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Full Length Article

Identification of Differentially Expressed Transcripts in Ovule Development during Free Nuclear Mitosis in *Pinus tabulaeformis*

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Abstract

Ovule development is an important step in the life cycle of sexually reproducing organism. In *Pinus tabulaeformis*, within the ovule, the female gametophyte undergoes dozens of syncytial mitotic divisions, followed by cellularization to form archegonium. However, at the stage of free nuclear mitosis of female gametophyte, the molecular mechanism is still unknown. In the present study, suppression subtractive hybridization (SSH) was used to identify rare and differentially expressed transcripts in developing (fertile clone, FC) and aborted (sterile clone, SC) *P. tabulaeformis* ovules. Both forward and reverse SSH libraries were constructed, and756 clones were randomly selected from the libraries and sequenced, yielding valid expressed sequenced tags (ESTs) ranging from 105 bp to 763 bp. Out of them, 233 ESTs from FC and 197 ESTs from SC SSH libraries showed high similarity to BLAST hits, and 336 (47.9%) proteins were annotated to gene ontology (GO) terms. The putative functions of differentially expressed transcripts were involved in most aspects of biological processes. The reliability of SSH libraries and expression patterns of 10 differentially expressed transcripts were confirmed by real-time quantitative PCR. We identified hundreds of differentially expressed transcripts mainly involved in cellular processes, other metabolism, developmental processes and protein metabolism. Furthermore, 27 candidate genes were focused, which might be responsible for mitotic progression, phytohormone regulation and defense response during ovule development. Taken together, this study provides transcriptional information on ovule development in *P. tabulaeformis*, offers a group of potential developmental-related regulators and poses hypothesis for the abortion of *P. tabulaeformis* ovules. © 2018 Friends Science Publishers

Keywords: Conifer; Female gametophyte abortion; Megagametogenesis; Suppression subtractive hybridization (SSH)

Introduction

Ovules are female reproductive organs that support and protect female gametophytes. An ovule is generally composed of four parts, the nucellus, the embryo sac, the integuments and the funiculus (Kagi and Gross-Hardt, 2010). As the core of the ovule, the female gametophyte arises from the archespore in the nucellus. The archespore then differentiates into megaspore mother cell, which undergoes meiosis to generate four megaspores. Only one of them, the functional megaspore, undergoes three rounds of nuclear mitosis to produce a coenocyte with eight nuclei. After nuclear migration, polar nuclei fusion and cellularization, the female gametophyte finally becomes a seven-celled structure (Yang *et al*., 2010). Ovule development is indispensable for seed formation, and plays essential role in plant reproduction. Due to the importance of ovule development in the plant life cycle, many studies have focused on the molecular mechanism underlying ovule development, and most researches are conducted by taking advantage of mutants.

To identify genes expressed in ovules, *Arabidopsis* mutants without integument(s) or embryo sac are selected for transcriptome analysis. It has been proposed that genes, such as *AINTEGUMENTA* (*ANT*), *BELL1* (*BEL1*), and *INNER NO OUTER* (*INO*) not only play a central role in integument development, but also are important for organ formation in *Arabidopsis*. The analysis of transcriptome between *ino* and *ant* reveals the genes controlling integument ontogeny (Skinner and Gasser, 2009; Brown *et al*., 2010). Also, the transcriptome comparison of ovules from *ino*, *ant* mutants and wild type identified 207 genes specifically expressed in integument (Brown *et al*., 2010). In addition, comparative analysis between mutant *sporocyteless* (*spl*), which has no embryo sac in ovules, and wild type revealed 225 ovule-expressed genes. *Pinus tabulaeformis* Carr. is one of the most important afforestation and landscaping tree in boreal area of China. As a woody gymnosperm, the ovule of *P. tabulaeformis* is a large and multicellular structure serving the important function as protection and nutrients provision for the developing female gametophyte, which generate gametes

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(Cheng *et al*., 2003; Wang *et al*., 2011). This differs from the situation in angiosperms, whose female gametophyte is generally structure with seven cells and eight nuclei with a single functional gamete (Yadegari and Drews, 2004; Shi and Yang, 2011). Therefore, it is necessary to explore the molecular mechanism underlying ovule development of *P. tabulaeformis*.

In the last decade, many studies have been focused on the ovule development of *P. tabulaeformis*. A female sterile mutant of *P. tabulaeformis* (sterile clone, SC), growing in a *P. tabulaeformis* seed orchard in Xingcheng, Liaoning in China, is identified (Li *et al*., 1992). The ovule development of conifer endures ovule initiation, megaspore formation, free nuclear mitosis, female megagametophyte cellularization and archegonia differentiation (Cheng *et al*., 2003). The free nuclear mitosis in female gametophyte is a key stage for ovule development. It lasts for about six months without the cell division. Moreover, the number of free nuclei increases from about one dozen to about one thousand (Cheng *et al*., 2003; Zhou *et al*., 2006). Also, anatomic study shows that the number of free nuclei in SC didn't increase when it reached to several dozens. Moreover, both the metabolic disorder and nutrient reduction (Li and Zheng, 1990) exists in the SC ovules, and the abnormal content changes of three endogenous plant hormones are shown in the sterile clone (Bao and Zheng, 2005). Recently, a single gene, *SLOW WALKER2* homologue, is found differentially expressed in the sterile mutant (Guo *et al*., 2014). Proteomic analysis reveals the different profiles of expressed proteins between SC and fertile clone (FC) (Lv *et al*., 2015; Zhang *et al*., 2017). These evidences suggest the complexity of transcriptional network in ovule development, and the abnormal gene expression may lead to the abortion of ovules.

In this study, we investigated the key genes expressed in ovules during free nuclear mitosis. We applied light microscopy to show the situation of free nuclear mitosis of female gametophyte and confirm the developmental stage. We also construct the suppression subtractive hybridization (SSH) libraries to explore key genes expressed in the stage of free nuclear mitosis. Furthermore, we screened the candidate genes in related to ovule development based on bioinformatic analysis. These findings will increase our understanding of the molecular mechanism underlying ovule development in woody gymnosperm.

Materials and Methods

Plant Materials

Cones of *P. tabulaeformis* in the period of free nuclear mitosis were gathered from both (FC) and SC that were cultivated in a *P. tabulaeformis* seed orchard (40°43'N, 120°34'E) in Xingcheng, Liaoning Province (The collection is permitted by forestry administration of Xingcheng). Trees in the *P. tabulaeformis* seed orchard are planted in 1974. The collection season was the spring of the year in February and April. Early and late stage in the period of free nuclear mitosis was estimated based on former research (Zhou *et al*., 2006). Former research (Cheng *et al*., 2003) has proved that the ovules with same size were in same developmental stage, and these ovules take up the middle-upper part of one cone. Thus, same-sized Ovules were dissected from the placenta of scales with knives and needles under the anatomical lens, and their bracts and scales were carefully removed. The ovules were immediately put into liquid nitrogen overnight, and then were stored at -80℃.

The early-staged ovules were chosen as our experimental material for SSH library construction and real-time quantitative PCR (RT-qPCR). Both early- and late-staged ovules were further confirmed though anatomic observation.

Developmental Stage Confirmation

Samples were prepared as paraffin sections by conventional method. Ovules were taken out of the fixative, dehydrated with an ethanol gradient (from 70 to 100%), treated with xylene, embedded in paraffin, and cut as sections with a thickness of 5 μm using a microtome (RM2016, Leica). The sections were stained with hematoxylin, mounted on slides with neutral balsam, and observed and photographed using a light microscope (Olympus BH2).

RNA Extraction

For each sample, 0.10 g of ovule tissue was ground into powder in liquid nitrogen with a pestle and mortar and total RNA was isolated from *P. tabulaeformis* SC and FC ovules using Plant RNA assistant kit (Kebaiao, Beijing, China) according to manufacturer's instruction. The concentration and purity of the extracted total RNA was confirmed by NanoDrop 2000 spectrophotometer and gel electrophoresis. To start SSH library construction, 5 μg of total RNA from three replicates of either SC or FC were pooled.

SSH Library Construction

cDNA was synthesized from 1 μg of total RNA of the SC and FC ovules using the Super SMART cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA). SSH was performed using the PCR-Select^{TM} Subtractive Hybridization Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's recommendations. Forward subtracted cDNA library was performed using the FC cDNA as tester and SC cDNA as driver. Reverse subtracted cDNA library was performed using the SC cDNA as tester and FC cDNA as driver. The two libraries were stored at -80℃. The reference gene used in analysis of subtraction efficiency was *TUBULIN* (forward: 5´-ACAGCTGATCTCGGGCAAAGAAGA -3´, reverse: 5´-TGCAGTTATCGGCTAGCTTCCTCA-3´).

Amplification of cDNA Inserts

The subtracted PCR products were cloned into the vector pGEM-T (Promega) and transformed into *Escherichia coli* Top 10 to obtain the forward and reverse subtracted libraries. A total of 756 white clones were randomly selected from the forward and reverse SSH libraries and cultured overnight at 37℃ in 2 mL centrifuge tubes in 1 mL Luria-Bertani medium with ampicillin (100 mg/mL) and glycerol (15%) as additives. To estimate the size range of cDNA inserts from these clones, PCR amplification was employed using primers (forward: 5′-GGGTTTTCCCAGTCACGAC-3′, reverse: 5′-TCACACAGGAAACAGCTATGAC-3′) in 25 μL PCR mixtures containing 1.0 μL bacterial culture (template), 2.5 μL 10 × PCR buffer, 1.0 μL of each primer (10 mM each), 0.6 μL dNTPs mix (10 μM each), 18.65 μL sterile water and 0.25 μL of *Taq* DNA polymerase (TaKaRa Biotechnology, Dalian, China). The PCR reactions were performed using the following parameters: an initial denaturation at 94℃ for 5 min, followed by 30 cycles of 94℃ for 30 s, 58℃ for 30 s, 72℃ for 2 min, with a final extension at 72℃ for 2 min. PCR products were analyzed using 1.0% agarose gel electrophoresis. All positive colonies containing inserted fragments with a single band over 200 bp were sent to Sangon Biotech Co. Ltd (Beijing, China) for sequencing. Sequencing was performed on an ABI 3700 automated sequencer (Perkin-Elmer, Foster City, CA, USA).

Bioinformatics Analysis

The obtained sequences were screened to remove vector, adaptor and poor quality sequences using the National Center of Biotechnology Information (NCBI) UniVec database (NCBI VecScreen, The UniVec Database 2013). ESTs longer than 100 bp were considered for further analysis. Similarity analysis of the resulting *P. tabulaeformis* unigene sequences was performed against the Uniprot Knowledgebase in EBI (http://www.ebi.ac.uk/Tools/sss/ncbiblast/) and TAIR (http://www.ebi.ac.uk/Tools/sss/ncbiblast/) using the BLASTX and BLASTN program respectively. cDNAs with a threshold E-value under 10^{-4} were designated as having significant homology. The putative or hypothetical functional categories of all the BLAST results were assigned based on Gene Ontology (GO) annotations (http://www.arabidopsis.org/tools/ bulk/go/index.jsp).

Real-time Quantitative PCR Analysis

According to the generated ESTs, the reliability of SSH libraries was confirmed by RT-qPCR. Based on the putative functions, transcripts from the SSH libraries were evaluated by RT-qPCR. *EF1*, which has been successfully applied in former research (Guo *et al*., 2014), was used as a reference gene for the normalization of gene expression. Primers were designed using the Primer6.0 software, and details of gene specific primers are listed in Table 1. Total RNA was purified and first-strand cDNA was generated using the Super SMART cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. Synthesized cDNAs were diluted 1/10 with sterile water before use. RT-qPCR was performed in a total volume of 20 μL containing diluted cDNA (2 μL), 10 μL of SYBR Premix ExTaq (Tiangen, China), 0.4 μL of each primer (10 mM final concentration), and 6.8 μL of deionized water. PCR reactions were run on a Mini Opticon Two-Color Real-Time PCR Detection System (BIO-RAD, USA). The cycling conditions used were 95℃ for 30 s, followed by 45 cycles of 95℃ for 3 s and 60℃ for 30 s. A melting curve was performed to confirm the amplification of specific products. A no template control was included on each reaction plate. Relative expression levels of the target genes were normalized, and then calculated using comparative Ct ($2^{-\Delta\Delta Ct}$) method (Schmittgen and Livak, 2008). Each sample had three replicates.

Results

Verification of Developmental Stage

Through paraffin sections, early- and late-staged ovules were observed (shown in Fig. 1). Visible morphological changes, from dozens of free nuclei to hundreds of them in the FC, were clearly observed (Fig. 1a and c). However, in the SC group, degradation process of the female gametophyte was found (Fig. 1b and d). In the section of early-stage FC (Fig. 1a), the number of free nuclei was about fifty, and the nuclei seemed larger than that in the section of SC at the same stage (Fig. 1b). In addition, fewer free nuclei were formed in Fig. 1b. The female gametophyte in SC ovules seemed degenerating at this stage. Subsequently, the free nuclear in the FC ovules underwent extensive mitosis (Fig. 1c), while the position of the female gametophyte in the SC finally became a hollow cavity (Fig. 1d) instead of the beginning of cellularization. The fate of FC and SC ovules has been diverged from the early stage of free nuclear mitosis. To acquire the key genes in developmental stage of free nuclear mitosis, we chose ovules in the early stage as our plant material for SSH library construction. By the observation of female gametophyte, the developmental stage is confirmed.

Evaluation of Subtraction Efficiency

After the RNA extraction, cDNA synthesis and subtractive hybridization, the subtracted cDNAs were evaluated by PCR analysis using *TUBULIN* as an indicator. The amount of *TUBULIN* transcript decreased significantly after subtraction. In subtracted cDNAs, *TUBULIN* products were observed after 23 cycles, while the amplified products were seen at 18 cycles in the unsubtracted cDNAs. The results

Table 1: List of Genes and Primers Used in the Real-time Quantitative PCR Experiment

Gene description	Accession number	Primer sequence	Product size (bp)
Guanine nucleotide exchange family protein	HX969100	F 5´-AATAAATTGCGGCGAGATGCGTTT-3´ R 5´-CGAACCTTCTTGCGATGGAGTCTT-3´	81
TIR/P-loop/LRR	HX969198	F 5´-TTGATGGCACAACGATGTTTGAAGT-3´ R 5´-AACCACGTACAATGTTCGACACCTT-3´	87
Nuclear factor NO VEIN	HX969106	F 5´-GAATGATGGAATCGCAATGGACAGT-3´ R 5´-GCAGGCTTTGTTGAGAAGAATGTGA-3´	85
Zinc finger protein	HX969133	F 5´-TCCAATACCCAAGGCACCGAAGA-3´ R 5´-TCACTGTTGTTGTTGCTGCTCTGT-3´	87
Asparaginyl-tRNAsynthetase	HX969144	F 5´-GGTAAATGATGGGTCCTGTCTTTCA-3´ R 5´-AGCCAGACTCAACCTTGTCATATCC-3´	83
tRNA ligase 2	HX969158	F 5´-AACGGAACCTCTGTGCCTACATTTC-3´ R 5´-CCAACCTCTGTATTGCTGCTCTGC-3´	104
Heat shock factor protein HSF24	HX969339	F 5´-CACCTTCATAGTGTGGCGACCTG-3´ R 5´-TGCCGGACGAAGCTGGAGAA-3´	90
Pectinesterase/pectinesterase inhibitor	HX969308	F 5´-ACCAACTGCGGATCTGGCTGAG-3´ R 5´-CTCCTTCACCTTGTCCTTGAACTCC-3´	95
Catalase isozyme	HX969370	F 5´-GGAATCCCATCCGACTTTAGGCATA-3´ R 5´-CCTCCAACAATTACAGCCTCCTCAT-3´	155
Amino acid permease	HX969406	F 5´-AGCTCAAATTCCAGACTTCGACCAA-3´ R 5´-ACTGAGGCCGAGACCTATTGATGAA-3´	94

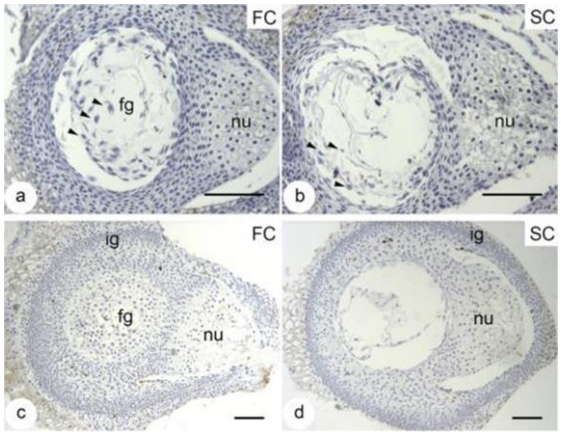

Fig. 1: Anatomic observation of ovule development in both FC and SC during the period of free nuclear mitosis. (a, b) The early stage of free nuclear mitosis. (c, d) The end of free nuclear mitosis. (a, c) The optical images of the FC show the maturation process of a multi-cellular female gametophyte (fg). (b, d) The female gametophyte of the SC. (d) The development of integuments (ig) and nucellus (nu) is well underway in SC. The free nuclei are indicated by the arrowheads. fg: female gametophyte; ig: integuments; nu: nucellus. Scale bars, 200 μm

indicated that the presence of the *TUBULIN* gene was reduced by up to 2^5-fold after subtraction, suggesting that the subtractive hybridization procedure was successful, and that SSH between the FC and SC effectively removed non-differentially expressed genes.

Annotation and Functional Analysis

The subtracted cDNA library generated in this study contained the upregulated and downregulated genes in *P. tabulaeformis* compared with the FC. After transformation and cloning, a total of 756 bacterial clones were isolated and screened by PCR. After analysis by electrophoresis, 18 clones (2.4%) were excluded due to the presence of multiple amplifications. The remaining clones were sequenced, and after removing low quality sequencing data, vector and adaptor sequences, 701 high-quality expressed sequence tags (ESTs), ranging from 105 bp to 763 bp, were obtained. These ESTs were used in BLASTX searches against the EBI UniProt Knowledgebase. The results revealed that, out of 701 clones, 430 sequences (61.3%) had strong similarity with the contents in the database with an E-value below 10^{-4}, including 234 ESTs in FC library, and 196 ESTs in SC library, respectively (shown in Supplementary Sheet 1). Of the 430 genes, 51 (11.8%) encode proteins with an EST match but without any specific function (uncharacterized protein). The remaining genes were distributed among all the major classification groups, including metabolism, stress response, cell structure organization, transport, signal transduction, and transcriptional regulation. All of these sequences were submitted to the DDBJ database (http://www.ddbj.nig.ac.jp/sub) with accession number HX969077-HX969506. The ESTs without hits comprised the main portion (45.8%) of all sequences, which suggests that there may be a number of gymnosperm-specific genes with unknown properties and functions.

To further evaluate the putative functions of the identified ESTs based on the BLASTX search results against the database, gene ontology (GO) annotation was employed. Genes for putative proteins were categorized into cellular component, molecular function and biological process, respectively (Fig. 2).

In the ontology of cellular component, other

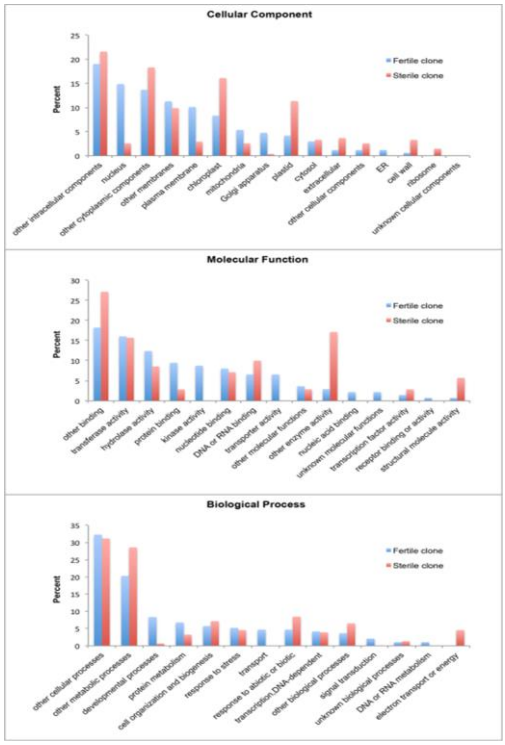

Fig. 2: Classification of identified genes by protein functions. Genes were categorized using GO annotation and the percentage of genes in each category from both SC and FC are shown

intracellular components, nuclear, and other cytoplasmic components had the highest representation in the fertile clone library. Compared with sterile clone library, nucleus had an obvious higher representation in fertile clone library. Conversely, plastid and chloroplast had higher representation in sterile clone library. In the ontology of molecular function, other binding and transferase activity were highly represented in both female fertile and sterile clones. Distinctively, other enzyme activity in SC library had a significant high representation than that in FC library. In biological progress, the other cellular process and other metabolic process had high percentage in both FC and SC libraries. Interestingly, the developmental process in FC had higher percentage than that in SC library.

A group of candidate genes were screened from forward or reverse libraries based on the putative functions acquired from BLASTX and GO analysis results. The function of these genes included auxin regulation, mitotic progression and defense response (shown in Table 2).

Real-time Quantitative PCR Verification

In order to confirm the dependability of the SSH libraryies, RT-qPCR was utilized to validate ten genes, which were involved in the development process (HX969100, HX969106), signal transduction (HX969198), transcriptional regulation (HX969133, HX969339, HX969370), metabolism (HX969144, HX969158, HX969308), and cellular component related categories (HX969406) from the forward library or reverse library. The relative expression of these genes was determined by using primers shown in Table 1. The relative gene expression levels are shown in Fig. 3, with a relative fold change from 1.32 to 4.41. Genes encoding cytohesin 1,2,3 (HX969100, Fig. 3a), nuclear factor NO VEIN (HX969106, Fig. 3c), and zinc finger protein (HX969133, Fig. 3d) are responsible for auxin response and cell growth. They showed high transcript level in the FC. The Also, the gene encoding Asparaginyl-tRNA synthetase (HX969144, Fig. 3e), which takes part in the metabolic activity of ovule development, showed a high transcriptional level in FC ovules. On the contrary, the transcript abundance of Heat shock transcription factor (HX969339, Fig. 3g), Catalase (HX969370, Fig. 3i) and Amino acid permease (HX969406, Fig. 3j) from the reverse library were suggested to have a high level in SC. In addition, the relative expression of genes encoding TIR/P-loop/LRR protein (HX969198, Fig. 3b), tRNA ligase 2 (HX969158, Fig. 3f) and Pectinesterase (HX969308, Fig. 3h) were upregulated in FC ovules, which was in accordance with the SSH results. It is validated that the SSH libraries were reliable.

Discussion

Pinus tabulaeformis Carr. is one of the most important afforestation and landscaping tree in boreal area of China. Its propagation mainly relies on seeds, but heavy losses of seed production in the seed orchards attracts researchers' attention (Wang and Shen, 1989), which is mainly caused by ovule abortion, pests and selfing. Research indicates that its ovule development is a complicated and long duration process, which lasts for nearly three years. The period of free nuclear mitosis lasts for nearly 6 months, including 2 months of dormancy (Jiang *et al*., 2006). FL and SC ovules in the early stage of free nuclear mitosis (Fig. 1a and b) were selected for SSH library construction and RT-qPCR verification. Previous studies eliminated the possibility that the sterility is caused by insufficient pollination and pest damage (Li and Zheng, 1990). Other studies on specific protein and hormone levels showed that the ovule abortion might be related to metabolic disorder (Bao and Zheng, 2005).

Ovule offers protection and structural support for female gametophyte, and develops into seed after fertilization. Intensive morphological research has been conducted on some species in gymnosperm (Cheng *et al*., 2003; Wang *et al*., 2011). Less is known about gene expression in this course though ovule development in gymnosperm is a complicated and important biological process. To explore the gene expression in *P. tabulaeformis* ovules during the process of free nuclear mitosis, we constructed forward and reverse SSH libraries from ovules in female FC and SC. Based on the application of selective

Table 2: Candidate genes that may be involved in ovule development in *P. tabulaeformis*

Accession number	Nr annotation	Putative functions	E value	Expression pattern (FL vs. SL)
HX969387	BEL1-like protein	unknown	2.00E-16	downregulated
HX969377	Cytokinin dehydrogenase	restrict cytokinin in ovules	5E-31	downregulated
HX969391	MYB transcriptional factor-like protein	Mitotic progression	9E-21	downregulated
HX969425	MYB transcriptional factor-like protein	Mitotic progression	5.00E-16	downregulated
HX969084	auxin-induced GH3 protein	Auxincontrol and regulation	7.00E-125	upregulated
HX969114	Auxin response factor	Auxin control and regulation	2.00E-18	upregulated
HX969163	Auxin transport protein	Auxin control and regulation	2.00E-05	upregulated
HX969276	Dormancy/auxin associated-like protein	Auxin control and regulation	1.00E-10	upregulated
HX969398	Auxinresponsive family protein	Auxin control and regulation	3.00E-16	downregulated
HX969234	B1-type CDK-like protein	Mitotic progression	1.00E-30	upregulated
HX969235	B1-type CDK-like protein	Mitotic progression	2.10E-31	upregulated
HX969090	ubiquitin carboxyl-terminal hydrolases	Mitotic progression	6.00E-06	upregulated
HX969186	ubiquitin carboxyl-terminal hydrolases	Mitotic progression	6.00E-25	upregulated
HX969213	ubiquitin carboxyl-terminal hydrolases	Mitotic progression	7.00E-05	upregulated
HX969093	E3 ubiquitin-protein ligases	Mitotic progression	1.00E-45	upregulated
HX969094	E3 ubiquitin-protein ligases	Mitotic progression	4.00E-31	upregulated
HX969095	E3 ubiquitin-protein ligases	Mitotic progression	4.30E-94	upregulated
HX969155	E3 ubiquitin-protein ligases	Mitotic progression	1.00E-44	upregulated
HX969210	E3 ubiquitin-protein ligases	Mitotic progression	1.00E-49	upregulated
HX969099	ligase subunit F-box	Mitotic progression	1E-44	upregulated
HX969157	ligase subunit F-box	Mitotic progression	1.00E-35	upregulated
HX969120	kinesin-1	Mitotic progression	6.00E-12	upregulated
HX969447	Heat shock protein	Defense response	1.00E-18	downregulated
HX969252	Heat shock protein	Defense response	9.00E-15	upregulated
HX969206	Heat shock protein	Defense response	6.00E-20	upregulated
HX969337	Heat shock protein	Defense response	1.00E-18	downregulated
HX969339	Heat shock transcription factor HSF29 family protein	Defense response	8.00E-25	downregulated

amplification of differentially expressed sequences, SSH leads to the enrichment of specifically expressed genes and overcomes technical limitations involved in traditional subtraction methods (Diatchenko *et al*., 1996). During the last decade, SSH has been considered a powerful and efficient tool for the analysis of different gene expression in researches related to plant development (Kalidhasan *et al*., 2015). Our results showed that the reference gene *TUBULIN* was reduced 2^5 fold after subtraction hybridization between FC and SC cDNA, suggesting that non-specifically expressed gene cDNAs had been greatly decreased, while specifically expressed gene cDNAs had been enriched in our SSH libraries. Therefore, our library construction was successful. In the present study, we found that the specifically expressed genes had potential functions involved in the regulation of ovular development, especially cell cycle-related process. These ESTs in the libraries will be helpful to understand the mechanisms of free nuclear mitosis and ovule development.

BEL1 is one of the most important transcriptional factor and participates in various developmental processes in plants, including ovule genesis (Schneitz *et al*., 1997). Screening of ovule defective mutants is an important method to identify genes involved in ovule development, including both sporophytic and gametophytic tissues. Among them, *bel1* lacks regular integuments initiation and cannot facilitate megagametogenesis. The *bel1* nucellus contains no embryo sac (Schneitz *et al*., 1997), which indicates that the development of the haploid generation is regulated by the factors in the maternal surrounding tissues. As a key transcriptional factor, BEL1 interacts with the MADS-box dimer and plays a role in determination of ovule identity (Brambilla *et al*., 2008). However, we observed that there was no distinction between FC and SC integuments (Fig. 1), and the development of integument in proceeded normally. In our study, a gene (HX969387) encoding a BEL1-like protein was significantly upregulated in SC (Table 2). Analysis of BEL1-like homeodomain 1 (BLH1) and BLH5 showed that they may not be involved in gametophyte development or that a broader functional redundancy among the members of the BELL1 family exists (Pagnussat *et al*., 2007). In addition, other studies have found that the overexpression of *TaqSH1*, encoding a BEL1-like protein, downregulated known abscission related genes (Zhang *et al*., 2013). Similarly, previous study showed that the level of ABA in SC was always much higher than that in FC (Bao and Zheng, 2005). Whether the biosynthesis of ABA is regulated by BEL1-like protein in *P. tabulaeformis* requires further study. Bencivenga *et al*. (2012) found that the homeodomain transcriptional factor BEL1 is required in the process of ovule patterning, indicating the pleiotropism of *BEL1-like* genes. These results indicated that function of BEL1-like protein might play a different role in *P. tabulaeformis* ovule development.

Promoting cell proliferation and specification, cytokinins play important roles in the process of ovule development. Reduction of cytokinin gradient in plants induces a decrease in ovule numbers or female fertility

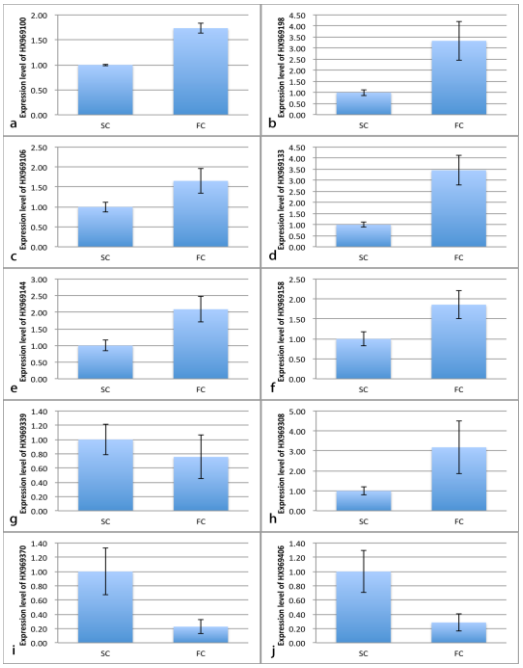

Fig. 3: Comparison of relative expression level of transcriptsin *P. tabulaeformis*ovules using RT-qPCR. cDNA from FC and SC ovules was utilized as templates for RT-qPCR, and expression level were normalized to the *EF1* gene. Error bars for RT-qPCR values are standard deviation (n = 3)

(Hejatko *et al*., 2003). Cytokinin oxidases/dehydrogenases (CKX) are responsible for the irreversible degradation of cytokinin and inhibition of hormone concentrations in tissues (Schmulling *et al*., 2003). We identified a CKX-like gene (HX969377), which was upregulated in SC (Table 2), which may restrict cytokinin in ovules and, therefore, cause sterility. Based on the function of CKX enzymes, alteration of *CKX* gene expression influences the regulation of cytokinin levels and controls the developmental process of ovule. This result is similar to those of Bartrina *et al*. (2011). Dual mutation of *CKX3* and *CKX5* genes in *Arabidopsis* established supernumerary ovules, further confirming the relationship between cytokinin and ovule development. Based on the results we inferred that the upregulation of the CKX-like gene might contribute to the steritlity of female gametophyte in *P. tabulaeformis*.

The auxin is also important for developmental processes, and the level of auxin is regulated by its biosynthesis and transport. Manipulation of levels of auxin have been proved a method to change cell fate (Pagnussat *et al*., 2009). In our libraries, the ectopic expressions of five sequences (HX969084, HX969114, HX969163, HX969276, HX969398) (Table 2), which may encode auxin-related genes, were detected. Of the five genes, four of them were specifically expressed in FC, and they might be involved in auxin induction (HX969084), response (HX969114, HX969276) and transport (HX969163). Prior work on endogenous plant hormones in the ovule of *P. tabulaeformis* demonstrated that the IAA content in FC had a certain change throughout the entire stage of free nuclear mitosis in the female gametophyte, while IAA level in SC remained stable (Bao and Zheng, 2005). Therefore, our findings were consistent with our former research on hormone levels. Auxin also regulates ovule development, as a transient application of N-1-naphthylphthalamic acid (an auxin efflux inhibitor) causes significant loss of ovules (Nemhauser *et al*., 2000). Accordingly, Note-Wilson *et al*. (2010) associated ovule loss with a severe reduction in local auxin biosynthesis. It was suggested that synthesis of auxin might also function in megagametogenesis. Concomitantly, the transcriptional factors BEL1 and SPL/NZZ are needed to participate in the cytokinin and auxin signaling pathways for the precise initiation of ovules (Bencivenga *et al*., 2012). Therefore, in the *P. tabulaeformis*, the silencing of these specific auxin-related factors may contribute to the abortion of female gametophyte in SC ovules.

From the observation of paraffin sections, we found that the SC embryo sac stopped developing at during the period of free nuclear (Fig. 1d). The free nuclei failed to complete mitosis process, and DNA within the embryo sac appeared diffuse and broken (Fig. 1d). These deleterious events may also arouse cell death by mitotic interruption (Surova and Zhivotovsky, 2013). These suggested that there may be cell-cycle-related genes that are differentially expressed in SC, and that interfere with the division of free nuclei in female gametophyte.

MYB transcriptional factor is supposed to control cell development, cell cycling, and respond to varieties of hormones and environmental signals. Two MYB transcriptional factor genes were found expressed in the female gametophyte (Wang *et al*., 2010), which may be related to the development of the female gametophyte. In our study, the upregulation of genes (HX969391, HX969425) encoding MYB transcriptional factor-like protein were detected in the SC (Table 2). In *Arabidopsis*, attenuation of *MYB64* and *MYB119* expression, during transition to the FG5 phase, leads to failure of cellularization of the female gametophyte, resulting in supernumerary nuclei (Rabiger and Drews, 2013), as with female gametophyte in gymnosperm. Similarly, from the observation of sections, we found that the free nuclear were unable to undergo mitosis in SC ovules, and also that the expression of *MYB*-like sequences were downregulated in FC ovules. We speculated that the *MYB* genes might contribute to a block of free nuclear division in *P. tabulaeformis.*

The anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase, controls important transition during mitotic progression. The knockout of APC/C components interrupts the female gametophyte development (Kwee and Sundaresan, 2003). Also, cyclin-dependent kinases (CDKs) make a large contribution to the regulation of cell cycle progression and cell division.

Among these proteins, CDKB members are plant-specific kinases, and include the CDKB1 superfamily whose kinase activity reaches peak levels during mitosis (Boudolf *et al*., 2004). In the present study, 12 putative genes were related to regulatory mechanisms of the cell cycle. Two genes (HX969234, HX969235), encoding B1-type CDK-like proteins were highly expressed in FC, and ten genes, putatively encoding ubiquitin carboxyl-terminal hydrolases (HX969090, HX969186, HX969213), E3 ubiquitin-protein ligases (HX969093, HX969094, HX969095, HX969155, HX969210), and ligase subunit F-box (HX969099, HX969157), were downregulated in SC ovules (Table 2). Deficiency of CDKB1 and CDKA; 1 kinases lead to the absence of egg apparatus within the embryo sac (Nowack *et al*., 2012). CDK inhibitors negatively regulate CDKs, and, therefore, degradation of CDK inhibitors is also an essential component of cell cycle regulation (Zhou *et al*., 2003). The ubiquitin/proteasome pathway not only contributes to the degradation of CDK inhibitors (Liu *et al*., 2008), but also targets the disintegration of cyclin through recognition of D-box motifs, which are contained in all cyclins, by E3 ubiquitin ligase (Vandepoele, 2002). During megagametogenesis in *Arabidopsis thaliana*, two Ring-finger E3 ligases target the inhibitory protein, controlling mitotic cell cycle progression (Liu *et al*., 2008). It is tempting to speculate that the differential expression of CDKs and ubiquitin related proteins might be involved in the free nuclear mitosis in *P. tabulaeformis* ovules.

Plant kinesins, firstly identified in tobacco pollen (Tiezzi *et al*., 1992), are involved in many dynamic cellular processes of microtubules, including cell division (Zhu and Dixit, 2012). Mutation in *AtKin-1*, a member of kinesin-1, blocks the expansion of the megaspore, and nuclear division is arrested at the single-nucleate phase during embryo sac initiation (Wang *et al*., 2014). According to our data, a gene (HX969120) encoding kinesin-1 was specifically expressed in fertile ovules (Table 2). It is indicated that, in *P. tabulaeformis* ovules, the kinesin-1-like protein may play an important role in the regulation of nuclear division cycles during megagametogenesis.

Heat stress transcriptional factors are involved in control of variety of stress responses and in developmental progresses. In our study, four heat shock proteins (HX969447, HX969252, HX969206, HX969337) and one heat shock transcriptional factor (HX969339) were identified in both the forward and reverse libraries (Table 2). These may result from the dysfunction of some biological processes. Other studies have also found that the abnormal expression of heat stress transcription factor (HSF) gene *HSFB2a* in *Arabidopsis* ovule can increase sterility to nearly 50% from 2.2% in wild type (Wunderlich *et al*., 2014), by repression of vegetative growth and subsequently impaired development during megagametogenesis.

The genes discussed above have been intensely proved to be key regulators in female gametophyte development in *Arabidopsis*. However, in *P. tabulaeformis*, some genes may play different role in ovule development, such as *BEL1*. Whether the differentially expressed genes affect the process is still unknown, and the research on molecular mechanism needs further exploration.

Conclusion

Here, we reported the differentially expressed genes between FC and SC ovules in the period of free nuclear in *P. tabulaeformis*, and the SSH technique has been successfully applied to identify specifically expressed genes. These genes were involved in most aspects of plant biological processes. Our results provided an initial characterization of a group of candidate genes of *P. tabulaeformis* ovules with diverse functions related to ovule development. Taken together, these results provide new insight into the ovule development in woody gymnosperm.

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