Research Article

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Transcriptome analysis of *Pinus massoniana* Lamb. microstrobili during sexual reversal

Abstract: The normal megastrobilli and microstrobilli before and after the sexual reversal in *Pinus massoniana* Lamb. were studied and classified using a transcriptomic approach. In the analysis, a total of 190,023 unigenes were obtained with an average length of 595 bp. The annotated unigenes were divided into 56 functional groups and 130 metabolic pathways involved in the physiological and biochemical processes related to ribosome biogenesis, carbon metabolism, and amino acid biosynthesis. Analysis revealed 4,758 differentially expressed genes (DEGs) between the mega- and microstrobili from the polycone twig. The DEGs between the mega- and microstrobili from the normal twig were 5,550. In the polycone twig, 1,188 DEGs were identified between the microstrobili and the sexually reversed megastrobili. Concerning plant hormone signal transduction pathways, the DEGs from both the normal and polycone twigs displayed distinct male or female associated expression patterns. There were 36 common hormone-related DEGs from the two types of twigs of *P. massoniana*. Interestingly, expression of these DEGs was up-regulated in the bisexual strobili, which underwent the sexual reversal. A portion of MADS-box genes in the bisexual strobili were up-regulated relative to expression in microstrobili .

Keywords: *Pinus massoniana* Lamb., Microstrobilus, Sexual reversal, Transcriptome, Differentially expressed gene.

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1 Introduction

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 strobilli before and after the sexual reversal, and also the normal strobilli 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 A-F. Normal- and polycones of *P. massoniana*. A, normal microstobilli; B, normal megastrobilli; C, normal cones; D and E, sexual reversal of microstrobilli into megastrobilli and F, polycones.

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.

Table 1. A brief description of the collected samples from the same polycone twig of *P. massoniana*.

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

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 |log2 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. More specifically, the number of genes within the size ranges of 200-301, 301-500, 501-1,000, 1,001-2,000 bp, and >2,000 bp are 90,894, 45,307, 28,009, 15,329, and 10,484 unigenes, respectively. The number of unigene sequences decrease gradually with the length of the sequence, thus indicating good sequence quality.

Table 2. Summary of sequencing data quality of *P. massoniana* polycone

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 sitchenensis* (Bong) Carr. (24.9%), followed by *Gossypium raimondii* Ulbr. (7.4%), *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 it's entirety, there were 56 functional groups identified, among which, cellular process (GO: 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 in endoplasmic reticulum	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 metabolism	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 transduction	ko04075	468(1.59%)
15	Pyruvate metabolism	ko00620	464(1.58%)

The greatest proportion belonged to ribosome, with a gene number of 1,767 (6.01%), followed by carbon metabolism and biosynthesis of amino acids. The latter two metabolic pathways showed a number of 1,523 (5.18%) and 1,130 (3.84%), respectively. Plant hormone signal conduction involves a total of 468 genes (1.59%).

4   Expression analysis of DEGs

Common DEGs from the paired analysis of the ploycone of *P. massoniana* were subjected to stratified cluster analysis (Fig. 3). The PM_w from the polycone twig and the PM_m from the normal twig clustered together, while PM_q from the polycone twig and the PM_f from the normal twig clustered together. The difference between these two clusters was shown to be significant. The overall value of PM_b expression from the polycone twig also fell between the values of the microstrobili and the megastrobili. Principle component analysis (PCA) was performed for the expression of the unigenes of the ploycone of *P. massoniana*. The results demonstrated that the PM_b was located between the microtrobili and the megastrobili at the transition state of sexual reversal. This result is consistent with the actual situation and was observed with good reproducibility.

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 reversa.

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
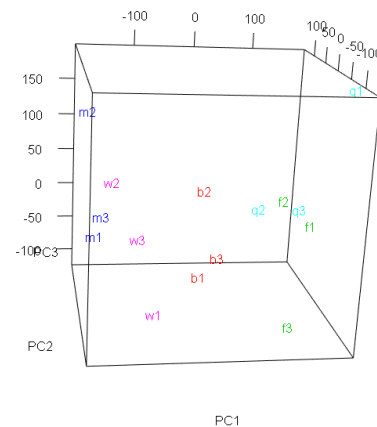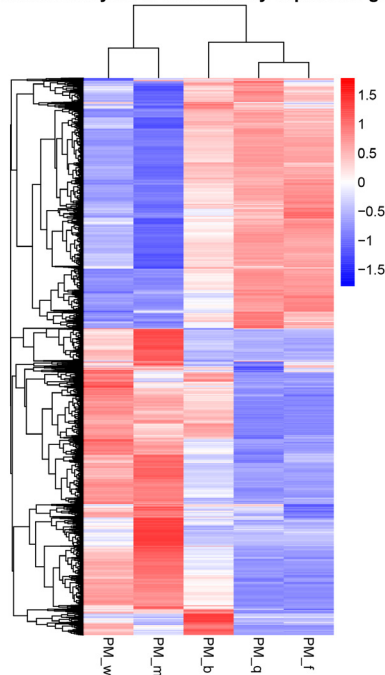
Figs. 3A-B. Clustering of transcriptome expression of the *P. massoniana*. A, stratified clustering of DEGs of the polycone of *P. massoniana*; B, three-dimensional PCA of transcriptome data.

enriched in the plant hormone signaling pathway (ko04075). Regarding the metabolic pathway of plant hormone signal transduction, there were 51 DEGs between the megastrobili and microstrobili from the polycone twig, with 26 up-regulated and 25 down-regulated. Also, there were 51 DEGs between the megastrobili and microstrobili from the normal twig, with 30 up-regulated and 21 down-regulated. Altogether there were 36 DEGs common to the polycone 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 megastrobili. The genes and their related metabolic pathways are listed in Table 5.

The DEGs between the megastrobili and microstrobili from the normal twig and those from the polycone 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 polycone twig.

4.3 Expression of MADS-box genes involved in the megastrobili and microstrobili of *P. massoniana*

According to our search using the conserved MADS-box protein domain of *Arabidopsis thaliana* in the transcriptome data of *P. massoniana*, with using the method of local blastp, a total of 63 unigenes were identified as homologues to the MADS box transcription factor. Their expression was analyzed using R software (Figure 5). It can be found that most of the MADS-box genes in the megastrobili and microstrobili of *P. massoniana* demonstrated either male or female preferred expression. For the expression of the MADS-box, there were two distinct expression patterns between the megastrobili and microstrobili. The genes from the megastrobili showed high expression in cluster 1 and the expression of PM_q was similar to that of PM_f. The genes from the microstrobili showed the high expression in cluster 2 and the expression of PM_w was similar to that of PM_m. The MADS-box genes of PM_b showed a higher expression level than the microstrobili on the cluster 1.

5 Discussion

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

Table 5. DEGs common to megastrobili and microstrobili from two different twigs that were involved in plant hormone signaling pathways.

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 peotein
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
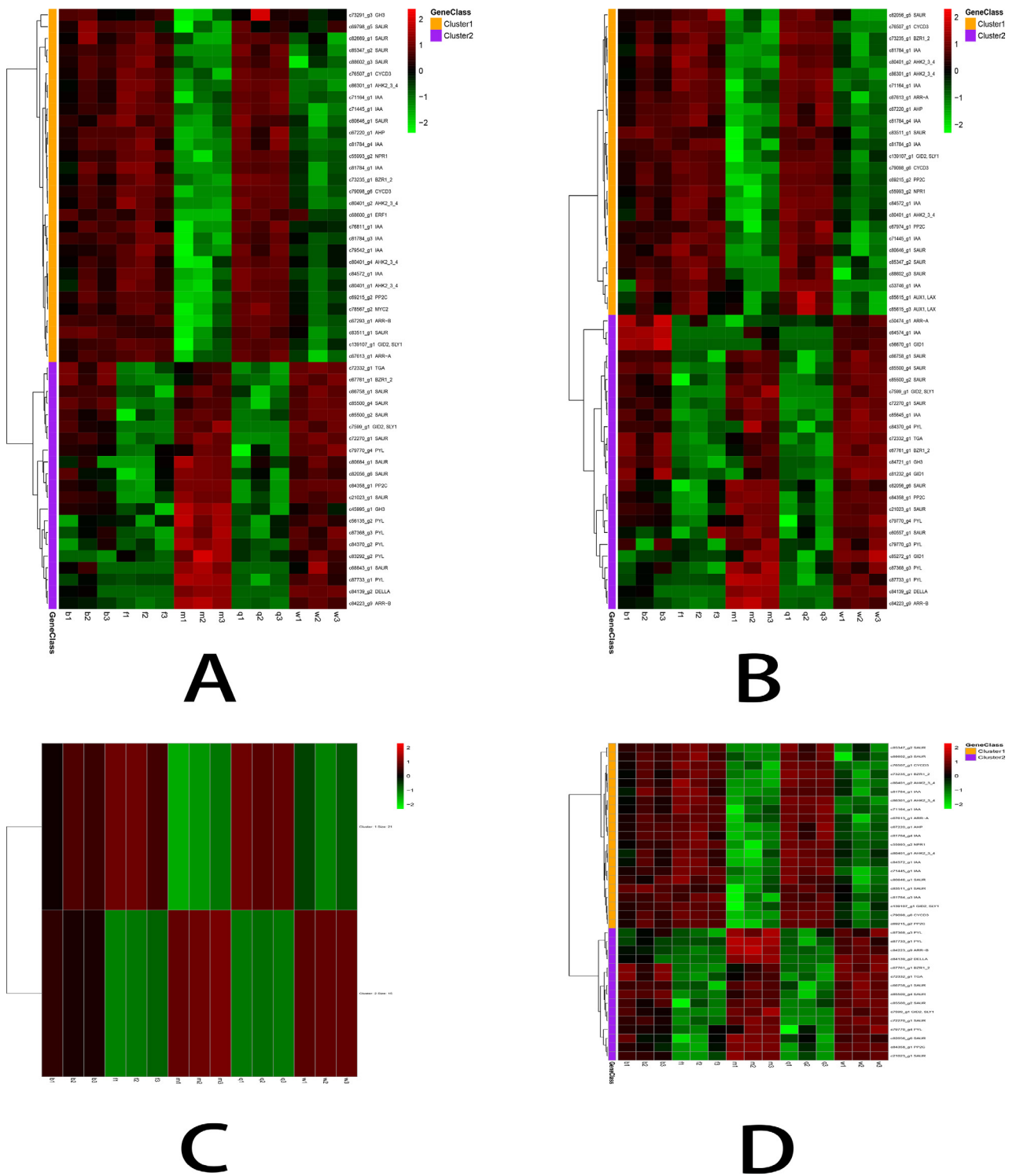
Figs. 4A-D. Heat map of unigene expression involved in plant hormone signal transduction of *P. massoniana*. A, DEGs of the megastrobili and microstrobili from the normal twig; B, DEGs of the megastrobili and microstrobili from the polycone twig; C, clustering of common DEGs from the two different types of twigs and D, common DEGs from the two different types of twigs.
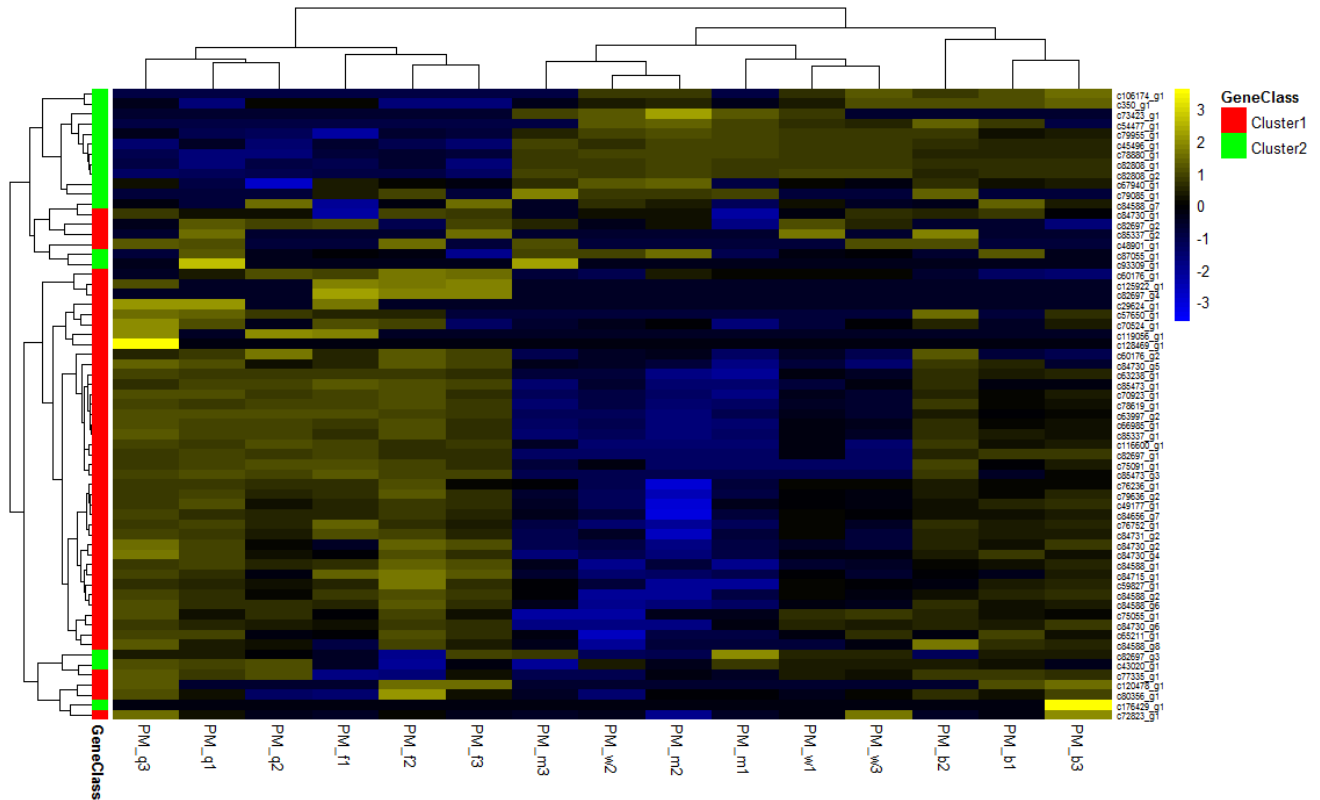
Fig. 5. Expression of MADS-box genes in *P. massoniana*.

desertification. Polycone development is a special phenomenon occurring in *P. massoniana*, in both seedling base and in natural forests. To date, there have been few studies examining the sexual reversal mechanism of *Pinus*, especially *P. massoniana*.

During the reproductive growth of plants, there are a number of external and internal factors affecting the flower bud differentiation. Six signaling pathways have been identified to regulate the flowering of *A. thaliana*: photoperiod, vernalization, autonomous, gibberellin (GA), temperature-sensitive, and age-dependent control [10]. The regulation and interaction of plant endogenous hormones in plant tissues have direct effects on plant bud differentiation and sex determination [11-13]. In angiosperms which are monoecism, endogenous GA played a feminine role in the sex determination of *Zea mays* L. [14]. Sex-determining genes and plant hormones have some connection with the sex determination of *Cucumis sativus* L [15-16]. The plant hormone, auxin, is at the core of many aspects of plant growth and development [17-18]. In the bisexual strobili during sexual reversal of the polycone twig expression of many hormone related genes is up-regulated, especially the expression of SAUR and AUX / IAA. These two kinds of hormones belong to the early response genes of auxin [19]. AUX / IAA is a

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.

Conflict of interest: Authors state no conflict of interest

References

[1] Zichang W., Hongyan W., Rengen Y., Kaiyue L., Zhide R., The intraspecific variation of *Pinus massoniana* – primary study on the polycone *Pinus massoniana*, J. Seed., 2001, 6, 66-68 [in Chinese]

[2] Caron G.E., Powell G.R., Morphological variation, frequency, and distribution of bisporangiate strobili in Picea mariana, J. Canadian Journal of Botany., 1990, 68 (8), 1826-1830,

[3] Matziris D., Hermaphrodism in black pine, J. Silvae genetica., 2002, 51 (2-3), 130-131,

[4] Flores-Rentería L., Vázquez-Lobo A., Whipple A V., Pinero D., Marquez-Guzman J., Dominquez C.A., Functional bisporangiate cones in *Pinus johannis* (*Pinaceae*): implications for the evolution of bisexuality in seed plants, J. American Journal of Botany,, 2011, 98 (1), 130-139,

[5] Yongsheng L., Shufen Z., Xiaomei Y., Observation on the sexual reversal of the microstrobili of *Pinus tabulaeformis*, J. Jilin Forestry Science and Technology, 2008, 37 (6), 1-6 [in Chinese]

[6] Xishun Z., Shijie L., Study on abnormal reproductive behavior of the sexual reversal of the microstrobili of *Pinus tabula-eformis*, J. Jilin Agriculture, 2015, 16, 58 [in Chinese]

[7] Shihui N., Huwei Y., Xiaoyang C., Wei L., Analysis of high-throughput gene expression profiles of male and female flowers of *Pinus tabulaeformis*, J. Forestry Science, 2013, 49 (9), 46-51 [in Chinese]

[8] Grabherr M.G., Haas B.J., Yassour M., Levin J.Z., Thompson D.A., Amit I., *et al.*, Full-length transcriptome assembly from RNA-Seq data without a reference genome, J. Nature biotechnology, 2011, 29 (7), 644-652

[9] Anders S., Huber W., Differential expression analysis for sequence count data, J. Genome biology, 2010, 11(10), R106

[10] Fornara F., de Montaigu A., Coupland G., SnapShot: control of flowering in *Arabidopsis*, J. Cell, 2010, 141(3), 550-550. e2

[11] To J.P.C., Kieber J.J., Cytokinin signaling: two-components and more, J. Trends in plant science., 2008, 13(2): 85-92.

[12] Pharis R.P., Ross S.D., McMullan E., Promotion of flowering in the *Pinaceae* by gibberellins, J. Physiologia Plantarum, 1980, 50(2), 119-126

[13] Bonnet-Masimbert M., Zaerr J.B., 2. The role of plant growth regulators in promotion of flowering, J. Plant Growth Regulation., 1987, 6(1-2), 13-35

[14] Rood S.B., Pharis R.P., Major D.J., Changes of endogenous gibberellin-like substances with sex reversal of the apical inflorescence of corn, J. Plant Physiology, 1980, 66(5), 793-796

[15] Pierce L.K., Wehner T.C., Review of genes and linkage groups in cucumber, J. Hort Science, 1990, 25(6), 605-615

[16] Perl-Treves R., Male to female conversion along the cucumber shoot: approaches to studying sex genes and floral development in *Cucumis sativus*, J. Sex determination in plants., 1999, 189-215

[17] Wang H., Jones B., Li Z., Frasse P., Delalande C., Regad F., et al. The tomato Aux/IAA transcription factor IAA9 is involved in fruit development and leaf morphogenesis, J. The Plant Cell Online, 2005, 17(10), 2676-2692

[18] Davies. P. (ed.), Plant hormones and their role in plant growth and development, M. Springer Science & Business Media, 2012

[19] Hagen G., Guilfoyle T., Auxin-responsive gene expression: genes, promoters and regulatory factors, J. Plant molecular biology, 2002, 49(3-4), 373-385

[20] Remington D.L., Vision T.J., Guilfoyle T.J, Reed J.W., Contrasting modes of diversification in the Aux/IAA and ARF gene families, J. Plant physiology, 2004, 135(3), 1738-1752

[21] Tian Q., Reed J.W., Control of auxin-regulated root development by the *Arabidopsis thaliana* SHY2/IAA3 gene, J. Development., 1999, 126(4), 711-721

[22] Yang X., Lee S., So J., Dharmasiri S., Dharmasiri N., Ge L., et al., The IAA1 protein is encoded by AXR5 and is a substrate of SCFTIR1, J. The Plant Journal, 2004, 40(5), 772-782

[23] Tatematsu K., Kumagai S., Muto H., Sato A., Watahiki M.K., Harper, R M., et al., MASSUGU2 encodes Aux/IAA19, an auxin-regulated protein that functions together with the transcriptional activator NPH4/ARF7 to regulate differential growth responses of hypocotyl and formation of lateral roots in *Arabidopsis thaliana*, J. The Plant Cell, 2004, 16(2), 379-393

[24] Harper R.M., Stowe-Evans E.L., Luesse D.R., Muto H., Tatematsu K., Watahiki M.K., et al., The NPH4 locus encodes the auxin response factor ARF7, a conditional regulator of differential growth in aerial *Arabidopsis* tissue, J. The Plant Cell, 2000, 12(5), 757-770

[25] Wakushima S., Yoshioka H., Sakurai N., Promotion of lateral female strobili production in Pinus densiflora by cytokinin application at a specific stage, J. Journal of Forest Research, 1997, 2(1), 51-57

[26] Flores-Rentería L., Molina-Freaner F., Whipple A.V., Gehring C.A., Dominguez C.A., Sexual stability in the nearly dioecious *Pinus johannis* (*Pinaceae*), J. American journal of botany, 2013, 100(3), 602-612

[27] Scutt C.P., Vinauger-Douard M., Fourquin C., Finet C., Dumas C., An evolutionary perspective on the regulation of carpel development, J. Journal of Experimental Botany, 2006, 57(10), 2143-2152

[28] Coen E.S., Meyerowitz E.M., The war of the whorls: genetic interactions controlling flower development, J., nature., 1991, 353(6339), 31-37

[29] Ditta G., Pinyopich A., Robles P., Pelaz S., Yanofsky M.F., The SEP4 gene of *Arabidopsis thaliana* functions in floral organ and meristem identity, J. Current Biology, 2004, 14(21), 1935-1940

[30] Theißen G., Development of floral organ identity: stories from the MADS house, J. Current opinion in plant biology, 2001, 4(1), 75-85

[31] Wang Y.Q., Melzer R., Theißen G., Molecular interactions of orthologues of floral homeotic proteins from the gymnosperm Gnetum gnemon provide a clue to the evolutionary origin of 'floral quartets', J. The Plant Journal, 2010, 64(2), 177-190

[32] Theißen G., Becker A., Gymnosperm orthologues of class B floral homeotic genes and their impact on understanding flower origin, J. Critical Reviews in Plant Sciences, 2004, 23(2), 129-148

[33] Theissen G., Becker A., Di Rosa A., Kanno A., Kim J.T., Münster, T., et al. A short history of MADS-box genes in plants, J. Plant molecular biology., 2000, 42(1), 115-149

[34] Groth E., Functional diversification among MADS-Box genes and the evolution of conifer seed cone development, D. Acta Universitatis Upsaliensis, 2010

[35] Tandre K., Albert V A., Sundås A., Engström P., Conifer homologues to genes that control floral development in angiosperms, J. Plant molecular biology, 1995, 27(1), 69-78

[36] Niu S., Yuan H., Sun X., Porth I., Li Y., El-Kassaby Y.A., et al., A transcriptomics investigation into pine reproductive organ development, J. New Phytologist, 2016, 209(3), 1278-1289