Response to Reviewer 1 Comments

Point 1: The authors focus much of the introduction and discussion on the process of somatic embryogenesis. And they often interpret the regulatory pathways involved in this process on the basis of the results obtained here without documenting somatic embryogenesis in this paper (obtaining embryogenic callus does not prove that regeneration by somatic embryogenesis occurs!!!) Hence, the question is whether the authors are able to document the formation of somatic embryos from transgenic callus? If not such conclusion should be omitted and clearly written that it is only speculation.

Response 1: Thank you very much for your comments. We have modified them throughout the manuscript.

Point 2: If somatic embryogenesis can be induced in this spices why Authors didn't use somatic embryo to expression analysis of GPT1?

Response 2: Thank you very much for your comments. We have modified it to embryogenic callus formation.

Point 3: As the authors conducted studies on different fragments of the plant, zygotic embryos and callus please clearly specify the purpose of the presented work.

Response 3: Thank you very much for your comments. We have added and modified it in the manuscript.

Point 4: Please explain all abbreviations when used for the first time.

Response 4: Thank you very much for your comments. We have modified them throughout the manuscript.

Point 5: Please make it clear when you are writing about zygotic versus somatic embryogenesis, and somatic embryogenesis versus other in vitro regeneration processes. In many places in the work, the reader loses clarity about what the particular fragment is about.

Response 5: Thank you very much for your comments. We have modified them throughout the manuscript.

Point 6: Given the results obtained, the introduction should not place so much emphasis on improving the efficiency of somatic embryogenesis and conversion of somatic embryos because this was ultimately not demonstrated in this study.

Response 6: Thank you very much for your comments. We have modified them throughout the manuscript.

TITLE

Point 7: The title is inadequate to the overall work and omits completely: functional analysis of GPT1, its localization in the plant and expression in zygotic embryo, and expression of transcription

factors in calli. So 4 of the 6 points of the results were not included in the title. Moreover, the use of the phrase "formation of embryogenic cells" in the title and not showing the ultrastructure of embryogenic and non-embryogenic cells is unjustified. That's why I propose to change the title and bring it in line with all aspects undertaken in the work.

Response 7: Thank you very much for your comments. We have modified it in the manuscript.

ABSTRACT

Point 8: Line 21: Throughout the manuscript, there is no information on how the development of embryos in Peonia ostia proceeds. Also absent is any photographic documentation showing the embryos. On what basis do the authors conclude that PoGPT1 transcription increases during embryo development????

Response 8: Thank you very much for your comments. We have modified it in the manuscript.

Point 9: Line 26-28: As the authors do not document in the paper the regeneration of somatic embryos they obtained, or the efficiency of somatic embryogenesis (only embryogenic callus is presented) this sentence does not hold true.

Response 9: Thank you very much for your comments. We have modified it in the manuscript.

INTRODUCTION

Point 10: Line 49-50: Please explain the difference between vegetative and somatic. From my point of view means the same.

Response 10: Thank you very much for your comments. We have modified it in the manuscript.

RESULT

Point 11: My biggest objection in this part is to the lack of visualization of the statistical analysis in the graphs included in the Results. Please supplement the graphs with information on which means are different from each other. Without this the results cannot be interpreted!

Response 11: Thank you very much for your comments. We have modified them throughout the manuscript.

REFERENCES

Point 12: This section should be examined again In terms of italics for species and gene names (some marked in the pdf).

Response 12: Thank you very much for your comments. We have modified it in the manuscript.

Point 13: 25 and 44 are the same publication!!!

Response 13: Thank you very much for your comments. We have modified it.

FIGURES

Point 14: Figures are not very precisely described: there are no information on day of culture, the titles of the figures often do not refer to all the pictures on them.

Response 14: Thank you very much for your comments. We have modified them throughout the manuscript.

Point 15: All figures on which quantitative analyses are presented (Figs. 5, 7, 8) , do not contain statistics only deviations are marked. No information on which samples are statistically different from each other. Please mark the differences. Without this, the results cannot be interpreted and all conclusions drawn are unjustified.

Response 15: Thank you very much for your comments. We have modified them throughout the manuscript.

Other Points: peer-review-21915863.v1.pdf

Responses: The revision comments in the PDF file are also revised and answered one by one in the original file.

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Article

Glucose-6-phosphate translocator *PoGPT1* promotes embryogenic cell formation of tree peony by mediating dynamic changes in endogenous hormones

Yinglong Song [1,†], Wenqian Shang [1,†], Zheng Wang [1,*], Songlin He [1,2,*], Yuke Sun [1], Liyun Shi [1], Yuxiao Shen [1], Dan He [1]

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[1] College of Landscape Architecture and Art, Henan Agricultural University, Zhengzhou, 450002, P.R.China; edward_song1989@163.com (Y.L.S.); qianqian656@163.com (W.Q.S.); yukesun1@163.com (Y.K.S.); sisyrin@henau.edu.cn (L.Y.S.); yxshen@henau.edu.cn (Y.X.S.); dandan990111@163.com (D.H.)
[2] School of Horticulture Landscape Architecture, Henan Institute of Science and Technology, Xinxiang, 453003, P.R.China
* Correspondence: wzhengt@163.com, +86-13643846891 (Z.W.); hsl213@yeah.net, +86-0373-3040569 (S.L.H.)
† Contributed equally to this work and are co-first authors

Abstract: Tree peony (*Paeonia ostii*) is an important ornamental flowering plant, but is generally recalcitrant to establishment of a mature somatic embryo regeneration system *in vitro*. Glucose-6-phosphate translocator (GPT) plays an important regulatory role in embryogenesis of plants. In this study, *PoGPT1* was cloned, and a bioinformatic analysis and functional verification of the gene were performed. The results showed that *PoGPT1* encoded a polypeptide of 392 amino acids, which was a basic non-secreted hydrophobic transmembrane protein, and was localized in the plastids. *PoGPT1* was highly expressed in tree peony leaves and its transcript abundance increased with the progression of embryo development. Overexpression of *PoGPT1* caused up-regulation of *PoLEC2*, *PoSERK*, and *PoABI3L15* in tree peony callus and enhanced embryogenesis in the calli. In addition, *PoGPT1* overexpression promoted the increase in IAA, 5DS, and BL contents, especially of IAA, but reduced the contents of ABA, BARP, and ACC. The present research showed that *PoGPT1* synergistically regulated the contents of endogenous hormones and expression levels of embryogenesis-related genes to promote the early embryonic development of tree peony. The results provide theoretical and technical support for the establishment of a tree peony somatic embryo regeneration system.

Keywords: *Paeonia ostii*; *GPT1*; functional analysis; homologous transformation; hormone regulation

Abbreviations: IAA, indole-3-acetic acid; ABA, abscisic acid; BARP, 6-benzyladenosine; ACC, 1-aminocyclopropanecarboxylic acid; 5DS, 5-deoxystrigol; BL, brassinolide; GPT1, glucose-6-phosphate translocator; RT-qPCR, quantitative reverse-transcription PCR; MS, Murashige–Skoog medium; WPM, woody plant medium; 6-BA, 6-benzylaminopurine; IBA, 3-indolebutyric acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; PVP, polyvinylpyrrolidone; LH, lactalbumin hydrolysate; CTAB, cetyltrimethylammonium Ammonium Bromide.

1. Introduction

Tree peony (*Paeonia* sect. *Moutan*) is among the oldest and most culturally important horticultural and medicinal plants in China. It is known for its large and colorful flowers, and is widely regarded as the national flower of China [1–5]. To date, tree peony has been propagated mainly using asexual methods, such as plant division and grafting. However, these methods have a low propagation efficiency, which limits large-scale plant production [6–9]. Tissue culture methods have the potential to solve this problem, but there are some problems when using such methods to produce tree peony plantlets by direct organogenesis. For example, it is difficult to induce explants to form roots, and a large proportion of the roots that do form are of poor quality, with a disconnect between the adventitious root and the stem. This results in low survival rates of plantlets at the transplanting stage [6,10–12]. Somatic (non-zygotic) embryos develop from vegetative or somatic cells that form proembryogenic masses, and have the same ability as zygotic embryos to germinate and develop into a complete plant [13,14]. Thus, tree peony plantlet regeneration *via* the somatic embryogenesis pathway may overcome the above-mentioned problems.

Previous studies of embryo culture and somatic embryo regeneration of tree peony have shown that the regeneration ability of tree peony somatic embryos is strongly associated with the explant genotype and hormones [10,11,15]. However, the embryogenic cell

induction rate of tree peony is low and the differentiation of somatic embryos from proembryogenic masses is poor [16]. Even if seedlings are formed, many irregularities are observed, such as plant deformity and nonconnection of the root duct and sieve tubes [6,17]. Thus, an efficient and stable somatic embryo regeneration system for tree peony has not been reported previously. In essence, the low efficiency of the transformation from non-embryogenic callus to embryogenic callus is a major restrictive factor in the early stage of somatic embryo development [10,12,16]. Early-stage embryos are heterotrophic tissues that absorb, transport, and assimilate carbohydrates from plastids in non-photosynthetic cells [18,19]. *GPT1*, the gene encoding glucose-6-phosphate translocator 1 (GPT1), is mainly expressed in heterotrophic tissues, where its product mediates glucose metabolism and transport between source and sink cells [20,21]. GPT1 also participates in starch and fatty acid synthesis and/or the oxidative pentose phosphate pathway to produce NADPH [22–24], as well as playing an important role in the synthesis, storage, and transportation of materials essential for embryonic structure and development [25,26]. Previous research has shown that GPT1 is essential for pollen fitness and successful reproduction during pollen maturation [27,28]. The distribution of sugars and auxin is severely affected in the *gpt1* homozygous mutant of *Arabidopsis thaliana*, resulting in embryonic death [26,29]. Therefore, *GPT1* is crucial for early embryo development. However, it is unknown whether *GPT1* plays a related regulatory role through glucose metabolism and transport in the process of tree peony somatic embryogenesis.

In this study, we cloned *PoGPT1* and analyzed its function. Our findings showed that *PoGPT1* is expressed in different organs and at different stages of embryo development in tree peony. Given that *SERK*, *LEC1*, and *AGL15* are crucial genes in plant somatic embryo development [30,31,32], we analyzed the regulatory effects of *PoGPT1* on these genes and endogenous hormones. Homologous overexpression of *PoGPT1* significantly affected the transcript levels of *PoSERK*, *PoLEC1*, and *PoAGL15*, and the contents of endogenous hormones. Our analyses show that the expression of *PoGPT1* is strongly associated with auxin content. These results provide insight into the role of *PoGPT1* during early somatic embryogenesis in tree peony.

2. Materials and Methods

2.1. Plant Materials and Culture Conditions

Tree peony (*Paeonia ostii*) was used as the test material. All plants were grown in a nursery at the Henan Agricultural University in Zhengzhou, China. The plants were approximately 10 years old, cultivated in sandy loam soil, and of healthy, vigorous growth.

Pods were collected at 5, 60, 65, 75, 90, and 110 d after pollination, from the beginning of pod development until full maturity (from April to August, 2021). The pericarp of the pod was removed after collection, and the RNA was isolated specifically from developing embryos. In addition, roots, stems, leaves, flowers, and fully mature seeds were collected on the same days as specified for pods. The samples were used for real-time quantitative PCR (RT-qPCR) analysis. Axillary buds (Figure 1A) were selected for total RNA extraction and gene cloning. The samples were quickly frozen in liquid nitrogen and stored in a −80°C refrigerator (Thermo Forma 991, Thermo Fisher Scientific, Shanghai, China). Each sample comprised 0.5 g tissue with three replicates. The RNAiso Plus Kit (TaKaRa, Dalian, China) was used to extract total RNA from the tree peony materials.

Axillary buds were selected for tissue culture on plantlet induction medium (MS + Ca^{2+}(WPM) + 6-BA 0.3 mg·L^{-1} + IBA 0.5 mg·L^{-1} + PVP 1.0 g·L^{-1} + Sucrose 30 g·L^{-1} + Agar 7 g·L^{-1}, pH 5.8). After 30 d of culture (Figure 1B), the stems of plantlets *in vitro* were collected for induction of non-embryonic callus (1/2 MS + Ca^{2+}(WPM) + 6-BA 1.0 mg·L^{-1} + 2.4-D 1.0 mg·L^{-1} + PVP 1.0 g·L^{-1} + LH 0.5 g·L^{-1} + Sucrose 30 g·L^{-1} + Agar 7 g·L^{-1}, pH 5.8) (Figure 1C). The calli were used for transformation after 35 d of culture (Figure 1D).

The plantlets and calli were cultured in glass Erlenmeyer flasks (100 mL, Sichuan Shubo Group Co., Ltd., Sichuan, China) in a tissue culture room at the Laboratory of

Ornamental Plants, Henan Agricultural University, Zhengzhou, China, under the following conditions: air temperature, 23 ± 1°C; relative humidity, 70% ± 5%; photoperiod, 16 h light/8 h dark, with photosynthetically active radiation (45 ± 2 μmol·m^{-2}·s^{-1}) supplied by plant growth fluorescent lamps (TLD-type, Philips Co., Shanghai, China).

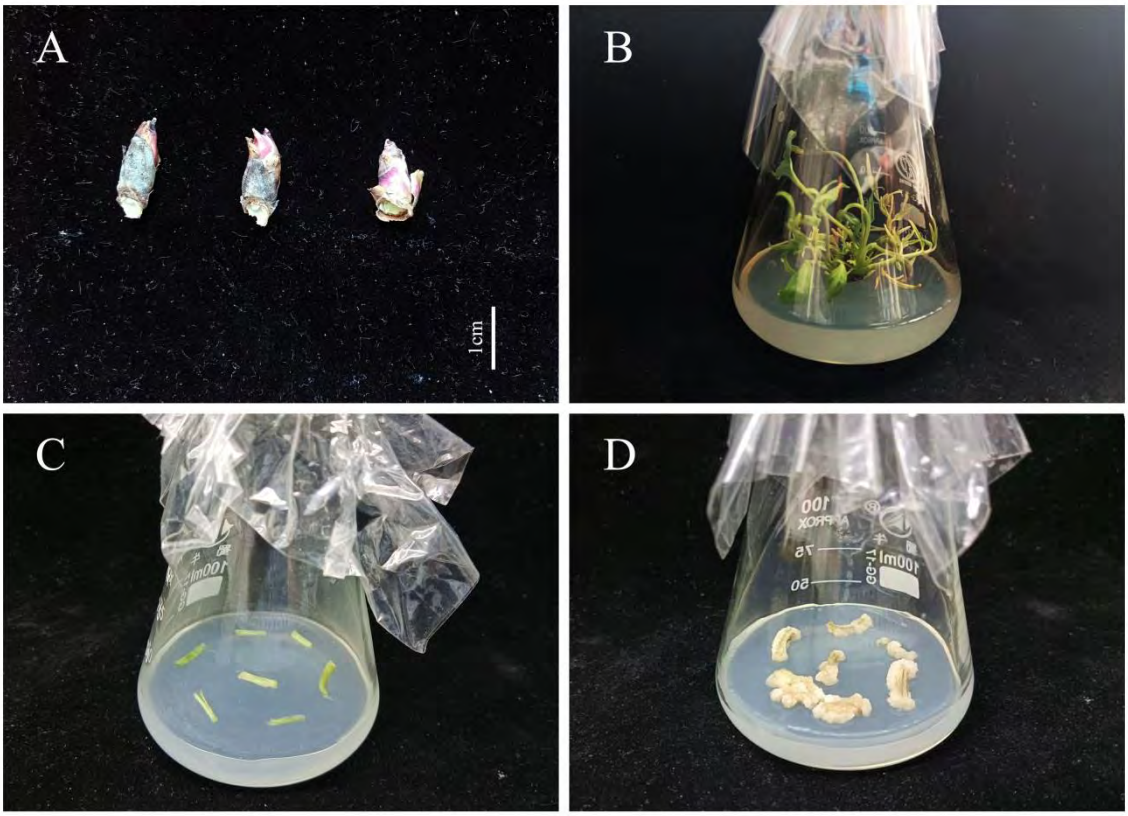

Figure 1. Test materials of tree peony (A, Axillary buds; B, Plantlets *in vitro*; C, Callus induction; D, Non-embryonic callus). Scale bar: 1 cm.

2.2. Cloning and Analysis of *GPT1*

Total RNA was extracted from 0.5 g of the tree peony materials (stems and leaves) using the modified CTAB method. The cDNA was synthesized using the Prime Script RT Reagent Kit (TaKaRa). Gene-specific primers (Table 1) were designed to clone the full-length coding sequence (CDS) of *PoGPT1* (GenBank: ON392713). BioXM2.7 was used to translate the open reading frame (ORF) of *PoGPT1*. The physicochemical properties and conserved domains of the PoGPT1 protein were estimated using analytical tools accessible on the NCBI website (https://www.ncbi.nlm.nih.gov/). The online software SMART (http://smart.embl-heidelberg.de) was used for domain analysis. DNAMAN8 was used for multiple sequence alignment and MEGA7.0 was used to construct phylogenetic trees with the neighbor-joining method.

Table 1. Summary of primers used in the study.

Primer Name	Forward Primer Sequence (5′-3′)	Reverse Primer Sequence (5′-3′)
PoGPT1-Clone	ATGATTTGCTCTGCAAAACAAGC	TCACTGCTTTGCCTGGGAAT
PoGPT1-OE	ACGGGGGACTCTTGACCATGGATGATTTGCTCTGCAAAACAAGC	AAGTTCTTCTCCTTTACTAGTTCACTGCTTTGCCTGGGAAT

PoGPT1-GFP	GGGGTACCATGATTTGCTCTGCAAAACAAGC	GCTCTAGACTGCTTTGCCTGGGAATACAA
β-tubulin	TGAGCACCAAAGAAGTGGACGAAC	CACACGCCTGAACATCTCCTGAA
PoGPT1-RT	GCAGTTTCGTTCACCCATATCATCAAG	CAGCAAGAGCACATCCACCAATTATC
PoLEC1-RT	TCACGGCTGAGGACTTACTATGGG	TAATGTTCTCGGCACGCAAGGAAG
PoSERK-RT	GGCGAAGGAAACCACAAGAA	CCAACGAACCATCAGCAAGG
PoAGL15-RT	GGATAATTCACTGCCGTCTT	CCATGCCAATTTGACTTCC

2.3. Subcellular Localization Analysis

The ORF of *PoGPT1* (without the stop codon) was amplified with gene-specific primers (Table 1) and cloned into the pCAMBIA1300 vector to generate 35S::*PoGPT1*:GFP. The construct was introduced into lower epidermis cells of *Nicotiana benthamiana* leaves as described previously [33]. The GFP fluorescence was observed at 40× magnification with a confocal laser scanning microscope (A1+, Nikon, Shanghai, China). The filter settings for visualization of the fluorescence were as follows: chloroplast fluorescence: excitation light wavelength 640 nm, emission light wavelength 675 nm; GFP fluorescence: excitation light wavelength 488 nm, emission light wavelength 510 nm.

2.4. Plasmid Construction and Transformation of Tree Peony Callus

To construct the overexpression vector, the ORF of *PoGPT1* (without the stop codon) was amplified with gene-specific primers (Table 1) and cloned into pCAMBIA1300 to generate 35S::*PoGPT1*. The complete vector was verified by sequencing, and the construct was transformed into *Agrobacterium tumefaciens* strain LBA4404. The pCAMBIA1300 vector was transformed into *A. tumifaciens* cells separately as the control (CK). Nontransformed callus was used as the blank control (WT). The *A. tumefaciens* cells were used to transform calli of tree peony and were removed before expression analysis. The specific protocol was based on our previous research results [34]. The transformed calli were analyzed to determine gene transcript levels and endogenous hormone contents, and paraffin-embedded thin sections were observed.

2.5. Determination of Plant Hormone Content

The transgenic calli with different genotypes (CK and 35S::*PoGPT1*) were finely ground in liquid nitrogen and the powder was transferred to 10 mL centrifuge tubes. The contents of endogenous plant hormones (abscisic acid, ABA; 1-aminocyclopropanecarboxylic acid, ACC; 6-benzyladenosine, BARP; indole-3-acetic acid, IAA; 5-deoxystrigol, 5DS; and brassinolide, BL) in the transformed calli were determined using ultra-high-performance liquid chromatography (LC-30A, Shimadzu, Kyoto, Japan)–mass spectrometry (Triple TOF 5600+, AB SCIEX, Foster City, CA, USA) following a previously described method [6]. The plant hormone standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). The extraction and measurement methods were as described in our previous study [6].

2.6. Morphological and Anatomical Observation

The transgenic calli with different genotypes (CK and 35S::*PoGPT1*) were transferred to somatic embryogenesis induction medium (1/2 MS + Ca^{2+}(WPM) + 6-BA 0.5 mg·L^{-1} + TDZ 0.5 mg·L^{-1} + PVP 1.0 g·L^{-1} + LH 0.5 g·L^{-1} + Sucrose 20 g·L^{-1} + Agar 7 g·L^{-1}, pH 5.8) and cultured for 20 d. Embryogenic calli were collected for morphological and anatomical observations. After fixation in formalin/ acetic acid/ alcohol=1/ 1/ 18 (v/v/v), paraffin embedded sections (thickness, 10 μm; prepared by Wuhan Service Biotechnology Co., Ltd., Wuhan, China) were examined to observe the cellular structure of the different transgenic calli.

2.7. Real-Time Quantitative PCR (RT-qPCR) Analysis

Expression of the somatic embryogenesis-related genes *PoLEC1* (GenBank: ON454840), *PoSERK* (GenBank: KF876175), and *PoAGL15* (GenBank: ON454841) was analyzed to verify the establishment of embryonic properties of the calli by RT-qPCR. Forward and reverse primers were designed using Primer-BLAST online tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi). A tree peony β-tubulin gene (EF608942) was used as the internal reference gene (Table 1). Analyses of gene transcript levels were conducted using the SYBR Premix Ex Taq™ II Kit (TaKaRa) in accordance with the manufacturer's instructions. A standard two-step method was used with three biological replicates [35,36]. The quantitative analysis was performed on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative transcript levels were calculated using the $\Delta\Delta C_t$ method.

2.8. Statistical Analysis

Analysis of variance (ANOVA) was performed with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Significant differences between means were detected using Duncan's multiple range test at $P \leq 0.05$.

3. Results

3.1. Sequence Analysis of PoGPT1

The ORF of *PoGPT1* was 1179 bp, encoding a polypeptide of 392 amino acids with a predicted molecular weight of 42.80 kDa (Table 2). The amino acid sequence included a highly conserved triose phosphate translocator (TPT) superfamily domain (Figure 2A, B).

The online prediction analysis revealed that PoGPT1 lacked a signal peptide, indicating that it was a non-secretory protein (Figure 2C). The transmembrane domain prediction analysis revealed that PoGPT1 was a typical transmembrane protein with seven transmembrane domains (Figure 2D). The physicochemical properties of the predicted PoGPT1 protein were analyzed using online software; the protein instability index of 49.04, fat solubility index of 102.04, and total average hydrophilicity index of 0.464 indicated that PoGPT1 was an unstable hydrophobic liposoluble protein (Table 2). The predicted isoelectric point of the protein was 9.4. The protein contained more basic amino acid residues than acidic residues, thus it was an alkaline protein (Table 2). It showed high homology with homologs in *Carya cathayensis*, *Camellia sinensis*, citrus, oak, and rubber tree. Therefore, the gene was designated *PoGPT1* (Figure 2B, E).

Table 2. Characteristics and physicochemical properties of the nucleotide and deduced amino acid sequences of the *PoGPT1* gene.

Gene	ORF (bp)	Number of amino acids	Molecular weight (kDa)	Theoretical pI	Total number of negatively charged residues (Asp + Glu)	Total number of positively charged residues (Arg + Lys)	Instability index	Aliphatic index	Grand average of hydropathicity (GRAVY)
PoGPT1	1179	392	42.80	9.43	18	30	49.04	102.04	0.464

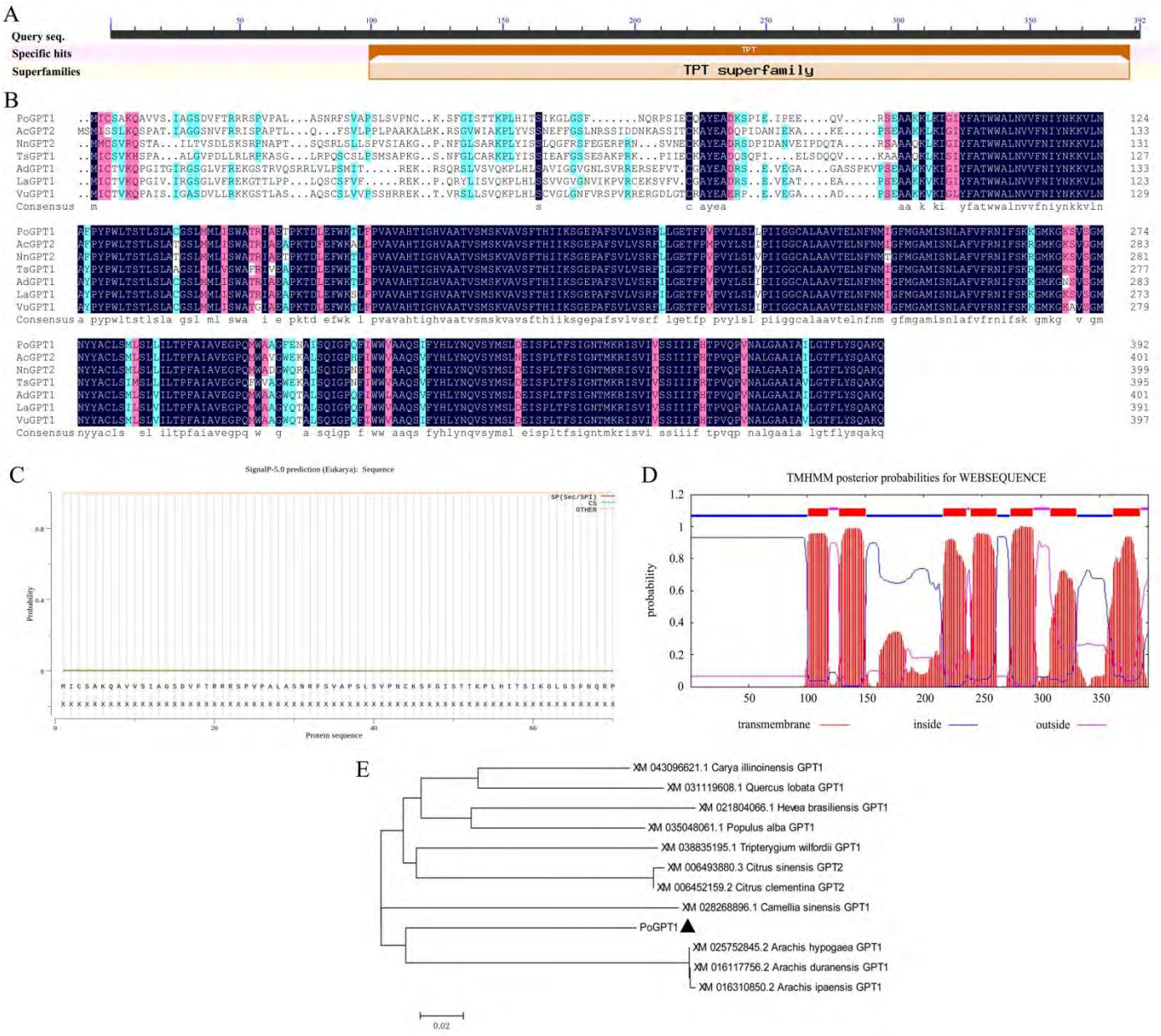

Figure 2. Protein structure and phylogenetic analysis of PoGPT1 (A, Conserved domains; B, Sequence alignment; C, Signal peptide analysis; D, Transmembrane domain analysis; E, Phylogenetic analysis).

3.2. Subcellular Localization of PoGPT1

The subcellular localization vector GFP-*PoGPT1* was constructed and transfected into the lower epidermis cells of *N. benthamiana* leaves. Blue fluorescence was observed after DAPI staining (Figure 3B), and red autofluorescence was emitted from the plastids in the leaf (Figure 3D). The green fluorescence signal from the GFP-PoGPT1 construct precisely overlapped with the red autofluorescence emitted from the plastids (Figure 3A,C), indicating that PoGPT1 was localized in the plastids.

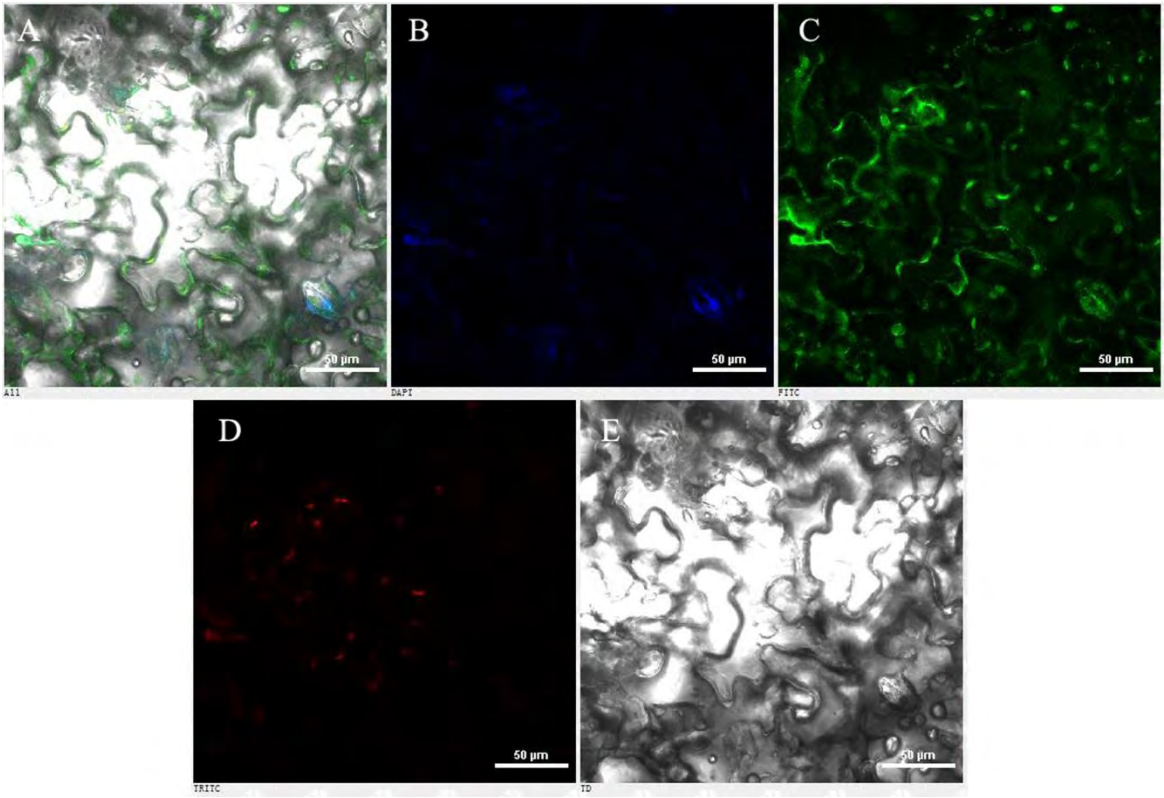

Figure 3. Subcellular localization of PoGPT1 (A, All; B, DAPI; C, FITC; D, TRITC; E, TD). *Nicotiana benthamiana* leaf lower epidermis cells were i[illegible] with the 35::*PoGPT1*-GFP construct mediated by *Agrobacterium tumefaciens* strain GV3101. Green fluorescence indicates the fusion protein location and blue fluorescence is the nuclear signal localized by DAPI. Scale bar: 50 μm.

3.3. Spatiotemporal Expression Analysis of PoGPT1

To determine the spatiotemporal-specific expression patterns of *PoGPT1* and its transcript levels in different organs of tree peony and at different stages of embryonic development, RT-qPCR analysis was performed on cDNAs synthesized from RNA isolated from the roots, stems, leaves, flowers, and seeds, and from pods at different developmental stages. The *PoGPT1* transcript abundances were ranked, from highest to lowest, as follows: leaf > flower > root > seed > stem (Figure 4A). The RT-qPCR analyses also revealed a gradual increase in the abundance of *PoGPT1* transcripts during embryo development (Figure 4B).

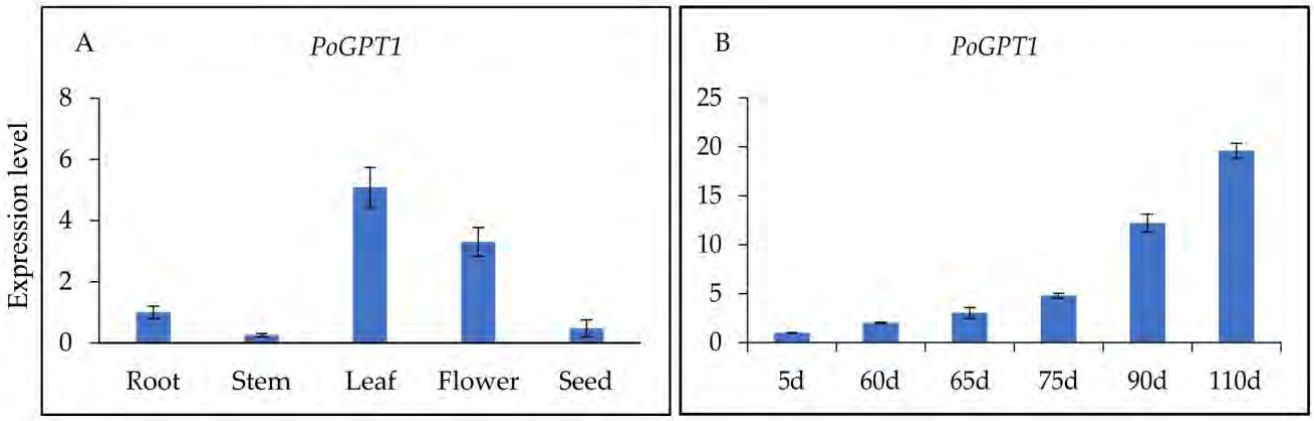

Figure 4. Spatiotemporal expression analysis of *PoGPT1* in tree peony (A, Expression levels in different tissues of tree peony; B, Expression levels at different stages of tree peony embryo development). Vertical bars indicate standard error ($n = 3$).

3.4. RT-qPCR Analysis of PoGPT1 and Genes Associated with Somatic Embryogenesis

The *PoGPT1* overexpression vector pCAMBIA1302-*PoGPT1* was transformed into tree peony calli by *Agrobacterium* infection. Significantly increased expression of *PoGPT1* was confirmed in the transgenic calli compared with that in WT and CK calli (Figure 5A). Further RT-qPCR analyses confirmed significant up-regulation of *PoLEC1* (Figure 5B), *PoSERK* (Figure 5C), and *PoAGL15* (Figure 5D) in the transgenic calli overexpressing *PoGPT1*. The empty vector pCAMBIA1302 had no significant effect on the expression of these genes in tree peony callus.

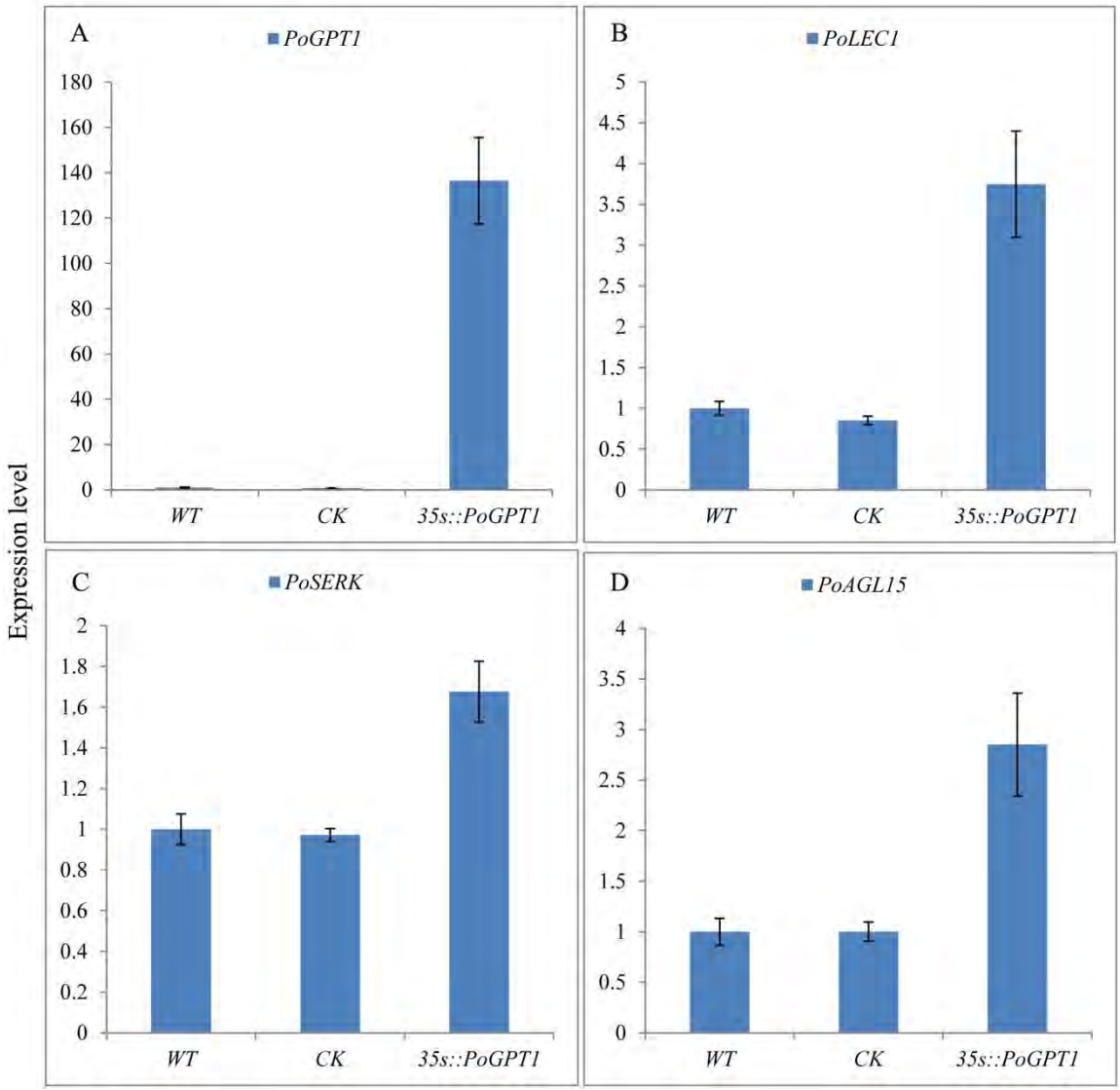

Figure 5. RT-qPCR positive identification and expression level of embryogenesis-related genes in *PoGPT1*-overexpressing transgenic calli of tree peony (A, Positive identification; B, Expression level of *PoLEC1*; C, Expression level of *PoSERK*; D, Expression level of *PoAGL15*). Vertical bars indicate standard error (n = 3).

3.5. Anatomical Observations

Observation of paraffin-embedded sections revealed that the transgenic calli overexpressing *PoGPT1* comprised more compact cells and a large embryogenic cell mass compared with the WT and CK calli after 25 d of culture (Figure 6A, B, C, *a*, *b*, *c*), indicating that increased *PoGPT1* expression effectively promoted the formation of embryogenic cell clusters in tree peony calli. No difference in the number of embryogenic cell masses was observed between the WT and CK calli.

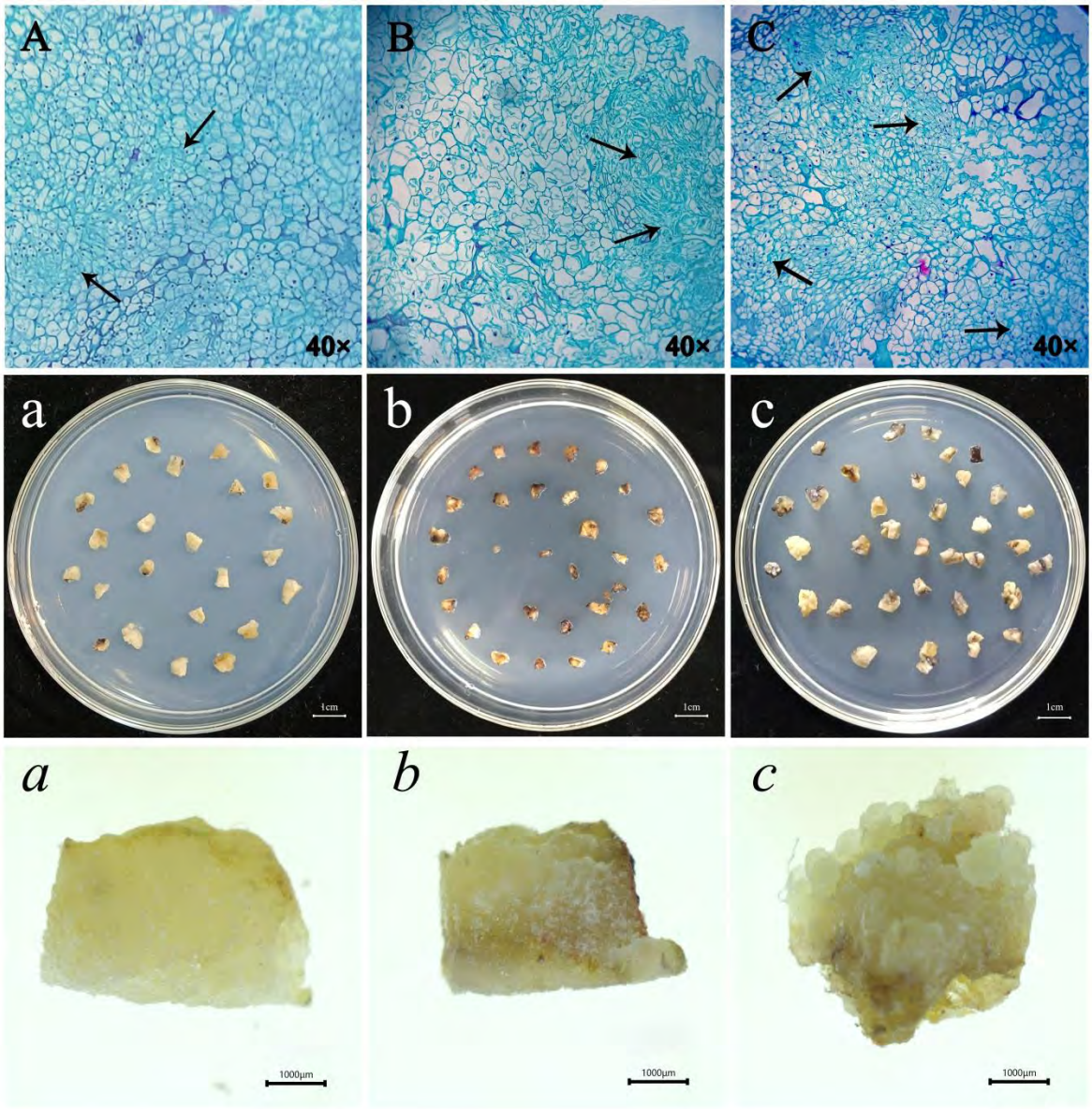

Figure 6. Paraffin-embedded sections of *PoGPT1*-overexpressing transgenic callus of tree peony (A, a, *a*, WT: non-transformed callus; B, b, *b*, CK: pCAMBIA1302; C, c, *c*, *35s::PoGPT1*; Black arrow indicates embryogenic callus mass. Scale bar: 1cm, 1000 μm).

3.6. Endogenous Plant Hormone Contents

Given that no anatomical difference was observed between WT and CK calli, CK with the same transformation background as 35s::*PoGPT1* was used as the control to analyze the effect of *PoGPT1* overexpression on endogenous hormone contents in tree peony callus. Overexpression of *PoGPT1* differentially affected the contents of various endogenous hormones during embryogenesis in tree peony calli (Figure 7). The ABA content in transformed calli gradually increased during the culture period (Figure 7B). The ABA content was significantly lower than that of the CK from day 0 to day 15, then increased on day 20. In contrast, the BARP content was significantly higher in transformed calli than in the CK on day 0, then decreased sharply and remained at a lower level than that of the CK from day 5 to day 20 (Figure 7C). The ACC content was higher in transformed calli than in the CK at days 0, 5, and 20, but was lower than that of the CK on days 10 and 15 (Figure 7D). The 5DS content first increased and then decreased in the transformed calli (Figure 7E). The 5DS content was lower in the transformed calli than in the CK from day 0 to day

10, then increased sharply to peak on day 15 at a level significantly higher than that of the CK before decreasing. The BL content in the transformed calli gradually increased over time, but was lower in the transformed calli than in the CK at all time points except for day 20, when it was higher than that of the CK (Figure 7F).

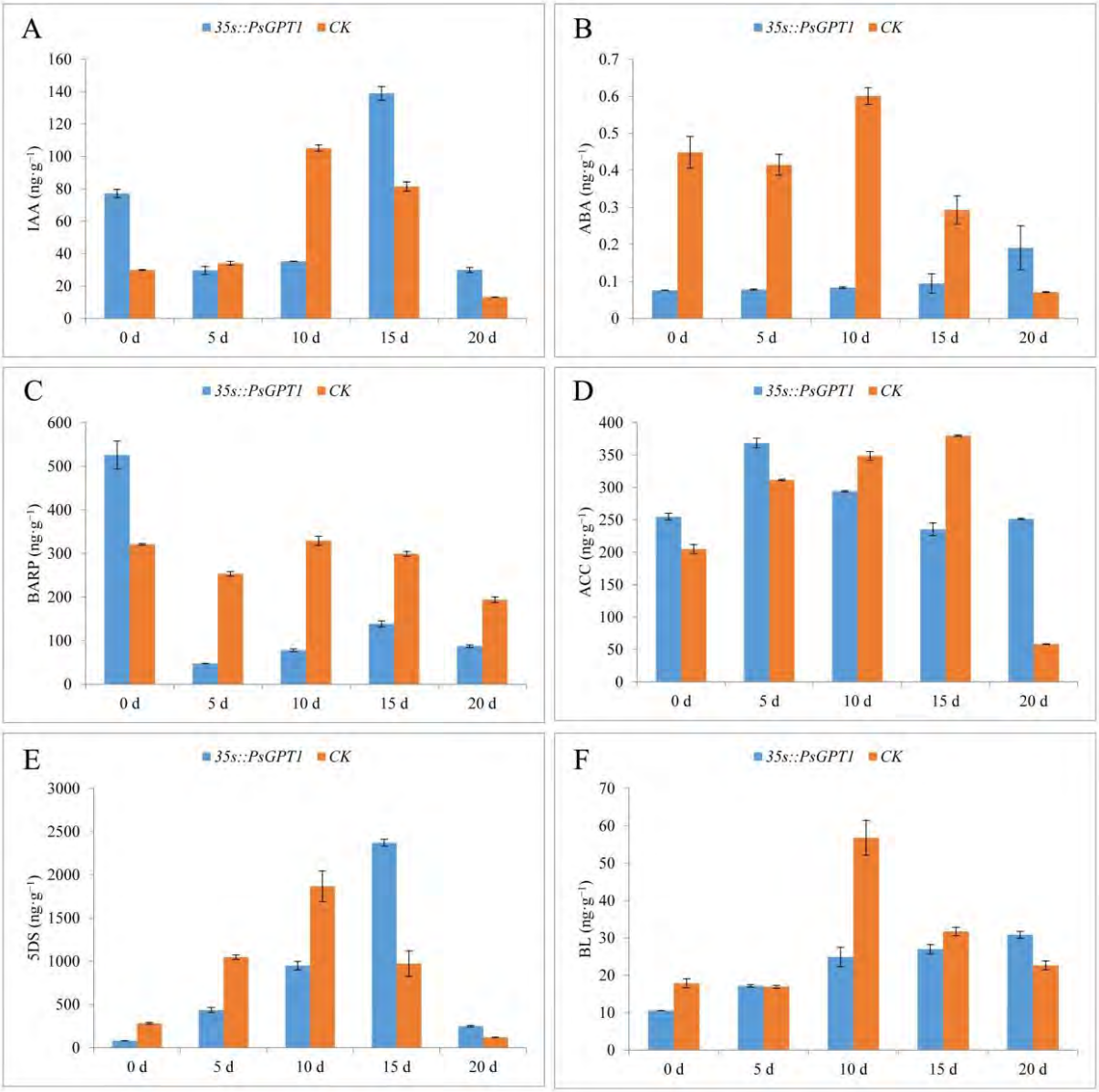

Figure 7. Endogenous plant hormone contents in tree peony callus (A, IAA; B, ABA; C, BARP; D, ACC; E, 5DS; F, BL). Vertical bars indicate standard error ($n = 3$).

Both the IAA/ABA and IAA/BARP ratios were higher in calli overexpressing *PoGPT1* than in the CK (Figure 8A, B). The ratio values of these plant hormones peaked on day 15. In contrast, the IAA/ACC ratio was significantly higher than that in the CK only on days 0 and 15, and peaked on day 15 (Figure 8C).

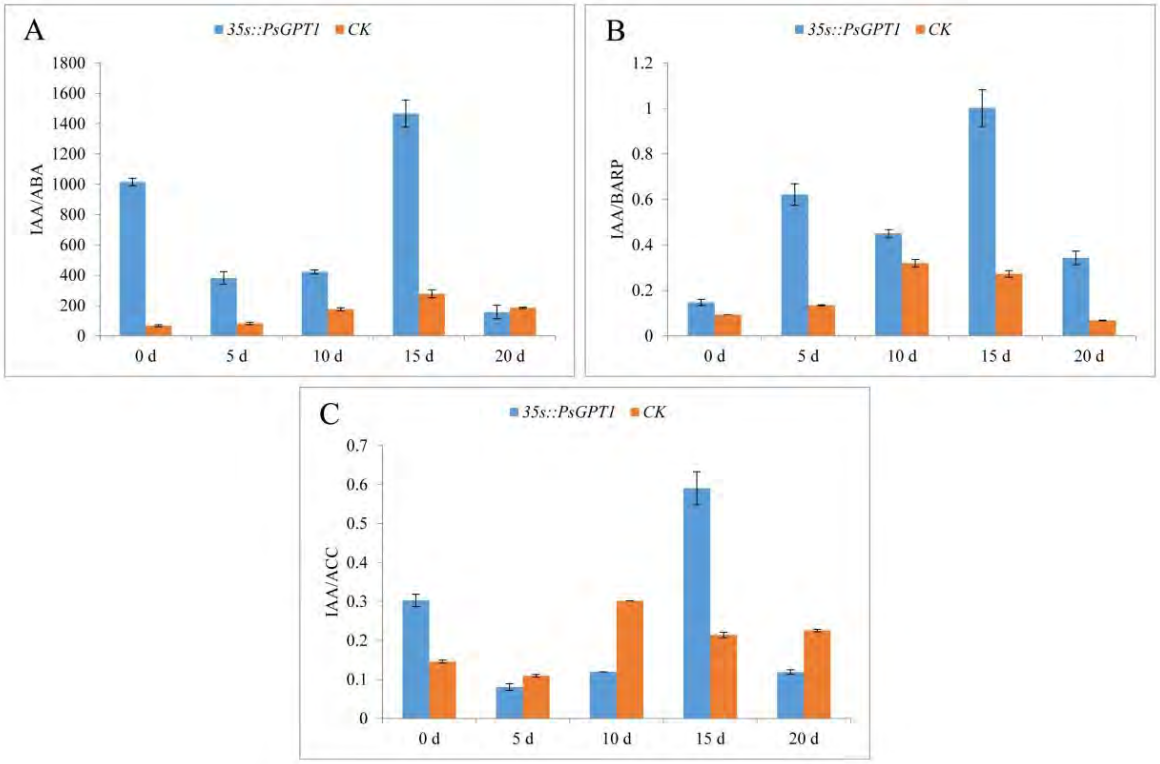

Figure 8. Endogenous plant hormone ratios in tree peony callus (A, IAA/ABA; B, IAA/BARP; C, IAA/ACC). Vertical bars indicate standard error ($n = 3$).

4. Discussion

Somatic embryogenesis is the process of embryo regeneration in which somatic cells develop into complete plants, without the involvement of sexual reproduction [37–40]. The photosynthetic system has not yet been established at the early stage of plant somatic embryogenesis, so the growth and differentiation of plant cells and tissues relies on heterotrophic processes [29]. Most of the glucose taken up by plant cells is phosphorylated to form glucose-6-phosphate (G6P) by hexokinase, and then G6P is translocated from the cytosol into the plastids by GPT. Inside the plastid, G6P is converted into starch and fatty acids or enters the oxidative pentose phosphate pathway [41–43]. Because GPT1 plays important roles in glucose metabolism and transport in heterotrophic tissues, it is a crucial participant in the regulation of plant growth and embryonic development [21,25,44]. Preliminary studies have observed many irregularities, such as delayed development of heart-shaped embryos, plant deformities, and poor root development, during tree peony somatic embryogenesis, which might be associated with metabolite synthesis and metabolism, endogenous hormone contents, and embryogenesis-related gene regulation [6–8, 11]. Given that *GPT1* is an important nutrient transport factor [25], it might perform an important role in the regulation of tree peony somatic embryogenesis. In the present study, the *PoGPT1* gene was successfully cloned, and bioinformatic analyses revealed that the 1179 bp ORF encodes a polypeptide of 392 amino acids. The PoGPT1 protein was predicted to be a basic non-secreted hydrophobic transmembrane protein with seven transmembrane domains. Subcellular localization analysis confirmed that the protein was localized to the plastids, consistent with the results of previous studies on GPT proteins [19,26].

Previous investigations have observed that *GPT1* is highly expressed in pollen, that there are significant temporal-specific differences in its expression during pollen development, and that it is important for both male and female gametogenesis [27,29]. In the present study, we detected high transcript levels of *PoGPT1* in the leaves and flowers, and a gradual increase in its transcript levels during embryo development of tree peony. These

results showed that *PoGPT1* was highly expressed in vegetative and reproductive organs and tissues of tree peony, and that it was co-expressed with other important embryogenesis-related genes during embryonic development. These expression patterns may reflect that PoGPT1 is required to provide metabolic substrates and energy sources during the growth and development of embryos in tree peony [19,25].

Embryonic development is a complex process coordinated by multiple genes, especially *SERK*, *LEC1*, and *AGL15* [45–47]. Previous research has shown that *GPT1* is required for early embryonic development [25,26]. Therefore, we generated tree peony calli overexpressing *PoGPT1* to explore its effect on key genes in somatic embryogenesis. We observed that overexpression of *PoGPT1* resulted in significant up-regulation of *PoLEC1*, *PoSERK*, and *PoAGL15*, all of which are involved in embryogenesis. In addition, overexpression of *PoGPT1* resulted in a significant increase in the embryogenic cell mass in calli, indicating that it has the ability to enhance somatic embryogenesis in tree peony.

Embryo induction and development relies on interactions among endogenous hormones [48,49]. Endogenous auxin, cytokinin, ethylene, ABA, jasmonates, and gibberellins are involved in the acquisition of embryogenic competence by somatic cells [39,49,50]. Among these hormones, auxin is the most important endogenous hormone for somatic embryo induction, and high IAA and low ABA contents have been shown to favor somatic embryogenesis [38,51,52]. In the present study, we observed that overexpression of *PoGPT1* significantly decreased the contents of ABA and BARP in the embryogenic calli of tree peony, significantly increased the contents of IAA and 5DS on day 15, and led to a continuous increase in BL content. We also observed that overexpression of *PoGPT1* resulted in significant increase in the IAA/ABA and IAA/BARP ratios, and increase in the IAA/ABA, IAA/BARP, and IAA/ACC ratios at day 15 of culture. The transcript level of *PoGPT1* was positively correlated with the contents of endogenous IAA and other associated hormones, consistent with the reported role of PoGPT1 in the polar transport and distribution of auxin [26]. We determined that the transcript levels of *PoLEC1*, *PoSERK*, and *PoAGL15* were associated with changes in hormone contents. These findings suggest that *PoGPT1* mediates dynamic changes in endogenous hormone contents, coordinates key genes in somatic embryogenesis, and regulates the process of somatic embryogenesis in tree peony.

5. Conclusions

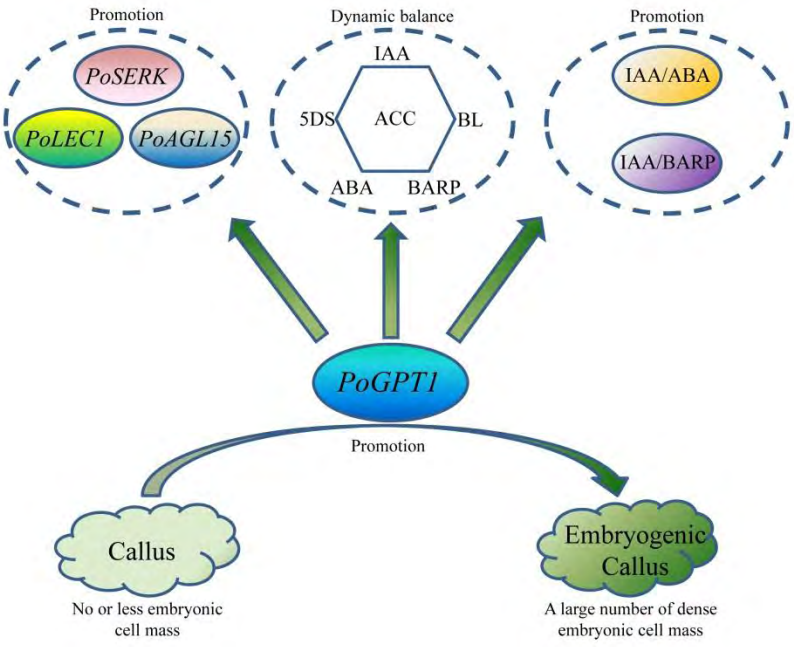

Figure 9. Model map of *PoGPT1* promotion of embryogenic cell formation in tree peony (*Paeonia ostii*).

The present results show that PoGPT1 is localized to the plastids and is highly expressed in tree peony leaves. Overexpression analyses showed that it is able to up-regulate expression of embryogenesis-related genes in tree peony callus, enhance embryogenesis in calli, and mediate dynamic changes in IAA, ABA, BARP, ACC, 5DS, and BL contents to regulate the early embryonic development of tree peony. On the basis of these results, we speculate that *PoGPT1* might coordinate the expression of endogenous hormones, especially IAA, thereby stimulating the remodeling of somatic totipotency and promoting the establishment of embryogenesis (Figure 9). Further research is required to determine how this process is regulated and the specific mechanism of action.

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