

Genome-wide identification of transcripts and miRNAs during somatic embryogenesis in *Pinellia ternata*

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Abstract

Pinellia ternata(Thunb.) Breit, a well-known Chinese medicinal plants, is mainly produced by clonal propagation. Somatic embryogenesis (SEG) is a major process for plant regeneration, however, the molecular mechanism of SEG in *P. ternata* is still unclear. In this study, genome-wide mRNAs and small RNAs analysis were performed on the samples from three key stages of *P. ternata* SEG, including explant, calli and protocorm-like bodies. The results showed that the increase in the content of auxins, especially I3CA and ICA1, contributed to the regeneration of *P. ternata*; *PtAUX/IAA2*, *PtSAUR4*, *PtAEC1*, *PtAEC2*, *PtAUT1*and *PtAUT2* were important regulatory genes for auxins sythesis and signal transduction in *P. ternata* SEG. For the first time, we employed small RNA sequencing to investigate *P. ternata* SEG, and we discovered that miR396 were the key miRNAs involved in regulating *P. ternata* SEG by targeting GRF genes.

Key message

***PtAUX/IAA2*, *PtSAUR4*, *PtAEC1*, *PtAEC2*, *PtAUT1* and *PtAUT2* were important regulatory genes for auxins sythesis and signal transduction in *P. ternata* SEG, and miR396 were the key miRNAs involved in regulating *P. ternata* SEG by targeting GRF genes**

Introduction

Somatic embryogenesis (SEG) is the process by which somatic cells first differentiate and then re-differentiate to produce embryos that develop into plants. Embryos can be formed directly from somatic cells, a process called direct SEG, or indirectly through an intermediate calli stage(Smertenko and Bozhkov 2014). SEG has the advantages of high reproduction frequency and strong controllability, which provides important technical support for rapid propagation of seedlings, artificial seed growth and genetic engineering breeding.

The process of SEG involves cell reprogramming, activation of the cell cycle, cell differentiation and organ development, and is regulated by a complex signaling transduction network, transcriptome reprogramming, and epigenetic modifications. Many transcription factors (TF) play an important role in the process of SEG. The representative TFs are AP2-domain PLETHORA (PLT) TFs, NF-Y (nuclear factor of the Y box) TF LEAFY COTYLEDON1 (LEC1), B3 TF LEC2, RWP-RK DOMAIN-CONTAINING4(RKD4)/GROUNDED (GRD), AT-HOOK MOTIF CONTAINING NUCLEAR LOCALIZED15(AHL15), and WOX TF WUSCHEL (WUS)(Brand, et al. 2019; Horstman, et al. 2017; Marsch-Martinez, et al. ; Paul, et al. 2022; Xiao, et al. 2018; Yang, et al. 2020). Given their ability to induce SEG, all of these TFs can be considered totipotency-related TFs(Wang, et al. 2022). Notably, it has been confirmed that the regeneration potential of some plants can be increased by regulating the expression levels of *BABY BOOM* (*BBM/PLT4*), *WUS* and *LEC2* transcription factors related to somatic embryogenesis(Lowe, et al. 2016). In addition, plant hormones, especially auxin, cytokinin and abscisic acid also play an important role in regulating the induction of plant SEG. It has been confirmed that auxin

treatment of explants was an indispensable inducer of SEG, which activates numerous totipotency-related TF, including the LEC1, LEC2, BBM (PLTs), AGL15 and WUS/WOX genes(Wójcik, et al. 2020). Conversely, SE-involved TFs could also control auxin metabolism and signalling pathway. Thus, the extensive crosstalk between the hormones, in particular, auxin and the TFs, was revealed to play a central role in the SE-regulatory network.

MicroRNAs (miRNAs) are small, 19–24 nucleotides (nt), non-coding small RNA regulatory molecules controlling a large number of biological processes. In recent years, many studies have shown that many types of miRNAs are involved in the regulation of plant SEG, including miR156, miR162, miR166a, miR167, miR168, miR393, miR396, miR397, miR398 and so on. For examples, miR393 plays an important role in the transition of somatic cells to embryonic cells in Arabidopsis by controlling the expression of the auxin receptor genes *TIR1* and *AFB2*(Bai, et al. 2023). miR167 is a negative regulator of SE induction in Arabidopsis, and overexpression of miR167 can reduce transcription levels of auxin response factor genes ARF6 and ARF8, thereby inhibiting the formation of somatic embryos(Hua, et al. 2016). miR396 might contribute to SE induction by controlling the sensitivity of plant tissues to auxin and regulating the expression level of *GROWTH-REGULATING FACTOR* (GRF)(Szczygieł-Sommer and Gaj 2019; Xia, et al. 2016). Therefore, the study of the regulatory mechanism of miRNAs in somatic embryogenesis is helpful to elucidate the regulatory network of plant somatic embryogenesis and regeneration.

P. ternata is an important medicinal plant, and the corms of *P. ternata* could be used to treat inflammation, relieving headaches, and relieving cough. It has been used as traditional Chinese medicine in countries such as China, Japan, and South Korea for more than 2000 years(Xu, et al. 2023). Asexual reproduction is the main mode of production, so it is very important to understand the process of SEG in *P. ternata*. In recent years, studies on SEG of *P. ternata* mainly focus on the screening of different genotypes of explants, induction of embryogenic calli and optimization of somatic embryogenesis conditions(He, et al. 1996; Wang, et al. 2009). However, there are few reports about the molecular mechanism of somatic embryogenesis in *P. ternata*, especially the molecular mechanism and related regulatory network of SEG induced by leaves as explants.

In the regeneration process of *P. ternata* explants, three stages are very important. Firstly, the tissue around the wound of the explant expanded to form calli, then the expanded calli can regenerate into multiple protocorm-like bodies, and finally each protocorm-like bodies can form one plant. In this study, the contents of endogenous auxin and cytokinin were compared at three key stages of *P. ternata* SEG: explant (Ex) stage, calli (Ca) stage and protocorm-like bodies (Pr) stage. And the transcriptome and small RNA profile were performed to identify the relevant functional genes, transcription factors (TFs), and miRNAs involved in the SEG process of *P. ternata*. These results provide a reference for depicting the regulatory network of miRNA-target genes in *P. ternata* early somatic embryogenesis, and provide a theoretical basis for discovering key genes regulating somatic embryogenesis in *P. ternata*.

Materials and methods

Plant materials

P. ternata used in this study was collected from the *P. ternata* germplasm resource nursery of Hubei University of Chinese Medicine. *P. ternata* leaves were washed, sterilized, and tissue cultured to obtain sterile seedlings. The leaves of sterile seedlings were cut into small squares with side lengths of 0.5 ~ 1 cm to obtain the Ex. After 15 days of cultivation, Ca of *P. ternata* can be obtained; the Ca were further cultured for 20–30 d to obtain the Pr of *P. ternata*. The above tissue cultures were grown using MS medium (6-Benzylaminopurine, 2.0 mg/L, α -Naphthalene acetic acid, 0.5 mg/L). The temperature in the tissue culture room is 25°C, the light intensity is 8000 Lux, and the photoperiod was 13 h light/11 h dark. After obtaining the experimental materials, immediately frozen in liquid nitrogen, and then stored in a freezer at – 80°C.

Determination of auxin and cytokinin.

The stock solution of individual phytohormone was mixed and prepared in phytohormone-free matrix to obtain a series of phytohormone calibrators. The stock solution of all of these and working solution were stored in refrigerator of -20°C.

Samples of 0.5 g were weighed, resuspended with liquid nitrogen, and then added to 500 μ L water by well vortexing as the diluted sample. Then 100 μ L of them were taken respectively and homogenized with 400 μ L of acetonitrile (50%) which contained mixed internal standards and extracted at 4°C. Then centrifuged at 12,000 rpm for 10 min. The supernatant (300 μ L) passed through the HLB sorbent (first flow-through fraction) and then was eluted subsequently with 500 μ L of acetonitrile (30%)(second flow-through fraction). These two fractions were combined into the same centrifuge tube and mixed well. Finally, these solutions were injected into the LC-MS/MS system for analysis.

An ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) system (ExionLC™ AD UHPLC-QTRAP 6500+, AB SCIEX Corp., Boston, MA, USA) was used to quantitate phytohormone. Separation was performed on a Waters XSelect HSS T3 column (2.1×150mm, 2.5 μ m) which was maintained at 45°C. The mobile phase, consisting of 0.01% formic acid in water (solvent A) and 0.01% formic acid in acetonitrile (solvent B), was delivered at a flow rate of 0.30 mL/min. The solvent gradient was set as follows: initial 10% B, 1min; 10–50% B, 3min; 50–65% B, 4 min; 65–70% B, 6min; 70–100% B, 7min; 100 – 10% B, 9.1min; 10% B, 12min. The mass spectrometer was operated in multiple reaction mode (MRM) mode. Parameters were as follows: IonSpray Voltage (Negative mode:-4500 V, Positive mode: 4500V), Curtain Gas (35 psi), Ion Source Temp (550°C), Ion Source Gas of 1 and 2 (60 psi). LC-MS was used to detect the concentration series of standard solution. The ratio of concentration of standard to internal standard as abscissa, and the ratio of peak area of standard to internal standard as ordinate to investigate the linearity of standard solution. The correlation coefficient (r) > 0.99 of each metabolites were the necessary condition (Supplementary document A).

Total RNA isolation, RNA quantification, and qualification

Total RNAs from the samples of three cultivation stages were extracted using the miRcute miRNA isolation kit(DP501) (TIANGEN Biotech (Beijing) CO., Ltd, Beijing, China). RNA degradation and contamination were monitored visually on 1% agarose gels. RNA purity was determined using the NanoPhotometer® spectrophotometer (Im-pen Inc., Munich, Germany). RNA concentration was measured using the Qubit® RNA Assay Kit in the Qubit 2.0 Fluorometer (Thermo Fisher Scientific Inc., San Jose,CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit f the Agilent Bioanalyzer 2100 system (Agilent Technologies, Co., Ltd., Santa Clara,CA, USA).

Transcriptome Small RNA sequencing and Library preparation and sequencing

Samples of Ex, Ca and Gl stages were collected in three replicates each, for a total of nine samples for transcriptome sequencing. Sequencing library construction using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, USA) according to manufacturer's instructions. In order to ensure the quality of the library, the library needs to be tested. After the construction of the library, the library was initially quantified by Qubit2.0 Fluorometer, then diluted to 1.5ng/ul, and the insert size of the library is detected by Agilent 2100 bioanalyzer. After insert size meets the expectation, qRT-PCR is used to accurately quantify the effective concentration of the library (the effective concentration of the library is higher than that of 2nM) to ensure the quality of the library. After the library is qualified, the different libraries are pooling according to the effective concentration and the target amount of data off the machine, then being sequenced by the Illumina NovaSeq 6000.

Small RNA sequencing using the same samples as transcriptome sequencing. The small RNA libraries were prepared from a total of 2 µg total RNA isolated from each sample using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (New England Biolabs, Ipswich, USA) according to the manufacturer's instruction. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq SR Cluster Kit v3-cBot-HS (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on a illumina Novaseq 6000 platform. All transcriptome and miRNAome data sets in this study have been uploaded to the NCBI SRA database (PRJNA1090974).

Differentially Expressed Gene Analysis

Differential expression analysis of two groups was performed using the DESeq2 R package (1.24.0). DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. $\text{P}_{\text{adj}} < 0.05$ and $|\log_2(\text{foldchange})| > 1$ were set as the threshold for significantly differential expression.

Differentially Expressed miRNAs Analysis

Differential expression analysis of two groups was performed using the DESeq2 R package (1.24.0). The P-values was adjusted using the Benjamini and Hochberg's method. Corrected P-value < 0.05 was set as

the threshold for screening differentially expressed genes.

Target gene prediction

The target gene prediction of miRNA was performed by Targetfinder(Fahlgren, et al. 2007).

Fahlgren N, Howell MD, Kasschau KD, et al. High-throughput sequencing of Arabidopsis microRNAs: evidence for frequent birth and death of MIRNA genes. PLoS One. 2007;2(2):e219. Published 2007 Feb 14. doi:10.1371/journal.pone.0000219

Results

Dynamic changes of endogenous hormones contents in different regeneration stages of *P. ternata*.

Morphology changes in explants during SEG were observed over 30 days following phytohormone induction [Naphthylacetic acid (NAA) + 6-benzylaminopurine (6-BA)]. Leaf explants (Ex) were used as control. After 15 days post induction, the explants showed significant swelling at the wound site and calli (Ca) were formed. Then, after 30 days of induction, protocorm-like bodies (Pr) were produced from the compact calli clumps. Thus, we divided the SEG process of *P. ternata* into three different stages: Ex stage, Ca stage and Pr stage (Fig. 1A). To monitor hormone changes during the three SEG stages, the content of endogenous auxin, cytokinin and their analogs were measured by liquid chromatograph mass spectrometer (LC-MS). The result showed that the content of indole-3-acetic acid (IAA) was highest in Ex, but significantly decreased in Ca and Pr. On the contrary, the contents of indole-3-carboxylic acid (I3CA) and indole-3-carboxaldehyde (ICA1d) were lowest in Ex, but significantly increased in Ca and Pr. There was no significant difference in IBA content in the three stages. Although the endogenous content changes of different kinds of auxin were different during *P. ternata* SEG, the content of total auxin was increased in Ca and Pr compared with Ex. Moreover, there was no significant change in auxin content from Ca to Pr. (Fig. 1B). For cytokinin, it was found that the content of isopentenyl adenosine (iPA) decreased sharply in Ex and Pr compared with that in Ca, and the contents of trans-zeatin-riboside (ZR) showed no significance in the three stages. In total, the endogenous cytokinin content decreased during *P. ternata* SEG. (Fig. 1C).

Transcriptome analysis of differential gene expression during *P. ternata* SEG

Transcriptome sequencing were performed to analysis the changes of gene expression at the whole genome-wide level in three stages of *P. ternata* SEG. A total of 56 Gb clean reads were generated, with at least 5.9 Gb per sample (Table S1). Clean reads were assembled by the Trinity software (v2.6.6), and then hierarchical clustered by Corset (version 4.6), 266,220 transcripts and 84,499 unigenes were obtained (Table S2). A total of 15,401 differentially expressed genes (DEGs) (FPKM > 1 in either of two pairwise combination samples, false discovery rate (FDR) < 0.05, and $|\log_2(\text{FoldChange})| > 2$) were obtained in two comparative groups (Ca Vs. Ex and Pr Vs. Ca). Furthermore, 15,089 DEGs were identified between Ca and Ex, including 6,388 upregulated and 8,701 downregulated DEGs; 1,586 DEGs were identified between Pr and Ca, containing 596 upregulated DEGs and 990 downregulated DEGs

(Fig. 2A, Table S3). Hierarchical cluster analysis was used to classify the expression and variation model of DEGs in the samples of three SEG stages. The results showed that the expression patterns of DEGs of Pr and Ca were similar, but significantly different from Ex (Fig. 2B). Principal component analysis (PCA) was used to reveal the relative separation between the Ex, Ca and Pr samples (Fig. 2C). The first principal component could explain 75.18% of the total variance and separated the Ex from Ca and Pr samples. The second principal component explained 7.89% of the total variance. In addition, the correlation analysis between samples also showed that Ca and Pr had stronger correlation among the samples of the three periods during SEG (Fig. 2D). Furthermore, we performed GO and KEGG enrichment analysis on the DEGs in Ca vs. Ex and Pr vs. Ca comparative components, respectively. As shown in Fig. 2E and 2F, the DEGs function of Ca vs. Ex were most enriched in single-organism metabolic process(GO:0044710), oxidation-reduction process(GO:0055114), oxidoreductase activity (GO:0016491) and Ribosome(ko03010), Glyoxylate and dicarboxylate metabolism(ko00630), Carbon fixation in photosynthetic organisms(ko00710). The DEGs function of Pr vs. Ca were most enriched in metal ion binding(GO:0046872), cation binding(GO:0043169), regulation of RNA biosynthetic process(GO:2001141), oxidoreductase activity(GO:0016491), regulation of transcription, DNA-templated(GO:0006355), regulation of nucleic acid-templated transcription(GO:1903506) and Phenylpropanoid biosynthesis(ko00940), Protein processing in endoplasmic reticulum(ko04141), Plant-pathogen interaction(ko04626), Glutathione metabolism(ko00480).

Analysis of the expression patterns of transcription factors and auxin signalling pathway genes during *P. ternata* SEG

Endogenous hormones, particularly the ratio of auxin to cytokinin, play a pivotal role in determining the embryogenic capacity of regenerating plant organs during tissue culture. To gain deeper understanding of the associated mechanisms, we performed a rigorous statistical analysis of differentially expressed genes (DEGs) involved in auxin and cytokinin metabolism and signal transduction. In the two comparisons of DEGs, Ca vs. Ex and Pr vs. Ca, a total of 142 and 42 genes related to auxin synthesis and signal transduction, including *auxin-responsive* genes *IAA Aux/IAA*, (Ca vs. Ex 23 and Pr vs. Ca 7), *small auxin up-regulated RNA (SAUR)* family genes (Ca vs. Ex 35 and Pr vs. Ca 19), *indole-3-acetic acid-amido synthetase GH3-2* (Ca vs. Ex 13 and Pr vs. Ca 2), *Auxin induced protein AIP* (Ca vs. Ex 5 and Pr vs. Ca 1), *Auxin response factor ARF* (Ca vs. Ex 24 and Pr vs. Ca 4), *Auxin efflux carrier component AEC* (Ca vs. Ex 15 and Pr vs. Ca 3), *Auxin transporter-like protein AUT* (Ca vs. Ex 13 and Pr vs. Ca 4), *Auxin-repressed protein ARP* (Ca vs. Ex 4 and Pr vs. Ca 2), *Auxin binding protein AB* (Ca vs. Ex 7), were identified, respectively; 26 and 10 genes related to cytokinin production and signal transduction, including *cytokinin riboside 5'-monophosphate phosphoribohydrolase LOG* (Ca vs. Ex 14 and Pr vs. Ca 4), *cytokinin receptor* genes (Ca vs. Ex 7 and Pr vs. Ca 2), *cytokinin synthase* gene (Ca vs. Ex 1), *cytokinin dehydrogenase* genes (Pr vs. Ca 4). (Table S4), were identified, respectively.

We eliminated the genes with low expression levels (FPKM<10) in each group and finally, we totally identified 17 auxin-related DEGs and 4 cytokinin-related DEGs in Ca vs. Ex and Pr vs. Ca comparisons (Fig. 3A). We performed a correlation analysis to investigate the association between 17 auxin-related

genes and auxins. The results showed that *PtAux/IAA4*, *PtSAUR1*, *PtGH3-2*, *PtAEC1*, *PtAEC2*, *PtAUT1* and *PtAUT2* were significantly positively correlated with I3CA, ICA1d and total auxins, but they were significantly negatively correlated with IAA. In addition, *PtAux/IAA2*, *PtSAUR4*, *PtAIP*, *PtAUT3*, *Pt-Auxin-repressed protein1 (PtARP1)* and *PtARP2* were significantly positively correlated with IAA and negatively correlated with I3CA, ICA1d and total auxins. *PtAux/IAA1*, *PtAux/IAA3* and *PtSAUR3* were significantly positively correlated with IBA. *PtLOG1* were significantly positively correlated with iPA, while *PtLOG2*, *PtLOG3* and *PtLOG4* were negatively correlated. (Fig. 3B). Furthermore, we identified 35 transcription factors (TFs) in DEGs, including 6 *PtNACs*, 4 *PtAP2/ERFs*, 3 *PtMYB* and other TFs (Fig. 3A). Interestingly, we identified 6 *Late organic boundaries domain (LBD)* genes, which could establish a molecular link between auxin signal transduction and embryonic regeneration of cells, and play an important role in the initiation of cell division in the formation of calli (Berckmans, et al. 2011; Fan, et al. 2012; Lee, et al. 2013; Okushima, et al. 2007). The expression levels of 6 *LBD* genes first increased and then decreased during SEG, and reached the peak in Ca stage. Additionally, 3 *Somatic embryogenesis receptor kinases (SERK)*, a marker gene of embryogenic cells (Hecht, et al. 2001; Huang, et al. 2010; Pérez-Núñez, et al. 2009; Schmidt, et al. 1997; Zhang, et al. 2011), and 8 *WUSCHELs (WUS)*, which could regulate stem cells in shoot apical meristem (Bhalla and Singh 2006; Busch and Benfey 2010), were also identified in Ca vs. Ex (Table S5).

Global analysis of differentially expressed miRNAs and their targets during *P. ternata* SEG

To characterize sRNA profiles in *P. ternata* during SEG, 9 high-depth sRNA libraries were constructed and more than 11.31 million clean reads were generated from each samples. The percentage of Q20 and Q30 exceeded 99.18% and 97.19%, respectively, and the percentage of GC was between 49.24% and 52.31% (Table S6). In the 9 libraries, an average of 62.11% of reads were successfully mapped to the reference genome (Table S7). The small RNA (sRNA) reads were typically 17 nt-25 nt in length. For three SEG data sets, the sRNA distribution all showed a major peak at 24 nt and the second major peak at 21 nt (Fig S1). A total of 1,368 miRNAs, including 1234 (414 mature RNAs, 820 hairpin RNAs) known miRNAs and 134 (61 mature RNAs, 73 hairpin RNAs) novel miRNAs, were identified in *P. ternata* genome. They belonged to 51 conserved families. Among them, MIR159 family was the largest family with 60 members, followed by MIR156 (59), MIR166 (48), MIR395(42), and MIR171_1 (42) (Table S8).

A total of 412 (48 families), 353 (48 families) and 358 (48 families) miRNAs were expressed (FPKM > 1) in at least one of the samples from the Ex, Ca and Pr groups, respectively. Specifically, MIR156 (38 miRNAs), MIR166 (24 miRNAs), MIR139 (28 miRNAs), MIR159 (26 miRNAs), and MIR395 (18 miRNAs) are the families with the most members in Ex; MIR156 (32 miRNAs), MIR166 (30 miRNAs), MIR396 (26 miRNAs), MIR159 (24 miRNAs), and MIR397 (18 miRNAs) are the families with the most members in Ca; MIR166 (30 miRNAs), MIR159 (27 miRNAs), MIR156 (23 miRNAs), MIR169 (23 miRNAs), and MIR396 (20 miRNAs) are the families with the most members in Pr (Table S9). Detailed analysis revealed that a total of 112 miRNAs were differentially expressed (FDR<0.05, and $|\log_2(FC)| \geq 1$) among the two pairwise combinations; 95 (56 upregulated, 39 downregulated) and 36 (16 upregulated, 20 downregulated)

miRNAs were differentially expressed in the Ca vs. Ex and Pr vs. Ca comparisons, respectively (Table S10).

Veen analysis showed that a total of 19 miRNAs, including MIR156 (2 members), MIR169(3 members), MIR395(2 members), MIR396(7 members), MIR5139 (1 members), and 4 novel members, were differentially expressed miRNAs in both Ca vs. Ex and Pr vs. Ca comparison groups (Fig. 4A), which indicated that these miRNAs maybe the key miRNAs involved in SEG of *P. ternata*. Upon detailed analysis, the expression levels of the 19 miRNAs were found to be distinctively grouped into three categories. 5 miRNAs, including novel_16, novel_17, gma-miR169v, ath-miR169b-5p and ptc-miR169s, showed a continuous increase in expression levels in three stages and were classified as category U. The expressions of 6 miRNAs, including mdm-miR396a, pe-miR396a, novel_73, novel_74, csi-miR159b-5p and rgl-miR5139 were firstly increased and then decreased during SEG, belonging to category UD. The expressions of 8 miRNAs, including vi-miR396b, ath-miR396b-5p, zma-miR396g-3p, ath-miR396a-5p, gma-miR396h, ath-miR156a-5p, ath-miR395a, tae-miR395b, showed a gradual downward trend in the three stages of *P. ternata* SEG, classified as category D. (Fig. 4B and C, Fig S2)

Furthermore, to elucidate the functions of key miRNAs identified in *P. ternata* SEG, we predicted their target genes using TargetFinder. A total of 1,742 distinct miRNA–mRNA interaction pairs were identified. Correlation analysis between miRNAs and their predicted target genes showed that a total of 528 (category U 54, category UD 220, category D 254) miRNA-mRNA pairs exhibiting significant negative correlations, involving 342 genes in transcriptome (Table S11, Fig. 4C). In addition, there was no significant correlation between the expression levels of novel_17, ath-miR169b-5p, and novel_74, and their respective predicted target genes.

We then constructed a cytoscape regulatory networks of the 16 miRNAs and the 342 target genes, and found that miR396, miR5139, miR169, and miR395 were the most relevant miRNAs, suggesting that they may play a crucial role in the regeneration of calli and protocorm-like bodies of *P. ternata* (Fig. 4C, D). Moreover, we identified 4 *PtGRFs* in the 342 target genes, which were the target genes of vvi-miR396b. miR396 can attenuate the sensitivity of *Arabidopsis* explants to exogenous auxin treatment and suppress the contents of endogenous auxin in SE by regulating the expression of GRF (Szczygieł-Sommer and Gaj 2019). Correlation analysis showed that the relative expression level of *PtGRF1* was significantly positively correlated with the content of ICA1d, while the relative expression levels of *PtGRF2* and *PtGRF3* were significantly positively correlated with the content of I3CA(Fig. 4E).

qRT-PCR validations of key genes and miRNAs regulating *P. ternata* SEG

To verify the accuracy of the gene expression data obtained by RNA-seq and miR-seq, 4 *PtGRFs* 4 miRNAs were selected for qRT-PCR expression analysis. The expression levels of the 4 *PtGRFs* and 4 miRNAs obtained by qRT-PCR were highly consistent with the results of RNA-seq and miR-seq (Fig. 5), which confirmed the reliability of our data analysis. Furthermore, we observed a distinct and consistent upward trend in the expression levels of *GRFs* within *P. ternata* SEG, which exhibited a notable contrast,

being inversely proportional to the expression pattern of vvi-miRNA396b, which demonstrated a decreasing trend.

Discussion

Auxins, including IAA, IBA, I3CA and ICA1d, are pivotal regulators of plant SEG, exhibits distinct patterns of profile among various plant species. Particularly, IAA plays a crucial role in inducing most plants SEG. During the SEG process of *Capsicum chinense*, the IAA content progressively increased in the SEG, peaking at 40 days of cultivation(Pérez-Pastrana, et al. 2021). In Cork Oak, the IAA content increased upon the induction of embryogenesis, but decreased in developed embryos(Carneros, et al. Dynamics of Endogenous Auxin and Its Role in Somatic Embryogenesis Induction and Progression in Cork Oak). In *Avena sativa*(Dziurka, et al. 2022) and *Cyathea delgadii*(Mikuła, et al. 2021) SEG, the content of I3CA was observed to be higher in the later stages of SEG compared to the initial stages. In this study, a total of 4 auxins were detected, and the content of IBA remained relatively stable throughout the three stages of *P. ternata* SEG. However, IAA content was significantly decreased in the Ca and Pr stages of *P. ternata* SEG. In contrast, I3CA and ICA1d exhibited significantly higher contents in Ca and Pr stages compared to Ex stage in *P. ternata*. Noteworthy, the contents of I3CA in Ca and Pr were more than 50 times higher than that of IAA, inferred that I3CA may serve as an important endogenous auxin in inducing *P. ternata* SEG.

In the in vitro tissue culture of plants, the harmonious interplay between endogenous hormones within the explant and exogenous hormones is crucial for effectively inducing SEG. We observed that during *P. ternata* SEG, there was a significant negative correlation between the expression of *PtSAUR4* and the contents of I3CA and ICA1d. SAUR functions not only as a responder to the initial auxin signal but also as a modulator of auxin content. Therefore, we speculate that NAA, serving as an exogenous auxin in the culture medium, exerts an inhibitory effect on *PtSAUR4* expression(Wang, et al. 2020; Xu, et al. 2017). The suppression subsequently facilitated an increase in the endogenous levels of I3CA and ICA1d. Furthermore, our observations revealed a significant negative correlation between the expression of *PtAUX/IAA2* and the content of I3CA and ICA1d, as well as a significant negative association between *PtGH3-2* expression and IAA content. We speculated that *PtAUX/IAA2* and *PtGH3-2* served as key primary response genes to auxin during the *P. ternata* SEG(Smet, et al. 2010; Winkler, et al. 2017). Additionally, it appeared that *PtGH3-2* may specifically amino-conjugate IAA, without exerting a similar effect on I3CA and ICA1. Consequently, this may contribute to the significantly higher content of I3CA and ICA1 observed in comparison to IAA(Zažímalová1, et al. 2010; Zhou and Luo 2018). Endogenous auxin is synthesized within plant cells and subsequently exported via transport carriers, serving to regulate plant growth(Zažímalová1, et al. 2010; Zhou and Luo 2018). There were 4 DEGs, *PtAEC1*, *PtAEC2*, *PtAUT1* and *PtAUT2*, consistent with the content change pattern of I3CA and ICA1d, may be involved in the efflux process of I3CA and ICA1d.

In this study, we identified 1234 known miRNAs (414 mature miRNAs, 820 hairpin miRNAs) and 134 novel miRNAs (61 mature miRNAs, 73 hairpin miRNAs) from 9 sequencing libraries. The quantity of miRNAs identified in both Ca and Pr were fewer than that in Ex, suggesting that the expression of

unnecessary miRNA may be inhibited during *P. ternata* SEG. There were 16 miRNAs were identified as potential regulators during *P. ternata* SEG. Notably, the expression of vvi-miR396b was found to have a significant negative correlation with its 4 target genes, *GRFs*. Additionally, a significant positive correlation was observed between the expression of *PtGRF1* and ICA1d content, as well as between the expression of *PtGRF2* and *PtGRF3* and the I3CA content. GRF not only enhanced auxins biosynthesis but also contribute to the induction of SEG by modulating tissue sensitivity to auxins(Cai, et al. 2022; Szczygieł-Sommer and Gaj 2019). These results indicated that the miRNA396-GRFs regulatory module was a pivotal factor in controlling *P. ternata* SEG via auxin-associated pathways.

Conclusions

In this paper, the mechanism of somatic embryogenesis in *P. ternata* were studied. We found that I3CA may serve as an important endogenous auxin in inducing *P. ternata* SEG and key genes and miRNAs were screened by high throughput sequencing technology during *P. ternata* SEG. Under the induction of exogenous auxin, the higher expression of *PtSAUR4* in Ca and Pr may serve as initial signal transducers for endogenous I3CA and ICA1 synthesis, while *PtAEC1*, *PtAEC2*, *PtAUT1*, and *PtAUT2* function as critical regulatory genes involved in the transport of I3CA and ICA1. A total of 1368 miRNAs were identified in our study, of which 134 were novel miRNAs. Notably, miR396 emerged as a key regulator of *P. ternata* SEG by regulating the expression of *PtGRFs*. The findings of this study were crucial for enhancing the efficiency of tissue culture, genetic improvement, and the development of transgenic technologies in *P. ternata*.

Declarations

Supplementary Information The following supporting information can be downloaded at: <https://doi.org/10.6084/m9.figshare.25465462.v1>.

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Author contributions D.L. R.X. and M.W. designed the research project. R.X., M.L., L.W., M.W., and D.L. performed most of the experiments. D.L. and Y.M. supervised the experiments. R.X. wrote the article. D.L. and Y.M. supervised and complemented the writing of the article. All authors have read and agreed to the published version of the manuscript.

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Figures

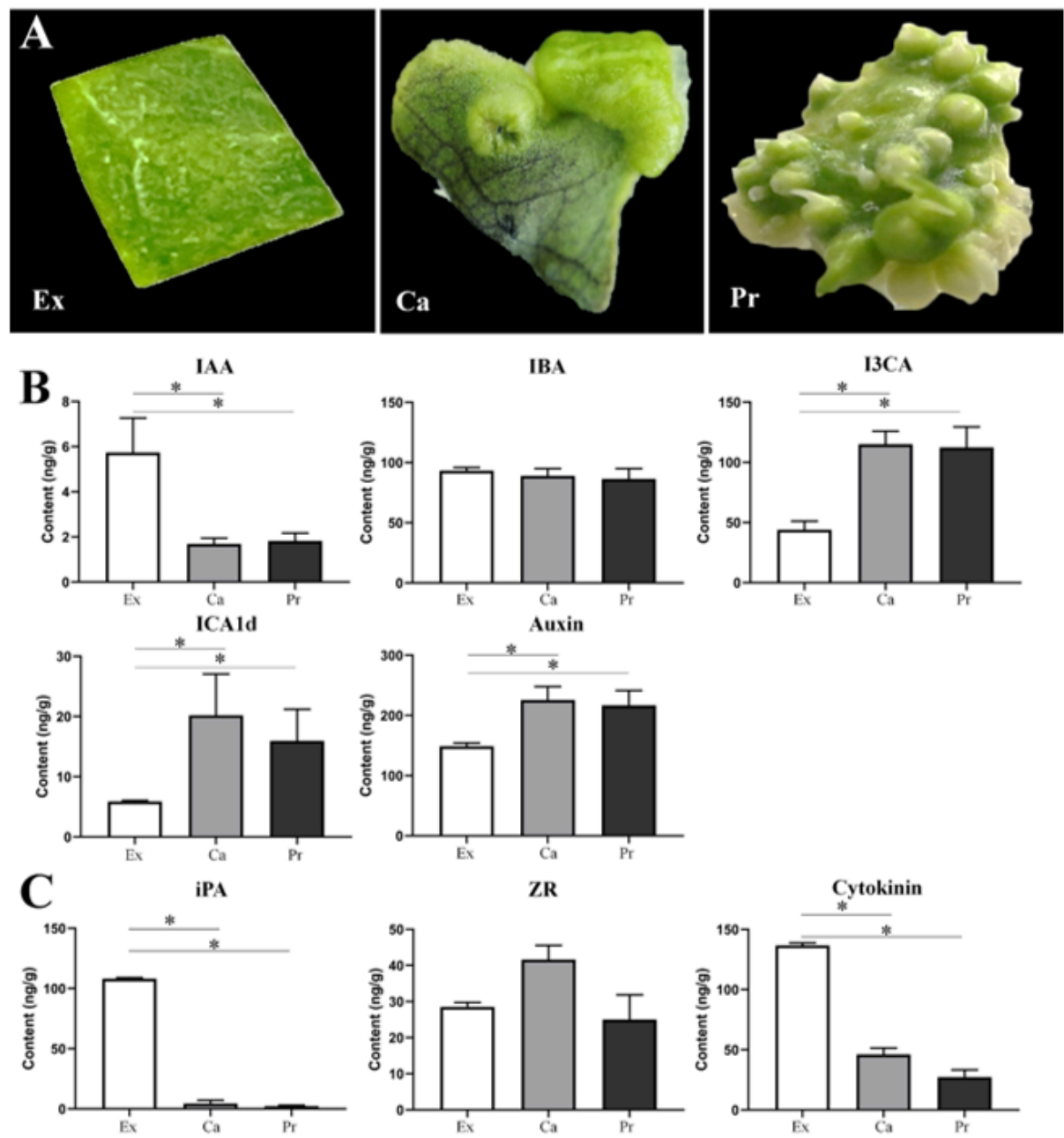


Figure 1

The phenotype and contents of endogenous auxins and cytokinins in three growth stages of *P. ternata*. (A) The three growth stages of in vitro regeneration culture of *P. ternata*. (B) Contents of endogenous auxins in explants of *P. ternata* at different stages of regeneration culture. (C) Contents of endogenous cytokinins in explants of *P. ternata* at different stages of regeneration culture. (* $p < 0.05$, Student's t-test).

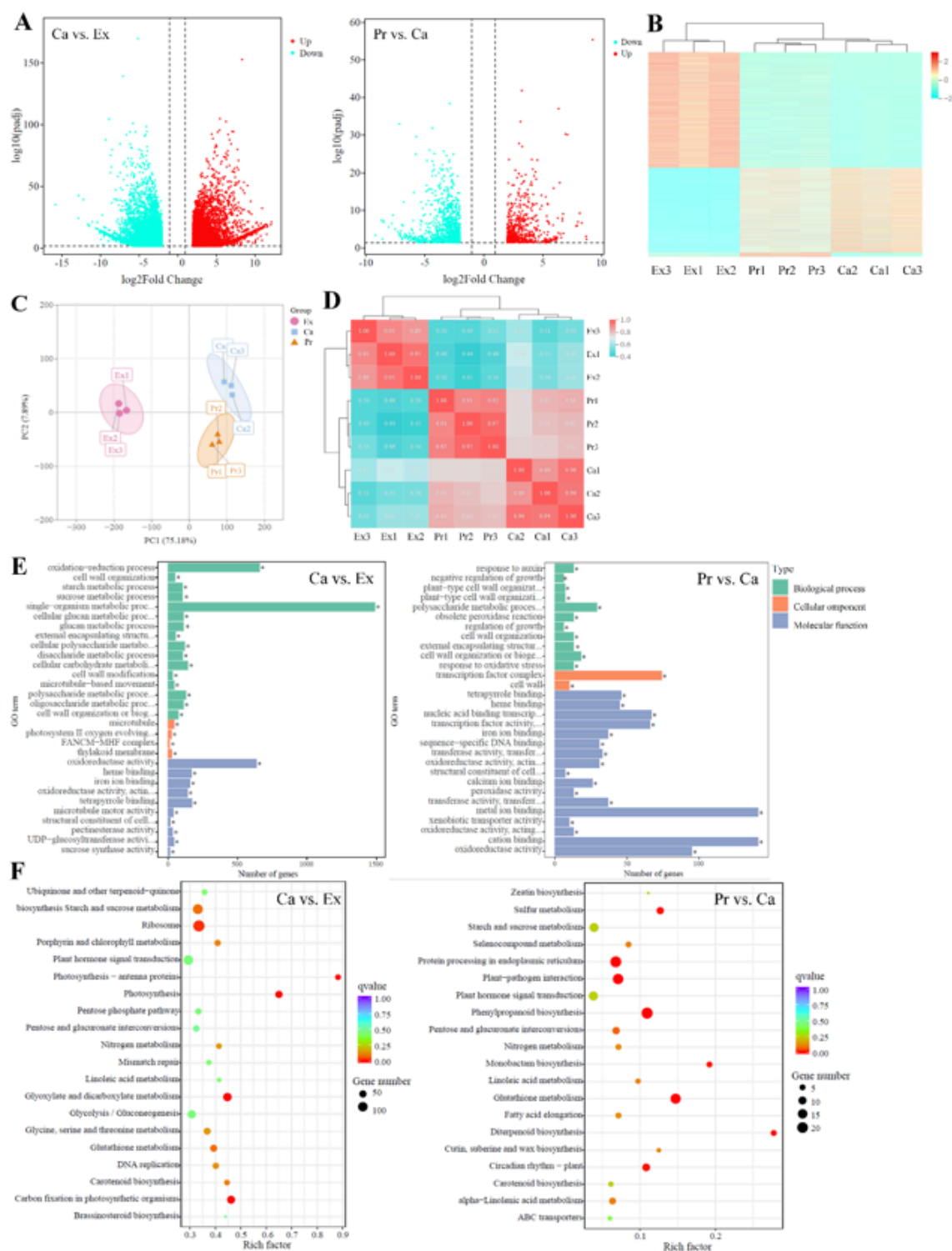


Figure 2

Transcriptome analysis of three stages of regeneration culture of *P. ternata* during SEG. (A) Volcano plots displaying the DEGs in Ca vs. Ex and Pr vs. Ca. (B) Hierarchical clustering heat map of DEGs in samples of three culture stages. (C) PCA of DEGs in samples of three culture stages. (D) Correlation analysis of DEGs in samples of three cultivation stages. (E) GO enrichment analysis of DEGs. (F) KEGG enrichment analysis of DEGs.

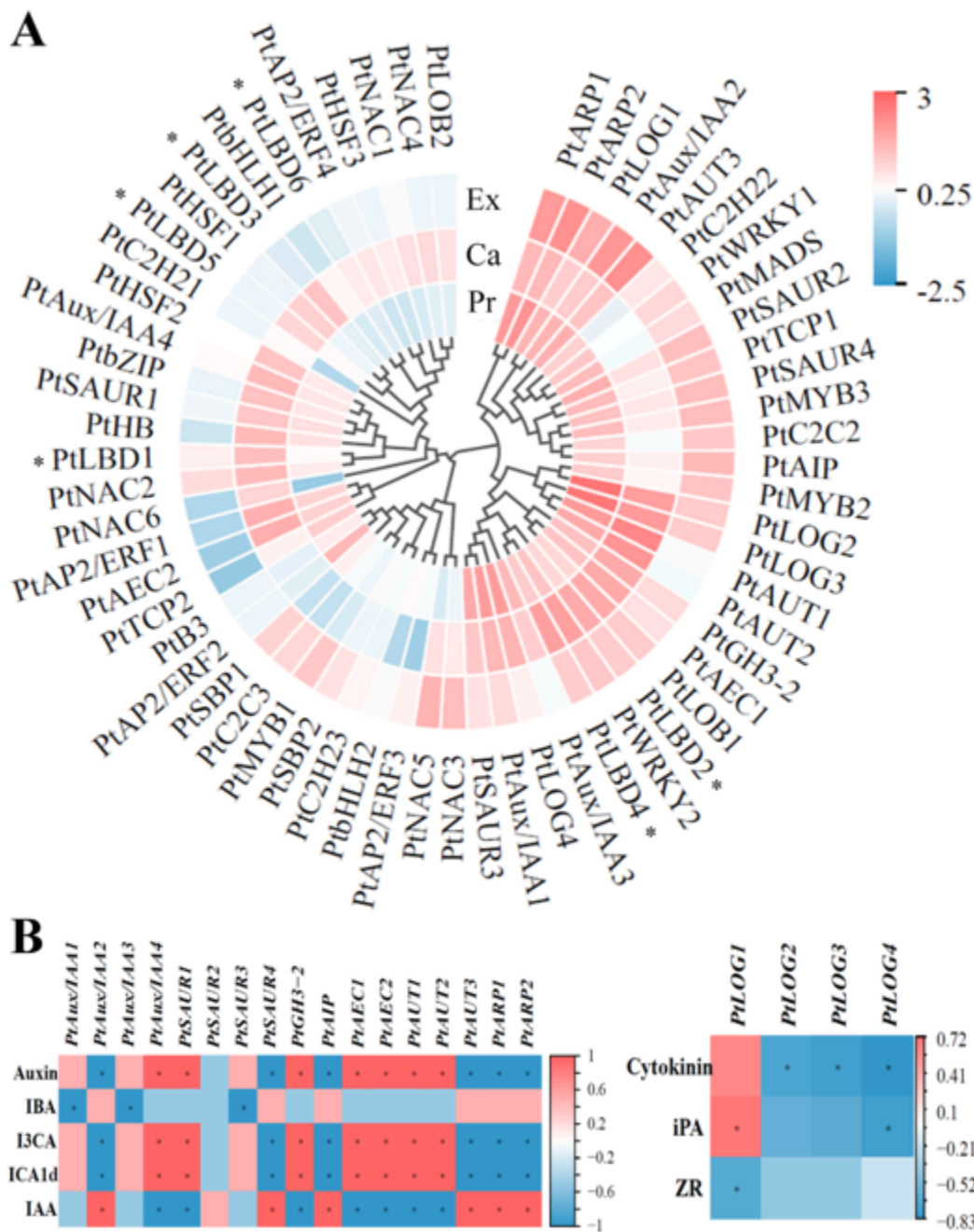


Figure 3

Statistical analysis of SEG-related genes *P. ternata*. (A) Heat map of genes related to SEG. (B) Correlation analysis of endogenous auxin, cytokinin and their respective related genes in three culture stages. *, significant correlation, $p < 0.05$, $R > 0.8$).

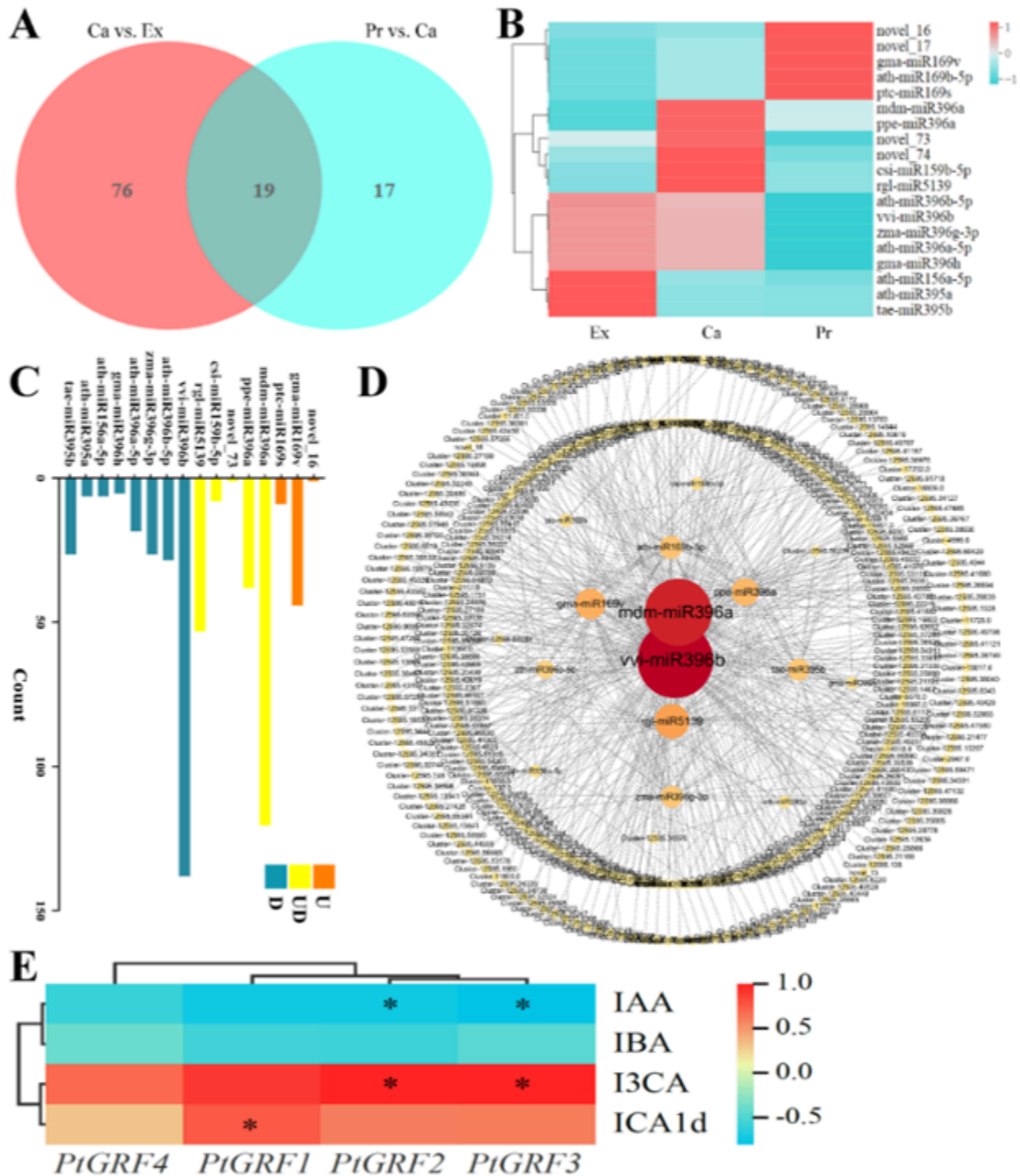


Figure 4

Differentially expressed miRNAs in SEG of *P. ternata*. (A) Venn diagram of DEmiRs of Ca vs. Ex and Pr vs. Ca. (B) Heat map of DEmiRs of Ca vs. Ex and Pr vs. Ca. (C) Statistics of target genes negatively correlated with the miRNA expression levels of DEmiRs. (D) Cytoscape regulatory networks of active miRNAs and their target genes. The size of dots in the networks was proportional to the number of miRNAs and genes. (E) Correlation analysis between *PtGRFs* and the contents of auxins. *, significant correlation, $p < 0.05$, $R > 0.8$).

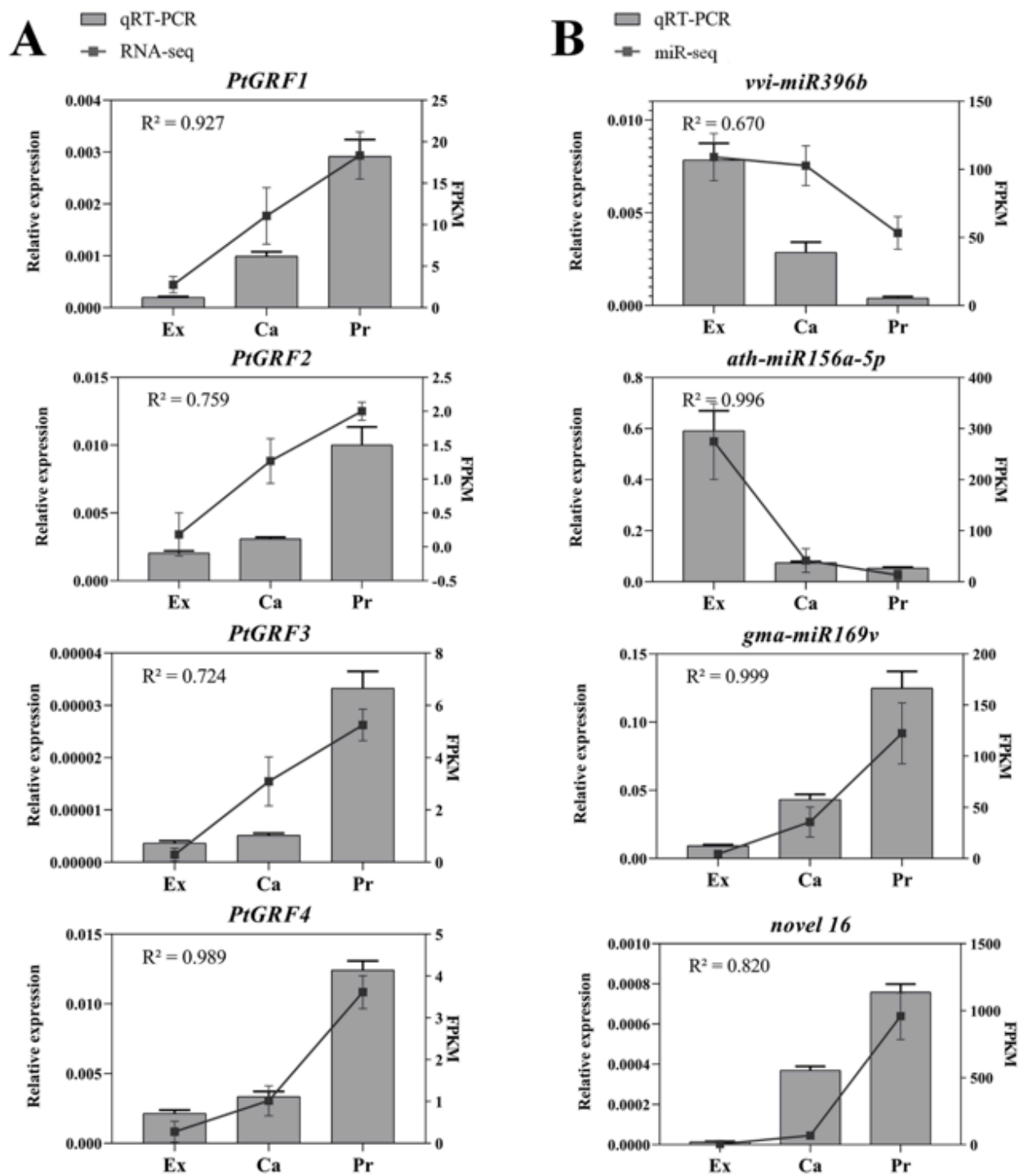


Figure 5

qRT-PCR confirmation of genes and miRNAs identified by transcriptome and miRNA analysis. (A) Transcriptome sequencing validation. (B) miRNAome sequencing validation.