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Gene-expression profile of developing pollen tube of *Pyrus bretschneideri*

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ABSTRACT

Pollen is an ideal model system for investigation of cell growth. In order to better understand the molecular biology mechanisms of the process of pear pollen tube development, RNA sequencing (RNA-Seq) technology was used to characterize the expression of genes during four development stages of pear pollen, including mature pollen grains (MP), hydrated pollen grains (HP), growing pollen tubes (PT) and stopped-growth pollen tubes (SPT). The four libraries generated a total of 47,072,151 clean reads that were mapped and assembled into 21,394 genes. Transcripts from the four stages were classified into 38 functional subcategories. Between MP and HP, 305 genes were differentially expressed, and 502 genes were differentially expressed between HP and PT. More importantly, we have observed that 2208 genes were differentially expressed between PT and SPT, and this is the first report of the gene expression comparison between the two development stages. Eight of the differentially expressed genes were randomly selected to confirm the RNA-Seq results by quantitative real-time PCR (qRT-PCR). Taken together, this research provides a platform for future research on pear pollen tube growth and growth cessation.

Pollen germination and pollen tube growth are important for sexual reproduction in flowering plants. Similar to other species, pear pollen germination and pollen tube growth has tip-growth features; pollen tube extension is very rapid and death of the pollen tube occurs within 24 h when cultured *in vitro* (Gao et al., 2014). Therefore, the pollen and pollen tube represent an ideal model system for studying the molecular mechanisms of cellular processes, such as cell growth and death (Gao et al., 2014).

Several genes involved in the regulation of pollen germination and pollen tube growth have been discovered by means of biochemical and molecular genetic studies in *Arabidopsis thaliana*. It is well known that the calcium gradient within the pollen tube tip, vesicle transport, cell wall biosynthesis, ion fluxes and actin microfilaments are important biochemical factors during pollen germination and tube elongation (Hepler et al., 2012; Steinhorst and Kudla, 2013). Traditionally, using biochemical assays and mutant analysis, such as RNA interference and genetic transformation, researchers have verified the functions of several genes (Guan et al., 2013). These methods are very useful, but it is difficult to characterize many genes in a single experiment, especially genes expressed in specific development stage. Thus only a small percentage of genes have been functional identified during pollen germination and pollen tube growth.

Transcriptome analysis can provide comprehensive insights into the molecular mechanisms of the growth and development in plants. In recent years, it has been used to analyze the gene expression changes during pollen development, such as *arabidopsis* (Honys and Twell, 2004; Loraine et al., 2013; Qin et al., 2009; Wang

Abbreviations: CaM, calmodulin; CHXs, Cation/H^+ exchangers; CMLs, Calmodulin-like proteins; COX, Cytochrome c oxidase; DEGs, Differentially expressed genes; FDA, Fluorescein diacetate; KEGG, Kyoto Encyclopedia of Genes and Genomes; KFB, Kelch containing F-box protein; MP, Mature pollen grains; HP, Hydrated pollen grains; PT, Growing pollen tubes; SPT, Stopped-growth pollen tubes; PCD, Programmed cell death; qRT-PCR, Quantitative real-time PCR; ROS, Reactive oxygen species; RPKM, Reads per kilobase per million reads; SAM, S-adenosylmethionine; SAMS, S-adenosylmethionine synthase; PI-PLC, Phosphoinositide phospholipase C; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; VPE, Vacuolar processing enzyme; V-ATPase, Vacuolar H^+-ATPase; WEGO, Web Gene Ontology Annotation Plot.

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et al., 2008), *Nicotiana tabacum* (Bokvaj et al., 2015; Hafidh et al., 2012), *Lilium longiflorum* (Lang et al., 2015) and *Oryza sativa* (Wei et al., 2010). Although the transcriptome changes during pollen tube growth *in vitro* have been studied in several model species (Lang et al., 2015; Wang et al., 2008), little attention has been paid to the mechanisms underlying the cessation of pollen tube growth *in vitro*. Pear (*Pyrus bretschneideri* Rehd.) belongs to the Rosaceae family, has a S-RNase-based self-incompatibility system during the fertilization; it indicates that cessation of pollen tubes growth natural occurred in the pistil (Wang et al., 2009; Wu et al., 2010), but we know little about the molecular mechanisms of pear pollen germination, pollen tube growth and growth cessation until now. RNA-Seq is an accurate and sensitive approach for expression analysis of the transcriptome, which has been successfully used for indentifying differentially expressed genes (DEGs) in pear (Qi et al., 2013). Thus, this technology was used to characterize the gene expression patterns, and screen out DEGs during pear pollen germination, pollen tube growth and growth cessation in this study.

The cDNA libraries of the four developmental stages of 'Dangshansuli' pollen tube development were sequenced, including mature pollen grains (MP), hydrated pollen grains (HP), growing pollen tubes (PT) and stopped-growth pollen tubes (SPT) stages. Approximately 47 million high-quality reads were obtained and assembled into 21,394 genes and gene expression patterns during pollen tube developmental stages were analyzed. In addition to providing valuable sequence resources for the identification of DEGs and specific genes, we also discussed the potential mechanisms of pear pollen tube growth cessation *in vitro*.

1. Results

1.1. Illumina sequencing and data assembly

To analyze the changes in expression profiles of pollen during its germination and tube elongation, pollen samples from four different stages were prepared for RNA extraction: MP (0 min post-cultured), HP (40 min post-cultured), PT (6 h post-cultured) and SPT (15 h post-cultured) (Fig. 1). The growth cessation status of pollen tubes in 'Dangshansuli' must be defined. We found that pollen tubes almost stop growing at 15 h post-cultured *in vitro* (Fig. S1A). More than 90% of MP, HP and PT were positive for fluorescein diacetate (FDA) staining, indicating the high viability in these stages; more than 15% of pollen tubes were negative for FDA staining at 15 h post-cultured, indicating that pollen tube viability decreased remarkably (Fig. S1B). The pollen tubes displayed the lowest growth rate and drastically decreased viability at 15 h post-cultured (Fig. S1), thus we defined the pollen tubes at 15 h post-cultured as the stopped-growth pollen tubes (SPT).

The RNA-Seq technique was used to analyze the changes in gene expression of the MP, HP, PT and SPT stages of pear pollen, giving corresponding totals of 11,698,192, 11,656,492, 11,634,854 and 12,082,613 clean reads (95.78, 93.52, 92.68 and 99.39% of the raw data, respectively) (Fig. S2). The tag sequences of the four digital transcript abundance measurement libraries were mapped to the reference pear genome sequences of 'Dangshansuli' (http://peargenome.njau.edu.cn/) (Wu et al., 2013). Finally, for the four libraries, the numbers of mapped reads were 8,426,736 (72.03%), 8,393,579 (72.01%), 8,405,661 (72.25%) and 9,012,711 (74.59%), respectively (Table 1).

A total of 21,394 genes were assembled by mapping the reads to the reference genome sequences. Of these, 16,517, 16,218, 16,668 and 17,096 genes were identified in the MP, HP, PT and SPT libraries, respectively (Fig. 2). The expression of 12,482 genes was detected in all four libraries. There were 14,099, 14,170 and 14,459 genes that were expressed both in MP and HP, HP and PT, and PT and SPT, respectively. Furthermore, 923, 767, 816 and 1264 genes were specifically expressed in MP, HP, PT and SPT, respectively (Fig. 2). These stage-specific expressed genes could be used to find key genes correlated with pollen germination and pollen tube growth.

Gene coverage is the percentage of a gene covered by reads, which is equal to the ratio of the base number of a gene covered by unique mapping reads to the total number of bases of the gene, and can reflect the quality of sequencing. The gene coverage over the four libraries was highly reproducible and quite uniform. The similarity distribution had a comparable pattern with about 20% of the sequences, with a similarity of 70% (Fig. S3).

To confirm whether there was a proportional increase of the number of detected genes to the sequencing amount (total tag number), a saturation analysis was performed. The saturation trend showed that the number of detected genes almost ceased increasing when the number of reads reached 8 million (Fig. S4).

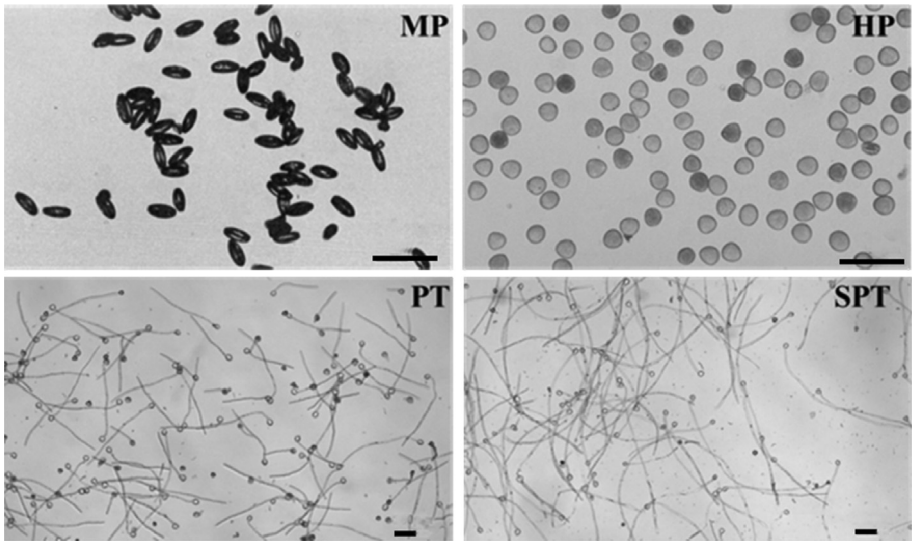

Fig. 1. Developmental stages during pollen germination and pollen tube growth. Pollen grains were incubated in medium for various times before RNA isolation. Bar = 100 μm. MP (0 min post-cultured); HP (40 min post-cultured); PT (6 h post-cultured); SPT (15 h post-cultured).