RESEARCH ARTICLE

Comparative transcriptome analyses reveal genes related to pigmentation in the petals of red and white *Primula vulgaris* cultivars

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Abstract *Primula vulgaris* is an important ornamental plant species with various flower color. To explore the molecular mechanism of its color formation, comparative transcriptome analyses of the petals in red and white cultivars was performed. A total of 4451 differentially expressed genes were identified and annotated into 128 metabolic pathways. Candidate genes *FLS*, *F3'H*, *DFR*, *ANS* and *AOMT* in the anthocyanin pathway were expressed significantly higher in the red cultivar than the white and may be responsible for the red coloration. In the red petals, a putative transcription factors bHLH (c52273.graph_c0) was up-regulated about 14-fold, while a R2R3-MYB unigene (c36140.graph_c0) was identified as a repressor involved in anthocyanin regulation and was significantly down-regulated. In addition, the anatomy analyses and pigments composition in the red and white petals were also analyzed. The papillae on the adaxial epidermis of the red petals of *P. vulgaris* display a triangle-shapes, in contrast with a spherical shape for the white petals. Although flavonoids were detected in both cultivars, anthocyanins could only be identified in the red cultivar. Gossypetin and peonidin/rosinin were the most abundant

pigments in red petals. This study shed light on the genetic and biochemistry mechanisms underlying the flower coloration in *Primula*.

Keywords *Primula vulgaris* · Red petal · Anthocyanin biosynthesis · Flower coloration · R2R3-MYB · Epigenetic analysis

Abbreviations

3GT	Anthocyanin 3- <i>O</i> -glucosyltransferase
5GT	Anthocyanin 5- <i>O</i> -glucosyltransferase
a*, b*	Chromatic components
ABP	Anthocyanin biosynthetic pathway
ANS	Anthocyanin synthase
AOMT	Anthocyanin <i>O</i> -methyltransferase
bHLH	Basic helix-loop-helix
C*	Chroma (brightness)
CHI	Chalcone isomerase
CHS	Chalcone synthase
CIE	Three-Dimensional International Commission on Illumination
COG	Cluster of orthologous groups of proteins database
DEGs	Differentially expressed genes
DFR	Dihydroflavonol 4-reductase
F3'H	Flavonoid 3'-hydroxylase
F3H	Flavanone 3-hydroxylase
FDR	False discovery rates
FLS	Flavonol synthase
FPKM	Fragments per kilobase per million mapped reads
GO	Gene ontology database
KEGG	Kyoto encyclopedia of genes and genomes database
KOG	EuKaryotic Orthologous Groups database

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L*	Lightness
NCBI	National Center for Biotechnology Information
NR	Non-redundant protein database
Pfam	Protein family
qRT-PCR	Quantitative reverse transcription PCR
R	Pearson's correlation coefficient
RNA-Seq	RNA sequencing
SEM	Scanning electron microscopy
SDs	Standard deviations
SwissProt	SwissProt protein database
TFs	Transcription factors
UHPSFC–MS	Ultra-high performance supercritical fluid chromatography–mass spectrometry
WD40	WD40 proteins

Introduction

Primula vulgaris is an important flowering plant for potting or bedding during winter and early spring. It has a relatively long blooming period, bright flower colors, profuse flowering and higher environmental adaptability. Until now, *P. vulgaris* cultivars with various flower colors (dark red, fresh red, black pink, deep yellow, light red, red-rimmed white, light pink and black) have been produced by conventional breeding and are currently available in the market (Karlsson 2002). In plants, flavonoids/anthocyanins, carotenoids and betalains are the three major classes of pigments responsible for coloration. Flavonoids/anthocyanins and carotenoids are widely distributed in flowers, fruits, leaves and other tissues and are responsible for the pale-yellow to blue colors, while betalains have been found only in some Caryophyllales families (Strack et al. 2003; Hatlestad et al. 2012).

The biosynthesis of flavonoid/anthocyanin is one of the best characterized secondary metabolic pathways and is highly conserved across different organisms (Feller et al. 2011; Hichri et al. 2011). To date, the genes involved in flavonoid biosynthesis and transportation of pigments have been well characterized in many plants. In particular, the expression of anthocyanin biosynthesis enzymes are primarily regulated by complexes comprising R2R3-MYB transcription factors (TFs), WD40 proteins (WD40) and basic helix-loop-helix (bHLH) TFs (Xu et al. 2015). Many R2R3-MYB TFs have been identified predominantly responsible for various color patterns, such as *Rosea1*, *Rosea2* and *Venosa* in snapdragons (Schwinn et al. 2006; Shang et al. 2011), *CgMYB1* in *Clarkia gracilis* (Martins et al. 2016), *LhMYB12* in Asiatic hybrid lilies (*Lilium* spp.) (Suzuki et al. 2016), *LrMYB15* in *L. regale* (Yamagishi 2016) and *PeMYB11* in *Phalaenopsis* spp. (Hsu et al. 2015). However, the molecular mechanisms the

corresponding candidate genes underlying flower pigmentation in *Primula* remain unclear.

RNA sequencing (RNA-Seq) is an effective tool for transcriptome study on complicated floral traits in plants when a reference genome is not available. It has been successfully applied to many species including *Lilium* spp., *Paeonia suffruticosa* Andr. and *P. rockii* (Suzuki et al. 2016; Zhang et al. 2015; Shi et al. 2017). Until now, no RNA-seq study on the coloration of *P. vulgaris* flowers have been reported.

In the present study, we performed comparative transcriptome analyses on the red-colored ‘Star Fire’ (R) and white-colored ‘White Lover’ (W) cultivars of *P. vulgaris* using illumina sequencing. Anatomical and pigments profiling studies were also carried out to investigate the most likely causes of red coloration. This work reveals how transcriptional changes influenced the physiological and biochemical characteristics responsible for the flower coloration in *Primula*. Our results contribute to the understanding of the genetic mechanism underlying the flower coloration and provide a valuable resource for identifying candidate genes in *Primula*.

Materials and methods

Plant material

Seeds of *P. vulgaris* cultivars with white and red phenotype (‘White Lover’ and ‘Star Fire’) were obtained from Shanghai Hemei Gardening Co., Ltd. (Shanghai, China) and were planted in a greenhouse at Northwest A&F University, Yangling, Shaanxi, China in 2016 (Fig. 1a, e). In January of 2017, the fresh petals from ten plants of each cultivar were sampled just after anthesis and were cut off immediately the middle yellow part for morphological and anatomy observations as well as protoplast preparation. Meanwhile, petals were collected from ten plants of each cultivar, immediately frozen in liquid nitrogen and stored at -80°C for pigment measurement and RNA-seq analyses.

Petal color measurement

Three color parameters, L^* , a^* and b^* , were measured by a chroma meter (CR-400, Konica Minolta Sensing, Inc., Osaka, Japan). L^* , a^* , and b^* represent the brightness (ranging from black to white), the ratio of red and green, and the balance between yellow and blue, respectively (Zhang et al. 2007). Six biological replicates were recorded for each cultivar.

Fig. 1 Cellular features of the flower materials. **a–d, e–h** Fully open flowers of individuals selected for sequencing, the adaxial and abaxial epidermis and cross sections of the petals of W and R petals, respectively. Bar, 100 μ m

Microscopic observation

Fresh petals were cross-sectioned. The adaxial and abaxial epidermal layers were peeled off manually with a razor blade. Then the fresh sections were placed on glass slides with a drop of water and were photographed immediately under a light microscope (Eclipse 50i, Nikon, Tokyo, Japan).

Scanning electron microscopy (SEM)

The petals were cut into small pieces and were then fixed in glutaraldehyde buffer with vacuum treatment for 30 min. The samples were then incubated for 10 h at 4 °C. Next, the fixed petals were dehydrated in a series of ascending aqueous ethyl alcohols (30, 50, 70, 80, 90, and 100%). The solvents in the samples were then replaced with liquid carbon dioxide using a critical-point drying method (Qi et al. 2013). The dry samples were mounted on a specimen stub and were sputter-coated with gold before examination under a scanning electron microscope (JSM-6360LV SEM, JEOL Ltd., Tokyo, Japan).

Pigment measurement

One milligram of lyophilized petal powder from each sample was extracted with 1 ml of 0.1% acetic acid/methanol at 4 °C for 10 h. The samples were then centrifuged at 10,000 rpm for 10 min. The supernatants were then decanted and dried using a vacuum centrifuge concentrator (CV100-DNA, Aijimu, Beijing, China). The

anthocyanin compositions were characterised and quantified using the ultra-high performance liquid chromatography–mass spectrometer (UPLC-MS/MS, Waters, Milford, MA, USA) method, coupled with a triple-quadrupole mass spectrometer (XEVO®-TQ) according to a previous method (Barbara 1980; Veberic et al. 2015).

Three milligrams of lyophilized petal powder from each sample was extracted for the analysis of carotenoid components with acetone-hexane (1:2) for 30 min three times and analyzed using ultra-high performance supercritical fluid chromatography–mass spectrometry (UHPSFC-MS) techniques (Jumaah et al. 2016).

Anthocyanin compounds were identified by comparing their retention time of the standards. The UV–Vis peaks spectra characteristics and the mass spectrometric information were analyzed using the Mass Hunter qualitative software. The relative quantity of anthocyanin and flavonoids were calculated from the peak sample areas.

Pigment extraction was performed as previously described (Hashimoto et al. 2000). Semi-quantification of flavonoids and carotenoids were carried out using linear regression method by comparing to the signal of malvidin-3,5-*di-O*-glucoside (Mv3G5G), rutin and β -carotene standards at 520 nm, 350 nm and 440 nm, respectively. Anthocyanins and flavonoids concentrations were calculated in milligrams per gram of fresh weight (Mv3G5G mg/g and rutin mg/g, respectively) (Shi et al. 2017). The mean values and SDs (standard deviations) were calculated for four biological replicates.

RNA extraction, library construction, and RNA-seq

Total RNA was extracted from 1 mg of mixed petals powder from ten plants using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was assessed using NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The integrity of RNA was evaluated using Agilent 2100 Bioanalyzer (Santa Clara, CA, USA) with an RIN (RNA integrity number) > 8.0. Three biological replicates were included for each variety. Library construction and RNA-seq analyses were performed by Biomarker Biotechnology Corporation (Beijing, China) using Illumina HiSeqTM 2500 platform. All sequencing data were deposited at NCBI Sequence Read Archive under accession number SRP6279556.

De novo assembly, gene annotation and differentially expressed gene (DEG) analyses

After removing the poor-quality reads (adaptor reads, ambiguous nucleotides and low-quality reads), the clean reads were assembled using Trinity software (Grabherr et al. 2011). The expression levels of unigenes were calculated as FPKM (Trapnell et al. 2010). Next, the edgeR package was utilized to normalize the expression levels of each unigene in two samples to identify DEGs. The false discovery rate (FDR) control and ratio of FPKM values from two samples were used to compute the differences at the gene expression level. DEGs (FDR value ≤ 0.05 and ≥ 1 -fold change) were determined and categorized automatically using the COG database, GO database and KEGG database.

Quantitative real-time PCR verification

In order to estimate the validity of the RNA-Seq results, we selected thirty pigmentation related unigenes for real-time quantitative PCR (qPCR) tests using gene specific primers. Primers were designed using Oligo dT7 and Primer 3 software. The housekeeping gene *actin* (c55882.graph_c0) was selected as the internal control. All primer sequences are listed in Table S1. Total RNA was extracted using TRIzol reagent (Invitrogen). After the removal of genomic DNA, first-strand cDNA synthesis was conducted using reverse transcriptase (Promega, Madison, WI, USA) with 2 μ g of RNA as template. qPCR was performed using Light Cycler 480 SYBR Green I Master Mix (Roche, Mannheim, Germany; Roche, USA). The cycling conditions were 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and at 72 °C for 20 s. All samples were performed in technical and biological triplicates to ensure reproducibility and reliability.

Results

Anatomical observations of the two cultivars

To elucidate the mechanism underlying flower coloration in R, we examined the spatial location of the pigment in the two cultivars. Colored cells in R petals were found to be mainly located on the adaxial and abaxial epidermises. The colors of adaxial epidermal cells appeared much deeper than the abaxial (Fig. 1f–h). In contrast, the epidermal cells were colorless for the W petals (Fig. 1b–d).

SEM analyses indicated that papillae were pronounced and distributed on both adaxial and abaxial layers in both cultivars. Papillae on the adaxial and abaxial epidermis of W appeared to be spherical. The papillae margins in the abaxial epidermal layer were clearly smoother than those in the adaxial epidermis. In contrast, papillae on the adaxial epidermis of R displayed a triangle-shape. However, papillae on the abaxial epidermis were also spherical, similar to that for W. In addition, the density of papillae on the adaxial epidermis in R was lower than that in W (Fig. 2).

Qualitative and quantitative pigments analyses

The color indices (L^* , a^* and b^*) and flavonoid concentrations were measured for the petals in R and W. The L^* and b^* values of the W petals were significantly higher than that of R, while the a^* values were significantly lower (Fig. 3a). The total anthocyanins and flavonoids accumulated at significantly higher levels in R petals (Fig. 3b, c) in comparison with W. In contrast, the carotenoid contents in R and W were similar (Fig. 3d). The results indicated that anthocyanins and flavonoids may play vital roles in red flower color formation. Therefore, we went further to characterize the anthocyanin and flavonoid composition in these two cultivars. In total, six anthocyanins were identified in R petals, which include cyanidin 3,5-diglucoside, peonidin 3,5-diglucoside, cyanidin-3-glucoside, peonidin-3-sophoroside, peonidin-3-glucoside and rosinin (Table 1).

Fig. 2 Scanning electron microscope (SEM) analysis of papillate cells from petals of the adaxial (a, b) and abaxial (c, d) epidermis. W and R represent ‘White Lover’ and ‘Star Fire’, respectively. Bars, 40 μ m (a, c) and 20 μ m (b, d)

Fig. 3 **a** Tepal colors and color parameters of white and red flowers of *P. vulgaris*. L^* lightness; a^* , b^* chromatic components. A high L^* value indicate a lighter color. The parameter a^* determined the balance between red and green, and the parameter b^* determined the balance between yellow and blue. **b–d** Concentrations of anthocyanin, flavonoids and carotenoids (mg/g) in two *P. vulgaris* cultivars at the fully open stage. W and R represent ‘White Lover’ and ‘Star Fire’, respectively

Table 1 Anthocyanins in contents in ‘White lover’ and ‘Star Fire’ at the fully open stage

No. of peaks	Tentative identification	Retention time (min)	λ_{max} (nm)	MS ²	‘Star Fire’ (μg/g)	‘White Lover’ (μg/g)
1	Cyaniding 3,5-diglucoside	5.07	278, 515	449, 287	2118.4 ± 74.3	0 ± 0
2	Peonidin,3,5-diglucoside	5.63	280, 522	463, 301	37,072.9 ± 304.8	0 ± 0
3	Cyanidin-3-glucoside	6.07	280, 524	287	6069.6 ± 327.9	0 ± 0
4	Peonidin-3,5-sophoroside	6.26	275, 520	301, 463	2699.7 ± 143.9	0 ± 0
5	Peonidin-3-glucoside	6.73	280, 526	301	11,067.9 ± 340.5	0 ± 0
6	Rosinin	7.11	280, 523	315, 477	3196.1 ± 240.7	0 ± 0

In contrast, no anthocyanins could be found in the petals of W (Table 1).

A total of 20 flavonoids were identified in the petals of W and R (Table 2). All of the flavonoids detected in R, with the except of gossypetin-7-methoxy-3-glucoside, were found present in the petals of W. Among these flavonoids, the concentrations of quercetin and kaempferol were much higher in W than those in R. In addition, the concentrations of kaempferol-3-gentiatrioside and quercetin-7-methoxy-3-gentiatrioside were comparable in these two cultivars. Furthermore, the other 16 flavonoids showed much higher concentrations in R than that in W. Overall, the concentrations of all four flavonoid derivatives, including gossypetin-, quercetin-, kaempferol- and isorhamnetin-derivatives, were much higher in R. In particular, gossypetin- and isorhamnetin-derived flavonoids were

significantly higher in R. These results suggested that the color variation of the petals in R and W may be related with anthocyanins and flavonoids, accumulation, which prompts us to further investigate the transcriptional variation of the candidate genes in the anthocyanin biosynthesis pathway.

RNA sequencing and gene annotation

A comparative transcriptome analyse between W and R were performed with three biological replicates using the Illumina HiSeqTM 2500 technique. The total numbers of clean reads in W and R samples were approximately 23.4 million and 24.3 million, respectively. The GC% and the Q30% (percentage of bases with quality value > 30) were about 43% and 93%, respectively (Table S2). These data

Table 2 Flavone and flavonol contents in ‘White lover’ and ‘Star Fire’ at the fully open stage

No. of peaks	Tentative identification	Retention time (min)	λ_{max} (nm)	MS ²	‘Star Fire’ (μg/g)	‘White Lover’ (μg/g)
1	Gossypetin-3,5-diglucoside-8-caffeic ester	6.17	278, 344	643, 481, 319	455.2 ± 34.2a	13.6 ± 2.7b
2	Gossypetin-7-methoxy-3,5-diglucoside-8-caffeic ester	6.92	279, 343	657, 495, 333	893.3 ± 102.1a	118 ± 13.6b
3	Quercetin-3-gentiobiosiden-5-glucoside	7.07	257, 358	627, 465, 303	5265.3 ± 246.0a	4985.8 ± 516.3a
4	Gossypetin-7-methoxy-3,5-diglucoside-8-caffeic ester	7.22	274, 361	657, 495, 333	2080.3 ± 16.3a	193.2 ± 18.5a
5	Quercetin-3-gentiobiosiden-acetate-7-glucoside	7.5	257, 358	465, 367, 303	5090.2 ± 270.8a	2980.8 ± 230.1b
6	Quercetin 3,5-diglucoside	7.62	268, 349	465, 303	1886.4 ± 101.6a	925.1 ± 140.8b
7	Kaempferol-3-gentiotrioside	7.65	255, 353	611, 449, 287	3844.9 ± 250.3a	4206.3 ± 208.8a
8	Quercetin-7-methoxy-3-gentiotrioside	7.74	254, 359	671, 479, 317	3903.8 ± 304a	3298.2 ± 166.6a
9	Gossypetin-7,3'-dimethoxy-3,5-diglucoside-8-caffeic ester	7.89	255, 359	671, 509, 347	721.7 ± 169.6a	1710 ± 145.4b
10	Kaempferol-3-sophoroside-5glu-7-acetic	8.05	268, 349	653, 611, 449, 287	3873.6 ± 200.7a	3091.0 ± 199.2b
11	Quercetin-7-methoxy-3-glucoside-5-sophoroside-acetate	8.22	268, 350	683, 641, 479, 317	3963.8 ± 175.6a	2837.6 ± 182.8b
12	Kaempferol-3,5-diglucoside	8.24	255, 356	449, 287	986.7 ± 108.2a	306.9 ± 139.0b
13	Isorhamnetin-3-glucoside-caffeic ester	8.35	255, 355	479, 317	1437.7 ± 175.5a	394.0 ± 36.8b
14	Gossypetin-7-methoxy-3-glucoside	8.56	269, 358	333	149.9 ± 12.6a	0 ± 0b
15	Quercetin-3-glucoside	8.64	256, 353	303	2418.7 ± 25.3a	329.8 ± 6.8b
16	Kaempferol-3-glucoside	9.09	265, 348	287	167.4 ± 24.5a	58.4 ± 27.3b
17	Kaempferol-5-glucoside	9.31	265, 348	287	406.1 ± 17.8a	77.5 ± 14.3b
18	Quercetin-7-methoxy-3-glucoside	9.45	254, 355	317	278.0 ± 29.3a	87.5 ± 9.2b
19	Quercetin	12.28	254, 370		414.3 ± 67.5b	796.2 ± 176.3a
20	Kaempferol	14.15	264, 365		204.8 ± 21.3b	575.0 ± 59.2a

suggested that the sequencing results were sufficient for further analysis. A total of 101,112 unigenes from six libraries were obtained with a N50 length of 1184 bp and a mean length of 733 bp (Table S2).

Sequence similarity search was carried out against protein sequences available in various databases using the BLASTX algorithm with an *E* value threshold of $1e^{-10}$. Among the 101,112 unigenes, 45,173 (44.7%), 29,231 (28.9%), 27,020 (26.7%), 14,514 (14.4%), 27,202 (26.9%), 31,044 (30.7%) and 18,728 (18.5%) were blasted into the NCBI, Non-redundant (NR) protein database, SwissProt protein database (SwissProt), Gene Ontology database (GO), Cluster of Orthologous Groups of proteins database (COG), euKaryotic Orthologous Groups database (KOG), Pfam database (protein family), and Kyoto Encyclopedia of Genes and Genomes (KEGG), respectively (Figure S1). Based on the NR annotations, the highest homology of sequences (21.89%) was found with *Cucumis melo*, followed by *C. sativus* (21.01%), *Vitis vinifera* (8.69%), *Sesamum indicum* (3.15%), *Coffea canephora* (3.1%), *Theobroma cacao* (2.62%), *Nicotiana glauca* (2.22%), *N.*

tomentosiformis (2.03%), *Nelumbo nucifera* (1.7%) and *Jatropha curcas* (1.56%); the remaining 14,463 unigenes (32.03% of all transcripts) contained sequence homologs from other species (Fig. 4a).

Based on homology search against the COG database, 14,514 unigenes (14.4%) were annotated and classified into 25 different functional classes (Fig. 4b). The most dominant group, “General function prediction”, contained 4963 all-unigenes (18.36%), followed by “Replication, recombination and repair” (9.04%) and “Transcription” (8.25%). Based on GO analysis, 27,020 unigenes were categorized into three main GO ontologies: molecular function, cellular component and biological process. Under the cellular component category, “cell part”, “organelle” and “membrane” were the most frequent terms. In the molecular function category, “catalytic activity”, “binding” and “transporter activity” were predominant. In the biological process category, the dominant groups were “metabolic process”, “cellular process” and “single-organism process” (Fig. 4c). A total of 18,728 unigenes were mapped to 129 KEGG pathways, of which “Carbon metabolism”,

Fig. 4 **a** Characteristics of the unigene homology search. **b** Classification of the clusters of Orthologous Groups (COG) for the *P. vulgaris* transcriptome. The unigenes were annotated and divided into

25 specific categories. **c** Gene ontology (GO) classification for the *P. vulgaris* transcriptome

“Ribosome” and “Biosynthesis of amino acids” were the three largest pathways, which included 852, 744 and 729 unigenes, respectively.

Annotation analysis of DEGs

The differentially expressed gene (DEG) was identified as FDR value ≤ 0.05 and ≥ 1 -fold change. Compared with W, 4451 unigenes were differentially expressed in R, including 2056 up-regulated unigenes and 2395 down-regulated unigenes (Figure S2). These DEGs were categorized into 128 pathways using the KEGG database. Of these, “Flavonoid biosynthesis” (ko00941), “Phenylpropanoid biosynthesis” (ko00940), “Starch and sucrose metabolism” (ko00500) and “Anthocyanin biosynthesis” (ko00942) were the four most enriched pathways (Fig. 5). These identified DEGs were verified using qPCR (correlation $R^2 = 0.8453$, $P < 0.0001$) (Figure S3), which indicated the high reliability of the transcriptome data. According to the genes annotation and homology search with previously characterized genes related with flower color in other plants (Shi et al. 2017), the most likely candidate DEGs responsible for the red flower color in *P. vulgaris* were identified (Table 3).

Genes related to flower color formation

Based on the flavonoid characterization and DEGs results, a tentative ischematic diagram for the red coloration was developed (Fig. 6). Among the four flavonoid derivatives identified in R and W petals, gossypetin- and isorhamnetin-flavonoids accumulated two to three times higher in R than that in W. In the flavonoid biosynthetic pathway, isorhamnetin is synthesized from quercetin methylated by

AOMT, of which c47583.graph_c0 and c44905.graph_c0 showed higher expression levels in R. Subsequently, quercetin and gossypetin were synthesized from the common substrate dihydrokaempferol under F3'H and FLS. c48339.graph_c0 (*F3'H*) was expressed fivefold higher in R. In addition, c15244.graph_c0, encoding FLS, was expressed more than 100-fold higher in R. Then, a F3H unigene (c48030.graph_c0) showed threefold higher transcription in R and may contribute to the synthesis of dihydrokaempferol, which is the common substrate for kaempferol, quercetin and gossypetin biosynthesis. Moreover, c40107.graph_c0 encoding CHI was expressed much higher in R, which may be the main enzyme responsible for the formation of naringenin. More than half of CHS, the first key flavonoid biosynthesis enzyme, were up-regulated in R petals. Obviously, the expression of c43168.graph_c0 and c46201.graph_c0 were more than ten-fold higher in R than those in W. In addition, c46201.graph_c0 showed close homology with the anthocyanin-regulated gene *CHS* in *Rhododendron simsii* (accession no. CAC88858, e-value = 0.0). c50255.graph_c0, encoding DFR expressed 18-fold more highly in R and was considered as *P. vulgaris* candidate anthocyanin biosynthesis gene. Moreover, c50255.graph_c0 showed high homology to the anthocyanin-related protein DFR (accession no. BAJ08042) of *Cyclamen graecum* (e-value = $2e-10^5$) (Akita et al. 2010). Cyanidin is synthesized from leucocyanidin, catalyzed by ANS (c48790.graph_c0), which was expressed significantly higher in R. Subsequently, c47583.graph_c0 and c44905.graph_c0, encoding candidates for AOMT, showed two- to four-fold higher expression levels in R. c47583.graph_c0 was highly similar to the AOMT (accession no. BAK74804) of cyclamen (*Cyclamen persicum* $\times$ *C. purpurascens*) (ident = 80%) while c44905.graph_c0 was

Fig. 5 The q-values coloring indicates the significance of the rich factor ranging from 0 to 1, and lower Q-values indicate greater intensiveness. The rich factor is the ratio of DEG numbers annotated in a given pathway term to all gene numbers that were annotated in the pathway term, and greater rich factor values indicate greater intensiveness. The top 20 pathway terms enriched in the KEGG database are listed in this figure. The circle size represents the quantity of DEGs

Table 3 Candidate DEGs involved in *Primula vulgaris* red color formation

Gene	Enzyme	Enzyme no.	No. all	No. up in R	No. down in R
<i>CHS</i>	Chalcone synthase	[EC:4.3.1.24]	11	6	5
<i>CHI</i>	Chalcone isomerase	[EC:5.5.1.6]	2	1	1
<i>F3H</i>	Flavanone 3-hydroxylase	[EC:1.14.11.9]	3	1	2
<i>F3'H</i>	Flavonoid 3'-hydroxylase	[EC:1.14.13.21]	5	3	2
<i>F3'5'H</i>	Flavonoid 3'/5'-hydroxylase	[EC:1.14.13.88]	3	1	2
<i>FLS</i>	Flavonol synthase	[EC:1.14.11.23]	8	2	6
<i>DFR</i>	Dihydroflavonol 4-reductase	[EC:1.1.1.219]	2	1	1
<i>ANS</i>	Anthocyanidin synthase	[EC:1.14.11.19]	1	1	0
<i>3GT</i>	Anthocyanidin 3-O-glucosyltransferase	[EC:2.4.1.115]	9	2	7
<i>5GT</i>	Anthocyanidin 5-O-glucosyltransferase	[EC:2.4.1.298]	1	0	1
<i>AOMT</i>	Anthocyanin O-methyltransferase	[EC:2.1.1.104]	3	2	1

highly homologous to the caffeoyl-CoA-methyltransferase (accession no. AFY97679) of *Camellia sinensis* (ident = 94%) (Akita et al. 2011). c50034.graph_c0, c52483.-graph_c0, c39825.graph_c0, c31006.graph_c1, c51479.-graph_c0 and c17924.graph_c0 were highly expressed in R, while the other five members showed high abundances in W (Fig. 6).

Thirty-five unigenes in the R2R3-MYB family were divided into four groups based on their expression profiles in the two cultivars (Fig. 7a). The expression levels of R2R3-MYBs in clusters 1 and 3 were more abundant in W than in R. By contrast, the transcription of clusters 2 and 4 were significantly higher in R compared to those in W. Nine unigenes were differentially expressed with absolute

$\log_2$ (FC values) > 2, including five up-regulated genes (c38443.graph_c0, c47013.graph_c0, c48106.graph_c0, c40864.graph_c0 and c39429.graph_c0) and four down-regulated genes (c19192.graph_c0, c18654.graph_c0, c36140.graph_c0 and c48646.graph_c0). Notably, c36140.graph_c0 was found to be closely related to *VvMYBA1* and *VvMYBA2* in grapevine (Figure S4) (Koyama et al. 2014; Walker et al. 2007).

The expression profiles of 33 predicted bHLH genes were divided into four clusters (Fig. 7b). Notably, the candidate genes in Cluster 1 showed low abundance in both cultivars. Those in cluster 2 showed similar expression levels in the two cultivars. In contrast, the candidate genes in Cluster 3 and 4 exhibited high expression levels in R,

Fig. 6 Detailed schematic of anthocyanin metabolism related to flower pigmentation in ‘White Lover’ and ‘Star Fire’. The enzyme names and expression patterns are shown beside each step. The expression pattern of each gene is shown in a heatmap. The color

scale represents log2-transformed FPKM (fragments per kilobase of exon per million mapped reads) values. Red represents high expression, and green represents low expression. W and R represent ‘White Lover’ and ‘Star Fire’, respectively

while the genes in Cluster 5 genes showed much higher expression levels in W than in R. Specifically, c52273-graph_c0 in the cluster 4 was found to be closely related to *anthocyanin 1* (*Petunia hybrid*, accession no. AAG25927, Figure S5) (Spelt et al. 2002). This gene was expressed significantly higher in R. The expression levels of WD40 repeat genes could be divided into two clusters (Fig. 7c).

WD 40 repeat genes in Cluster 1, which accounted for two-thirds of the entire WD40 family, were highly expressed in R, while those in Cluster 2 were expressed highly in W. Unfortunately, no WD40 genes were found to be involved in anthocyanin regulation.

Fig. 7 All unigenes encoding the R2R3-MYB (a), bHLH (b) and WD40 (c) transcription factors were hierarchically clustered and mapped using FPKM (fragments per kilobase of exon per million mapped reads) values. The numbers in the heat map represent

Expression analysis of candidate genes related to flower color formation

Based on homology search for the previously characterized anthocyanin genes in other flower plants (Tan et al. 2013; Nakatsuka et al. 2008), 11 candidate genes were identified from DEGs. These include *CHS* (c46201.graph_c0), *CHI* (c27920.graph_c0), *DFR* (c50255.graph_c0), *FLS* (c15244.graph_c0), *F3H* (c48030.graph_c0), *F3'H* (c53714.graph_c0 and c48339.graph_c0), *ANS* (c48790.graph_c0), *AOMT* (c47583.graph_c0), *bHLH* (c52273.graph_c0) and *R2R3-MYB* (c36140.graph_c0). The expression profiles of these 11 genes were further verified using qRT-PCR. Results showed that the expression levels of 10 genes were higher in R than those in W, with the exception of c36140.graph_c0. Specially, *DFR* (c50255.graph_c0), *FLS* (c15244.graph_c0), *F3'H*

(c48339.graph_c0), *ANS* (c48790.graph_c0) and *bHLH* (c52273.graph_c0) were expressed over tenfold higher in R. On the other hand, one *R2R3-MYB* showed much higher expression in W (Fig. 8).

Discussion

P. vulgaris is widely cultivated all over the world. It has a diverse variety of flower colors including red, white, and yellow. However, the genetic basis underlying the flower coloration is very complex and remains to be characterized (Cao and Liang, 2008; Freyre and Griesbach, 2004; Harborne 1968, 1969; Quintana et al. 2007). The uneven distribution of different pigments has been known as the primary contributor to color formation in the petals (Suzuki et al. 2016). In this study, we found that the colored cells in

Fig. 8 qPCR analysis of anthocyanin structural genes and transcriptional gene expression in ‘White lover’ and ‘Star Fire’ petals. Relative mRNA (y-axis) expression levels are presented as a ratio and were normalized to *actin* (c55882.graph_c0)

the petals were primarily located in the adaxial and abaxial epidermises. Furthermore, the adaxial epidermal cells had a much deeper color based on microscopic observation. In addition, the papillae on the adaxial epidermis of the red petals were triangle-shaped, while those of the white petals were spherical. The observations were consistent with a previous report (Quintana et al. 2007). Furthermore, we found that the petals of red and white *P. vulgaris* displayed distinct accumulations of gossypetin- and isorhamnetin-derived flavonoids and several anthocyanins, which provides a likely explanation for the flower color variation.

Due to the absence of a reference genome of *P. vulgaris*, the identification of the candidate genes responsible for the flower color was hindered. Here, we performed a comparative transcriptomic analysis of the red and white cultivars based on the Illumina platform. The transcriptional differences responsible for flavonoid and anthocyanin biosynthesis in the red and white petals were revealed. We assembled 101,112 unigenes and identified 4451 DEGs, which are relatively higher than those previously studies in *P. poissonii* and *P. wilsonii* (Zhang et al. 2013). The expression profiles of DEGs were confirmed by qPCR. Our study provide a valuable resource for future molecular study of *Primula* flowers.

Clear differences in the flavonoid and anthocyanin biosynthesis and gene transcription between red and white flowers were observed in the present study. In particular, the red flower showed significant up-regulation of all

anthocyanin biosynthetic genes, including *DFR* (c50255.graph_c0), *ANS* (c48790.graph_c0) and *AOMT* (c47583.graph_c0). As expected, these transcriptional differences were coincident with the pigment accumulation. Cyanidin, peonidin and rosinin appeared to be the principal pigments in red petals. *DFR*, a crucial later gene in the anthocyanin biosynthesis pathway, is able to reduce dihydroflavonols to colorless leucoanthocyanidins. leucoanthocyanidins could be converted by *ANS* into colored cyanidins (Lou et al. 2014). Indeed, *ANS* showed significant up-regulation in R. The identified *DFR* (c50255.graph_c0) and *ANS* (c48790.graph_c0) displayed strong homology to the anthocyanin-related genes reported in *C. graecum* (Akita et al. 2010). Rosinin is a rare pigment synthesized from the peonidin series via *AOMT* (Harborne 1968). Peonidin could be synthesized from cyanidin by 3'-*O*-methyltransferase (Fournier-level et al. 2011; Jaakola 2013). As we expected, c47583.graph_c0 was very similar to the anthocyanin-related *AOMT* of cyclamen and was highly expressed in R (Akita et al. 2011).

Among the identified flavonoid components in *P. vulgaris* flowers, gossypetin and isorhamnetin showed the most significant differences between R and W. *CHS* is the first key enzyme to associate one 4-coumaroyl-CoA molecule with three malonyl-CoA molecules to produce trihydroxy-chalcone, the biosynthesis precursor of flavonoids and anthocyanins (Hichri et al. 2011). In this study, we found that c46201.graph_c0 was expressed at significantly higher levels in R and showed high homology with the anthocyanin-regulated gene *CHS* in *Rhododendron simsii*. In addition, *CHI* (c40107.graph_c0) and *F3H* (c48030.graph_c0) are able to convert tetrahydroxy-chalcone to dihydrokaempferol, which serves as the common substrate for *F3'H* and *FLS*, resulting in the production of different flavonoids and anthocyanins (Hichri et al. 2011). *FLS* is the main enzyme responsible for the formation of quercetin and gossypetin, therefore *FLS* was expressed at a much higher levels than other flavonoid biosynthesis-related genes, such as c15244.graph_c0. Notably, *FLS* exhibited 100-fold higher expression levels in R than W. In addition, isorhamnetin is synthesized by quercetin under *AOMT*. c47583.graph_c0 was considered as the candidate *AOMT* that produce quercetin. In summary, we speculated that *FLS*, *F3'H*, *DFR*, *ANS* and *AOMT* were the most likely candidate responsible for flower coloration in *P. vulgaris*.

R2R3-MYB, bHLH and WD40 proteins are the main TFs responsible for the regulation of the structural genes in the anthocyanin biosynthesis pathway (Shin et al. 2013). The different combinations of MYB and bHLH with WD40 could either activate and/or repress the expression of a set of target genes and thus regulate the production of anthocyanin (Lepiniec et al. 2006). In Asiatic hybrid lilies, *LhMYB12* has been reported to be responsible for the

coordinated expression of *LhCHSa*, *LhCHSb*, *LhF3H*, *LhF3'H*, *LhDFR* and *LhANS*. In addition, *PeMYB11* has been shown to activate the expression of the anthocyanin biosynthetic genes *PeF3H5*, *PeDFR1* and *PeANS3* in *Phalaenopsis* spp (Suzuki et al. 2016; Yamagishi 2016; Hsu et al. 2015). Moreover, several anthocyanin repressors of R2R3-MYB TFs have also been characterized in plants, including *FaMYB1* and *FcMYB1* from strawberry (*Fragaria* spp.), *PhMYB27* from petunia (*Petunia hybrida*) and *MtMYB2* from *Medicago truncatula* (Aharoni et al. 2001; Salvatierra et al. 2013; Albert et al. 2014; Nemie-Feyissa et al. 2014; Jun et al. 2015). Overexpression of these repressors resulted in reduced anthocyanin biosynthesis (Aharoni et al. 2001; Salvatierra et al. 2013; Albert et al. 2014; Nemie-Feyissa et al. 2014; Jun et al. 2015). In the present study, 35 R2R3-MYBs were annotated and were used to construct a phylogenetic tree with those anthocyanin-related R2R3-MYBs in other plants. One R2R3-MYB TF (c36140.graph_c0), down-regulated significantly in R, was found to be closely related to the anthocyanin-regulated genes *VvMYBA1* and *VvMYBA2* (Koyama et al. 2014; Walker et al. 2007). In addition, a bHLH unigene (c52273.graph_c0) was also found to be closely related to the known plant bHLHs involved in anthocyanin biosynthesis regulation. This gene is up-regulated significantly in R. Further study on these candidate TFs is needed to verify our hypothesis.

In conclusion, a combination of anatomy, analytical chemistry and transcriptome analyses were performed to uncover the molecular basis underlying the red and white pigmentation in the flowers of *P. vulgaris*. Gossypetin and peonidin/rosinin were deemed to be the main contributors to red color formation. We identified the potential candidate genes encoding key enzymes in the anthocyanin biosynthetic pathway, such as *FLS*, *F3'H*, *DFR*, *ANS* and *AOMT*, which are significantly differently expressed in red and white flowers of *P. vulgaris*. In particular, a bHLH TF and a R2R3-MYB repressor that might be involved in red flower coloration regulation in *P. vulgaris* were also identified. The most likely cause of the color variation in the flowers of *P. vulgaris* was proposed and discussed.

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Authors contribution LL performed the experiments, analyzed the data and wrote the manuscript. YZ, XL, YZ helped prepare the plant materials and performed some experiments. QS designed the study. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest All the authors have declared no conflict of interest.

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