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Identification of key genes related to flowering by transcriptome of flowering and nonflowering *Prunella vulgaris*

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Abstract

Prunella vulgaris L. have high medicinal and ornamental values. It blooms in summer and then quickly withers, and there is no research on the regulatory mechanisms of flowering-related genes. Therefore, in this study, the flowering (leaves and spicas) and nonflowering (leaves) parts of *P. vulgaris* were sequenced with an *Illumina HiSeq 4000*, and 187 387 transcripts were obtained using *Trinity* software package. A total of 10 158 differentially expressed genes (DEGs) were found in the leaves of flowering and nonflowering *P. vulgaris*, with 6 294 upregulated genes and 3 864 downregulated genes. DEGs in leaves of flowering and nonflowering *P. vulgaris* were mainly annotated for metabolic processes (4 207) in *Gene Ontology (GO)* and ribosomal pathways (416) in *Kyoto Encyclopedia of Genes and Genomes (KEGG)*. Screening of this set of genes yielded 50 flowering-related unigenes homologous to *Arabidopsis* genes involved in multiple regulatory pathways related to plant flowering, including autonomous, vernalization, gibberellin, and age pathways. In addition, there are significant differences in the expressions of genes related to flowering, such as *PvFLC*, *PvSOC1*, and *PvFY*, as well as genes involved in plant hormone signal transduction and sugar metabolism. The accuracy and reliability of the transcriptome results were verified by quantitative real-time RT-qPCR analysis of *PvGA20OX*, *PvSVP*, *PvELF3*, *PvCRY1*, and *PvSOC1*. Thus, we speculate that the flowering of *P. vulgaris* is regulated by certain genes related to the flowering regulation pathway and sugar and hormone metabolism pathways. Analysis of the *P. vulgaris* transcriptome will provide a basis for revealing its flowering mechanism.

Keywords: *Prunella vulgaris*, transcriptome, differentially expressed genes, flowering-related genes, RT-qPCR.

Introduction

Prunella vulgaris L. is known as ‘Xiaku-cao’, because it withers and dies after the summer solstice (Chen *et al.* 2013). It is a perennial herb in the family *Lamiaceae*, and its dried spicas are used in medicine (Liao *et al.* 2012). The Pharmacopoeia of the People’s Republic of China (2020) states that *P. vulgaris* has drug efficacy in removing liver-fire for improving eyesight, subsiding swelling to dissipate indurated mass. Modern research shows that *P. vulgaris* contains a variety of chemical constituents, such as phenylpropanoids, flavonoids, triterpenes, organic acids, sugars, coumarins, and steroids (Bai *et al.* 2016). Studies show that *P. vulgaris* exerts antitumour (Feng *et al.* 2010), anti-inflammatory (Hwang *et al.* 2013), and hypoglycaemic activity (Raafat *et al.* 2016). In addition, the use of *P. vulgaris* in herbal tea has been continuously developed, resulting in high demand. Flowering *P. vulgaris*

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Abbreviations: A-S-L - leaf of *P. vulgaris* sown in autumn; A-S-S - spicas of *P. vulgaris* sown in autumn; *BUSCO* - Benchmarking Universal Single-Copy Orthologs; *GO* - Gene Ontology; GC - numbers of bases G and C in the total number of bases; *KEGG* - Kyoto Encyclopedia of Genes and Genomes; *COG/KOG* - Clusters of Orthologous Groups of proteins; Ref - reference sequence; *Nr* - non-redundant protein sequences; *Nt* - nucleotide sequences; *Pfam* - protein family; Q20 - rate of bases where the quality is greater than 20; Q30 - the rate of bases where the quality is greater than 30; real time RT-qPCR - real-time reverse transcription quantitative PCR; S-S-L - leaf of *P. vulgaris* sown in spring; *SWISS-PROT* - protein sequence database.
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has seasonal ornamental value in southern Chinese cities.

A previous study found that flowering is a complex process that includes flower induction, flower initiation, flower differentiation, and flowering stages. It is believed that the induction of flowers is triggered by environmental and endogenous stimuli, and its mechanism has been thoroughly studied in model plants (Liu *et al.* 2020b). Studies have found that the formation of floral organs is affected by a variety of hormones, of which auxin plays an important role in general floral-organ formation (Huang *et al.* 2016) and the arrangement of different flower wheel patterns (Lampugnani *et al.* 2013). Gibberellin (GA) and cytokinin (CK) affect inflorescence formation by regulating cell proliferation and differentiation (Liu *et al.* 2020a). Furthermore, hormones can regulate flowering by inducing the expression of flowering genes (Guan *et al.* 2019).

During flower development, the expression of genes involved in hormone signalling and in carbon and nitrogen metabolism show obvious differences (Wang *et al.* 2019). Koch *et al.* (2000) and Mornya *et al.* (2011) believe that sugar may exist in the form of a certain signalling molecule, regulating plant growth, development and maturity in a manner similar to the action of plant hormones. Sugar signal transduction is also involved in the process of flowering regulation, with sucrose and trehalose-6-phosphate extensively studied and found to promote or delay the flowering of *Arabidopsis* (Ponnu *et al.* 2011, Tognetti *et al.* 2013, Wahl *et al.* 2013), and starch also has a role in this process (Yu *et al.* 2000, Seo *et al.* 2011). Furthermore, studies have found that carbon and nitrogen compounds affect plant flowering by regulating gene expression (Paparelli *et al.* 2013, Zhang *et al.* 2019).

Based on research with *Arabidopsis*, there are six pathways in the regulation of flowering, including the photoperiod, gibberellin, autonomous, temperature-sensitive, vernalization, and age pathways. These pathways are independent and cross-linked with each other, forming a flowering regulatory network with precise regulatory functions (Khan *et al.* 2014). For example, *GIGANTEA* (*GI*); *FLAVIN, KELCH REPEAT, F-BOX 1* (*FKF1*); *CONSTANS (CO*) and *FLOWERING LOCUS T* (*FT*) genes play important regulatory roles in the photoperiod pathway (Yeon *et al.* 2019). *CRYPTOCHROMES* (*CRY2*) inhibit the activity of *CONSTITUTIVELY PHOTOMORPHOGENIC 1* (*COP1*) and *SUPPRESSOR OF PHYA-105* (*SPA1*) at night so that the CO protein is not degraded and eventually functions to promote flowering (Zuo *et al.* 2011). In *Arabidopsis*, many genes that inhibit flowering and two conserved miRNAs, miR156 and miR172, play important roles in the regulation of flowering in the age dependent pathway (Wang *et al.* 2014). The results of transgenic experiments show that overexpression of miR156 maintains plants in an immature stage, while overexpression of miR172 leads to early flowering, which indicates that these two small RNAs play opposite roles (Yu *et al.* 2013). The flowering of plants is regulated by many genes.

Through three years of field experiments, it was found that *P. vulgaris* sown in autumn in the first year will flower the next summer but will not bloom in the same year when planted in the spring of the second year (Fig. 1 Suppl.). This phenomenon provides a favourable opportunity to explore the mechanisms of flowering regulation in *P. vulgaris.* Therefore, this study used RNA-seq technology to perform transcriptome analysis on flowering (leaves and spicas) and nonflowering (leaves) *P. vulgaris* plants in summer. The analysis of differentially expressed genes (DEGs) has great utility for the application of *P. vulgaris* flowering regulation. The mining of data on genes related to flowering regulation pathways and the analysis of genes related to sugar and hormone synthesis pathways can further elucidate the flowering mechanism of *P. vulgaris*. In addition, quantitative real-time RT-qPCR analysis of seven genes can also confirm transcriptome analysis results. Thus, our data may provide valuable resources to enhance the study of the flowering process in *P. vulgaris*, and at the same time provide the possibility to regulate the flowering of *P. vulgaris* to solve the problem of market supply and demand.

Materials and methods

Plants and cultivation: In this study, seeds from *Prunella vulgaris* L. purchased in Queshen County, Henan Province, in June 2018 were identified as mature *P. vulgaris* seeds by Prof. Qiaosheng Guo from the Institute of Chinese Medicinal Materials of Nanjing Agricultural University. The seeds were sown in the medicinal garden of Chengdu Medical College (latitude: 30°49'32.41" N, longitude: 104°12'7.3" E, altitude: 471 metres) on September 20, 2018 and March 10, 2019. Seedlings were grown naturally under the same conditions of fertilizer and water supply. In this experiment, the planting in September 2018 was called autumn sowing (flowering), and the planting in March 2019 was called spring sowing (nonflowering). Based on characterization of plant growth and development, the plants in the two sowing periods were defined as flowering plants and nonflowering plants (Fig. 1 Suppl.). On May 20, 2019, the leaves and spicas of autumn-sown *P. vulgaris* were picked and denoted as leaf of *P. vulgaris* sown in autumn (A-S-L) and spicas of *P. vulgaris* sown in autumn (A-S-S), respectively, and the leaves of *P. vulgaris* sown in spring in the blooming period were picked and denoted as leaf of *P. vulgaris* sown in spring (S-S-L). Samples were frozen in nitrogen for 5 min and transferred to a -80 °C refrigerator for future use.

RNA extraction: Total RNA was extracted from leaves and spicas using *TRIzol* (*Invitrogen*, Massachusetts, USA) and treated with RNase-free DNase I to remove residual DNA, absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} were detected using a *NanoPhotometer* spectrophotometer to determine RNA purity, and RNA integrity was accurately measured using an *Agilent 2100* (*Agilent*, Palo Alto, USA) bioanalyser. The RNA extracted from all samples was complete, with A_{260}/A_{280} ratios between 1.8 and 2.1 and an A_{260}/A_{230} ratio greater than 1.8. Three replicates were

completed for each sample. Results of measurements met the requirements for further analysis.

Library construction and sequencing: Oligo (dT) magnetic beads were used to enrich mRNA molecules with polyA tails in the total RNA meeting the requirements, and then the mRNA obtained was randomly interrupted with divalent cations in *NEB* fragmentation buffer. Ordinary library construction was performed according to the NEBNext Ultra RNA Library Prep Kit (*NEB*, Ipswich, MA, USA). Using fragmented mRNA as a template and random oligonucleotides as primers, the first strand of cDNA was synthesized in the *M-MuLV* (*NEB*) reverse transcriptase system, and then the RNA strand was degraded with *RNaseH*. The purified double-stranded cDNA was repaired at the ends, tails were added, and the sequencing adapter was connected to the system. cDNA of approximately 250 - 300 bp was screened with *AMPure XP* (*NEB*) beads, PCR amplification was performed, and the PCR product was purified again with *AMPure XP* beads to finally obtain the library.

After library construction was completed, a *Qubit2.0* (*ThermoFisher*, MA, USA) fluorometer was used for preliminary quantification, the cDNA library was diluted to 1.5 ng·mm^{-3}, and then the *Agilent 2100* bioanalyser was applied to detect the insert size of the library. After the insert size was confirmed at the value expected, RT-qPCR was used to determine the effective concentration of cDNA library. Accurate quantification (effective library concentration exceeded 2 nM) was performed to ensure library quality. After the library inspection was qualified, different libraries were pooled according to the requirements of effective concentration and target offline data volume for *Illumina* sequencing.

Data quality testing and unigene assembly and annotation: To ensure the quality and reliability of the data analysis, the original data were filtered, the sequence error rate was checked, and the GC content distribution was checked to obtain clean reads. Then, clean reads were stitched using *Trinity* (Grabherr *et al.* 2011). *BUSCO* (Benchmarking Universal Single-Copy Orthologs) software was used to evaluate the stitching quality of *Trinity FASTA*, *unigene.fa*, and cluster *FASTA* sequences obtained by stitching . To obtain comprehensive functional information for genes, the obtained transcripts were compared and annotated in different databases: Kyoto Encyclopedia of Genes and Genomes (*KEGG*), Clusters of Orthologous Groups of proteins (*COG/KOG*), non-redundant protein sequences (*Nr*), nucleotide sequences (*Nt*), protein family (*Pfam*), protein sequence database (*SWISS-PROT*). *BLASTx* and *Blast2GO* was used to annotate Gene ontology (*GO*).

Analysis of differentially expressed genes (DEGs): Using the transcript set obtained by *Trinity* as the reference sequence (Ref), clean reads of each sample were mapped to Ref, and reads with alignment quality values less than 10 were filtered out. In the comparison process, quantitative analysis of gene expression was performed on each sample by *RSEM* software (Li and Dewey 2011), and expressions were estimated using the most commonly used *FPKM*. Finally, using *DESeq2* software for analysis, if a gene's log2Foldchange > 1 and $p_{adj} < 0.05$, the differential gene was considered to be upregulated, and if log2Foldchange < 1 and $p_{adj} > 0.05$, the differential gene was considered to be downregulated (Love *et al.* 2014).

Real-time RT-qPCR validation: The total RNA of each sample was extracted with a *TaKaRa MiniBEST* plant RNA extraction kit (*Takara*, Dalian, China), and the mass and concentration of the total RNA were determined by 1.0 % (m/v) agarose gel electrophoresis and ultrafine spectrophotometry. Seven flowering genes were randomly selected, with *Actin* as the internal reference gene, and primers were designed by *Primer Premier 5.0*, as shown in Table 1 Suppl. The *PrimeScript™ RT Reagent* kit and the gDNA eraser (*Perfect Real Time*) kit (*Takara*) were used for the synthesis of cDNA. The cDNA was diluted 5 times, and PCR was performed with the *TB Green®Premix Ex Taq™ II* (*Tli RNaseH Plus*) kit (Takara, Dalian, China). Step One PlusTM real-time fluorescence quantitative PCR was applied to each 20 mm^3 of reaction system containing 10 mm^3 of 2×*TB Green Premix Ex Taq II*, 0.8 mm^3 of each upstream and downstream primer, 2 mm^3 of cDNA, 0.4 mm^3 of *ROX* reference dye (50×), and 6 mm^3 of ddH_2O. The RT-qPCR procedure was as follows: pre-denaturation at 95 °C for 30 s followed by 40 cycles of denaturation at 95 °C for 5 s, and annealing at 60 °C for 30 s, set to 3 biological repeats. The comparative cycle threshold ($2^{-\Delta\Delta Ct}$) was used to calculate the relative fold difference, and differences among values were tested using one-way *ANOVA* with Student's *t*-test at $P < 0.05$.

Result

A total of 470 681 244 raw reads were obtained from the transcriptome of *P. vulgaris*. After filtering out 2 679 659 (1.14 %) reads with adapters, 639 813 (0.28 %) reads with bases, and 288 997 (0.13 %) reads with low sequencing quality, 463 464 306 (98.45 %) clean reads were obtained. A total of 69.54 Gb of data were obtained through sequencing. The average error rate was 0.025. The average

Table 1. Distribution of stitching length.

Sort	Min. length	Mean length	Median length	Max length	N50	N90	Total nucleotides
Transcripts	301	1698	1352	18455	2494	819	318207776
Genes	301	1303	771	18455	2147	516	75320896

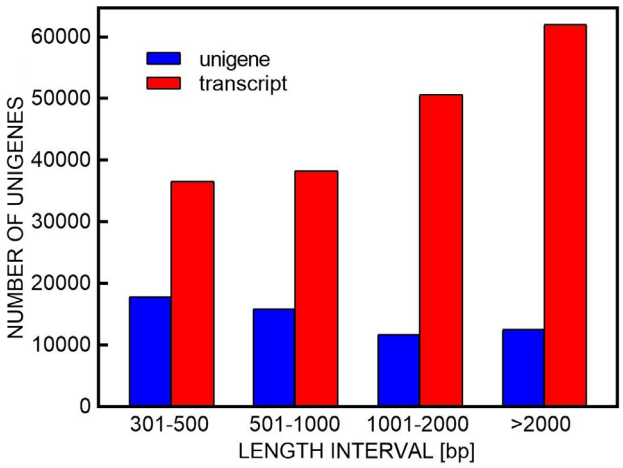

Fig. 1. Length frequency distribution of transcript/unigene for all samples pooled.

values of Q30 and Q20 were 98.01 and 94.14, respectively, and the GC content was 47.19 (Table 1).

A total of 187 387 transcripts and 57 621 unigenes were obtained through *Trinity*. The length distribution is shown in Fig. 1. The numbers of transcripts and unigenes from 300 - 500 bp in length were 36 580 and 17 815, respectively. There were 38 235 transcripts and 15 816 unigenes between 500 and 1 000 bp, and 62 001 and 12 521 longer than 2 000 bp, respectively. The minimum read lengths, maximum read lengths, and average read lengths of transcripts and genes were 301 and 301 bp, 1352 and 771 bp, and 1698 and 1302 bp, respectively. Their total sequencing lengths, N50, and N90 were 318 207 776 and 75 320 896 bp, 2 494 and 2 147 bp, and 819 and 516 bp, respectively (Table 2).

Under the condition of E value < $1e^{-5}$, gene function annotation was performed in the database using software such as *Blast2GO* and *KAAS*, and the annotation result is shown in Fig. 2. There were 57 621 unigene annotations to *Nr*, *Nt*, *KEGG*, *SWISS-PROT*, *Pfam*, *GO*, and *KOG*/*COG*; the numbers of annotated unigenes in each database were 33 789 (58.43 %), 25 095 (43.4 %), 13 327 (23.04 %), 28 563 (49.39 %), 27 851 (48.16 %), 27 851 (48.16 %), and 10 161 (17.57 %), respectively. There were 7 132 unigenes that could be annotated into *Nr*, *Nt*, *Pfam*, *GO*, and *KOG*/*COG* at the same time. The numbers of the unique annotated unigenes in *Nr*, *Nt*, *Pfam*, *GO*, and *KOG*/*COG* were 4 159, 1 328, 0, 0, and 14, respectively (Fig. 2).

By analysing the gene expression with *DESeq2*, a total of 10 158 differential genes were found in the leaves of flowering and nonflowering *P. vulgaris* that met the criteria of $p_{adj} < 0.05$ and $|\log2FoldChange| > 1$, with 6 294 upregulated genes and 3 864 downregulated genes. There were 12 982 genes differentially expressed between leaves and spicas of flowering *P. vulgaris*, 4 896 upregulated and 8 086 downregulated. There were 13 231 genes differentially expressed between leaves of nonflowering *P. vulgaris* and spicas of flowering *P. vulgaris*, 6 323 upregulated and 6 908 downregulated (Fig. 3).

There were 6 626 DEGs annotated for biological processes, cell components, and molecular functions in *GO* for the leaves of flowering *P. vulgaris*. Within the biological process category, differentially expressed genes, upregulated genes and downregulated genes annotated for metabolic processes reached maxima of 4 207 (63.49 %), 2 646 (26.45 %), and 1 561 (65.34 %), respectively.

Table 2. Number of up- and down-regulated DEGs in specific *KEGG* pathways. S-S-L denotes the leaves of nonflowering *P. vulgaris*; A-S-L and A-S-S denote the leaves and spicas of flowering *P. vulgaris*, respectively.

Compare	DEGs	*KEGG* Pathway	Gene (eg)	Number
S-S-L *vs*. A-S-L	up DEGs	plant-pathogen interaction	*PvCML PvFLS2 PvPTI1*	92
		starch and sucrose metabolism	*PvGLGC PvBGLX PvGAUT*	71
		glycolysis/gluconeogenesis	*PvPFKA PvPFK PvIPT*	24
		amino sugar and nucleotide sugar metabolism	*PvRGP PvUTM PvUGD*	54
		plant hormone signal transduction	*PvIAA PvGH3 PvPYL*	70
	down DEGs	ribosome	*PvRPL24 PvRPL12 PvRPSC*	218
		plant hormone signal transduction	*PvSAUR PvIAA PvTGA*	26
		starch and sucrose metabolism	*PvGLGC PvUXS1 PvGAUT*	17
		glycolysis/gluconeogenesis	*PvPFKA PvPFK PvHK*	50
		amino sugar and nucleotide sugar metabolism	*PvRGP PvUTM PvUXS1*	77
A-S-L *vs*. A-S-S	up DEGs	plant-pathogen interaction	*PvRPS3 PvCKF PvFLS2*	67
		starch and sucrose metabolism	*PvTPS PvGPI PvPGI*	26
		glycolysis/gluconeogenesis	*PvACS PvPGK PvHK*	47
		amino sugar and nucleotide sugar metabolism	*PvGPI PvSPDB PvMPI*	17
		plant hormone signal transduction	*PvTIR1 PvABF PvIAA*	92
	down DEGs	starch and sucrose metabolism	*PvGLGP PvPYG PvBGLX*	137
		glycolysis/gluconeogenesis	*PvADH PvPGM PvFBP*	50
		amino sugar and nucleotide sugar metabolism	*PvMPI PvGAUT PvGME*	77
		plant hormone signal transduction	*PvPYL PvBSK PvIAA*	44

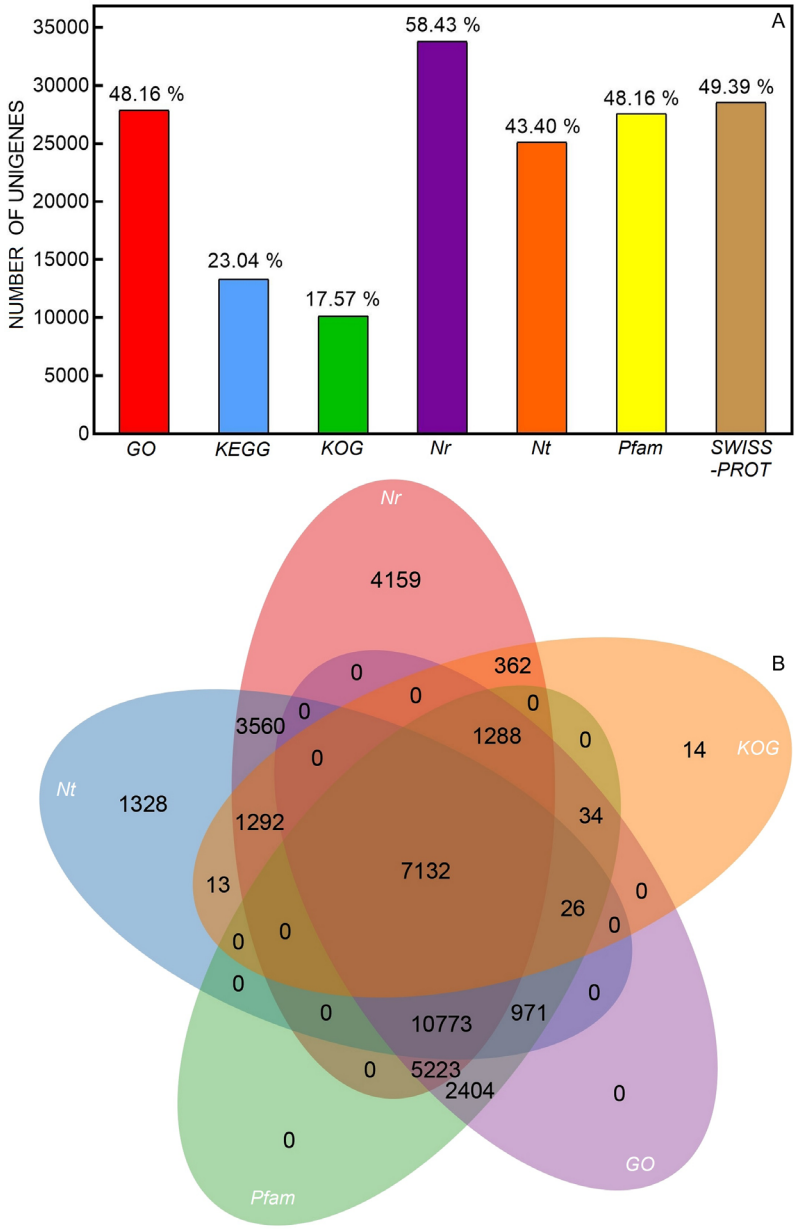

Fig. 2. Distribution frequency of unigenes in the databases. *A* - Distribution frequency of unigene histogram in the databases; *B* - Distribution frequency of unigene Veen diagram in the databases.

There were 1 958 differentially expressed genes for the intracellular complex under the cell components category, and there were 1 241 and 717 upregulated and downregulated differentially expressed genes, respectively. For catalytic activity under the molecular function category, there were 3 471 annotations significantly higher than other functions, and the upregulated and downregulated genes annotated for catalytic activity numbered 2 281 and 1 190, respectively (Fig. 2*A* Suppl.).

There were 8 597 DEGs annotated for biological processes, cell components, and molecular functions in *GO* for the leaves and spicas of flowering *P. vulgaris*. In the biological process category, numbers of differentially expressed genes, upregulated genes and downregulated genes annotated for metabolic processes reached maxima of 1 158, 534, and 624, respectively. There were 332 differentially expressed genes for the intracellular complex under the cell components category, and there were 80 and 252 upregulated and downregulated differentially expressed genes, respectively. For catalytic activity under molecular function, there were 4 481 annotations significantly higher than other functions, and numbers of upregulated and downregulated genes annotated for catalytic activity were 1 670 and 2 811, respectively (Fig. 2*B* Suppl.).

DEGs in the leaves of flowering and nonflowering *P. vulgaris* were annotated into 119 *KEGG* pathways. The 20 pathways with the highest numbers of annotated genes are shown in Fig. 4*A*: ribosome (416), plant-pathogen interaction (111), plant hormone signal

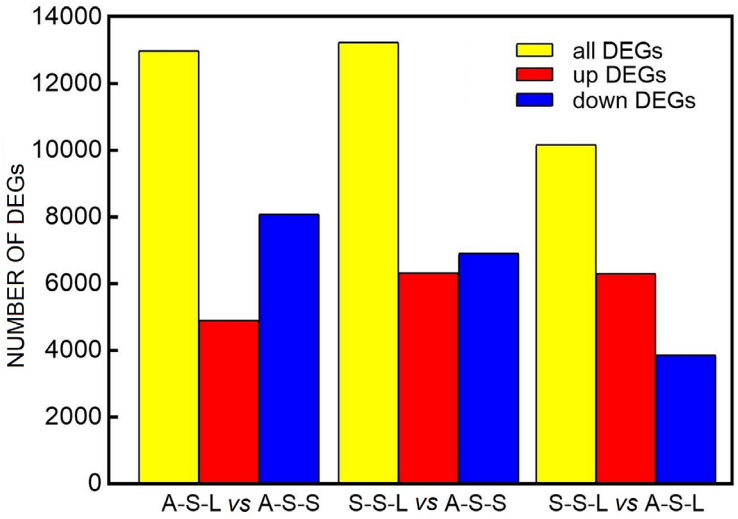

Fig. 3. Cluster analysis of DEGs. S-S-L denotes the leaves of nonflowering *P. vulgaris*; A-S-L and A-S-S denote the leaves and spicas of flowering *P. vulgaris*, respectively.

transduction (100), starch and sucrose metabolism (96), phenylpropanoid biosynthesis (88), protein processing in endoplasmic reticulum (76), endocytosis (66), amino sugar and nucleotide sugar metabolism (65), and pentose and glucuronate interconversions (64). Upregulated genes were enriched in plant pathogen interaction pathways with a maximum number of 92, and number of downregulated genes in the ribosome pathway attained a maximum

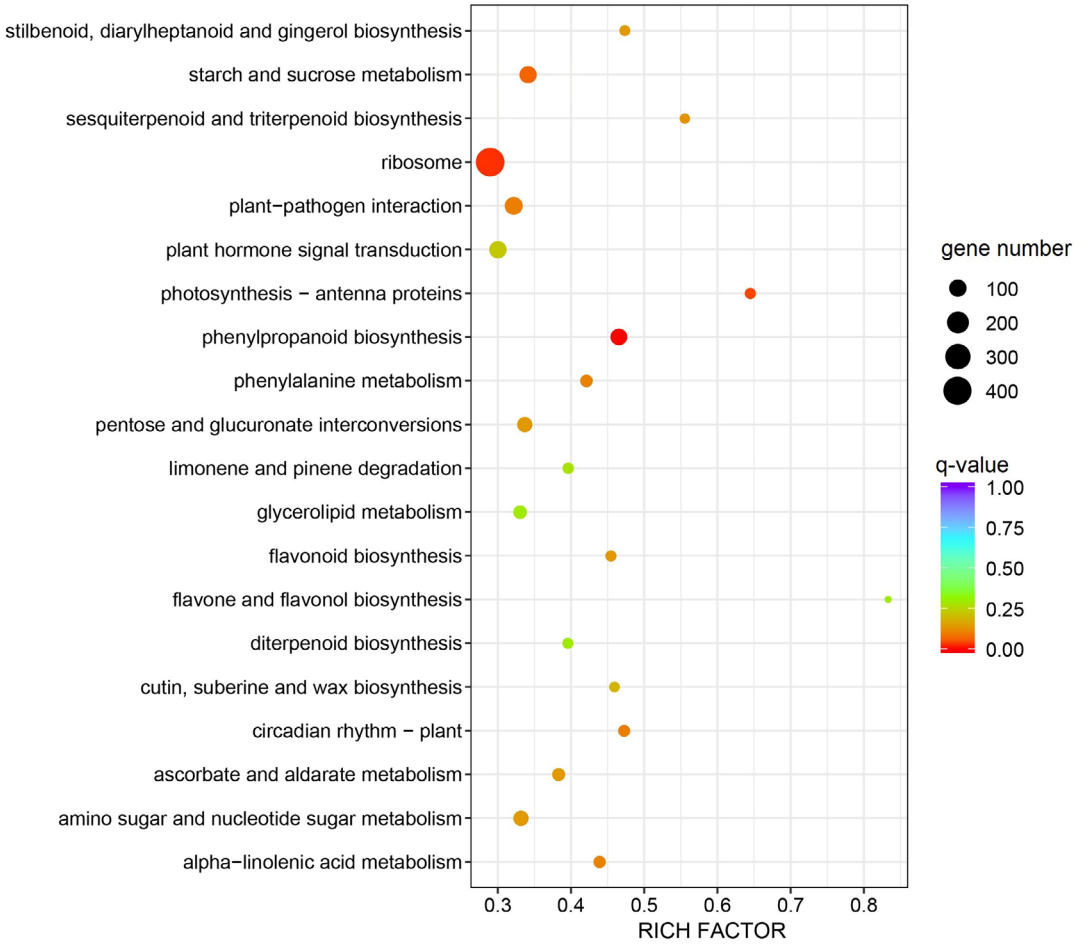

Fig. 4*A*. Distribution of DEGs of S-S-L *vs*. A-S-L in the top 20 *KEGG* pathways.

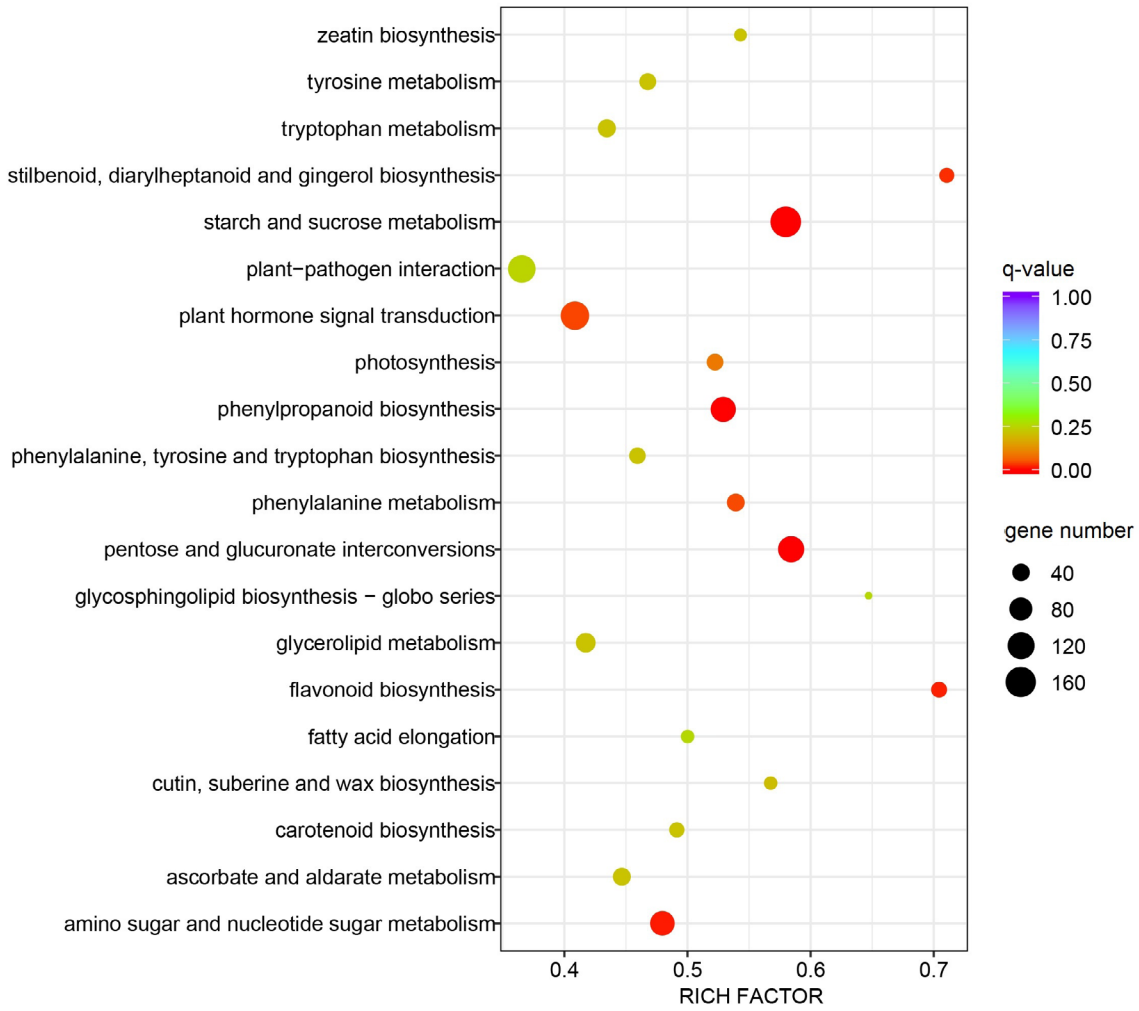

Fig. 4*B*. Distribution of DEGs of A-S-L *vs*. A-S-S in the top 20 *KEGG* pathways.

of 218. DEGs in the leaves and spicas of flowering *P. vulgaris* were annotated into 118 *KEGG* pathways. The 20 pathways with the highest numbers of annotated genes are shown in Fig. 4*B*: ribosome (310), starch and sucrose metabolism (163), plant hormone signal transduction (136), plant-pathogen interaction (126), pentose and glucuronate interconversions (111), phenylpropanoid biosynthesis (100), glycolysis/gluconeogenesis (97), and amino sugar and nucleotide sugar metabolism (94). The number of upregulated genes in the plant pathogen interaction pathway reached a maximum of 67, and the number of downregulated genes in the starch and sucrose metabolism pathways was as high as 137.

By analysing the genes enriched in the *KEGG* pathway, it was found that DEGs were significantly enriched in the "glycolysis/gluconeogenesis", "amino sugar and nucleotide sugar metabolism", and "starch and sucrose metabolism" pathways (Table 3). Compared to the leaves of flowering *P. vulgaris*, upregulated genes in the leaves of nonflowering *P. vulgaris* were enriched in the above three pathways by 24 (such as *PvPFKA PvPFK* and *PvIPT*), 54 (such as *PvRGP PvUTM* and *PvUGD*), and 71(such as *PvGLGC PvBGLX* and *PvGAUT*), and downregulated genes were enriched by 50 (such as *PvPFKA PvPFK* and *PvHK*), 77 (such as *PvRGP PvUTM* and *PvUXSL*) and 17 (such as *PvGLGC PvUXSL* and *PvGAUT*), respectively. Compared to the spicas of flowering *P. vulgaris*, upregulated genes in the leaves were enriched in the above three pathways by 47 (such as *PvACS PvPGK* and *PvHK*), 17 (such as *PvGPI PvSPDB* and *PvMPI*), and 26 (such as *PvTPS PvGPI* and *PvPGI*), and downregulated genes were enriched by 50 (such as *PvADH PvPGM* and *PvFBP*), 77 (such as *PvMPI PvGAUT* and *PvGME*) and 137 (such as *PvGLGP PvPYG* and *PvBGLX*), respectively. Furthermore, compared to the leaves of flowering *P. vulgaris*, there were 70 (such as *PvIAA PvGH3* and *PvPYL*) upregulated and 26 (such as *PvSAUR PvIAA* and *PvTGA*) downregulated genes in the leaves of nonflowering *P. vulgaris*, which were significantly enriched in "plant hormone signal transduction" pathways. Compared to the spicas of flowering *P. vulgaris*, there were 92 (such as *PvTIRL PvABF* and *PvIAA*) upregulated and 44 (such as *PvPYL PvBSK* and *PvIAA*) downregulated genes in the leaves, which were significantly enriched in "plant hormone signal transduction" pathways.

To identify genes related to flowering of *P. vulgaris*, we

Table 3. Information about flowering genes, up - upregulated genes, down - down-regulated genes.

Gene name	Gene ID	S-S-L *vs*. A-S-L	A-S-L *vs*. A-S-S
cry1	cluster-7524.15863	up	up
cry2	cluster-7524.16006	-	-
gi	cluster-7524.10051	up	-
elf3	cluster-7524.11783	down	-
	cluster-7524.11785	down	up
	cluster-7524.4484	-	-
cop1	cluster-7524.29327	up	-
	cluster-7524.16311/ cluster-7524.11671	-	-
soc1	cluster-7524.34093/ cluster-7524.33052	down	-
	cluster-7524.15099	up	-
	cluster-7524.33732 / cluster-7524.29382	down	-
	cluster-7524.29380	-	up
svp	cluster-9289.0	up	-
agl24	cluster-7524.5336/	down	-
ga20ox	cluster-7524.32968/ cluster-7524.32729	down	-
	cluster-3993.0/ cluster-16939.0	-	-
rgl	cluster-7524.11892	up	-
	cluster-3473.0	down	-
	cluster-4171.0	-	down
	cluster-17329.1	-	-
ap2	cluster-7524.13185/ cluster-7524.32833	-	down
	cluster-7524.32832	up	-
ag1	cluster-8035.0	-	-
fy	cluster-7524.7978/ cluster-7524.7977	-	-
fca	cluster-7524.22049/ cluster-7524.15362	-	-
	cluster-7524.15362	-	-
ld	cluster-7524.9223	-	-
fpa	cluster-7524.3912/ cluster-7524.14858	-	-
fld	cluster-7524.14857	-	-
flk	cluster-7524.2721/ cluster-7524.13558	-	-
	cluster-7524.27334/ cluster-7524.13382	-	-
	cluster-7524.11303	-	-
fri	cluster-7524.4005	-	-
flc	cluster-7524.6948	-	-
fca	cluster-7524.22049/ cluster-7524.9223	-	-
	cluster-7524.15362	-	-

collected the genes known to be involved in the flowering regulation pathway of *A. thaliana* (Srikanth and Schmid 2011). A search of our annotated *Nr* and *SWISS-PROT* databases based on these gene names revealed a total of 55 genes, of which 50 were homologous to those of *A. thaliana*. These flowering genes of *P. vulgaris* can be divided into five pathways (Fig. 5), including photoperiod pathway genes (*PvCRY1* 1, *PvCRY2* 1, *PvGI* 2, *PvELF3* 3, *PvCOP1* 3, *PvAP2* 3), autonomous pathway genes (*PvFY* 2, *PvFCA* 3, *PvLD* 1, *PvFPA* 2, *PvFLD* 1, *PvFLK* 4), vernalization pathway genes (*PvFRI* 1, *PvFLC* 1, *PvSOC1* 6, *PvFCA* 3), ageing pathway genes (*PvAP2* 13), and GA signalling pathway genes (*PvGAI* 2, *PvSVP* 1, *PvAGL24* 1, *PvGA20OX* 5, *PvRGL* 4, *PvGA1* 1). In total, a further comparison of the expression profiles of these genes identified 21 differentially expressed unigenes in our transcriptome data (Table 3). Compared to the leaves of flowering *P. vulgaris*, the unigenes *PvCRY1* (Cluster-7524.15863), *PvGI* (Cluster-7524.10051), *PvCOP1* (Cluster-7524.29327), *PvSOC1* (Cluster-7524.15099), *PvSVP* (Cluster-9289.0), *PvRGL* (Cluster-7524.11892), and *PvAP2* (Cluster-7524.32832) were significantly upregulated. in the leaves of nonflowering *P. vulgaris*. The unigenes *PvELF3* (Cluster-7524.11783), *PvELF3* (Cluster-7524.11785), *PvSOC1* (Cluster-7524.34093), *PvSOC1* (Cluster-7524.33052), *PvSOC1* (Cluster-7524.33732), *PvSOC1* (Cluster-7524.29382), *PvAGL24* (Cluster-7524.5336), *PvGA20OX* (Cluster-7524.32968), *PvGA20OX* (Cluster-7524.32729) and *PvRGL* (Cluster-3473.0)

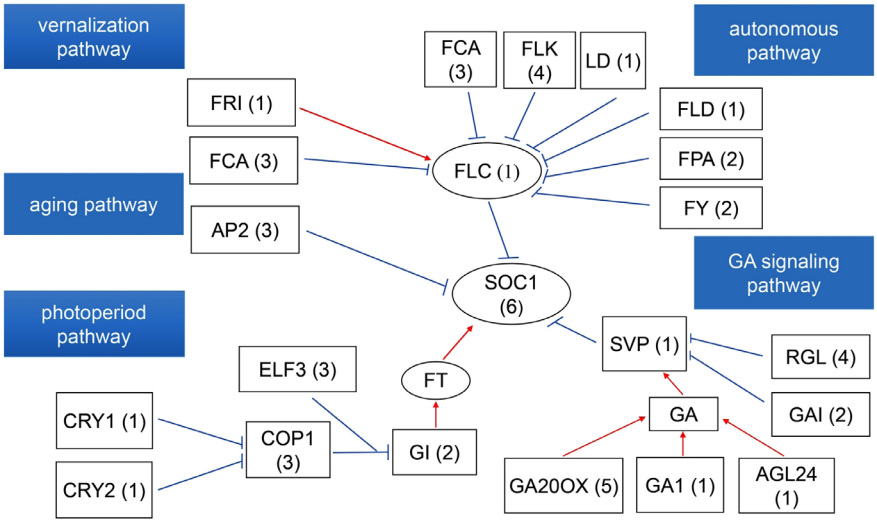

Fig. 5. Putative genes involved in flowering time and their expression levels in *P. vulgaris*. The *red arrow* indicates promotion, and the *blue line* indicates inhibition; FCA - flowering time control protein FCA, FLK - flowering locus K homology domain, LD - homeobox protein LUMINIDEPENDENS, FRI - inactive protein FRIGIDA, AP2 - aspartyl protease 2, FLD - protein FLOWERING LOCUS D, FPA - flowering time control protein FPA, FY - flowering time control protein FY, SOC1 - MADS-box protein SOC1, CRY1 - cryptochrome 1, CRY2 - cryptochrome 2, ELF3 - protein EARLY FLOWERING 3, COP1 - E3 ubiquitin-protein ligase COP1, FT - protein FLOWERING LOCUS T, GI - protein GIGANTEA, SVP - MADS-box protein SVP, GA - gibberellin, GA20OX - gibberellin 20 oxidase, RGL - DELLA protein RGL, AGL24 - MADS-box protein AGL24.

were significantly dowregulated. Compared to the spicas of flowering *P. vulgaris*, the unigenes *PvCRY1* (Cluster-7524.15863), *PvELF3* (Cluster-7524.11785), and *PvSOC1* (Cluster-7524.29380) were significantly upregulated in the leaves of flowering *P. vulgaris*. In contrast, the unigenes *PvRGL* (Cluster-4171.0), *PvAP2* (Cluster-7524.13185), *PvAP2* (Cluster-7524. 32833) were significantly downregulated (Table 3).

To confirm the gene expression patterns of flowering-related genes by transcriptome analysis, seven randomly selected candidate flowering genes and their isoforms were determined by RT-qPCR analysis. Except for *SVP* (Cluster-9289.0), the expressions of selected genes based on RT-qPCR were consistent with the RNA-seq data (Fig. 6). Specifically, the expression of *PvGA20OX* (Cluster-7524.32729), *PvSOC1* (Cluster-7524.33732 and Cluster-7524.33052), and *PvELF3* (Cluster-7524.11783) was significantly upregulated from flowering *P. vulgaris* to nonflowering *P. vulgaris*, while the expression of *PvCRY1* (Cluster-7524.15863) was significantly downregulated from flowering *P. vulgaris* to nonflowering *P. vulgaris*. There were no significant differences in the expression of *PvSVP* (Cluster-9289.0) or *PvSOC1* (Cluster-7524.29380) from flowering *P. vulgaris* to nonflowering *P. vulgaris*.

Discussion

With the continuous development of sequencing technology, RNA-Seq can generate a large number of sequences at a lower cost and in a shorter time (Mutz *et al.* 2013) and can provide a basis for understanding gene regulation and complex networks (Hua *et al.* 2011). To date, RNA-seq has been widely used for *de novo* transcriptome sequencing of many medicinal and ornamental species, such as *Salvia miltiorrhiza* (Gao *et al.* 2014), *Camellia sasanqua* (Huang *et al.* 2017), and *Dendrobium* (Chen *et al.* 2017), and has also been used with *P. vulgaris* (Li *et al.* 2020). Three earlier cycles of field experimental observations revealed that the flowering habits of *P. vulgaris* sowed in spring and autumn are different. It is speculated that this phenomenon is related to the effects of the ageing, vernalization, and photoperiod pathways and nutrient transformation on flowering. In this study, *Illumina HiSeq* was used to sequence *P. vulgaris* grown from different sowing dates to reveal this phenomenon at the molecular level. A total of 187 387 transcripts and 57 621 unigenes were obtained through experiments. The mean lengths of the transcripts and unigenes were 1 698 bp and 1 302 bp, respectively. The numbers of annotated genes in the *Nr*, *Nt*, *KEGG*, *SWISS-PROT*, *Pfam*, *GO*, and *KOG*/*COG* public databases were 33 789, 25 095, 13 327, 28 563, 27 851, and 10 161, respectively. Enrichment analysis revealed that DEGs in leaves of flowering and nonflowering *P. vulgaris* were mainly annotated for metabolic processes (4 207) in *GO* and ribosomal pathways (416) in *KEGG*. The expression of 7 flowering genes was verified by RT-qPCR, and the value 85.71 % was found to be consistent with the transcriptome results, which confirmed the accuracy and reliability of the transcriptome results. This provides the basis for further molecular-level research on *P. vulgaris*.

Sugar, as an energy metabolite, regulates the transformation of plants from vegetative growth to reproductive growth (Shalom *et al.* 2014). Recent studies have also shown that trehalose-6-phosphate plays a regulatory role in the flowering process of *Arabidopsis* (Wahl *et al.* 2013), and sucrose transport genes also regulate flowering (Gu *et al.* 2020). In this study, a large

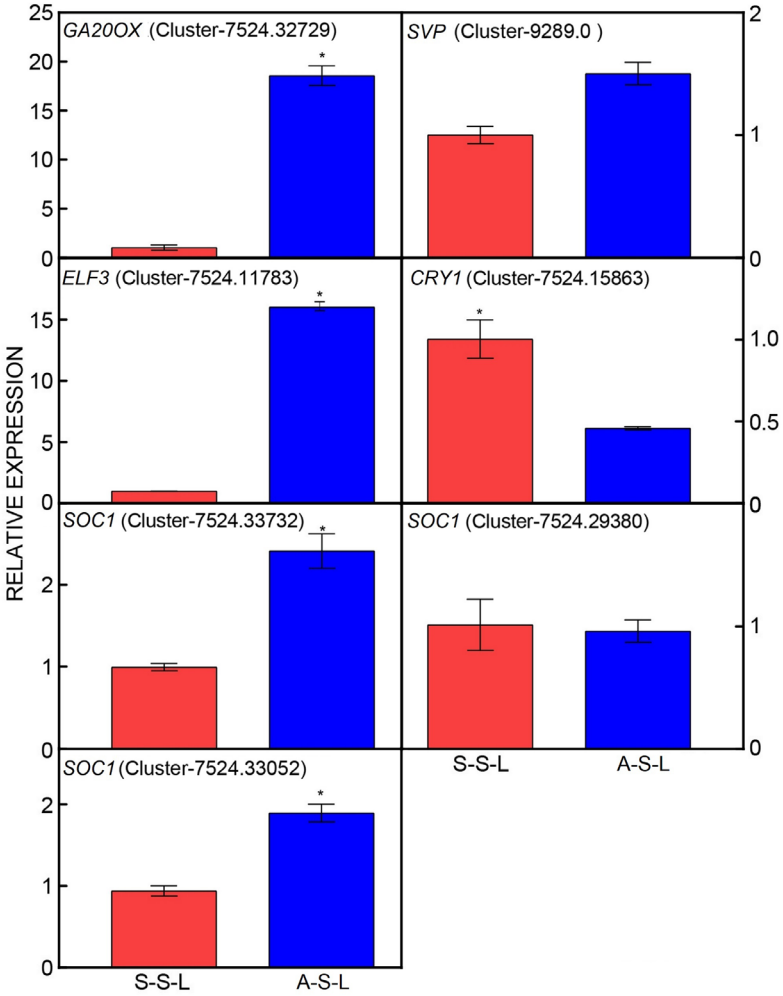

Fig. 6. Expressions of the genes involved in the flowering process. Significant changes ($P < 0.05$) in the expressions of these genes between S-S-L and A-S-L are indicated by *, S-S-L denotes the leaves of nonflowering *P. vulgaris*, and A-S-L denotes the leaves of flowering *P. vulgaris*, respectively. GA20OX - gibberellin 20 oxidase, SVP - MADS-box protein SVP, ELF3 - protein EARLY FLOWERING 3, CRY1 - cryptochrome-1, SOC1 - MADS-box protein SOC1.

number of DEGs were associated with most *KEGG* energy-metabolism pathways, such as "glycolysis/ gluconeogenesis", "amino sugar and nucleotide sugar metabolism", "starch and sucrose metabolism". Chen *et al.* (2017) studies have shown that flowering genes of *Dendrobium* are associated with most *KEGG* energy-metabolism pathways. Further analysis showed that many genes involved in energy-metabolism such as *PvGLGC*, *PvBGLX*, *PvGAUT*, *PvRGP*, *PvUTM*, *PvUGD*, *PvUXSL*, *PvTPS*, *PvGPI*, *PvPGI*, *PvACS* and *PvHK*, *etc*. are all differentially expressed genes. And sugar, as an energy metabolite, regulates the transformation of plants from vegetative growth to reproductive growth (Shalom *et al.* 2014). Thus, it can be speculated that sugars play an important role in the flowering process of *P. vulgaris*. The result also showed that hormones play an important role in regulating flowering in the medicinal plant *Lonicera japonica* (Li *et al.* 2019). In this study, the upregulated genes (70) enriched in phytohormone transduction pathways in the leaves of flowering and nonflowering *P. vulgaris* were almost three times as numerous as the downregulated genes (26). Upregulated genes (92) enriched in plant hormone pathways in leaves and spicas of flowering *P. vulgaris* were almost twice as numerous as downregulated genes (44). Among them, *PvIAA*, *PvGH3*, *PvPYL*, *PvSAUR*, *PvTGA*, *PvTIR1*, *PvABF*, and *PvBSK*, *etc.,* regulate hormone synthesis pathway to affect hormone content. Guan *et al.* (2019) have shown that changes in hormone concentration can promote the expression of *PsSOC1*, *PsSPL9*, and *PsSVP* in the flowering base of *Paeonia suffruticosa* to promote its flowering. Therefore, it is speculated that hormones play an important role in the flowering process of *P. vulgaris*. This analysis provided the basis for the in-depth study of the mechanism of flowering in *P. vulgaris*.

Studies have shown that multiple pathways related to plant flowering (photoperiod pathway, vernalization pathway, gibberellin pathway, autonomous pathway, and ageing pathway) are regulated by environmental factors (irradiance and temperature) (Fornara *et al.* 2010) and internal signals (Hong *et al.* 2019, Yan *et al.* 2019). With in-depth study at the molecular level, the flowering

machinery of model plants has been continually revealed, and many flowering genes have been repeatedly verified (Amasino 2010). From previous research, we have learned that *FLC* and *FRIGIDA* (*FRI*) play important regulatory roles in flowering in the vernalization pathway (Yu *et al.* 2020). *FRI* promotes the transcription of *FLC* by encoding a frizzled protein (Johanson *et al.* 2000). In addition, *FLC* directly interacts with *CONSTANS* overexpression factors, and *FT* overexpression factor binds to suppress its transcription (Searle *et al.* 2006). There are many genes known to participate in the regulation of flowering in the gibberellin pathway, including genes related to GA biosynthesis such as *GA20OX*, *GA2OX*, and *GA3OX* (Bao *et al.* 2020). In the model plant *A. thaliana*, genes involved in the flowering signalling pathway have been well characterized (Andres and Coupland 2012), but the flowering genes of *P. vulgaris* have not been studied. In this study, further analysis revealed flowering genes homologous to those in *A. thaliana*: *PvCRY1*(1), *PvCRY2*(1), *PvGI*(2), *PvELF3*(3), *PvCOP1*(3), *PvFRI*(1), *PvFLC*(1), *PvSOC1*(6), *PvFCA*(3), *PvFY*(2), *PvFCA*(3), *PvLD*(1), *PvFPA*(2), *PvFLD*(1), *PvFLK*(4), *PvGAI*(2), *PvSVP*(1), *PvAGL24*(1), *PvGA20OX*(5), *PvRGL*(4), *PvGA1*(1), and *PvAP2*(3). These genes are mainly involved in the vernalization pathway, the autonomous pathway, the ageing pathway, and the gibberellin pathway that regulate plant flowering, and nearly half of them exhibit significant expression differences. Studies have found that gene regulation genetic networks are largely conserved in plant species (Benlloch *et al*. 2007). Therefore, based on the *Arabidopsis* flowering regulatory network, we predict that the *PvGI, PvFRI, PvAGL24, PvGA20OX*, and *PvGA1* will promote the flowering, and the *PvCRY1*, *PvCRY2*, *PvELF3*, *PvCOP1*, *PvFLC*, *PvSOC1*, *PvFCA*, *PvFY*, *PvFCA*, *PvLD*, *PvFPA*, *PvFLD*, *PvFLK*, *PvGAI*, *PvSVP*, *PvRGL*, and *PvAP2* will inhibit the flowering of *P. vulgaris*. Real-time RT-qPCR results for flowering genes (*PvGA20OX*, *PvSVP*, *PvELF3*, *PvCRY1*, *PvSOC1*) showed that the transcriptomic results were extremely reliable. These differentially expressed genes and their isoforms may be involved in the control of flowering. This study provides the basis for further research on the mechanism and regulation of flowering in *P. vulgaris*.

Conclusion

In this study, 187 387 transcripts were obtained by sequencing flowering and nonflowering *P. vulgaris*. The numbers of annotated genes in the *Nr*, *Nt*, *KEGG*, *SWISS-PROT*, *Pfam*, *GO*, and *KOG*/*COG* public databases were 33 789, 25 095, 13 327, 28 563, 27 851, 27 851, and 10 161, respectively. Furthermore, 6 626 and 119 DEGs from the leaves of flowering and nonflowering *P. vulgaris* were annotated into *GO* and *KEGG*, respectively. In addition, 8 597 and 118 DEGs from the leaves and spicas of flowering *P. vulgaris* were annotated into *GO* and *KEGG*, respectively. In-depth analysis determined that there were large numbers of DEGs in the plant hormone signal transduction and sugar metabolism pathways, providing the basis for the study of the flowering mechanism. Fifty genes homologous to the flowering genes of *A. thaliana* were identified in our transcripts. These genes included *PvCRY1, PvCRY2, PvGI, PvELF3, PvCOP1, PvFRI, PvFLC, PvSOC1, PvFCA, PvFY, PvFCA, PvLD, PvFPA, PvFLD, PvFLK, PvGAI, PvSVP, PvAGL24, PvGA20OX, PvRGL, PvGA1,* and *PvAP2*, and nearly half of them showed significant expression differences. Fluorescence quantification was used to further verify the accuracy and reliability of the transcriptomic results. The flowering of *P. vulgaris* is clearly regulated by a complex gene network. This study provides the basis for further research on the mechanism and regulation of flowering in *P. vulgaris*, with the specific functions of flowering genes in *P. vulgaris* requiring further research.

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