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CmHY5 functions in apigenin biosynthesis by regulating Flavone synthase II expression in chrysanthemum flowers

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Research Article

Keywords: Chrysanthemum, flower, apigenin, HY5, flavone synthase II

Posted Date: August 29th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1789992/v1

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Abstract

Chrysanthemum (*Chrysanthemum morifolium*) flowers have been used for centuries as functional food and in herbal tea and traditional medicine. The chrysanthemum flower contains significant amounts of the biologically active compound flavones, which has medicinal properties. However, the mechanism regulating flavones biosynthesis in chrysanthemum flowers organs is still unclear. Here, we compared the transcriptomes and metabolomes of different floral organs between two cultivars with contrasting flavone levels in their flowers. We identified that the predominant flavones in the ray florets of chrysanthemum flowers are apigenin and its derivatives, of which the contents are highly correlated with the expression of flavones synthase II gene *CmFNSII-1*. We also determined that CmHY5 is a direct upstream regulator of *CmFNSII-1* transcription. We showed that *CmHY5* RNAi interference lines in chrysanthemum have lower contents of apigenin compared to wild-type chrysanthemum. Our results demonstrated that CmHY5 participates in flavone biosynthesis by directly regulating the expression of *FNSII-1* in chrysanthemum.

Key Message

Our results determined that CmHY5 is a direct upstream regulator of *CmFNSII-1* transcription. Meanwhile, *CmHY5* RNAi interference lines in chrysanthemum have lower contents of apigenin compared to wild-type chrysanthemum.

1. Introduction

Flavonoids are secondary metabolites that exist in various parts of plants, especially in flowers and leaves. Flavonoids can be divided into several classes according to their structures: flavones, flavonols, flavanones, flavanols, isoflavones, and anthocyanins (Andersen & Markham, 2006). As one of the largest groups of flavonoids, flavones have diverse functions and play important roles in protection against ultraviolet (UV) irradiation (Schmitz-Hoerner & Weissenböck, 2003), interaction with other organisms (Peters et al., 1986; Kong et al., 2007), auxin transport (Mathesius et al., 1998), copigmentation with anthocyanins (Yoshida et al., 2009), and lignification (Lan et al., 2015). In addition, flavones provide nutritional value and are beneficial to human health since they have many antioxidant properties, as well as anticancer, antimicrobial, anti-inflammatory, and antiviral activities (Hostetler et al., 2017).

Apigenin is one of the most widely distributed flavones and is abundant in a variety of vegetables and fruits, such as parsley (*Petroselinum crispum*), celery (*Apium graveolens*), chamomile (*Matricaria chamomilla*), oranges (*Citrus* × *sinensis*), thyme (*Thymus vulgaris*), and onions (*Allium cepa*), as well as in beverages derived from plants, such as tea, beer, and wine (Min et al., 2016; Zhou et al., 2016). Apigenin in plants typically exist in glycosidic forms, which are catalyzed by glycosyltransferases. The modification confers structural complexity, and is critical for the storage and transportation of apigenin in

plants (Hostetler et al., 2017). Many studies conducted over the past several years have demonstrated that apigenin has potential antioxidant, anti-inflammatory, and anticancer properties (Shukla & Gupta, 2010).

As a branch of the flavonoid biosynthesis pathway, the apigenin biosynthesis pathway (ABP) initiates from the formation of naringenin chalcone, which is catalysed by chalcone synthase (CHS). Naringenin chalcone is then converted to naringenin by chalcone isomerase (CHI). Flavone synthases (FNSs) then convert naringenin into apigenin. FNS enzymes include FNSI and FNSII in dicots. FNSI has only been reported in the Apiaceae, rice (*Oryza sativa*), maize (*Zea mays*), and Arabidopsis (*Arabidopsis thaliana*) (Kim et al., 2008; Ferreyra et al., 2015; Qi et al., 2018; Righini et al., 2019), while FNSII is widely distributed in dicots (Martens and Mithöfer, 2005). FNSII is an oxygen- and NADPH-dependent cytochrome P450 (CYPs) membrane-bound monooxygenase (Martens & Mithöfer, 2005). The participation of FNSII in apigenin biosynthesis has been demonstrated in various plant species, including *Lonicera japonica*, *Lonicera macranthoides*, *Glycine max*, and *Chrysanthemum* (Fliegmann et al., 2010; Wu et al., 2016; Jiang et al., 2019; Wang et al., 2021). Extensive studies have been showed that the expression of structural genes in flavonoids biosynthesis is regulated in an independent or combinatorial manner by MYB and basic helix-loop-helix (bHLH) transcription factors, as well as WD40 proteins (Hichri et al., 2011). GtMYBP3 and GtMYBP4 in gentian flower, and MYB transcription factors P1 and P2 in maize positively regulate flavone biosynthesis (zhang et al., 2003; Li et al., 2016). In chrysanthemum, CmMYB012 negatively regulate flavone biosynthesis in response to high temperature (Zhou et al., 2021).

The basic leucine-zipper (bZIP) transcription factor ELONGATED HYPOCOTYL5 (HY5) plays diverse roles in plant development, including cell proliferation, chloroplast development, and pigment accumulation (Koornneef et al., 1980; Oyama et al., 1997a; Jing et al., 2013; Gangappa & Botto, 2016). In addition, HY5 has been reported to promote anthocyanin accumulation by directly regulating the expression of anthocyanin biosynthetic genes, including *CHS*, *CHI*, and *FLS* (Shin et al., 2007; Oyama et al., 1997b; Holm et al., 2002; Shin et al., 2007; Song et al., 2008; Stracke et al., 2010). However, whether HY5 contributes to the accumulation of flavone is still unknown.

Chrysanthemum (*Chrysanthemum morifolium*) is an important ornamental crop worldwide whose flowers have been used in Chinese herbal medicine, for making tea, and as functional food for thousands of years (Do Yeon et al., 2009; Yuan-Yuan et al., 2012; Li et al., 2019). Apigenin is the major active component in chrysanthemum flowers. Here, we investigated how apigenin biosynthesis is regulated in chrysanthemum flowers. Our results demonstrated that CmHY5 can directly regulate the expression of *CmFNSII-1* to modulate flavone biosynthesis.

2. Materials And Methods

2.1.1 Plant materials and growth conditions

Chrysanthemum cultivars (*Chrysanthemum morifolium*) and wild chrysanthemum (*Chrysanthemum indicum*) were propagated by tissue culture. Chrysanthemum young branch cuttings were grown on 1/2 Murashige and Skoog (MS) medium for 45 days. The plantlets of wild chrysanthemum were planted into a mixture of 1:1 (v/v) peat and vermiculite and growth in a growth chamber (22°C ± 2°C, 20–45% relative humidity, and an 8/16 h light/dark photoperiod).

For transcriptome and metabolomic analyses, two cultivars were cultivated in a greenhouse at day/night temperatures of 24°C/18°C, 40% relative humidity, and 8/16 h light/dark photoperiod. Floral bud and organs (ray florets and disk florets) at different developmental stages (S1, S2, S3) were harvested as three biological replicates. The samples were divided into two parts: one for transcriptome analysis and the other for metabolomic analysis.

2.2. Metabolite profiling

The sample preparation, extraction, metabolites identification and quantification were carried out according to the standard procedures described by Peng et al., 2017 and performed at Wuhan MetWare Biotechnology Co., Ltc. (www.metware.cn). Metabolites were qualified following the method described by Chen et al., 2013. Flavonoids were quantified according to standards of apigenin. The detailed information of apigenin is showed in Supplemental Table S1.

2.3. Transcriptome deep sequencing (RNA-seq) analysis

Total RNA was extracted from the flowers of the cultivars ‘14_C_1’ and ‘Yutai’ at three developmental stages after flowering (three independent plants for each stage and replicate) using the Plant RNA Extraction Kit (TaKaRa, Japan). Thirty libraries were prepared and sequenced on an Illumina HiSeq 4000 instrument by Gene Denovo Biotechnology Co. (Guangzhou, China) (Zhong et al., 2011). The workflow of data processing, assembling, and annotation was described by Yang et al., 2014.

2.4. Genes cloning and phylogenetic analysis

Total RNA was extracted from chrysanthemum flowers at different developmental stages using the Plant RNA Extraction kit (TaKaRa, Japan). cDNAs for cloning were synthesised using PrimeScript™ RT Master Mix (TaKaRa, Japan). Genes were amplified with PrimeSTAR HS DNA Polymerase (TaKaRa, Japan), according to the manufacturer’s instructions. Amino acid sequence alignment and phylogenetic analysis were performed in MEGA 6.0 software with neighbour-joining method running 1,000 bootstrap replications (Tamura et al., 2013).

2.5. Reverse transcription quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from chrysanthemum flowers using the Plant RNA Extraction kit (TaKaRa, Japan). 1μg of total RNA was used to synthesise first-strand cDNAs following the manufacturer’s instructions of the PrimeScript™ RT Master Mix (TaKaRa, Japan). RT-qPCR reactions (using 2 μL cDNA as the template) were performed using the StepOne Real-Time PCR System (Applied Biosystems) with the

TB Green Fast qPCR Mix (TaKaRa, Japan). The chrysanthemum *Ubiquitin* gene (GenBank accession NM_112764) was used as an internal control.

2.6. Yeast one-hybrid (Y1H) assay

Y1H assays were performed according to the Yeast Protocols Handbook (Clontech Laboratories, USA). *CmFNSII-1* promoter fragments were inserted into the pLacZi vector, and the coding sequences of *CmMYB32*, *CmMYB330*, *CmbHLH79*, *CmHY5*, *CmP*, and *CmBIM1* were individually cloned into the pJG4-5 vector. The plasmids were then transformed into yeast strain EGY48. Individual colonies were selected on synthetic defined (SD) medium lacking uracil and tryptophan and containing X-*β*-gal for blue/white screening.

2.7. Dual-Luciferase Assays

According to a previous protocol (Yin et al., 2010), the full-length coding sequences of *CmHY5* or *CmbHLH79* were cloned individually into the pGreen II 62-SK vector. The promoter fragment of *CmFNSII-1* was inserted into upstream of the firefly luciferase (*LUC*) report gene in the pGreen II 0800-LUC vector. The internal control invector pGreen II 0800-LUC is Renilla luciferase (*REN*) gene promoted by the cauliflower mosaic virus (CaMV) 35S promoter. The above plasmids were transiently infiltrated in pairs in *Nicotiana benthamiana* leaves via Agrobacterium (*Agrobacterium tumefaciens*)-mediated infiltration (strain GV3101). The activation of transcription was determined 3 days after infiltration and expressed as the ratio between LUC and REN activity, as measured on a Modulus Luminometer (Promega, USA). For each reporter/effector pair, each Agrobacterium culture was resuspended in infiltration buffer (10 mM 2-(N-Morpholino)-ethanesulfonic acid, 10 mM $MgCl_2$, 0.2 mM acetosyringone, pH 5.7) to a final OD_{600} of 1.0, before mixing 10 mL of the effector cell suspension with 1 mL of reporter cell suspension. As a negative control and for calibration, the empty vector SK was co-infiltrated with the reporter plasmids, and the resulting LUC/REN ratio was set to 1. After 48 h infiltration, the leaves were treated in the dark for 5 min before measuring LUC-derived luminescence by a CDD camera (CHEMIPROHT 1300B/LND, 16 bits; Roper Scientific, Sarasota, FLS, USA).

2.8. Chrysanthemum transformation

To construct the RNA interference (RNAi) plasmid, two 288-bp *CmHY5* fragments containing *Asc*I/*Swa*I and *BamH*I/*Pac*I sites were prepared respectively. Each fragment was cutted with the corresponding restriction enzymes and cloned into the pFGC1008 vector to form an intron-containing “hairpin” RNA (iphRNA) construct. The resulting plasmid was transformed into chrysanthemum according to the method described by Hong et al., 2006. Hygromycin-resistant primary transformants were screened by PCR to confirm the presence of the transgene.

2.9. Data availability

Raw RNA-seq data used in this study have been deposited at the NCBI SRA database under BioProject accession number PRJNA798227. All the primer sequences used in this study are listed in Supplementary Table S2.

3. Results

3.1. Transcriptomics and metabolomics analyses reveal the floral organ-specific accumulation pattern of flavone

To uncover the molecular network associated with flavone biosynthesis in chrysanthemum, we tested nine chrysanthemum cultivars for their contents of several flavones in flowers. We selected two cultivars ('14_C_1' and 'Yutai') with extreme flavone levels as materials in this study: high levels in 14_C_1 and low levels in Yutai (Fig. S1). We performed transcriptomic and untargeted metabolomic analyses of flower buds, and ray and disk florets at different opening stages (Fig. 1A). For transcriptomics, we assembled the clean RNA-seq reads into 67,285 unigenes. After filtering with expression levels rpkm > 5, 15350 unigenes were yeilded (Dataset S1). For metabolite profiling, we extracted compounds in methanol and analysed them using liquid chromatography-mass spectrometry (LC-MS), yielding 186 flavonoids including 103 flavones, 36 flavonols, 21 flavanones, 13 anthocyanins, and 13 isoflavone metabolites (Dataset S2). Separate principal component analyses (PCA) on the gene expression and metabolite datasets showed a primary separation of samples along floral organs, suggesting that the accumulation of flavonoids is organ specific (Fig. 1B). Among the 186 flavonoids detected here, we found that apigenin O-malonylhexoside, apigenin 7-O-glucoside, and apigenin were the most accumulated in ray floret (Fig. 1C). We also found that apigenin and its derivatives were specifically accumulated in ray floret, and had higher levels in 14_C_1 than Yutai. In addition, acacetin and luteolin also exhibited higher accumulation in 14_C_1. Moreover, luteolin were specifically accumulated in disk floret at stage 3 (Fig. 2).

3.2. Integrative analysis of the transcriptome and metabolome revealed the correlation of structural genes expression with the major flavonoids contents

Based on the KEGG pathway annotation of Unigenes and metabolites from transcriptome and metabolome datasets, we identified eight genes encoding enzymes associated with the flavonoid biosynthetic pathway (Fig. 3A). Among the eight putative structural genes, *CHS* and *FNSII* (Unigene0057769) were more highly expressed in ray florets than in disk florets, with higher expression levels in stage 3 ray florets of the cultivar 14_C_1 relative to Yutai. Moreover, the expression of *FLS* and *F3H* was higher in disk florets than in ray florets (Fig. 3B). Gene-to-metabolite correlations indicated that contents of apigenin and its derivative were tightly correlated with *FNSII* expression levels (Fig. 3C). The phylogenetic analysis of the predicted protein encoded by Unigene0057769 with other FNSII proteins determined that Unigene0057769 is most closely related to CmFNSII and CiFNSII, two known FNSII enzyme from *C. morifolium* and *C. indicum* (Jiang et al., 2019; Wang et al., 2021) (Fig. 4A). Accordingly, we renamed Unigene0057769 as *CmFNSII-1*. We also validated the RNA-seq results by RT-qPCR (Fig. 4B). Moreover, RT-qPCR results showed that *CmFNSII-1* reaches its highest expression levels in ray florets compared to other organs, such as roots, stems, leaves, and floral organs (Fig. 4C).

3.3. CmHY5 is a direct upstream regulator of *CmFNSII-1* transcription

To investigate the regulatory mechanism of *CmFNSII-1*, we performed a co-expression analysis between genes encoding transcription factors and *CmFNSII-1*. Genes from the transcription factor gene families MYB, bZIP, basic helix-loop-helix (bHLH), WRKY, and MADS showed relatively high co-expression (Pearson's correlation coefficient $r > 0.6$) with *CmFNSII-1* (Fig. 5A, Dataset S3). To narrow down which transcription factor(s) can directly regulate the expression of *CmFNSII-1*, we cloned a 2-kb *CmFNSII-1* promoter fragment and looked for putative *cis*-acting regulatory elements in the PlantCARE database (Fig. S2) (Lescot et al., 2002). The promoter of *CmFNSII-1* contained MYB-binding sites, MYC-binding sites, and *cis*-acting regulatory elements involved in light responsiveness, such as G-box and GATA-motif (Fig. S2). Based on co-expression analysis and putative *cis*-elements along the *CmFNSII-1* promoter, we selected six candidate transcription factors whose gene expression patterns positively correlated with apigenin contents (Fig. 5B). We tested their binding to the *CmFNSII-1* promoter by Y1H assay. We established that CmbHLH79 and CmHY5 can bind to the proximal 1-kb fragment (P1) of the *CmFNSII-1* promoter (Fig. 5C). We also conducted a dual-luciferase (LUC) reporter assay in *N. benthamiana* leaves to assess the regulation of the *CmFNSII-1* promoter by CmbHLH79 and CmHY5 *in vivo*. We observed that leaf cells expressing *CmHY5* dramatically activate the transcription of the *CmFNSII-1pro:LUC* reporter (Fig. 5D). Indeed, expressing the *CmHY5* effector construct resulted in a 2.2-fold increase in LUC activity from the *CmFNSII-1pro:LUC* reporter construct, whereas the *CmbHLH79* effector construct can not increase LUC activity (Fig. 5D).

3.4. Silencing *CmHY5* reduces apigenin contents in floral organs

To investigate whether CmHY5 influences the production of flavones in chrysanthemum, we silenced *CmHY5* in wild chrysanthemum (*C. indicum*), leading to the isolation of 10 *CmHY5*-RNAi lines. We confirmed lower *CmHY5* transcript levels in the RNAi lines by RT-qPCR (Fig. 6A). We noticed that the RNAi lines were taller than wild-type (WT) non-transgenic plants (Fig. 6B and 6C), while we observed no significant difference in flower diameter between WT and RNAi lines (Fig. S3). In addition, RT-qPCR results indicated that except of *FLS*, the expression of *CmFNSII-1* and other structural genes was significantly reduced in *CmHY5*-RNAi plants compared to WT plants (Fig. 6D). We also measured the contents of apigenin and apigenin-7-O-glucoside by LC-MS, which revealed that RNAi lines accumulated less apigenin and apigenin-7-O-glucoside in their leaves and flowers than the WT (Fig. 7). Taken together, our results demonstrated that CmHY5 is involved in flavone biosynthesis by regulating the expression of *CmFNSII-1* in chrysanthemum flowers.

4. Discussion

The expression of genes participating in the same biosynthesis pathway is often coordinately regulated, which can be assessed by generating co-expression modules. If the compounds of interest of these gene

products exhibit spatial, temporal, and/or condition-specific expression, the underlying co-expression patterns would become apparent after quantification of expression, as done here by RNA-seq. Therefore, the integration of transcriptomic and metabolomic datasets is a powerful tool to identify candidate genes responsible for the accumulation of specific metabolites. In this study, we implemented this approach to identify candidate regulatory genes involved in flavone biosynthesis in chrysanthemum flowers. We showed that flavonoids accumulated distinctively in disc florets and ray florets. In addition, apigenin and its derivatives accumulated abundantly in ray florets (Fig. 1B). Organ-specific accumulation of metabolites is of special importance for the survival and adaptation of plant species. Mounting evidence has illustrated the crosstalk between flavonoids and various phytohormones in various developmental processes including seed size and fertility (Ferreyra et al., 2012; Buer CS, 2013 Jul; Doughty et al., 2014; Maloney et al., 2014). Jang et al., 2019 and Wang et al., 2021 showed that the expression of *FNSII* and *FLS* in chrysanthemum were higher in flower than other tissues. However, the details of their expression patterns in flower organs have not been studied. In this work, we noted that *CHS* and *FNSII* transcripts reach high accumulation levels in ray florets, while those of *F3H* and *FLS* accumulated in disk florets (Fig. 3B), suggesting that flavonoids may play different roles in the development of disk and ray florets. Since chrysanthemum flower is composed of multiple individual ray florets and disk florets, and the details of the expression patterns of structural genes in florets will be beneficial for breeding of high flavonoids chrysanthemum cultivars.

Among the regulators of flavonoid biosynthesis, MYB transcription factors have been comprehensively studied (Liu et al., 2015). Recently, in chrysanthemum, a novel transcription factor *CmMYBB012* has been shown to inhibit flavone and anthocyanin biosynthesis by directly regulating *CmFNS* and other structural genes in response to high temperature (Zhou et al., 2021). In our co-expression analysis, we found that the expression of MYB gene families was highly correlated with the expression of *CmFNSII-1* (Fig. 5A). However, none of the MYB transcription factors that we tested, directly bind to the promoter of *CmFNSII-1*(Fig. 5B).

In addition, other potential regulators, which have been reported to be involved in the flavonoid pathway, such as bHLH, WRKY, MADS, and bZIP transcription factors (Ishida et al., 2007; Jaakola et al., 2010; Wang et al., 2016; Shi et al., 2018), are also found in the co-expression network (Fig. 5A). Yeast one-hybrid experiments showed that CmbHLH79 and CmHY5 can directly bind to the promoter P1 of *CmFNSII-1*. However, only CmHY5 can activate the transcription of *CmFNSII-1* (Fig. 5C and 5D), suggesting a role of CmHY5 in flavonoid biosynthesis.

To date, most of the genes and transcription factor genes involved in anthocyanin biosynthesis appear to be controlled by HY5 (Gangappa and Botto, 2016). By contrast, the functions and regulatory mechanisms of HY5 in flavone biosynthesis remain uncharacterised, perhaps because Arabidopsis does not harbour *FNS* genes in its genome (Martens and Mithöfer, 2005). In maize, two FNS enzymes participating in the biosynthesis of apigenin are regulated by UV-B, suggesting that FNS-catalysed apigenin accumulation might be dependent on the HY5 signalling pathway (Righini et al., 2019). In this study, yeast one-hybrid and dual-luciferase assays revealed that CmHY5 can activate the transcription of *CmFNSII-1* via directly

bind to its promoter, indicating that HY5 positively regulates flavone biosynthesis. The expression of *CHS*, *CHI*, *F3H*, and *F3'H* was downregulated in *CmHY5*-RNAi lines (Fig. 6D), which were in agreement with previous studies (Shin et al., 2007; Oyama et al., 1997b; Holm et al., 2002; Song et al., 2008; Stracke et al., 2010). In addition, *CmFNSII-1* was also downregulated in *CmHY5*-RNAi lines, which was consistent with the reduction of apigenin accumulation in *CmHY5*-RNAi lines (Fig. 6D, Fig. 7).

5. Conclusion

In this study, we compared the transcriptomes and metabolomes of different flora organs between two cultivars with contrasting flavone levels in their flowers. The analysis of the metabolome revealed the major flavonoids in the ray florets were apigenin and its derivatives, which were accumulated higher in 14_C_1 than Yutai. We further established that CmHY5 participates in flavone biosynthesis by directly regulating the expression of *CmFNSII-1* in chrysanthemum. Silencing of *CmHY5* reduced apigenin contents in floral organs and leaves. Our results provide a strategy of molecular breeding for improving the quality of medicinal chrysanthemum.

Declarations

Conflict of interest

The authors declare no competing financial interests.

Contributions

CM, CL, and CH conceived and designed the experiments. CL, LL, JZ, and YX performed experiments. HL, DC, XC, and JG provided technical support and conceptual advice. CM, CL, BH, and CH analysed the data and wrote the manuscript.

Funding

This work was supported by The Project of Science and Technology of Beijing Academy of Agriculture and Forestry Sciences (QNJJ202015) and the National Natural Science Foundation of China (Grant no. grant 31822045, 32072611), the Construction of Beijing Science and Technology Innovation and Service Capacity in Top Subjects (Grant no. CEFF-PXM2019_014207_000032, Beijing Municipal Education Commission).

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Figures

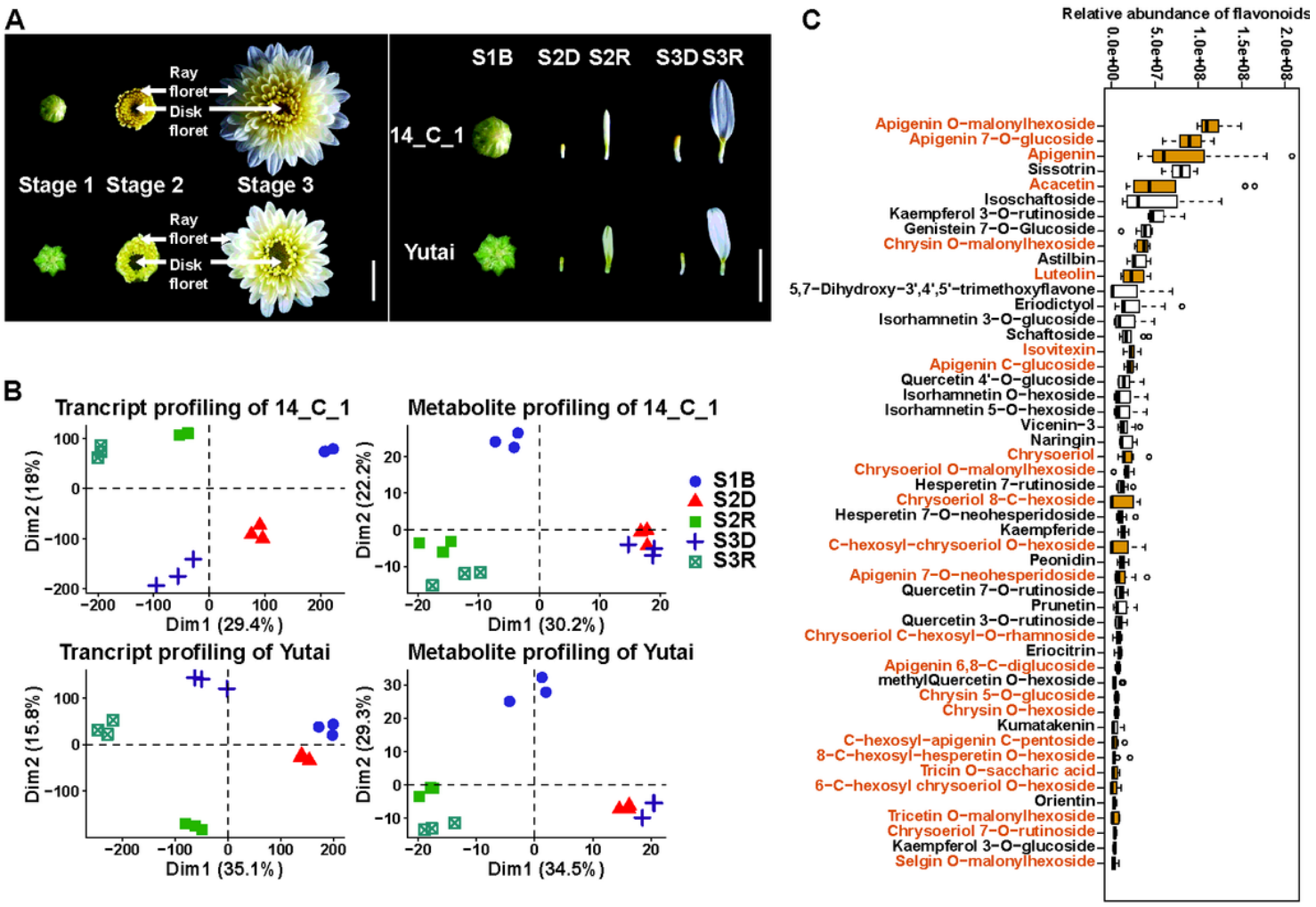


Figure 1

Transcriptomic and metabolic profiling of different floral organs. (A) Different opening stages and floral organs in chrysanthemum. S1B, bud at opening stage 1; S2D, disk floret at opening stage 2; S2R, ray floret at opening stage 2; S3D, disk floret at opening stage 3; S3R, ray floret at opening stage 3. Scale bars, 1 cm. (B) Principal component analysis (PCA) of transcriptomic and metabolic profiling datasets. Note: all organs form distinct clusters. (C) Relative abundant of top 50 flavonoids metabolites in ray florets of the two cultivars. Orange boxplots represent flavones. Line shows the median of flavonoids abundant. The bottom and top of the box show the 25th and 75th percentiles, respectively. Points are outliers. Whiskers indicate the data less than 1.5 times the interquartile range above the 75th percentile or below the 25th percentile.

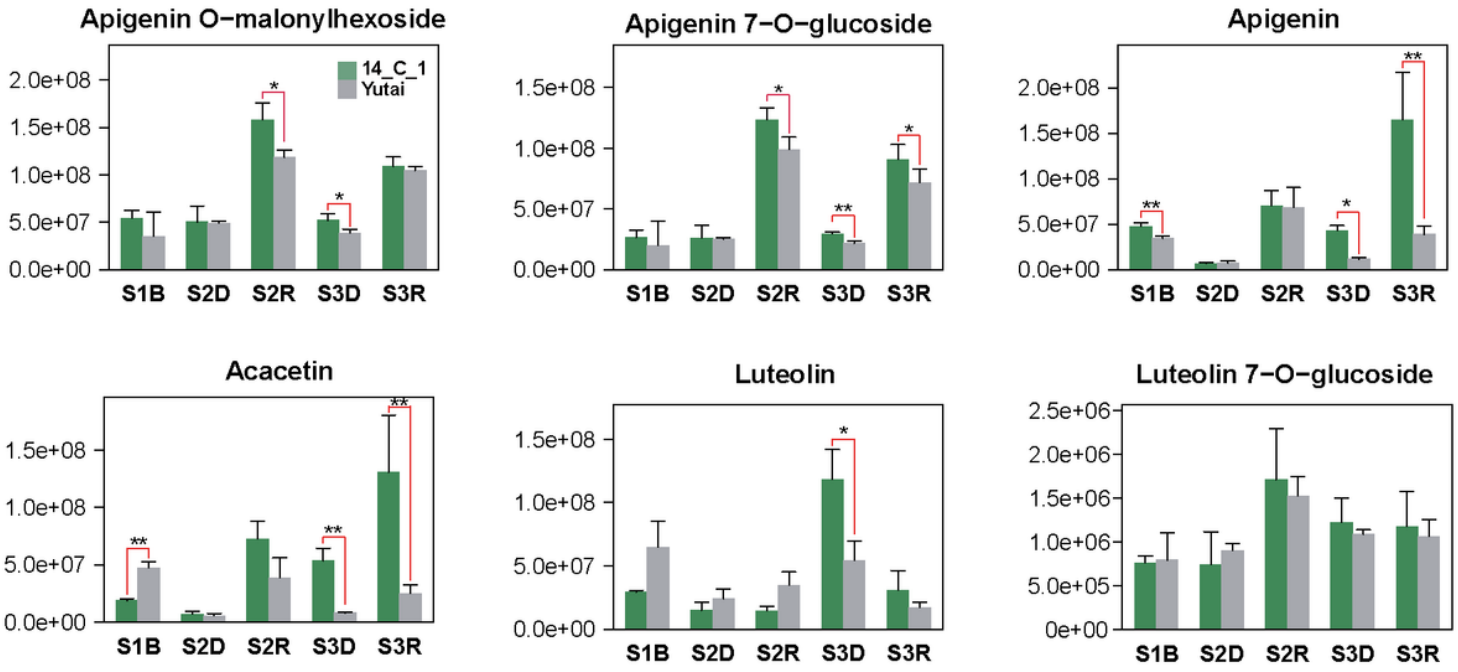


Figure 2

Levels of the major flavones in different floral organs of the two cultivars. Asterisks indicate statistically significant differences between the two cultivars, as determined by two-tailed Student's *t*-test (*P< 0.05,**P < 0.01).

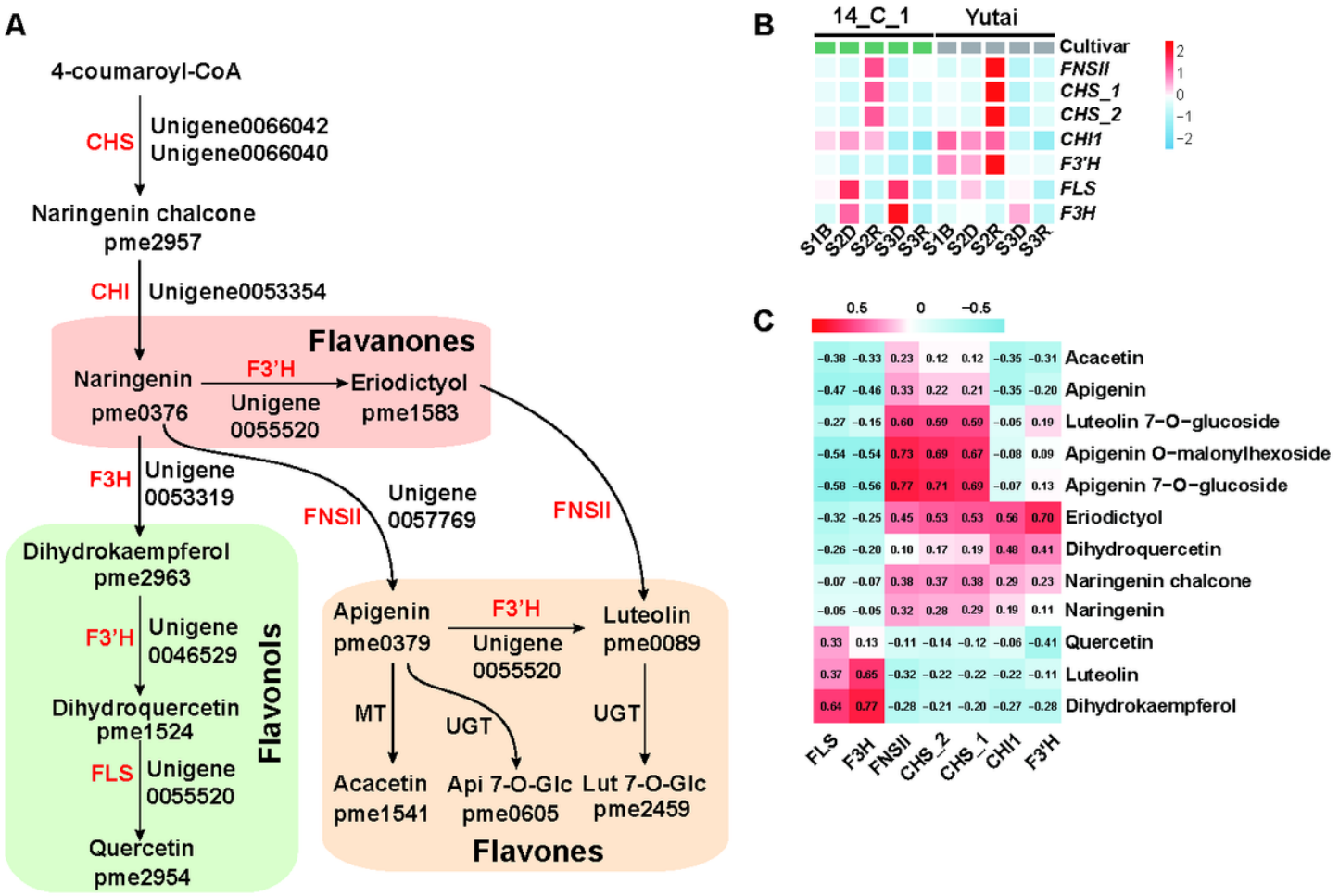


Figure 3

Integrative analysis of genes and metabolites of the flavonoid biosynthetic pathway. (A) Flavonoid biosynthetic pathway. Putative structural genes (red) identified by RNA-seq are indicated by their Unigene ID. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavanone 3'-hydroxylase; FNSII flavone synthase II; FLS, flavonol synthase; The metabolites identified by metabolomics are also listed with their index number below their respective dedicated enzymes: pme2957 (naringenin chalcone), pme0376 (naringenin), pme0379 (apigenin), pme0089 (luteolin), pme2963 (dihydrokaempferol), pme2954 (quercetin), pme1583 (eriodictyol), pme1541 (acacetin), pme0605 (apigenin 7-*O*-glucoside), pme2459 (luteolin 7-*O*-glucoside), pme 1524 (dihydroquercetin). (B) Heatmap representation of gene expression levels (in reads per kilobase of transcript, per million mapped reads [RPKMs]) of structural genes in different floral organs of the two cultivars. The heatmap represents Z-score normalised expression values. (C) Correlation analysis between the expression of structural genes and metabolite contents in the flavonoid biosynthetic pathway.

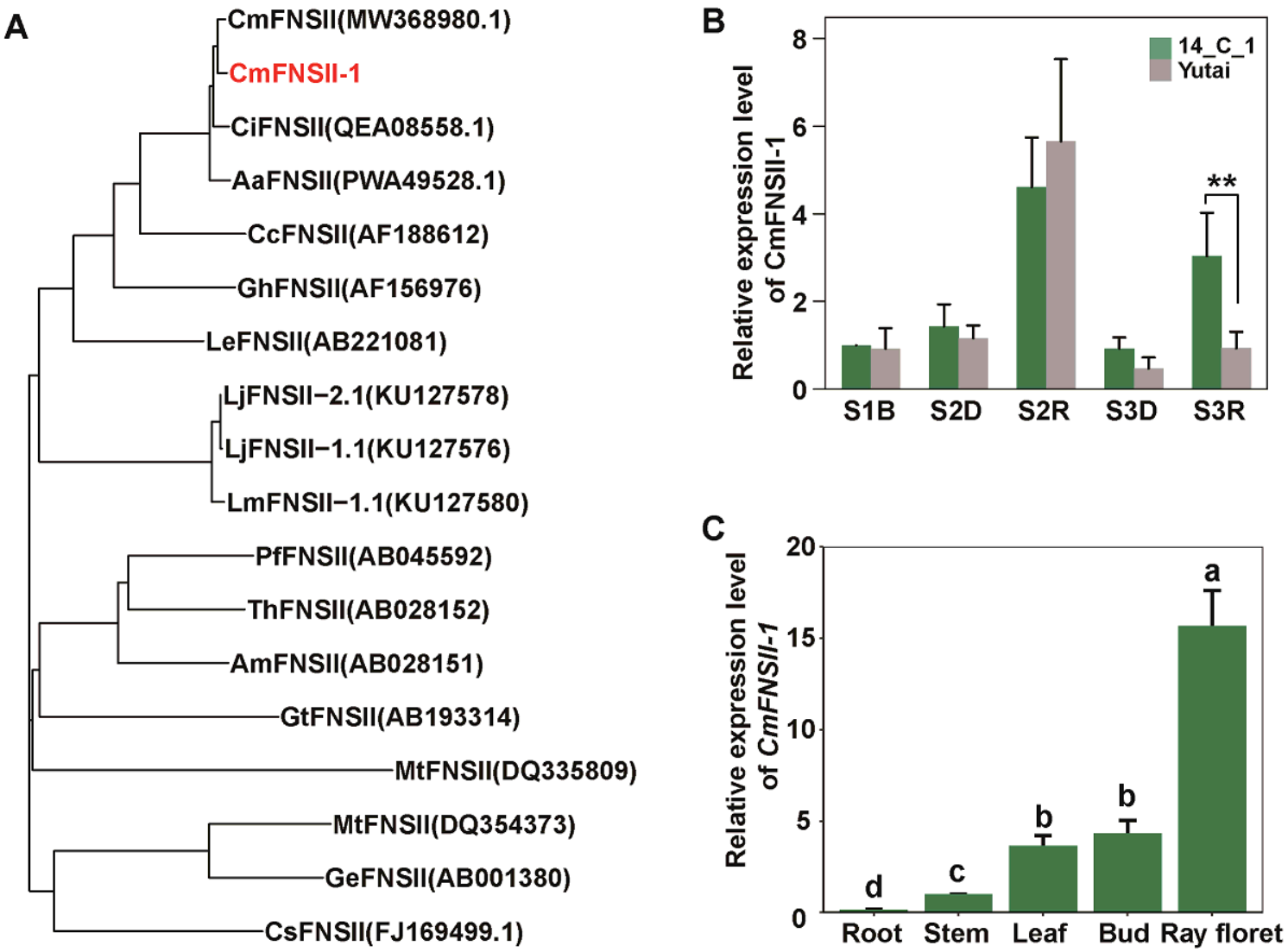


Figure 4

Phylogenetic and expression pattern analsyis of the *FNSII* from chrysanthemum.

(A) The evolutionary relationships of CmFNSII-1 and other FNSII proteins. (B) Relative

CmFNSII-1 expression levels in different floral organs of the two cultivars, as determined by RT-qPCR. (C) Relative *CmFNSII-1* expression levels in different floral organs of cultivar 14_C_1, determined by RT-qPCR analysis. Chrysanthemum *Ubiquitin* was used as the internal control. Asterisks indicate statistically significant differences between the two cultivars, as determined by two-tailed Student's *t*-test (**$P < 0.01$). Letters indicate significant differences according to Tukey-Kramer test ($P < 0.05$).

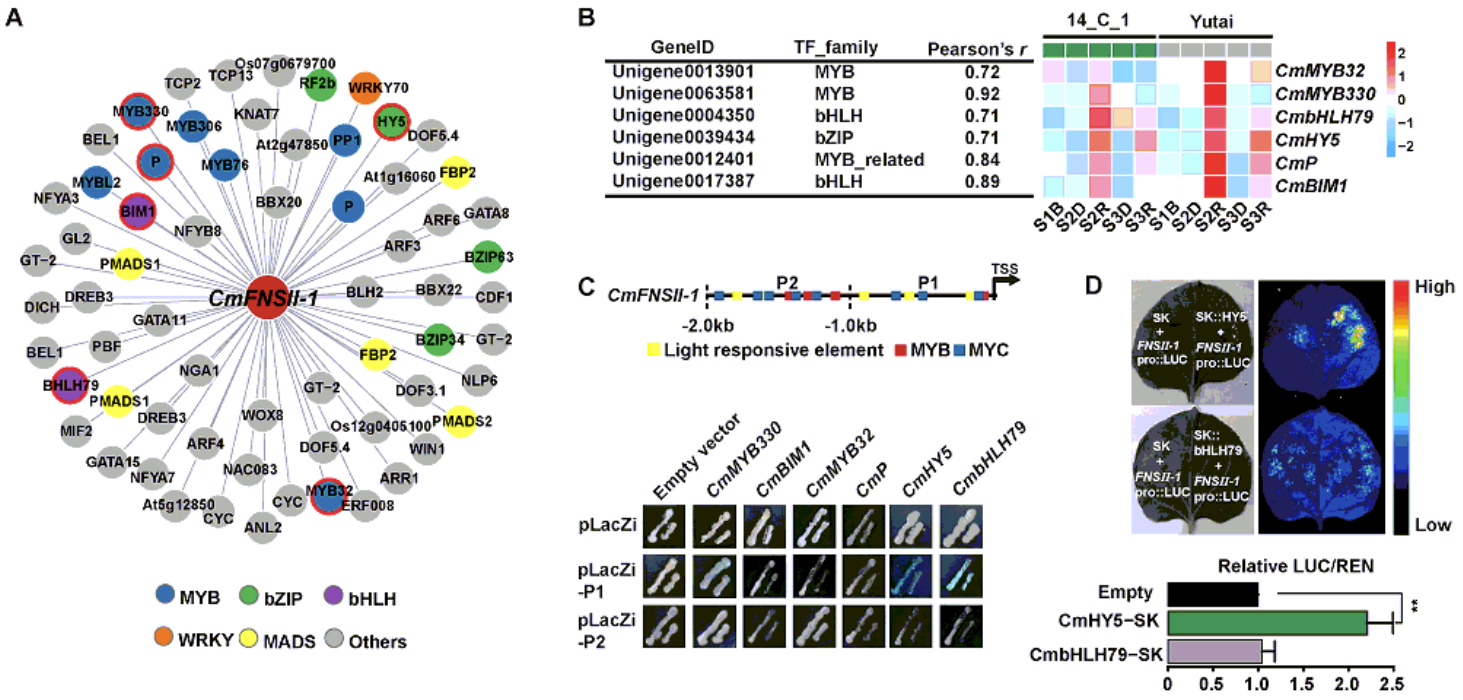


Figure 5

CmHY5 binds to the *CmFNSII-1* promoter. (A) Transcriptional regulatory network of *CmFNSII-1*. The large nodes represent the structural genes. The smaller nodes represent transcription factors. Different transcription factor families are shown in different colours. The grey lines indicate high correlation between the expression of *CmFNSII-1* and transcription factor genes (Pearson's correlation coefficient $r > 0.6$). Nodes with red circles were identified as candidate *CmFNSII-1* regulators. (B) Candidate *CmFNSII-1* regulators identified from co-expression analysis (left) and expression of the candidate regulator genes in different floral organs in the two cultivars, shown as a heatmap. (C) CmHY5 binds to the *CmFNSII-1* promoter, as shown by yeast one-hybrid assay. The *CmFNSII-1* promoter was divided into two fragments (P1, P2). Interactions were determined by yeast cell growth and confirmed by colour indication on SD –Trp –Ura medium containing X-β-gal. (D) Dual luciferase assays using different combinations of CmHY5 and bHLH79 proteins as effectors and a *CmFNSII-1* promoter construct in *N. benthamiana* leaves. LUC activity was tested 3 d after infiltration. Asterisks indicate statistically significant differences, Student's *t*-test (**$P < 0.01$).

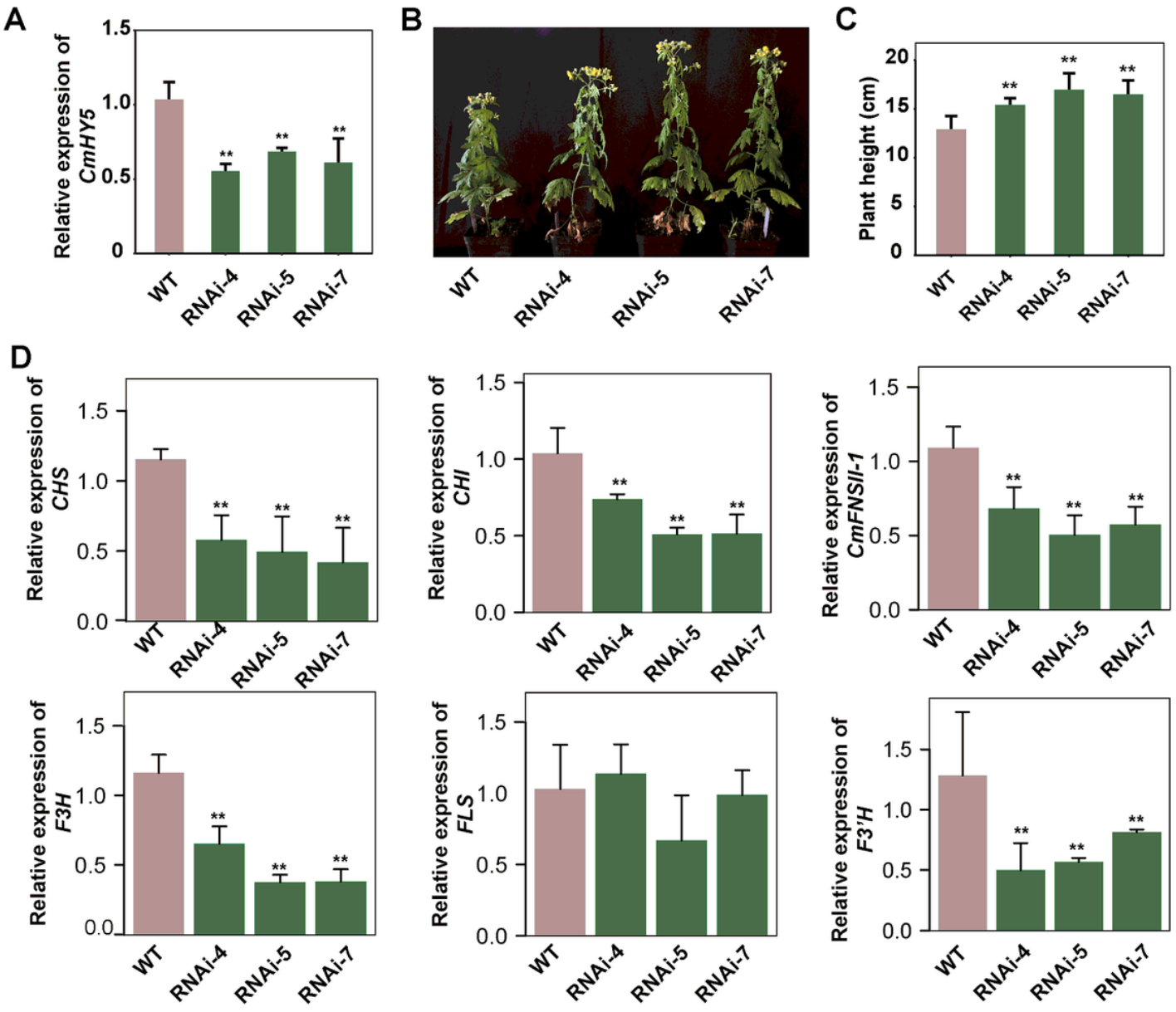


Figure 6

Phenotypes and differentially expressed structural genes from WT and *CmHY5*-RNAi chrysanthemum flowers. (A) Relative *CmHY5* transcript levels in *CmHY5*-RNAi chrysanthemum lines by RT-qPCR. (B) Representative phenotypes of 47-d-old wild-type (WT) and transgenic plants. (C) Plant height of WT and *CmHY5*-RNAi chrysanthemum plants. (D) Relative structural genes transcript levels in WT and *CmHY5*-RNAi chrysanthemum plants by RT-qPCR. RNAi-4, -5, and -7 are three independent *CmHY5*-RNAi lines. Asterisks indicate statistically significant differences between the WT and each RNAi line, Student's *t*-test (**$P < 0.01$).

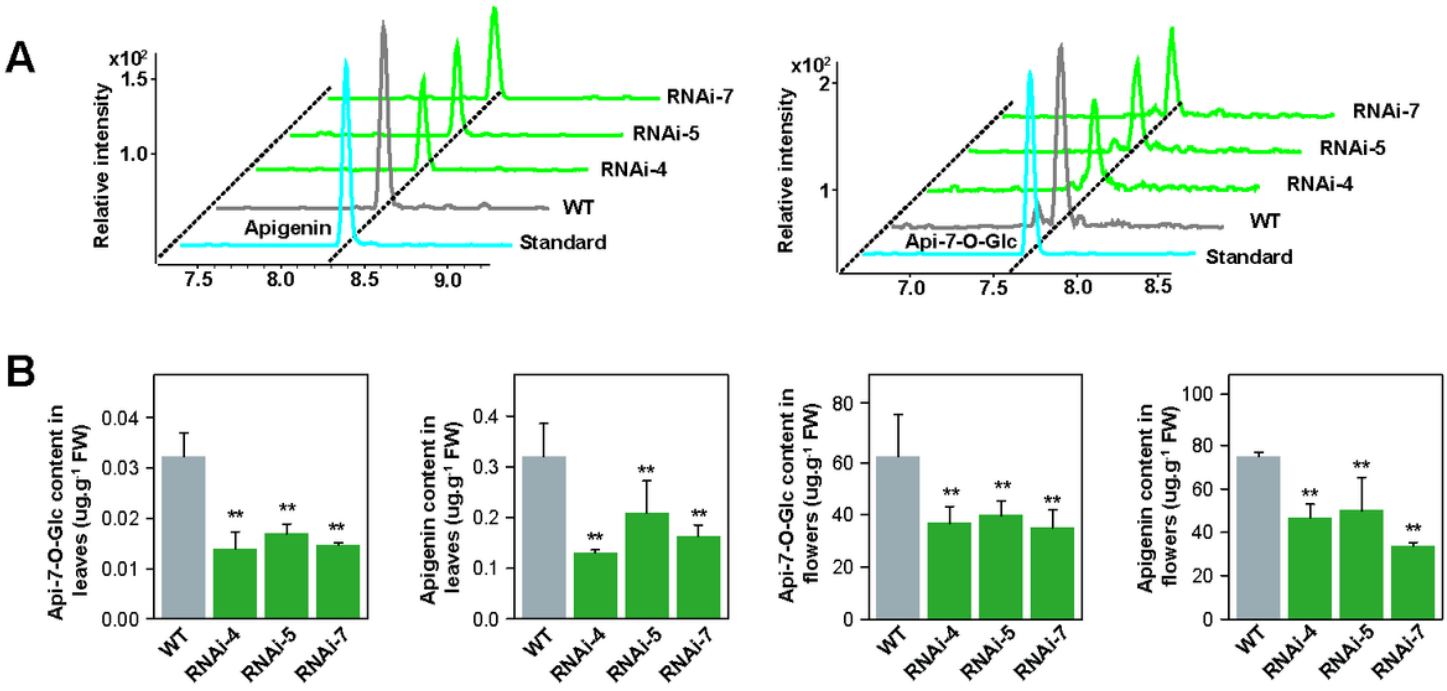


Figure 7

Effect of *CmHY5* silencing on apigenin contents in chrysanthemum.

(A) HPLC-MS chromatographic profiles of apigenin and apigenin 7-O-glucoside in WT and *CmHY5*-RNAi.

(B) Apigenin and apigenin 7-*O*-glucoside contents in leaves and flowers of WT and *CmHY5*-RNAi chrysanthemum plants. RNAi-4, -5, and -7 are three independent *CmHY5*-RNAi lines. Asterisks indicate statistically significant differences between the WT and each RNAi line, Student's *t*-test (**$P < 0.01$).

Supplementary Files

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