Plant Physiology and Biochemistry 142 (2019) 510–518

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Transcriptome analysis during floral organ development provides insights into stamen petaloidy in *Lagerstroemia speciosa*

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ARTICLE INFO

Keywords:
Lagerstroemia speciosa
RNA-seq
Stamen petaloidy
Floral organ development
Co-expression analysis
Floral homeotic gene

ABSTRACT

As one of the most popular woody species that blooms in summer, *Lagerstroemia speciosa* has been used abundantly in urban landscape for its excellent floral beauty. For the first time, we discovered a double-flower variant with all petaloid stamens. To understand the molecular basis of this variation, we contrasted the transcriptomes of single- and double-flower buds at three stamen development stages. In total, 73,536 unigenes were mapped and 30,714 differently expressed genes (DEGs) were identified in the tissues. We focused on the DEGs expressing in both phenotypes and investigated the association of their expression profiles with their functions in transcription pathways. Furthermore, we performed WGCNA and identified co-expressed genes with four floral homeotic genes as hubs (*MADS16*, Unigene0026169; *AP2*, Unigene0042732; *SOC1*, Unigene0046314; *AG*, Unigene0056437). The expression of these hub genes has been conserved across the three developmental stages but significantly different between the two floral phenotypes. As a result, the robust transcriptional regulation of stamen petaloidy in double flowers was deduced. These findings will help to unravel the regulatory mechanisms of several specific genes, thereby providing a basis to study double-flower molecular breeding in *L. speciosa*.

1. Introduction

Lagerstroemia speciosa L. (Lythraceae) is a small to medium-sized (rarely large) deciduous or semi-deciduous tree species. It is abundantly grown in tropical or sub-tropical urban landscapes and bears purple flowers that bloom in summer (Sharmin et al., 2018). The studies have been conducted on the pharmaceutical use of this species (Stohs et al., 2012). However, little is documented on its ornamental value, especially the flowers (Khanduri, 2014; Ren et al., 2009). Normally, there are four whorls of floral organs in its flower (diameter of 10.6 ± 0.76 cm), which include six fleshy brownish sepals in whorl 1 (5.2 ± 0.86 cm in length), six pink to pinkish white petals (4.8 ± 0.64 cm in width) in whorl 2, numerous yellow stamens in whorl 3, and one green stigma with a style length of 2.2 ± 0.14 cm in whorl 4 (Khanduri, 2014). However, we recently found a natural variant of double-flower *L. speciosa* in Guangxi Zhuang Autonomous Region, China. In its flowers, there are also four whorls of floral organs, but all the stamens are homeotically converted into petal-like organs (Fig. 1A and B). This variant provides unique material to study the regulatory mechanism of double flowering in the genus of *Lagerstroemia* and thus supports our ornamental breeding research by improving its floral phenotypes.

As a complex process, flower development is regulated by multiple biological processes. Due to its contribution to both plant reproductive and ornamental value, flower patterning has gained significant attention and has been systematically studied in the model plants *Arabidopsis* and *Antirrhinum* in particular. In *Arabidopsis*, the morphology of petals is under the control of complicated genetic pathways. Generally, the genes involved in signal transduction of phytohormones or regulation of transcription factors (TF) have a primary function in these processes (Huang and Irish, 2016). The mechanism of floral organ identity has been elucidated through the typical ‘ABCE model’ in *Arabidopsis*

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https://doi.org/10.1016/j.plaphy.2019.08.012
Received 3 February 2019; Received in revised form 14 August 2019; Accepted 15 August 2019

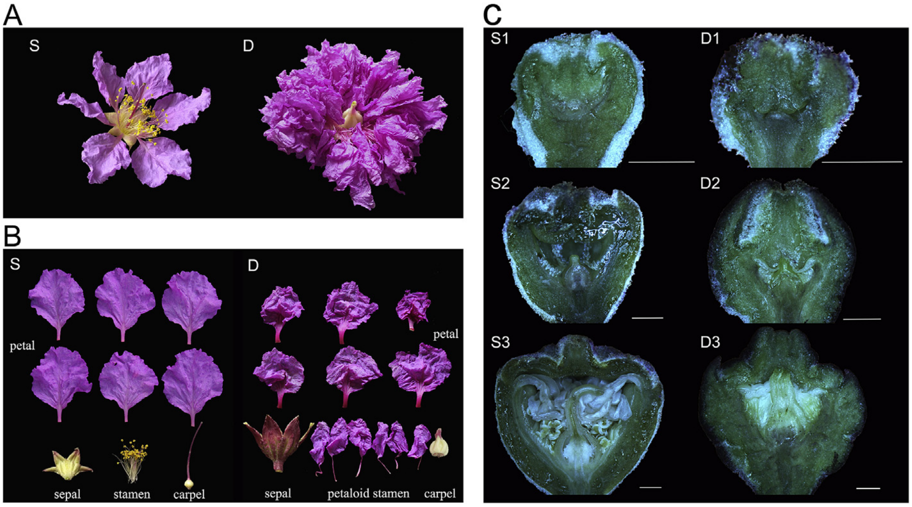

Fig. 1. Phenotypic comparison between single- (S) and double-flower (D) *L. speciosa*.
(A) Whole flower comparison between S and D. **(B)** Floral organ comparison between S and D. **(C)** Axial section of flower bud under stereomicroscope at three stages of stamen development in S and D. Scale bar = 1 mm.

(Theißen et al., 2016). Petal development is regulated by Class A (*AP1*, *APETALA 1*; *AP2*, *APETALA 2*) and E (*SEP1-4*, *SEPALLATA*) genes along with class B genes (*AP3*, *APETALA3*; *PI*, *PISITTALA*), while the stamen is controlled by the interaction of genes in class B with E and C (*AG*, *AGAMOUS*). The insufficiency of C gene function may stimulate the stamen petaloidy (Noor et al., 2014). All these genes belong to the MADS-box family with the only exception being *AP2*, but they all compose the floral homeotic genes. In accordance with the 'floral quartet model', these genes form tetrameric complexes by interacting with each other, and bind to target DNA to promote organogenesis (Theißen et al., 2016). The flower morphogenic function of floral homeotic genes consisting of MADS-box and AP2/EREBP (ethylene-responsive element binding proteins) genes (Riechmann and Meyerowitz, 1998) has been well elucidated. The morphology of petaloid stamens is regulated through a complicated genetic network. In several ornamental plants, including woody plants such as *Catalpa bungei* and *Camellia*, the conversion of stamen petaloidy has been found, and the functions of some regulatory genes have been investigated (Jing et al., 2015; Li et al., 2017). The expression level of *AG* homologs has a crucial role in developing the petaloidy of stamens in double flowers (Liu et al., 2013; Zhang et al., 2015).

Recently, RNA-Seq has been used to identify the regulation of promising genes in stamen petaloidy in some ornamental crops, such as rose, chrysanthemum and lotus (Lin et al., 2018; Wang et al., 2017). Floral development genes have a conserved role in angiosperms with proven differences in regulation, redundancy and function among species (Annick et al., 2010). However, the molecular basis of flower development is lacking in woody plants, and there is no evidence that the 'ABCE model' is also applicable in *Lagerstroemia*. Therefore, to get a better understanding of the mechanism of double-flower formation and the stamen petaloidy, we applied RNA-seq technology to comparatively analyse the transcriptomes of single and double flower buds. Several candidate genes, that were likely to be involved in stamen petaloidy in *L. speciosa*, were investigated. This dataset will serve as the foundation for double-flower breeding in *Lagerstroemia.*

2. Materials and methods

2.1. Plant materials

Single- and double-flower plants of *L. speciosa* were cultivated in the nursery of Guangxi Forestry Research Institute, Nanning, China (22°92' N, 108°36' E). The double-flower plants were cultivated by grafting branches onto single-flower rootstocks. The flower bud samples were collected in May 2018 and identified morphologically under stereomicroscope at three different stages of flower bud development: early-stage bud (diameter 0.1–0.2 cm) with only floral primordia; mid-stage bud (diameter 0.3–0.4 cm) with developing petals and stamens; mature bud (diameter 0.5–0.6 cm) with formed stamens and carpels, which were named as S1 and D1, S2 and D2, S3 and D3 in single and double flower buds, respectively (Fig. 1C). Buds at separate stages were dissected for each biological replicate (~ 0.30 g) with three biological replicates at each stage. All samples were instantly frozen in liquid nitrogen and stored at −80 °C.

2.2. Illumina sequencing, transcriptome assembly and gene functional annotation

A total of > 3 μg RNA was extracted using an RNA reagent (OminiPlant RNA Kit, CWBIO, China) and each sample was standardized at an integrity number > 7.3 for library construction. The isolated mRNA was enriched by Oligo (dT) beads and broken into short fragments. The cDNA fragments were sequenced on an Illumina HiSeq 4000 platform by Gene Denovo Biotechnology Co. (Guangzhou, China). Reads containing adapters, poly-N, or low-quality sequences were removed and only clean reads of high quality were mapped to ribosome RNA (rRNA) to identify residual rRNA reads. The high-quality reads with rRNA removed were mapped to the reference transcriptome using the short read alignment tool Bowtie2 at default parameters, and the mapping ratio was calculated. As there has been no genome sequence of *L. speciosa* published to date, transcriptome *de novo* assembly was carried out with Trinity. The unigene expression was calculated and normalized to reads per kb per million reads (RPKM). All sequence data of S and D have been deposited in the Sequence Read Archive (SRA) as BioProject PRJNA513250 (https://www.ncbi.nlm.nih.gov/sra/PRJNA513250). For gene functional annotation, we performed the BLASTx program (http://www.ncbi.nlm.nih.gov/BLAST/) at an E-value threshold of $1e^{-5}$ to NCBI Non-redundant protein sequences (Nr, http://www.ncbi.nlm.nih.gov), Clusters of orthologous groups of proteins database (COG, http://www.ncbi.nlm.nih.gov/COG), Swiss-Prot protein sequence database (http://www.expasy.ch/sprot) and Kyoto Encyclopaedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg). Protein coding sequences of unigenes were aligned by BLASTp to Plant TFdb (http://planttfdb.cbi.pku.edu.cn/) to predict TF families.

2.3. Differential gene expression, GO and KEGG enrichment analysis

Principal component analysis (PCA) was performed with R package models (http://www.r-project.org/) to evaluate repeatability between replicates. Identified genes at fold change > 2 ($|\log_2 FC| > 1$) and false discovery rate (FDR) < 0.05 in a comparison were recognized as significant differentially expressed genes (DEGs) across the groups. On the basis of Wallenius non-central hyper-geometric distribution, GO enrichment analysis of DEGs was performed using GOseq R packages 1.10.0 with adjustment for gene length bias. Regulatory pathways were enriched using KEGG orthology-based annotation system (KOBAS) (FDR < 0.05).

2.4. RNA-seq data validation through quantitative real-time PCR

The RNA-seq results were validated *via* quantitative real-time PCR (qRT-PCR) with the expression of 22 DEGs found to be involved in floral development. An RNA prep Pure Kit (for polysaccharides & polyphenolics-rich plants) (DP441; Tiangen Biotech CO., LTD, Beijing, China) was used to extract total RNA in the buds corresponding to the RNA-seq samples according to the manual. Subsequently, first-strand cDNA was synthesized using Prime Script^{TM}RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) with the gene-specific primers listed in Table S1. The PCR protocol and procedure were followed as per a previous study in our lab (Han et al., 2017). In three biological replicates, the data were collected from three technical replicates for each and normalized with *elongation factor-1-alpha* (*EF-1α*) as an internal reference (Zheng et al., 2018). The calculation of relative expression was based on the $2^{-\Delta\Delta CT}$ method.

2.5. Co-expression network analysis for module construction

Co-expression networks were constructed using the WGCNA (v1.47) package in R. The genes expressing in at least 40% of samples were selected and their expression values were introduced to WGCNA to create co-expression modules with default settings, except that the power was 12, TOM Type was unsigned, merge Cut Height was 0.2, and min Module Size was 50. Samples were clustered into 13 correlated modules. To identify the relationship between module and stage-specific expression in S and D, a correlation coefficient was calculated as module eigenvalue with samples. Genes with high intra-modular connectivity tended to be hub genes which probably have significant functions. Pathway enrichment of DEGs was annotated by Mercator (http://www.plabipd.de/portal/mercator-sequence-annotation) and visualized *via* MapMan. Co-expression networks were visualized using Cytoscape (v3.7.0).

2.6. Prediction of the transcriptional regulation in double-flower development

To discover the significant module with a close relationship to sample-specific expression between two phenotypes, we followed a cross-tabulation approach and constructed 13 modules. Among them, we selected the genes in module brown, in which the module eigenvalue between S and D samples was remarkably different at a stable level throughout the three stages. Floral homeotic genes are well-known to regulate the development of floral organs. We performed a cross comparison between the overlapped DEGs and the co-expressed genes of floral homeotic genes (*MADS16*, Unigene0026169; *AIL5*, Unigene0042732; *SOC1*, Unigene0046314; *AG*, Unigene0056437) in module brown. A co-expression network of floral organ development at the highest connectivity was generated to predict the regulation of double-flower variation in *L. speciosa*.

3. Results

3.1. Phenotypic difference between single- and double-flower L. speciosa

The arrangement of floral organs determines flower appearance and therefore affects the ornamental value. Normally, there are four distinct whorls of organs in the flower of *L. speciosa*, which are sepals, petals, stamens and carpels. In the double-flower variant, all stamens are converted into petals (Fig. 1A and B), which results in male sterility, increases the ornamental value of the flowers and provides a good model for research on stamen petaloidy. Three stages of bud development from stamen primordia to maturation were designated as S1-S3 and D1-D3, respectively (Fig. 1C). At the first stage, there was negligible inner difference between two buds as it was too early to develop their structures. However, from the second stage, as the stamen tissues differentiated into filaments and anthers/petals, the phenotype of the double flower became noteworthy at the morphological level. By analysing the transcriptomes of buds at different stages, we can acquire a better understanding of the molecular mechanisms underlying the conversion of stamen petaloidy in double flowers.

3.2. Overall transcriptome analysis of single- and double-flower buds in L. speciosa

Total RNA-seq analysis was conducted into 18 libraries at the three stages of stamen development. In total, 784,114,784 clean reads were filtered from 849,097,572 raw reads. According to the results of *de novo* assembly of the Trinity method, 73,536 unigenes with a mean size of 849 bp were ultimately assembled, in which the lengths ranged from 201 to 11,383 bp (Fig. S1 and Table S2). Homology analysis of the top 10 species is shown in Fig. S2. Using BLAST comparison, we found that the unigenes were annotated to match the most homologs in *Eucalyptus grandis* (23.63%). In total, 53.94% of the annotated unigenes could be detected minimally in one of the four databases (KOG, SwissProt, KEGG, Nr) at an E-value $< 10^{-5}$ (Fig. S3 and Table S3). Within these databases, 39,521 genes (99.64%) were annotated in Nr; 28,786 genes (72.58%) in SwissProt; 23,546 genes (59.37%) in KOG and 15,827 genes (39.90%) in KEGG. Altogether, 39,663 unigenes were annotated. In the category of biological processes (Fig. S4A and Table S4), the most frequent terms were found in 'metabolic process' (9094 unigenes), 'cellular process' (8530) and 'single-organism process' (6709), which revealed the dominate regulation of most genes for floral organ development in these processes. Referring to the KOG database, 4380 unigenes were regulated in 'signal transduction mechanisms' (Fig. S4B and Table S5). In the putative biological pathways identified by the KO database, 477 unigenes were attributed to 'Plant hormone signal transduction' (Table S6).

3.3. Differential gene expression between single- and double-flower buds

To investigate the transcriptional differences that result in stamen petaloidy in double flowers, we preferentially identified the DEGs between S and D. Among the three biological replicates at each stage, the principal component analysis (PCA) showed their close correlation in expression and the correlation coefficient between samples showed high repeatability (Fig. S5). Gene expression was estimated with RPKM. Unigenes at RPKM < 1 in both S and D were removed from different gene expression analyses. In sum, 30,714 DEGs were identified at a fold change > 2 ($|\log_2 FC| > 1$) and FDR < 0.05. Among them, 8630 DEGs overlapped in all pairwise comparisons (S1 vs. D1, S2 vs. D2, S3 vs. D3) (Fig. 2A). In three comparisons, the downregulated DEGs were detected more than the upregulated DEGs, and this trend was most notable at the third stage (S3 vs. D3), with an over 2-fold change in DEG number. There were 6660 and 11,475 DEGs in S1 vs. D1, 7486 and 11,342 in S2 vs. D2, and 6046 and 13,084 in S3 vs. D3 that were up- and down-regulated, respectively (Fig. 2B). We performed qRT-PCR validation for

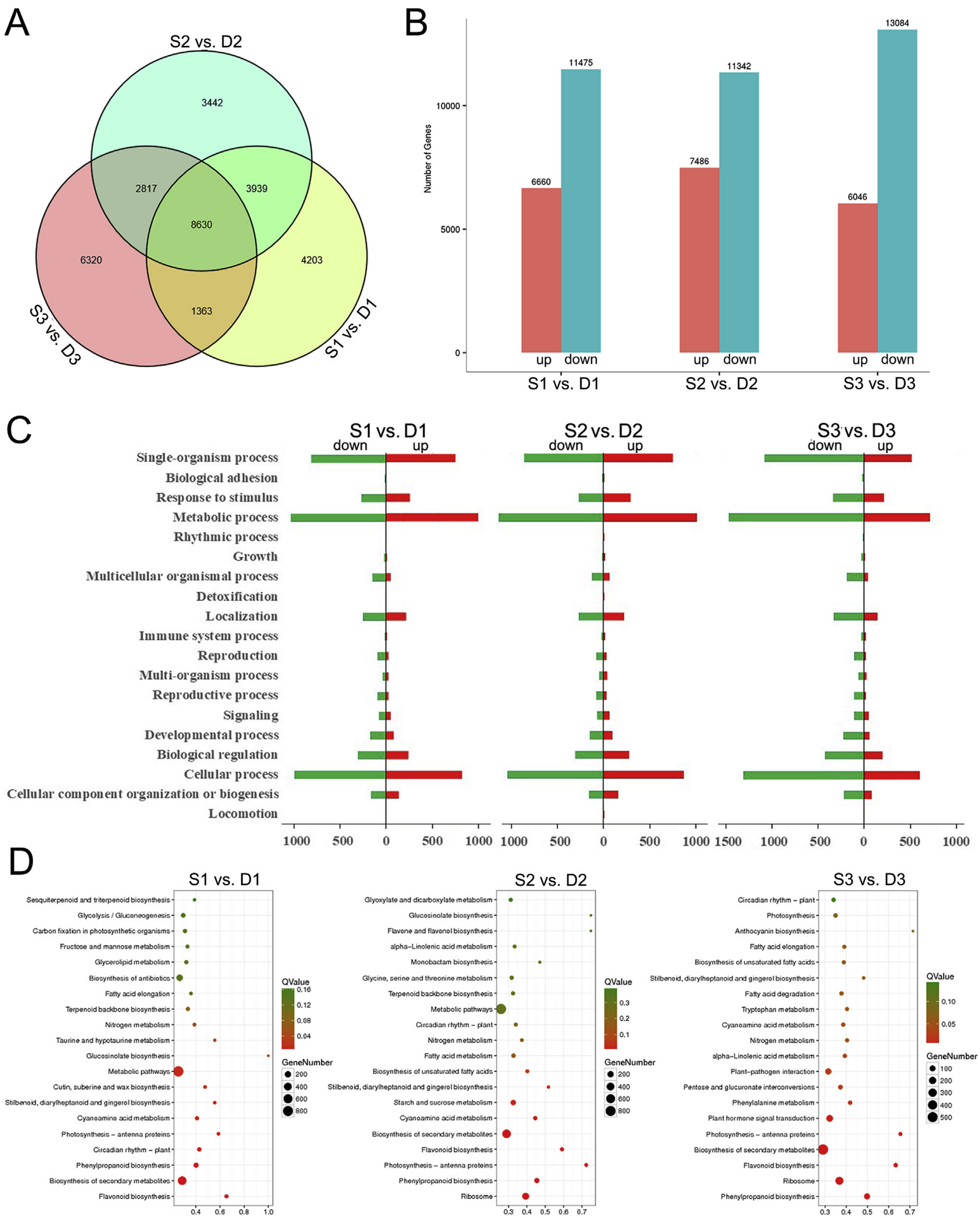

Fig. 2. DEGs at the three stages of stamen development between S and D.
(A) Venn diagram of the number of DEGs in stage comparisons: S1 vs. D1, S2 vs. D2 and S3 vs. D3. **(B)** The number of up- and downregulated DEGs in three comparisons. **(C)** The involved GO biological processes of up- and downregulated DEGs in three comparisons. **(D)** KO pathway enrichment of the DEGs. The number of genes in each category is equal to the size of the corresponding dot which represents the q-value. The top 20 for each pathway enrichment are shown in each comparison. The X-axis represents the rich factor. The Y-axis represents pathway.

22 DEGs in all samples. The results of the RNA-seq showed a correlation coefficient ≥ 0.70 with qRT-PCR analyses for most of the verified genes (Fig. S6), indicating a high reliability of the RNA-seq data.

We conducted GO enrichment for function classification and KO for pathway verification in pairwise comparisons between S and D. It revealed that 19 terms for biological processes, 15 for cellular components, and 11 for molecular functions were concentrated in all DEGs (Fig. S7). It showed representation of genes related to cellular processes, metabolic processes, single-organism processes, biological regulation and response to stimulus, which are known to be involved in flower development (Fig. 2C). In KO enrichment, significant DEGs (q-value < 0.5) were enriched in 12, 8 and 13 pathways in the three comparisons, respectively. Five pathways were enriched in all comparisons, which were phenylpropanoid biosynthesis, photosynthesis-antenna proteins, flavonoid biosynthesis, biosynthesis of secondary metabolites and cyanoamino acid metabolism (Fig. 2D).

3.4. Transcriptomic analysis of phytohormone signal pathway genes regulating floral organ development

Plant development is regulated by hormones such as auxin (AUX), abscisic acid (ABA), brassinosteroid (BR), cytokinin (CK), ethylene (ETH), gibberellin (GA), jasmonic acid (JA) and salicylic acid (SA) (Wang and Irving, 2011). To discover the genes involved in phytohormone regulation of the differentiation of floral organ development between S and D, we focused on the overlapping DEGs in all pairwise comparisons. 109 DEGs were regulated in plant hormone signal biosynthesis (55 DEGs) and signalling (54 DEGs) pathways (Fig. 3 and Table S7).

AUX, JA and GA are known to be involved in organ identity and development. In our study, DEGs were involved in auxin pathways most, including the genes *ALDH* (Unigene0009818-Unigene0044154) and *katE* (Unigene0012981-Unigene0032224) in the auxin biosynthesis pathway, and *ARF* (Unigene 0012000-Unigene0063170), *IAA* (Unigene0022532-Unigene0069410, *AUX1* (Unigene0014725-Unigene0057841), *GH3* (Unigene0005816-Unigene0039630), *SAUR* (Unigene0015588-Unigene0072929), and *TIR1* (Unigene0023268) in the auxin signalling pathway. The major homologs of *ALDH*, *ARF*, *IAA*, *AUX1* and *katE* were downregulated, except for Unigene0032224 (*katE*), Unigene0022532 (*IAA*), and Unigene0014725 (*AUX1*) which were upregulated. *GH3* and *SAUR* were upregulated during the whole period in double-flower buds. Among the DEGs involved in GA pathways, *GA2ox* (Unigene0002850-Unigene0036518) in GA biosynthesis and *PIF4* (Unigne0021729-Unigene0043791) in GA signalling were up- and downregulated, respectively, while *PIF4* (Unigene0021729) poorly expressed in double-flower buds. In the JA biosynthesis pathway, *ACOX1* (Unigene0005980-Unigene0043467) was downregulated while other DEGs were upregulated; *OPCL1* (Unigene0009125 and Unigene0049156) showed a higher expression. There was only one DEG, *MYC2* (Unigene0071866), being annotated in the JA signalling pathway. Other DEGs involved in ABA, BR, CK, ETH and SA also had different transcript levels. In particular, *CYP707A* (Unigene0046896)

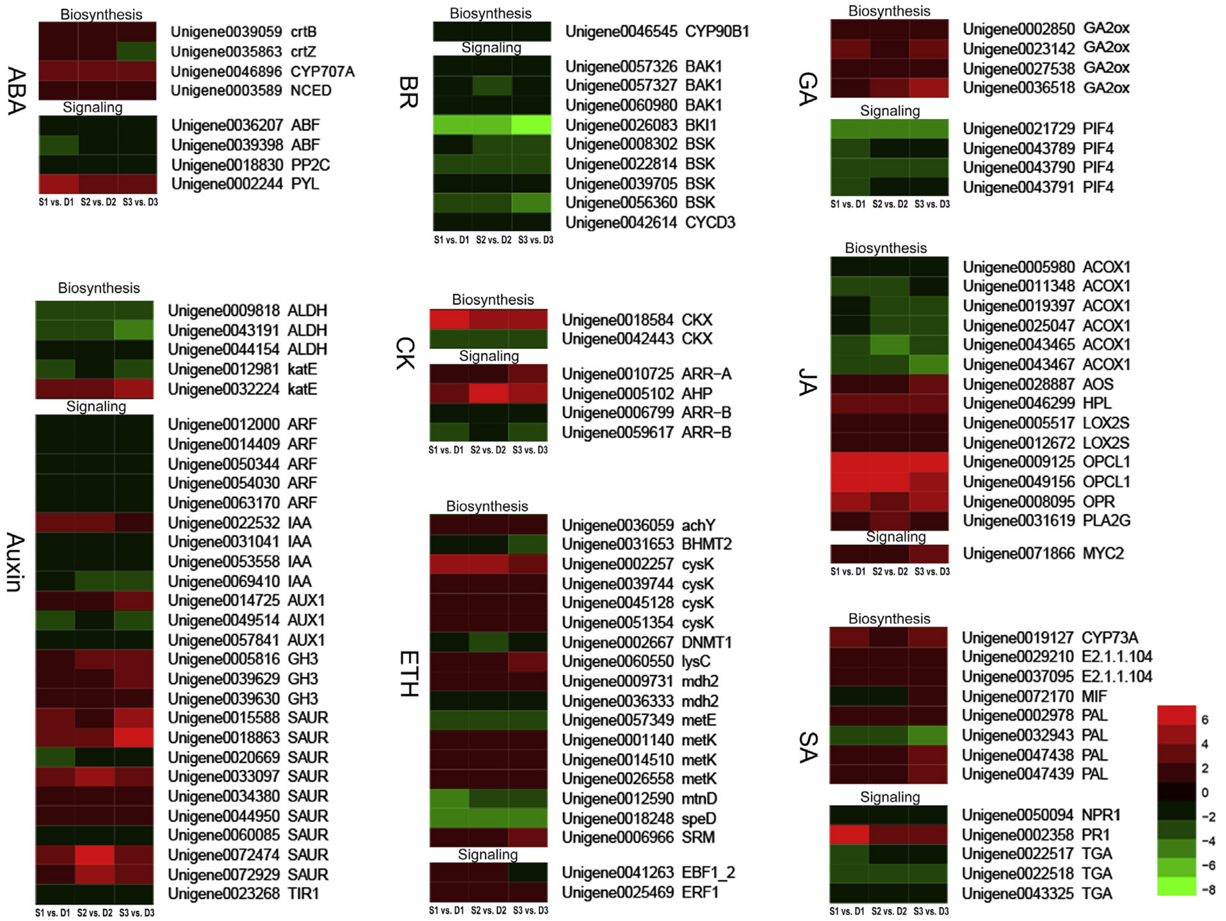

Fig. 3. Expression heat maps of DEGs involved in the biosynthesis and signalling pathways of different phytohormones. The DEGs are selected from the overlapping genes in three pairwise comparisons (S1 vs. D1, S2 vs. D2, and S3 vs. D3) during bud development. ABA, abscisic acid; Auxin, auxin; BR, brassinosteroid; CK, cytokinin; ETH, ethylene; GA, gibberellic acid; JA, jasmonic acid; SA, salicylic acid. Red and green represent up- and downregulated in gene expression, respectively. The scale represents the $\log_2$FC value in three comparisons. The labels beside the unigene IDs are gene symbols. All DEGs are listed in Supplementary Table S6. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

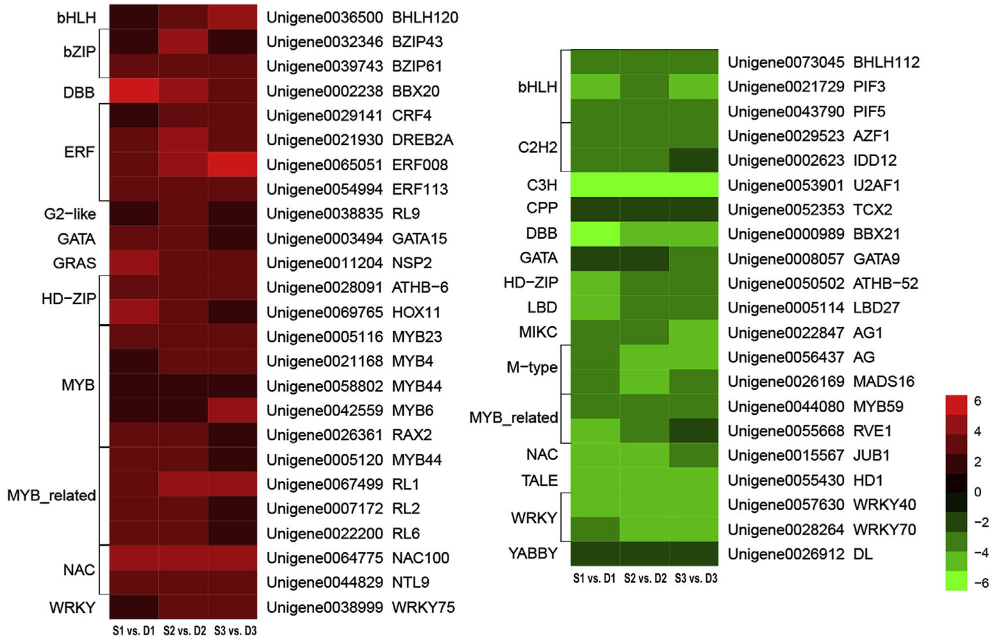

Fig. 4. Expression heat maps of transcription factor (TF) encode DEGs possibly regulating stamen petaloidy in *L. speciosa*. The DEGs are selected from the overlapping genes with significant expression changes ($|\log_2FC| > 2$) in three pairwise comparisons (S1 vs. D1, S2 vs. D2, and S3 vs. D3) throughout bud development. Red and green represent up- and downregulation in gene expression, respectively, and the scale represents the $\log_2FC$ value in three comparisons. The label to the left of the heat map represents TF family and the label to the right of the unigene ID represents the gene symbol. All TFs are listed in Supplementary Table S7. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and *PYL* (Unigene0002244) were continuously showing higher expressed in D during bud development. In the BR signalling pathway, *BK1* (Unigene0026083) was obviously downregulated much more than other genes. Among the DEGs involved in CK pathways, *CKX* (Unigene0018584) and *AHP* (Unigene005102) were significantly upregulated as compared to other genes.

3.5. Transcriptomic analysis of transcription factors regulating floral organ development

A large number of TFs (Table S8) regulated the differentiation of floral organ development during the three stages; we recognized those at $|\log_2FC| > 2$ to be significant. In our 46 significant TF coding DEGs out of the 8,630, there were 25 upregulated and 21 downregulated TFs (Fig. 4). Their expression profiles remained up or down across bud development, indicating that these genes had stable regulation to stamen petaloidy in double flowers. The DEGs encoding MYBs (MYB and MYB_related) were the richest. Among the 4 upregulated ERF DEGs, *ERF008* (Unigene0065051) had the most obvious expression differentiation. The DEGs encoding the bHLH family were downregulated, except for *BHLH120* (Unigene0036500). In the GATA family, *GATA15* (Unigene0003494) and *GATA9* (Unigene0008057) were up- and downregulated, respectively. In the NAC family, *NAC100* (Unigene0064775) was relatively more upregulated than *NTL9* (Unigene0044829), while *JUB1* (Unigene0015567) was downregulated. Two WRKY DEGs (*WRKY40*, Unigene0057630; *WRKY70*, Unigeen0028264) exhibited higher differentiation in downregulated expression than the level of *WRKY75* (Unigene0038999) with up-regulation. Furthermore, *U2AF1* (Unigene0053901) from the C3H family exhibited continuously downregulated expression during the whole period. Notably, the expression profiles of only three floral homeotic DEGs, *MADS16* (Unigene0026169), *AG1* (Unigene0022847) and *AG* (Unigene0056437), were found to have continuously significant differentiation during the three stages at $|\log_2FC| > 3.36$.

3.6. Identification of conserved DEGs regulating stamen petaloidy in double flowers via WGCNA

We sought to identify co-expressed genes through weighted gene co-expression network analysis (WGCNA). In the main subnetworks, representative genes displaying a similar expression pattern were separated in certain co-expression modules. The genes showing very low expression levels were not considered for this analysis, and a total of 13 modules were identified (Fig. S8). Further, we associated the gene expression profiles in each module with the samples from all stages to generate a heat map of the module-sample matrix (Fig. 5A). As anticipated, we found that the gene expression profiles in module brown were remarkably different between D and S, and they remained relatively steady throughout the whole period (Fig. 5B). This may suggest that the genes in this module have conserved regulation related to stamen petaloidy, as their expression levels were scarcely effected in double–flower organ development. Mapman was used to investigate the functional differences responsible for the stamen variation in *L. speciosa*. We provide the regulation (Fig. 5C) and metabolism overview maps (Fig. 5D) representative of S3 and D3 by referencing the PCA result (Fig. S5). In the regulation overview, there were many genes related to plant hormones, TF and protein regulation that were differentially expressed in S and D. In the metabolism overview, the activities of secondary metabolism, cell wall and lipids had a major part in transcriptional regulation. More DEGs were annotated to be downregulated versus upregulated in both maps.

Further, to investigate the co-expression network in the decisive regulation of stamen petaloidy, we observed the DEGs that were co-expressed with the floral homeotic genes in module brown. Four homeotic genes were found in this module, and Unigene0056437 (*AG*) and Unigene0046314 (*SOC1*) both had a close interaction with Unigene0026169 (*MADS16*), while Unigene0042732 (*AIL5*) was separately related to these genes (Table S9). As hub genes, we explored their directly co-expressed DEGs involved in phytohormone signal transduction and TF regulation during floral organ development (Tables S7 and S8). In this subnetwork (Fig. 6A), all of the co-expressed genes of *AIL5* (Unigene42732), *SOC1* (Unigene46314) and *AG* (Unigene56437) had a cross regulation with *MADS16* (Unigene26169), except for *TIR1* (Unigene23268) and *HDG5* (Unigene59693) which only had co-expression with *AIL5*. Almost all of these overlapping co-expressed genes were downregulated, which suggests that the downregulation of the key genes has a vital role in stamen petaloidy in double flowers. *MADS*16 had many other co-expressed genes, revealing it to be highly important in this regulation. These results contribute to the prediction of significant transcriptional regulation of stamen petaloidy in double flowers, which has been correlated with floral homeotic genes and conserved throughout the floral organ development (Fig. 6B).

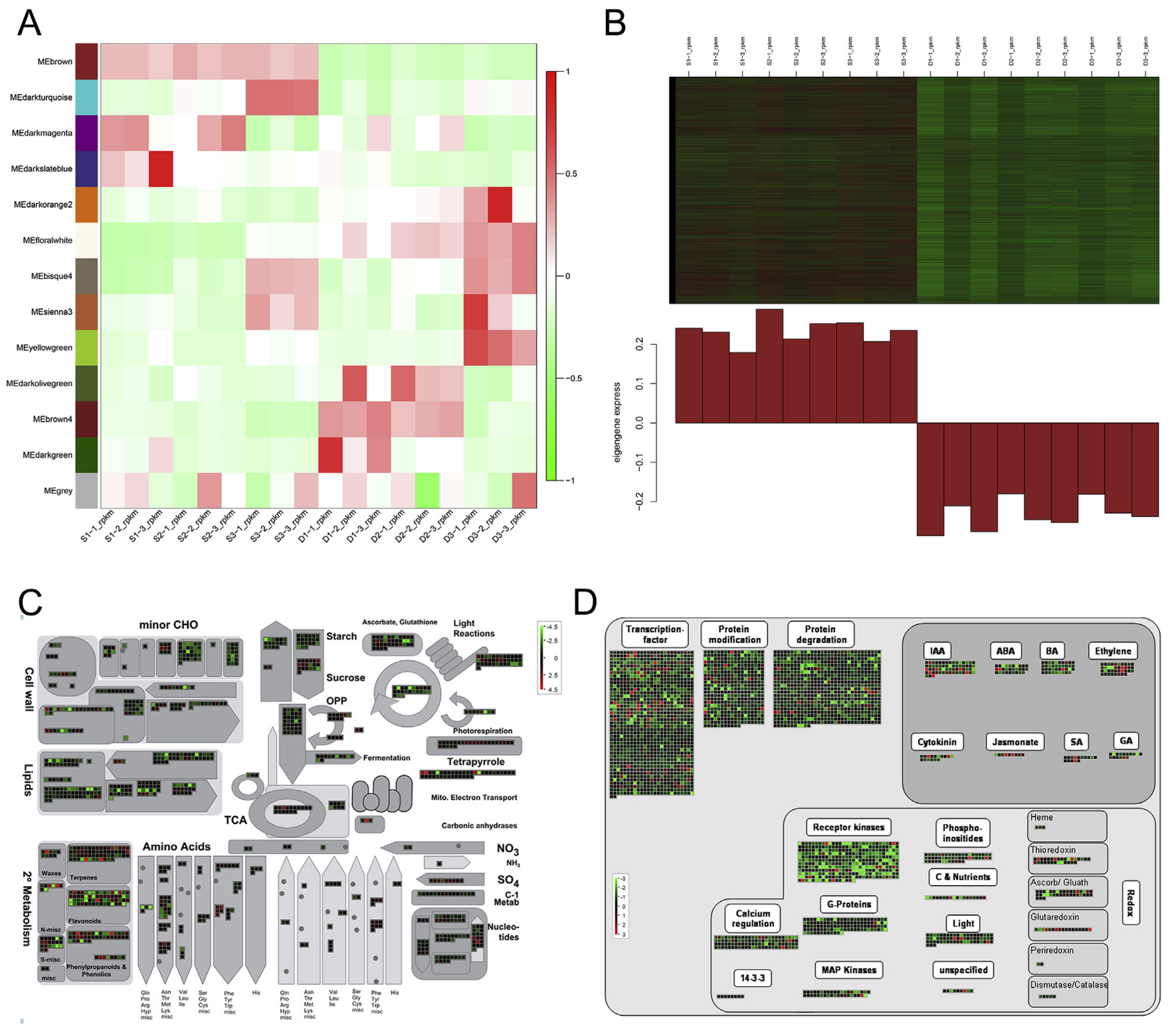

Fig. 5. Transcriptional module revealing conserved regulatory genes in stamen petaloidy in *L. speciosa*.
(A) Heat map showing the sample expression patterns in the modules. The colour scale represents Z-score. **(B)** The expression profile of all the co-expressed genes in module brown. The colour scale represents the Z-score. The bar graph shows the consensus expression pattern of the corresponding co-expressed genes in this module. **(C)** The metabolism overview of all the annotated genes in module brown. **(D)** The regulation overview of all the annotated genes in module brown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Floral architecture is one of the essential ornamental characteristics of flowering plants. Many commercial cultivars are double-flower mutants with petaloid stamens. This phenotype has been studied at the molecular level. On a morphological basis of stamen, petal and petaloid stamen, the formation of petaloid stamen suggests the conversion from stamen to petal. Therefore, it is imperative to explore the molecular mechanism of double flowering in *Lagerstroemia*. Comparing the non-reference transcriptomes between single and double flower buds, we discovered 30,714 DEGs throughout bud development. About half of the detected genes were found to be differently expressed and more of them were downregulated, suggesting that the notable reduction in the expression of some genes probably has a significant contribution in the variation from single to double flowers. During flower bud development, a complex regulatory system may exist to control changes and therefore ensures a total conversion of stamen petaloidy. With the help of transcriptome analysis including transcriptional modules and co-expression networks, several specific transcriptional programs across the two phenotypes were investigated to provide potential targets.

Floral organ primordia are initiated and developed with dependence on the activities of multiple phytohormones (Wellmer et al., 2014). In particular, the biosynthesis, transport, and response of auxin often has an effect on the formation of floral organs, and helps to establish the largely variable arrangement of the different floral whorls (Lampugnani et al., 2013). In our datasets, the DEGs related to AUX biosynthesis and signalling were suggested to have a key role in this activity. BR plays a major role in promoting petal growth with cell expansion and lengthening (Huang et al., 2017). CK related genes regulate reproductive development by delaying cellular differentiation and promoting the abnormal development of flowers (Isabel et al., 2011). In both the GO enrichment and Mapman pathway identification, numerous significant DEGs participated in cellular processes, which suggested the potential involvement of BR and CK in the regulation of cell development for the growth of petal-like tissues. In addition, other hormonal activities are associated with the expression of floral organ identity genes as well,

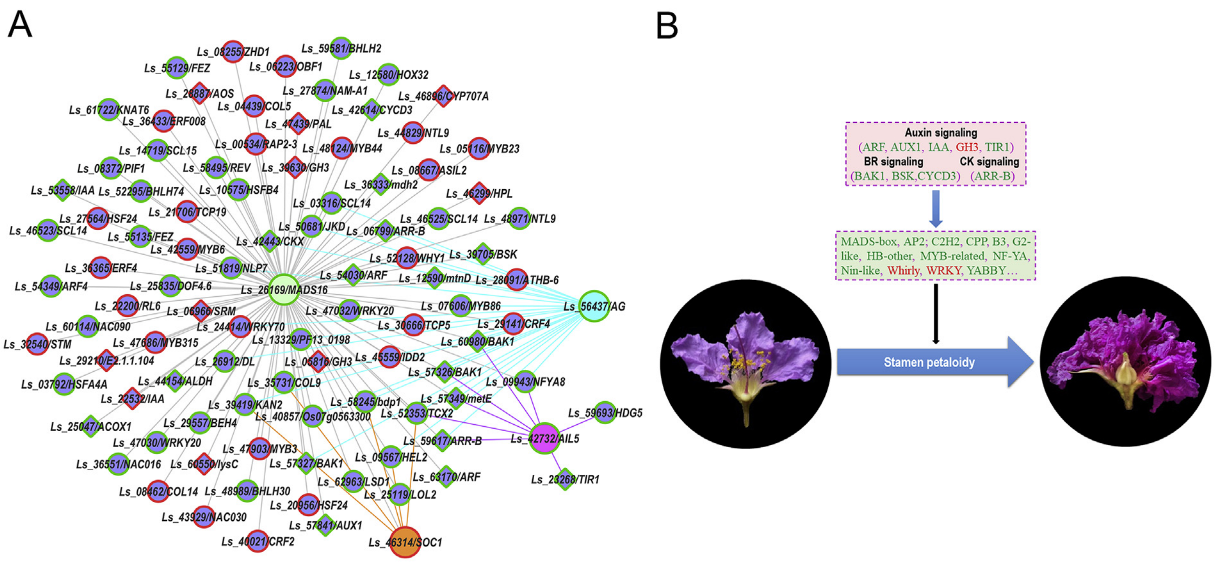

Fig. 6. Prediction of conserved transcriptional regulation of stamen petaloidy in *L. speciosa*. **(A)** DEGs highly co-expressed with floral homeotic genes Ls_26169/*MADS16*, Ls_42732/*AIL5*, Ls_46314/*SOC1* and Ls_56437/*AG* in module brown overlapping in three comparisons. The edges of their co-expressed genes are divided into different colours. The diamond node represents the gene involved in phytohormones while the circular node annotates those involved in TF. The green border of the nodes represents downregulation while red represents up regulation of the corresponding gene. The gene label ID corresponds to its unigene ID. **(B)** Summary of candidate DEGs involved in plant hormone signals and TFs regulating stamen petaloidy in double-flower *L. speciosa*. Genes were either upregulated (red) or downregulated (green) in this conversion. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

including AG with JA synthesis, which are necessary in anther development and petal maturation (Ito et al., 2007). In our results, the synthesis of hydroperoxide dehydratase (*AOS*) and hydroperoxide lyase (*HPL*) together contributed to JA metabolism, probably resulting in the maturation of petal-like stamens.

Some members of the TF families, including MYB, NAC, WRKY, bHLH and DBB, showed drastic expression difference between the two flower bud samples, suggesting their potential regulation of floral organ development in *L. speciosa*. MYB has a crucial responsibility during floral organ and pollen development in multiple species, including rice, cotton and *Arabidopsis* (Miyuki et al., 2004; Xu et al., 2014). NAC participates in floral-boundary morphogenesis in tomato and ethylene-regulated cell expansion in petals (Anat et al., 2013; Haixia et al., 2013). AP2/ERF coding genes showed high expression throughout the rapid enlargement stage before bud blooming, thus promoting petal development for late blooming (Tuan et al., 2016). bHLH and bZIP proteins bind together with G-box (Sablowski, 2010). The bHLH TFs might mediate organ, region and flower type specific signals in the inflorescence of *L. speciosa* (Elomaa et al., 2010). B-box zinc finger protein (DBB, DOUBLE B-BOX) genes are commonly implicated in light signal transduction during early photomorphogenesis. The expression of DBB family genes might be regulated by the circadian rhythm and thus have an effect on floral morphogenesis (Kumagai et al., 2008), but further investigations are needed. The C2H2 family genes coding for zinc finger proteins might regulate floral organ identity and the development of basal pattern elements of gynoecium (Payne et al., 2004). In contrast, the functions of other TFs in floral organ development are unclear although we know that they are involved in flower development, including TCX2 (Andersen et al., 2007) and YABBY (Gross et al., 2018), which may be responsible for both male and female reproductive development. These TFs may connect the signalling from upstream to the functional downstream genes during the stamen determination of *L. speciosa*. The differential regulation of members from certain families in the two flower buds lead to different regulatory networks that can determine the differentiation in floral organ development. The homologs of some TFs had opposing expression differentiation during flower organ development, indicating that their development was regulated by a complex transcriptional network.

We discovered transcriptional modules to investigate candidate genes with decisive regulation of stamen petaloidy in double flowers. Only in module brown, the remarkable and conserved difference in eigenvalue between S and D suggests a vital function of the genes included in this variation. The functions of MADS-box genes along with AP2 family members are of high interest due to their key regulation of floral organ identity. These regulators have persistent expression throughout floral organ development including early determination (Bowman et al., 1991). Multiple processes are related to these genes within floral organ morphogenesis (Ito et al., 2007; Wuest et al., 2012). Four members of floral homeotic protein coding genes in our study (*MADS16*, Unigene0026169; *AP2*, Unigene0042732; *SOC1*, Unigene0046314; *AG*, Unigene0056437) are likely to play a vital role in regulating the differentiation of stamens with prolonged downregulated expression. Using these as hubs, we discovered the key co-expressed genes that are supposed to determine differential floral organ development at a transcriptional level, specifically the stamen petaloidy. These data provide a basis to study the regulation of these key genes involved in plant hormone signals and TFs within the whole period of floral organ development. More comprehensive relationships with other genes and functional studies on the individuals in the co-expression network are required to elucidate a thorough regulatory picture of the candidate genes. However, it should be mentioned that as this analysis has been conducted with whole flower buds, the expression of any floral organ has a possible contribution to differential regulation. Further spatial and temporal verification of gene expression is needed, which should reflect the systematic regulatory network.

Author contributions

LH, TZ and MC conceived and designed the research. LH performed the entire study and prepared the first draft of the manuscript. TZ supervised the research and provided edits on the manuscript. MC discovered the original double-flower individual and assisted in data analysis; HP provided comments and edits on the proposal and manuscript; JW took charged of field sampling and cooperation with RNA-seq company; QZ acquired funding, supervised the research, and provided comments on the manuscript.

Acknowledgments

The research was supported by the program for Science and Technology of Beijing (No. Z181100002418006), and Special Fund for Beijing Common Construction Project. The authors would like to thank Mao Lin, Kaidao Sun and Bing Li in Guangxi Forestry Research Institute (Nanning, China) for the assistance with sample collection.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.08.012.

Conflicts of interest

The authors have declared that no competing interests exist.

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