Transcriptional regulation of flavonoid biosynthesis in *Artemisia annua* by AaYABBY5

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**Abstract**

*Artemisia annua* is a medicinal plant rich in terpenes and flavonoids with useful biological activities such as antioxidant, antitumor, and antimalarial activities. The transcriptional regulation of flavonoid biosynthesis in *A. annua* has not been well-studied. In this study, we identified a YABBY family transcription factor, AaYABBY5, as a positive regulator of anthocyanin and total flavonoid contents in *A. annua*. AaYABBY5 was selected based on its similar expression pattern to the phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), and flavonol synthase (FLS) genes. A transient dual-luciferase assay in *Nicotiana bethamiana* with the AaYABBY5 effector showed a significant increase in the activity of the downstream LUC gene, with reporters AaPAL, AaCHS, AaCHI, and AaUFGT. The yeast one-hybrid system further confirmed the direct activation of these promoters by AaYABBY5. Gene expression analysis of stably transformed AaYABBY5 overexpression, AaYABBY5 antisense, and control plants revealed a significant increase in the expression of AaPAL, AaCHS, AaCHI, AaFLS, AaFSII, AaLDOX, and AaUFGT in AaYABBY5 overexpression plants. Moreover, their total flavonoid content and anthocyanin content were also found to increase. AaYABBY5 antisense plants showed a significant decrease in the expression of flavonoid biosynthetic genes, as well as a decrease in anthocyanin and total flavonoid contents. In addition, phenotypic analysis revealed deep purple-pigmented stems, an increase in the leaf lamina size, and higher trichome densities in AaYABBY5 overexpression plants. Together, these data proved that AaYABBY5 is a positive regulator of flavonoid biosynthesis in *A. annua*. Our study provides candidate transcription factors for the improvement of flavonoid concentrations in *A. annua* and can be further extended to elucidate its mechanism of regulating trichome development.

**Introduction**

*Artemisia annua* is a renowned plant rich in terpenes and flavonoids. Flavonoids are polyphenolic plant secondary metabolites. Anthocyanins, flavonols, flavanols, and proanthocyanidins (PAs) or condensed tannins are the major classes of flavonoids. These compounds show differential expression patterns according to plant growth or developmental stages and in a species-specific manner. Flavonoids perform various functions in plants, including antioxidant activity, protection against UV light, defensive responses against plant pathogens, activation of nodulation genes in legumes, fertility, and auxin transport. Flavonoids are also beneficial for human health in many aspects as a nutritional source, as well as for the treatment of various diseases.

Increasing evidence recommends using the dried leaves of *A. annua*, which contain both artemisinin and flavonoids, to cure malaria more effectively than using only artemisinin. A recent study showed that the combination of kaempferol and artesunate exerts potent antimalarial activity synergistically in mice infected with malarial plasmodium. In a recent study, the mechanism for increased bioavailability of artemisinin when using dried leaves that supply artemisinin and flavonoids synergistically was shown. A comparative study indicated the
parallel biosynthesis of flavonoids and artemisinin at three growth stages, except for a few flavonoids that showed altered behavior. Furthermore, flavonoids of *A. annua* also exhibit efficacious antioxidant effects\(^6\), strong anti-inflammatory properties\(^7\), and potent anticancer characteristics\(^8\). To cure cancer, various flavonoids of *A. annua* have been reported to synergize with many anticancer drugs. The reported flavonoids that aid anticancer drugs include apigenin, eupatin, luteolin, silyben, kaempferol, and quercetin\(^9\)–\(^14\). In a recent study, potent anti-inflammatory activities of the flavonoids of *A. annua* were reported\(^7\).

*A. annua* can produce ~40 different flavonoids, which may vary depending upon the cultivar or Line\(^15\). Despite the massive abundance of flavonoid varieties in *A. annua* and their significant therapeutic potential, the flavonoid biosynthetic pathway and its transcriptional regulation have not been well-elucidated. A few studies have been reported on the regulation of flavonoid biosynthesis in *A. annua*\(^16,17\). In other plant species, including *A. thaliana*, the flavonoid biosynthetic pathway has been thoroughly studied\(^18\) (Fig. 1). The upstream shikimate pathway provides the precursor amino acid phenylalanine, which is catalyzed by phenylalanine ammonia lyase (PAL), to generate cinnamate\(^19\). Cinnamate is then transformed into 4-coumaroyl-CoA in the presence of cinnamate 4-hydroxylase (C4H) and 4-coumarate-CoA ligase (4CL)\(^20\). The first committed step toward flavonoid biosynthesis involves 3x malonyl-CoA and 1x 4-coumaroyl-CoA to generate chalcones (the basic skeleton of all flavonoids) in a condensation reaction catalyzed by chalcone synthase (CHS)\(^21,22\). In the next step, chalcone isomerized to the flavanone naringenin in the presence of chalcone isomerase (CHI)\(^23\). In the following step, which generates dihydroflavonones, naringenin is oxidized by flavanone-3-hydroxylase (F3H) to yield dihydrokaempferol, which is subsequently hydroxylated by flavonoid 3’-hydroxylase (F3’H) or flavonoid 3’,5’-hydroxylase (F3’5’H), producing dihydroquercetin or dihydromyricetin, respectively\(^24\).

F3’H or F3’5’H may also directly perform the hydroxylation of naringenin to yield flavonones called eriodictyol and penta-hydroxy-flavanone, which upon hydroxylation generate dihydroquercetin and dihydromyricetin, respectively. Next, three dihydroflavonols are converted to anthocyanidins in a two-step reaction catalyzed by dihydroflavonol reductase (DFR) and leucoanthocyanidin dioxygenase (LDOX), successively. In

![Fig. 1 The flavonoid biosynthetic pathway in Artemisia annua.](image)

PAL phenylalanine ammonia lyase, C4H cinnamate 4-hydroxylase, 4CL 4-coumarate-CoA ligase, CHS chalcone synthase, CHI chalcone isomerase, F3H flavanone-3-hydroxylase, F3’H flavonoid 3’-hydroxylase, F3’5’H flavonoid 3’,5’-hydroxylase, F3II flavone synthase II, FLS flavonol synthase, DFR dihydroflavonol reductase, LDOX leucoanthocyanidin dioxygenase, UFGT UDP-glucose flavonoid 3-O-glucosyl transferase, MTs methyltransferases are key enzymes involved in the pathway.
the first step, DFR is involved, which converts dihydroquercetin to leucocyanidin, dihydrokaempferol to leucopelargonidin, and dihydromyricetin to leucodelphinidin. The second step is involved in the LDOX-mediated catalytic oxidation of leucocyanidin to cyanidin, leucopelargonidin to pelargonidin, and leucodelphinidin to delphinidin. In the final step, cyanidin, pelargonidin, and delphinidin are glycosylated by the enzyme UDP-glucose flavonoid 3-O-glucosyl transferase (UGFT) to produce colorful and stable compounds called anthocyanins25. Anthocyanin:cyandin-3-glucoside may be further converted to peonidin-3-glucoside, and delphinidin-3-glucoside is converted to petunidin or malvidin-3-glucoside after being methylated by methyltransferases (MTs). Molecules called tannins or PAs are generated by the enzymes leucohyanidin reductase (LAR) and anthocyanidin reductase (ANR), which catalyze the reduction of leucocyanidin to catechin or cyanidin to epicatechin, respectively.

Recently, several genes from the A. annua flavonoid biosynthetic pathway, including chalcone isomerase (AaCHI) phenylalanine ammonia lyase (AaPAL1), flavone-3-hydroxylase (AaF3H), and AaFLS1, have been cloned and characterized16,17,26,27. A recent study showed that the overexpression of AaCHI in A. annua provides not only increased levels of flavonoids but also artemisinin27. The transcriptional regulation of flavonoid biosynthesis in A. annua has not been well-elucidated. The overexpression of MYC2 in A. annua resulted in an increased level of both anthocyanins and artemisinin28. Most of the R2R3-MYB genes have been shown to play regulatory roles in flavonoid biosynthesis in A. annua. Recently, the MYB family transcription factor AaTAR2 from A. annua was reported to synergistically regulate both artemisinin and flavonoids29; however, the mechanism of the regulation of flavonoid biosynthesis was not found. In plants, flavonoid biosynthesis is tightly regulated by MBW complexes that interact with late biosynthetic genes. In Arabidopsis thaliana, this ternary complex comprises specific R2R3-MYB (PAP1, PAP2, MYB113, or MYB114) and bHLH transcription factors (GLABRA 3, GL3 or Enhancer of GLABRA 3, ECL3) that interact with WDR proteins (TTG1; TRANSPARENT TESTA GLABRA 1)30–34. However, these complexes have not yet been identified in A. annua. The identification of transcription factors that regulate flavonoid biosynthesis (by direct means or via indirect interactions), the discovery of MBW complexes and their transcriptional control is important to obtain a better understanding of flavonoid biosynthesis in A. annua.

The YABBY gene family is a small family of transcription factors that are specifically found in only seed plants. Due to their small group and their role in primary and secondary plant metabolism, they are of great interest to researchers. Over the last decade, several studies have been conducted on YABBY proteins; however, the function of YABBY family transcription factors in flavonoid biosynthesis in A. annua was not previously characterized. The characteristic features of the YABBY family are the N-terminal C2C2 zinc finger-like domain and C-terminal HMG domain35,36. The zinc finger domain mediates protein–protein interactions, whereas the YAB domain mediates DNA binding. YABBY protein complexes generate homo or heterodimers between the YABBY proteins and form complexes with other proteins37. Angiosperms such as Arabidopsis contain six YABBY genes in their genomes35,36, which are classified into six groups, FIL + YAB3, YAB2, YAB5, CRC, and INO, and are found to be transcriptional regulators38,39.

The primary function of YABBYs in plants is the regulation of laminar outgrowth and leaf development40. YABBY proteins interact with LEUNIG or its homologs, and YAB-LUC complexes regulate adaxial cell polarity in leaves and the initiation of the apical meristem in shoots41. It was first thought that these transcription factors were limited to the regulation of primary metabolic processes in plants, and massive data on YABBY-mediated leaf development in various seed plants are available. However, during the last decade, a few studies have reported the role of YABBY transcription factors in secondary metabolism, such as the regulation of anthocyanins and glucosinolates through AtFIL in A. thaliana and the control of monoterpenoid biosynthesis by MsYABBY5 in Mentha spicata42–44. YABBY transcription factors also work in response to various stresses and plant defense responses; for example, Arabidopsis YABBY10 is involved in drought and salt stress responses45, and AtFIL knockout lines provide good resistance to bacterial infections42.

In our previous study, we cloned and characterized a YABBY gene; AaYABBY5 is a positive regulator of artemisinin biosynthesis that directly binds to the promoters of AaCYP71AV1, and AaDBR2, activates their gene expression and results in a significant increase in the concentration of artemisinin. In this study, we elaborated on the role of AaYABBY5 in the biosynthesis of flavonoids, including anthocyanins, and their regulation. AaYABBY5 was selected for this study based on its similar expression pattern to that of the PAL, CHI, and FLS genes. YABBY5 was found to be a direct activator of promoters of the PAL, CHI, CHS, and UFGT genes in the transient Nicotiana benthamiana infiltration system and yeast transformation system, respectively. In addition, comparative analysis of transgenic A. annua plants that overexpressed the AaYABBY5 ORF, overexpressed the antisense RNA of AaYABBY5, plants transformed with empty pHB vector and untransformed/wild-type plants revealed a significant increase in the transcript levels of AaPAL, AaCHI, AaCHS, AaFLS, AaFSII, AaLDOX, and
AaUFGT, as well as enhanced flavonoid and anthocyanin production in AaYABBY5 overexpression plants but a significant decrease in gene expression, flavonoids, and anthocyanins in AaYABBY5 antisense plants compared to the control plants.

To our knowledge, in A. annua, the mechanism of the transcription factor-mediated direct activation of flavonoid biosynthesis genes has not been studied before. Previous data do not explain how flavonoids are regulated at the molecular level. Our study provided evidence of direct regulation of the PAL gene, which is present upstream of the flavonoid biosynthetic pathway; AaCHS and AaCHI, which regulate the first committed step of flavonoid biosynthesis and the next preceding step, respectively; and UFGT, which is involved in the conversion of precursor anthocyanidin molecules to anthocyanins. The present study broadens the knowledge on the direct regulation of flavonoids by AaYABBY5 in A. annua. To our knowledge, we provide the first molecular mechanism of flavonoid regulation in A. annua.

Results
Flavonoid biosynthetic genes contain YABBY-binding motifs

The cloned promoter sequences of flavonoid biosynthetic genes used in the present study, AaPAL, AaCHS, AaCHI, AaFLS, AaFSII, and genes regulating anthocyanins AaDFR, AaLDOX, and AaUFGT, were analyzed for putative YABBY-binding sequences37 using PlantPAN 3.0 (PlantPAN; http://PlantPAN.itps.ncku.edu.tw). Recent work, including ChIP and RNA-seq studies on YABBY-binding motifs present in soya bean and protein-binding microarrays in A. thaliana, has shown that these sites vary greatly among different species. YABBY-binding motifs are represented by AT-rich sites in Arabidopsis with consensus binding sequences defined as AATNATAA and AATNATTA. The homologous YABBY-binding motifs found in the promoter sequences are shown in Fig. 2a, with the positions marked by numbers. Except for DFR, YABBY-binding motifs were identified in all promoters.

![Fig. 2 Bioinformatic analysis of the promoters of flavonoid biosynthetic genes, as well as the expression profile of YABBY family genes and flavonoid-regulating genes in A. annua. a Putative YABBY-binding sites predicted by PLANTPAN3.0 are shown. Positions on plus and minus strands are represented by numbers above and below the promoter sequence, respectively. YABBY-binding sites were found in all promoters except the DFR promoter sequence, for which no predicted YABBY-binding site was predicted. b Heatmap showing the expression profile of YABBY family genes, as well as flavonoid biosynthetic genes in six tissues. The color scale at the top represents the RPKM (reads per kilobase per million mapped reads) values. AaYABBY5 (marked with a black dot) was selected as a potential transcription factor that might regulate flavonoid biosynthesis because of its similar expression pattern to the important flavonoid biosynthetic genes](image-url)
Global expression profile of YABBY family genes and selection of AaYABBY5 as a potential transcription factor regulating flavonoid biosynthesis

The transcriptome data of six different tissues of A. annua were previously generated by our lab. Plant secondary metabolites are usually synthesized in a species- or tissue-specific manner, and secondary metabolites, including flavonoids, are synthesized in trichomes. To identify potential YABBY family genes that might regulate flavonoid biosynthesis, a heatmap was constructed to compare the expression of YABBY genes and flavonoid biosynthetic genes across tissues; trichomes, buds, stems, roots, leaves, and seeds (Fig. 2b).

We found two YABBY genes clustered with flavonoid pathway genes, showing higher expression in trichomes and buds. Among the YABBY family genes, AaYABBY5 (marked with a black dot) was found to be a candidate transcription factor that showed a transcription profile parallel to that of PAL, CHI, DFR, and FLS and showed higher expression in trichome, bud, stem, and leaf tissues. In our previous findings, we found that AaYABBY5 regulates DBR2 and CYP71AV1 that are involved in the artemisinin biosynthetic pathway. Here, we found that AaYABBY5, DBR2, and CYP71AV1 showed similar expression patterns, i.e., higher expression in trichomes and buds, with a progressive decline in their expression in the stem tissues.

As this study focused on regulating flavonoid regulation, we hypothesized that AaYABBY5 might regulate PAL, CHS, CHI, and FLS, which showed similar expression patterns in trichomes and/or buds. Interestingly, PAL and DBR2 (the YABBY5 target gene) showed similar expression in trichomes, buds, stems, roots, and leaves. DFR and FSII expression was found more in buds than in trichomes. F3’H expression was very different from the other genes present in the flavonoid pathway. LDOX and UFGT are enzymes that are involved in anthocyanin biosynthesis. In A. annua, anthocyanins are limited to stem tissues, and the expression of LDOX and UFGT was found to be higher in stem tissues than in other tissues. In a previous study, real-time PCR analysis of different tissues revealed that AaYABBY5 transcripts are also found in the stem tissues of A. annua; therefore, we speculated that it might also regulate the LDOX and/or UFGT genes. Overall, it was speculated that YABBY5 might regulate PAL, CHI, CHS, and FLS, which are involved in early flavonoid biosynthesis, and LDOX and UFGT, which are present in the late flavonoid (anthocyanin) pathway. Further experiments were carried out to test this hypothesis.

AaYABBY5 significantly activates the promoters of AaPAL, AaCHI, AaCHS, and AaUFGT in transiently transformed N. benthamiana

Knowing putative YABBY-binding sites in the AaPAL, AaCHI, AaCHS, AaDFR, AaFLS, AaFSII, AaLDOX, and AaUFGT promoter sequences, a dual-luciferase assay was performed, where AaYABBY5 inserted in pEarleyGate 104-YFP was used as an effector and promoter sequences inserted into pGreenII 0800-LUC were used as reporters (Fig. 3a). Equal-sized infiltrated leaf discs for each combination of the reporter with the effector AaYABBY5 were analyzed by commercially available dual-LUC reagents (Promega, USA). Values greater than a twofold increase were taken into consideration, and lower values were negated. A significant increase in relative LUC/REN values was found for the AaPAL, AaCHS, AaCHI, and AaUFGT promoters. AaYABBY5 exhibited a 7.4-fold increase in the activity of the PAL promoter, a 3.2-fold increase in the activity of the CHS promoter, a 3.4-fold increase in the activity of the CHI promoter and a three fold increase in the activity of the UFGT promoter (Fig. 3b–d, i). The fold change was calculated from comparative values of each effector/reporter, and an empty vector was used as a negative control/reporter. The increase in the LUC/REN values corresponds to the intensity of LUC signals driven by the respective promoters in the presence of the AaYABBY5 protein. Based on these results, it was hypothesized that AaYABBY5 could activate these promoter sequences in A. annua either directly, by binding to putative YABBY-binding motifs, or indirectly, through some protein–protein interactions.

AaYABBY5 directly binds to promoter regions of AaPAL, AaCHS, AaCHI, and AaUFGT in the EGY48 yeast strain

Transactivation assays using N. benthamiana revealed that AaYABBY5 mediated a significant increase in the activities of PAL, CHS, CHI, and UFGT promoters in vivo. Therefore, to determine the molecular basis of this regulation and whether AaYABBY5 directly activates them, a Y1H assay was performed. The experiment demonstrated the binding of the pB42AD-AaYABBY5 fusion protein (blue color appearance), but not pB42AD alone (no color), to the PAL, CHS, CHI, and UFGT promoter sequences, indicated by the activation of the lacZ reporter gene, which produces β-galactosidase and cleaves the X-gal present in growth medium to a compound with a blue-colored phenotype. The experiment was repeated three times to validate the results. No colored phenotype was found for the DFR, FLS, FSII, and LDOX promoters (Fig. 4b).

The results of the Y1H assay were consistent with the findings of the dual-luciferase reporter assay. In other plants, including A. thaliana, flavonoid genes, and their transcriptional regulation are well-studied. The A. thaliana FIL gene has been previously reported to be a positive regulator of anthocyanins by activating the MYB75 gene. To our knowledge, for the first time, we found the molecular basis of the transcriptional regulation of flavonoid biosynthetic pathway genes, PAL, CHS, CHI, and UFGT in A. annua. These results indicated that AaYABBY5 might have the potential to regulate both flavonoid and anthocyanin biosynthesis in A. annua.
Therefore, these contents were measured and compared in AaYABBY5 OE and AaYABBY5 AnT. A. annua plants.

AaYABBY5-overexpressing A. annua plants showed a consistent significant increase in the expression of genes from AaPAL to AaUFGT

After analyzing the binding of AaYABBY5 to the promoters of early (flavanone) and late (anthocyanin) flavonoid biosynthetic genes, it was important to study its functions in A. annua. As expected, the gene expression analysis revealed an increase in the expression of all genes under study. The comparative expression of these genes indicated that AaCHS showed higher expression than all other genes under study. Similarly, the expression of DFR, FSII, and UFGT was lower than that of PAL, CHS, CHI, FLS, and LDOX (Fig. 4c–j).
Although DFR, FLS, FSII, and LDOX were not activated by AaYABBY5, a higher expression of these genes in AaYABBY5-OE plants was found. It is proposed that the increased flux provided by PAL, CHS, and CHI activates downstream pathway enzymes by increasing the concentration of substrates for the enzymes acting downstream: DFR, FLS, FSII, and LDOX. AaYABBY5 overexpression not only increased the expression of its direct target genes but also the flavonoid pathway under study.

AAvyABBY5 antisense A. annua plants showed a significant decrease in the expression of flavonoid biosynthetic genes

The AAvyABBY5 protein activates flavonoid biosynthetic genes, and the increased expression of AAvyABBY5 led to a dramatic increase in the expression of flavonoid-regulating genes. Transcript levels of PAL, CHI, CHS, DFR, FLS, FSII, LDOX, and UFGT were analyzed in AAvyABBY5 antisense RNA-containing plants. As expected, a significant decrease in the expression of the genes under study was found in AAvyABBY5 AnT. plants.
(Fig. 4c–j). Overall, from these findings, a clear understanding of AaYABBY5-regulated flavonoid biosynthesis was obtained. To validate the above findings, flavonoid and anthocyanin concentrations were measured and compared among AaYABBY5 OE, AaYABBY5 AnT, wild-type/control plants, and vector-containing plants.

**AaYABBY5 positively regulates flavonoid biosynthesis**

Flavonoids are polyphenolic plant secondary metabolites that are classified into different types. In this study, we found that AaYABBY5 positively regulates the PAL, CHI, CHS, and UFGT genes. The results of real-time PCR also verified the increased expression of these genes in AaYABBY5 overexpression plants. These findings revealed a positive behavior of AaYABBY5 toward flavonoid biosynthesis in *A. annua*. To justify this, flavonoid contents from AaYABBY5 overexpression plants, AaYABBY5 antisense plants, and control plants were measured using the aluminum chloride (AlCl3) colorimetric method with quercetin as a standard. As expected, the results revealed an increased concentration of flavonoids in AaYABBY5 overexpression plants compared to AaYABBY5 antisense or control plants (Fig. 5a), proving the function of AaYABBY5 as a positive regulator of flavonoid biosynthesis.

**AaYABBY5 promotes anthocyanin biosynthesis, displaying a purple phenotype in *A. annua* stems**

Anthocyanins are secondary metabolites widely present in plant species and are responsible for the purple, bluish, and pinkish pigmentation of different plant parts. In this study, we observed deep purple pigmentation in the stems
of AaYABBY5 overexpression plants, whereas AaYABBY5 antisense plants and control plants showed no purple phenotype (Fig. 5b, c).

To test whether AaYABBY5 is involved in the regulation of anthocyanin biosynthesis in A. annua, we measured and compared the total anthocyanin content of stem extracts of control plants and pHβ/wild-type, AaYABBY5-overexpressing, and AaYABBY5 antisense plants. It is known that anthocyanins produce a red color when treated with acids. Consistent with this, the anthocyanin extracts from purple-colored AaYABBY5-OE plants were red-colored in acidic medium (Fig. 5i). As expected, a significant increase in the concentration of anthocyanins was found in the stems of plants overexpressing AaYABBY5 compared to the control and AaYABBY5 antisense plants (Fig. 5e).

Previous investigations have demonstrated that the YABBY family TF AtFIL is a positive regulator of anthocyanin biosynthesis in the model plant A. thaliana through activating the MYB75 promoter; however, no direct link to the genes regulating anthocyanins or flavonoids was reported in A. annua. MYC2 has been reported to be responsible for an increase in the anthocyanin content, giving a purple phenotype to AaMYC2-overexpressing stems; however, the mechanism underlying this regulation was not studied. To our knowledge, we provide the first molecular basis of flavonoids, including anthocyanin regulation by the YABBY family transcription factor AaYABBY5. It was supposed that AaYABBY5 activates anthocyanin biosynthesis through the direct activation of UFGT and upstream pathway genes.

AaYABBY5 overexpression results in broader leaf lamina and increased trichome density

The primary function of YABBY family transcription factors found in seed plants is the control of leaf development, increasing the size of the leaf lamina, and maintaining organ polarity; therefore, it was important to determine whether AaYABBY5 overexpression and/or its downregulation in A. annua affected leaf morphology. Phenotypic analysis of AaYABBY5-OE plants, AaYABBY5 AnT. plants, and control plants showed that the leaves of AaYABBY5-OE plants have broader leaf lamina, whereas in AaYABBY5 AnT. plants, the leaf lamina was reduced. Leaves of AaYABBY5 AnT. plants were radialized compared to that of OE plants and/control plants (Fig. 5g, h). Trichomes are the sites of secondary metabolite synthesis, and studies have reported that flavonoids are synthesized in trichomes.

Trichome development is a part of leaf development. Knowing that AaYABBY5 regulates leaf lamina, the trichome densities on the leaf surfaces were compared among transgenic plants. The trichome densities on leaf surfaces from AaYABBY5 overexpression plants, AaYABBY5 antisense plants, and control plants with an empty vector were calculated. A significant increase in the trichome densities of AaYABBY5 overexpression plants was found compared to control plants, whereas for AaYABBY5 antisense plants, trichome density was found to significantly decrease. These results show that AaYABBY5 is also a positive regulator of trichome development (Fig. 6a, b).

AaHD1 (homeodomain protein 1) is involved in the initiation of both glandular and nonglandular trichomes in A. annua. However, no protein interactions between AaYABBY5 and AaHD1 were found. It is proposed that AaYABBY5 might activate the promoter of AaHD1, which regulates trichome development. This research opened paths for future research where the molecular mechanism of the regulation of trichome development by AaYABBY5 can be found.

Discussion

Artemisia annua is a renowned traditional Chinese medicinal plant that has been used in China for a long time as a treatment of fever, inflammation, and malaria, and its use now extends to Europe and North America. A. annua is rich in hydroxylated flavonoids and poly-methoxylated flavonoids with useful biological activities, such as antioxidant, anticancer, and antimarial activities. The most interesting feature of A. annua flavonoids found recently is to synergize antimarial and anticancer compounds, most importantly artemisinin. Despite the worthwhile effects of flavonoids, research on the molecular and transcriptional regulation of flavonoid biosynthesis in A. annua is limited. Recently, chalcone isomerase (AaCHI) phenylalanine ammonia lyase (AaPAL1), flavanone-3-hydroxylase (AaF3′H), and AaFLS1 have been cloned and characterized as enzymes that take part in flavonoid biosynthesis. A few transcription factors from the bHLH and MYB families have been recently reported as regulators of flavonoids in A. annua. Examples include the overexpression of MYC2 (which binds to G boxes present in the AaCYP71AV1 and AaDBR2 promoters) in A. annua, resulting in an increased level of both anthocyanins and artemisinin. Mostly, R2R3-MYB has been shown to play regulatory roles in flavonoid biosynthesis in A. annua. Recently, the MYB family transcription factor AaTAR2 from A. annua was reported to synergistically regulate both artemisinin and flavonoids; however, the molecular mechanism regulating flavonoid production was not elucidated.

Arabidopsis FIL activates MYB28, which activates aliphatic glucosinolate biosynthetic genes. AtYAB3 is a positive regulator of MYB75, an activator of anthocyanin biosynthesis. MsYABBY5 is a direct activator of WRKY75, which possibly represses terpene biosynthesis in M. spicata. OsYABBY4 is a negative regulator of the gibberellin (GA) 20-oxidase 2 gene (GA20ox2), and SLR1
is involved in gibberellic acid responses in rice. Because of the functions performed by YABBY genes in other plants and their role in anthocyanin biosynthesis in *A. thaliana*, it was important to determine the role of YABBY TFs in flavonoid biosynthesis. The role of YABBY family transcription factors in flavonoid biosynthesis in *A. annua* was not previously characterized. This study provides AaYABBY5 as a novel regulator of flavonoid biosynthesis in *A. annua*.

We screened the promoters of *PAL* (from upstream precursors of the flavonoids pathway), *CHS* (involved in the first committed step toward artemisinin biosynthesis), *CHI*, *DFR* (acting downstream of *CHS*) *FLS* and *FSII* as flavonols and flavones regulators, respectively, and *LDOX* and *UFGT* (which are responsible for anthocyanin and pigment formation), using dual-luciferase reporter assay and Y1H assay, and found that *PAL*, *CHS*, *CHI*, and *UFGT* are directly regulated by AaYABBY5 (Figs. 3 and 4b). The direct binding of AaYABBY5 to these promoter regions provided the molecular basis for the regulation of flavonoids in *A. annua*. The results of the Y1H assay were consistent with the results of the dual-Luc assay.

In *A. thaliana*, AtFIL was reported to be a target of JAZ3 and a regulator of anthocyanin biosynthesis by
activating the promoter of MYB75 (PAP1); however, no direct interaction with flavonoid pathway genes was reported. In plants, including A. thaliana, MYB family proteins have been proven to be candidate genes that regulate flavonoid biosynthesis. AtMYB12 has been found to be a regulator of CHS and FLS genes through Myb-recognition elements (MREs) present in the promoters of these genes. The R2R3-MYB transcription factor PAP1 displayed a purple phenotype in transgenic Arabidopsis by upregulating various genes involved in anthocyanin biosynthesis. Recently, the MYB family transcription factor AaTAR2 was found to synergistically regulate both artemisinin (indirectly) and flavonoids in A. annua; however, the molecular mechanism regulating flavonoid production was not clearly elucidated, and no direct interaction with the artemisinin biosynthetic pathway was given. Based on these findings, it was important to study the regulation of flavonoid biosynthetic pathway genes in AaYABBY5 overexpression plants, AaYABBY5 antisense plants, and control plants.

To confirm the role of AaYABBY5 in the regulation of flavonoid biosynthesis, comparative expression and biochemical analysis of transgenic A. annua containing pH35: AaYABBY5, pH35: anti-AaYABBY5, pH35, and wild-type plants revealed a significant upregulation of the AaPAL, AaCHI, AaCHS, AaFLS, AaFSII, AaLDOX, and AaUFGT genes under study (Fig. 4c–j). We propose that AaYABBY5 binds to the PAL promoter and activates PAL gene expression, which provides metabolic flux to the flavonoid pathway. The overexpression of AaYABBY5 in A. annua provides elevated levels of the AaYABBY5 protein, which binds to and activates the PAL gene. The increased activity of PAL converts more phenylalanine molecules to cinnamate, which provides precursor molecules for entry into the flavonoid pathway. This provides an increase in the concentration of the substrate molecules for the CHS enzyme. Knowing that AaCHS catalyzes the first committed step of the flavonoid pathway (Fig. 1) and considering our findings that AaYABBY5 has a strong binding capability to the AaCHS promoter, it is supposed that increased AaCHS expression accelerates the biosynthesis of chalcones, which provides flux to downstream reactions, providing an increase in the expression of genes lying downstream of AaCHS in the flavonoid biosynthesis in AaYABBY5-OE A. annua plants. When PAL and CHS are activated, they perform their function and trigger the activation of downstream reactions, providing accelerated substrate recovery, as a result of which more enzyme molecules are activated, as found by increases in the transcript levels of CHI, FLS, FSII, LDOX, and UFGT. Among these, CHI and UFGT are also activated directly by AaYABBY5. Therefore, it is concluded that the increase in the concentration of flavonoids in OE AaYABBY5 transgenic A. annua is governed by AaYABBY5, which directly activates the PAL, CHS, and CHI genes. The results were further validated by analyzing A. annua plants with suppressed AaYABBY5, wherein the expression of genes regulating flavonoid biosynthesis and flavonoid concentration were found to be significantly decreased (Figs. 4c–j and 5a).

In A. annua, chalcone isomerase (AaCHI), phenylalanine ammonia lyase (AaPAL1), flavanone-3-hydroxylase (AaF3H), and AaFLS1 have been characterized as enzymes that take part in the flavonoid biosynthesis pathway. The results obtained in this study are in accordance with these previous findings that, when the expression of PAL or CHI is increased, it thereby enhances the flavonoid concentration. The term flavonoids used in this study indicates the concentration of flavones and flavonols according to the principle of the AlCl3 method. Kaempferol and quercetin, are important flavonoids of A. annua that belong to the flavonol group, and a significant increase in the flavonoid concentration in AaYABBY5-OE plants might correspond to the enhanced accumulation of kaempferol and quercetin, which favors the synergistic role of AaYABBY5 toward the regulation of flavonoids (present study) and artemisinin biosynthesis.

Anthocyanins are a group of flavonoids that are synthesized downstream of the AaDFR gene, governed by AaLDOX and AaUFGT in two-step reactions. Physiological analysis of transgenic plants and control plants revealed the presence of purple pigmentation in the stems and petioles of AaYABBY5-OE plants, whereas no colored phenotype was observed in the control and AaYABBY5 AnT. plants (Fig. 5b, c). Gene expression analysis also revealed a significant increase in the expression of AaDFR, AaLDOX, and AaUFGT, as well as an increase in the concentration of anthocyanins in AaYABBY5-OE plants. As expected, the transcript levels of AaDFR, AaLDOX, and AaUFGT and the resulting anthocyanin concentrations were significantly decreased in AaYABBY5 AnT. plants (Fig. 4f, i, j). AaYABBY5 cannot directly bind to DFR and LDOX promoters. It is supposed that the activation of early flavonoid genes, CHI and CHS, results in the activation of later steps, which enables the rapid accumulation of anthocyanin precursor molecules; pelargonidin, delphinidin, and cyanidin are collectively named anthocyanidins. Precursor anthocyanidins called anthocyanidins are glycosylated by the enzyme UDP-glucose flavonoid 3-O-glucosyl transferase (UFGT) to produce colorful and stable compounds called anthocyanins. We hypothesized that the increase in the concentration of anthocyanidins, which are substrate molecules for the UFGT enzyme, and direct activation of AaUFGT by AaYABBY5 increases the rate of forward reaction toward anthocyanin biosynthesis governed by glycosylation. These findings prove that AaYABBY5 is a positive regulator of anthocyanins.
Regarding the basic role of YABBY genes in the regulation of leaf development, in this study, comparative phenotypic analysis of AaYABBY5-OE, AaYABBY5-AnT., and control plants showed that as expected, the leaf lamina was broader in overexpression plants (Fig. 5g–h (ii)), whereas radialized lamina was observed in antisense plants (Fig. 5g–h (ii)). The differences can also be seen in Fig. 5d (i–iii). These results showed that AaYABBY5, in addition to regulating secondary metabolite biosynthesis, also regulates primary metabolism associated with leaf growth. Considering these results, trichomes, which are the sites of secondary metabolite synthesis in plants, were studied. Comparative trichome densities of AaYABBY5-OE, AaYABBY5-AnT., and control plants revealed higher trichome densities in AaYABBY5-OE plants than in the control plants and AaYABBY5-AnT. plants; however, the molecular mechanism of its regulation is the target of future research (Fig. 6a, b). A model representing the functions of AaYABBY5 found in this study is given in Fig. 6c.

This study, for the first time, provided transcriptional regulation of flavonoids using YABBY family transcription factors, and this is the first report on the direct transcriptional regulation of flavonoid pathway genes in A. annua. Our previous data that AaYABBY5 directly activates artemisinin biosynthetic pathway genes and our present findings regarding AaYABBY5-mediated direct regulation of flavonoids, including anthocyanins, provide evidence of the parallel transcriptional regulation of artemisinin and flavonoid biosynthesis by AaYABBY5 in A. annua, thus proving that AaYABBY5 can be a good candidate gene to provide increasing concentrations of the two biologicals at the same time in A. annua.

Materials and methods
Cloning of promoters
The A. annua genomic assembly data present in NCBI were screened to find putative promoter sequences upstream of the initiation codon ATG using ORF sequences of AaPAL, AaCHS, AaCHI, AaFLS, AaFSII, AaDFR, AaLDOX, and AaUFGT as queries. Approximately 2 kb PAL promoter, 1.6 kb AaCHS promoter, 1.4 kb AaCHI promoter, 1.4 kb AaFLS promoter, 1.2 kb AaFSII promoter, 1.2 kb AaDFR promoter, 1.9 kb AaLDOX promoter, and 1.7 kb promoter sequence of AaUFGT were cloned. Genomic DNA extracted from young leaves of A. annua was used as a template, and amplification was performed using a KOD Plus PCR kit (Toyobo). DNA bands purified using the DNA Gel Extraction Kit (AxyPrep) were ligated to the PLB simple promoter, 1.4 kb AaCHI promoter, 1.4 kb AaFLS promoter, 1.2 kb AaFSII promoter, 1.2 kb AaDFR promoter, 1.9 kb AaLDOX promoter, and 1.7 kb promoter sequence of AaUFGT were cloned. Genomic DNA extracted from young leaves of A. annua was used as a template, and amplification was performed using a KOD Plus PCR kit (Toyobo). DNA bands purified using the DNA Gel Extraction Kit (AxyPrep) were ligated to the PLB simple vector (Tiangen Biotech, China) containing carbenicillin-resistance genes according to the manufacturer’s instructions provided with the Lethal Based Fast Cloning Kit (Tiangen), followed by transformation into DH5α-competent cells (Invitrogen).

Bioinformatic analysis of promoters of flavonoid biosynthetic genes
The cloned promoter sequences were confirmed by Sanger sequencing and analyzed for the prediction of putative YABBY-binding sites using PLANTPAN3.0.

Identification of AaYABBY5 as a potential flavonoid-regulating transcription factor
The transcriptomic data of six different tissues of A. annua generated by our research center were screened, and reads of each YABBY gene, flavonoid biosynthetic genes, and artemisinin biosynthetic genes in six different tissues (trichome, bud, stem, root, leaf, and seed) were obtained using a BLASTN search against each database with an E value <1 × 10^-6, and read counts were normalized by calculating the value of reads per kilobase per million (RPKM). To predict the YABBY transcription factor, which may regulate flavonoid-regulating genes, coexpression analysis was performed on the basis of RNA sequencing data using Multi-Experiment Viewer (MeV4.9.0) software.

Plant material and growth conditions
The seeds of the A. annua cultivar Hu Hao #1 used in this study were collected from our previous stably transformed transgenic A. annua plants with overexpressed AaYABBY5 (35S—AaYABBY5), silenced AaYABBY5 (35S-anti-AaYABBY5), empty vector-containing plants (35S), and wild-type or control plants (untransformed). Details about construct preparation and A. annua transformation are given in ref. 38. A. annua plants (transgenic and control) were also propagated from stem cuttings. For such cutting propagations, 8–10-cm long stems or side shoots were cut just below a leaf and propagated in plant trays supplemented with soil matrix. After extensive rooting development and proper growth as a complete plant, the cuttings were propagated to pots. In the transient agroinfiltration assay, N. benthamiana plants with young leaves and expanded lamina were used. Both plant types were grown under the same conditions of temperature: 25 °C ± 2 °C and photoperiod: 16 h light:8 h dark.

Transient N. benthamiana infiltration system
To perform a transient infiltration assay in the N. benthamiana system using a dual-LUC kit, effector and reporter strains were prepared as follows. For the effector strain, the open-reading frame of AaYABBY5 without its terminal codon was amplified using primer sequences specifically designed for cloning into the pENTR-TOPO vector, followed by subsequent recombination into the Gateway destination vector pEarleyGate 104-YFP (N-terminal YFP) (Invitrogen, USA). For the reporter construct, promoter sequences of PAL, CHS, CHI, FLS, FSII, LDOX, and UFGT were amplified with primer sequences
containing adapter sequences specific to the pGreenII 0800-LUC plasmid. The purified fragment was subsequently ligated into the pGreenII 0800-LUC plasmid through HindIII and PstI sites to generate prosLUC constructs, according to a previous protocol. The plasmids were transformed into A. tumefaciens GV3101 competent cells with pSoup-p19 to help suppress gene silencing. The process of agrobacterium culture preparation, infiltration into the N. benthamiana leaf tissues, sample collection, and preparation for the measurement of LUC activity was followed according to a previous protocol.

**Yeast one-hybrid system**

For the Y1H assay, bait and prey constructs were prepared as follows. The promoter regions of AaPAL, AaCHS, AaCHI, AaFLS, AaFSI, AaDFR, AaLDOX, and AaUFGT were amplified using primer sequences with 5’ adapter sequences specific to the placZ vector and subsequently ligated into placZ using one-step cloning, following the protocol given by ClonExpress II (Vazyme). Similarly, the open-reading frame of AaYABBY5 with its terminal codon was amplified using primer sequences with 5’ adapter sequences specific to the pB42AD vector (Addgene) and subsequently ligated into it. Both promoters were confirmed by sequencing. Promoter sequences cloned in placZ were used as bait against pB42AD-AaYABBY5 prey.

The assay was performed according to the manual of the Matchmaker Gold Y1H system given in the Yeast Protocols Handbook, Clontech, (Japan). The combination of pB42AD-AaYABBY5 with the respective promoter sequence in placZ was cotransformed into EGY48 competent cells. The combination of negative controls pB42AD-AaYABBY5 with empty lacZ (lacZ-0), pB42AD-0 with the placZ promoter, and empty pB42AD (pB42AD-0) with empty placZ (placZ-0) was also transformed into EGY48 cells. Transformed yeast cells were streaked on SD-Tryptophan-Uracil (SD/-Trp-Ura). After 3 days, colonies were shifted to SD/-Trp-Ura supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The experiment was repeated three times.

**RNA extraction and gene expression analysis**

To check the expression pattern using real-time PCR, RNA extraction and cDNA synthesis were performed using an RNAprep Pure Plant Kit (Tiangen) and PrimeScript RT Reagents (Takara), respectively. Pure and good quality 500 ng RNA was used as a template for cDNA synthesis using reverse transcriptase enzyme. The cDNA was diluted in RNase-free ddH2O at a 1:40 ratio. Six microliters of diluted cDNA was used as a template for real-time PCR analysis using the protocol of ref. with minor modifications.

SYBR Green Super real premix was used to prepare a master mix, and a Roche Light Cycler 96-well Real-Time PCR Machine (Roche, Switzerland) was used to quantify the expression. The PCR program was set up at 40 cycles, each containing 2 min at 95 °C for initial denaturation, 20 s at 95 °C for denaturation, 20 s at 54 °C for annealing, and 20 s at 72 °C for the extension. β-Actin was used as a standard. For each experimental and control sample, three repeats were used.

**Preparation of flavonoid extracts and measurement of total flavonoid content**

For quantitative determination of the total flavonoid content, leaf samples from each plant were collected and ground to powder form using liquid nitrogen (−70). Approximately 1 g of each leaf sample was placed in 50-ml glass flasks and mixed with 5 ml methanolic solution (70%). The extraction was performed twice using an ultrasonic processor (DL-720B) at a frequency of 55 Hz and a temperature of 30 °C for 35 min. The supernatants containing extracts were collected following centrifugation at 6000 rpm for 10 min. Approximately 3 ml of each extract was filtered through a 0.22-μm nitrocellulose filter and transferred to glass vials.

The total flavonoid content of A. annua exudates was calculated using the aluminum chloride (AlCl3) colorimetric method according to protocol. The working principle of the AlCl3 colorimetric method is that AlCl3 forms acid-stable complexes with the C-4 keto group and the C-3 or C-5 hydroxyl group of flavones and flavonols. It also forms complexes with the orthodihydroxy groups in the A- or B-ring of flavonoids. For sample preparation, 10 μl of each extract was diluted in 150 ml of deionized water, followed by the addition of 20 μl potassium acetate (1 M) and 20 μl of aluminum chloride (10%). The final volume of the 200 μl reaction mixture was incubated at 37 °C for 30 min. The absorbance was measured at 415 nm. The total flavonoid content was calculated as quercetin equivalents. The absorbance of three independent biological repeats from each plant type: AaYABBY5 OE, AaYABBY5 AnT., as well as control plants, was measured in triplicate. Error bars indicate the SD of the average flavonoid concentration.

**Preparation of anthocyanin extracts and measurement of anthocyanins**

To extract anthocyanins/pigments from the stem tissues of A. annua plants, the following protocol was used with minor modifications. Samples from each plant were collected and ground in liquid nitrogen to powder form. A 1 g sample was mixed with 10 ml of acidic methanol (70% methanol with 0.1% HCl) and processed by an ultrasonic processor (DL-720B) at 50 Hz for 40 min. The processed
samples were kept at 4 °C overnight mixing and incubation under dark conditions.

The extracts were purified by centrifugation at 12000 rpm for 10 min by filtration. The supernatants were separated and filtered through 0.22-μm nitrocellulose filters. Absorbance at 530 nm and 657 nm was measured using a microplate reader (BioTek, ELx 800). Relative anthocyanin content was found by

\[ \text{Absorbance at 530 nm} = \frac{\text{sample absorbance} - \text{blank absorbance}}{\text{A530} - 0.25 \times \text{A657}} / \text{FW} \]

FW (FW is the fresh weight of sample in grams). The average anthocyanin concentration.

Measuring trichome density

To calculate the trichome densities, mature and healthy leaves from three-month-old *A. annua* plants were used. To ensure comparison at the same growth level, the 8th leaf below the meristem was selected from transplanted and control plants. Images were captured using fluorescence microscopy (Olympus, Japan) under the following conditions: 5x magnification and 450–480 nm excitation wavelength. The images were analyzed by using ImageJ 1.51k software to measure the leaf area and calculate trichome numbers. Finally, trichome densities were calculated from the ratio of trichome number to the leaf area. The experiment was repeated three times, using the 8th leaf from three different plants of each line, to obtain statistically significant results.

Primer sequences

The primer sequences used in this study were prepared using Primer 3 software and are given in Supplementary Table S1.

Statistical analysis

Statistical analysis was performed using Student’s *t* test with paired and two-tailed distribution methods. ** and * represent statistically significant group differences for *P* < 0.01 and <0.05, respectively.

Acknowledgements

This work was supported by the National Key R&D Program of China (2018YFA0900600), the Bill & Melinda Gates Foundation (OPP1199872), the SJTU Global Strategic Partnership Fund (2020SJTU-CORNELL), and the SJTU Trans-med Awards Research Program (20190104).

Author contributions

S.K. and K.T. planned and designed the research. S.K. performed the experiments. Y.L., D.H., and Q.S. helped with promoter cloning and vector construction. X.F. helped with the measurement of flavonoid contents. C.W. helped with capturing plant images. S.K. wrote the manuscript. S.R., Q.S., and K.T. revised the manuscript. All authors approved the manuscript for submission.

Data availability

All data supporting this study are included in the article and its supplementary files.

Conflict of interest

The authors declare no competing interests.

**Supplementary information**

The online version contains supplementary material available at https://doi.org/10.1038/s41438-021-00693-x.

Received: 16 April 2021 Revised: 24 July 2021 Accepted: 15 August 2021

Published online: 01 December 2021

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