Introduction

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1 Introduction

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Pinus massoniana Lamb. (Fam.: Pinaceae) is a monoecious gymnosperm with unisexual flowers. It serves as an important afforestation and timber yielding species in the Peoples Republic of China. Usually in September to October, the axillary buds of the vegetative stem of *P. massoniana* begin to form the male cone primordia in the direction of development from bottom to top. Later, it produces nearly one hundred microstrobili per vegetative stem. In October, 2-4 female cone primordia develop at the apex of the twig. In the following months from February to April, 2-4 megastrobili (female flowers) are developed in the shoot apex. These form 2-4 female cones following pollination, fertilization and development (Fig. 1). However, the microstrobili in twigs of some plants experience sexual reversal. In those plants, the microsporangia develop into bracts and ovuliferous scales of the female cones basipetally. Gradually, they are converted into female flowers morphologically which develop further into long strings of cones (polycones). Previous studies have shown that this trait is genetically stable, with a great potential in increasing seed yield [1]. The sexual reversal of microstrobili to polycones has been discovered in other species of *Pinaceae* too [2-6].

Currently, few studies are available on the sexual reversal mechanism of unisexual flowers of gymnosperms. In *Pinus tabulaeformis* Carr., via transcriptome analysis, Shihui et al. [7] revealed that the expression of genes were dramatically different between male and female flowers. In the present investigation, transcriptome sequence analysis of strobili before and after the sexual reversal, and also the normal strobili of *P. massoniana* has been performed for the first time using this second generation sequencing technology. This will provide data on the induction factors the reproductive regulation of bud differentiation and the sexual reversal processes of the bisexual flower of *P. massoniana*.
2 Materials and Methods

2.1 Test material

On April 6, 2016, material for the present study was collected from the gene collection area of the national *P. massoniana* seedling base, located in Ma’anshan (26°16’ N and 107°31’ E), Duyun, Guizhou Province, China. One 14 year old *P. massoniana* plant with polycones was selected as the study subject. The same plant contained both the normal and polycone twigs. On the normal twigs, both mega- and microstrobili developed normally, without sexual reversal. Whereas on the polycone twigs, the microstrobili reversed sexually into megastrobili and produced polycones (Fig. 1). Five groups of samples were collected: (1) microstrobili before the sexual reversal (PM_w), (2) bisexual strobili during the sexual reversal (PM_b), (3) megastrobili formed by the sexual reversal (PM_q) from a polycone twig, (4) megastrobili (PM_f) and (5) microstrobili (PM_m) from a normal twig. From each group, three replicates were made and stored in liquid nitrogen. The description of the collected material and their images are shown in Table 1 and Fig. 2, respectively.

![Fig. 1](image1.png)

**Fig. 1 A-F. Normal- and polycones of *P. massoniana*. A, normal microstrobilli; B, normal megastrobilli; C, normal cones; D and E, sexual reversal of microstrobilli into megastrobilli and F, polycones.**

![Fig. 2](image2.png)

**Figs 2 m,f,w,b and q. Material sources of the normal and polycone twigs of the *P. massoniana*. m, microstrobili from a normal twig; f, megastrobili from a normal twig; w, microstrobili that had not yet been sexually reversed on the polycone twig; b, bisexual strobili during sexual reversal on the polycone twig and q, megastrobili formed from sexual reversal on the polycone twig.**

| Samples | Description                                                                 | Collection site    |
|---------|------------------------------------------------------------------------------|--------------------|
| PM_b    | Bisexual strobili during the sexual reversal (half red)                      | Polycone twig      |
| PM_q    | Megastrobili formed by the sexual reversal of microstrobili (all red)       | Polycone twig      |
| PM_w    | Microstrobili                                                               | Polycone twig      |
| PM_f    | Megastrobili                                                               | Normal twig        |
| PM_m    | Microstrobili                                                               | Normal twig        |

**Table 1. A brief description of the collected samples from the same polycone twig of *P. massoniana*.**
2.2 RNA extraction and library construction

A Trizol kit (Invitrogen) was used to extract the total RNA, then the total RNA was treated with RNase-free DNase I (Takara Bio, Japan) for 30 min at 37°C to remove residual DNA. RNA quality was verified using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and were also checked by RNase free agarose gel electrophoresis. The mRNA was enriched with oligo dT beads for total RNA whose quality met the requirements. Further, the mRNA was fragmented into short segments in the fragmentation buffer. Using mRNA as a template, cDNA was synthesized with reverse transcriptase (RT) using random hexamers. Purification was completed with end repair and addition of a poly-A tail to the double-stranded cDNA, thus establishing a cDNA library. The cDNA library was then sequenced on an Illumina sequencing platform after the qualification examination.

2.3 Transcriptome analysis of *P. massoniana* polycone

After filtering raw data to remove low-quality sequences, and reads with adapters that produced an N-ratio greater than 0.1%, clean reads were obtained and evaluated further. The Trinity system [8] was used to splice the clean reads. The longest transcript of each gene, obtained thereby, was used as the unigene for subsequent analysis. Unigenes then were queried against the major databases with BLASTX and were further classified into the Gene Ontology (GO), euKaryotic Ortholog Groups (KOG) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) according to the annotations. The clean reads of each sample were mapped to the longest transcript sequence, the resulting read counts were converted to FPKM (expected number of Fragments Per Kilobase of transcript sequence per millions base pairs sequenced) values to analyze the gene expression level. Using DEGseq [9], read-counts obtained from the gene expression analysis with \(|\log_2 \text{fold change} > 1\) and \(q\) value < 0.005 were taken as the differentially expressed gene (DEGs).

3 Results

### 3.1 Assembly and splicing of transcriptome

From the splicing quality as presented in Table 2, it was observed that the base error rates of all samples are below 0.01%. The averages of Q20, Q30 and GC were 97.88, 94.80 and ~45.2%, respectively. In total, 190,023 unigenes were obtained, with an average length of 595 bp. For N50 the length was 929 bp, and that for N90 it was 245 bp. The lengths of unigenes are predominantly within 200-300bp.

| Sample | Raw reads | Clean reads | Clean bases | Error (%) | Q20 (%) | Q30 (%) | GC Content (%) |
|--------|------------|-------------|-------------|-----------|---------|---------|----------------|
| PM_b1  | 58793018   | 57647452    | 8.65G       | 0.01      | 98.14   | 95.34   | 45.2           |
| PM_b2  | 47738824   | 46710370    | 7.01G       | 0.01      | 97.86   | 94.76   | 45.05          |
| PM_b3  | 47597638   | 46288816    | 6.94G       | 0.01      | 97.82   | 94.79   | 45.31          |
| PM_q1  | 52536546   | 50929904    | 7.64G       | 0.01      | 98.03   | 95.45   | 45.75          |
| PM_q2  | 45975644   | 44775990    | 6.72G       | 0.01      | 97.75   | 94.62   | 45.76          |
| PM_q3  | 44138302   | 42725456    | 6.41G       | 0.01      | 97.82   | 94.84   | 45.57          |
| PM_w1  | 68036142   | 66709378    | 10.01G      | 0.01      | 97.82   | 94.83   | 44.96          |
| PM_w2  | 52038922   | 50992778    | 7.65G       | 0.01      | 97.8    | 94.62   | 44.87          |
| PM_w3  | 45970238   | 44803048    | 6.72G       | 0.01      | 98.12   | 95.37   | 44.92          |
| PM_f1  | 59838294   | 58207864    | 8.73G       | 0.01      | 97.63   | 94.47   | 45.33          |
| PM_f2  | 51126784   | 49650744    | 7.45G       | 0.01      | 97.82   | 94.81   | 45.44          |
| PM_f3  | 41165852   | 40130986    | 6.02G       | 0.01      | 97.59   | 94.3    | 45.76          |
| PM_m1  | 49471722   | 48001564    | 7.2G        | 0.01      | 98.02   | 95.17   | 44.9           |
| PM_m2  | 48692894   | 47399458    | 7.11G       | 0.01      | 97.87   | 94.84   | 44.62          |
| PM_m3  | 47169460   | 45979646    | 6.9G        | 0.01      | 98.04   | 95.14   | 44.76          |
### 3.2 Unigene function annotation

According to the sequence similarity analysis, the resultant unigenes were queried in the NR, NT, KO, SwissProt, Pfam, GO, and KOG databases (Table 3). Due to the reproducibility and complexity of the sequence of the gymnospermic plants belonging to the family Pinaceae, the resources for the pine trees were relatively scarce compared to those of the other model organisms. The greatest number of annotations was retrieved from the NR database, with a value of 66,236 (34.85%), and the fewest from the KOG database, with a value of 28,025 (14.74%). Shared annotations between the seven databases are 12,198 (6.41%). From the NR database, the near-source species with the highest similarity is *Picea sitchensis* (Bong) Carr. (24.9%), followed by *Gossypium raimondii* Ulbr. (74%), *Amborella trichopoda* Baill. (4.7%), *Prunus persica* (L.) Batsch (4.2%), and *Prunus mume* Siebold & Succ (3.2%).

### 3.3 The GO, KOG, and KEGG classification of unigenes

The functional classification of unigenes was performed using the GO database. In its entirety, there were 56 functional groups identified, among which, cellular process (GO:0009987), the binding (GO:0005488), and metabolic process (GO:0008152) have most annotations. The number of genes of those important functional groups, namely binding, catalytic activity and cell part are 27,225, 21,903, and 14,994, respectively. In the KOG database, a total of 28,025 unigenes (14.74%) were annotated in 26 categories, with a maximal number of 4,742 in the class of General function prediction only. A minimum of two was obtained in the class of unnamed proteins, and varied gene expression abundance in other functional categories. In the KEGG database, a total of 25,863 unigenes were divided into 130 metabolic pathways, involving ribosome, carbon metabolism, biosynthesis of amino acids, and plant pathogen interactions. The number and proportion of metabolic pathways in the top 15 are listed in Table 4.

### Table 3. List of annotation of unigenes in different databases.

| Database        | Number of unigenes | Percentage (%) |
|-----------------|--------------------|----------------|
| NR              | 66,236             | 34.85          |
| NT              | 65,429             | 34.43          |
| KO              | 25,863             | 13.61          |
| SwissProt       | 55,836             | 29.38          |
| Pfam            | 51,428             | 27.06          |
| GO              | 52,472             | 27.61          |
| KOG             | 28,025             | 14.74          |
| Annotated in all databases | 12,198             | 6.41           |
| Annotated in at least one database | 96,476             | 50.77          |
| Total unigenes  | 190,023            | 100            |

### Table 4. KEGG metabolic pathway classification of unigenes of polycone Pinus.

| KEGG Pathway                  | Pathway ID | Quantity (percentage) |
|-------------------------------|------------|-----------------------|
| 1 Ribosome                    | ko03010    | 1767(6.01%)           |
| 2 Carbon metabolism           | ko01200    | 1523(5.18%)           |
| 3 Biosynthesis of amino acids | ko01230    | 1130(3.84%)           |
| 4 Protein processing          | ko04141    | 1041(3.54%)           |
| 5 Plant-pathogen interaction  | ko04626    | 875(2.98%)            |
| 6 Oxidative phosphorylation   | ko00190    | 791(2.69%)            |
| 7 Spliceosome                 | ko03040    | 706(2.40%)            |
| 8 Glycolysis/Gluconeogenesis  | ko00010    | 698(2.38%)            |
| 9 Starch and sucrose          | ko00500    | 643(2.19%)            |
| 10 RNA transport              | ko03013    | 615(2.09%)            |
| 11 Endocytosis                | ko04144    | 592(2.01%)            |
| 12 Purine metabolism          | ko00230    | 516(1.76%)            |
| 13 Phenylpropanoid biosynthesis| ko00940  | 481(1.64%)            |
| 14 Plant hormone signal       | ko04075    | 468(1.59%)            |
| 15 Pyruvate metabolism        | ko00620    | 464(1.58%)            |
4.1 Gene expression differences between microstrobili and megastrobili of *P. massoniana* polycone

According to screening criteria, 1,188 DEGs were found for the comparison between the PM_b and the PM_w samples. Of these, 715 genes were up-regulated and 473 were down-regulated. Further, a total of 4,768 DEGs were found for the comparison between the PM_q and the PM_w, of which, 2,075 genes were up-regulated and 2,717 were down-regulated. For the comparison between PM_f and PM_m, a total of 5,550 DEGs were identified, of which 2,651 genes were up-regulated and 2,899 were down-regulated. Among them, there are 69 differential genes specific to microstrobili to bisexual strobili (PM-bvsPM-w), and these genes may be related to the process of the sexual reversal.

4.2 Analysis of unigenes involved in plant hormone signal transduction of *P. massoniana* polycone

Identified DEGs were subjected to the pathway enrichment analysis. It was found that the majority of the combinations among the paired comparisons were significantly...
enriched in the plant hormone signaling pathway (ko04075). Regarding the metabolic pathway of plant hormone signal transduction, there were 51 DEGs between the megastroboli and microstrobili from the polycone twig, with 26 up-regulated and 25 down-regulated. Also, there were 51 DEGs between the megastroboli and microstrobili from the normal twig, with 30 up-regulated and 21 down-regulated. Altogether there were 36 DEGs common to the polycycle and normal twigs, among which, 15 were related to the auxin (indole-3-acetic acid, IAA), three to gibberellic acid (GA), five to abscisic acid (ABA), three to zeatin nucleoside (ZR), two to salicylic acid (SA), four to brassinosteroid (BR) and three to cytokinin (CTK). With respect to the IAA metabolic pathway, ten genes were related to the small auxin upregulated RNA (SAUR) family, six to auxin-reactive protein IAA, and the expression of the six genes were all up-regulated in the megastroboli. The genes and their related metabolic pathways are listed in Table 5.

The DEGs between the megastroboli and microstrobili from the normal twig and those from the polycycle twig, as well as their common DEGs which were related to the plant hormone signaling pathways are shown in Fig. 4. Interestingly, the involved genes demonstrated either male or female preferred expression, associated with the sex difference. However, the expression of 36 common DEGs were all up-regulated in the bisexual strobili during sexual reversal from the polycycle twig.

### 5 Discussion

*P. massoniana* is the main timber species in southern China as well as a pioneer afforestation plant to control

| Number | Gene ID | KO ID | KO Description |
|--------|---------|-------|----------------|
| 1      | c72332_g1 | K14431 | transcription factor TGA |
| 6      | c81784_g1, c84572_g1, c81784_g4, c71445_g1, c81784_g3, c71164_g1 | K14484 | auxin-responsive protein IAA |
| 10     | c66758_g1, c72270_g1, c80646_g1, c85500_g2, c85500_g4, c82056_g6, c21023_g1, c85347_g2, c88602_g3, c83511_g1 | K14488 | SAUR family protein |
| 3      | c80401_g2, c80401_g1, c86301_g1 | K14489 | arabidopsis histidine kinase 2/3/4 (cytokinin receptor) |
| 1      | c67220_g1 | K14490 | histidine-containing phosphotransfer protein |
| 1      | c84223_g9 | K14491 | two-component response regulator ARR-B family |
| 1      | c67613_g1 | K14492 | two-component response regulator ARR-A family |
| 1      | c84139_g2 | K14494 | DELLA protein |
| 2      | c7599_g1, c139107_g1 | K14495 | F-box protein GID2 |
| 3      | c87733_g1, c79770_g4, c87368_g3 | K14496 | abscisic acid receptor PYR/PYL family |
| 2      | c84358_g1, c69215_g2 | K14497 | protein phosphatase 2C |
| 2      | c67761_g1, c73235_g1 | K14503 | brassinosteroid resistant 1/2 |
| 2      | c76507_g1, c79098_g6 | K14505 | cyclin D3, plant |
| 1      | c55993_g2 | K14508 | regulatory protein NPR1 |
Transcriptome analysis of Pinus massoniana Lamb. microstrobili during sexual reversal

Figs. 4A-D. Heat map of unigene expression involved in plant hormone signal transduction of *P. massoniana*. A, DEGs of the megastrobili and microstrobili from the normal twig; B, DEGs of the megastrobili and microstrobili from the polycone twig; C, clustering of common DEGs from the two different types of twigs and D, common DEGs from the two different types of twigs.
transcription factor that is rapidly induced by auxin [20]. In *A. thaliana*, AUX / IAA gene-derived mutants induce auxin related phenotype abnormalities [21-23]. Recent genetic and molecular studies have shown auxin to be a major regulator of differential growth responses [24]. Previously, Wakushima et al. [25] were able to induce sex changes, and the production of bisexual strobili by administering exogenous hormones in conifers. The high expression of plant hormone related genes during the spontaneous reversal of *P. massoniana* indicated that the early response gene of IAA was related to the occurrence of sexual inversion.

The sex system of gymnosperms is very complex when compared to the system of angiosperms [26]. However, some aspects of the control of female reproductive development are conserved between flowering plants and their sister group, the gymnosperms, indicating the presence of these processes in a common ancestor of the extant seeds plants [27]. Besides, gymnosperms do not produce petals, and their male reproductive organs are different from angiosperms stamens. In the classical plant flowering 'ABCDE model' [28-29], all genes belong to the MIKC type MADS-box gene except the AP2 gene [30]. In this model, class B genes play a key role in specifying the identity of male reproductive organs (stamens) and petals.
during the development of flowers, while class C genes control female organ identity [31], the absence of B gene expression leads to the formation of female reproductive organs [32]. Theissen et al. [33] found that the phylogenetic development of the MADS-box gene is similar to the origin and evolution of plant reproductive structures such as the ovule and flower. MADS-box as an important transcription factor in seed plants (including flowering plants and conifers) [34], and plays an important role in controlling flower development and organ formation [35]. Comparing functions of the floral MADS-box genes in gymnosperms with their orthologues in the early angiosperm Amborella can improve our understanding of the transition of their control functions from cone to flower development in early angiosperm evolution [36]. According to our search of the conserved MADS-box protein domain of A. thaliana in the transcriptomic data of P. massoniana using the method of local blastp, a total of 63 unigenes were selected as homologues to the MADS-box transcription factor. Interestingly, the expression of MADS-box related genes in P. massoniana was found to be related to the gender difference. The MADS-box genes of PM_b that related to the process of sexual inversion showed higher expression than detected in the microstrobili in cluster 1. However, the expression of MADS-box genes in bisexual strobili was similar to that of microstrobili. The expression of many genes is regulated by transcription factors, and the different expression of MADS-box genes may be the first critical step during sex reversal.

At present, for the occurrence of P. massoniana inversion, there is no transcriptomic data available. Researches on the reversal of plant sex are still rare, and most of them only stay at the level of physiology and anatomy, many specific regulatory mechanisms are unclear. Questions remain, such as why the P. massoniana polycone can have both twigs of the polycone and normal cone, and yet, these twigs can inherit stably; how do plant hormones interact and respond to control the differentiation of flower buds; or the role of specific regulation factors in the phenomenon of the sexual reversal. However, the answers to these questions are not yet known, it need learning and exploring more deeply.

6 Conclusions

Results of the present study demonstrated that DEGs of the megastrobili and microstrobili of the normal and polycone twigs of P. massoniana exhibited male and female preferred expression in the plant hormone signal transduction pathways. A total of 36 common hormone-related DEGs between the two groups of DEGs (from the normal twig and the polycone twig) were all up-regulated in the bisexual strobili. This process involved a total of seven hormones, and the effect of IAA was the most significant. Among them, the expression of six auxin-related genes were up-regulated in the megastrobili and bisexual strobili. There was a significant positive correlation between IAA signal transduction pathway and the occurrence of sexual reversal in the P. massoniana. The expression of MADS-box related genes in P. massoniana was found to be related to sex difference. A part of the MADS-box genes of bisexual strobili showed a higher expression than measured in the microstrobili. However, the expression of MADS-box genes in the bisexual strobili was similar to that of microstrobili.

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