL1*

reduced the transcription level of *GhSus* and promoted sucrose accumulation (Fig. 5). *AGPase B*-silenced lines of potato also have lower amount of starch, higher amount of glucose and sucrose, and lower expression of *Sus* (Müller-Röber et al., 1992).

In this study, *GhAGPL1* was mostly expressed in sink organs, while being expressed lower in source organs [leaf (Fig. 3A)]. This expression profile promotes sucrose-to-starch conversion in sink organs (Yang et al., 2004). *GhAGPL1* expression was suppressed by ABA (Fig. 3B), which is consistent with other research that shows ABA alone can reduce starch accumulation in *O. sativa* (Akihiro et al., 2005). Furthermore, sucrose and glucose induced *GhAGPL1* expression (Fig. 3B), which is similar to its homologs in arabidopsis (Sokolov et al., 1998).

In summary, we have isolated and characterized a large subunit of AGPase in gladiolus. *GhAGPL1* was highly expressed in sink organs (cormel and corm), and was induced by sucrose, glucose, and mannitol, while being repressed by ABA. Silencing *GhAGPL1* in gladiolus resulted in reduction of corm yield and number of cormels.

Literature Cited

- Adikini, S., S.B. Mukasa, R.O.M. Mwanga, and R.W. Gibson. 2015. Sweet potato cultivar degeneration rate under high and low sweet potato virus disease pressure zones in Uganda. *Can. J. Plant Pathol.* 37:136–147.
- Akihiro, T., K. Mizuno, and T. Fujimura. 2005. Gene expression of ADP-glucose pyrophosphorylase and starch contents in rice cultured cells are cooperatively regulated by sucrose and ABA. *Plant Cell Physiol.* 46:937–946.
- Butt, S.J. 2005. Effect of N, P, K on some flower quality and corm yield characteristics of gladiolus. *J. Tekirdag Agr. Faculty* 2:212–214.
- Caspar, T., S.C. Huber, and C. Somerville. 1985. Alterations in growth, photosynthesis, and respiration in a starchless mutant of *Arabidopsis thaliana* L. deficient in chloroplast phosphoglucomutase activity. *Plant Physiol.* 79:11–17.
- Chen, J. and M.F. Yi. 2004. Effects of different planting time on growth and development of gladiolus stolon and cormel. *Acta Hort.* 31:767–772.
- Clough, S.J. and A.F. Bent. 1998. Floral dip: A simplified method for agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16:735–743.
- Crevillén, P., M.A. Ballicora, Á. Mérida, J. Preiss, and J.M. Romero. 2003. The different large subunit isoforms of *Arabidopsis thaliana* ADP-glucose pyrophosphorylase confer distinct kinetic and regulatory properties to the heterotetrameric enzyme. *J. Biol. Chem.* 278:28508–28515.
- Crevillén, P., T. Ventriglia, F. Pinto, A. Orea, Á. Mérida, and J.M. Romero. 2005. Differential pattern of expression and sugar regulation of *Arabidopsis thaliana* ADP-glucose pyrophosphorylase-encoding genes. *J. Biol. Chem.* 280:8143–8149.
- Cross, J.M., M. Clancy, J.R. Shaw, T.W. Greene, R.R. Schmidt, T.W. Okita, and L.C. Hannah. 2004. Both subunits of ADP-glucose pyrophosphorylase are regulatory. *Plant Physiol.* 135:137–144.
- Dantu, P.K. and S.S. Bhojwani. 1995. In vitro corm formation and field evaluation of corm-derived plants of gladiolus. *Sci. Hort.* 61:115–129.
- Gonzalez-Melendi, P., M. Uyttewaal, C.N. Morcillo, J.R.H. Mora, S. Fajardo, F. Budar, and M.M. Lucas. 2008. A light and electron microscopy analysis of the events leading to male sterility in Ogu-INRA CMS of rapeseed (*Brassica napus*). *J. Expt. Bot.* 59:827–838.
- Hall, T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser.* 41:95–98.

- Han, S.S., A.H. Halevy, R.M. Sachs, and M.S. Reid. 1991. Flowering and corm yield of brodiaea in response to temperature, photoperiod, corm size, and planting depth. *J. Amer. Soc. Hort. Sci.* 116:19–22.
- Hendriks, J.H.M., A. Kolbe, Y. Gibon, M. Stitt, and P. Geigenberger. 2003. ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of arabidopsis and other plant species. *Plant Physiol.* 133:838–849.
- Huang, B.Q., J. Chen, J.J. Zhang, H.M. Liu, M.L. Tian, Y. Gu, Y.F. Hu, Y.P. Li, Y.H. Liu, and Y.B. Huang. 2011. Characterization of ADP-glucose pyrophosphorylase encoding genes in source and sink organs of maize. *Plant Mol. Biol. Rpt.* 29:563–572.
- Kang, G., G. Liu, X. Peng, L. Wei, C. Wang, Y. Zhu, Y. Ma, Y. Jiang, and T. Guo. 2013. Increasing the starch content and grain weight of common wheat by overexpression of the cytosolic AGPase large subunit gene. *Plant Physiol. Biochem.* 73:93–98.
- Larionov, A., A. Krause, and W. Miller. 2005. A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics* 6:62.
- Livak, K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 25:402–408.
- Meyer, F.D., E.D. Smidansky, B. Beecher, T.W. Greene, and M.J. Giroux. 2004. The maize Sh2r6hs ADP-glucose pyrophosphorylase (AGP) large subunit confers enhanced AGP properties in transgenic wheat (*Triticum aestivum*). *Plant Sci.* 167:899–911.
- Müller-Röber, B., U. Sonnewald, and L. Willmitzer. 1992. Inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. *EMBO J.* 11:1229–1238.
- Roldan, I., F. Wattedled, M.M. Lucas, D. Delvalle, V. Planchot, S. Jimenez, R. Perez, S. Ball, C. D'Hulst, and A. Merida. 2007. The phenotype of soluble starch synthase IV defective mutants of *Arabidopsis thaliana* suggests a novel function of elongation enzymes in the control of starch granule formation. *Plant J.* 49:492–504.
- Saripalli, G. and P.K. Gupta. 2015. AGPase: Its role in crop productivity with emphasis on heat tolerance in cereals. *Theor. Appl. Genet.* 128:1893–1916.
- Seng, S.S., J. Wu, J.J. Sui, C.Y. Wu, X.H. Zhong, C. Liu, C. Liu, B.H. Gong, F.Q. Zhang, J.N. He, and M.F. Yi. 2016. ADP-glucose pyrophosphorylase gene plays a key role in the quality of corm and yield of corms in gladiolus. *Biochem. Biophys. Res. Commun.* 474:206–212.
- Sharma, J.R. and R.B. Gupta. 2003. Effect of corm size and spacing on growth, flowering and corm production in gladiolus. *J. Ornamental Hort.* 6:352–356.
- Sokolov, L.N., A. Dejardin, and L.A. Kleczkowski. 1998. Sugars and light/dark exposure trigger differential regulation of ADP-glucose pyrophosphorylase genes in *Arabidopsis thaliana* (thale cress). *Biochem. J.* 336:681–687.
- Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28:2731–2739.
- Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 24:4876–4882.
- Tuncel, A., B. Cakir, S.K. Hwang, and T.W. Okita. 2014a. The role of the large subunit in redox regulation of the rice endosperm ADP-glucose pyrophosphorylase. *FEBS J.* 281:4951–4963.
- Tuncel, A., J. Kawaguchi, Y. Ihara, H. Matsusaka, A. Nishi, T. Nakamura, S. Kuhara, H. Hirakawa, Y. Nakamura, B. Cakir, A. Nagamine, T.W. Okita, S.K. Hwang, and H. Satoh. 2014b. The rice endosperm ADP-glucose pyrophosphorylase large subunit is essential for optimal catalysis and allosteric regulation of the heterotetrameric enzyme. *Plant Cell Physiol.* 55:1169–1183.
- Ventriglia, T., M.L. Kuhn, M.T. Ruiz, M.R. Pedro, F. Valverde, M.A. Ballicora, J. Preiss, and J.M. Romero. 2008. Two arabidopsis ADP-glucose pyrophosphorylase large subunits (APL1 and APL2) are catalytic. *Plant Physiol.* 148:65–76.
- Wang, G.L. and H.Y. Fang. 2002. Gene engineering in plant. 2nd ed. Press Sci., Beijing, China.
- Wu, J., S.S. Seng, J.J. Sui, V. Eliana, X. Luo, B.H. Gong, C. Liu, C.Y. Wu, C. Liu, F.Q. Zhang, J.N. He, and M.F. Yi. 2015. *Gladiolus hybridus* abscisic acid insensitive 5 (*GhABI5*) is an important transcription factor in ABA signaling that can enhance gladiolus corm dormancy and arabidopsis seed dormancy. *Front. Plant Sci.* 6:749–753.
- Yang, J.C., J.H. Zhang, Z.Q. Wang, G.W. Xu, and Q.S. Zhu. 2004. Activities of key enzymes in sucrose-to-starch conversion in wheat grains subjected to water deficit during grain filling. *Plant Physiol.* 135:1621–1629.
- Yu, S.M., S.F. Lo, and T.H. Ho. 2015. Source-sink communication: Regulated by hormone, nutrient, and stress cross-signaling. *Trends Plant Sci.* 20:844–857.
- Zhong, X.H., X. Yuan, Z. Wu, M.A. Khan, J. Chen, X. Li, B.H. Gong, Y. Zhao, J. Wu, C. Wu, and M.F. Yi. 2014. Virus-induced gene silencing for comparative functional studies in *Gladiolus hybridus*. *Plant Cell Rpt.* 33:301–312.

Supplemental Fig. 1. Iodine staining of arabidopsis leaves of *apl1* mutants and complementary lines. Rosette leaves from plants grown for 3 weeks were stained with lugol. (A) *apl1* mutant line: Salk-139228; (B) complementary line: 35S:*AGPL1/apl1-2*; (C) complementary line: 35S:*AGPL1/apl1-6*; and (D) complementary line: 35S:*AGPL1/apl1-12*.

Supplemental Table 1. Primers used in this study.

Gene name	Accession no.	Primer name	Sequence (5'-3')
<i>GhAGPL1</i>	KM023646	DP-AGPL1-F1	CCNRTDAGCAAYTGYHTNAACAG
		DP-AGPL1-R1	ATBGTHCCRATRTCYTCCCAGTA
		SP-AGPL1-F1	GAAAATTGACACCACGACTCTAGG
		SP-AGPL1-R1	GCTGCAAGGACCTCAACAAATCCAT
		SP-AGPL1-F2	GCTTCAATGGGAGTCTACGTATTTAG
		SP-AGPL1-R2	GAATTGAACTGGGTCATGATGAATATCTT
		SP-AGPL1-F3	GAGCTTGATCTGCTATTGGAAGTGC
		SP-AGPL1-R3	CATCTTCACAAAACCTGTGGAAATGTG
		Primers for vectors construction of overexpressing in arabidopsis	
		<i>Sal I</i> _for.	ACGCGT <u>CGAC</u> ATGGAGGTCAGCACAGTGTCTCTG
		<i>Kpn I</i> _rev.	CGGGGT <u>ACCCT</u> TATATGATGGTTCCATCCTTGATAATG
		Primers for vectors construction VIGS of in gladiolus	
		<i>XbaI</i> _for.	<u>CTCTAGAT</u> CACAATAATACCGAAGAACTCC
		<i>Sac I</i> _rev.	<u>GAGCTCGCCT</u> TATACAGCCTTCTACATTATG
<i>GhAGPL1</i>	KM023646	Primers for qRT-PCR	
		qF1	GAGTGTGAGGAGTAGGTGTCTG
<i>GhActin</i>	JF831193.1	qR1	GAGGAAACAGGTGAGTCCCA
		qF2	ACTGCAGAGCGGGAAATTGT
<i>AtActin2</i>	AT3G18780	qR2	CCAATCAGGGATGGCTGGAA
		qF3	CGCTCTTTCTTTCCAAGCTCAT
<i>GhSuSy</i>	KP698512	qR3	GCAAATCCAGCCTTCACCAT
		qF4	CATAGCGCTCGGGATAGCTT
		qR4	TCAGCTATGAGATGATGGGGC

VIGS = virus-induced gene silencing; qRT-PCR = quantitative real-time polymerase chain reaction.

Supplemental Table 2. *GhAGPL1* homologs used in the sequence analysis from NCBI database.

Species	Name	Accession no.
<i>Actinidia deliciosa</i>	<i>Actinidia deliciosa</i> 4	AGS94412.1
<i>Arabidopsis thaliana</i>	<i>Arabidopsis thaliana</i> 1	AAM14190
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i> 2	BAA76362
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i> 3	AAM20291
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i> 4	CAA77173
<i>Beta vulgaris</i>	<i>Beta vulgaris</i>	CAA55516
<i>B. vulgaris</i>	<i>Beta vulgaris</i> 1	AGB85111.1
<i>Brassica rapa</i>	<i>Brassica rapa</i>	AAK27685
<i>Cicer arietinum</i>	<i>Cicer arietinum</i> 1	AAK27719
<i>C. arietinum</i>	<i>Cicer arietinum</i> 2	AAK27718
<i>Citrullus lanatus</i>	<i>Citrullus lanatus</i> 1	AAB91468
<i>C. lanatus</i>	<i>Citrullus lanatus</i> 2	AAB91467
<i>Citrus unshiu</i>	<i>Citrus unshiu</i>	AAD56042
<i>Cucumis melo</i>	<i>Cucumis melo</i> 1	AAB91463
<i>C. melo</i>	<i>Cucumis melo</i> 2	AAB91464
<i>Fragaria ananassa</i>	<i>Fragaria ananassa</i> 1	AAS00542
<i>Gladiolus hybridus</i>	<i>GhAGPL1</i>	AIO11223.1
<i>Hordeum vulgare</i>	<i>Hordeum vulgare</i> 1	CAA47626
<i>H. vulgare</i>	<i>Hordeum vulgare</i> 2	AAC49729
<i>Ipomoea batatas</i>	<i>Ipomoea batatas</i> 2	AGB85110.1
<i>I. batatas</i>	<i>Ipomoea batatas</i> 3	AGB85111.1
<i>I. batatas</i>	<i>Ipomoea batatas</i> 4	AGB85112.1
<i>Oncidium goldiana</i>	<i>Oncidium goldiana</i>	AAM95945
<i>Oryza sativa</i>	<i>Oryza sativa</i> 1	BAA23490
<i>O. sativa</i>	<i>Oryza sativa</i> 2	AAB38781
<i>O. sativa</i>	<i>Oryza sativa</i> 3	NP_911710
<i>O. sativa</i>	<i>Oryza sativa</i> 4	AAT78793
<i>Ostreococcus tauri</i>	<i>Ostreococcus tauri</i>	AAS88891
<i>Perilla frutescens</i>	<i>Perilla frutescens</i>	AAF66436
<i>Phaseolus vulgaris</i>	<i>Phaseolus vulgaris</i> 1	BAC66692
<i>Pisum sativum</i>	<i>Pisum sativum</i>	CAA65541
<i>Populus trichocarpa</i>	<i>Populus trichocarpa</i> 2	XP002300758.1
<i>Solanum habrochaetes</i>	<i>Solanum habrochaetes</i>	AAD56405
<i>Solanum lycopersicum</i>	<i>Solanum lycopersicum</i> 1	AAC49942
<i>S. lycopersicum</i>	<i>Solanum lycopersicum</i> 2	AAC49941
<i>S. lycopersicum</i>	<i>Solanum lycopersicum</i> 3	AAC49943
<i>Solanum tuberosum</i>	<i>Solanum tuberosum</i> 1	CAA43490
<i>S. tuberosum</i>	<i>Solanum tuberosum</i> 2	CAA52917
<i>S. tuberosum</i>	<i>Solanum tuberosum</i> 3	CAA53741
<i>Sorghum bicolor</i>	<i>Sorghum bicolor</i>	AAB94012
<i>Triticum aestivum</i>	<i>Triticum aestivum</i>	CAA79980
<i>Vitis vinifera</i>	<i>Vitis vinifera</i> 1	XP_010664711.1
<i>V. vinifera</i>	<i>Vitis vinifera</i> 2	XP_010652349.1
<i>Zea mays</i>	<i>Zea mays</i> 1	JX462603.1
<i>Z. mays</i>	<i>Zea mays</i> 2	CAA86227
<i>Z. mays</i>	<i>Zea mays</i> 3	EU971227.1