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Transcriptome Sequencing Analysis of Flowering Related Genes in *Prunus sibirica*

Chen Chen, Xu Wanyu, Zhu Gaopu, Zhao Han, Liu Huimin, Wang Lin ✉, Wuyun Tana ✉
Non-timber Forest Research and Development Center, Chinese Academy of Forestry, Paulownia Research and Development Center of China, National Forestry and Grassland Administration, Key Laboratory of Non-timber Forest Germplasm Enhancement and Utilization of National Forestry and Grassland Administration, Zhengzhou, 450003, China
✉ Corresponding author email: wanglin1815@163.com; tanatanan@163.com
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Abstract In order to explore the molecular mechanism of regulating flowering in *Prunus sibirica*, the transcriptome of flower buds at two different stages was sequenced to investigate the candidate genes involved in the flowering. A total of 42.04 Gb clean data were obtained, and more than 92.00% of the clean reads were mapped to the reference genome. A total of 6 850 significantly differentially expressed genes (DEGs) were found, including 2 784 significantly up-regulated and 4 066 significantly down regulated genes. We further found 392 and 346 DEGs were special in sprouting stage and full-bloom stage, respectively. KEGG enrichment analysis showed that the first three DEGs were enriched in plant hormone signal transduction, phenylpropanoid biosynthesis, and starch and sucrose metabolism, respectively. Among these DEGs, 39 genes involved in the flowering regulation pathways were obtained, including 10, 17, 5, 1, and 2 genes in vernalization pathway, photoperiod pathway, autonomous pathway, gibberellin pathway, and temperature pathway, respectively. In addition, four integrators, including *SOC1*, *FT*, and *LFY*, were also identified. This study will provide the valuable information for studying the candidate genes involved in the flowering and for breeding in *P. sibirica*.
Keywords *Prunus sibirica*; Flowering regulation; Differentially expressed genes; Transcriptome

Siberian apricot (*Prunus sibirica*) belongs to *Armeniaca* genus of the subfamily Prunoideae in the family of Rosaceae (Zhang and Zhang, 2003), which is an important eco-economic tree species. *Prunus sibirica* is an important afforestation tree species with strong adaptability, cold resistance, drought resistance and sand resistance. It is widely distributed in northern and northeastern China, Russia and Mongolia (Wang and Yu, 2012). Almond protein of *Prunus sibirica* contains eighteen kinds of amino acids needed by human body (Yin et al., 2019), and almond oil contains high unsaturated fatty acids (Jiang et al., 2014). Due to the early flowering stage of *Prunus sibirica*, it is vulnerable to the "late spring coldness" in spring, resulting in a decline in yield (Song, 2011). At present, the traditional methods of anti-freezing measures at flowering stage, such as spraying water at flowering stage, cold-resistant agent treatment, smoking, and stem whitening, have been reported (Wei et al., 2008). However, with the increasing attention to *Prunus sibirica*, it is particularly important to cultivate excellent varieties of *Prunus sibirica* with late flowering and/or resistance to "late spring coldness".

The flowering process of plants is complex, which is affected by genetic factors and environment (Sun et al., 2007). Studies have shown that the five main pathways involved in the regulation of flowering in *A. thaliana* are vernalization, photoperiod, temperature, autonomous, gibberellin and age. These regulatory pathways are independent and interrelated to each other, jointly form a gene regulatory network for flowering (Srikanth and Schmid, 2011; Qi et al., 2018). FLOWERING LOCUS T (*FT*), SUPPRESSOR OF OVEREXPRESSION OF CO 1 (*SOC1*) and LEAFY (*LFY*) are the three important integration genes of Arabidopsis flowering signal, located in the cross position of multiple pathways, integrating signals from different pathways (Zhang, 2014; Yuan et al., 2017). At present, there are relatively few studies on flowering-related genes of *Prunus sibirica*. Wang et al. (2018) speculated that the expression of hormone synthesis and biological rhythm-related genes was related to flowering

through the analysis of flower bud expression of *Prunus sibirica* at different flowering stages. Transcriptome sequencing is one of the effective methods for studying differentially expressed genes. Therefore, in this study, transcriptome sequencing of flower bud samples from two different periods of *Prunus sibirica* was carried out to excavate differentially expressed genes and screen candidate genes related to flowering of *Prunus sibirica*, to provide theoretical basis for flowering regulation and directional cultivation of late flowering varieties of *Prunus sibirica*.

1 Results and Analysis

1.1 Analysis of the statistics of transcriptome database

Transcriptome sequencing of flower bud samples from two different periods of *P. sibirica* (Table 1) was carried out, a total of 292 851 358 raw reads and 43.93 Gb bases were obtained. After filtering, a total of 280 254 260 clean reads and 42.04 Gb bases were obtained, and the average amount of data per sample was 7.01 Gb. The accuracy of the filtered sequence is more than 99.9% and the base is more than 94%, which can be further used to compare the reference genome for further analysis.

Table 1 The statistics of transcriptome database in *P. sibirica*

Sample	Raw reads number	Raw bases number	Clean reads number	Clean bases number	Clean Q30 bases rate (%)
FA1	47 624 366	7 143 654 900	45 999 554	6 899 933 100	93.52
FA2	50 717 396	7 607 609 400	47 591 610	7 138 741 500	94.52
FA3	48 039 466	7 205 919 900	45 518 052	6 827 707 800	94.52
FB1	48 114 976	7 217 246 400	46 379 560	6 956 934 000	94.10
FB2	49 663 828	7 449 574 200	47 948 744	7 192 311 600	94.44
FB3	48 691 326	7 303 698 900	46 816 740	7 022 511 000	94.32

Note: FA: Germination period; FB: Blooming; 1-3: Biological replicates

The filtered sequences were aligned and mapped with the reference genome of *P. sibirica* (Figure 1). The results showed that more than 92.0% of the high-quality sequences could be localized, and the average alignment rate was 95.60%, indicating that the reference genome was suitable, and the sequencing samples had no exogenous species pollution. Furthermore, the distribution location of the sequence of the unique alignment genome in the genome region was analyzed, and the results showed that more than 92.00% of the data could be compared to the exon region, and only a small number of sequences were distributed in the intron region (2%~4%) and the intergenic region (3%~4%).

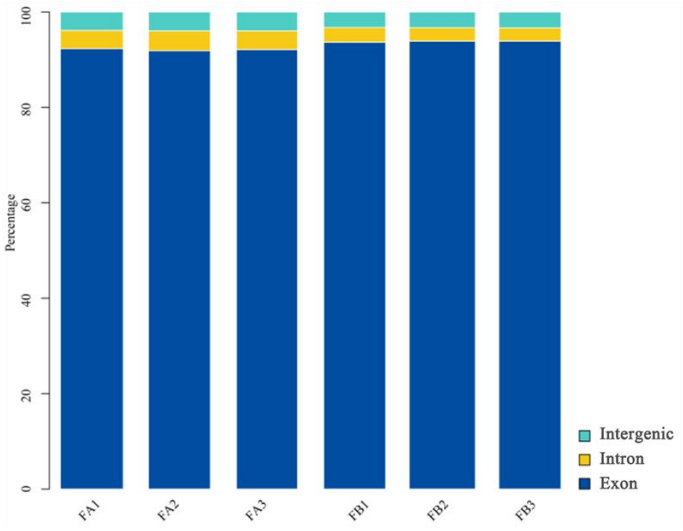

Figure 1 The distribution of transcriptome database in *P. sibirica* genome

1.2 Analysis of differentially expressed genes in flower buds at different stages

1.2.1 Pattern analysis of differentially expressed genes

A total of 24 785 differentially expressed genes (DEGs) were identified from flower bud transcriptome of *P. sibirica* at two different developmental stages. The volcano map of DEGs (Figure 2) showed that most of the DEGs were distributed between 1 and 4 of the |log2 (fold change)|, and a few of the DEGs with the |log2 (fold change)| of greater than 5. Further screening of these DEGs showed that a total of 6 850 significantly DEGs were found, including 2 784 significantly up-regulated and 4 066 significantly down-regulated in sprouting stage. And we found that 392 and 346 DEGs were special in sprouting stage and full-bloom stage, respectively.

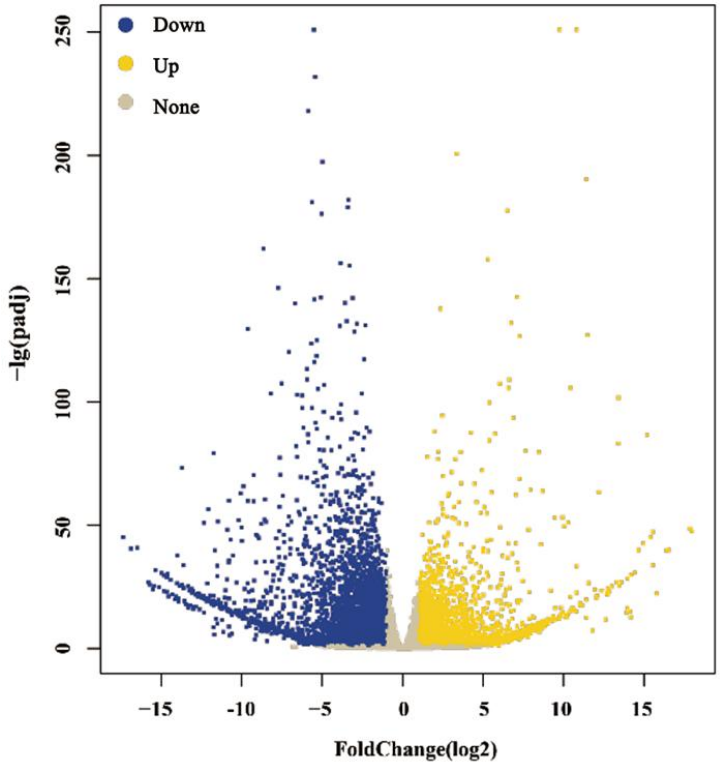

Figure 2 The volcano map of differentially expressed genes

1.2.2 Functional annotation (KEGG) of differentially expressed genes

In order to determine the function of flowering related genes, the DEGs were annotated by KEGG (Figure 3). The results showed that a total of 613 DEGs were enriched to 23 KEGGs, the most of which included plant hormone signal transduction with a total of 87 DEGs, accounting for 14.19%; Phenylpropanoid biosynthesis with a total of 72 DEGs, accounting for 11.75%; Starch and sucrose metabolism with a total of 56 DEGs, accounting for 9.14%. It is speculated that the above pathways play an important role in regulating the flowering period of *P. sibirica*.

1.2.3 GO classification of differentially expressed genes

GO classification was used to annotate the differentially expressed genes. The DEGs can be divided into three categories: biological process, cellular process and molecular function, and 54 subcategories (Figure 4). In the biological process category, it can be divided into 24 subcategories, and the proportion of up-regulated and down-regulated differentially expressed genes in cellular process is the highest. The category cellular process can be divided into 16 subcategories, among which the proportion of DEGs in cell part is the highest. In category molecular function, the obtained DEGs can be divided into 14 subcategories, among which the proportion of DEGs in binding is the highest.

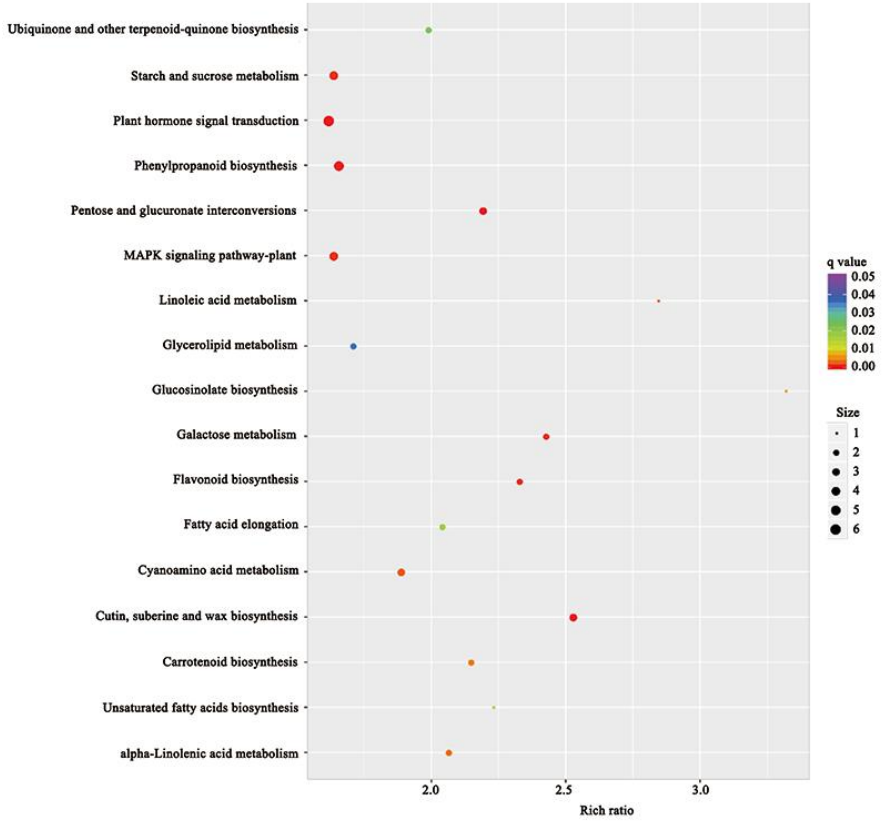

Figure 3 KEGG metabolic pathway of differentially expressed genes

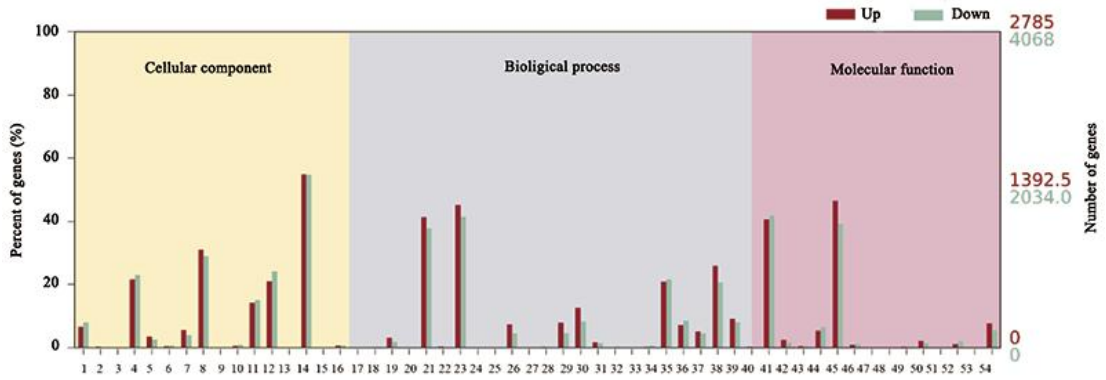

Figure 4 GO classification of differentially expressed genes

Note: 1: Extracellular region; 2: Cell; 3: Nucleoid; 4: Membrane; 5: Cell junction; 6: Membrane-enclosed lumen; 7: Macromolecular complex; 8: Organelle; 9: Other organism part; 10: Extracellular region part; 11: Organelle part; 12: Membrane part; 13: Synapse part; 14: Cell part; 15: Supramolecular complex; 16: Synapse; 17: Reproduction; 18: Cell killing; 19: Immune system process; 20: Behavior; 21: Metabolic process; 22:Cell proliferation; 23:Cellular process; 24:Carbon utilization; 25: Nitrogen utilization; 26: Reproductive process; 27: Biological adhesion; 28: Signaling; 29: Multicellular organismal process; 30: Developmental process; 31: Growth; 32: Locomotion; 33: Pigmentation; 34: Rhythmic process; 35: Response to stimulus; 36: Localization; 37: Multi-organism process; 38: Biological regulation; 39: Cellular component organization or biogenesis; 40: Detoxification; 41: Catalytic activity; 42: Signal transducer activity; 43: Structural molecule activity; 44: Transporter activity; 45: Binding; 46: Antioxidant activity; 47: Protein tag; 48: Translation regulator activity; 49: Nutrient reservoir activity; 50: Molecular transducer activity; 51: Toxin activity; 52: Molecular function regulator; 53: Molecular carrier activity; 54: Transcription regulator activity

1.3 Analysis of flowering related genes in *Prunus sibirica*

A total of 39 flowering pathway-related DEGs were screened from the flower bud transcriptome at two different stages of *Prunus sibirica* (Table 2), including 7 related DEGs involved in vernalization pathway (*VRN1* (*VERNALIZATION 1*)), all up-regulated in FA period compared with FB period; 1 *VIN3* (*VERNALIZATION*

INSENSITIVE 3) and *FRI* (*FRIGIDA*), respectively, were down-regulated, and 1 *FLC* (*FLOWERING LOCUS C*) was up-regulated. In autonomous pathway genes, a total of 4 *FCA* (*FLOWERING CONTROL LOCUS A*) genes and 1 *FY* gene were up-regulated. In photoperiod pathway genes, 1 *PHYA* (*PHYTOCHROMES A*) gene was up-regulated, 1 *ELF3* (*EARLY FLOWERING 3*), *KFK1* (*FLAVIN-BINDING KELCH REPEAT F-BOX 1*) and *LHY*(*LATE ELONGATED HYPOCOTYL*), respectively, were down-regulated, 1 *PIF* (*PHYTOCHROME INTERACTING FACTOR*)、*FD*(*FLOWERING LOCUS D*)、*NF-Y*(*NUCLEAR FACTOR Y*) respectively, were up-regulated. There were 7 *CO* (*CONSTANS*) genes, in which 6 genes were down-regulated. A total of 3 *COP1* (*CONSTITUTIVELY PHOTOMORPHOGENIC 1*) genes were down-regulated. In gibberellin pathway genes, *GAI* (*GIBBERELLIC ACID INSENSITIVE*) gene was down-regulated. In temperature pathway genes, a total of 2 *SVP* (*SHORT VEGETATIVE PHASE*)genes were up-regulated. One flowering-related integration factor *FT* and one *LFY* gene, and 2 *SOC1* genes were up-regulated.

Table 2 Analysis of flowering related genes in *Prunus sibirica*

Pathway	Gene name	Gene number	Log2Fold Change	Expression pattern
Vernalization pathway	*VRN1*	PaF106G0102070100.01	2.35	Up regulation
		PaF106G0201672600.01	2.00	Up regulation
		PaF106G0302624700.01	1.31	Up regulation
		PaF106G0705228000.01	1.64	Up regulation
		PaF106G0705462700.01	2.21	Up regulation
		PaF106G0705477300.01	2.51	Up regulation
		PaF106G0806086400.01	3.08	Up regulation
	VIN3	PaF106G0100185000.01	-1.37	Down regulation
	FLC	PaF106G0302119700.01	2.03	Up regulation
	FRI	PaF106G0604870000.01	-2.02	Down regulation
Autonomous pathway	*FCA*	PaF106G0202701800.01	1.47	Up regulation
		PaF106G0505946700.01	2.52	Up regulation
		PaF106G0505995000.01	1.72	Up regulation
		PaF106G0809510300.01	1.49	Up regulation
	FY	PaF106G0201276000.01	1.04	Up regulation
Photoperiod pathway	*PHYA*	PaF106G0604806300.01	1.46	Up regulation
	ELF3	PaF106G0302685900.01	-1.26	Down regulation
	KFK1	PaF106G0100756800.01	-1.17	Down regulation
	PIF	PaF106G0302394200.01	1.22	Up regulation
	LHY	PaF106G0201823200.01	-5.92	Down regulation
	CO	PaF106G0100204300.01	-1.68	Down regulation
		PaF106G0202060300.01	2.52	Up regulation
		PaF106G0202629300.01	-2.88	Down regulation
		PaF106G0302309800.01	-6.38	Down regulation
		PaF106G0302360000.01	-1.30	Up regulation
		PaF106G0604216800.01	-2.83	Down regulation
		PaF106G0604297000.01	-1.46	Down regulation
	COP1	PaF106G0202021100.01	-3.14	Down regulation
		PaF106G0403474200.01	-2.25	Down regulation
		PaF106G0503637400.01	-1.03	Down regulation
	bZIP(FD)	PaF106G0101404500.01	2.09	Up regulation
	NF-Y	PaF106G0705088200.01	1.01	Up regulation
Temperature pathway	*SVP*	PaF106G0806097200.01	2.88	Up regulation
		PaF106G0604585100.01	2.47	Up regulation
Gibberellin pathway	*GAI*	PaF106G0302116300.01	-7.91	Down regulation
Integration factor	*SOC1*	PaF106G0202010400.01	2.14	Up regulation
		PaF106G0503901800.01	1.17	Up regulation
	FT	PaF106G0604935800.01	1.40	Up regulation
	LFY	PaF106G0503884500.01	3.84	Up regulation

1.4 The statistics analysis of SNP and InDel sites

1.4.1 The statistics of SNP and InDel sites

After comparing the filtered high quality transcriptome data to the reference genome, SNP and InDel sites information were analyzed, respectively (Figure 5). The results showed that a total of 564 069 SNP sites, 53 253 homo sites and 510 816 hete sites were found. There were significant differences in the number of SNP sites among different samples. In which, FB2 samples had the most SNP sites with the number of 100 383, and FA2 had the least SNP sites of 89 269. Further statistics of Indel sites showed that there were 106 120 Indel sites, of which 14 790 homo sites, and 91 330 hete sites. Among them, the FB2 sample had the most Indel sites of 19 214, and the FA3 sample had the least Indel sites of 16 486.

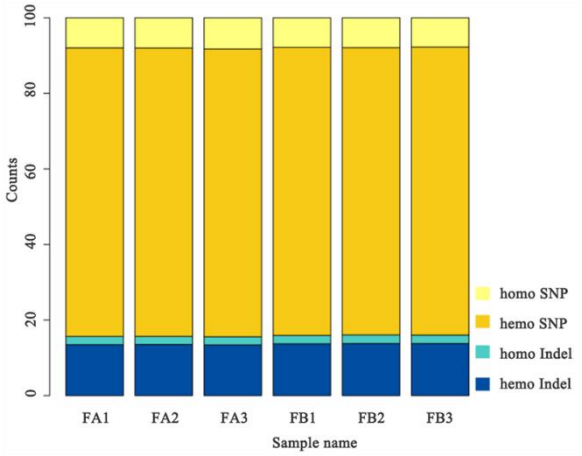

Figure 5 The statistics of SNP and InDel sites

1.4.2 The distribution of SNP and InDel

The statistical distribution of SNP and Indel was found to be distributed in 12 regions of the genome (Figure 6). SNP sites were most distributed in exon region, accounting for more than 40%, followed by intron region (17%~23%), 3'-untranslated region (UTR3) (13%~14%), 5'-untranslated region (UTR5) (8%~9%). And the InDel sites were most distributed in intron region, accounting for more than 20%, followed by exon region (18%~19%), UTR3 (17%~18%), UTR5 (17%~18%). SNP and InDel sites were less distributed in other regions.

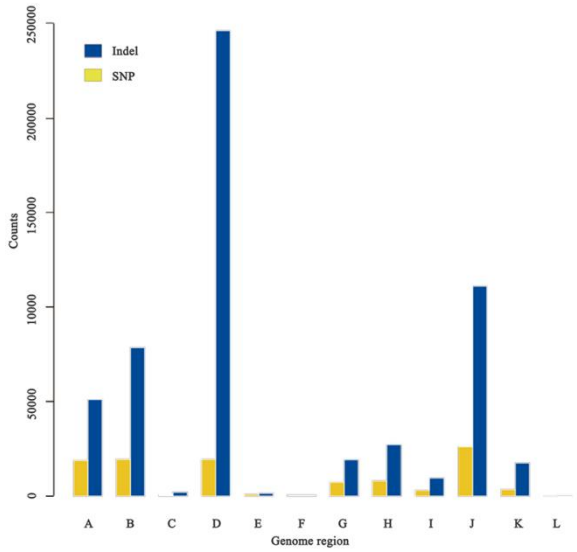

Figure 6 The distribution of SNP/InDel

Note: A: 5'-untranslated region of gene (UTR5); B: 3'-untranslated region of gene(UTR3); C: Overlap of 5' (UTR5)and 3' (UTR3)untranslated regions of genes; D: Exon region ; E: Splicing region; F: Exon adjacent to the splice site 2 bp region Exonic; G: The region upstream of the gene (1 000 bp) ; H: The region downstream of the gene (1 000 bp); I: The region upstream or downstream of a gene(1 000 bp) ; J: Intron region; K: Intergenic region ; L: Non-coding RNA exon region

1.4.3 Statistical analysis of SNP types

SNP sites can be divided into two types, that is, transitions (A/G and C/T) and transversions (A/C, A/T, C/G and G/T). In this study, the statistical analysis of obtained SNP genotypes was performed (Figure 7), and a total of 337 025 transitions (A/G and C/T) and 227 044 transversions (A/C, A/T, C/G and G/T) were found. The genotype with the smallest number was C/G (48 867), and the largest number was C/T (168 920).

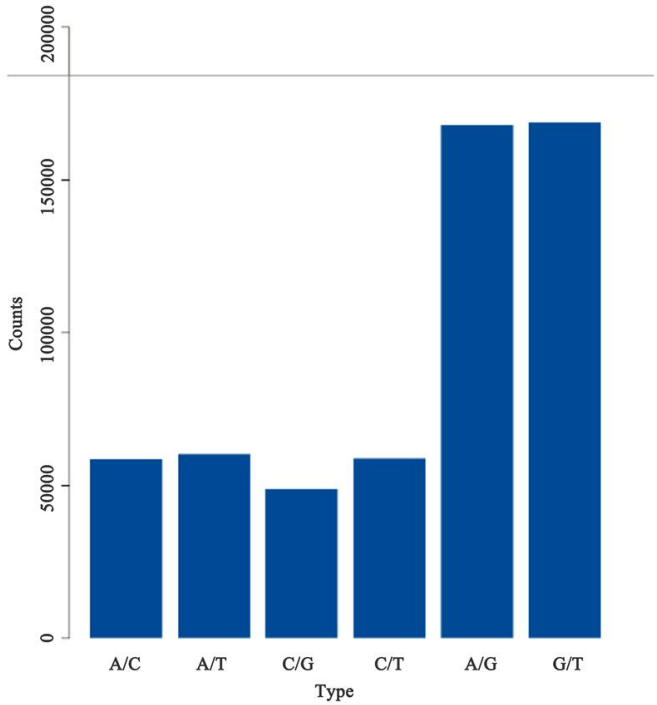

Figure 7 The statistics of transitions and transversions

2 Discussion

In this study, transcriptome sequencing was performed on flower bud samples from two different periods in *Prunus sibirica*. A total of 39 differentially expressed genes involved in the flowering regulation pathways were obtained, including 10, 5, 2, 1, 16 and 1 genes in vernalization pathway, autonomous pathway, temperature pathway, photoperiod pathway, biorhythm pathway, and gibberellin pathway, respectively. In addition, four integrators were also identified.

The vernalization pathway affects the flowering of plants by sensing the low temperature in the environment. Among them, *FLC* gene is an important flowering inhibitory gene in vernalization and autonomous pathways. In Arabidopsis, the expression of *FLC* gene promotes the growth of rosette leaves and inhibits the development of flower buds, thereby delaying flowering (Michaels and Amasino, 1999; Rouse et al., 2002). In this study, a *FLC* gene was annotated and down-regulated in full-bloom stage, indicating that *FLC* gene played a negative regulatory role in the flowering of *Prunus sibirica*, and its expression may affect the flowering of *Prunus sibirica*. In addition, in the vernalization pathway, *VRN1* and *VRN3* genes were screened out, but no *VIN2* gene. *VIN3* gene was up-regulated at full-bloom stage, while *VRN1* was down-regulated, indicating that *VIN3* gene may be involved in regulating the flowering process of *Prunus sibirica*. *VIN3* gene inhibits the expression of downstream *FLC* gene by up-regulating expression, thus promoting the flowering process. Duration of sunshine is one of the important factors affecting flowering of plants. *CO* gene is at the core of photoperiod pathway and biological rhythm pathway, which can transform light signal into flowering signal, affecting flowering time of plants (Suarez-lopez et al., 2001; Imaizumi and Kay., 2006). In Arabidopsis, *CO* gene affects flowering by regulating *FT* and *SOC1* genes, and increasing *CO* gene expression can promote flowering (Yoo et al., 2005). In this study, a total of seven *CO* genes were obtained, of which six *CO* genes were up-regulated at full-bloom stage, indicating that *CO* has a positive regulatory role in the flowering process of *Prunus sibirica*. Transcriptome data showed that

one *LFY* gene was up-regulated at full-bloom stage of *Prunus sibirica*, while *FT* and *SOC1* genes were down-regulated. As the key crossing point of multiple flowering pathways, the expression level is affected by many factors. Therefore, the regulatory mechanism of integration factor expression in the flowering process of *Prunus sibirica* needs further study. Plant flowering is affected by temperature, which is generally promoted by high temperature and inhibited by low temperature. Therefore, temperature pathway is of great significance to plant flowering (Qi et al., 2018). *SVP* gene is an important influencing factor of temperature pathway. In addition, *SVP* gene regulates plant flowering through autonomous and gibberellin pathway (Lee et al., 2007). The results showed that the *SVP* inhibits plant flowering by mediating the expression of *FT* and *SCO1* genes in Arabidopsis (Li et al., 2010; Li et al., 2019). Similarly, the expression levels of two *SVP* genes (*PmSVP1* and *PmSVP2*) in *Prunus mume* showed a downward trend during flower bud differentiation (Li et al., 2017). The expression of *SVP* gene was significantly down-regulated at full-bloom stage, suggesting that the expression of *SVP* gene plays a negative regulatory role in flowering of *Prunus sibirica*.

In addition, a total of 564 069 SNPs and 106 120 Indels were identified in the flower bud transcriptome data at two different stages in this study. Among them, SNP sites were most distributed in exon region (>40%), while InDel sites were most distributed in intron region (>20%). The development of these sites can be used for the identification of late flower resources of *Prunus sibirica*, and provide auxiliary technology for the cultivation of late flower varieties.

3 Materials and Methods

3.1 Research materials

The research materials were taken from Yuanyang Experimental Base of Paulownia Research and Development Center of China, National Forestry and Grassland Administration. *Prunus sibirica* ('F106') with good growth and normal flowering and fruiting was selected as the experimental materials. The samples of sprouting stage (FA) and full-bloom stage (FB) were collected respectively. Each sample was repeated three times, and stored after quick freezing with liquid nitrogen.

3.2 Library construction and sequencing for transcriptome

The total RNA of samples was extracted by phenol/chloroform method and diluted in proportion. The purity, concentration and integrity of samples were detected by micro spectrophotometer (NanoDrop 2000) and Agilent (2100 Bioanalyze, RNA 6000 Nano Kit), respectively. Library for different samples was constructed according to the instructions of NEBNext® Ultra™ RNA Library Prep Kit for Illumina®. After passing the library inspection, the raw data was obtained by sequencing.

3.3 Filtering and alignment (Reference genome)

The raw data was filtered to clean data. Bowtie (Langmead et al., 2009) was used for library construction of reference genome, and then clean data was compared to the reference genome by HISAT2 (Kim et al., 2015).

3.4 Screening and functional annotation of differentially expressed genes

Method of Fragments per Kilobase per Million Mapped Fragments (FPKM) was used to calculate gene expression levels. The differentially expressed genes in two samples of different periods were analyzed by DESeq2 (Love et al., 2014), and the main reference indexes for screening differentially expressed genes were |log2 Fold change|≥2 and $q<0.05$. The databases of EGG and GO were compared to obtain annotation information of differentially expressed genes.

3.5 Analysis of SNP and Indel

The SNP sites were searched by bcftool (Li et al., 2009). After sorting and filtering, the sequences were compared to the reference genome sequence, and the mutation detection results of each sample were obtained.

Authors' contributions

WYTN, WL and CC are the executors of the experimental design and research in this study. CC completed the data analysis and drafted the manuscript. XWY, ZGP, ZH, and LHM participated in the experimental design, experimental results analysis. WYTN and WL are the designers and directors of the project, guiding experimental design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

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