Cloning and Expression Analysis of Starch Synthesis-Related Enzyme Genes During Lily Bulb Development Postprint

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Abstract

Starch synthesis-related enzymes play a crucial role in starch accumulation and bulb bulking development in lily bulbs; investigating the genes of starch synthesis-related enzymes and exploring their expression regulation patterns holds significant promise for lily bulb propagation production. Three key lily starch synthesis enzyme genes—adenosine diphosphate glucose pyrophosphorylase (AGPase), granule-bound starch synthase (GBSS), and soluble starch synthase (SSS)—were cloned via homologous cloning technology, and the expression changes of these three key starch synthesis enzyme genes were analyzed; concurrently, starch content variations during lily bulb bulking development were measured. The results demonstrated: (1) AGPase possesses the protein structural characteristics of the GlgC family protein PLN02241 and the domains of cl11394 family protein ADP_Glucose_PP and NTP_transferase, with accession number KP751443; GBSS and SSS possess the cl10013 family protein Glyco_transf_5 and GT1_Glycogen_synthase_DULL1_like domains, with accession numbers KP751444 and KP751445, respectively. (2) During lily bulb formation and bulking development, starch content exhibited an increasing trend, reaching its maximum of 44.52% when the basal plate initiated stem differentiation. The expression levels of the three starch synthesis-related enzyme genes in both bulb and leaf tissues increased gradually; during the stem differentiation stage following bulb bulking, the expression levels of the three starch synthesis-related enzyme genes peaked, with expression levels of AGPase, GBSS, and SSS in scales being 10.79, 6.92, and 5.12, respectively, and in leaves being 6.79, 5.22, and 4.41, respectively, with expression levels in scales substantially higher than those in leaves; the changes in expression levels of starch synthesis-related enzyme genes were positively correlated with starch content and bulb bulking development. These findings provide a conceptual framework for bulb propagation production to promote lily bulb bulking development through regulating

the expression of key starch synthesis enzyme genes.

Full Text

Gene Expression of Starch Synthesis-Related Enzymes During the Bulblet Development of Lilium

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Abstract

Starch synthesis-related enzymes play a crucial role in starch accumulation and bulblet swelling development in lily. Investigating these genes and their expression regulation patterns is significant for lily bulb production. Through homologous cloning, we isolated three key lily starch synthesis enzyme genes: ADP-glucose pyrophosphorylase (AGPase), granule-bound starch synthase (GBSS), and soluble starch synthase (SSS), and analyzed their expression patterns. We also measured starch content changes during lily bulblet swelling development. The results showed: (1) AGPase possesses the PLN02241 protein domain characteristic of the GlgC family and the ADP_Glucose_PP and NTP_transferase domains of the cl11394 family (accession number KP751443). GBSS and SSS contain the Glyco_transf_5 and GT1_Glycogen_synthase_DULL1_like domains of the cl10013 family (accession numbers KP751444 and KP751445, respectively). (2) During lily bulblet formation and swelling, starch content exhibited an increasing trend, reaching its maximum of 44.52% when the basal plate began differentiating stems. Expression of all three starch synthesis-related enzyme genes in both bulblets and leaves gradually increased, peaking at the post-swelling stem differentiation stage. At this stage, AGPase, GBSS, and SSS expression levels in scales were 10.79, 6.92, and 5.12, respectively, while in leaves they were 6.79, 5.22, and 4.41—substantially higher in scales than in leaves. Changes in starch synthesis-related enzyme gene expression were positively correlated with both starch content and bulblet swelling development. These results provide a theoretical basis for developing bulblet swelling cultivation techniques and technical references for lily bulb production.

Key words: Lilium, starch synthesis, bulblet swelling, AGPase, fluorescence quantitative PCR

Introduction

Lilies (*Lilium*) are plants that reproduce via bulbs and can be categorized into ornamental and medicinal/edible types. China is a major production area for medicinal/edible lilies, with main varieties including Lanzhou lily, Longya lily, and *Lilium lancifolium*. In production, lilies are primarily propagated through bulb scales, stem bulblets, and bulbils, but these methods have the disadvantage of long breeding cycles, requiring completion of both basal and stem growth phases before commercial bulbs can be formed (Zhao et al., 2017). Lanzhou lily requires 2–3 years to develop stem seedlings and 3 years for commercial production. Therefore, regulating development to induce rapid bulblet swelling and shorten the growth cycle is crucial for lily bulb production. Studies have shown that starch synthesis and accumulation promote swelling development of new lily bulblets (Zhang et al., 2016), while starch synthesis and metabolism are regulated by multiple genes. Among them, ADP-glucose pyrophosphorylase (AGPase) is the rate-limiting enzyme in the starch synthesis pathway, participating in the first step of starch synthesis, while granule-bound starch synthase (GBSS) and soluble starch synthase (SSS) are involved in amylose and amylopectin synthesis, respectively (Wang et al., 2017), affecting crop quality. These three starch synthesis enzymes play important roles in starch synthesis, yet no studies on cloning and expression analysis of AGPase, GBSS, and SSS genes in lily have been reported.

Therefore, this study aims to clone lily bulb starch synthesis-related enzyme genes, analyze expression changes of key enzyme genes at different bulblet formation and swelling stages using fluorescence quantitative PCR, measure starch content changes during lily bulblet swelling development, and explore expression patterns of key starch synthesis enzyme genes in combination with starch content changes to understand mechanisms promoting bulblet swelling development. The results will provide a theoretical foundation for developing bulblet swelling cultivation techniques and technical references for lily bulb production.

Materials and Methods

1.1 Plant Materials

Plant materials consisted of *Lilium davidii* var. *unicolor* (Lanzhou lily) tissue culture seedlings. Based on the developmental progression of bulblets during tissue culture, four stages were defined: cluster bud stage (A), bulblet formation stage (B), bulblet swelling stage (C), and stem differentiation stage after bulblet swelling (D). Tissue samples comprising bulb scales and leaves were collected at each stage [Figure 1: see original paper]. For stage A, scale material was taken from callus tissue forming bulb scales. Fresh samples were snap-frozen in liquid nitrogen and stored at -80°C. Plant materials were provided by the Institute of Biotechnology, Guangxi Academy of Agricultural Sciences.

1.2.2 Homologous Cloning of Starch Synthesis Key Enzyme Gene cDNA Sequences

Using homologous cloning, we first identified complete open reading frames of these three genes from closely related species based on previously reported AGPase, GBSS, and SSS gene cDNA sequences from other crops. These ORF sequences were then aligned on Blastn, and all related species ORF sequences were collected and globally aligned using MegAlign software to identify the most conserved regions for designing degenerate primers. The final degenerate primers capable of amplifying lily AGPase, GBSS, and SSS genes are listed in .

Total RNA was extracted from each tissue using the Trizol method, and DNA was removed using DNase I (RNase-free). cDNA was synthesized by reverse transcription, and PCR amplification was performed using degenerate primers. Amplified fragments were gel-purified, ligated into pGEM-T vector, and transformed into *E. coli* TOP10 competent cells for positive clone screening. Positive clones were identified by colony PCR, and correct clones were selected for sequencing.

1.2.3 Fluorescence Quantitative PCR

Gene-specific primers were designed using Primer Premier 5.0 (Premier Biosoft Interpairs, Palo Alto, CA) and are listed in . Scale and leaf tissues from different developmental stages (A, B, C, and D) were used for RNA extraction with Trizol reagent. mRNA was reverse-transcribed into cDNA using PrimeScript RT Master Mix (Perfect Real Time), and the synthesized cDNA was used for target gene expression analysis. Using 18S rRNA as an internal reference, SYBR Green fluorescence quantitative PCR was performed to amplify target gene fragments and 18S rRNA. The reaction program was: denaturation at 95°C for 7 min, followed by 40 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s. Each sample was run in triplicate, ΔCt values for target and reference genes were calculated, and relative gene expression was determined using the 2^(-$\Delta\Delta$Ct) method (Rajeevan et al., 2001).

1.2.4 Statistical Analysis

Statistical analysis was performed using SPSS 17.0 software. All measurement data were analyzed by t-test, with $P < 0.05$ indicating significant differences.

Results

2.1 Starch Content at Different Lily Bulblet Developmental Stages

During lily tissue-cultured bulblet formation and swelling development, starch content at each stage showed an increasing trend. As shown in , starch content gradually increased with bulblet swelling development, reaching its maximum of 44.52% when the basal plate began differentiating stems after bulblet swelling

(stage D). This indicates a positive correlation between bulblet swelling and starch content, consistent with previous reports (Zhang et al., 2016).

2.2 cDNA Cloning of Lily Starch Synthesis Key Enzyme Genes AGPase, SSS, and GBSS

Through Blast alignment of related amino acid sequences from closely related species, appropriate conserved regions were identified for designing degenerate primers. Using these primers, we amplified cDNA sequences of lily starch synthesis-related enzyme genes AGPase, SSS, and GBSS [Figure 2: see original paper], obtaining fragments of 918 bp, 567 bp, and 1257 bp, respectively. Sequence analysis and alignment revealed that these three sequences showed highest homology with starch synthesis key enzyme genes AGP small subunit, SSSIII, and GBSSI, at 100%, 99%, and 99% identity, respectively. The cloned sequences were submitted to NCBI with accession numbers KP751443, KP751445, and KP751444. Nucleotide sequences were translated into amino acid sequences for Blast alignment and conserved domain analysis. As shown in [Figure 3: see original paper], AGPase possesses the PLN02241 family protein domain characteristic of the cl28238 (GlgC) superfamily and the ADP_Glucose_PP and NTP_transferase domains of the cl11394 family. As shown in [Figure 4: see original paper] and [Figure 5: see original paper], GBSS and SSS contain the Glyco_transf_5 and GT1_Glycogen_synthase_DULL1_like domains of the cl10013 family.

2.3 Expression of Key Starch Synthesis Enzyme Genes at Different Lily Bulblet Developmental Stages

[Figure 6: see original paper] shows expression patterns of starch synthesis enzyme genes during lily bulblet differentiation, formation, and swelling development. Expression levels of the three lily starch synthesis-related enzyme genes (AGPase, SSS, and GBSS) were lowest at stage A, gradually increasing with bulblet swelling development and peaking at stage D. Expression differences among the four developmental stages were all significant ($P < 0.05$). AGPase, encoding the rate-limiting enzyme in starch synthesis, showed the highest expression at stage D (10.79), representing 10.75-, 6.95-, and 2.69-fold increases over stages A, B, and C, respectively. GBSS expression at stage D was 6.92, representing 6.81-, 4.03-, and 1.75-fold increases over stages A, B, and C, respectively. SSS expression at stage D was 5.12, representing 5.09-, 1.51-, and 1.09-fold increases over stages A, B, and C, respectively. These expression changes correlated with increased starch granule formation and progressively higher starch content across the four stages.

[Figure 7: see original paper] shows expression of starch synthesis enzyme genes in leaves during bulblet swelling development. Expression of all three genes in leaves also increased gradually with developmental progression, peaking at stage D. Expression differences among the four stages were all significant ($P < 0.05$). AGPase showed the highest expression at stage D (6.79), while GBSS and SSS

expression reached 5.23 and 4.41, respectively. Stage D AGPase expression was 2.18-, 3.10-, and 6.73-fold higher than stages C, B, and A, respectively; GBSS expression was 1.97-, 2.45-, and 5.21-fold higher; and SSS expression was 1.74-, 2.77-, and 4.40-fold higher. This indicates that starch synthesis enzyme gene expression patterns in leaves were consistent with those in bulblets.

Discussion and Conclusion

Lily is a high-starch crop. Our experiments observed that tissue-cultured bulblet swelling development involves dramatic changes in starch content, with the swelling process representing a period of starch accumulation (Zhang et al., 2016), indicating that starch synthesis and metabolism are important for bulb swelling development. Previous studies have reported on lily bulblet swelling, showing that treatment with appropriate concentrations of the exogenous regulator brassinolide can increase bulb fresh weight in *Lilium brownii* var. *viridulum* (Qiu et al., 2017). In *Lilium dauricum* tissue culture, sucrose, NAA, IBA, activated carbon, and paclobutrazol all affected test-tube bulblet formation and swelling, while salicylic acid had minimal effect (Zhang et al., 2016). In studies on Lanzhou lily bulblet swelling, activated carbon, sucrose, and paclobutrazol promoted bulblet swelling (Qin et al., 2015). Bulbils are a reproductive mode in lily, representing stem-derived small bulbs; studies on axillary bulbil formation in *Lilium lancifolium* found that starch and sugar metabolism and plant hormone signal transduction pathways play important roles in bulbil formation, with starch synthesis and accumulation promoting bulbil initiation (Yang et al., 2017). These findings demonstrate that starch synthesis and metabolism are important for bulblet formation.

During lily tissue-cultured bulblet development, exogenous factors (sucrose, plant growth regulators) induce bulblet swelling development. Electron microscopy observations revealed a positive correlation between bulblet swelling and starch granule formation and starch content increase (Zhang et al., 2014). We used real-time fluorescence quantitative PCR to analyze expression of major starch synthesis key enzyme genes to understand their expression patterns during bulblet development and explore regulatory mechanisms. Temporal and spatial expression of AGPase showed positive correlation with bulb formation, swelling development, and starch content (Zhang, 2016). Expression occurred in both bulblets and leaves, with higher expression in bulblets than leaves, and consistent expression changes across developmental stages. GBSS and SSS play major catalytic roles in amylose and amylopectin synthesis (Miao et al., 2016), with expression patterns similar to AGPase. They use products catalyzed by AGPase as substrates to extend glycoside chains. AGPase gene regulation is typically considered to be modulated by the activator 3-PGA and inhibitor Pi (Gao and Huang, 1998). The different developmental stage materials used in this study were formed after treatment with different concentrations of sucrose and plant growth regulators, and this process of promoting bulblet swelling development was consistent with enhanced AGPase expression, suggesting that

the exogenous factors used may positively regulate AGPase gene expression. This has positive significance for lily bulb swelling production, though further research is needed.

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Note: Figure translations are in progress. See original paper for figures.