Metabolomic and Transcriptional Analysis Provides Insights into Flavonoids Variation between two Artemisia Cultivars

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Research Article

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Abstract

Background

Artemisia plants are widely distributed in East Asia. Though the Artemisia cultivars are commonly used as raw materials of medicine, food or moxibustion in China, most of the potential medical and nutritional ingredients, especially flavonoids ingredients are largely unknown, which imped further utilization of these cultivars.

Results

In this study, two Artemisia cultivars usually used as the source of moxibustion or food, named NYSY and NYYY were investigated. NYSY and NYYY were authenticated as *A.argyi* based on molecular data. In all detected 882 metabolic ingredients, nearly half of biological ingredients varied between two cultivars. Most of potential medical ingredients, especially flavonoids (flavone, flavonol, isoflavone, and anthocyanin) were up regulated in NYYY. The contents of total flavonoids and their antioxidant activities were also higher in NYYY. In addition, transcriptional evidences showed that more than half of flavonoids synthesis genes were up regulated in NYYY, and their putative transcription factors members of MYB, bHLH, bZIP, WRKY, NAC and MADS families were uncovered.

Conclusions

In all, our results provide metabolome and transcriptome evidence to underly the biochemical ingredients, especially flavonoids variation in two Artemisia cultivars. This study provides scientific evidence for the utilizing and improving the quality of Artemisia cultivars in the future.

Background

The genus Artemisia, widely distributed in temperate regions of Asia, Europe and North America, is one of the largest genera of Compositae and comprises over more than 500 species [1]. There are more than 180 species of Artemisia in China, which belong to two subtypes (*subgenus Artemisia* and *subgenus Dracunculus*) [2]. For many years, the Artemisia species, including *A. annua*, *A. argyi*, *A. lavandulaefolia, A. montana*, *A.princeps*, *A.selengensis*, *A.sylvatica*, *A. roxburghiana*, *A. qinlingensis*, *A. mongolica*, *A. indica*, *A. leucophylla*, *A. igniaria*, *A. atrovirens*, *A. vulgaris*, *etc*., have been used as ethnomedicines to treat different diseases such as malaria, hepatitis, cancer, inflammation, infections by fungi, bacteria, and viruses, or as food [2-3].

Bioactive compounds are experiencing a growing interest in wide range of applications: geo-medicine, plant science, modern pharmacology, agrochemicals, cosmetics, food industry, nano-bio-science, and so on [3,4]. Artemisia plants are rich in active substances. For example，artemisinin, the most famous medicine used for antimalarial treatment, is derived from *A.annua*[5]. Except that, many other phytochemicals, such as essential oils [6], flavonoids [7], terpenoids [7], phenolic acids [8], lignans coumarins [9], organic acids [10], alkaloids [11], tannin [12], and other groups of metabolites were found in Artemisia species. Previous evidence showed that many ingredients have pharmacological activities including antioxidant [13-14], antiobesity [14], anti-inflammatory [14], antifungal [15], antibacterial [16], and anticoagulation [17] activities, *etc*. These bioactive compounds from Artemisia species represent rich sources for potential medicine and nutrition.

Differences in qualitative and quantitative composition of bioactive compounds might be correlated with species variation [3]. As we known, though *A. argyi*, *A. princeps* and *A. montana* are all used as sources of material for moxibustion or medicine, only *A. argyi* were used as medicine [2,4-5,27]. Currently, many Artemisia cultivars are used as the raw materials of medicine, moxa, food and cosmetics in China. However, the researches on different cultivars are limited. The authentication, most of chemical composition, nutritional and functional properties varieties of different cultivars, especially the flavonoids and their synthesis pathway in Artemisia cultivars remain largely unknown. This imped further utilization of these plants.

In this study, two Artemisia cultivars usually used as the material food (named NYSY) or moxibustion (named NYYY), were investigated. NYSY and NYYY were authenticated as *A.argyi* based on molecular data though their morphological traits was distinct at vegetative growth stage. Metabolic evidence showed that 882 metabolites were identified in two cultivars. Most of medical ingredients, especially flavonoids, were up regulated in NYYY. Both the contents of total flavonoids and their antioxidant activities were higher in NYYY. In addition, transcriptional evidences showed that most of flavonoid synthesis

genes were up regulated in NYYY, and their putative transcription factors members of R2R3-MYB, bHLH, bZIP, WRKY, NAC and MADS families were uncovered. Our work enriches the understanding of the chemical components of Artemisia cultivars, and provide molecular and metabolic mechanisms for flavonoids variation between two cultivars. This provides the basis for further utilization of increasing demand of health-promoting components in medical, diet or daily cosmetics, and genetic improvement of Artemisia in the furfure.

Results

NYSY and NYYY were authenticated as A.argyi based on ITS2 sequences.

As we known, the taxonomic relationships of the Artemisia genus species based on morphological traits are controversial because of their diverse morphological types, ploidy, and complicated genetic relationships [28]. It is difficult to authenticate these cultivars only through morphological traits, especially at vegetative growth stage.

At the vegetative growth stage, significant morphological phenotypes differences were observed between NYYY and NYSY. NYSY exhibits pinnately compound leaf while NYSY exhibits palmatipartite leaves (Fig. 1A).

ITS2 sequence was used to aid authentication species in Artemisia [28-29]. The phylogenetic tree was constructed using the ITS2 sequences of NYYS, NYYY, and *Compositae* species of *Section Artemisia* (*A. montana*, *A. stolonifera*, *A. princeps*, *A. argyi*, *A. feddei*), *Section Absinthium* (*A. frigida*, *A. absinthium*, *A. selengensis*) ， *Section Dracunculus* (*A. scoparia* and *A. capillaris*), *Section Abrotanum* (*A. fukudo*), and *Aster spathulifolius.* The phylogenetic tree shows that the sequence of NYSY and NYYY were all clustered to *Sect*. *Artemisia* with *A. princeps, A.montana, A argyi, A. feddei*, and *A.stolonifera* (Fig. 1B). In addition, both NYSY and NYYY showed highest similarity to *A. argyi* (Accession Number: DQ925700) with 100% in NYYY, or a degenerate site V (C/G/T) at position 107 in NYSY. According to DNA barcode of *A. argyi* [29], our results suggest that both the two cultivars are *A.argyi* though significant morphological

phenotypes differences were observed between NYYY and NYSY at the vegetative growth stage（Fig. 1A）.

Biochemical ingredients with medical or nutritional properties varied between NYSY and NYYY.

To investigate the possible medical and nutritional ingredients in two cultivated varieties, metabolism was performed. Based on the local metabolite database MWDB (Metware database), qualitative and quantitative mass spectrometry analyses were conducted on the metabolites in the samples. In total, 882 metabolites, including 160 flavonoids (5 chalcones, 1 anthocyanin, 4 dihydroflavones, 1 dihydroflavonol, 100 flavones, 22 flavonols, 14 flavonoid carbonosides, 12 isoflavones, and 1 sinensetin) , 154 phenolic acids, 125 lipids, 99 organic acids and derivatives, 80 amino acids and derivatives, 70 terpenes, 53 saccharides and alcohols, 52 nucleotides and derivatives, 27 lignans and coumarins,14 alkaloids, 13 vitamins, 4 quinones，4 tannins，2 steroids， and 25 others metabolites were identified in two Artemisia cultivars (Fig 2A, Table S1).

To understand the possible variation between NYSY and NYYY, metabolite data was further analyzed. Correlation heat map showed that the biological repeatability between the samples is sufficient for further analysis($R2 > 0.8$) (Fig. S1A). Two principal components (PC1 and PC2) were extracted as 68.16% and 13.84% by PCA score plot analysis, suggesting that significant differences in the contents of metabolites between the two varieties caused by PC1 (Fig. S1B). This also means genetic variation strongly influenced the metabolite profiles of different Artemisia cultivated varieties. Detailed information of all identified in Table S1.

Based on fold change≥2 or≤0.5 and VIP ≥1, 430 metabolites were significantly up (229) or down (131) regulated, while 452 metabolites were insignificantly regulated between NYSY and NYYY (Fig S2, Table S1). In all detected metabolites ingredients，more than half of flavonoids (145 significant regulated, 45 insignificantly regulated), terpenoids (52 significant regulated, 18

insignificantly regulated), and lignans and coumarins (15 significant regulated, 12 insignificantly regulated) varies between two varieties. Less than half of other biochemical ingredients, including phenolic acids, organic acids, saccharides and alcohols, quinones, alkaloids, amino acids and derivatives, nucleotides and derivatives, lipids, and vitamin varied between NYSY and NYYY（Fig 2B）

Among the significantly regulated metabolites, most of flavonoids (91 up regulated, 79%), phenolic acids (44 up regulated, 67%), lipids（50 up regulated, 88%）,terpenoids (35 up regulated, 67%), organic acids (21 up regulated, 62%), saccharides and alcohols (14 up regulated, 78%), vitamin (5 up regulated, 83%) and quinones (2 up regulated,100%)， were up regulated in NYYY. The proportion of up or down regulated alkaloids (3 up regulated, 50%) and lignans and coumarins (7 up regulated, 47%) were almost equal between NYSY and NYYY. Less than half of amino acids and derivatives (9 up regulated, 41%), nucleotides and derivatives (8 up regulated,40%) were up regulated in NYYY (Fig 2C).

The contents of total flavonoids , antioxidant activity , and most ingredients of flavonoids were up regulated in NYYY.

Flavonoids are the most abundant different metabolites, and a lot of flavonoids ingredients are used as medical, pharmaceutical and nutritional compounds because of their medical and nutritional properties. Antioxidant ability is important for medical, food and cosmetic use. Flavonoids are also important substance with antioxidant ability [30]. Then, the contents of total flavonoids and their antioxidant abilities were performed to understand possible antioxidant capacity variation between NYSY and NYYY. Our results showed the contents of flavonoids in NYYY (5.0 mg/g) were much higher than that in NYSY (3.8 mg /g) (p <0.05) (Fig. 3A). The scavenging abilities of DPPH free radicals, Free radical ABTS+ scavenging abilities, and FRAP are much higher in NYYY (86.2%, 62.7 μmol Trolox/g and 52.7 μmol Trolox/g) than that of NYSY (64%,48.9.0 μmol Trolox/g, and 43.6 μmol Trolox/g) (Fig.3B-4D). This means

that the antioxidant capacity between NYSY and NYYY is significantly different ($P<0.05$). Combining the above results, our results indicate that the contents of total flavonoids are positively correlated with the antioxidant activity.

Further analysis was performed to understand the flavonoids variation between NYSY and NYYY. In 145 significant regulated flavonoids, all the chalcones (4 up regulated,100%), anthocyanin（1 up regulated, 100%）, dihydroflavonol (1 up regulatd, 100%) and dihydroflavone (4 up regulated,100%), most of isoflavones (10 up regulated, 91%), flavones (57 up regulated, 83%), flavonoid carbonoside (7 up regulated, 54%) and flavonols (7 up regulated, 54%) were up regulated in NYYY (Table 1，Table S1).

Transcriptomes provide the molecular basis of flavonoids diversity between two Artemisia cultivars.

To explore the molecular basis of flavonoids diversity between Artemisia cultivars, the transcriptome analysis was performed. Based on FC≥2 or ≤0.5 and VIP≥1, a total of 58,947 common differential expression gene clusters were found between NYSY and NYYY. All the differential expression gene clusters were functionally annotated in NR, Swiss Prot, Gene Ontology (GO), COG, KOG, Pfam, KEGG, and other databases. KEGG enrichment analysis showed 37 metabolic pathways ($P<0.01$), were significantly regulated between NYSY and NYYY (Fig S3, Table S2).

A total of 710 genes related to “Phenylpropanoid biosynthesis”, “Anthocyanin biosynthesis”, “Flavonoid biosynthesis”, “Isoflavonoid biosynthesis”, and “Flavone and flavanol biosynthesis” were identified (Table S3). As expected, GSEA analysis showed that most genes related to “Flavonoid biosynthesis” and “anthocyanin biosynthesis”’ were up regulated in NYYY (Fig. 4). Our data suggest that the flavonoid biosynthesis pathway genes were activated in different level, then lead to the variation of total flavonoid contents between two cultivars.

Combined metabolite and transcriptional analysis reveal the flavonoid ingredients possible synthesis pathway.

Our above flavonoid contents and widely targeted metabolomics results indicated that the ingredients and the synthesis related genes diverse between Artemisia cultivars. Most of the flavonoid ingredients, for example, naringenin, apigenin, vitexin, quercetin, eriodictyol and luteoforol have anti-cancer, anti-oxidant, anti-inflammatory, antiviral, antimicrobial, and anti-bibetic activities [31]. To identify the candidate synthesis genes of differently expressed flavonoids metabolites, we conducted correlation analyses between selected transcripts and metabolites.

Based on coefficient≥0.8 or ≤0.8, 238 different expressed gene clusters encoding 23 flavonoid synthesis enzymes related to 19 flavonoids metabolites, including 8 flavones (apigenin, acacetin, luteolin, ayanin, rhoifolin, cynaroside, lonicerin and lonicerin), one dihydroflavonol (pinobanksin), one flavonols (quercetin), 2 dihydroflavone (naringenin and eriodictyol), one chalcone (naringenin chalcone), one anthocyanins(cyanidin-3-O-glucoside), 2 flavonoid carbonoside (isovitexin and vitexin) , and 4 isoflavones (prunetin, 2'-hydroxygenistein, genistin, and 6''-O-Malonylgenistin) were uncovered(Table S4) .

The flavonoids synthesis starts from plant phenylpropane metabolism pathway. The metabolic intermediates and end product flavonoid biosynthesis pathway were mapped to a known KEGG pathway. Consist with up regulation of flavonoids and their precursors metabolites, a total of a total of one C4H, 28 HCT(shikimate O-hydroxycinnamoyl transferase,), 26 CCoAOMT (Caffeoyl-CoA O-methyltransferase), one C3'H gene clusters (5-O-(4-coumaroyl)-D-quinate 3'-monooxygenase),14 CHS (chalcone synthase), 23 CHI (chalcone isomerase), 2 F3H (naringenin 3-dioxygenase), 3 FSII (Flavone synthase II), 3 FLS (flavonol synthase), 6 PGT1(phlorizin synthase) , one F3’H and one C12RT1 (flavanone 7-O-glucoside 2''-O-beta-L-rhamnosyltransferase) regulated to flavones ingredients were up regulated in NYYY (Fig.5A,Table S3 and Table S4). In cyanidin biosynthesis pathway, consist with regulated isoflavones metabolites, one 1 ANS (Anthocyanidin synthase) and one BZ1 (anthocyanidin 3-O-glucosyltransferase) related to cyanidin 3-glucoside were

up regulated in NYYY. In isoflavones synthesis pathway,16 HIDH (2-hydroxyisoflavanone dehydratase), 2 I2'H (isoflavone/4'-methoxyisoflavone 2'-hydroxylase), and one IF7MAT (isoflavone 7-O-glucoside-6''-O-malonyltransferase) synthesis gene clusters were up regulated in NYYY (Fig 5A, Table S3 and Table S4). We also noticed that part of gene clusters encoding C4H, CCoAOMT, C3'H, F3'H, FLS, CHS, CHI, FSII, BZ1, HIDH, ANS, HCT, PGT1, and I2'H gene clusters down regulated in NYYY (Fig 5A, Table S3 and Table S4). The opposite expression patterns between flavonoids ingredients and synthesis genes indicated the existence of multigene families that may differentially control different flavonoid biosynthesis in Artemisia cultivars.

To validate the transcriptomic results, the expression of 6 differently expressed gene clusters encoding CHI (Gene cluster 12810.216674), F3H (Gene cluster 12810.173163), F3′H (Gene cluster 12810.6764), FSII (Gene cluster 12810.213557), CHS (Gene cluster 12810.170191), and I2′H (Gene cluster 12810.175245) were detected with qRT-PCR. The qRT-PCR data exhibited similar expression patterns to the RNA-Seq data between the two cultivars (Fig. 5B).

Possible Transcription Factors (TFs) regulate flavonoids ingredient synthesis were uncovered.

The above research shows the expression of genes related to the synthesis of flavonoids is different between the two materials. The above results showed that F3H, CCoAOMT, CHS, CHI, ANS, FLS, C3'H, HCT, C12RT1, DFR, HIDH, I2'H, IF7MAT, PGT1, FSII enzymes are the most possible synthesis genes in these flavonoids ingredients biosynthesis. These up regulated synthesis genes are the most possible genes for the regulated flavonoids ingredients biosynthesis in NYYY. Transcription factors (TFs) are essential regulators that bind to specific DNA sequences to activate or inhibit the expression of target genes, thereby influencing multiple biological processes. Nowadays, a large number activators and repressors, including R2R3-MYB [32-35], NAC [36],

bZIP[37-39], MADS-box[40] , WRKY[41-42] and SPL[43] TFs have been confirmed to be involved in regulating the expression of flavonoid biosynthetic genes according to the study of Arabidopsis, grapes, gerbera, eggplant, tomato, loquat, populous , apple , *etc*.

Based on coefficient≥0.8 or ≤0.8, possible 569 different expressed TFs encoding bHLH(94), MYB(204), NAC(71), bZIP(42), MADS(32), WRKY(126) related to the up regulated CHS gene clusters（10）, CHI(15), F3H (2), ANS(1), and FLS(1) transcription were uncovered (Table S5, Table S6). Among these TFs, most of bHLH (58, 62%) , MYB (120, 59%), WRKY(99,79%), NAC(45, 63%), MADS(23,72%) and bZIP (23，55%) were up regulated in NYYY (Fig 6，Table S6). These FTs may directly or indirectly regulate the expression of these genes for the synthesis of the up regulated flavonoids ingredients in NYYY.

Discussion

Morphological diversity was found in *A. argyi* cultivars.

Morphological difference was found between two cultivars (Fig 1A). As reported, the taxonomic relationships of the Artemisia genus species based on morphological traits are controversial because of their diverse morphological types, ploidy, and complicated genetic relationships [28]. In addition，there are many morphological types of variation after cultivated. Based on morphological traits, we could not discern which species the two cultivars belong at vegetative growth stage.

Single gene data (such as ITS regions of ribosomal genes, ITS2) and chloroplast genes (psbA-trnH, matK, rbcL, *rpl32-trn*L, and trnQ-5'-rps16 regions) are commonly used as molecular methods to aid plant authentication [28-29,44-45]. It is reported that ITS sequence is efficient to authentication species in Artemisia [28-29,44]. Indeed, based on the sequece of ITS2, we found that NYSY, NYYY, *A. princeps*, *A.montana, A argyi, and A.stolonifera* are clustered

to *Sect*. *Artemisia*(Fig. 1B). A degenerate site was detected in the *ITS sequence* from NYSY. This is probably caused by the polyploidization in breeding.

The two Artemisia cultivars are rich in potential medical and nutritional ingredients.

Artemisia plants were used as the source of medicine, food with a long history. Now, they also used as the resource of cosmetics. Based on UPLC-MS/MS technology, 882 kinds of metabolites, including flavonoids, phenolic acids, organic acids and their derivatives, amino acids and their derivatives, terpenes, alkaloids, lignans medicinal and nutritional ingredients were detected in two Artemisia cultivars (Fig 2). Flavonoid ingredients, such as naringenin, apigenin, eriodictyol, luteolin, quercetin, luteoloside have the activities of antioxidants, anti-inflammatory, inhibit platelet aggregation, anti-tumor, control blood sugar and improve insulin sensitivity, neuroprotection, and other effects, which provide resources for drug development. Our results suggest that the two materials are rich in potential medicinal and nutritional ingredients, and could be used as the source of medicine.

However, our combined PCA statistical analysis showed that there are large differences between the two materials. In significantly regulated metabolic compounds, most of potential medicinal ingredients were up regulated in NYYY, such as flavonoids (70.6%), terpenoids (70.6%), lignans and coumarins (55.6%), and phenolic acids (66.7%). It seems that NYYY is more suitable for medicinal use.

Besides used for medicinal purposes, Artemisia plants also used as food for a long history. The taste, flavors, and toxicity, are important for food. Many of Artemisia plants have an aromatic, bitter taste [3]. Astringency and bitterness cause uncomfortable taste. Polyphenols, including non-flavonoids and flavonoids, are related to the sensorial property of astringency and bitterness [46-47]. Our results showed that most of the flavonoid ingredients are down regulated in NYSY (Table S1, Fig.3C). The less flavonoids may relieve the uncomfortable taste in NYSY. In addition, santonin, a lactone compound used

for deworming drug during a long time and now already phased out for its toxicity, was only detected in NYYY, but not in NYSY [48] (Table S1). Considering the taste and toxicity, it seems that NYSY is more suitable as the source of food.

In addition, inflammation and oxidation of skin cell are the main factors leading to aging and skin problems. Anti-inflammatory and anti-oxidation are the two important functions of cosmetics. The two Artemisia cultivars have higher antioxidant activities (Fig. 3B-3D) though the antioxidant activities are different, suggesting the two cultivars also have the potential for using in the field of cosmetics.

Flavonoid synthesis gene and possible transcription factors is useful for the molecular breeding of Artemisia cultivar.

Flavonoids is a group of most investigated plant secondary metabolites, and widely present in plant leaves, flowers, fruits and other tissues [18]. Flavonoids possess various types with variable phenol structure, including flavone, anthocyanin, flavanone, flavanols, and isoflavone with wide range of pharmacological and biological activities, including anti-cancer, anti-allergic, anti-inflammatory, antioxidant, anti-microbial, anti-fungal and anti-diarrheal activities [18-20]. Flavonoids are widely found in Artemisia plants, such as, *A. argyi* [16], *A.montana* [21], *A. tenuisecta* [22], *A. annua* [23], *A.santolinifolia* [24], *A. frigida* [25], and *A. absinthiuma* [26], *etc*. Based on combing analysis of transcriptome and metabolome data, 14 flavonoid synthesis gene clusters encoding trans-cinnamate 4-monooxygenas, C3′H, HCT, FLS, F3′H, FSII, CHS, CHI, HDHF, F3′5′H, F3H, and I2′H，DFR，and BZ1 genes were up regulated or down regulated in NYYY, indicating that these genes is related to the synthesis of flavonoids substances.

According to the research of Arabidopsis, flavonoids biosynthetic genes (CHS, CHI, F3H, F3′H, and FLS, DFR, ANS/LDOX, UFGT) are directly or indirectly regulated by R2R3-MYB transcription factors [49-50] MBW complex (R2R3-MYBs such as MYB75, MYB90, MYB113, and MYB114, bHLH, and WD40) [51-

52], NAC [53], WRKY [41], SPL [43] transcription factors in response to various stimulus, such as stresses [54], light [55], UV [56], temperature [57].

Differential combinatorial interactions of cis-acting elements recognized by R2R3-MYB, bZIP, and bHLH factors control light-responsive and tissue-specific activation of phenylpropanoid biosynthesis genes

Recently, a lot of R2R3-MYB [58-59], NAC [36], MADS [60], bZIP [38], WRKY [61-62] and SPL transcription factors [63] are also found to participate in flavonoind synthesis in economic crops, such as apple, Gerbera hybrida, eggplant, populus tomentosa, and poplar. In our results, 7 R2R3-MYB, 94 WRKY, 57 NAC, 44 bHLH and 20 bZIP transcription factors expressed differently between NYSY and NYYY. Most of WRKY, R2R3- MYB, bHLH and MADS are up regulated, while most NAC and bZIP are down regulated in NYYY (Fig 7). In previous study, AtMYB11, AtMYB12 and AtMYB111 are all independently capable of activating the genes encoding chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H) and flavonol synthase (FLS), which together determine flavonol content. WRKY genes worked with MYB and bHLH to positively or negatively regulated in flavonoinds synthesis [42,61]. For example, PyWRKY26 and PybHLH3 could co-target the PyMYB114 promoter, which resulted in anthocyanin accumulation in red-skinned [61].

This suggest that these TFs may directly or indirectly coordinate the expression of flavonoids synthesis genes through a complex and precise manner, then lead to the accumulation of flavonoids metabolites, such as naringenin, apigenin, eriodictyol, luteolin, quercetin, luteoloside, *etc*. The roles of these TFs in the metabolic pathway of flavonoids and the mutual regulation network response to different stimulus need to be further studied in the future.

Conclusion

Taken together, our metabolomics results provide evidence for further medical and nutritional use of the two Artemisia Cultivars. We also provide insight into

the regulatory mechanisms of flavonoid biosynthesis between the two Artemisia cultivars. Our results will facilize the utilization of the two Artemisia Cultivars and for molecular breeding of Artemisia cultivar in the future.

Materials and Methods

Plant materials

Two Artemisia cultivars, NYYY and NYSY, were kindly provided by Nanyang Guoyizhongjing Wormwood Industry Co. Ltd, China and collected from Qiaotou, Sheqi, Nangyang, Henan, China with permission. The two materials were authenticated by Gu HK and Qiao YC and have been deposited in Institute of Radiation Technology, Beijing Academy of Science and Technology.

The two cultivars were grown in soil under a 16 h light/8 h dark photoperiod (about 200 μmol m^{-2} s^{-1} light intensity) at 25℃. The leaves of 2-month-old plants were harvested, frozen immediately in liquid nitrogen, and stored at -80°C for further study.

DNA extraction, sequencing, and phylogenetic analysis

Total genomic DNA was extracted from leaves using TGuide plant Genomic DNA Extraction Kit (Tiangen, OSR-M301). ITS sequences were amplified using primers ITS2F (5'-ATGCGATACTTGGTGTGAAT-3') and ITS3R (5'-GACG CTTC TCCA GACT ACAAT-3'). DNA sequence were conducted by Sangon Biotech (Shanghai) Co.,Ltd. Thirteen ITS2 sequences of Artemisia species deposited in GenBank were down loaded. The ITS2 sequences of 14 Artemisia samples, including NYYY, NYSY, A. *montana* (FJ643027.1), *A. stolonifera* (KU555697.1), *A. princeps* (KU855168.1), *A. argyi* (DQ925700.1), *A. feddei* (FJ643013.1), *A. frigida* (JN861917.1), *A. absinthium* (KX581838.1), *A. sieversiana* (KU855170.1), *A. scoparia* (KU555638.1), *A. capillaris* (KX675134.1), *A. fukudo* (IM150921. 1), and *Aster spathulifolius* (HQ154050.1) were aligned using UPGMA method. Phylogenetic trees based on ITS sequences of 15 Artemisia were generated by the MEGAX [64]. Among them,

Aster spathulifolius was chosen as an out-group species due to its distinct separation from the ingroup taxa.

Sample preparation and extraction for widely targeted metabolome analysis

All samples are freeze-dried by vacuum freeze-dryer (Scientz-100F). The freeze-dried sample was crushed using a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 Hz. Dissolve 100 mg of lyophilized powder with 1.2 ml 70% methanol solution, vortex 30 seconds every 30 minutes for 6 times in total, place the sample in a refrigerator at 4°C overnight. Following centrifugation at 12000 rpm for 10 min, the extracts were filtrated (SCAA-104, 0.22μm pore size; ANPEL, Shanghai, China, http://www.anpel.com.cn/) before UPLC-MS/MS analysis. Three biological replicates were performed for each variety. Quality control (QC) samples were prepared by mixing sample extracts.

Flavonoid content analysis

Flavonoids of Artemisia samples were extracted according to the following protocol. Briefly, 100 mg of powder was extracted with 70% methanol(v/v) at 4℃ overnight and vortexed three times. The samples were centrifuged at 12000 g for 10 min. The supernatant fractions were filtrated through a micropore filter membrane (0.22 μm pore size). Three biological replicates were performed for each variety.

Flavonoids content in the sample extract was assayed using spectrophotometric method as described by with slight modifications. Briefly, 40 μL test sample stock solution or rutin (0.2mg mL^{-1}) were mixed with 40 μL 5% $NaNO_2$ and incubated at room temperature for 6 min. Then, 40 μL 10% Al $(NO3)_3$ were added to the above mixture and incubated at room temperature for 6 min. Finally, 400 μL 4% NaOH were added to the above mixture, dilute with 70% methanol to volume 1ml, and incubated at room temperature for 30 min. The absorbance was measured at 510 nm using SpectraMax M_3. A calibration curve (y = 1.4682X -0.0061, R^2 = 0.9975) of quercetin was drawn at

final concentrations of 0.08,0.24,0.32,0.40, and 0.48 μg/mL and the resultant flavonoid content is expressed in microgram equivalents of quercetin per gram dry weight (mg /g).

Determination of antioxidant capacity

DPPH radical scavenging activities were determined using a total antioxidant capacity. Briefly, 400 μL DPPH solution (absolute ethyl alcohol) and 20 μL of the supernatant were added to a 96-well microplate. The mixture was mixed and left for 30 min at room temperature under darkness, and then the absorbance at 517 nm was measured using SpectraMax M3. The DPPH radical scavenging activities of each sample were calculated as the percent inhibition according to the following equation: The DPPH radical scavenging activity of positive control (%) = [1-(A1-A2) ÷ A3] ×100%. Note: A1 is the absorbance of the test sample, A2 is the absorbance of the positive control, A3 is the absorbance of the blank.

Scavenging capacity of 2,2′-azino-bis-[3-ethylbenzthiazoline-6-sulfonic] (ABTS radical cations) was measured using a total antioxidant capacity assay kit (Suzhou Grace Biotechnology Co.,Ltd，G0127W). Briefly, 190 μL peroxidase solution, 10 μL of the supernatant were added to a 96-well microplate. The mixture was mixed and left for 6 min at room temperature under dim light, and then the absorbance at 414 nm was measured using Spectra Max M3. The results were reflected in mmol Trolox/g of extracts (umol/g).

The FRAP assay was conducted using a total antioxidant capacity assay kit (Suzhou Grace Biotechnology Co.,Ltd， G0115W). Briefly, 170 μL FRAP solution, 25ul distilled water and 5 μL of the supernatant and were added to a 96-well microplate. The mixture was mixed and left for 10 min at room temperature under dim light, then the absorbance at 590 nm was measured using Spectra Max M3. The results were expressed as the Trolox of extracts (umol/g).

RNA isolation and transcriptome sequencing

RNA was isolated from leaves harvested from two-month-old plants grown in the greenhouse. Three biological replicates were sampled per variety. The cDNA libraries were sequenced on the Illuminase sequencing platform by Metware Biotechnology Co.,Ltd. (Wuhan, China). The raw data were filtered to ensure the quality and reliability. The obtained clean data were spliced *de novo* using Trinity software. The unigene annotations were obtained based on seven major databases including NR (NCBI non-redundant protein sequences), PFAM (Protein family), Swiss-Prot (A manually annotated and reviewed protein sequence database), KEGG (Kyoto Encyclopedia of Genes and Genomes), and GO (Gene Ontology).

Quantitative real-time PCR (qRT-PCR) Analysis

To validate the reliability of transcriptome results, qRT-PCR tests were performed. cDNA synthesis and qRT-PCR were performed according to a previously reported method. The AaActin was selected as an internal control, and the primers of validated genes used for qRT-PCR analysis were showed in Supplementary Table S7. All samples were analyzed in three biological replicates. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Unsupervised PCA (principal component analysis) was performed by statistics function prcomp within R. The data was unit variance scaled before unsupervised PCA. The contents of total flavonoids and antioxidant activity were analyzed by one-way analysis of variance (ANOVA) and a Fisher's least significant difference (LSD) test at the 5% level.

GSEA analysis

GSEA was conducted using cluster Profiler package (version 4.2.3). The fold change of gene expression between NYSY and NYYY group was calculated, and the gene list was generated according to the change of |log2FC|.The adjusted p-value < 0.05 was set as the cut-off criteria.

Declarations

Ethics approval and consent to participate

Experimental research and field studies on plants, including the collection of plant material, complied with relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable

Availability of data and material

The RNA sequence data are in the NCBI's Sequence Read Archive (SRA) under the accession number PRJNA821649.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Song M., Qiao Y., Wu L., Wei L. and Wang Q. prepared the main manuscript text and figures, Gu H, Liu G. , Zhou S and Wang P. prepared the materials. All authors have read the manuscript.

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Figures

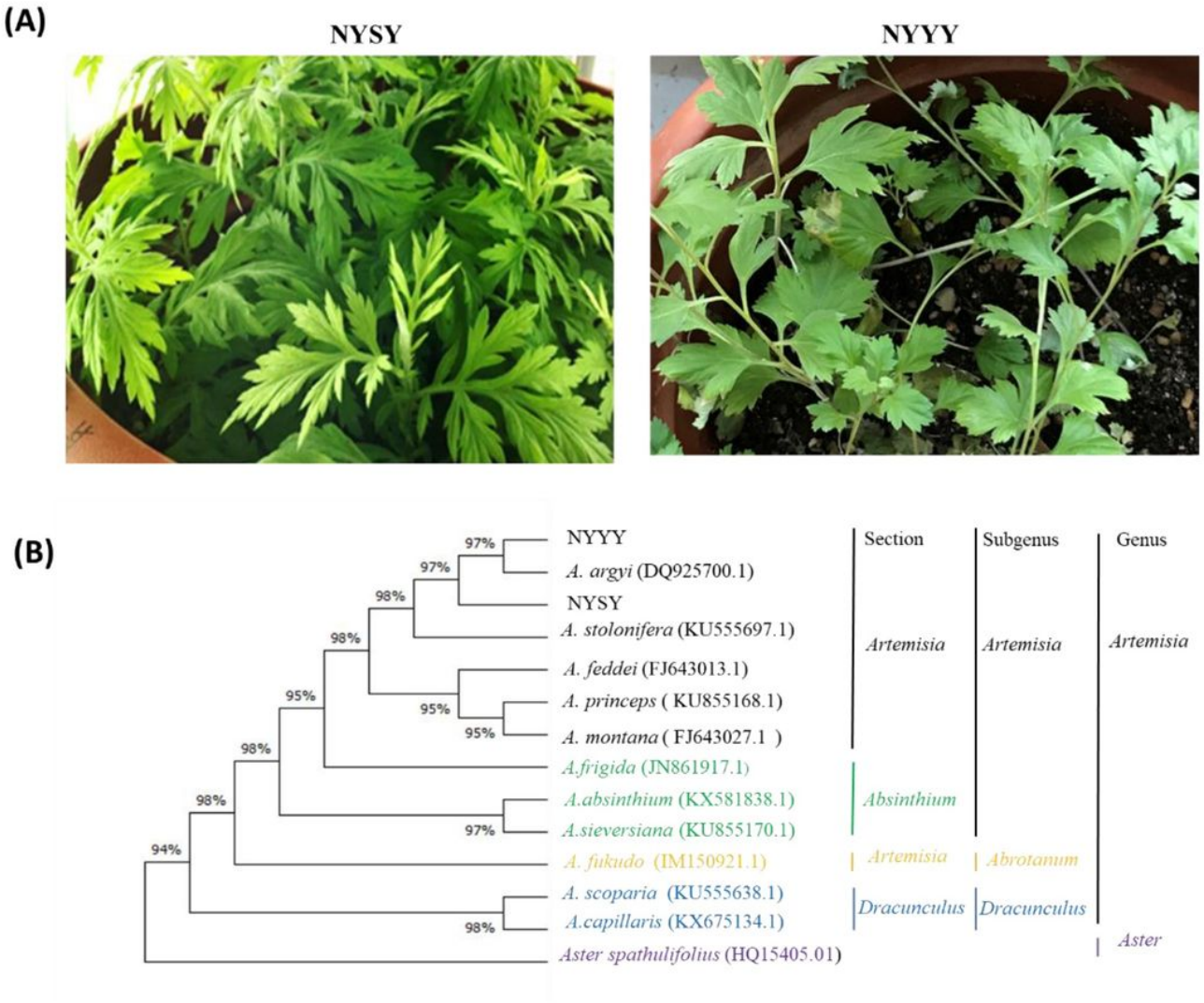

Figure 1

Morphological characterization and evolutionary relationships of NYSY and NYYY. (A) The morphological phenotypes of NYSY and NYYY grown under long day condition for two months. (B) Phylogenetic tree constructed from 14 Composite plants nucleotide sequences including NYYY, NYSY, A. montana (FJ643027.1), A. stolonifera (KU555697.1), A. princeps (KU855168.1), A. argyi (DQ925700.1), A. feddei (FJ643013.1), A. frigida (JN861917.1), A. absinthium (KX581838.1), A. A. sieversiana KU855170.1), A. scoparia (KU555638.1), A.capillaris (KX675134.1), A.fukudo (IM150921_1), and Aster spathulifolius (HQ154050.1).

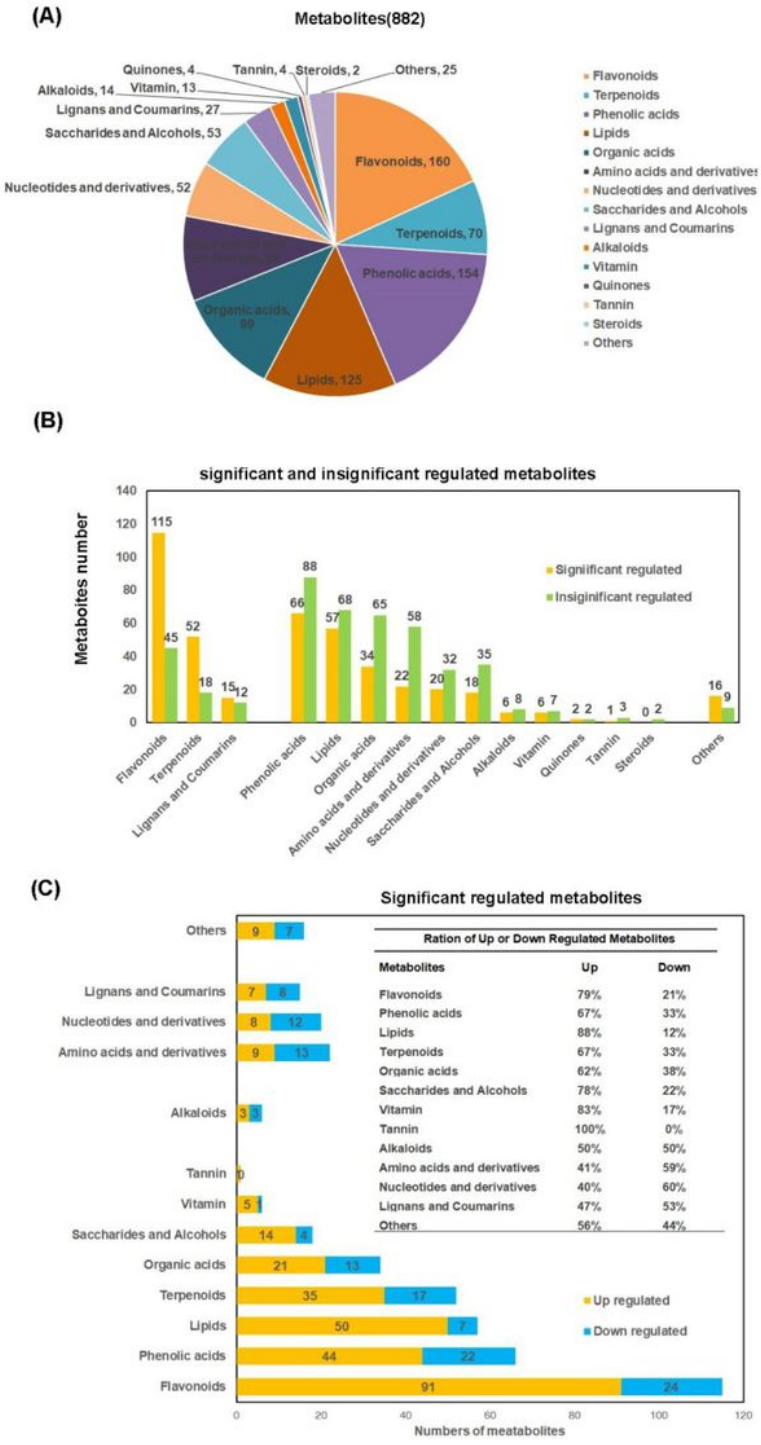

Figure 2

Metabolomic profiling of NYSY and NYYY. (A) Metabolites distribution in two cultivars.(B) Profile of significant and insignificant regulated metabolites between NYSY and NYYY. (C) Profile of significant regulated metabolites between NYSY and NYYY.

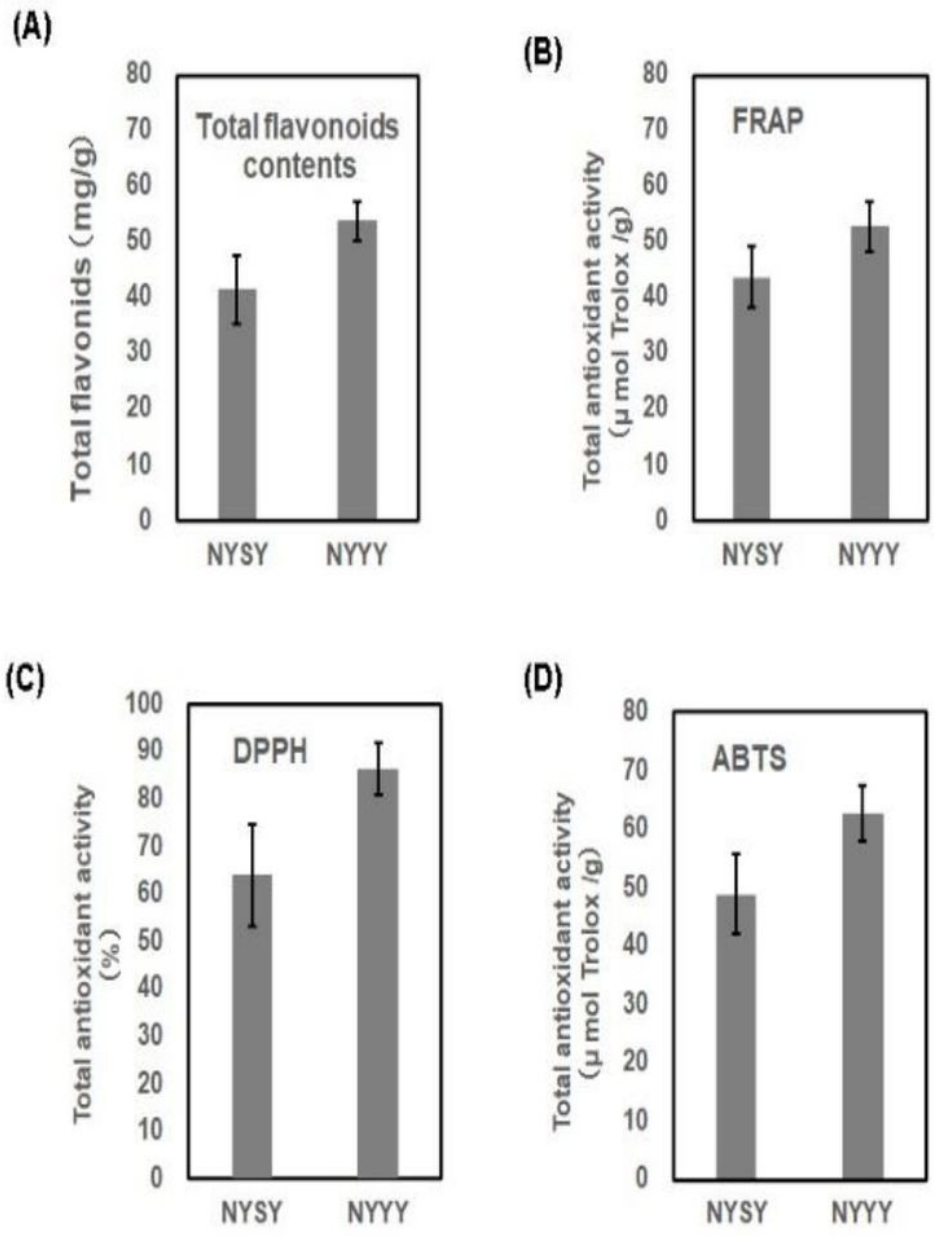

Figure 3

The total flavonoids content and antioxidant activity of NYSY and NYYY. (A)Total flavonoids contents of two cultivars(B-D) Antioxidant ability of NYSY and NYYY. (B)FRAP scavenging activities, (C) DPPH radical scavenging activities, and (D) ABTS scavenging capacity of 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic of two cultivars. Both the two cultivars grown under long-day condition for two months.

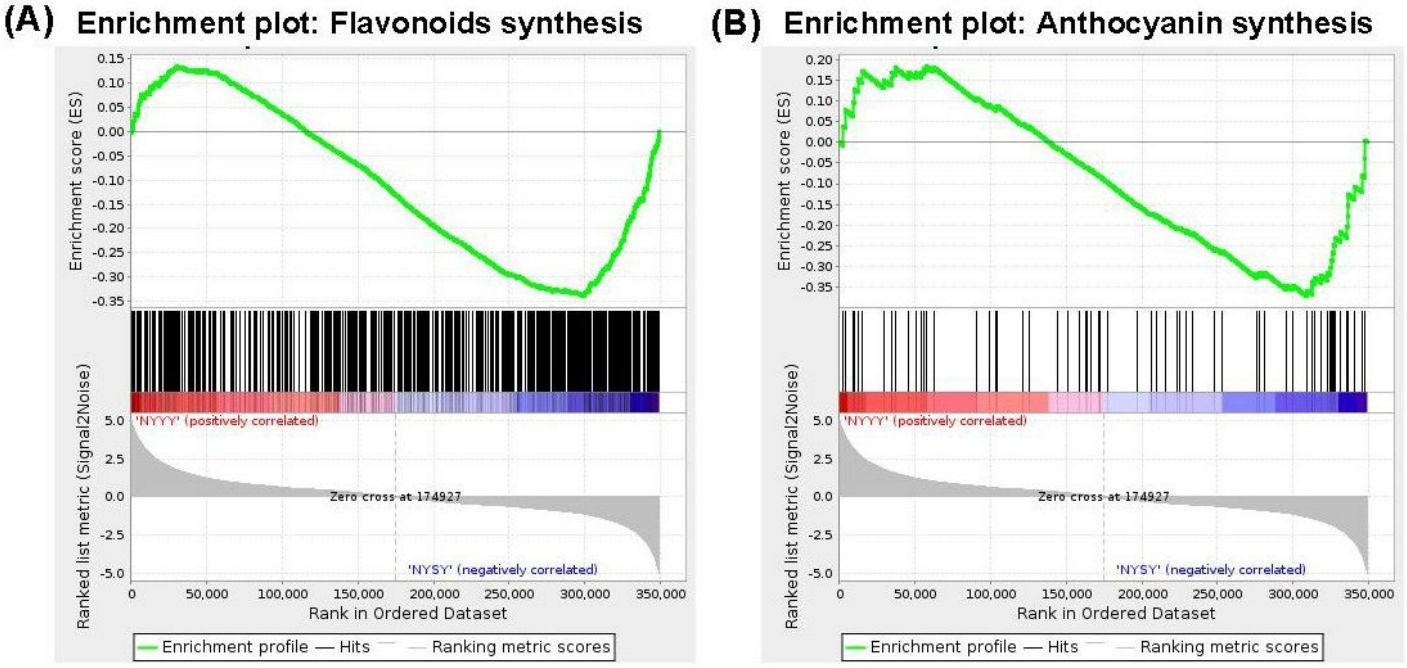

Figure 4

Gene set enrichment analysis the flavonoids synthesis regulated genes transcription between NYSY and NYYY. (A) KEGG flavonoids metabolism and (B) anthocyanin metabolism gene sets.

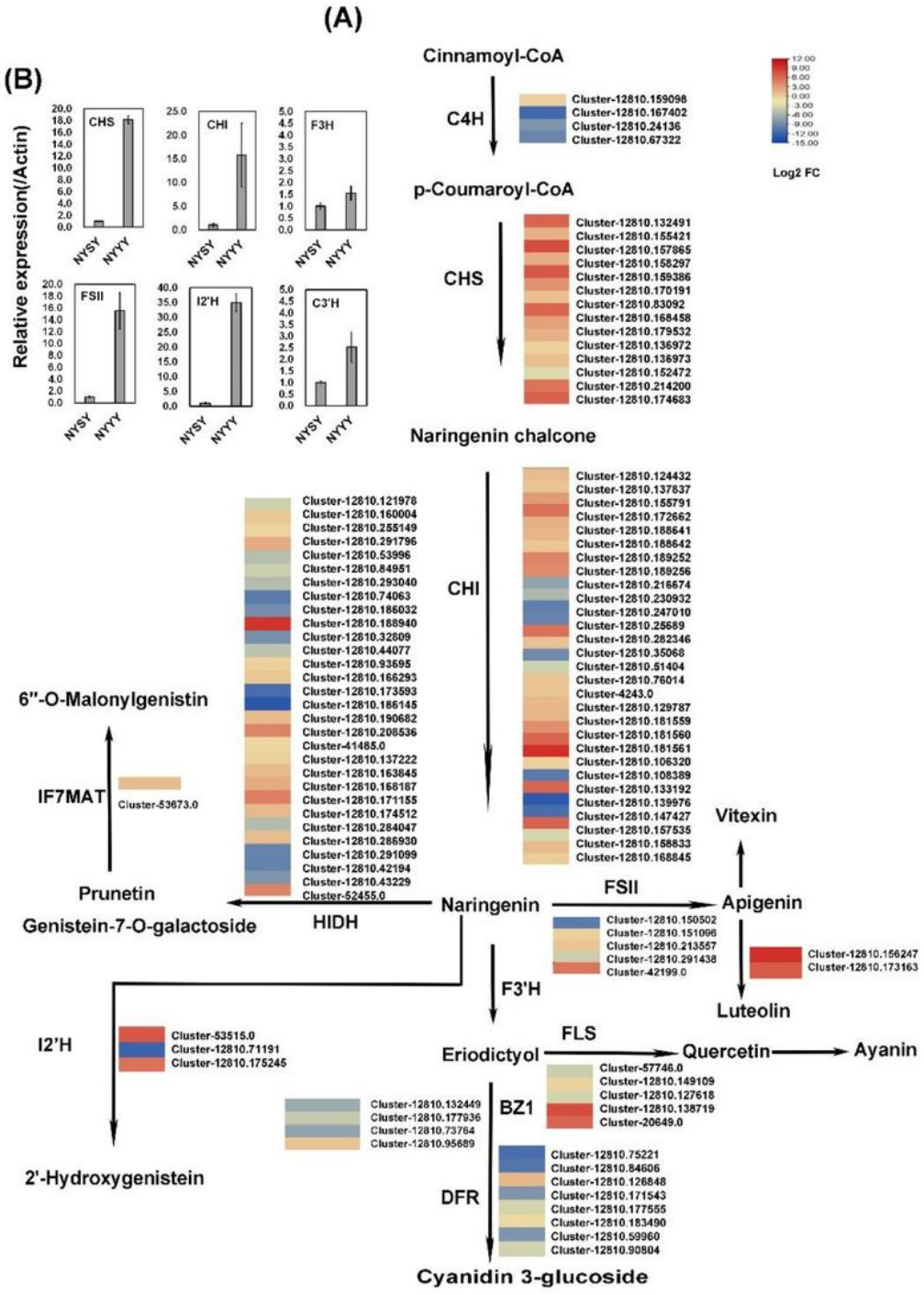

Figure 5

Possible Flavonoid synthesis pathway of the two Artemisia cultivars. (A) Synthesis pathway of part flavonoid ingredients in the two Artemisia cultivars. C4H, transcinnamate 4-monooxygenase; CHS, chalcone synthase; CHI, chalcone isomerase; FSII, flavone synthase II; F3H, naringenin 3-dioxygenas; FLS, flavanol synthase; F3'H, flavonoid 3'-monooxygenase; I2'H, isoflavone/4'-methoxyisoflavone 2'-hydroxylase; HIDH, 2- hydroxyisoflavanone dehydratase; IF7MAT, isoflavone 7-O-glucoside-6"-

Omalonyltransferase, BZ1,anthocyanidin3-O-glucosyltransferase and C12RT1, flavanone 7- O-glucoside 2"-O-beta-L-rhamnosyltransferase, DFR, bifunctional dihydroflavonol 4- reductase/flavanone 4-reductase. B Quantitative real time PCR validation of selected candidate genes predicted to differentially affect the synthesis of above flavonoids ingredients synthesis. CHI (Gene cluster 12810.216674), CHS (Gene cluster 12810.170191), F3H (Gene cluster 12810.173163), FSII (Gene cluster 12810.213557), C3'H (Gene Cluster-12810.193865), I2'H (Gene cluster 12810.210239) in NYSY and NYYY. The data represent the mean from three replicates with three biological repeats. Error bars indicate SE.

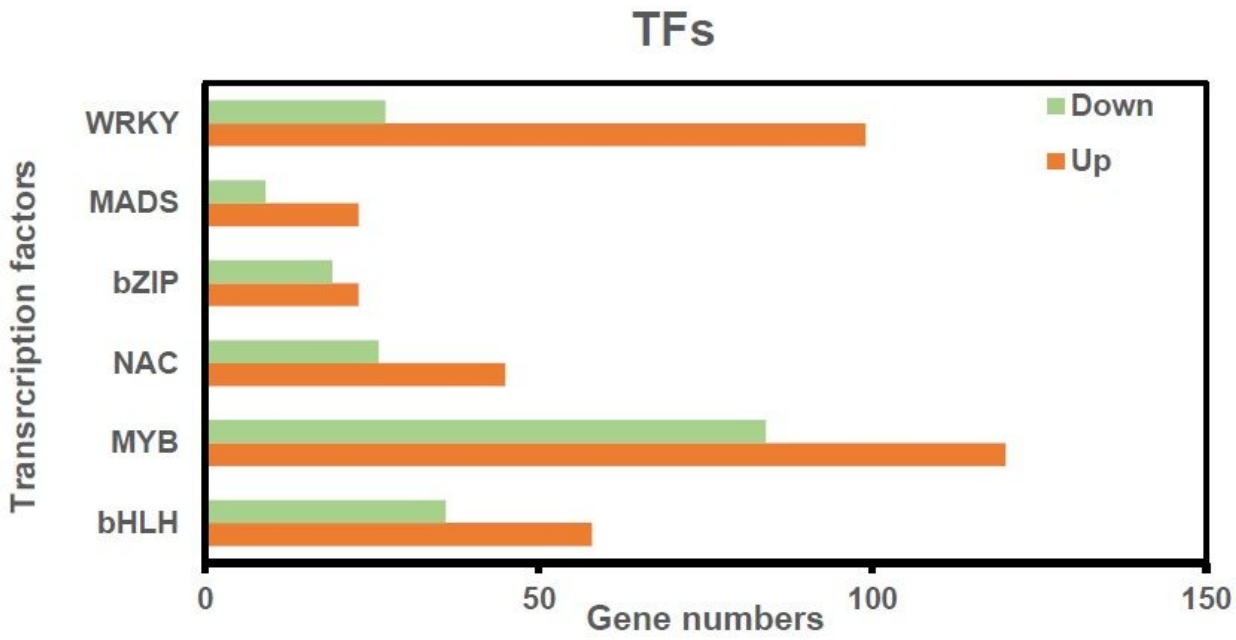

Figure 6

Distribution of significant regulated transcription factors TFs possibly regulate to 5 flavonoids synthesis genes between NYSY and NYYY.

Supplementary Files

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