TCP15 negatively regulates anthocyanin biosynthesis under low light in Brassica

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Article

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Abstract

Low light caused by industrial development and environment change has become a limitation in crop production. This condition inhibits the stalk anthocyanin synthesis and even its tastes in *brassica rapa* L; however, the molecular basis on the inhibitory effects by low light on anthocyanin synthesis in brassica stalk is less reported. In this study, we combined transcriptomes and metabolisms to decipher key genes and /or metabolites that responsible for shading acclimation in a brassica cultivar, XH1. Results shows that anthocyanin is obviously repressed in low light condition, and consistently the structural genes related to anthocyanin biosynthesis pathway is significantly enriched in the list of differentially expressed genes according to both GO and KEGG analysis. Furthermore, amounts of some metabolites related to anthocyanin are dramatically decreased under shading treatment, such as cyanindin 3-O-glucoside chloride, cyanindin O-syringic acid, and cyanidin 3-O-rutinoside. In addition, we found that some transcription factors of interest such as TCP15 is stimulated by shading treatments, while acts as a negative regulator anthocyanin biosynthesis by low light. The mechanistic explanation of inhibitory effects of anthocyanin by low light condition through TCP15 was proposed. This study extend our knowledge on transcriptional regulation mechanism for anthocyanin biosynthesis in response to shading condition, and provides an evidence that a potential role of TCP15 in low light inhibitory effects in anthocyanin synthesis in brassica.

Introduction

Brassica rapa comprises a variety of vegetables. Among them, Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) and pakchoi (*Brassica rapa* L. ssp. *chinensis*) are the two most consumed vegetables in China and throughout East Asia. *B. rapa* vegetables provide dietary fiber, vitamin C, and anti-cancer glucosinolates (Fahey et al., 1995), and are also a potentially important source of dietary flavonols (Rochfort et al., 2006). Therefore, plants of the *Brassicaceae* family are important sources of vital natural biologically active substances—enzymes, pigments, vitamins, as well as specific secondary metabolites, especially anthocyanin (Guo et al., 2014). However, the production of these natural biologically active substances always experiences many environmental factors.

Light regime represents one of most important environmental factors that affect crop production (Bell et al., 2000). This phenomenon exacerbates especially in some regions characterized with regular precipitation and cloudy cover, for example, in Sichuan, a province in Southwest China. Along with original low-radiation environments, shading has become one of the main constraints to rice or other crop production (Deng et al., 2009; Wang et al., 2013). In addition, low light leads to the changes of other agronomic traits, including canopy architecture, photosynthetic physiology, biomass accumulation, and crops quality (Möller and Assouline, 2007; Mu et al., 2010; Li et al., 2010; Wang et al., 2013). During past decades, effects of low light on molecular profilings, such as secondary metabolites, including flavonol, anthocyanin, and phenylalanine has been becoming a hot topic (Fukuoka et al., 2014). Anthocyanin is an important natural biologically active substance, and it is inhibited under low light condition (Ichimura et

al., 2021). Therefore, exploring key genes/metabolites could help to provide indicators for precise molecular breeding in the improvements of anthocyanin production in adaption to low light.

The anthocyanin biosynthetic pathway has been extensively reported. It begins with the chalcone synthase (CHS) mediated synthesis of naringenin chalcone from 4-coumaroyl-CoA and malonyl-CoA. Then, naringenin chalcone can be isomerized by chalcone isomerase (CHI) to naringenin. In addition, the anthocyanin biosynthetic pathway constitutes an extension of the global flavonoid pathway, mainly regulated by MYB–bHLH–WD40 (MBW) transcription complexes (Ramsay et al., 2005). In Arabidopsis, there are four MYBs subgroup, three IIIf bHLHs subgroups, and a TTG1 protein (Zhang et al., 2003; Gonzalez et al., 2008). Interestingly, there are still in need of other transcription factors that involved in anthocyanin biosynthetic pathway.

Currently, multiple-omics studies encompassing metabolome, transcriptome and proteome have been extensively reported in plants to better understand the reprogrammed biological pathways related to anthocyanin biosynthetic pathway by heat, drought and salt stress etc (Qu et al., 2018; Li et al., 2020). However, less study on low light effects on the degradation of anthocyanin especially in *Brassica rapa* L. stalk were reported.

In current study, in order to uncover the key biological pathways and/or genes that responsible for the degradation of anthocyanin under low light in red cabbage stalks, we combined transcriptomes and non-targeted metabolism in a stalk of *brassica rapa* L species, XH1. Enriched biological pathways are comparable to biochemical analyses of genes and metabolites associated with antioxidant systems. The transcription factors of interest were further discussed with known regulatory gene MBW complex in light of the expression levels responded to low light. This study could extend our knowledge on regulatory mechanism of anthocyanin biosynthetic pathway in response to low light in *Brassica rapa* L.

Materials And Methods

Plants growth condition

A commercial variety, Xianghong No. 1 (XH1, *Brassica rapa* L) was sown in pots on April 1, 2019. Three plants were grown in each pot, and cultivated in a light incubator on April 19, and the temperature of the incubator was set to 15 ℃/11 ℃ (day and night). Two conditions of light intensity were set up, i.e., normal growth light (500 mmol $m^{-2}s^{-1}$, CK) and shading (100 mmol $m^{-2}s^{-1}$, S) under long-day illumination (light/dark cycle is 14 h/10 h). There are three biological replicates for each light treatment. The stalk of the plants on the 29th, weigh 5 grams and 2 grams of materials were taken, respectively, wrap them in tin foil, wrap them in a fresh-keeping bag, store them in a freezer at -80℃, and send samples for LC-MS/MS analysis.

RNA extraction,

To identify the effects of low light treatments on stalk anthocyanin degradation, we performed RNA-sequencing using XH1. Stalk of XH1 from 3 days shading treatment (100 μmol $m^{-2}s^{-1}$) and control (500 μmol $m^{-2}s^{-1}$) were harvested and applied to anthocyanin determination, transcriptomes and metabolism analysis. The ~5g stalks were collected for transcriptome analysis described as followings. Total RNA was extracted from the tissue using TRIzol Reagent according to the instructions of manufacturer (Invitrogen) and genomic DNA was then removed with DNase I (Takara). Then RNA quality was determined by 2100 Bioanalyser (Agilent) and quantified using the ND-2000 (NanoDrop Technologies). In this study, only high-quality RNA sample (OD260/280=1.8~2.2, RIN>6.5, 28S:18S>1.0) was used to construct the sequencing library.

Library preparation and Illumina Hiseq4000 Sequencing

~5μg of total RNA was used to construct the transcriptome library for RNA-seq using TruSeq RNA sample preparation Kit (Illumina, San Diego, CA). Shortly, mRNA was first isolated by oligo(dT) beads, and double-stranded cDNA was synthesized with a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA). Then the cDNA was synthesized to end-repair following the standard protocol of Illumina's library construction. The target cDNA fragments with 200~400 bp were selected for library and Phusion DNA polymerase (NEB) was used for PCR with at least 15 PCR cycles. Paired-end RNA-seq library was finally sequenced via Illumina HiSeq 4000 platform.

Read mapping and differential expression analysis

For read mapping step, the raw paired-end reads were first quality controlled by FastQC software (https://github.com/s-andrews/FastQC) with default parameters. Then we aligned the clean reads were mapped to reference genome using HISAT2software (https://github.com/DaehwanKimLab/hisat2) (Trapnell et al. 2009). To identify DEGs (differentially expressed genes) induced by low light, a transcript per million (TPM) method was used to calculate the expression abundance of each transcript. This method takes the gene length for normalization, and it is reported to be an appropriate tool for sequencing (Wagner et al., 2012). In addition, we used RSEM software (http://deweylab.biostat.wisc.edu/rsem/) to estimate the abundances of the gene (Li et al. 2011). R statistical package software EdgeR (http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html) was finally applied for performing differential expression analysis as documented earlier (Robinson et al. 2010).

GO and KEGG analysis

To identify the changed biological pathways induced by low light, we used an in-house Perl script UniProtKB GOA file (ftp.ebi.ac.uk/pub/databases/GO/goa) to perform gene ontologies (GO) annotation. In addition, KOBAS (KEGG Orthology Based Annotation System, v2.0) was also applied to identify reprogrammed biochemical pathways of each pathway as previously reported (Xie et al., 2011).

Non-targeted metabolism analysis

To perform the changes of metabolites induced by low light, ~2gBrassicastalks *were sampled from 3 days shading treatments and control, and were extracted using pre-cooled metal beads in a 2 mL Eppendorf tube, at 30 Hz for 3 min. We then* dissolved *the extracted powder with 1.3 m*L methanol/chloroform, followed by incubation of the powder at -20 °C for 4 h, and centrifuged the mixture at 2,000 *g* at 4 ^{o}C for 10 min. The mixture was then filtered using 0.43 μm organic phase medium (GE Healthcare, 6789-0404), and the 10 μL mixture was loaded to HPLC for analysis. Mobile phase: 15% buffer A (95% H_2O and 5% ACN, pH = 9.5) + 85% buffer B (100% ACN), 0.2 ml.min^{-1}. Three biological replicates were conducted for non-targeted metabolic measurements.

q-RT-PCR analysis

Total RNA was isolated from XH1 stalks with Ultra-Pure RNA Kit (cwbiotech) following the instructions of manufacturer, and cDNA was synthesized using ExScript RT kit (Takara). We performed q-RT-PCR on a Realplex 4 Master Cycler using Syber Green kit (TIANGEN). Calculation of relative gene expression was followed as reported previously (Livak and Schmittgen 2001), where *actin1* gene was used as a reference. At least three biological replicates were performed.

Results

Anthocyanin is inhibited by shading in stalk of XH1

Based on previous observations, we found that shading stress inhibited dramatically anthocyanin biosynthesis following 6 days, in stalk of XH1 (*Brassica rapa* L), but didn't affect the biomass accumulation (Fig. 1A). Therefore, we sampled the stalk of XH1 from 3 days shading treatments for transcriptomes and metabolite analysis to decipher the molecular mechanism that responsible for anthocyanin accumulation in *Brassica rapa* L. The samples within same treatment perform well repeatability either for transcriptome and metabolism (Fig. 1B-C).

Scaffolds assemble and quality control of transcriptomes

Based on transcriptome analysis, we found 46,836,588 raw reads and 44,703,004 clean reads, with 47% GC content, 98% Q20 and 96% Q30 in average across different samples (Table S2). The reads mapped and unique reads account 87.9% and 85.6% for total reads, respectively (Table S3). The reads error rates maintain 2% (Fig. S1A), and base distribution on different position along reads is ~150 bp (Fig. S1B). Frequencies of nucleotide A and T were higher than C and G (Fig. S1B). After reads assemble, clean reads account for 95%, while only 3% events characterized with low quality (Figs. S1C). For reads alignments, we found that the coverage depts upon different chromosomes are 20× (Fig. S2A), and 80% percentile covers at downstream part of gene body (Fig. S2B). There are 98% reads mapped to the exon of genes and only 2% reads mapped to intergenic region (Fig. S2C). After gene annotation process, we found that more than 4000 genes are related to general function prediction only, posttranslational modification, protein turnover, signal transduction mechanisms (Fig. S3A). In addition, more than 70% genes are annotated to brassica rapa #28437, while only 22% are mapped to brassica napus #8961 and only 0.6%

are mapped to Arabidopsis thaliana according to NR database (Fig. S3B). The values of FPKM across global genes show normal distribution (Fig. S4B).

Global gene expression induced by shading treatments

Principal component analysis shows that the samples within either control or shading treatments were clustered closely, and the explanation rates for top PC1 and PC2 are 29.7% and 27.4%, respectively (Fig. 2A). In addition, we found that there are 1906 significantly differently expressed genes (DEGs) in total (two fold up or down), and among these genes, 886 genes are upregulated and 1020 genes are down-regulated caused by shading treatments on the stalk of XH1 (Fig. 2B, Table S4).

Top 50 terms of GO analysis shows that circadian rhythm, rhythmic process, cellular response to light stimulus, detection of light stimulus, pigment biosynthetic process, response to gibberellin, starch catabolic process, anthocyanin-containing compound biosynthetic process are significantly enriched in the list of DEGs (Fig. 3A). From KEGG enrichments analysis, we found that zeatin biosynthesis, circadian rhythm, biosynthesis of secondary metabolites, anthocyanin biosynthesis and plant hormone signal transduction were significantly enriched in the list of DEGs (Fig. 3B). These extensive biological pathways were altered suggest that transcription factors could act key roles. Consistently, we found that there are 236 transcription factors are differentially expressed due to shading effects, and majority of them include AP2/ERF, bHLH, bZIP, C2C2-Dof, MYB, NAC, WRKY, TCP and DBB gene family (Fig. 4A-B. Table S5). We found that the top two ranking in upregulated transcription factors with account for 25% and 18% belongs to bHLH and C2C2-Dof gene family, respectively (Fig. 4A), while the top two ranking in downregulated transcription factors with account for 24% and 20% belongs to MYB and bHLH gene family, respectively (Fig. 4B). Interestingly, we found that all *TCP* gene family was up-regulated due to shading treatments (Fig. 4B). Therefore, we further detected the known genes related to anthocyanin biosynthesis pathway (Fig. 4C). Results show that there are 16 DEGs related to anthocyanin biosynthetic pathway including 7 transcription factors of interest that used for further analysis (Fig. 4C, Table S6). These transcription factors include *MYB4* (Bra008570) gene and *TCP15* (Bra007875) gene that were downregulated and upregulated, respectively (Fig. 4C, Table S6).

To confirm the expression of genes of interest inhibited by shading treatment, we conducted qRT-PCR experiments on the six downregulated genes related to anthocyanidin biosynthesis as shown by transcriptomes analysis (Table S6). Results show that the expression levels of genes such as BrLDOX (Bra019350), BrDFR (Bra027457), Br5MAT (Bra036208) and BrUGT75C1 (Bra038445) were inhibited by 2~4 times, in contrast, the expression level of BrTCP15 (Bra007875) was stimulated by two times (Fig. 5A-F). These results further confirm that transcript abundances from transcriptome data regarding the downregulated genes related to anthocyanidin biosynthesis.

Extremely differentially abundant metabolites due to shading effects

Considering anthocyanin metabolic pathways were detected by both GO and KEGG analysis, we further performed non-targeted metabolism analysis. Results first show that samples within same treatment

were tightly closed and the explanation rates for top PC1 and PC2 are 45.71% and 18.83%, respectively (Fig. 6A), and volcano plot shows that there are 290 differentially abundant metabolites (DAMs), and 118 and 172 were down and up-regulated, respectively (Fig. 6B-C, Table S7). According to KEGG classification analysis, we found that the pathways of biosynthesis of secondary metabolites, phenylalanine metabolism, lysine degradation, glutathione metabolism, fructose and mannose metabolism, flavone and flavanol biosynthesis, citrate cycle, anthocyanin biosynthesis were significantly enriched in the list of DAMs (Fig. 6D).

For DAMs related to anthocyanin biosynthesis or anthocyanin related pathway according to GO analysis, we found 19 DAMs from anthocyanins, flavanone, terpene, amino acids and derivatives class (Fig. 7, Table S8). In particular, peonidin 3-O-glucoside chloride, and cyanindin O-syringic acid were upregulated, while Cyanidin 3-O-rutinoside, Quercetin 7-O-rutinoside and rutin were downregulated due to shading effects (Fig. 7, Table S8).

Discussion

Anthocyanin metabolites arise extensive attentions during last past decades, majorly due to it contains huge antioxidant compound that is helpful for healthy. Brassica is a typical vegetable that is famous in China, while it is very sensitive to light and temperature, low light not only induces decrease of anthocyanin, but also influence its tastes (Jaakola et al., 2017). Therefore, understanding the molecular mechanism that underlying low light acclimation is essential for the improvement of high-quality Brassica breeding. In this study, we integrated transcriptome and metabolic analysis in the stalk of brassica exposed to 3 days shading treatments. Here we discussed the potential casual genes and biological pathway, with aiming towards gene engineering of high- anthocyanin brassica in breeding.

Recently, the draft genome sequence of brassica was completed, which will be an important resource for genetic research of brasscia (Wang et al., 2011) and the genome sequence was updated later (Cai et al., 2017). For the brasscia transcriptome, 45 GB clean reads of data were generated and assembled into 40 GB high-quality unique reads and Q30% were all 95% (Tables S2-S3), and around 78% mapped reads were aligned with the *B. rapa* genome reference sequence (Version 1.2, http://brassicadb.org), which is similar as reported previously (Chen et al., 2016). Many genes related to photosynthetic process were inhibited by shading treatments from bioinformatic analysis upon transcriptome dataset, such as proton gradient regulation 5 (PGR5, Bra000757) (Table S4), PGR5 is very important for photoprotection by increasing cyclic electron flow and ΔpH across the thylakoid membrane (Munekage et al., 2002; Johnson, 2011), and it transiently activates non-photochemical quenching at the onset of low light levels (Wolf et al., 2020). In addition, the expression of glyceraldehyde-3-phosphate dehydrogenase (Bra008416) involved in Calvin cycle is dramatically down-regulated due to shading effects (Table S4), which is reported to promote the shading tolerance in rice in its overexpression lines (Liu et al., 2020).

Metabolomics and transcriptomics are extensively used to uncover the effects of different light regimes on anthocyanins synthesis in horticultural plants. There are many horticultural plants reported that

accumulated anthocyanins in the light including blood orange, purple pomelo lemon fruits, and purple aloes (Huang et al., 2019; Dong et al., 2018). For example, purple aloes do not synthesize anthocyanins in dark environments, but it can restore the synthesis of purple pigments under light conditions (Dong et al. 2018), which is consistent with our observations in the current study in brassica stalk (Fig. 1A). In terms of DEGs in purple aloe under dark and light conditions, they found that the genes encoding bHLH protein and MYB family genes are closely related to anthocyanin contents (Dong et al. 2018), these genes together with WD40 protein form a MBW complex regulating anthocyanin biosynthesis (Ramsay and Glover 2005). Importantly, these genes are downregulated by shading treatments as observed in our study, suggesting MYB and bHLH transcription factors are crucial for anthocyanins metabolism (Fig. 5A-B; Table S5). These findings confirm that the anthocyanin regulatory genes are crucial for anthocyanin accumulation in brassica stalk.

Generally, anthocyanin is synthesized by an anthocyanin synthase (ANS) in its biosynthesis pathway (Broun 2005). In this study, we find that the anthocyanin synthase in brassica rapa, i.e., BrLDOX (Bra019350) was decreased almost 5 times based on transcriptome (Table S6), suggesting anthocyanin biosynthetic pathway is dramatically inhibited by low light. Many transcription factors have function in anthocyanin biosynthetic process, such as R2R3-MYB, bZIP, and bHLH (Stracke et al., 2007; Zhou et al., 2020), which is in line with our observations regarding MYB and TCP transcription factors involved in anthocyanin biosynthesis (Table S6).

In addition, some genes related to flavones synthase are also downregulated following the changes of anthocyanin induced by shading treatments, such as chalcone synthase 3 (Bra023441), flavonone isomerase (Bra009101), flavanone 3-hydroxylase (Bra012862), dihydroflavonol-4-reductase (Bra027457), 2-oxoglutarate 3-dioxygenase (Bra036828) with fold change ranging 2 to 8 (Table S6). Gene expression levels of leucoanthocyanidin dioxygenase (Bra013652), dihydroflavonol-4-reductase (Bra027457), anthocyanidin 5-O-glucoside (Bra036208) and anthocyanin 5-O-glucosyltransferase (Bra038445) were further tested by qPCR (Fig. 5B-F; Table S4). For metabolites related to anthocyanin, we also found 80% metabolites are downregulated, including naringenin 7-O-glucoside (pme0371), Naringenin(pme0376), phloretin (pme1201), quercetin (pme0199), kaempferol 3-O-rutinoside (pme0370), except for quercetin 3-O-rutinoside (pmb0711) (Table S8). This is also observed in other studies under other abiotic stress, such as ABA treatments (Zhu and Assmann 2017), heat stress (de Leonardis et al., 2015) and drought stress (Leonardis et al., 2021).

Brassica is very sensitive to light intensity and quality, which leads to changes of anthocyanins accumulation. This change is very likely associated with circadian rhythm. First, we found that circadian rhythm biological pathway was the most significantly enriched in the list of DEGs according to both GO and KEGG analysis (Fig. 3A-B). In Arabidopsis, it is much known about molecular basis of circadian clock (McClung 2008; Harmer 2009). The key loop includes two partially reductant MYB domain transcription factors, CIRCADIAN CLOCK ASSOCAITED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY). These three proteins form a negative arm that binds to TIMING OF CAB EXPRESSION1 (TOC1) promoter (Harmer 2009). Interestingly, TOC1 could interact with CCA1 HIKING EXPENITION (CHE, i.e., TCP15

reported in our study listed in Table S6), hence inhibits CCA1 gene expression (Pruneda-Paz et al., 2009). Consistently, we found that the expression of TCP15 is dramatically enhanced by shading treatment by transcriptomes analysis and qPCR (Fig. 5A; Table S4). In addition, TCP15 is reported as a negative regulator in anthocynins accumulation (Viola et al., 2016), which is also related to the alteration of circadian rhythm pathway, and hence the changes of metabolites contents involving in carbon metabolism and citrate cycle (Kim et al., 2017; Carroll et al., 2019). The enhanced TCP15 gene is related to increased expression of some genes involved in GA pathway, such as Bra002790, Bra005177, Bra005899 and Bra009207 (Table S4; Fig. 8B). The anthocynins degradation under shading condition is mainly ascribed to inhibitory effects of TCP15. Notably, some transcription factors are also reported as negative regulators, such as MYB3, MYBL2 and CPC for anthocynins accumulation (Dubos et al., 2008; Zhu et al., 2009; Table S6).

In summary, we proposed a mechanistic model that underlies anthocyanin accumulation in the stalk of XH1 due to shading treatments. Most metabolites and regulatory genes involving in anthocyanins biosynthesis metabolic pathway were downregulated by shading treatments, which explains well the inhibition of anthocyanin by shading treatment (Fig. 8A). In addition, shading treatments inhibits the expression of some genes related to circadian rhythm MYBs modules. This module containing CCA and LHY inhibits the TOC1 gene expression by targeting its ci-element on promoter (Harmer 2009). TOC1 gene inhibits TCP15 expression. Genes related to Gibberellin (GA) signaling promotes TCP15 the expression. TCP15 is a negative regulator of anthocyanin. Therefore, low light condition stimulates the expression of TCP15 leading to the inhibition of anthocyanin biosynthesis.

Declarations

Competing Interests

The authors declare no competing interests.

Author contributions

Conceptualization, G.J., W.T, K.Y., Methodology, W.T., W.T., F.M., Investigation, W.T., W.T., F.M., L.G., L.W., Statistical analysis, F.M., L.G., L.W., K.Y., Writing, G.J., W.T., Funding Acquisition, G.J., W.T., K.Y., Resources, G.J., W.T, Supervision, G.J., W.T, K.Y.

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Data availability

The sequencing data generated in this study have been deposited in the NCBI GEO (Gene Expression Omnibus) database under accession code SRR18150630.

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Figures

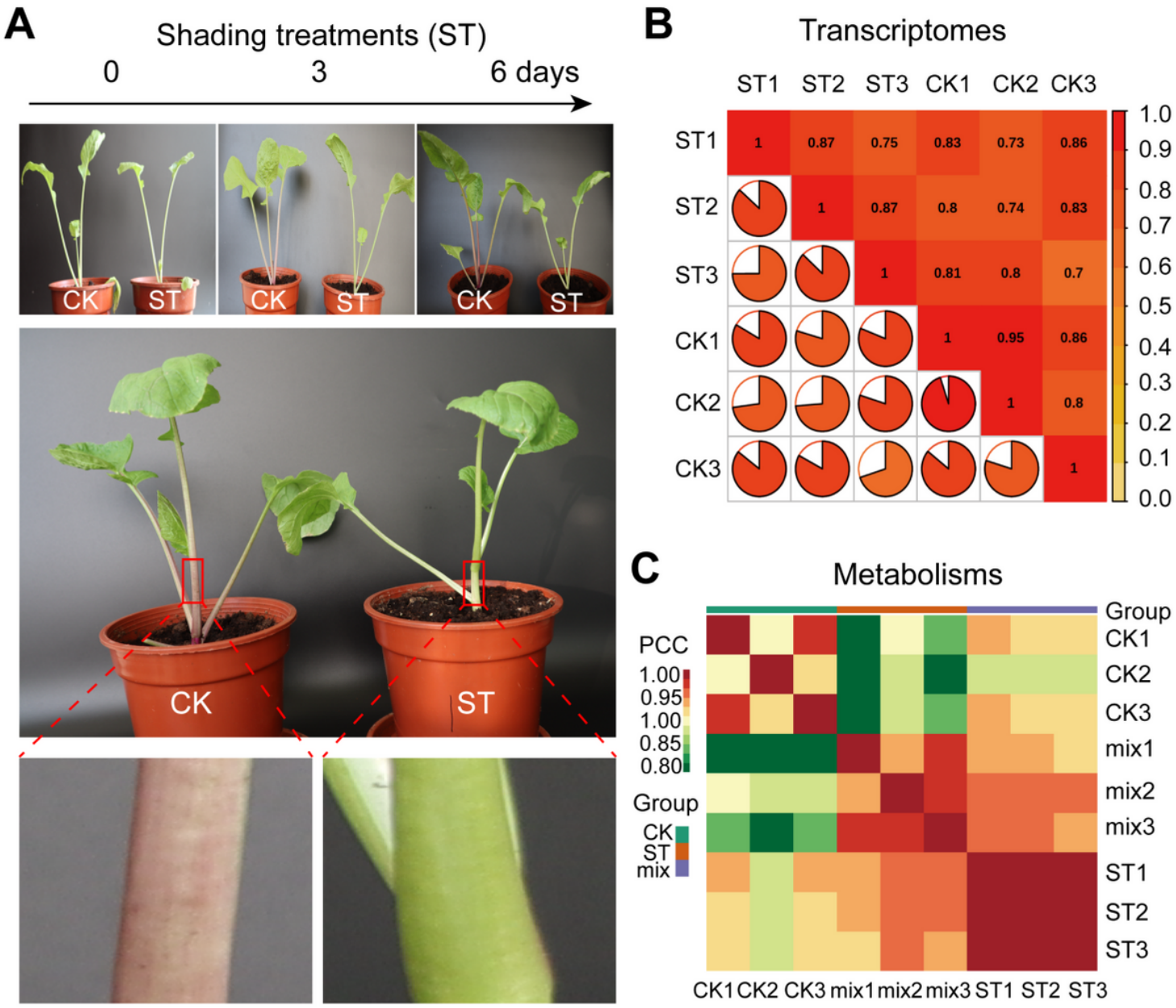

Figure 1

Transcriptome and metabolic analysis in shading treated stalk of *brassica rapa* L. **A**, images of XH1 exposed to shading treatments (ST) for different days and enlarged images showing the differences of anthocyanin accumulation in the stalk of XH1. Normal light promotes anthocyanin accumulation rather than shading treatments. **B-C**, Pearson correlation plot for transcriptome and metabolism, respectively. Different color represents the ranged values of Pearson correlation coefficient between samples.

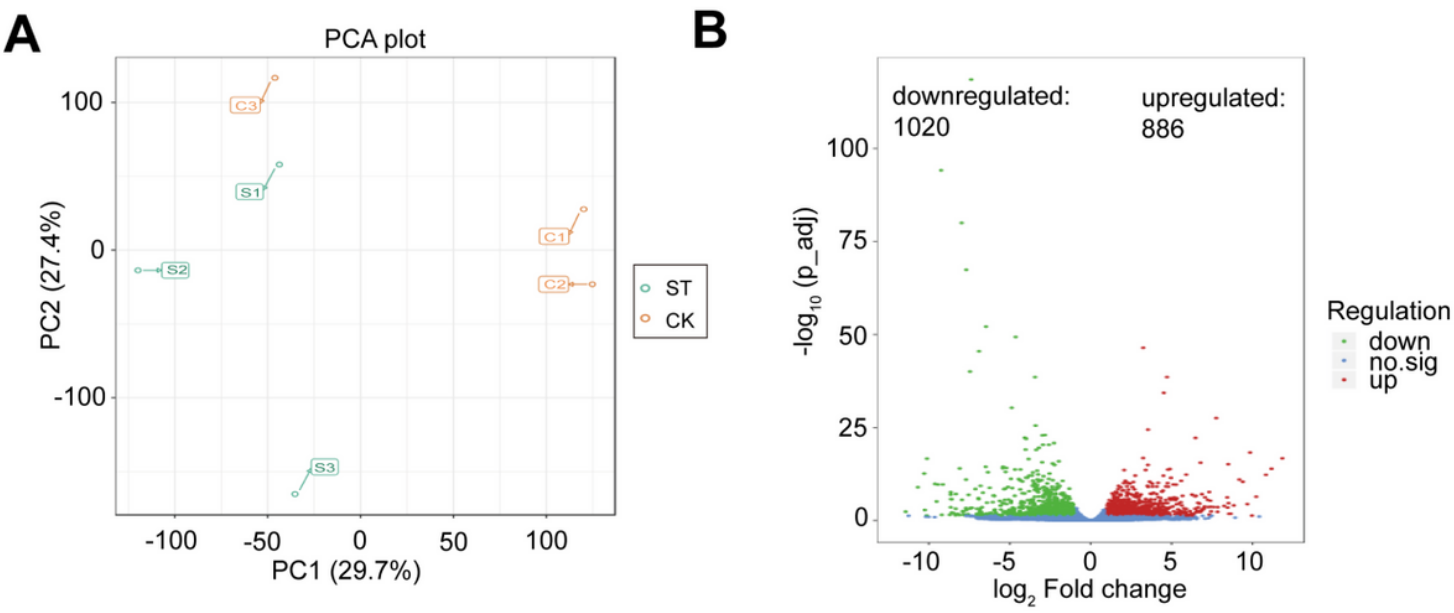

Figure 2

Shading treatment induced gene expression in the stalk of XH1. **A,** Principal component analysis on the global gene expression in the stem of XH11 induced by shading treatment. **B,** volcano plot representing the differentially expressed genes induced by shading treatment.

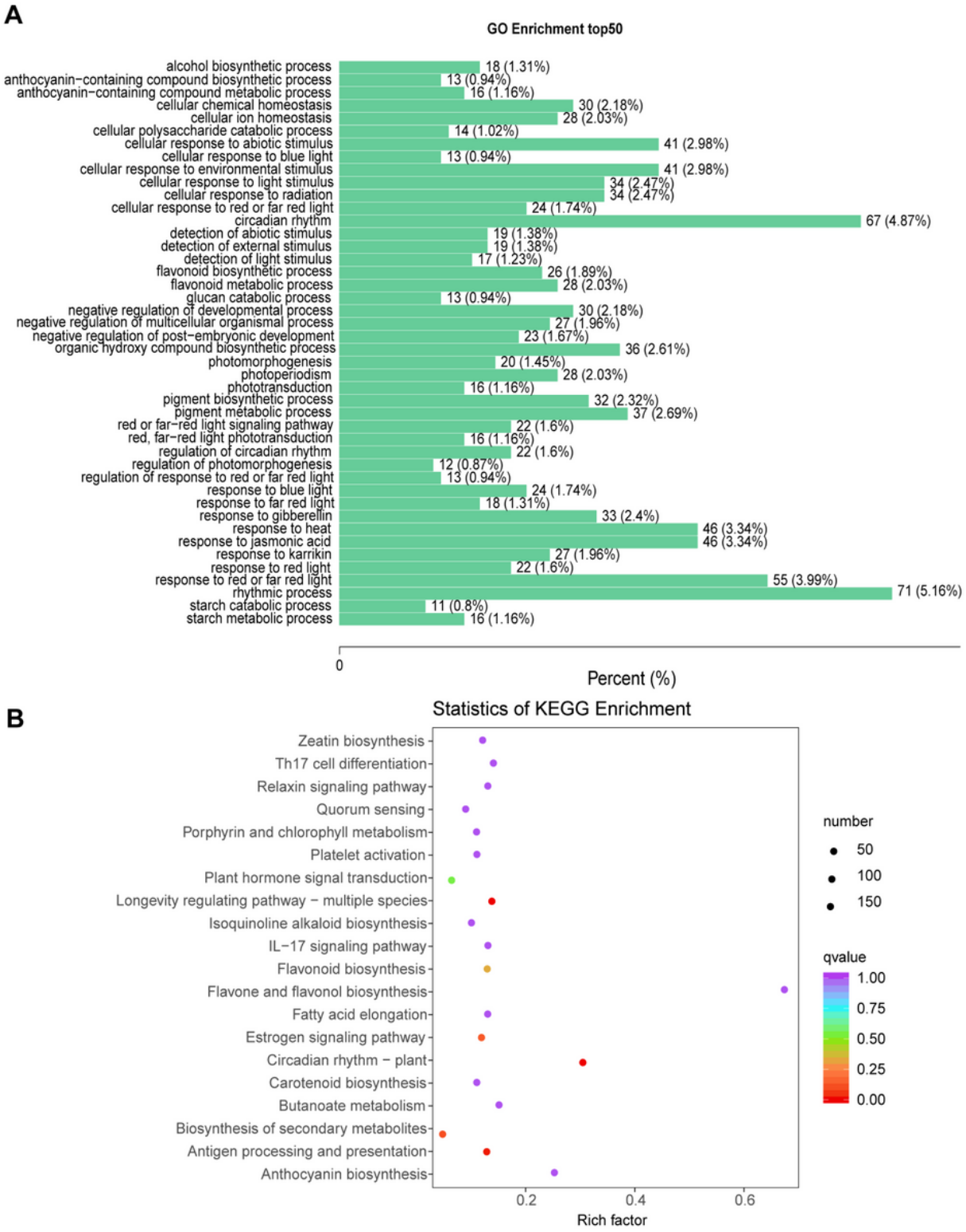

Figure 3

Bioinformatic analysis on DEGs induced by shading treatments in the stalk of XH1. **A,** top 50 terms based on Gene Ontolog enrichment analysis. **B,** KEGG analysis on differentially abundant metabolites.

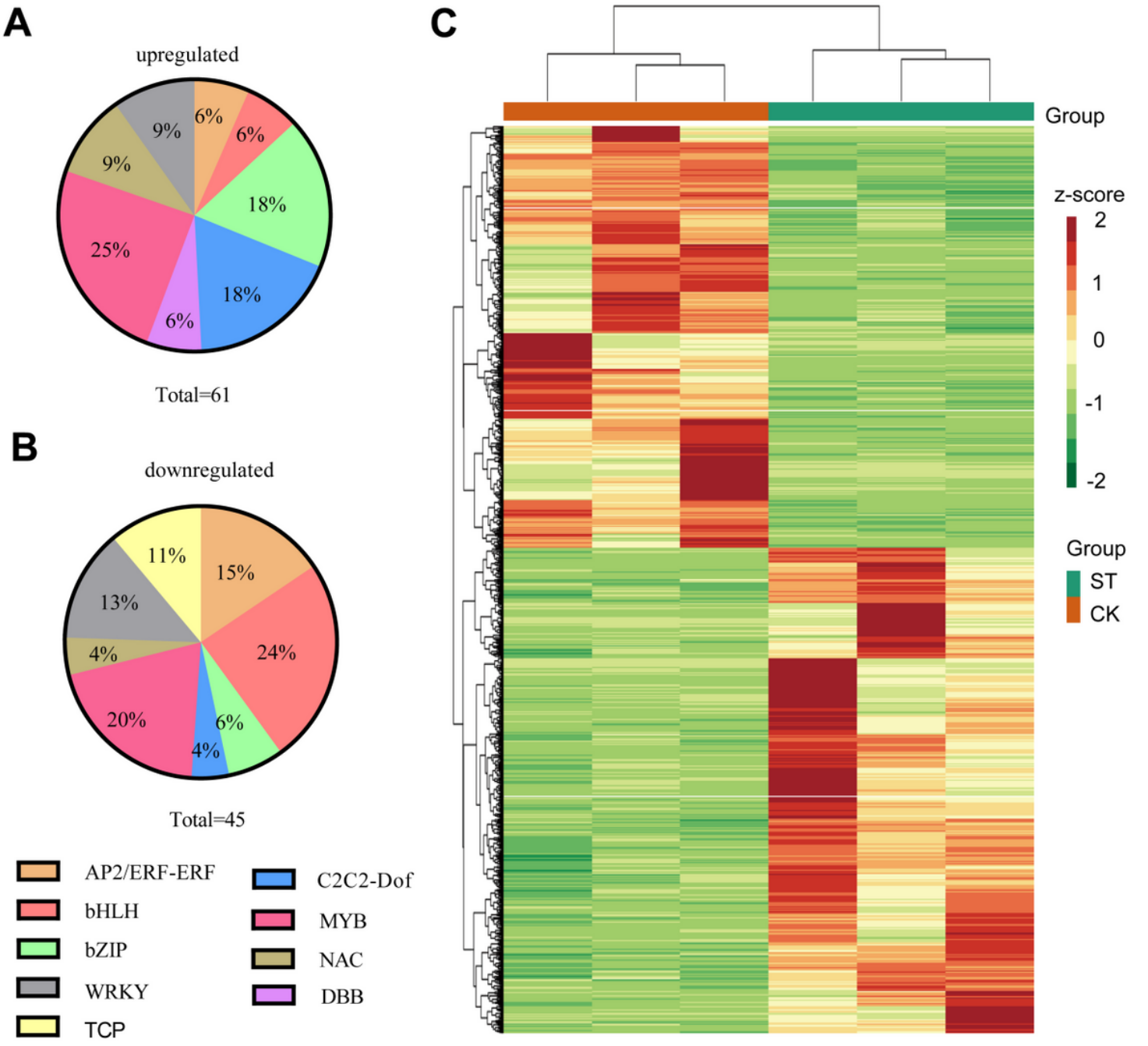

Figure 4

Distribution of transcription factors gene family with differential expression induced by shading treatments. **A,** upregulated. **B**, downregulated. **C**, heatmap representing the differentially expressed genes related to anthocyanin biosynthesis pathway induced by shading treatment (ST) and control (CK).

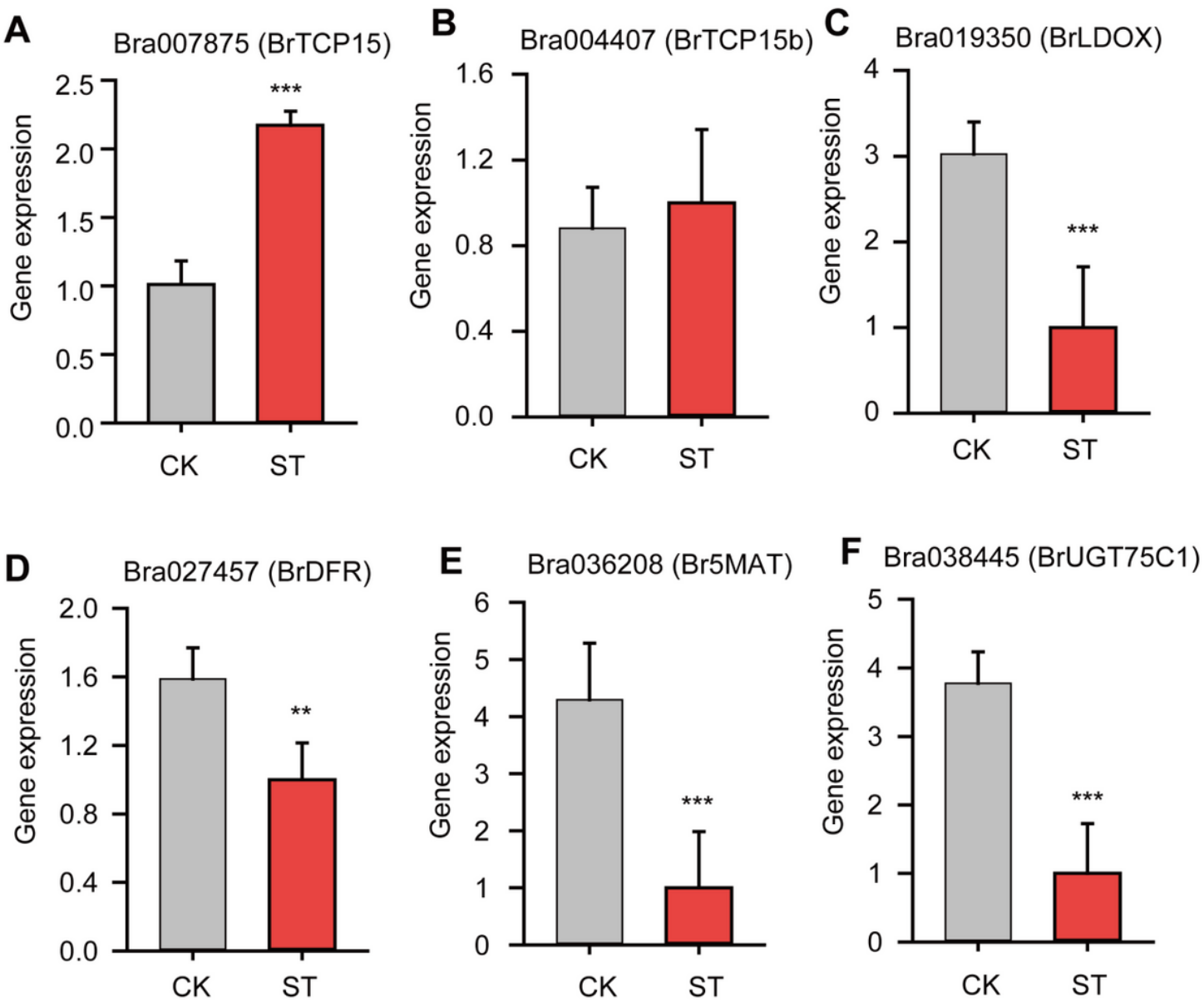

Figure 5

Differential expression of key genes induced by shading treatments in *brassica rapa* L. A-F, qPCR testing the expression of genes, including Bra007875, Bra004407, Bra019350, Bra027457, Bra036208 and Bra038445, respectively. The qPCR experiments were conducted with three biological replicates, and three technical replicates for each biological replicate. The significant levels were determined between control and shading treated samples using t-test, at $P<0.01$, and 0.001 indicated by "**" and "***", respectively. The primer of each gene was listed in Table S1.

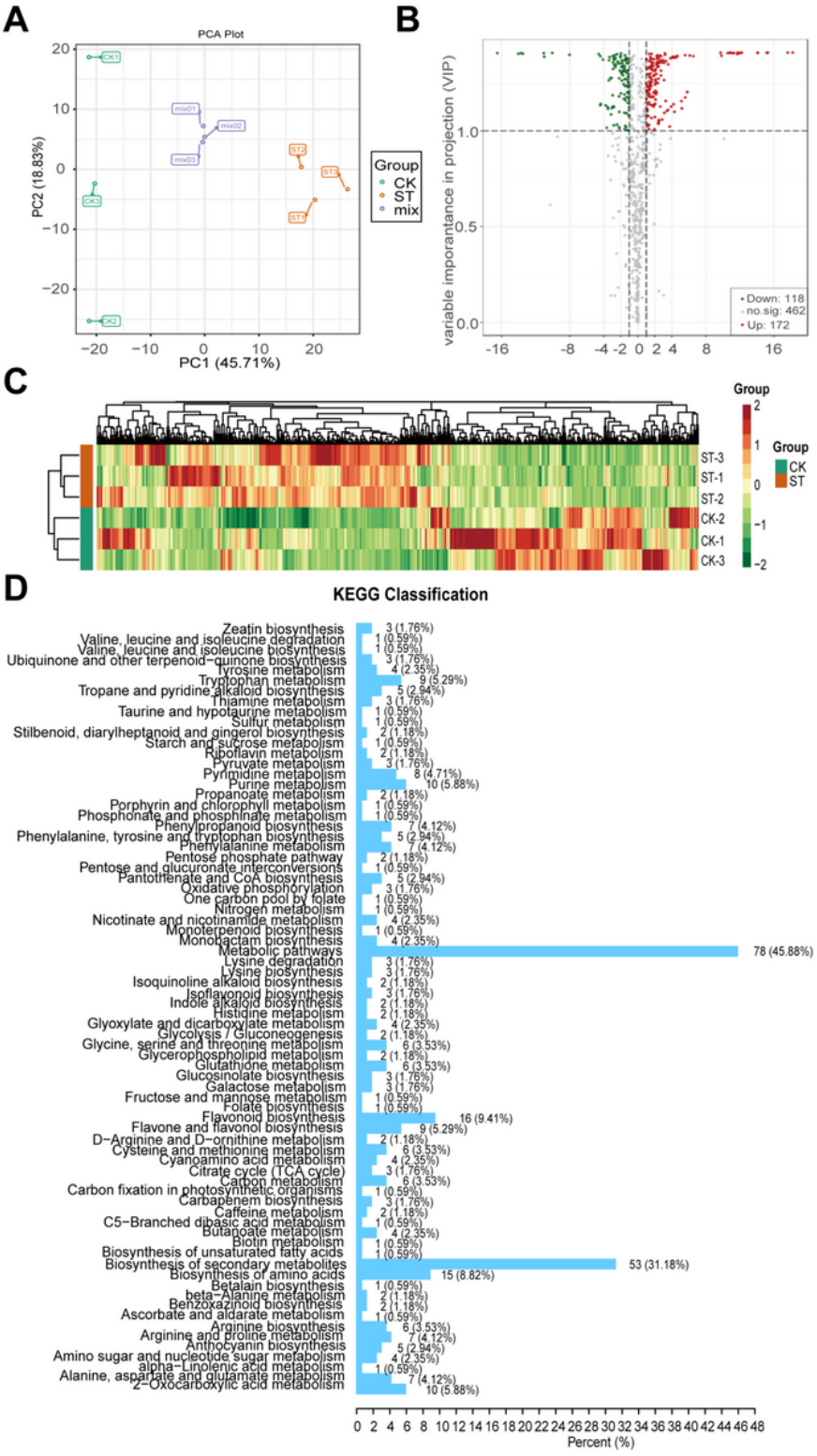

Figure 6

Differentially abundant metabolites induced by shading treatments in the stalk of XH1. **A,** principal component analysis across samples. **B,** valcono plot representing the differentially abuntant metabolites (DAMs). **C,** heatmap showing DAM induced by shading treatments. **D,** KEGG analysis on DAMs induced by shading treatments.

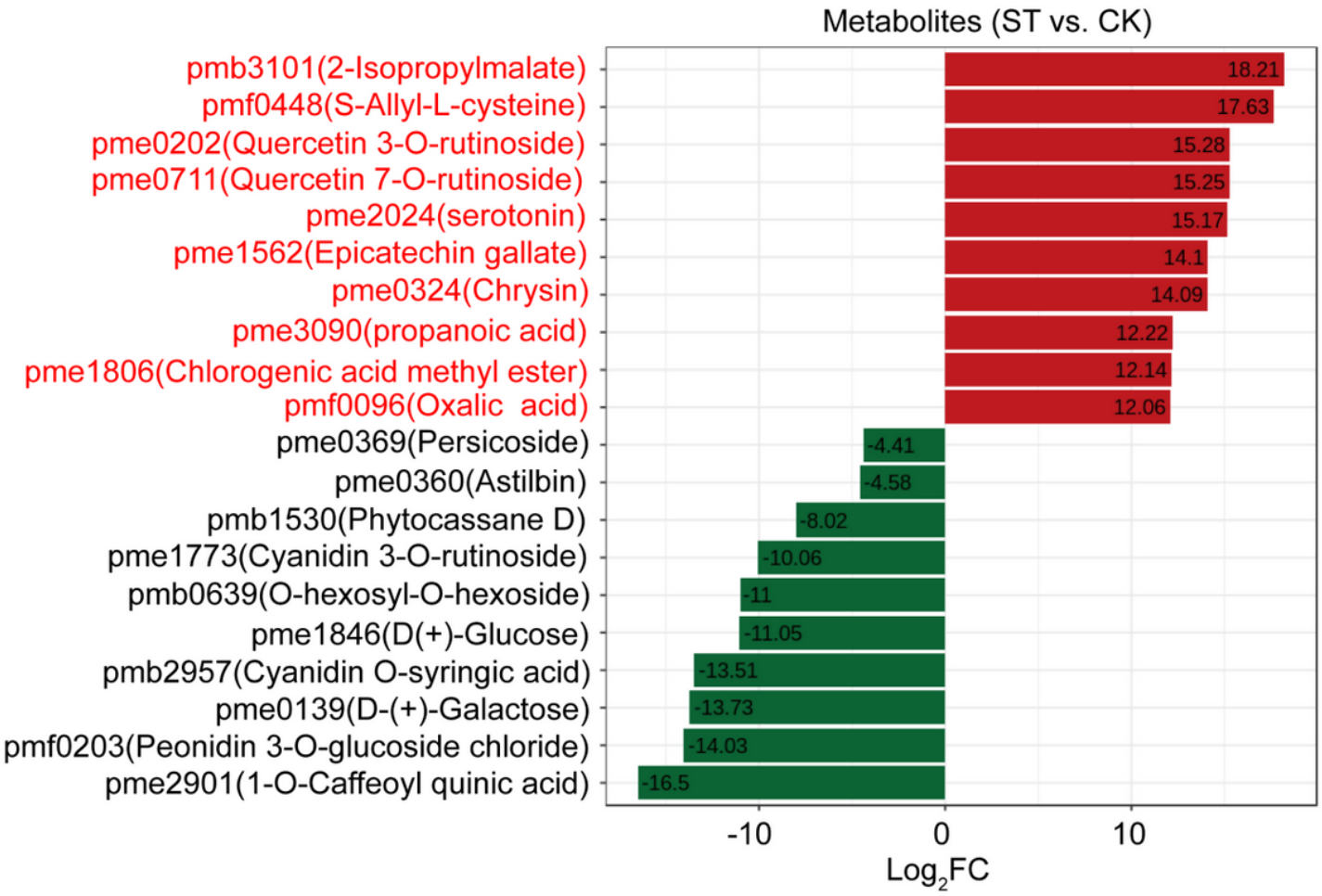

Figure 7

heatmap representing the changes of metabolites related to anthocyanin metabolic pathway in response to shading treatments. The detailed abundance of metabolites was referred to Table S8.

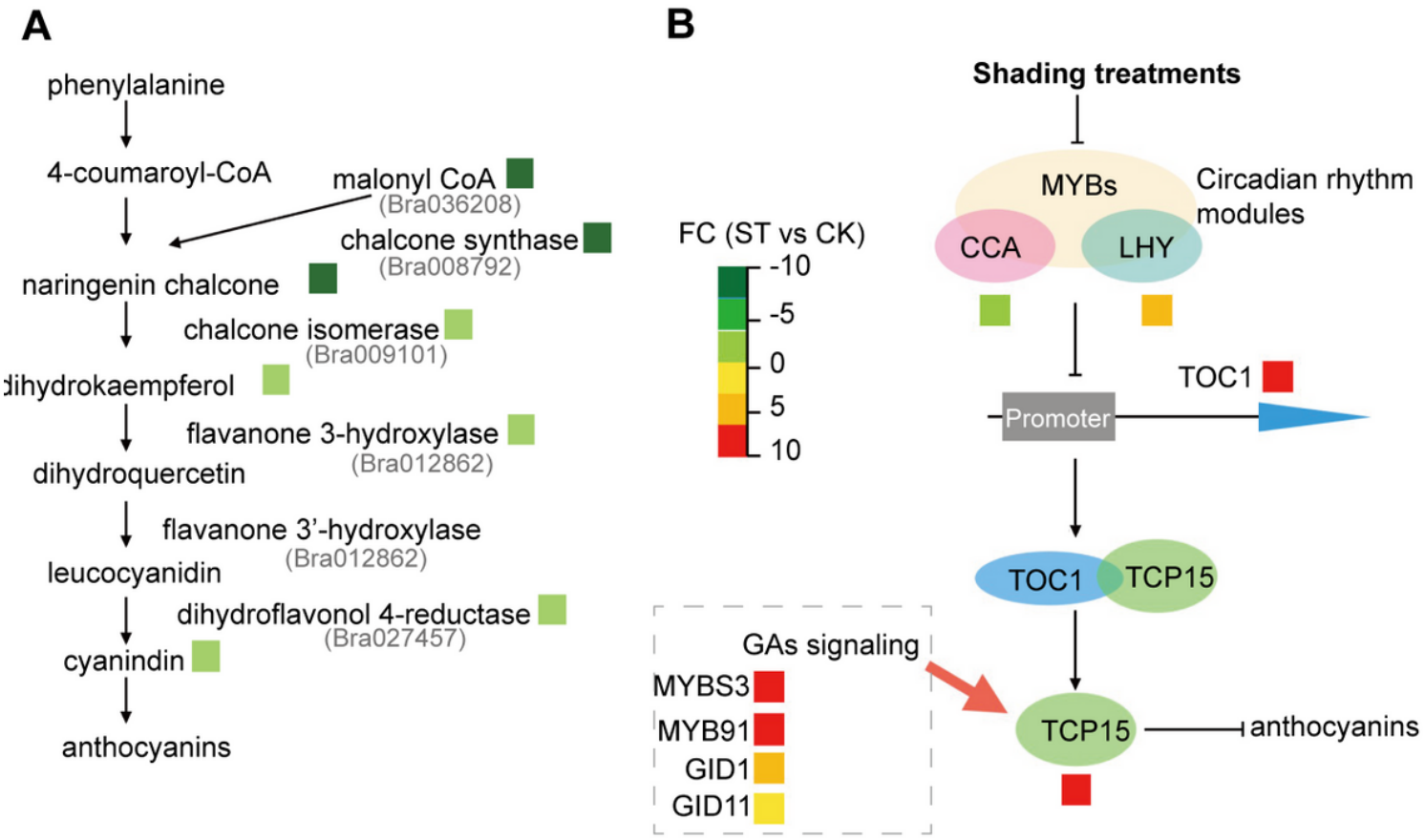

Figure 8

working model showing anthocyanin metabolic pathway in response to shading treatments. **A**, Anthocyanin biosynthetic pathway in response to shading treatments in *brassica rapa* L. **B**, Proposed molecular mechanism that circadian rhythm participles in anthocyanins metabolism through TCP15 interaction. The gene ID of MYBS3, MYB91, GID1 and GID11 are Bra002790, Bra005177, Bra005899 and Bra009207, respectively. CCA, LHY and TOC1 are Bra004503, Bra028258 and Bra035933, respectively. All the differentially expressed genes were listed in Table S4.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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