Research Square

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Comparative analysis of flower development, active ingredients and transcriptomic response revealed mechanisms of Chrysanthemum morifolium cv. 'Hangju' responding to flooding stress

Hong Zhang
Nanjing Agricultural University
Feng Yang
Nanjing Agricultural University
Qiaosheng Guo
Nanjing Agricultural University
Shuyan Yu
Nanjing Agricultural University
Jingzhe Pu
NMPA Key Laboratory for Quality Research and Evaluation of Traditional Chinese Medicine
Minhua Liu
Nanjing Agricultural University
Qingjun Zou
Nanjing Agricultural University
Wenyan Zhang
Nanjing Agricultural University
Tao Wang (wt1344@njau.edu.cn)
Nanjing Agricultural University

Research Article

Keywords: flooding stress, flower-bud differentiation, luteoloside, Chrysanthemum morifolium

Posted Date: April 18th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2800953/v1

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Abstract

C. morifolium cv. 'Hangju' is used as material medicine and herbal tea in China for several centuries. In the previous observation, it was found that sufficient rainfall conditions can affect the flower buds differentiation process of 'Hangju'. In order to study the effects of flooding stress on 'Hangju', this study simulated the flooding stress during the reproductive growth of 'Hangju' and detected the morphological difference of the capitulum and luteoloside content change in different growth stages. RNA-seq was also used to contrast transcriptomes of pre- and post-treatment performance. In total, 101,806 unigenes were obtained and 968, 624, 964, and 582 differentially expressed genes (DEGs) were identified between flooding groups and control groups in the 4 stages. GO enrichment analysis of DEGs was performed to derive the main enrichment entries, KEGG enrichment analysis reveals a significant enrichment of DEGs in the biosynthetic pathway of secondary metabolites, which include flavonoid, flavone, and flavonol biosynthesis pathways. Overall, our research provides reasonable insights into the flower bud differentiation response mechanisms of 'Hangju' is subjected to flooding stress, and will expand our understanding of the transcriptional response to flood stress. These results will provide a better understanding of the formation of quality in 'Hangju' when subjected to flooding stress and will expand our understanding of the transcriptional response to flooding stress. These results will provide greater understanding of the formation of quality in 'Hangju'.

Key Message

Chrysanthemum morifolium cv. 'Hangju' is an important medicinal plant. Investigated the contents of luteoloside and capitulum morphological of 'Hangju' with flooding stress in flower buds differentiation process, RNA-seq identified DEGs associated with flooding resistance, which provided insight into the flooding resistance mechanism and the formation of road quality in 'Hangju'

Introduction

Chrysanthemum morifolium cv. 'Hangju' is used as material medicine and herbal tea in China for several centuries, which has an important economic value. Many pharmacological studies have shown that it has a wide range of antioxidant activity (Lii et al. 2010; Cui et al. 2012; He et al. 2012; Zheng et al. 2015; Zhan et al. 2022), antimicrobial (Kuang et al. 2018) and anti-inflammatory (Ukiya et al. 2001; Tao et al. 2017), and cardiovascular protection functions (Gao et al. 2016; Xu et al. 2019), which benefits from the main components, such as flavonoids (Xie et al. 2012; Lin et al. 2015), organic acids (Clifford et al. 2007; Wang et al. 2013) and volatile oils (Guo et al. 2008; Yu et al. 2023). Therefore, luteolin and chlorogenic acid have been made indicator components in the Chinese Pharmacopoeia as indicator ingredients for the quality control of Flos Chrysanthemi.

Flavonoids is a important class of secondary metabolites, which are mainly controlled by plant genes. It has been reported that CmMYB3a and CmMYB3b transcription factors isolated from *C. morifolium* can regulate flavonol biosynthesis in Arabidopsis (Yang et al. 2023). And their synthesis is regulated by

environmental stimuli, including light (Hong et al. 2015; Pan et al. 2017), temperature (Olsen et al. 2009; Petridis et al. 2016), moisture (Castellarin et al. 2007; Nakabayashi et al. 2014) and other factors. All of these environmental conditions are the key factors that regulate and control the high quality of genuine medicinal materials. Based on our previous research, 'Hangju' among Flos Chrysanthemi has a long history of cultivation and has gradually developed into multiple genotypes (Shao et al. 2010). Meanwhile, 'Hangju' from Tongxiang County, Zhejiang Province, which is a traditional area, is considered to have an advantage over other growing areas in terms of luteolin content (Wang et al. 2014). Analyzing the environmental conditions of different producing areas, the moisture has a significant relationship with the content of luteolin. So, whether moisture is the key environmental factor to determine the high quality of *C. morifolium* needs to be verified.

In the entire life activity of plants, water plays an important role as an active substrate for cellular life; nevertheless, excess water can disrupt the water balance of the organism, causing numerous variations in the morphological characteristics, structure, physiology and metabolism of plants, eventually influencing the growth and development, yield and quality of plants (Liu et al. 2010; Anee et al. 2019; Gao et al. 2022; Gong et al. 2022). When plants are stressed by flooding, the oxygen supply to the root system decreases and aerobic respiration gradually changes to anaerobic respiration, thus influencing the natural growth and development for the root system (Peng et al. 2020). Harmful metabolites, as ethanol and lactic acid, also accumulate in the anaerobic respiration process, disrupting the plants' normal metabolic pathways (Liao and Lin 2001; van Veen et al. 2014). Finally, it affects the photosynthesis of the plant (Xiao et al. 2020). 'Hangju' is a medicinal herb with dried capitulum, and the morphology and active ingredient content of its capitulum are important indicators of its quality composition. The formation of capitulum undergoes the process of flowering transformation the transition from nutritional to reproductive growth (Poethig 2003; Baurle and Dean 2006), which is an important process of plant growth and development and is related to the continuation of species for generations. Water is one of the factors affecting the differentiation of flower buds in plants, and some scholars have found that flooding stress prolongs the time of flower bud differentiation, reduces the number of inflorescences, and leads to the occurrence of asymmetrical and incomplete inflorescences in 'Hangju' (Zhang et al. 2020). In addition to affecting the ornamental value of the plant itself, the change of flower shape may also affect the quality of flowering herbs like 'Hangju', which deserves further study.

In this study, RNA-seq was performed to identify these molecular mechanisms in genotype ‘Zaoyang’ of *C. morifolium* cv. 'Hangju' with flooding stress at the beginning of the flower bud development. Then, we compared the morphology of the capitulum in the developing stages by paraffin section. The content of luteolin was detected and quantified by HPLC, and genes related to luteolin biosynthesis were screened and verified by transcriptome analysis. Our study illuminated the molecular mechanism of moisture-regulating luteolin biosynthesis in *C. morifolium* development. Furthermore, the study offered a research method to analyze the genuine character of medicinal materials and a foundation for the introduction of *C. morifolium*.

Methods

Plant materials and flooding treatment

The genotype ‘Zaoyang’ of *C. morifolium* cv. 'Hangju' was obtained from the chrysanthemum germplasm resource preserving center, Tongxiang County, Zhejiang province, and was grown in a greenhouse at Nanjing Agricultural University under natural daylight conditions. When the flower buds initiate, the material in the first stage were collected. Then, the flooding treatment was performed for about 72 h, while the control group was treated with the normal amount of water. Hereafter, the capitulum in two groups was collected every 12 days until mid-October. The samples were divided into three parts, one part was stored in the FAA(70% ethanol: formaldehyde solution: glacial acetic acid = 18:1:1), one part was dried and the rest were frozen in the − 80℃

Plant sections of C. morifolium **capitulum**

The apical buds at different developmental stages were obtained from five *C. morifolium* individual plants after flooding treatment. Then, the plant materials were fixed in FAA and dehydrated in an ascending series of ethanol. The flower buds were embedded in paraffin, and the materials were cut into 8 μm slices using a rotary microtome. After being stained using safranine-fast green, the observations and photomicrographs were carried out using a microscope (Olympus BX 60, Tokyo, Japan) with an auto-photo system.

Quantitative analysis of flavonoid

The dried capitulum was crushed into powder, then a 0.25 g sample was accurately weighed and suspended in 25 mL of 70% methanol. After ultrasonication (300 W, 45 kHz) for 40 min, the extracted solution was filtrated and analyzed by Agilent HPLC 1260 series system (Agilent, USA), which is equipped with a C_{18} reversed-phase column (4.6 mm × 250 mm, 5 μm, Agilent, USA). The column temperature and wavelength were kept at 25℃ and 348 nm, the mobile phases consisted of (A) acetonitrile and (B) 0.1% phosphoric acid. The 20 μL of each sample solution were injected and eluted at a flow rate of 1.0 $mL{\cdot}min^{-1}$ with the conditions: 10→18% of A from 0 to 5.5 min, 18→20% of A from 5.5 to 15 min, and 20% of A from 15 to 20 min.

RNA extraction and transcriptome sequencing

Total RNA was isolated from the sample in different stages and treatments by using the TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, China). The quality of RNA was assessed on Nanodrop 2000 Spectrophotometer and agarose gel. For transcriptome sequencing, equal quantities of RNA from each sample were pooled. The mRNA was enriched with Oligo (dT) magnetic beads and was broken into short fragments, which were used as a template to synthesize cDNA. After purification, the cDNA was end-repaired and connected with a single nucleotide of adenine at the 3’ terminal. Finally, the cDNA library was constructed using PCR, followed by quality evaluation and sequencing with Illumina HiSeq TM 2000 system.

De novo assembly and functional annotation

Raw data were collected and processed to generate clean reads by removing low-quality reads and reads containing adapters or more than 5% unknown nucleotides. The clean data were then assembled into unigenes with eliminating redundant sequences. For functional annotation, the resulting unigenes were searched against the NCBI non-redundant nucleotide (Nt), NCBI non-redundant protein (Nr), SwissProt, Clusters of Orthologous Groups of proteins (COG), Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genome (KEGG) database by BLAST.

DGE sequencing and gene expression analysis

After extracting the total RNA, the DGE libraries were constructed and sequenced as described for the transcriptome sequencing above. The clean data were mapped to our chrysanthemum transcriptome database using Bowtie2 software, and gene expression levels were calculated by the Reads per Kb per Million reads (RPKM) method. Differential gene expression (DGE) analyses were performed in a Poisson distribution model. The unigenes comply with the criteria of change ratio ≥ 2 and false discovery rate (FDR) ≤ 0.001 were assigned as differential expression genes (DEGs) which also were mapped to terms in the GO and KEGG databases. To further analyze the expression profiling of genes involved in the flavonoids biosynthesis pathway, the candidate unigenes were identified. The RPKM values of the genes were visualized in a heatmap by R package.

Results

The morphological development of the capitulum after flooding treatment

To further investigate the pre- and post-treatment phenotypic of flower buds, four-petal primordium differentiation phases of flower buds were selected to analyze the influences of flooding treatment at early stages of inflorescence development in *C. morifolium* (Fig. 1A, C). The results showed that the initiation time of flower bud differentiation was delayed after flooding treatment, the four periods (Stage B Stage C Stage D, and Stage E) of flower bud differentiation were all delayed compared with the control group after the flooding treatment. In addition, comparing the two groups of sections, their microscopic characteristics were obvious differences in flower buds, for example, the treatment group had more sepals than the untreated group, the tip of the inflorescence was sharper, and so on. Compared to the control, the capitulum tended to be more compact and smaller in flower shape, and the number of disc florets and the diameter of flower clusters became smaller after the flooding treatment.

The luteoloside change in the C. morifolium **capitulum**

To further analyze the pre- and post-treatment performance of the active component, the luteoloside was extracted and quantified in different stages (Fig. 1B). We found that the content of luteoloside increase with the capitulum development. Interestingly, the buds contain more luteoloside in stages B and C after

flooding, which may be a result of the plants producing secondary metabolites (such as flavonoids) to defend against waterlogged stress. While the growth and developments of the buds in treatment groups were retards at the last stages, there is a lower active ingredient.

Transcriptome data and unigene annotation

To analyze the molecular mechanism of the changes between the flooding and unflooding groups, the total cDNA library of *C. morifolium* capitulum was constructed and sequenced. A total of 53,397,608 clean reads with Q20 and GC percentages of 98.9% and 42.73% were generated after filtering the low-quality reads (Supplementary Table 1). And all the clean reads were de novo assembled into 101,806 unigenes with an average length of 670 bp and N50 of 1184 bp. Then, all unigenes were compared against the public database for functional annotation. The results showed that a total of 54,702 unigenes were annotated, of which, 52,480 unigenes were matched into the Nr database with high blast hit against *Vitis vinifera* (24.97%) and *Lycopersicon esculentum* (16.40%). Furthermore, there are 34,742, 30,857, 18,030, and 38,728 unigenes that showed significant similarity to sequences in the Swiss-Prot, KEGG, COG, and GO databases, respectively (Supplementary Tables 2 and 3, Supplementary Fig. 1).

DGE library and functional analysis

To analyze the gene expression profiling in the *C. morifolium* after flooding, the DGE library was constructed and sequenced. A total of 11,738,261, 12,448,013, 11,719,089, and 12,054,980 clean reads were obtained from the flooding groups at four developing stages, while 12,621,025, 12,601,115, 12,451,596 and 12,488,478 reads were isolated from the control groups (Supplementary Table 4), which have more than 75% mapping rate to the reference sequence. After calculating the gene expression level by RPKM value, there are 968, 624, 964, and 582 DEGs between flooding groups and control groups in the 4 stages, compared to Stage B of flooding groups and control groups, a total of 968 differentially expressed genes were identified, among which 537 (55.5%) were differentially expressed up-regulated genes and 431 (44.5%) down-regulated genes (Fig. 2A, Supplementary Fig. 2). At Stage C of flooding groups and control groups, 624 DEGs were found, of which 470 (75.3%) were up-regulated genes and 154 (24.7%) were down-regulated genes (Fig. 2A). At Stage D of flooding groups and control groups, 964 DEGs were found, of which 650 (67.4%) were up-regulated genes and 314 (32.6%) were down-regulated genes (Fig. 2A). At Stage E of flooding groups and control groups, 582 DEGs were found, of which 265 (45.5%) were up-regulated genes and 317 (54.5%) were down-regulated genes (Fig. 2A). Although there were no common DEGs in all stages according to the Venn results, different DEGs could belong to one key enzyme gene (Fig. 2B).

To understand their functions, all the DEGs were searched against the GO database (Supplementary Fig. 3), of which, 531, 346, 620, and 357 DEGs were annotated and classified into biological process, cellular component, and molecular function. And the representative subclasses such as metabolic process, cellular process, cell, cell part, and catalytic activity were significantly enriched in four stages. While comparing the DEGs to the KEGG database, a total of 468, 330, 518, and 290 DEGs were enriched into 98, 83, 97, and 73 pathways, respectively. We found that the metabolic pathway or the pathway of

biosynthesis of secondary metabolites is the main pathway to enrich DEGs (Fig. 3), of which, there were 21, 27, 22, and 15 DEGs involved in these two pathways (Supplementary Table 5), which indicated that flooding could influence the biosynthesis of the flavonoid component.

In the biosynthesis of flavonoids, the highest number of genes were annotated as metabolic pathways in stages B, C and E, in addition to stage D; the biosynthetic pathways of secondary metabolites were significantly enriched and had higher gene abundance in all four groups, indicating that the activities of related differential proteins were more vigorous, and Stage D nodes had the highest number of genes; the biosynthesis of isoflavones, flavonoids and flavonols were mainly enriched in Stage B, C, and D. The genes of anthocyanin biosynthesis pathway were mainly enriched in Stage B and C (Fig. 4).

The expression profiling of genes involved in the luteolin biosynthesis pathway

According to the KEGG database, there were more than 7 key genes in the luteolin biosynthesis pathway including *PAL*, *C4H*, *4CL*, *CHS*, *CHI*, *FNS*, and *F3'H*. According to the unigenes sequence similarity and expressional abundance, we obtained 14 unique sequences, whose expression profiling was shown in the heatmap (Fig. 5). The gene expression was a similar tendency with the luteoloside content, most of the unigenes in the flooding group had a higher expressive level at Stage C and a lower level at Stage D and E.

Discussion

The capitulum morphological responses of 'Hangju' to early flooding treatments

Under drought conditions, plants can respond through morphology, physiology, and metabolism. Whereas excess water is likewise one of the adverse plant stresses, the degree of inundation at different depths and durations also exerted selection pressure on various plant traits (Avramova 2019). 'Hangju' is the most widely cultivated and highest-yielding strain of Flos Chrysanthemi among medicinal plants in China, and Tongxiang country, Zhejiang Province is considered to be the authentic production area of 'Hangju' (Huang et al. 2011). Earlier studies found that floral bud differentiation of 'Hangju' occurred in mid to late August, Coincidentally, at this time, the traditional cultivation area of Tongxiang is in the typhoon season, and has significantly higher precipitation in August than other cultivation areas (Zou et al. 2018), which most likely affected the bud differentiation process of 'Hangju'. The results showed that short flooding during the pre-flower bud differentiation period induced morphological adaptations in 'Hangju' chrysanthemum, which manifested as changes in the capitulum, the whole head tended to be more compact, while the flower shape became smaller, especially the number of tubular flowers decreased and the diameter of flower clusters became smaller, indicating that the flooding treatment negatively regulated the development of 'Hangju' capitulum. Under nutrient-rich conditions, nutrient

uptake and utilization by plants is an important means of resisting flooding stress (Qiu et al. 2020). The effect of flooding stress on 'Hangju' capitulum development may be due to the influx of energy and nutrients into the developmental pathway of the ray flowers and the promotion of its differentiation under higher soil moisture conditions, while the disc florets are relatively inhibited. The experiment simulated the soil moisture conditions of 'Hangju' in its native habitat, which is of practical significance for further exploring the mechanism of quality formation of the native medicinal herbs.

Effect of flooding stress on the active ingredients in the capitulum of 'Hangju'

Plants live in complex natural environments where their growth and developmental processes are affected by a variety of environmental stresses (also called adversity). The main medicinal components of Chinese medicines are usually secondary metabolites, and stress stimulation affects the synthesis of secondary metabolites in plants (Huang and Guo 2007). Previous studies have shown that the accumulation and synthesis of secondary metabolites are influenced by physical, chemical and biological stresses (Ramawat and Mérillon 2013; Pereira 2016). Soil moisture levels as a key factor in the plant's development, are closely related to the formation, accumulation, and transport of secondary metabolites in plants (Coutinho et al. 2018; Copolovici et al. 2021; Grof-Tisza et al. 2022). The growth and development of plants can be impacted by soil moisture content (Peng et al. 2020) and the effect of its content on plant secondary metabolites varies according to species and specific pathways. Studies have reported results in component accumulation differences in *Isatis tinctoria* in response to flooding stress and found that too high or too low water content in the soil was detrimental to the accumulation of indole indirubin. Previous studies have found most of the total flavonoid content of 'Hangju' significantly enhanced after flooding stress and affected secondary metabolic biosynthesis (Zou et al. 2018). Some scholars have found that the aliphatic glucosinolate and flavonoid contents of *Arabidopsis thaliana* under normal conditions were lower than that under flooding stress (Mewis et al. 2012). Consistent with the results of previous studies, the present study showed that flower buds in stages B and C after flooding contained more luteoloside. Taken together, flooding stress promotes the increase of flavonoids in 'Hangju' as a way to resist the environmental stress. This phenomenon complements the reasons for the formation of the medicinal qualities of 'Hangju'.

KEGG pathways for flavonoid component biosynthesis are associated with 'Hangju' in response to flooding stress

With the continuous updating and development of transcriptome sequencing technology, it is gradually playing an important role in the field of biology (He and Jiao 2014; Arismendi et al. 2015; Hou et al. 2021). This technique can powerfully reveal transcriptomic changes in plants in response to flooding stress, providing a basis for further elaboration of the molecular mechanisms by which plants respond to flooding stress. In the present study, the capitulum of experimental and control plants was collected at 12 days intervals after 3 days of flooding treatment at the beginning of flower bud differentiation, and the transcriptomes were sequenced using Illumina HiSeq TM 2000. The sequencing results yielded a total of

53,397,608 clean reads, with 968, 624, 964, and 582 DEGs between the four-stage flooding groups and the control group, respectively. These differentially expressed genes may play an important regulatory role in the morphological and structural changes, and related metabolic regulation in response to flooding stress in 'Hangju'. In the GO annotation, DEGs were significantly enriched in cellular processes, cells, cellular fractions and catalytically active fractions. KEGG enrichment analysis further demonstrated the metabolic pathways of 'Hangju' under flooding stress and provided a reference for further revealing the biological functions of these DEGs. The results revealed that some differentially expressed genes in the treated groups were enriched in the metabolic pathways related to flavonoids, indicating that flooding stress promoted gene expression in the biosynthetic pathways of flavonoids in 'Hangju' as a positive adaptation to the occurrence of flooding stress. Flavonoids are important components for plants to resist multiple extreme environments such as cold, drought, and UV-B radiation by providing OH groups to reduce oxidative stress (Liu et al. 2014), but there are fewer studies on the possible role of flavonoids in flooding stress. Previous studies have explored that flooding stress can alter flavonoid accumulation by influencing the expression of appropriate key enzymes in the synthetic pathway of flavonoids (Wang et al. 2019). In addition, it was found that *Pterocarya stenoptera* could enhance tolerance to flooding stress by increasing the synthesis of flavonoids and related signaling pathways (Li et al. 2021). Collectively, our results confirmed our hypothesis that the biosynthetic pathway of flavonoid components may be related to the response to flooding stress in 'Hangju'.

Conclusion

In this study, we investigated the luteoloside and transcriptional changes in capitulum of *C. morifolium* cv. 'Hangju' to determine the effects of flooding stress on above-ground parts. Our results suggest that the timing and morphological structure of flower bud differentiation and the content of luteoloside, a key active ingredient in the capitulum of 'Hangju', will be affected by flooding stress. Transcriptome sequencing analysis on the basis of Illumina sequencing technology determined the expression of genes related to the biosynthesis of flavonoid components affected in 'Hangju' under flooding conditions. This transcriptome analysis offered significant information for the continued study of flooding resistance-related genes in 'Hangju' and provided a foundation for future functional genomics studies of 'Hangju' and other chrysanthemum cultivars. This study has important implications for cultivation practices and will help to promote the formation of medicinal qualities of 'Hangju'.

Declarations

Ethical Approval: Not applicable.

Competing interests: The authors declare no competing interests.

Authors' contributions: Tao Wang and Qiaosheng Guo devised the research project; Wenyan Zhang, Qingjun Zou and Feng Yang conducted the research; Hong Zhang and Feng Yang analyzed data and

wrote the paper; Shuyan Yu, Jingzhe Pu and Minhua Liu revised the paper. All the authors read and approved the manuscript.

Funding: This study was financially supported by grants from the National Natural Science Foundation of China (grant number: 81503180), the Opening Project of NMPA Key Laboratory for Quality Research and Evaluation of Traditional Chinese Medicine (grant number: AHYJ-KFKT-202101), and the Earmarked fund for Jiangsu Agricultural Industry Technology System (grant number: JATS [2022] 460).

Availability of data and materials: Data available on request from the authors. The data that support the findings of this study are available from the corresponding author, upon reasonable request.

References

1. Anee TI, Nahar K, Rahman A, Al Mahmud J, Bhuiyan TF, Ul Alam M, Fujita M, Hasanuzzaman M (2019) Oxidative damage and antioxidant defense in *Sesamum indicum* after different waterlogging durations. Plants-Basel 8(7):196. https://doi.org/10.3390/plants8070196
2. Arismendi MJ, Almada R, Pimentel P, Bastias A, Salvatierra A, Rojas P, Hinrichsen P, Pinto M, Di Genova A, Travisany D, Maass A, Sagredo B (2015) Transcriptome sequencing of Prunus sp rootstocks roots to identify candidate genes involved in the response to root hypoxia. Tree Genet Genomes 11(1):11. https://doi.org/10.1007/s11295-015-0838-1
3. Avramova Z (2019) Defence-related priming and responses to recurring drought: Two manifestations of plant transcriptional memory mediated by the ABA and JA signalling pathways. Plant Cell Environ 42(3):983–997. https://doi.org/10.1111/pce.13458
4. Baurle I, Dean C (2006) The timing of developmental transitions in plants. Cell 125(4):655–664. https://doi.org/10.1016/j.cell.2006.05.005
5. Castellarin SD, Matthews MA, Di Gaspero G, Gambetta GA (2007) Water deficits accelerate ripening and induce changes in gene expression regulating flavonoid biosynthesis in grape berries. Planta 227(1):101–112. https://doi.org/10.1007/s00425-007-0598-8
6. Clifford MN, Wu WG, Kirkpatrick J, Kuhnert N (2007) Profiling the chlorogenic acids and other caffeic acid derivatives of herbal chrysanthemum by LC-MSn. J Agr Food Chem 55(3):929–936. https://doi.org/10.1021/jf062314x
7. Copolovici L, Lupitu A, Moisa C, Taschina M, Copolovici DM (2021) The effect of antagonist abiotic stress on bioactive compounds from basil (*Ocimum basilicum*). Appl Sci-Basel 11(19):9282. https://doi.org/10.3390/app11199282
8. Coutinho ID, Henning L, Dopp SA, Nepomuceno A, Moraes L, Marcolino-Gomes J, Richter C, Schwalbe H, Colnago LA (2018) Flooded soybean metabolomic analysis reveals important primary and secondary metabolites involved in the hypoxia stress response and tolerance. Environ Exp Bot 153:176–187. https://doi.org/10.1016/j.envexpbot.2018.05.018

9. Cui G, Niu YR, Wang H, Dong J, Yuki H, Chen SZ (2012) Rapid isolation and identification of active antioxidant ingredients from Gongju using HPLC-DAD-ESI-MSn and postcolumn derivatization. J Agr Food Chem 60(21):5407–5413. https://doi.org/10.1021/jf300938e
10. Gao M, Chen YC, Zhao YX, Wang YD (2022) Sex-specific physiological and biochemical responses of *Litsea cubeba* under waterlogging stress. Environ Exp Bot 202:105018. https://doi.org/10.1016/j.envexpbot.2022.105018
11. Gao T, Zhu ZY, Zhou X, Xie ML (2016) *Chrysanthemum morifolium* extract improves hypertension-induced cardiac hypertrophy in rats by reduction of blood pressure and inhibition of myocardial hypoxia inducible factor-1alpha expression. Pharm Biol 54(12):2895–2900. https://doi.org/10.1080/13880209.2016.1190764
12. Gong XF, Xu Y, Li H, Chen X, Song ZF (2022) Antioxidant activation, cell wall reinforcement, and reactive oxygen species regulation promote resistance to waterlogging stress in hot pepper (*Capsicum annuum* L.). Bmc Plant Biol 22(1):425. https://doi.org/10.1186/s12870-022-03807-2
13. Grof-Tisza P, Kruizenga N, Tervahauta AI, Blande JD (2022) Volatile-mediated induced and passively acquired resistance in sagebrush (*Artemisia tridentata*). J Chem Ecol 48(9–10):730–745. https://doi.org/10.1007/s10886-022-01378-y
14. Guo Q, Wang Y, Yang X, Xu W (2008) Characterization of chemical components of essential oil from cupitulum of *Chrysanthemum morifolium* cultivated in Tongxiang city. Zhongguo Zhong yao za zhi = Zhongguo zhongyao zazhi = China journal of Chinese materia medica 33(6):624–627.
15. He DX, Ru XC, Wen L, Wen YC, Jiang HD, Bruce IC, Jin J, Ma X, Xia Q (2012) Total flavonoids of Flos Chrysanthemi protect arterial endothelial cells against oxidative stress. J Ethnopharmacol 139(1):68–73. https://doi.org/10.1016/j.jep.2011.10.043
16. He J, Jiao Y (2014) Next-generation sequencing applied to flower development: RNA-seq. Methods in molecular biology (Clifton, N.J.) 1110:401–411. https://doi.org/10.1007/978-1-4614-9408-9_23
17. Hong Y, Tang XJ, Huang H, Zhang Y, Dai SL (2015) Transcriptomic analyses reveal species-specific light-induced anthocyanin biosynthesis in chrysanthemum. Bmc Genomics 16:202. https://doi.org/10.1186/s12864-015-1428-1
18. Hou C, Tian YX, Wang YL, Lian HM, Liang DC, Shi SQ, Deng N, He BX (2021) Revealing the developmental dynamics in male strobilus transcriptome of Gnetum luofuense using nanopore sequencing technology. Sci Rep-Uk 11(1):10516. https://doi.org/10.1038/s41598-021-90082-0
19. Huang L, Guo L (2007) Secondary metabolites accumulating and geoherbs formation under enviromental stress. Zhongguo Zhong yao za zhi = Zhongguo zhongyao zazhi = China journal of Chinese materia medica 32(4):277–280.
20. Huang L, Guo L, Ma C, Gao W, Yuan Q (2011) Top-geoherbs of traditional Chinese medicine: common traits, quality characteristics and formation. Front Med-Prc 5(2):185–194. https://doi.org/10.1007/s11684-011-0141-y
21. Kuang CL, Lv D, Shen GH, Li SS, Luo QY, Zhang ZQ (2018) Chemical composition and antimicrobial activities of volatile oil extracted from *Chrysanthemum morifolium* Ramat. J Food Sci Tech Mys

55(7):2786–2794. https://doi.org/10.1007/s13197-018-3203-1

22. Li Y, Shi LC, Yang J, Qian ZH, He YX, Li MW (2021) Physiological and transcriptional changes provide insights into the effect of root waterlogging on the aboveground part of *Pterocarya stenoptera*. Genomics 113(4):2583–2590. https://doi.org/10.1016/j.ygeno.2021.06.005
23. Liao C, Lin C (2001) Physiological adaptation of crop plants to flooding stress. Proceedings of the National Science Council Republic of China Part B Life Sciences 25(3):148–157.
24. Lii CK, Lei YP, Yao HT, Hsieh YS, Tsai CW, Liu KL, Chen HW (2010) *Chrysanthemum morifolium* Ramat. reduces the oxidized LDL-induced expression of intercellular adhesion molecule-1 and E-selectin in human umbilical vein endothelial cells. J Ethnopharmacol 128(1):213–220. https://doi.org/10.1016/j.jep.2010.01.018
25. Lin LC, Pai YF, Tsai TH (2015) Isolation of luteolin and luteolin-7-O-glucoside from *Dendranthema morifolium* Ramat Tzvel and their pharmacokinetics in rats. J Agr Food Chem 63(35):7700–7706. https://doi.org/10.1021/jf505848z
26. Liu SH, Ju JF, Xia GM (2014) Identification of the flavonoid 3 '-hydroxylase and flavonoid 3 ',5 '-hydroxylase genes from Antarctic moss and their regulation during abiotic stress. Gene 543(1):145–152. https://doi.org/10.1016/j.gene.2014.03.026
27. Liu Y, Li T, Sun Z, Chen Y (2010) Impacts of root-zone hypoxia stress on muskmelon growth, its root respiratory metabolism, and antioxidative enzyme activities. Yingyong Shengtai Xuebao 21(6):1439–1445.
28. Mewis I, Khan MA, Glawischnig E, Schreiner M, Ulrichs C (2012) Water stress and aphid feeding differentially influence metabolite composition in *Arabidopsis thaliana* (L.). Plos One 7(11):e48661. https://doi.org/10.1371/journal.pone.0048661
29. Nakabayashi R, Mori T, Saito K (2014) Alternation of flavonoid accumulation under drought stress in *Arabidopsis thaliana*. Plant Signal Behav 9(8):e29518. https://doi.org/10.4161/psb.29518
30. Olsen KM, Slimestad R, Lea US, Brede C, Lovdal T, Ruoff P, Verheul M, Lillo C (2009) Temperature and nitrogen effects on regulators and products of the flavonoid pathway: experimental and kinetic model studies. Plant Cell Environ 32(3):286–299. https://doi.org/10.1111/j.1365-3040.2008.01920.x
31. Pan JQ, Chen HM, Guo BL, Liu C (2017) Understanding the molecular mechanisms underlying the effects of light intensity on flavonoid production by RNA-seq analysis in *Epimedium pseudowushanense* BLGuo. Plos One 12(8):e182348. https://doi.org/10.1371/journal.pone.0182348
32. Peng YQ, Zhu J, Li WJ, Gao W, Shen RY, Meng LJ (2020) Effects of grafting on root growth, anaerobic respiration enzyme activity and aerenchyma of bitter melon under waterlogging stress. Sci Hortic-Amsterdam 261:108977. https://doi.org/10.1016/j.scienta.2019.108977
33. Pereira A (2016) Plant Abiotic Stress Challenges from the Changing Environment. Front Plant Sci 7:1123. https://doi.org/10.3389/fpls.2016.01123
34. Petridis A, Doll S, Nichelmann L, Bilger W, Mock HP (2016) *Arabidopsis thaliana* G2-LIKE FLAVONOID REGULATOR and BRASSINOSTEROID ENHANCED EXPRESSION1 are low-temperature regulators of flavonoid accumulation. New Phytol 211(3):912–925. https://doi.org/10.1111/nph.13986

35. Poethig RS (2003) Phase change and the regulation of developmental timing in plants. Science 301(5631):334–336. https://doi.org/10.1126/science.1085328
36. Qiu SY, Liu SS, Wei SJ, Cui XH, Nie M, Huang JX, He Q, Ju RT, Li B (2020) Changes in multiple environmental factors additively enhance the dominance of an exotic plant with a novel trade-off pattern. J Ecol 108(5):1989–1999. https://doi.org/10.1111/1365-2745.13386
37. Ramawat KG, Mérillon JM. Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes[M]. Springer, 2013.
38. Shao QS, Guo QS, Deng YM, Guo HP (2010) A comparative analysis of genetic diversity in medicinal *Chrysanthemum morifolium* based on morphology, ISSR and SRAP markers. Biochem Syst Ecol 38(6):1160–1169. https://doi.org/10.1016/j.bse.2010.11.002
39. Tao JH, Duan JA, Jiang S, Feng NN, Qiu WQ, Ling Y (2017) Polysaccharides from *Chrysanthemum morifolium* Ramat ameliorate colitis rats by modulating the intestinal microbiota community. ONCOTARGET 8(46):80790–80803. https://doi.org/10.18632/oncotarget.20477
40. Ukiya M, Akihisa T, Yasukawa K, Kasahara Y, Kimura Y, Koike K, Nikaido T, Takido M (2001) Constituents of compositae plants. 2. Triterpene diols, triols, and their 3-O-fatty acid esters from edible chrysanthemum flower extract and their anti-inflammatory effects. J Agr Food Chem 49(7):3187–3197. https://doi.org/10.1021/jf010164e
41. van Veen H, Akman M, Jamar D, Vreugdenhil D, Kooiker M, van Tienderen P, Voesenek L, Schranz ME, Sasidharan R (2014) Group VII Ethylene Response Factor diversification and regulation in four species from flood-prone environments. Plant Cell Environ 37(10):2421–2432. https://doi.org/10.1111/pce.12302
42. Wang L, Wang T, Guo Q, Huang Y, Xu H (2013) Comparative study on four major active compounds of Sanvitalia procumbens and *Chrysanthemum morifolium* cv 'Hangju' and 'Gongju'. Zhongguo Zhong yao za zhi = Zhongguo zhongyao zazhi = China journal of Chinese materia medica 38(20):3442–3445.
43. Wang T, Guo QS, Mao PF (2014) Flavonoid accumulation during florescence in three *Chrysanthemum morifolium* Ramat cv. 'Hangju' genotypes. Biochem Syst Ecol 55:79–83. https://doi.org/10.1016/j.bse.2014.02.023
44. Wang T, Zou QJ, Guo QS, Yang F, Wu LW, Zhang WY (2019) Widely targeted metabolomics analysis reveals the effect of flooding stress on the synthesis of flavonoids in *Chrysanthemum morifolium*. Molecules 24(20):3695. https://doi.org/10.3390/molecules24203695
45. Xiao YS, Wu XL, Sun MX, Peng FT (2020) Hydrogen sulfide alleviates waterlogging-induced damage in peach seedlings via enhancing antioxidative system and inhibiting ethylene synthesis. Front Plant Sci 11:696. https://doi.org/10.3389/fpls.2020.00696
46. Xie YY, Qu JL, Wang QL, Wang Y, Yoshikawa M, Yuan D (2012) Comparative evaluation of cultivars of *Chrysanthemum morifolium* flowers by HPLC-DAD-ESI/MS analysis and antiallergic assay. J Agr Food Chem 60(51):12574–12583. https://doi.org/10.1021/jf304080v

47. Xu R, Fan H, Zhang L, Wang H, Tong Y, Chen J, Chang G, Feng W, Zheng X (2019) Caffeoyl quinic acids in *Chrysanthemum morifolium* improve LPS-induced HUVEC vascular endothelial cell injury by regulating ERK/MAPK signaling pathway. Yaoxue Xuebao 54(7):1207–1213. https://doi.org/10.16438/j.0513-4870.2018-1060
48. Yang F, Wang T, Guo QS, Zou QJ, Yu SY (2023) The CmMYB3 transcription factors isolated from the *Chrysanthemum morifolium* regulate flavonol biosynthesis in Arabidopsis thaliana. Plant Cell Rep https://doi.org/10.1007/s00299-023-02991-1
49. Yu SY, Yang F, Pu JZ, Guo QS, Zou QJ, Zhang H, Liu MH, Zheng Y, Wang T (2023) Integrated transcriptomic and metabolomic analyses reveal the characteristics of volatile oils in *Chrysanthemum morifolium*. Sci Hortic-Amsterdam 315: 111983 https://doi.org/10.1016/j.scienta.2023.111983
50. Zhan G, Long M, Shan K, Xie C, Yang RQ (2022) Antioxidant effect of *Chrysanthemum morifolium* (Chuju) extract on H_2O_2-treated L-O_2 cells as revealed by LC/MS-based metabolic profiling. Antioxidants-Basel 11(6):1068. https://doi.org/10.3390/antiox11061068
51. Zhang WY, Wang T, Guo QS, Zou QJ, Yang F, Lu DH, Liu J (2020) Effect of soil moisture regimes in the early flowering stage on inflorescence morphology and medicinal ingredients of *Chrysanthemum morifolium* Ramat. Cv. 'Hangju'. Sci Hortic-Amsterdam 260:108849. https://doi.org/10.1016/j.scienta.2019.108849
52. Zheng CP, Dong Q, Chen HJ, Cong QF, Ding K (2015) Structural characterization of a polysaccharide from *Chrysanthemum morifolium* flowers and its antioxidant activity. Carbohyd Polym 130:113–121. https://doi.org/10.1016/j.carbpol.2015.05.004
53. Zou QJ, Wang T, Guo QS, Xiao YM, Wu LW (2018) Cloning and expression analysis of F3'H and quantification of downstream products in *Chrysanthemum morifolium* under flooding stress. Zhongguo Zhong yao za zhi = Zhongguo zhongyao zazhi = China journal of Chinese materia medica 43(1):52–57. https://doi.org/10.19540/j.cnki.cjcmm.20171030.008

Figures

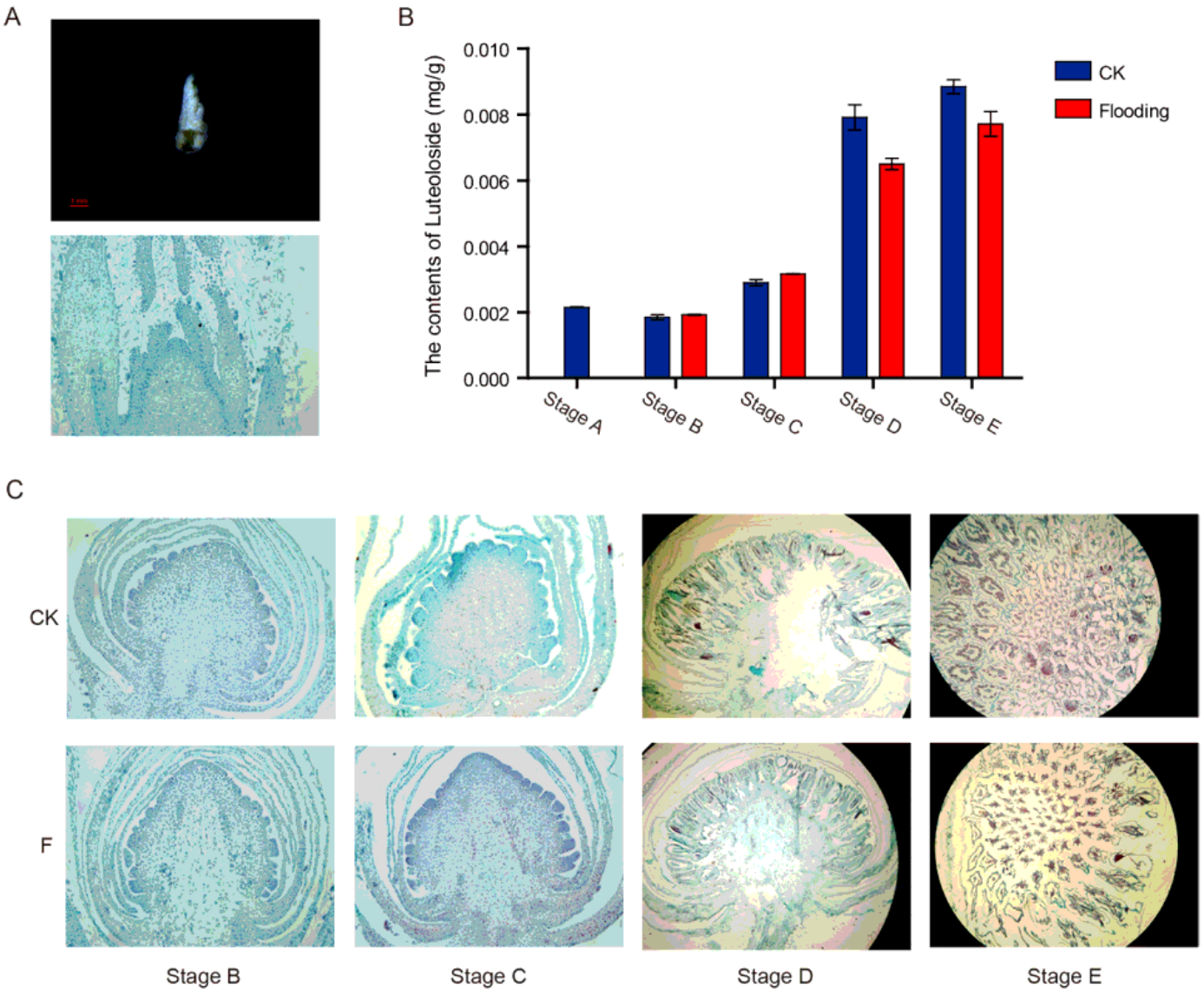


Figure 1

Dynamic changes of flower bud differentiation and luteolin content of 'Hangju' in different stages. A Diagram of flower bud differentiation of 'Hangju' before flooding treatment, **B** Luteolin content of each treatment stage, **C** Flower bud differentiation of'Hangju' after flooding treatment. (Stage B) petal primordium differentiation early; (Stage C) petal primordium differentiation middle; (Stage D) petal formation period; (Stage E) petal formation period

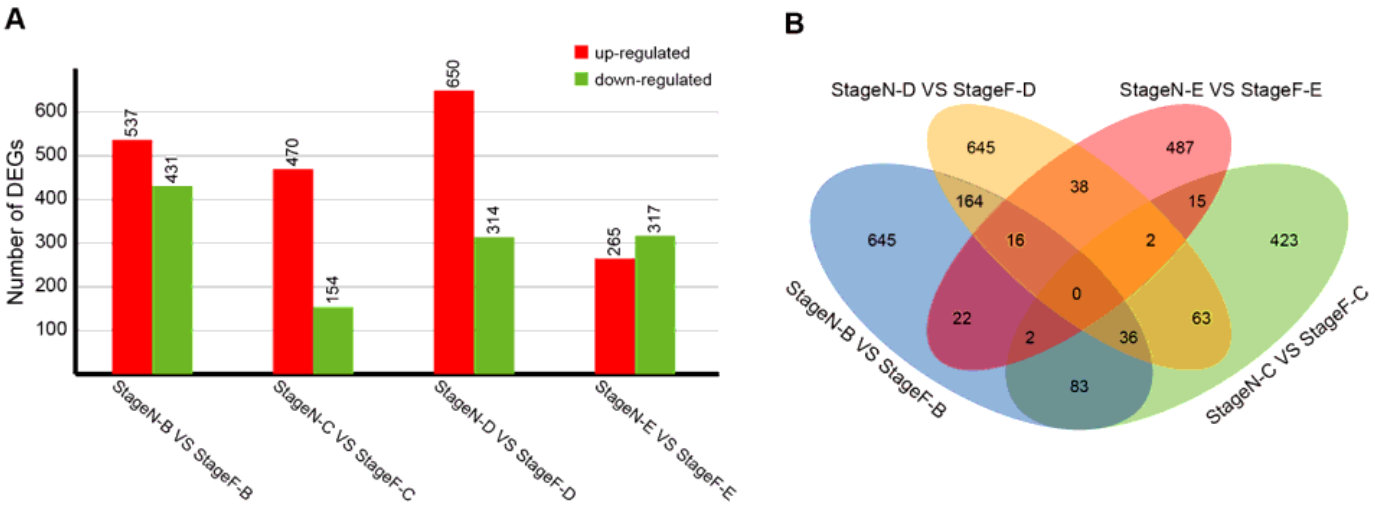


Figure 2

Differentially expressed genes (DEGs) between flooding groups and control groups in the 4 stages. A The horizontal axis represents the sample comparison result N-VS-F, where N is the control and F is the treatment. Number of DEGs up-regulated (red) or down-regulated (green) at four different stages. B Venn diagram of unique and common DEGs at four different stages. The horizontal axis indicates the size of the enrichment factor, the vertical axis indicates

the name of the metabolic pathway, and the size of the dots in the graph indicates the number of genes

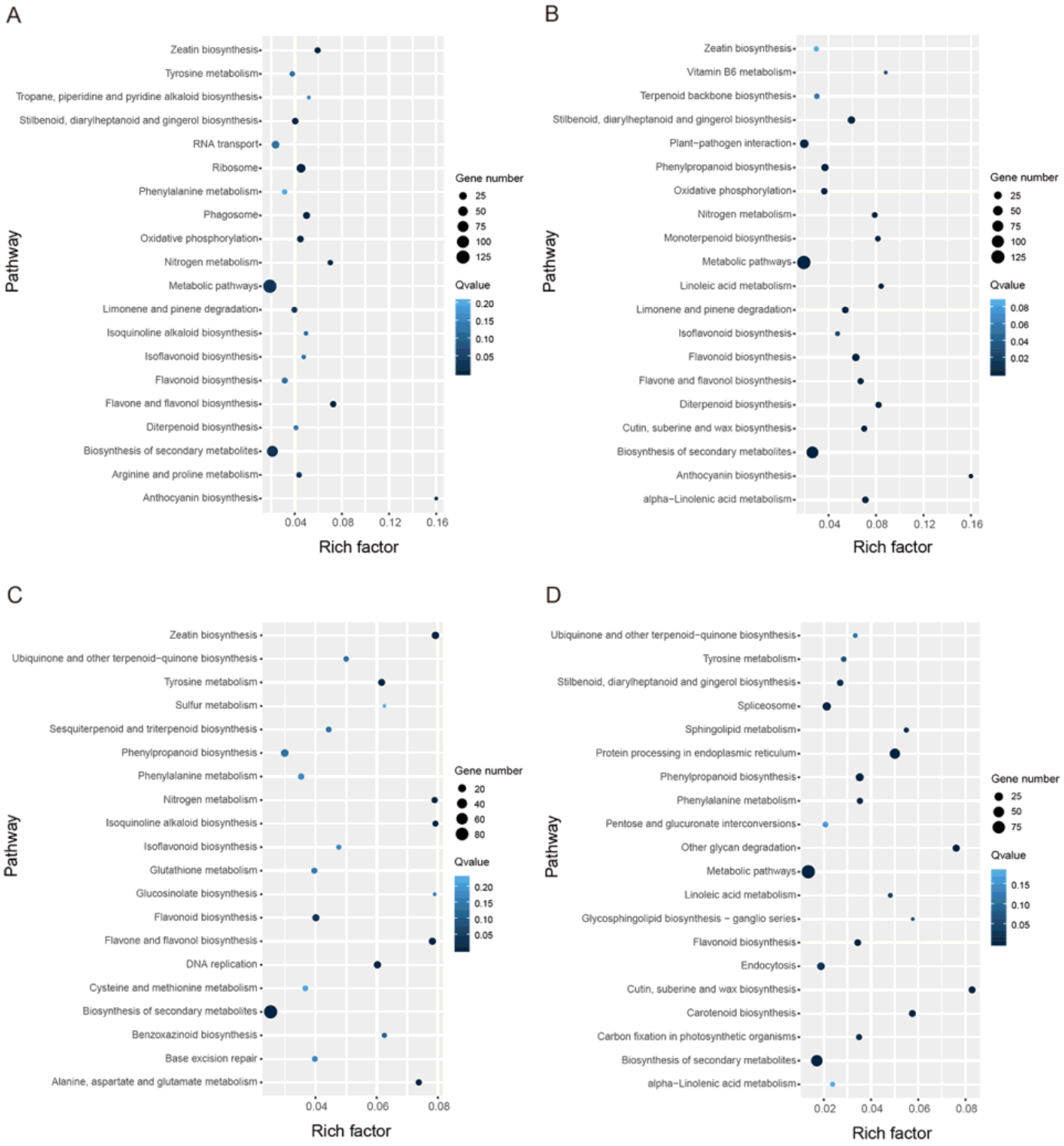


Figure 3

3 Scatter plot of KEGG enrichment statistics. The horizontal axis represents the size of the enrichment factor, the vertical axis represents the name of the metabolic pathway, and the dot size in the figure represents the number of genes

In the biosynthesis of flavonoids, the highest number of genes were annotated as metabolic pathways in stages B, C and E, in addition to stage D; the biosynthetic pathways of secondary metabolites were significantly enriched and had higher gene abundance in all four groups, indicating that the activities of related differential proteins were more vigorous, and Stage D nodes had the highest number of genes; the biosynthesis of isoflavones, flavonoids and flavonols were mainly enriched in Stage B, C, and D.

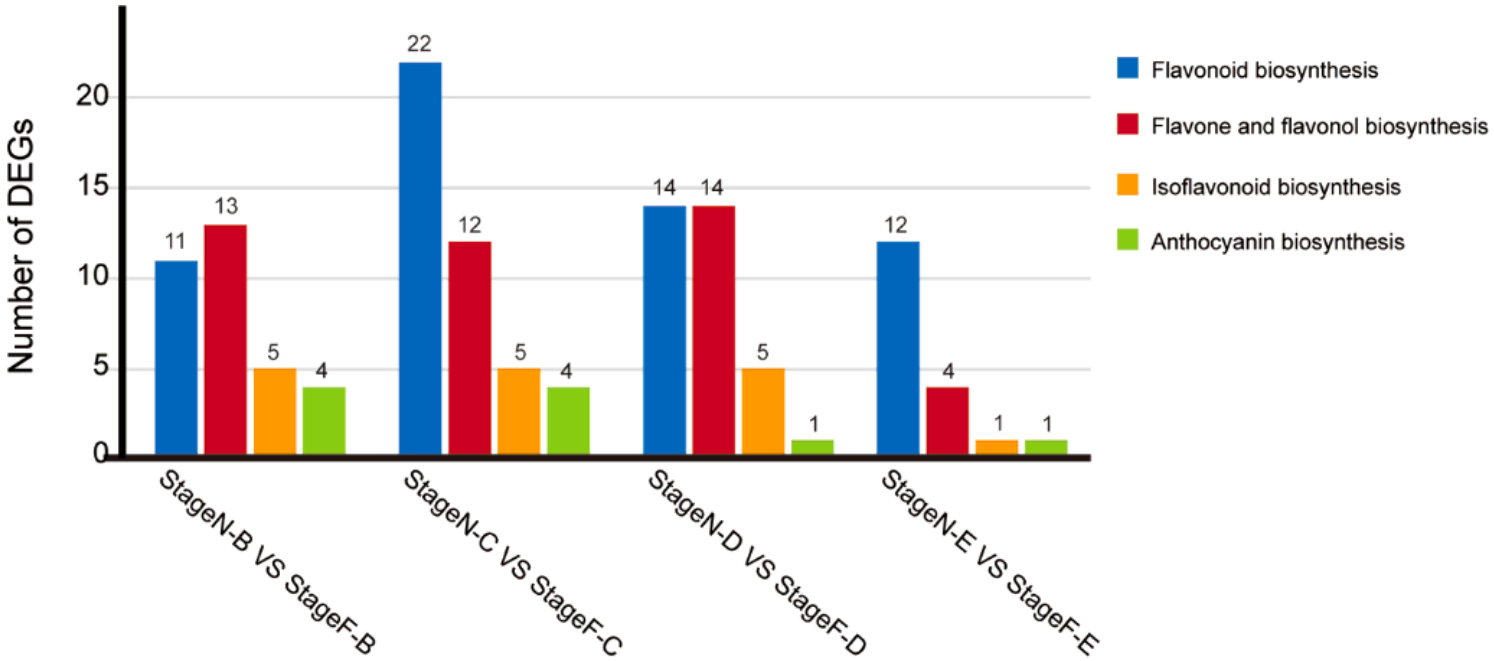


Figure 4

Number of DEGs associated with flavonoid biosynthesis in four stages

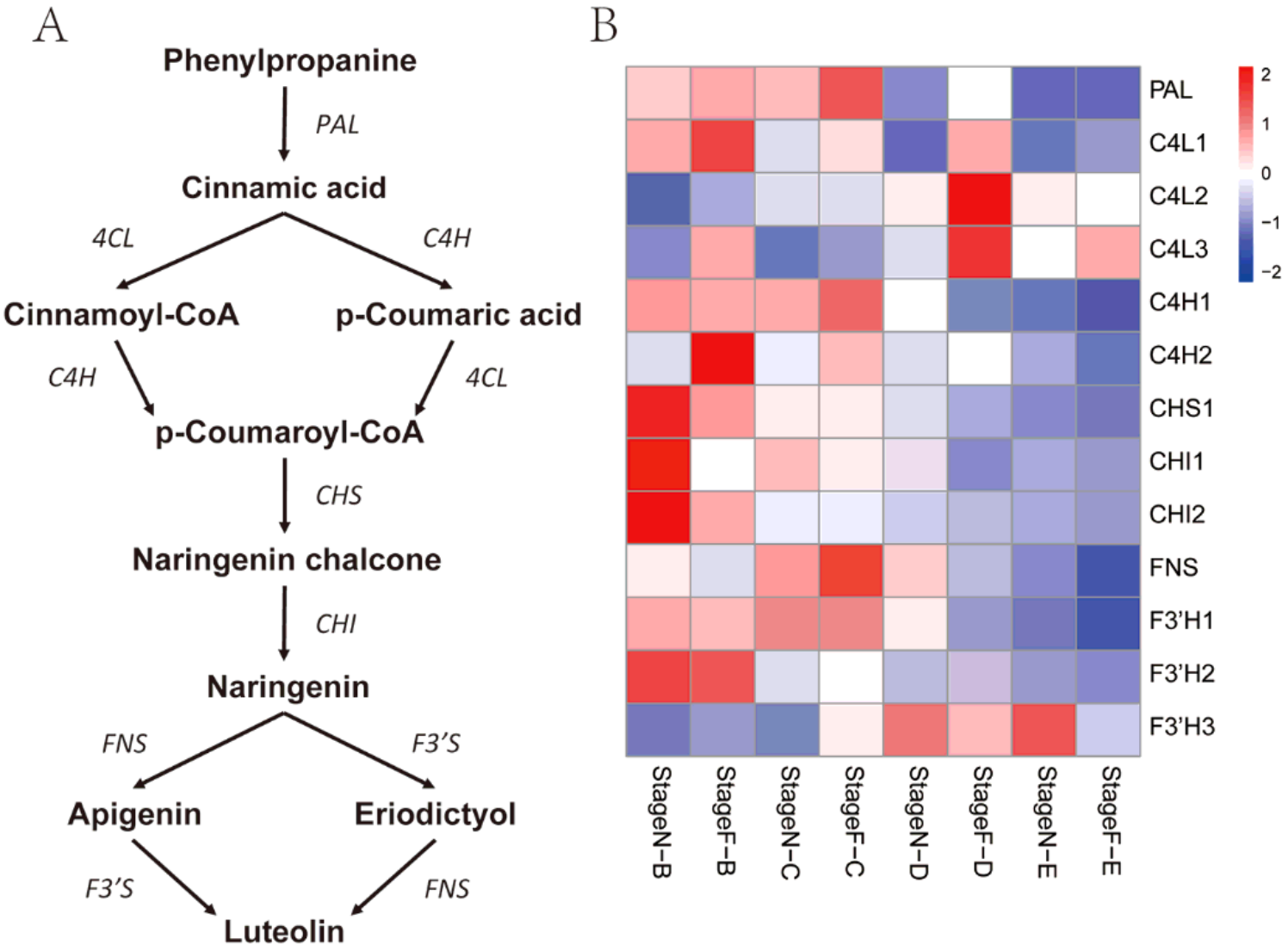


Figure 5

Luteolin metabolism biosynthesis pathway and heat map of 'Hangju'. A Luteolin biosynthesis pathway, B Heat map of pathway-related differentially expressed genes. The red box indicates upregulated genes, and the blue box indicates downregulated genes

Supplementary Files

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

- Table1.xlsx
- SupplementaryFig1.pdf
- SupplementaryFig.2.pdf
- Supplementaryfig.3.pdf