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Cloning and Expression Analysis of *EdAGPL1* in *Eleocharis dulcis*

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Abstract In order to clone the cDNA sequence of water chestnut AGPase large subunit gene (*EdAGPL1*) and analyze its structural characteristics and its expression in different tissues and corm development of water chestnut. Using water chestnut 'Guilin water chestnut ' as research material. The *EdAGPL1* gene was cloned by RT-PCR and analyzed by bioinformatics. The expression of *EdAGPL1* in different tissues and corm development process was analyzed by real-time fluorescence quantitative PCR technology. The results showed that the length of the cloned *EdAGPL1* gene was 1 744 bp, its open reading frame was 1 599 bp, it encoded 532 amino acids, the protein molecular mass was 58.84 kD, the isoelectric point was 7.54, and it had NTP_transferase and PbH1 domains. The secondary structure was composed of helix (13.72%), for the strand (25.19%) and the loop (61.09%). The tertiary structure consists of 17 α-helices, 34 β-sheets, and 51 β-turn (curl) composition. Homology and phylogenetic analysis showed that the homology of amino acid sequence of EdAGPL1 and other plants AGPL protein was 57.54%~61.64%, and has far evolutionary kinship with other plants. Fluorescence quantitative PCR analysis showed that the expression of *EdAGPL1* in different tissues was corm> leaf stem > stolon > root. The expression of *EdAGPL1* was the highest in the initial stage of corm development, and then it decreased and remainedstable. The study of this gene will help to clarify the regulation mechanism of starch synthesis in water chestnut and provide a theoretical basis for the breeding of high-starch varieties.
Keywords *Eleocharis dulcis*; Starch synthase; Gene cloning; Expression analysis

Eleocharis dulcis ((N. L. Burman) Trinius ex Henschel), commonly known as Chinese water chestnut, belongs to Cyperaceae, a perennial herb in shallow water. It is native to southern China and India, with its underground corms as edible organs. It has been cultivated for more than 2000 years in China (Li et al., 2006; Changjiang Vegetables, (8): 39-43). The corm of water chestnut is crisp and juicy, containing 65~85 g water per 100 g fresh corm, 21.8 g carbohydrate, 1.5 g protein, 0.1 g fat and 0.6 g crude fiber (Kong, 2004, Hubei Science and Technology Press, pp.250-269). Carbohydrate is the main nutrient of the corm, while starch is its main storage form, which varies greatly among different varieties (Jiang et al., 2009). At least 4 enzymes are involved in plant starch biosynthesis, including ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching enzyme (BE) and debranching enzyme (DBE) (Abe et al., 2014). Among them, the activity of AGPase affects the synthesis rate of starch. It was significantly positively correlated with starch content (Min et al., 2010).

AGPase in higher plants consists of two large subunits (AGPL) and two small subunits (AGPS) to form a heterotetramer (α2β2). It is widely recognized that the small subunit plays a catalytic role while the large subunit focuses on the conformation regulation of the enzyme activity (Cheng et al., 2015). However, with the further study, the large subunit was also shown to have catalytic functions (Ventriglia et al., 2008). Due to the importance of AGPase in starch biosynthesis, many studies have focused on improving the catalytic activity of AGPase to improve starch content and yield (Tuncel and Okita, 2013). For example, overexpression of AGPase-related genes in rice, maize, wheat, potato and other crops can improve starch content in seeds or tubers (Smidansky et al., 2003; Song et al., 2005; Li et al., 2011; Kang et al., 2013).

Currently, there are few studies on starch biosynthesis in water chestnut, and only a few annotated information of genes involved in starch biosynthesis was obtained by high-throughput sequencing of leaves, corms and other

tissues (Liu et al., 2015; Cheng et al., 2016; Song et al., 2019). However, there were no reports on the cloning of water chestnut *AGPase* gene, and the molecular biological characteristics such as sequence structure and expression pattern of water chestnut *AGPase* gene are still unclear. Therefore, the cDNA sequence of water chestnut *AGPase* gene (*EdAGPL1*) was cloned and bioinformatics analysis was conducted. The expression of *EdAGPL1* gene in different corm development stages and tissues was detected by real-time quantitative PCR, which provided theoretical basis for studying the starch synthesis mechanism of water chestnut.

1 Results and Analysis

1.1 The gene squence encoding the large subunit gene *AGPase* was cloned

The cDNA obtained by reverse transcription of total RNA of water chestnut corm was used as the template for PCR amplification and the target gene fragment with length of 1 744 bp was obtained (Figure 1). Positive clones were obtained by ligating, transforming and colony PCR identification. The nucleotide sequence encoding the large subunit of water chestnut AGPase was obtained by sequencing, and it was named *EdAGPL1* (GenBank Accession: MT151684).

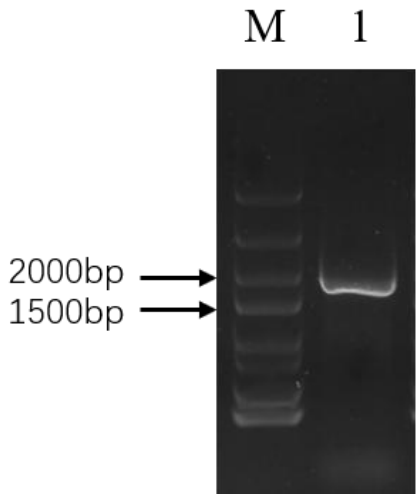


Figure 1 PCR amplification of *EdAGPL1* gene of *Eleocharis dulcis*
Note: M: *Trans*5K DNA Marker; 1: The fragment of PCR

1.2 Bioinformatics analysis of *EdAGPL1* gene

According to NCBI online analysis, the cDNA sequence of *EdAGPL1* gene contained a 1 599 bp open reading frame encoding 532 amino acids (Figure 2A). The predicted molecular weight of EdAGPL1 protein was 58.84 kD, and the theoretical isoelectric point was 7.54. The total number of negatively charged residues (Asp+Glu) and positively charged residues (Arg+Lys) were 60 and 61, respectively. The total atomic number is 8 244, the molecular formula is $C_{2595}H_{4112}N7_{20}O_{794}S_{23}$; The extinction coefficient is 0.669 (assuming cysteine forms cystine) or 0.660 (assuming cysteine is reduced). The estimated half-life *in vitro* is 30 h, >20 h in yeast and >10 h in *Escherichia coli*. The instability index of the protein was 40.53, which was classified as unstable protein. The lipid index of the protein was 81.67, and the total mean hydrophilicity was -0.227. According to hydrophilic prediction, EdAGPL1 showed hydrophilicity, with the lowest negative value appearing at the 67th position of amino acid sequence (-2.922) and the highest hydrophilicity. The highest positive value occurred at the 107th position of amino acid sequence (2.156), and the hydrophobicity was the strongest (Figure 2B). The physicochemical properties predicted above have reference significance for studying the physiology and biochemistry of the protein.

The results of signal peptide prediction showed that EdAGPL1 had no signal peptide (Figure 2C). Subcellular localization indicated that the protein might be localized in cytoplasm (0.450), microbody (0.368), mitochondrial stromal space (0.360) and chloroplast thylakoid membrane (0.100).

The conserved domain of EdAGPL1 was predicted in NCBI, and it was found that the amino acid sequence of EdAGPL1 was highly conserved and similar to that of Glucose-1-phosphate adenylyltransferase, indicating that EdAGPL1 had similar functions. It belongs to the PLN02241 superfamily (Figure 2D). According to SMART online database analysis, EdAGPL1 contains one NTP_transferase domain (Ala^{104}~Glu^{379}) and two PbH1 domains (Ser^{415}~Asp^{444}, Gly^{473}~Ser^{515}) (Figure 2E). The NTP_transferase domain could transfer nucleotides to sugar phosphate. PbH1 is a parallel β-helix repeat sequence, which contributes to the overall stability of proteins. Proteins with this domain are usually enzymes based on polysaccharides.

The secondary structure and solvent accessibility of EdAGPL1 were predicted (Figure 2F), and the results showed that the secondary structure was composed of helix (13.72%), strand (25.19%) and loop (61.09%). Solvent accessibility results showed that exposed accounted for 34.77%, buried accounted for 59.77%, intermediate accounted for 5.45%. It has one DNA and six protein binding regions. Phyre2 was used to model 423 residues of EdAGPL1 protein (covering 80% of the sequence) with 100.0% confidence and potato tuber AGPase (c1yp3C_) as template. The results showed that tertiary structure of the protein consists of 17 α-helices, 34 β-folds, and 51 β-turns (curls) (Figure 2G).

1.3 Homology alignment and phylogenetic analysis of *EdAGPL1* amino acid sequences

The amino acid sequence of *EdAGPL1* was submitted to NCBI for comparative analysis of BlastP homology. The results showed that the amino acid sequence of *EdAGPL1* had low homology with AGPL protein of other plants, and the highest consistency was the amino acid sequence of AGPL of Jujube (*Phoenix dactylifera* L.), which was only 68.94%. DNANAN was used to perform multiple comparison analysis on the amino acid sequences of AGPL proteins in 11 plants. The results showed (Figure 3) that the homology of the amino acid sequences of the AGPL proteins in water chestnut EdAGPL1 and other plants was low, ranging from 57.54%~61.64%. The results indicated that EdAGPL was relatively conserved in the conserved domain such as NTP_transferase and PbH1, but had a large variation range in other regions of the sequence.

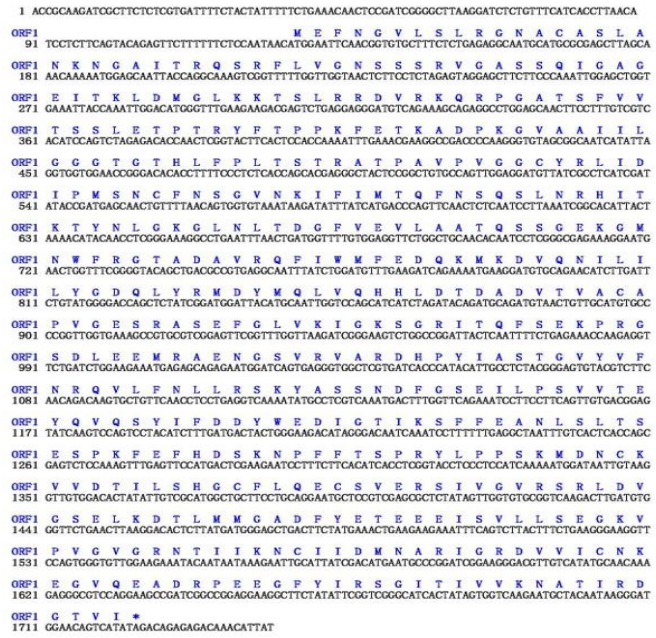
MEGA 5.1 was used to construct the phylogenetic evolutionary tree of water chestnut EdAGPL1 and other plants AGPL. The results showed that 5 monocotyledons such as oil palm and jujube clustered into one group (Figure 4), 5 dicotyledons such as pomegranate and balmoma clustered into one group, and water chestnut became a separate branch. The results suggested that EdAGP had a distant evolutionary relationship with other plants, suggesting that water chestnut *EdAGPL1* gene had a different evolutionary mode from other species.

1.4 Expression level analysis of *EdAGPL1*

Fluorescence quantitative PCR results showed that *EdAGPL1* gene was expressed in the roots, leaf stems, stolons and corms of water chestnut, with the highest expression level in corms, followed by leaf stems and the lowest expression level in roots (Figure 5). During corm development, the expression level of corm was higher at the early stage of corm development (90 d), then decreased and maintained a relatively stable expression level (Figure 6). The expression of EdAGPL was mainly concentrated in corm, which was the main organ of starch accumulation in water chestnut. The high expression of this gene at the early stage of corm development indicates that this gene is more active in the early stage, initiating starch synthesis.

2 Discussion

In this study, *EdAGPL1* cDNA sequence 1 744 bp was cloned from water chestnut, containing a 1 599 bp ORF (532 aa) encoding the large subunit of water chestnut AGPase. The molecular weight of EdAGPL1 protein was 58.84 kD and the theoretical isoelectric point was 7.54. It has typical NTP_transferase and PbH1 domains and is most likely to be located in the cytoplasm. The secondary structure was composed of helix (13.72%), strand (25.19%) and loop (61.09%). The tertiary structure consists of 17 α-helices, 34 β-folds and 51 β-turns (crimp). EdAGPL1 has a homology of 57.54%~61.64% with AGPL protein of other plants, and has a distant evolutionary relationship with other plants. The expression of *EdAGPL1* gene was mainly concentrated in corm and was highest in the early stage of corm development.



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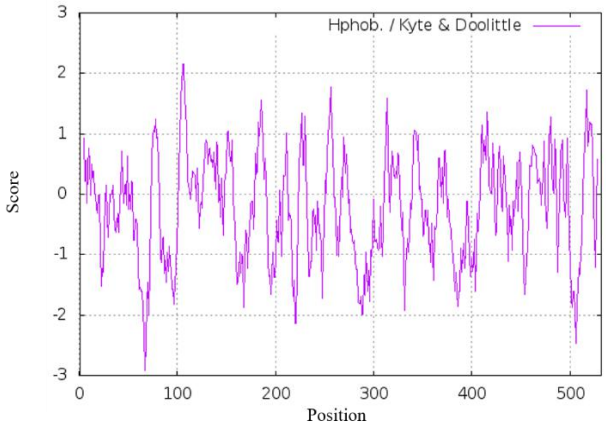

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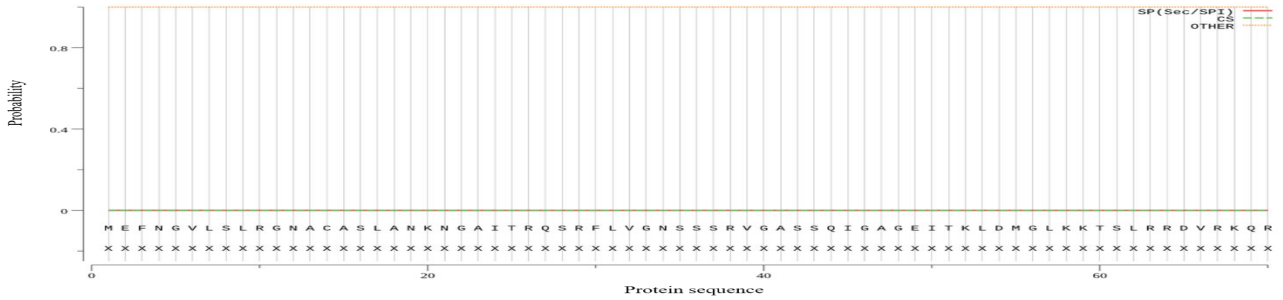

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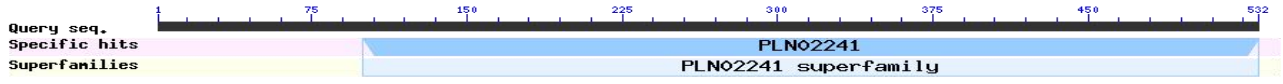

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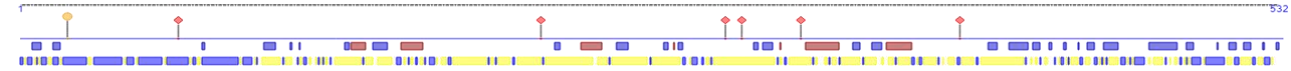

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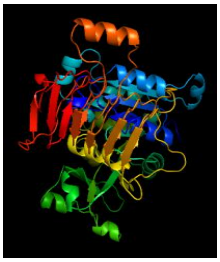

G

Figure 2 Bioinformatics analysis of *EdAGPL1* gene

Note A: *EdAGPL1* gene cDNA ribotide sequence and its deduced amino acid sequence; B: Analysis of hydrophobicity / hydrophilicity of amino acid sequence of EdAGPL1; C: Prediction of EdAGPL1 signal peptide; D: Prediction of *EdAGPL1* amino acid conserved domain; E: Analysis of EdAGPL1 protein domain; F: Secondary structure and solvent accessibility prediction for EdAGPL1 protein (The circle represents the DNA binding region and the rhombus represents the protein binding region; The blue represents Strand / Exposed, and the kermesinus represents helix and the yellow represents buried); G: Prediction of EdAGPL1 protein tertiary structure

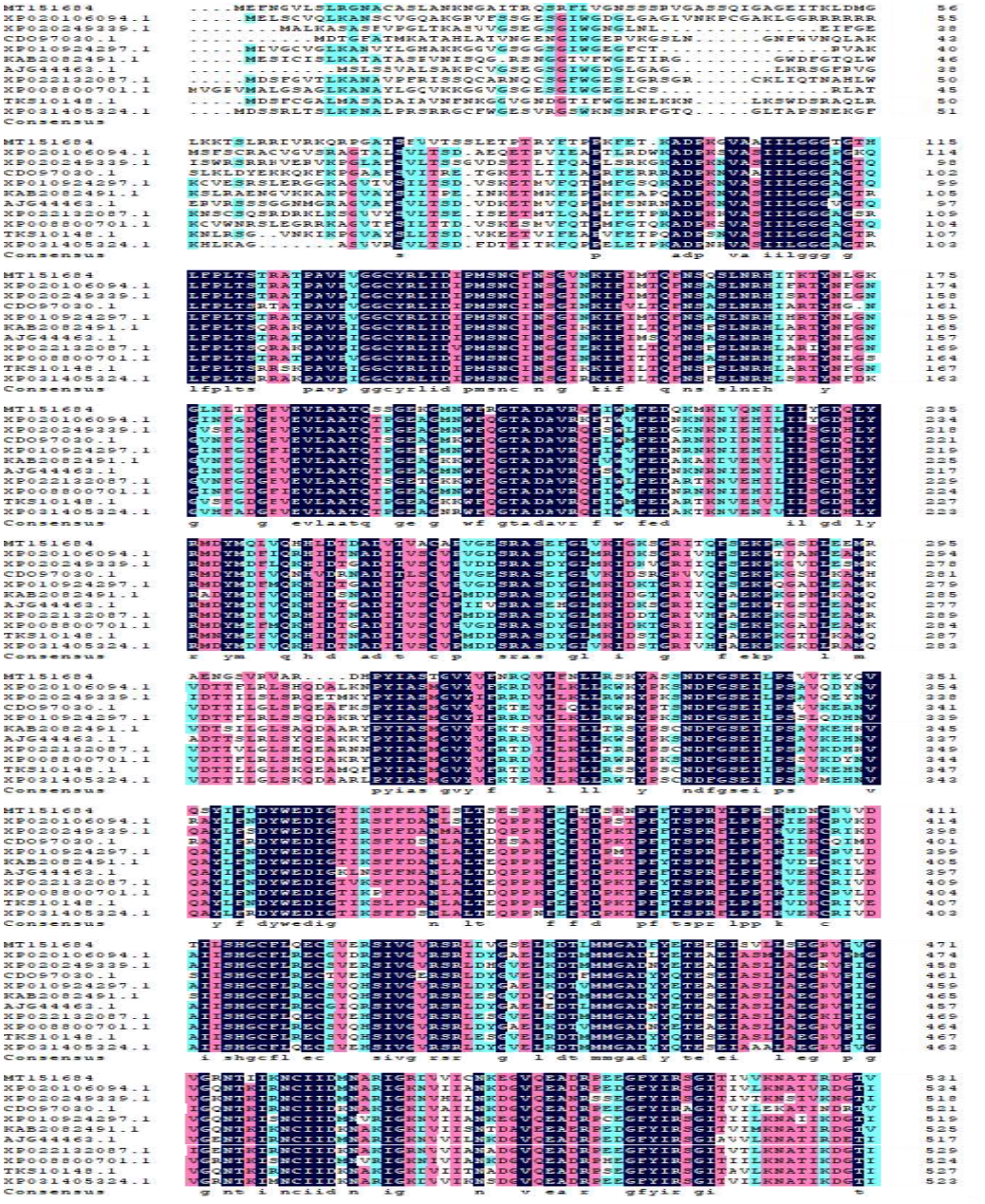

Figure 3 Multiple alignment of the amino acid sequence of AGPL protein between *E. dulcis* and other plants

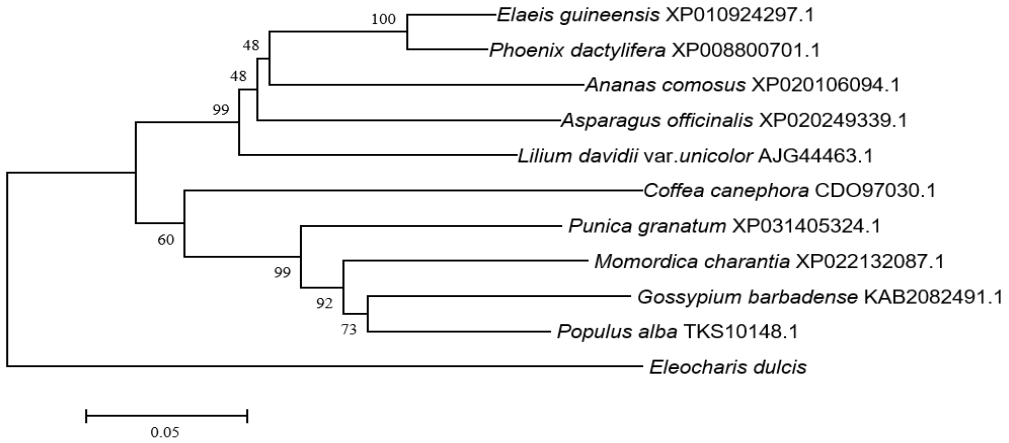

Figure 4 Phylogenetic tree constructed based on amino acid sequence of EdAGPL1 protein

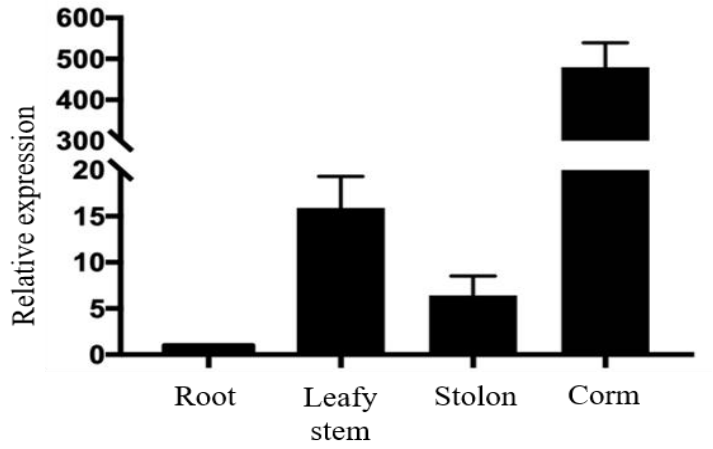

Figure 5 Tissue-specific expression of *EdAGPL1* gene in *Eleocharis dulcis*

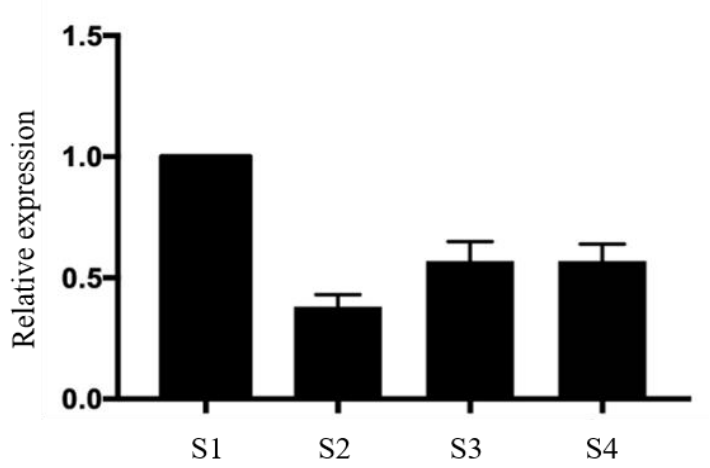

Figure 6 Changes of *EdAGPL1* gene expression during the corms expansion process of *Eleocharis dulcis*
Note: S1 ~ S4 respectively represent the corm samples of transplanted field 90 d, 100 d, 110 d and 120 d

A large number of studies have shown that AGPase plays a key role in starch synthesis (Abe et al., 2014). *EdAGPL1* gene cloned in this study belongs to the PLN02241 superfamily, which has the function of transferring nucleotides to sugar phosphate. Involved in anabolic pathways such as starch and sucrose, amino acids and nucleotides (Jensen and Reeves, 1998). Bioinformatics analysis of EdAGPL1 protein showed that its structural characteristics and physicochemical properties were similar to that of potato sAGP protein, with NTP_transferase and PbH1 domains, indicating that EdAGPL1 had similar functions. Subsequent studies will further verify the function of this gene through transgenic technology.

The EdAGPL1 protein had low homology with AGPL proteins of other plants in GenBank database. Phylogenetic results showed that water chestnut EdAGPL1 became a separate branch and had a distant relationship with other plants. It is speculated that the *EdAGPL1* gene of water chestnut has a different evolutionary mode from that of other species. Meanwhile, the large subunit of AGPase is later than the small subunit in evolution and less conservative than the small subunit. It has more variability and has great differences in different plant families and genera (Ballicora et al., 2004). Therefore, more conserved water chestnut AGPase subunit genes should be further explored for systematic analysis to clarify its evolutionary position.

Many studies have shown that AGPase-related genes have different expression patterns in different plants (Xu et al., 2011; Cheng et al., 2015; Li et al., 2018). Fluorescence quantitative analysis showed that *EdAGPL1* expression was relatively concentrated in the corm, and this expression pattern had many similarities with that of bare barley and lotus root, which were all concentrated in the starch accumulation site (Cheng et al., 2015; Li et al., 2018). During corm development, *EdAGPL1* was expressed at all growth stages, with the highest expression level

at the early stage (90 d), and remained stable at other growth stages, which was different from the expression pattern in lotus root and bare barley. In lotus root, the expression level of *AGPL* gene was basically stable at the early and late expansion stages.In hull-free barley, *APL* gene expression peaked 15 days after flowering and then decreased (Cheng et al., 2015; Li et al., 2018). The starch accumulation of water chestnut corm increased steadily during development, while *EdAGPL1* gene was the highest at the early stage of the development of corm, and maintained a relatively stable level in other periods. It is speculated that the gene plays a conformational regulation role in starch synthesis,, catalysis is completed by AGPase small subunit. The expression pattern of water chestnut AGPase small subunit should be further studied to clarify the role of water chestnut AGPase small subunit in starch synthesis.

In this study, *EdAGPL1* gene was successfully cloned from water chestnut, and its bioinformatics and expression analysis were carried out, which provided a basis for research on the regulation mechanism of starch biosynthesis and breeding of high-starch varieties in the later stage.

3 Materials and Methods

3.1 Experimental materials

The experimental material 'Guilin water chestnut' was planted in the demonstration base of Nian Village, Xiuren Town, Lipu City, Guilin City, Guangxi Province in late July 2019. Samples were taken at 90, 100, 110 and 120 days after transplanting according to the development stage of water chestnut corm. The aboveground part is leaf stem, the underground part is corm, stolon and root. Liquid nitrogen is quick-frozen and stored in a refrigerator at -80℃.

3.2 RNA extraction and cDNA synthesis

Total RNA was extracted from tissue samples using the Polysaccharide polyphenol plant total RNA extraction Kit (DP441) of Tiangen Biotech (Beijing) Co., Ltd. The extraction method referred to kit instruction. The extracted total RNA was reversely transcribed into the first strand of cDNA using the TIANScript II cDNA First Strand Synthesis Kit (KR107) of Tiangen Biotech (Beijing) Co., Ltd. cDNA synthesis reaction was carried out according to the kit instruction.

3.3 Cloning of *EdAGPL1* gene

Using cDNA of water chestnut corm as template, the annotation information of *EdAGPL1* gene of water chestnut was obtained according to transcriptome. Specific cloning primers EdAGPL1-F (5′-ACCGCAAGATCGCTTCTCTCGTG-3′) and EdAGPL1-R (5′-ATAATGTTTGTCTCTCTGTC-3′) were designed.

PCR amplification reaction system was as follows: 5×Phusion HF Buffer 4.0 μL, dNTPs (10 mmol/L) 0.4 μL, upstream and downstream primer (10 μmol/L) 1.0 μL, Phusion DNA Polymerase (2 U/μL) 0.2 μL, cDNA 1.0 μL, supplemented by double steam water to 20.0 μL. The PCR reaction procedure was as follows: pre-denaturation at 98℃ for 30 s, 30 cycles (denaturation at 98℃ for 10 s, annealing at 55℃ for 20 s, extension at 72℃ for 1 min); Finally, it was extended at 72℃ for 5 min. The amplified products were detected by electrophoresis in 1.0% agarose gel. The *pEASY*-Blunt Cloning Kit of Beijing TransGen Biotech Co., Ltd. was used to connect the target clip to the *pEASY*-Blunt carrier, and then transform it into trans-T1 Phage Resistant chemoreceptive cells. Coated on LB (100 mg/L Amp) plate. M13 Forward Primer and M13 Reverse Primer were used for PCR positive identification of the selected monoclonal clones, and the positive clones were sent to Guangzhou IGE Biotechnology Co., Ltd. for sequencing.

3.4 Bioinformatics analysis of *EdAGPL1* gene

Using NCBI ORF Finder online tools (https://www.ncbi.nlm.nih.gov/orffinder) for analysis of the open reading frame (ORF); DNAMAN 9 was used to compare the homology of amino acid sequences. The ExPASy - ProtParam (http://web.expasy.org/protparam/) and SignalP (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the physical and chemical properties of EdAGPL1 and signal peptide; Using the PSORT Prediction for subcellular localization (http://psort1.hgc.jp/form.html); Using the Conserved Domain Database of NCBI for

function structure domain analysis (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi); SMART database (http://smart.embl-heidelberg.de/) was used for protein domain prediction analysis. PredictPro software (https://www.predictprotein.org/home) was used to predict the secondary structure of protein. Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) was used to predict the tertiary structure of protein; Finally, MEGA 5.1 was used to construct the phylogenetic evolutionary tree.

3.5 Analysis of gene expression

Primer 5.0 was used to design fluorescence quantitative primers EDAGPL2-F (5′-CAAGGGTGTAGCGGCAATCAT-3′) and EdAGPL2-R (5′-GGCGATAACATCCTCCAACTGG-3′) based on the obtained *EdAGPL1* gene sequence. The reference primers CWC1-F (5′-GGCGATAACATCCTCCAACTGG-3′) and CWC1-R (5′-TCGCTCCACCAACTAAGAACGG-3′) were designed using water chestnut 18S rRNA (Registration number: MG742686.1) as the template. Fluorescence quantitative RT-PCR was performed using 2×ChamQ Universal SYBR qPCR Master Mix kit (Nanjing Vazyme Biotechnology Co., Ltd.), and the reaction system was 20 μL according to the kit instructions.

Quantitative RT-PCR reaction procedure was as follows: 95℃ for 3 min, 95℃ for 15 s, 59℃ for 15 s, 72℃ for 20 s, 45 cycles, 3 replicates per sample.

Authors' contributions

HFL was mainly responsible for experimental operation, data analysis and writing the first draft of the paper. QZY and LLL participated in sample collection; DWQ is the project proposer and the person in charge, who is responsible for directing the experiment and the writing and revision of the paper. All authors read and approved the final manuscript.

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