Parallel Regulation of Artemisinin and Flavonoid Biosynthesis in Medicinal Plants: Implications for Secondary Metabolite Production

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Abstract

Plant regulate the synthesis of a broad range of specialized compounds through the actions of individuals, or sets of transcription factors (TFs), often acting in complexes. One such compound, artemisinin, from *Artemisia annua*, and is widely used as a pharmacological product in the first-line treatment of malaria. However, the emergence of resistance to artemisinin in the malaria-causing *Plasmodium* species, and low rates of artemisinin production have required innovative treatments, such as exploiting the synergetic effects of flavonoids with artemisinin. Here we review the current knowledge of flavonoid and artemisinin transcriptional regulation in *A. annua* and review the dual action of TFs and structural genes that can regulate both pathways simultaneously. Understanding the concerted action of these TFs and associated structural genes can guide the development of strategies to further improve flavonoid and artemisinin production.

Plant secondary metabolites and their pharmacological applications

Plants are auxotrophs and use the energy and fixed carbon from photosynthesis to generate a core set of primary metabolites. These, in turn, are used as precursors to synthesize an estimated 200,000 types of specialized compounds, via so-called secondary metabolic pathways. The production of primary and secondary metabolites must be balanced to adapt to changing environments, involving trade-offs between growth/yield (often associated with primary metabolites) and survival, where secondary metabolites play key roles in responses and adaptations [1]. Secondary metabolism is not only important for plant success but also provides a large spectrum of phytochemicals that are of considerable value for human society. Indeed, over the past 20 years, > 70% of FDA-approved medicinal products have been based on plant specialized

metabolites [2, 3]. Many of these are derived from the phenylpropanoid pathway. An example is capsaicin, which accumulates in *Capsicum* spp. and acts as a rubefacient, or circulatory stimulant, and an analgesic [4]. Similarly, the cholagogue activity of *Cynara scolymus*, the anthelmintic activity of *Dryopteris filix-mas*, the anti-inflammatory analgesic activity of *Filipendula ulmaria* and the anti-catarrhal activities of *Solidago virgaurea*, are all attributed to the action of phenolic compounds [5].

Two important classes of secondary metabolites are terpenoids and flavonoids, many of which have been shown to have antiviral [6], antifungal and anticancer properties [7]. In addition, artemisinin, a sesquiterpene endo-peroxide lactone that is isolated commercially from *Artemisia annua* L., is currently the most effective drug used to treat malaria, a devastating disease caused by the protozoan parasite *Plasmodium* [8]. According to WHO report in 2015, about 3.3 billion people are facing the risk of malaria infection, and 1.2 billion are at high risk [9]. A range of flavonoids, including quercetin, silymarin, quercetin-3- glucoside, quercetrin, quercetin-3-galactoside, taxifolin, rhamnetin, and rutin, also show anti-plasmodial activities *in vitro* and *in vivo* [10]. In addition to the individual medicinal effects of these compounds, there is evidence that artemisinin and flavonoids have synergistic effects in the treatment of various diseases, including malaria. Methoxylated flavonoids, such as artemetin, casticin, chrysoplenetin, chrysosplenol-D 4, cirsilineol, and eupatorin, have been demonstrated to enhance the interaction of artemisinin with heme groups, releasing artemisinin peroxide, which has antimalarial effects [11, 12]. In addition, the inhibitory effect of some flavonoids on hepatic and intestinal cytochrome P450 enzymes may enhance the availability of artemisinin in blood serum [13, 14]. The results of anti-*Plasmodium* clinical trials involving the use of flavonoid-rich dried leaves from *A. annua* were consistent with a potentiating role for other compounds together with artemisinin in combatting malaria [15]. Such studies support the idea that the synergistic effects of flavonoids and artemisinin may be key to developing treatments to combat *Plasmodium* and counteracting emerging resistance to artemisinin.

Many of the enzymes and corresponding genes that constitute the artemisinin and flavonoid biosynthetic pathways have been characterized, often in association with attempts to increase product abundance [16]. However, much less is known about the transcription factors (TFs) that control these pathways. The discovery of these fundamental regulators and the binding motifs in

the promoter regions of their target genes, as well as understanding their combinatorial modes of action, represent important targets.

Here we review the intertwined nature of the flavonoid and artemisinin biosynthetic pathways in *A. annua*, with particular focus on the *cis*-regulatory elements and 'dual function' TFs that regulate both pathways, as well as the discovery that some structural genes can also simultaneously influence the accumulation of compounds, in what are ostensibly distinct branches of secondary metabolism. A better understanding of the underlying regulatory mechanisms for these pathways may enable enhanced flavonoid and artemisinin production and facilitate the development of a combinatorial treatment for malaria.

The artemisinin biosynthesis pathway and its regulation

The initial stages of terpenoid biosynthesis involve the generation of two 5-carbon precursors: isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP), via the cytosolic mevalonate (MVA) and plastid methylerythritol phosphate (MEP) pathways [17]. MVA-derived IPP and DMAPP are precursors for sesquiterpenes, triterpenes, and polyisoprenoids, whereas the MEP-derived products are usually incorporated into monoterpenes, diterpenes, and tetraterpenes [2, 17]. However, there appears to be cross-reactivity between plastidial IPP and cytosolic IPP and DMAPP, involving sesquiterpene cyclase, resulting in the production of farnesyl diphosphate (FPP) as the initial substrate of the artemisinin pathway. FPP is then further subjected to a series of cyclization, hydroxylation, oxidation and reduction reactions [3].

The artemisinin pathway begins with the lyase activity of amorpha-4,11-diene synthase, which converts FPP to amorpha-4,11-diene, This is then hydroxylated to artemisinic alcohol [18], followed by oxidation to artemisinic aldehyde and then to artemisinic acid. These coupled reactions are coordinated by cytochrome P450 monooxygenase (CYP71AV1) and cytochrome P450 oxidoreductase (CPR), respectively [19, 20]. Following the pathway, the reduction of artemisinic aldehyde by artemisinic aldehyde Δ11(13) reductase (DBR2) leads to dihydroartemisinic aldehyde, which is further converted to dihydroartemisinic acid (DHAA) by aldehyde dehydrogenase 1 (ALDH1) [20, 21]. In *A. annua*, DHAA is considered to be the final enzymatically generated compound in the pathway, but it undergoes photo-oxidation to form artemisinin in the glandular trichomes on the surfaces of the aerial parts of the plant [22, 23] (Figure 1).

The flavonoid biosynthesis pathway

The flavonoid biosynthetic pathway (see Figure. 1) is initiated by the catalytic actions of phenylalanine ammonia-lyase (PAL) on the precursor amino acid phenylalanine, and then cinnamate 4-hydroxylase (C4H) enzyme, leading to the production of the entry compound to flavonoid biosynthesis, chalcone [24, 25]. The first committed step in the flavonoid pathway is encoded by the enzyme chalcone synthase (CHS), catalyzing the formation of naringenin chalcone from three molecules of malonyl-CoA and one molecule of *p*-coumaroyl-CoA [26]. The pathway continues with the isomerization of naringenin chalcone to the flavanone naringenin through the activity of chalcone isomerase (CHI) [27, 28]. The sequential actions of other enzymes in the pathway, (Figure 1) result in the biosynthesis of diverse classes of flavonoid compounds, such as flavanones, dihydroflavonols and anthocyanins [29]. For example, flavanone 3-β-hydroxylase (F3H) synthesizes the common precursor for three major classes of end products: flavonols, anthocyanins, and proanthocyanins. Hence it holds a key position for flux distribution among the branches of the flavonoid pathway, each of which leads to end products with distinct physiological functions [30, 31]. Subsequently, dihydrokaempferol (DHK), the product of F3H can be either hydroxylated at the 3' position by (F3'H) or at both the 3' and 5' positions by (F3'5'H) to produce dihydroquercetin and dihydromyricetin, respectively [32]. DHK is then converted by dihydroflavonol reductase (DFR) in a stereospecific reduction of dihydroflavonol to leucoanthocyanidin. Subsequent reactions convert colorless leucoanthocyanidin to the colored anthocyanidins [33] (Figure 1).

TFs and the regulation of artemisinin and flavonoid biosynthesis

The expression of eukaryotic genes is influenced by numerous environmental factors through the action of TFs that bind to a specific region of their target genes promoters, referred to as *cis*-regulatory elements. Cytosolic post-translational modification of TFs directs them to the nucleus, where they can directly bind to their corresponding *cis* region or interact with other proteins to indirectly regulate their target gene [34, 35]. Transcriptional regulation often involves the combinatorial action of multiple TFs from the same, or different, families that modulate the expression of the same target gene [36]. The combinatorial action of TFs may involve three mechanisms: 1) two or more TFs bind to different *cis*-elements within the promoter region of the

same gene' 2) TFs compete for binding to the same *cis*-element; and 3) they display heteromeric or multimeric cooperative binding to a particular *cis*-element or a neighboring binding site [37]. In this review, we present five families of TFs families that act independently, or cooperatively, to simultaneously regulate the flavonoids and artemisinin biosynthesis pathways.

WRKY TF family

WRKY TFs regulate various processes of plants, including seed germination, seed dormancy, and responses to stress [38]. Like many other TFs, the specific DNA-binding domains in WRKY TFs define their functionality. A 60 amino residue motif at N-terminus and an atypical zinc-finger structure at the C-terminus, enable these proteins to bind to the W boxes in the promoters of their target genes. WRKY proteins are classified as double WRKY DBDs (DNA Binding Domains), single DBD with different C_2H_2 zinc finger, or single DBD with C_2HC zinc finger [38, 39].

Synthesis of several phenylpropanoid compounds, such as lignin, flavanols, and tannins have been reported to be regulated by the members of WRKY family [40]. For instance, WRKY23 (*TT7*) regulates the expression of F3'H, which catalyzes the conversion of dihydrokaempferol to dihydroquercetin [41]. This provides the precursor for quercetin, a negative regulator of auxin transport, which mediates growth and root development [42]. Other reports have described diverse roles for WRKY proteins, suggesting that they may have multiple regulatory functions. For example, a TTG2-like homolog protein belonging to the WRKY family a tonoplast P-ATPases in *Petunia hybrida*, whereas, in *A. thaliana*, it enhances proanthocyanin accumulation in the seed coat [43]. WRKY proteins are also known to function through interactions with other families of TFs, such as MYB, bHLH, and WD40, as exemplified by AtWRKY44, which regulates anthocyanin production in *Arabidopsis thaliana* [44].

In vivo and *in vitro* analyses have revealed the high affinity of AaWRKY1 from *A. annua* in binding to the W-box in the *ADS* promoter, following its overexpression in tobacco and *A. annua* leaves [45]. AaWRKY1 has been shown to act together with other families of TFs, such as AaERF1, AaERF2, AaORA, AabZIP1, and AabHLH1, in the regulation of enzyme coding genes in the artemisinin pathway in response to the phytohormones abscisic acid (ABA) and jasmonic acid (JA). Thus, there are parallels with the multi-TF family-based regulation of both flavonoid and artemisinin biosynthesis by WRKY TFs [46].

AP2/ERF TF family

The superfamily APELATA 2/ethylene response factor (AP2/ERF) comprises TFs that regulate plant development, fruit ripening, senescence, stress responses and specialized metabolite biosynthesis [47, 48]. AP2/ERF family proteins collectively recognize a variety of *cis*-element motifs, such as the GCC-box, DRE/CRT, VWRE, and CE1 and are categorized into four families based on the canonical AP2 DNA binding domain: AP2, ERF, RAV, and Soloist [48-51]. The involvement of AP2/ERF proteins in plant primary and secondary metabolism in response to environmental stimuli has been described. For instance, in *Catharanthus roseus*, the ORCA3 TF, belonging to AP2/ERF family, was found to induce the expression of multiple genes in the terpenoid indole alkaloids (TIAs) pathway [52, 53]. Similarly, AtORCA59 was shown to promote the accumulation of antimicrobial proteins or enzymes involved in the biosynthesis of protective secondary metabolites [54].

Flavonoid formation has been associated with AP2/ERF regulation in the pigmentation of floral organs and fruits. For example, the flavonoid biosynthetic pathway in plum (*Prunus salicina* Lindl.) is directly regulated by *PsERS1* and *PsETR1* [55, 56]. Moreover, RNAi-based repression of an AP2 gene in transgenic tomato resulted in reduced expression of genes involved in phenylpropanoid and flavonoid formation [57]. In addition to acting individually, there is also evidence that AP2/ERF TFs positively regulate anthocyanin and proanthocyanin accumulation in apple in a complex with MYB family TFs [58].

In addition to controlling flavonoid accumulation, AP2/ERF TFs are also known to be involved in the regulation of artemisinin biosynthesis [59, 60]. For example, the JA-inducible ERF1 and ERF2 TFs trigger the expression of *ADS* and *CYP71AV1* by targeting the CBF2 and RAA motifs [61]. Furthermore, the AP2/ERF AaORA, which is orthologous to ORCA3 and ORCA2 of *C. roseus* was found to be a trichome-specific TF and highly expressed in both filamentous and glandular trichomes, the site of artemisinin accumulation [62]. Furthermore, a recent study reported cooperative binding between the JA-responsive and trichome-specific AaORA with the TFAaTCP14 to activate *DBR2* and *ALDH1*, resulting in elevated artemisinin production [61].

bZIP TF family

Basic leucine zipper (bZIP) TFs have a specific DNA sequence binding domain and a leucine zipper motif for dimerization of DNA binding regions [63, 64]. bZIP proteins are also involved in

the regulation of plant development and signalling in response to a wide range of abiotic stresses [65-70]. Genome analysis of *A. thaliana* categorized 75-77 nuclear-encoded bZIP proteins into ten homologous groups, based on the bZIP domain similarities [71, 72]. Those from Group A are ABA-induced and have multiple ABA response *cis*-elements (ABRE) [73, 74]. Group C proteins have a CPFR2 motif with a highly conserved phosphorylation site, allowing this protein to participate in responses to biotic and abiotic stresses [75-77]. Group G bZIP proteins have a proline-rich domain at their N-terminus, which enables them to bind to G-box motifs genes [78, 79]. Similarly, members of Group H, such as HY5, regulates light-responsive genes through G-box interactions [80]. In secondary metabolites regulation, members of group A mainly target *cis*-regulatory motifs with the core sequence ACGT [81].

bZIP TFs have been shown to bind to ACGT-containing elements in the promoters of flavonoid biosynthesis pathway genes through interaction with mainly MYB TFs [82-84]. The heterodimerization capability gives them increases in their regulatory capacity [85]. In *A. thaliana*, the interaction between bZIP and MYB proteins and subsequent induction of genes such as *CHS* and *FLS*, enhances flavonol production [82]. Moreover, *A. thaliana* HY5 regulates numerous flavonoid pathway genes, such as *AtCHS*, *AtFLS,* and *AtMYB12*, during photomorphogenesis [84, 86]. Notably, to date, the regulation of the flavonoid pathway by a bZIP transcription factor has only been observed in combination with TFs from other families.

The enhancement of artemisinin production, as well as AabZIP1 overexpression in *A. annua* treated exogenously with ABA [87], suggests a relationship between this TF family and ABA signalling. AabZIP has been shown to form a complex with MYB and bHLH TFs and bind to the CANNTG motif in the *CYP71AV1* promoter in response to JA. This leads to tissue-specific activation of secondary metabolism, oxidative stress, dehydration- and wound-response genes [87]. Additionally, the bZIP TF AaHY5, interacts with AaGSW1, in the WRKY 1 family, to regulate the light-induced biosynthesis of artemisinin [8].

bHLH transcription factor

The basic helix-loop-helix (bHLH) is a dimerizing TF that is characterized by a double α-helix loop and basic amino acid residues that facilitate DNA binding [88], which occurs with E-box motifs in promoter regions of their target genes [89]. Due to the lack of a DNA binding region in their structure, they act to regulate transcription through binding to other TFs [90]. For example,

bHLH interaction with an R2R3 MYB and a WD40 protein is required for non-glandular trichome development and anthocyanin biosynthesis in *A. thaliana* and other plants[91]. There are many examples of bHLH TFs regulating secondary metabolite biosyntheses, such as tanshinone biosynthesis in *Salvia miltiorrhiza* [92], the JA-dependent iridoid pathway in *C. roseus* [93] and formation of the triterpene saponin in *Medicago truncatula* [94].

Many researchers have studied the role of bHLH proteins in flavonoid and JA-mediated anthocyanin production in *A. thaliana* [95], and CmbHLH from *Chrysanthemum morifolium* was shown to binds to the E-box promoter of DFR, thereby enhancing anthocyanin content [96]. Other studies in *Zea mays*, *Antirrhinum majus*, *A. thaliana, Malus domestica*, and *Vitis vinifera* have demonstrated flavonoid regulatory networks involving the combined actions of MYC-like (bHLH), R2R3 MYB and WD40 proteins [97, 98]. In addition, we found that the overexpression of *AaMYC2* (previously reported to positively regulate artemisinin content) significantly enhances anthocyanin levels in *A. annua* [99], again highlighting regulatory connections between these two pathways.

The abundance of E-box elements in the promoter regions of *ADS* and *CYP71AV1* from *A. annua* suggests that bHLH TFs are involved in their regulation. Consistent with this hypothesis, overexpression of *AabHLH* in *A. annua* was observed to enhance the transcript levels of the genes involved in artemisinin biosynthesis, including *ADS*, *CYP71AV1* and *HMGR*, leading to increased artemisinin content [100].

MYB transcription factor family

MYB TFs have a highly conserved 52 amino acid DNA binding domain, which is present in four repeats (R) that form three α helices, with a hydrophobic core and a DNA recognition site on its third helix [101, 102]. The proteins in this family are classified into four groups based on the number of neighboring repeats: c-MYB has three repeats (R1, R2, and R3); 4R-MYB, mostly found in plants, contains four R1/R2-like repeats; 3R-MYB, contains R1R2R3 repeats and is found in most eukaryotes [103, 104]; R3-MYB, mostly found in plants, falls into several subclasses, including R2R3-MYB involved in cellular morphogenesis and the regulation of secondary metabolism [105, 106]; and R2/R2 MYB, which functions in the central circadian oscillator [107], organ morphogenesis and chloroplast development [108].

Previous studies have described the regulation of secondary metabolite biosynthesis by members of the MYB family such as AtMYB*11/PFG1, AtMYB12/PFG1,* and, *AtMYB111/PFG3* [109], and their functional and expression variation [110]. For example, *AtMYB3, AtMYB4, AtMYB7,* and *AtMYB32* act as transcriptional repressors [111, 112], whereas *AtMYB4* controls sinapate ester biosynthesis [111] and *AtMYB32* regulates pollen wall composition. Notably, there are several examples of MYB proteins acting with other TFs to regulate terpenoid and flavonoid biosynthesis [91].

Among the MYB TFs, those in the R2R3-MYB class have been most closely associated with flavonoid biosynthesis. For instance, the regulators of proanthocyanin and anthocyanin production bear a specific R3 domain, allowing them to interact with bHLH TFs [113, 114], whereas MYB TFs governing flavanol biosynthesis have SG7 and SG7-2 motifs in their C- terminus, which enhances DNA binding capability [83, 115]. In contrast, some members such as R3 AtMYBL2 or R2R3 AtMYB60, act as repressors of anthocyanin biosynthesis [106, 116, 117].

MYB subgroups are highly conserved among different species, and the presence of consensus motifs in their C- terminus suggests that the specificity of this family in regulating different pathways is conferred via interactions with other TF protein families. As an example of regulating distinct pathways, overexpression of *VvMYB5b* in apple fruit was observed to affect both phenylpropanoid and carotenoid metabolism [118].

In the context of artemisinin biosynthesis, MYB1 from *A. thaliana* and its ortholog, MYB61 from *A. annua*, were both found to regulate terpene metabolism, as well as trichome initiation and development, root development, stomatal aperture and gibberellin biosynthesis [119]. In another study, over-expression of the MIXTA MYB transcription factor in *A. annua* in resulted in elevated artemisinin content [120]. An association between MYB TFs, and both flavonoid and artemisinin pathways was also suggested by the observation that it interacts with bHLH and WD40 proteins to regulate the development of non-glandular trichomes and anthocyanin biosynthesis[121].

The relationship between artemisinin and flavonoids biosynthesis

Flavonoids and terpenoids, the two largest groups of plant specialized metabolites, are derived from two distinct pathways. However, studies at the levels of TFs, structural genes, and biochemical compounds have all suggested associations between the pathways. First, prenylated flavonoids and terpenophenolics are both synthesized from the mutual precursor dimethylallyl

diphosphate [122-125]. At the level of TFs, overexpression of flavonoid and terpenoid enzyme coding genes, and an enhanced metabolic flux of both phenylpropanoids and isoprenoids were reported in *AtPAP1*-overexpressing *Rosa hybrida* flowers [126]. Similarly, the up-regulation of MYB14 was reported to result in the accumulation of both sesquiterpenes and flavonoids, as well as an isoprenoid-mediated response, in wounded and JA-treated *Picea glauca,* and *Pinus taeda* [127]. We also found that overexpression of a bHLH TF (*MYC2*) in *A. annua*, which was previously reported as a positive regulator of anthocyanin biosynthesis in *A. thaliana* [128, 129], resulted in the up-regulation of both terpenoid pathway genes and flavonoids, as well as higher artemisinin and anthocyanin levels [99]. Some TFs belonging to the WRKY and MYB family act as suppressors in both the flavonoid and terpenoid pathways. As an example, overexpression of *OsWRKY76* in rice resulted in the suppression of *CHS* and *CHI* and reduced diterpenoid and flavonoid production [130]. In addition to TFs, there are suggestions that enzyme coding genes may also link these two pathways. For example, overexpression of PAL (*AaPAL1*) in *A. annua* resulted in higher production of both artemisinin and the phenylpropanoid hormone salicylic acid [131]. Similarly, overexpression of *F3H*, encoding a flavonoid pathway-specific enzyme, resulted in elevated levels of both flavonoid and artemisinin, as well as the expression levels of *ADS, CYP71AV1,* and *DBR2* [132]. Tissue-specific flavonoid and artemisinin genes expression profiles showing similar expression patterns of genes in the early stages of the flavonoid and artemisinin pathways gene provides further support for a function association between the two pathways (Figure 2).

Concluding remarks and future perspectives

At present, artemisinin represents the most effective pharmacological agent with anti-*Plasmodium* activity, but low rates of artemisinin production in plants and emerging resistance to artemisinin are increasing the demand to develop alternative treatments, and synergism with other plant products, notably flavonoids, represents a promising path forward, potentially enhancing the accumulation of both compound classes [133, 134].

To achieve this goal, targets will include elevating the production of precursors in shared biosynthetic steps, as well as altering the identifying regulators and regulatory elements that are common to both pathways. Information about the *cis*-regulatory elements within putative promoter

regions of flavonoid and artemisinin biosynthesis genes (Figure 1), along with their expression profiles, will likely prove valuable (Figure 2). The recently published genome sequence of *A. annua* [135] will provide a useful resource for promoter characterization. Indeed, we have already identified many TF binding sites on the promoter regions *A. annua* flavonoid and artemisinin genes (Figure 1). This has revealed that MYB family TFS may act as key regulators as they have the highest number of binding sites in the promoter region of genes from both pathways.

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Figures legends

Figure 1. Flavonoid (right) and artemisinin (left) biosynthetic pathways and their regulatory elements in *Artemisia annua*. The shikimate pathway supplies both pathways with carbon skeletons generated via primary metabolism. The horizontally listed blue boxes represent the promoter regions containing the binding sites for the associated transcription factors. These are shown as the geometrical shape on top of the relevant blue box. The number of binding elements on each promoter region has been indicated by "X." The nucleotide sequences of the putative binding sites are listed. bZIP-associated ABRE and ABRE3a (GTGCA and GTGCAT, respectively); bHLH associated G-box1, 2, 3, 4 and G-box like (TACGTG, CACGTT, CACGAC, CACGTC, and CCACGTAA, respectively); W-box sequence recognized by WRKY (TTGACC); AP2-ERF recognizes GCC-box (AGCCGCC) and DRE/CRT (RCCGCC); MYB (CAACCA), MYB-like (TAACCA) and MBS (CAACTG) are associated with the MYB transcription factor.

Figure 2. Tissue-specific genes expression profile of flavonoid and artemisinin biosynthetic pathways in *A. annua.* The color scale at the top represents the value of transformed reads per kilobase per million mapped reads. The heat map was constructed using the Multi Experiment Viewer (MeV) V. 4.8.

Figure 1. Flavonoid (right) and artemisinin (left) biosynthetic pathways and their regulatory elements in *Artemisia annua*.

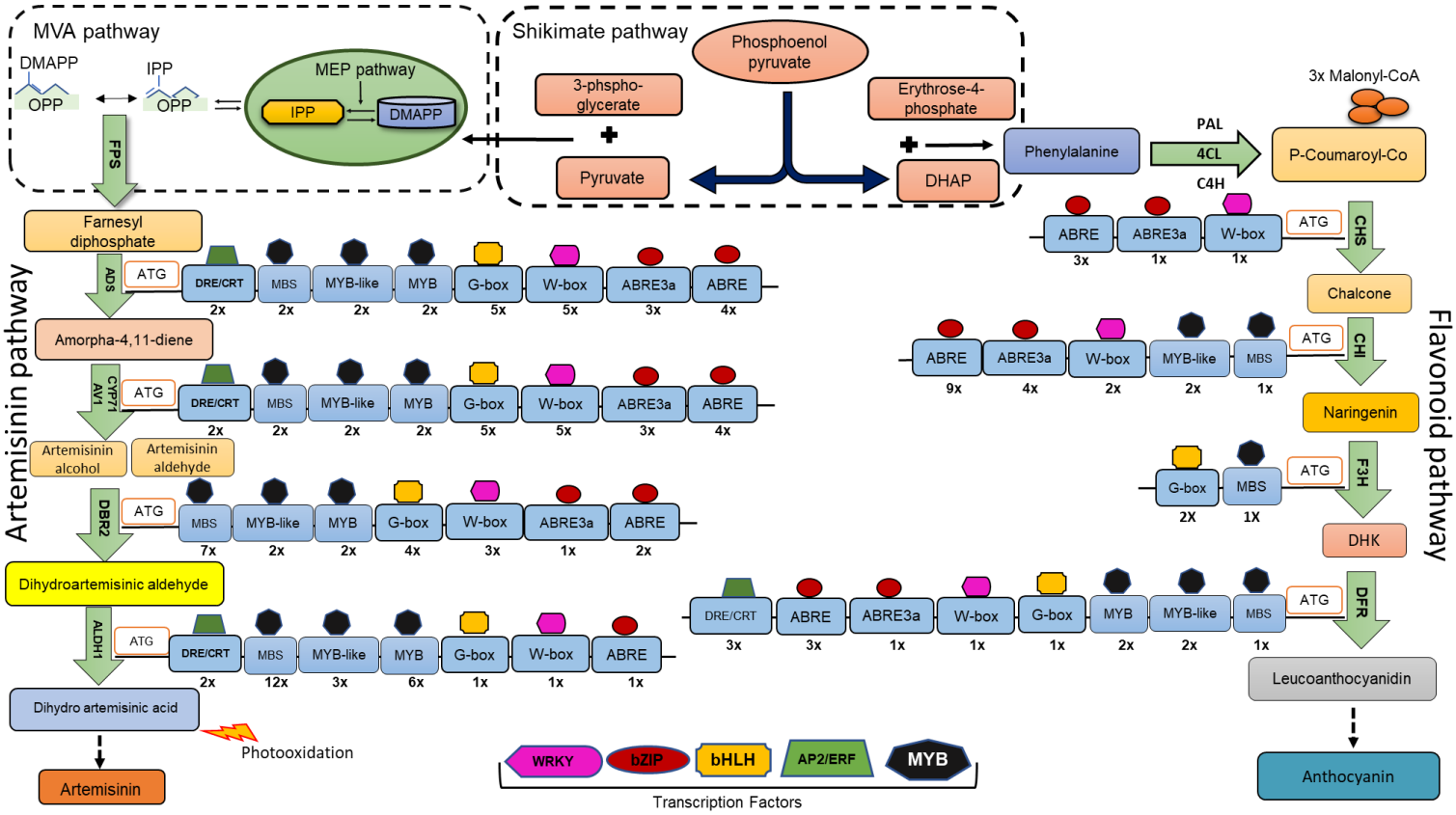

Figure 2. Tissue-specific genes expression profile of flavonoid and artemisinin biosynthetic pathways in *A. annua.*