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Two types of O-methyltransferase are involved in biosynthesis of anticancer methoxylated 4′-deoxyflavones in *Scutellaria baicalensis* Georgi

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Summary

The medicinal plant *Scutellaria baicalensis* Georgi is rich in specialized 4′-deoxyflavones, which are reported to have many health-promoting properties. We assayed *Scutellaria* flavones with different methoxyl groups on human cancer cell lines and found that polymethoxylated 4′-deoxyflavones, like skullcapflavone I and tenaxin I have stronger ability to induce apoptosis compared to unmethylated baicalein, showing that methoxylation enhances bioactivity as well as the physical properties of specialized flavones, while having no side-effects on healthy cells. We investigated the formation of methoxylated flavones and found that two *O*-methyltransferase (OMT) families are active in the roots of *S. baicalensis*. The Type II OMTs, SbPFOMT2 and SbPFOMT5, decorate one of two adjacent hydroxyl groups on flavones and are responsible for methylation on the C6, 8 and 3′-hydroxyl positions, to form oroxylin A, tenaxin II and chrysoeriol respectively. The Type I OMTs, SbFOMT3, SbFOMT5 and SbFOMT6 account mainly for C7-methoxylation of flavones, but SbFOMT5 can also methylate baicalein on its C5 and C6-hydroxyl positions. The dimethoxylated flavone, skullcapflavone I (found naturally in roots of *S. baicalensis*) can be produced in yeast by co-expressing SbPFOMT5 plus SbFOMT6 when the appropriately hydroxylated 4′-deoxyflavone substrates are supplied in the medium. Co-expression of SbPFOMT5 plus SbFOMT5 in yeast produced tenaxin I, also found in Scutellaria roots. This work showed that both type I and type II OMT enzymes are involved in biosynthesis of methoxylated flavones in *S. baicalensis*.

Introduction

Flavones are a class of flavonoids with a double bond between their 2 and 3 carbon positions on the C ring and without an additional substitution on the C3-position (Singh *et al.*, 2014). They are widely distributed in land plants, with functions in UV-protection, co-pigmentation or allelochemical activity (Martens and Mithöfer, 2005). Flavones are also important bioactive compounds used to promote human health. Dietary consumption of flavones reduces the risk of several types of cancer, chronic inflammation and coronary heart disease (Ajay *et al.*, 2019). These pharmacological activities have been attributed to their antioxidant, anticancerogenic, anti-inflammatory and immune modulatory properties (Dundar, 2015; Joana *et al.*, 2017). Although flavones are promising candidates for use as therapeutics, their adoption and development has been limited by their low stability, low uptake by cells and low bioavailability.

O-methylation is a common decoration of polyphenols including flavonoids and has been reported to increase stability and bioavailability (Koirala *et al.*, 2016). The relative bioefficacies of the polyphenol, resveratrol and its dimethoxylated derivative, pterostilbene, have been investigated extensively. Methylation results in pterostilbene being more bioavailable than resveratrol (Rimando *et al.*, 2002; Shin *et al.*, 2020), and also having greater cytotoxicity towards cancer cells than resveratrol (Kapetanovic *et al.*, 2011; Rimando *et al.*, 2002; Shin *et al.*, 2020). Methoxylated flavones are common in selected plant lineages, including the Lamiaceae, Asteraceae and Rutaceae (Wollenweber and H. Dietz, 1981). *Scutellaria baicalensis* Georgi is an important medicinal plant in the family Lamiaceae and has a long history of use in China as a traditional medicine (Huang Qin) (Zhao *et al.*, 2016). Scutellaria roots are the main ingredient of Lung Fufang, Fuzheng anti-cancer prescriptions and Huang Qin Tang, traditional Chinese medicines which have been used in combination with chemotherapy in China and shown to have significant

outcomes on primary bronchial pulmonary squamous cell carcinoma, NSCLD (non-small-cell lung cancer), compared to conventional chemotherapy alone (Duan *et al*., 2014; Li and Weng, 2017; Pan *et al*., 1990, 2000). Many studies have attributed the anticancer properties of extracts of *Scutellaria* to the major 4′-deoxyflavones found in the roots: baicalein, wogonin and their glucosides baicalin and wogonoside (Li-Weber, 2009; Woźniak *et al*., 2004).

Roots of several species of *Scutellaria* accumulate specialized 4′-deoxyflavones with distinct decorations (Qiao *et al*., 2016) and among the 70 flavones that have been detected in the dry roots of *Scutellaria baicalensis,* 43 of them are mono or polymethoxylated and will be referred to here as root methoxylated flavones (RMFs) (Wang *et al*., 2018). Consequently, this plant offers an ideal system to identify the biochemical mechanisms of RMF biosynthesis.

O-methylation is catalysed by *O*-methyltransferases that transfer a methyl group from S-adenosyl-L-methionine (SAM) to a hydroxyl group of their substrate. In plants, *O*-methylation is used in the decoration of many types of secondary metabolites, including alkaloids, terpenoids, phenylporpanoids and flavonoids (Ibdah *et al*., 2003; Ibrahim *et al*., 1998). *O*-methylation of flavonoids contributes greatly to structural diversification, and modifies solubility and bioactivity (Ibrahim and Anzellotti, 2003). *O*-methylation can change the intracellular location of flavonoids and increase their anti-microbial activity (Dakora and Phillips, 1996; Dixon *et al*., 1983; Yang *et al*., 2004).

Two types of plant *O*-methytransferase have been reported to decorate flavonoids (Noel *et al*., 2003). Type I OMTs have undergone considerable neofunctionalization in plants, including the formation of several sub-groups, namely; ChOMT (Chalcone OMT), COMT (Caffeic acid OMT), IOMT (Isoflavone OMT) and FOMT (Flavonoid OMT), depending on the different substrates they use (Noel *et al*., 2003). Mint (*Mentha x piperita*) FOMTs methylate flavonoids, but not caffeic acid or ferulic acid. These MpFOMTs are promiscuous with respect to their substrate flavones, but they usually transfer methyl groups to hydroxyl groups at specific positions on the aromatic rings of flavonoids. For example MpOMT1 methylates only the C7-hydroxyl group of flavones, MpOMT2 specifically methylates the C8-hydroxyl group, while MpOMT3 and MpOMT4 are specific for the 3′-hydroxyl and 4′-hydroxyl groups of the B ring respectively (Willits *et al*., 2004). Sweet basil FOMT (ObFOMT) showed similar specificities, except that the enzyme that is active on the C6-hydroxyl group can also methylate the 4′-hydroxyl group on the B ring of the flavone (Berim *et al*., 2012; Berim and Gang, 2013).

The activity of Type II OMTs was first described for *Chrysosplenum americamun* and *Lotus corniculatus*, and these enzymes are Mg^{2+}-dependent (Jay *et al*., 1985; Luca and Ibrahim, 1985). Similar enzymes from alfalfa and aspen have been shown to transfer a methyl group to caffeoyl-CoA as an intermediate in lignin biosynthesis, so they were named CCoAOMTs (caffeoyl-CoA OMTs) (Meng and Campbell, 1998; Parvathi *et al*., 2001). In 2003, Ibdah *et al* isolated a CCoAOMT-like enzyme from *Mesembryanthemum crystallumum,* which could also methylate quercetagetin (a flavonol) on its C6 and C3′ positions, in addition to its traditional substrate caffeoyl-CoA. Additional studies showed that Type II OMTs from different species could transfer methyl groups to both flavonoids and hydroxycinnamoyl CoA substrates (Ibdah *et al*., 2003) causing Type II OMTs to be renamed as PFOMTs (Phenylpropanoid and Flavonoid OMTs) to reflect more fully the multiple functions of these enzymes (Berim and Gang, 2013; Weng and Chapple, 2010). Substrates of PFOMTs include anthocyanins, flavonols, flavones, caffeoyl CoA and 5-hydroxyferuloyl CoA, but substrates have to present adjacent hydroxyl groups on carbons of the aromatic rings of the flavonoid (Lee *et al*., 2008; Thomas *et al*., 2011). Methylation of norwogonin to form wogonin is catalysed by a PFOMT, SbPFOMT 5 in roots of *S. baicalensis* (Zhao *et al*., 2019). However, the enzymes involved in the synthesis of the numerous other RMFs in Scutellaria, remain uncharacterized.

In this study, we found the di-methoxyflavone (skullcapflavone I) and the tri-methoxyflavone (Tenaxin I) from *S. baicalensis* have exceptional bioactivities in inducing apoptosis in human lung and liver cancer cells. Following up on this result, we describe methylation activities of both Type II OMTs and Type I OMTs on specialized 4′-deoxyflavone bioactives produced in the roots of *S. baicalensis*. The RMFs with elevated bioactivities could be semi-biosynthesized by co-expression of SbPOMT5 and selected SbFOMTs by supplementing the medium of yeast expressing these enzymes with the corresponding hydroxylated 4′-deoxyflavones.

Results

RMFs induce apoptosis in cancer cells

To evaluate the chemotherapeutic potential of methylation of 4′-deoxyflavones, we assayed the cytotoxicity in the form of their induction of apoptosis by the RMFs, wogonin, skullcapflavone I and tenaxin I on human cells. These flavones have between one and three methoxyl groups and have all been reported to be present in roots of *S. baicalensis* (Shen *et al*., 2020; Wang *et al*., 2018). Baicalein, a 4′-deoxyflavone without methoxyl groups, was used as a positive control, as it has been reported to induce apoptosis in several cancer cell lines (Li-Weber, 2009). The cell lines were grown with the treatment of the flavones for 24 h. Flow cytometric studies showed that all the compounds induced significant early apoptosis in liver cancer (SMMC-7721) cells and lung cancer (A549) cells compared with DMSO treatment alone. Tenaxin I showed the strongest promotion of apoptosis in both cell lines (Figure S1a–d). For A549 cells, the promotion of apoptosis seemed to be related to the number of methoxyl groups on the flavones. The lowest promotion of apoptosis was observed with baicalein, a non-methylated 4′-deoxyflavone, followed by wogonin, a monomethoxylated 4′-deoxyflavone, then skullcapflavone I, a dimethoylated 4′-deoxyflavone, and finally tenaxin I, a trimethoxylated 4′-deoxyflavone. Early apoptosis increased with the increasing number of methoxyl groups on the 4′-deoxyflavone (Figure S1b, d).

We then compared the effects of methoxylated flavones on the lung and liver cancer cell line to their effects on the primary vascular endothelial HUVEC line by assaying early and late apoptosis in the presence or absence of treatment with 100 μM flavones and extended the treatment time to 48 h. Flow cytometric analysis showed that all four 4′-deoxyflavones induced significant early apoptosis in A549 cells compared with the DMSO negative control and again, tenaxin I performed best; its induction of early apoptosis was twofold higher than that of baicalein over the same period (Figure 1a and Figure S2a). Baicalein, skullcapflavone I and tenaxin I also increased late apoptosis in A549 cells and tenaxin I showed 0.71-fold higher number of cells in late apoptosis compared to the value for baicalein over the same incubation period. In liver cancer cells, all the four flavones induced significant early and late apoptosis compared with the

negative control. Again, tenaxin I promoted the highest rate of early apoptosis, followed by skullcapflavone I (Figure 1b and Figure S2b). Despite their obvious cytotoxicity to cancer cells, none of the flavones showed significant effects on the non-cancerous primary cell line, HUVEC (Figure 1c and Figure S2c).

To evaluate the effects of the flavones on the cancer cells further, we carried out a series of dose response experiments. Each compound was tested at five concentrations on the cell cultures over 72 h, and cell death was measured using a multiscan spectrum. For all the compounds tested, the inhibition of cell proliferation increased with increasing concentrations of compounds (Figure 1d–k). IC50 values were calculated for each flavone based on these curves. The mono-methoxyflavone wogonin had a similar IC50 value of 62.9 μM to that of baicalein (62.2 μM) for the lung cancer cell line A549. The IC50 of skullcapflavone I was 68.5 μM, but the value of tenaxin I was 27.8 μM for the A549 cell line. The inhibition of growth of liver cancer cells increased with concentration of baicalein from 12.5 μM to 100 μM, but the value never rose above 50% with increases in concentration beyond 100 μM, although baicalein concentrations higher than 300 μM were insoluble in the culture media, so it was impossible to calculate the IC50 values (Figure 1h). The IC50 values of the RMFs wogonin, skullcapflavone I and tenaxin I to liver cancer cells were 104.59, 26.41 and 27.82 μM respectively (Figure 1i–k). Together, these results showed that tenaxin I inhibited cancer cell growth more than other 4′-deoxyflavones, and could be a strong candidate for future development of anti-cancer drugs.

Diverse RMFs are detected in the roots of *Scutellaria baicalensis*

To confirm that the methoxylated flavones of interest are present in the roots of *S. baicalensis* (the plant is shown in Figure 2a and the dried root is shown in Figure 2b), we undertook a widely targeted metabolite analysis of extracts from roots of 2-month-old seedlings and 2-year-old plants grown in Chenshan Botanical Garden, Shanghai. Samples were analysed using UPLC-MS/MS, which returned 400 distinct compounds based on the MS platform and our in house compound library. Among the compounds identified, flavonoids were the largest group (131), followed by phenolic acids (49) and lipids (46). In total, 72 flavones and 43 *O*-methylated flavones were detected (*O*-methylated flavones are listed in Table S1). The relative abundance of specific flavones was broadly equivalent in 2-month-old

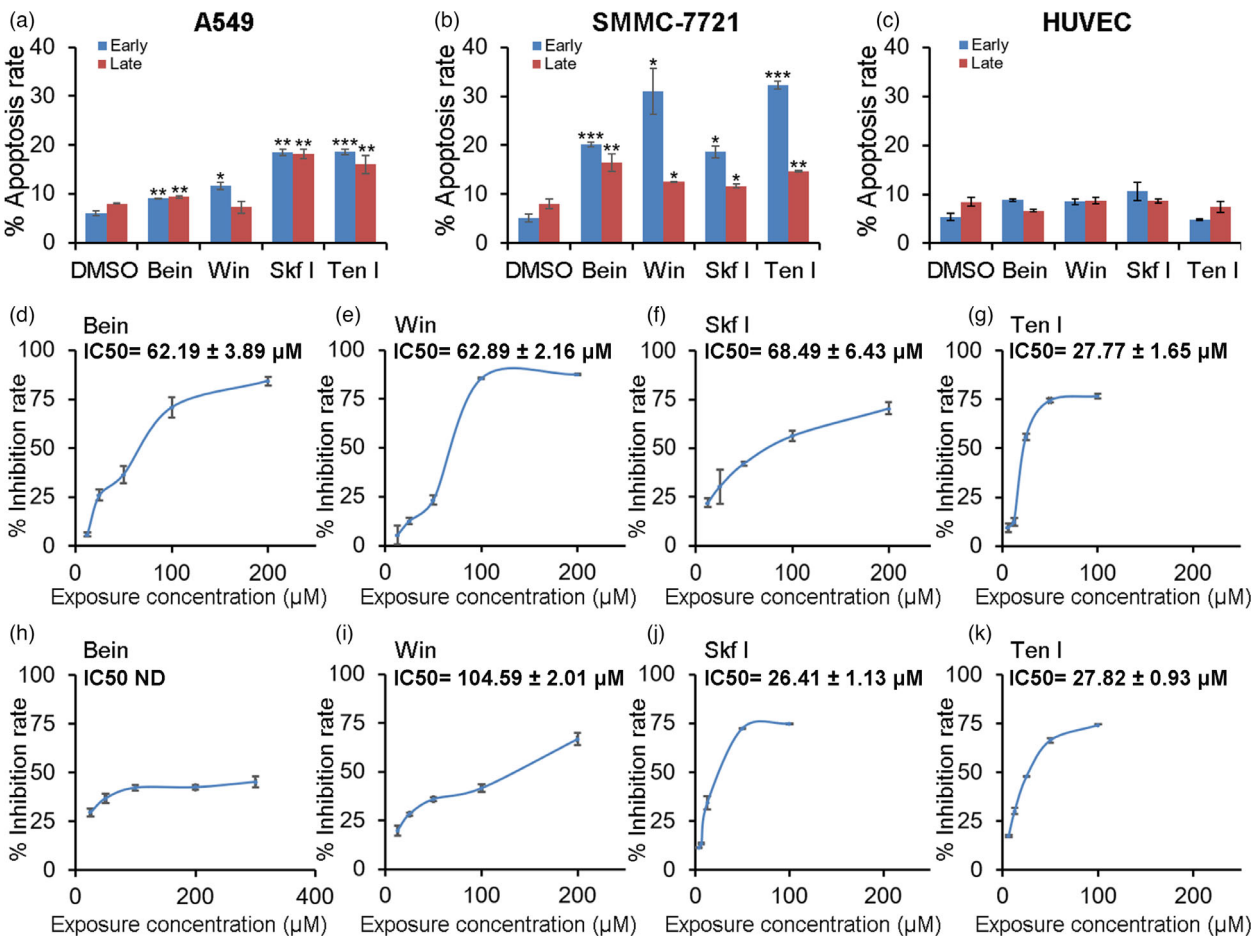


Figure 1 Effects of Scutellaria flavones on human cells. (a) Early and late apoptosis induced in A549 lung cancer cells, (b) SMMC-7721 liver cancer cells and (c) HUVEC healthy cells with and without baicalein (Bein), wogonin (Win), skullcapflavone I (Skf I) and tenaxin I (Ten I). Flavones were used at 100 μM for treatment of 48 h. (d–g) Dose–response curves for treatment of A549 cells with baicalein, wogonin, skullcapflavone I and tenaxin I. Each datapoint represents the mean ± SD. (h–k) Dose–response curves of SMMC-7721 for treatment of baicalein, wogonin, skullcapflavone I and tenaxin I. Each data represents the mean ± SD.

roots and 2-year-old roots. Among the flavones detected, baicalin had the strongest signal, which confirmed previous reports that baicalin is the most abundant flavonoid in the roots of *Scutellaria baicalensis*.

Various methoxylated flavones were found in Scutellaria roots (RMFs), with 25 mono-methoxyflavones, 9 di-methoxyflavones, 5 tri-methoxyflavones and 4 multiple-methoxyflavones (more than three O-methyl groups) (Table S1). The representative structures are shown in Figure 2c. Each hydroxyl group on the A ring of a flavone potentially can be methylated. Wogonin (1) and Tenaxin II (2) both have an methoxy group on their C8-position. Oroxylin A (3) and nepetin (4) are both methylated on the C6-hydroxyl group. Compound 5 (6,7,8-trihydroxy-5-methoxyflavone) and genkwanin (6) have methoxyl groups on their C5 and C7 positions respectively. Chrysoeriol (7) and acacetin (8) are mono-methoxyflavones methylated on C3′ and C4′ positions on their B rings respectively. Skullcapflavone I (9) and Rehderianin I (10) are di-methoxyflavones with methoxy modifications on C7 and C8-positions. Compound 11 (7,2′-dihydroxy-5,8-dimethoxyflavone) has methoxyl groups on its C5 and C8 positions. Compound 12 (5,7,2′,5′-tetrahydroxy-6,8-dimethoxyflavone) is methylated on its C6 and C8 positions. There are some di-methoxyflavones which have one methyl group on the A ring and another on the B ring, such as jaceosidin (13) and compound 14 (viscidulin III-6′-O-β-glucopyranoside). Tenaxin I (15) is a representative tri-methoxyflavone, with methoxyl groups on C6, 7 and 8 positions. Skullcapflavone II (16) is tetra-methoxyflavone with O-methylation on its C6, 7, 8 and 6′ positions.

SbPFOMTs can transfer methyl groups to flavones with adjacent hydroxyl groups on their aromatic rings

Having shown that *S. baicalensis* roots accumulate diverse RMFs which have comparable or even better bioactivities than non-methylated 4′-deoxyflavones, we attempted to identify the enzymes responsible for these decorations. Previously, we isolated and characterized 5 PFOMT enzymes from *S. baicalensis* (SbPFOMT1- SbPFOMT5) and demonstrated that SbPFOMT1, 2 and 5 can transfer a methyl group to the C8-hydroxyl group of norwogonin, producing wogonin *in vitro* (Zhao *et al*., 2019).

Baicalein (17) is an important 4′-deoxyflavone that accumulates in roots of *Scutellaria baicalensis*. Baicalein has three adjacent hydroxyl groups on the C5, C6 and C7 positions of its A ring and may be the precursor for oroxylin A (3) biosynthesis. We supplied baicalein to yeast strains expressing the different SbPFOMTs and grew the strains overnight. LC-MS analysis showed that both SbPFOMT2 and SbPFOMT5 could catalyse conversion of baicalein to oroxylin A (Figure 3a-c), but only tiny amounts of oroxylin A could be detected in strains expressing SbPFOMT1, 3 or 4.

To identify additional functions of the SbPFOMTs, we undertook *in vitro* enzyme assays using purified proteins with different flavone substrates. In addition to norwogonin, SbPFOMTs 2 and 5 could also methylate the C8 position of compound 18 (5,7,8,2′-tetra-hydroxyflavone) to produce tenaxin II (2) (Figure 3d–f). SbPFOMT1, 2 and 5 could methylate the 3′-hydroxyl group of luteolin (19) to produce chrysoeriol (7) (Figure 3g–i). Interestingly, only SbPFOMT1 could convert caffeoyl-CoA to feruloyl-CoA; neither SbPFOMT2 nor SbPFOMT5 had this activity (Figure 3j–l).

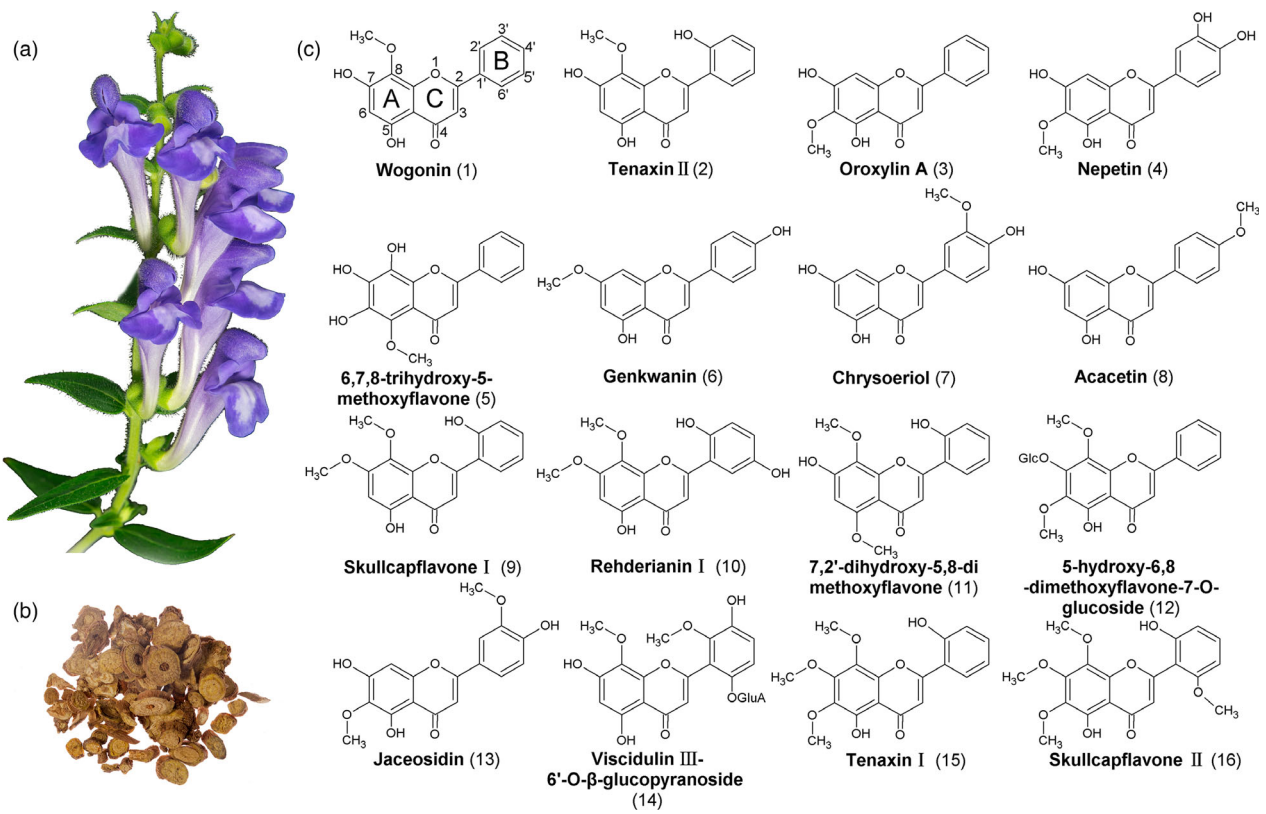


Figure 2 *Scutellaria baicalensis* and its root methoxylated flavones (RMFs). (a) *S. baicalensis* plant with flowers. (b) The dried root of *S. baicalensis* which is used in traditional Chinese medicine. (c) Representative methoxylated flavones detected in roots of *S. baicalensis*.

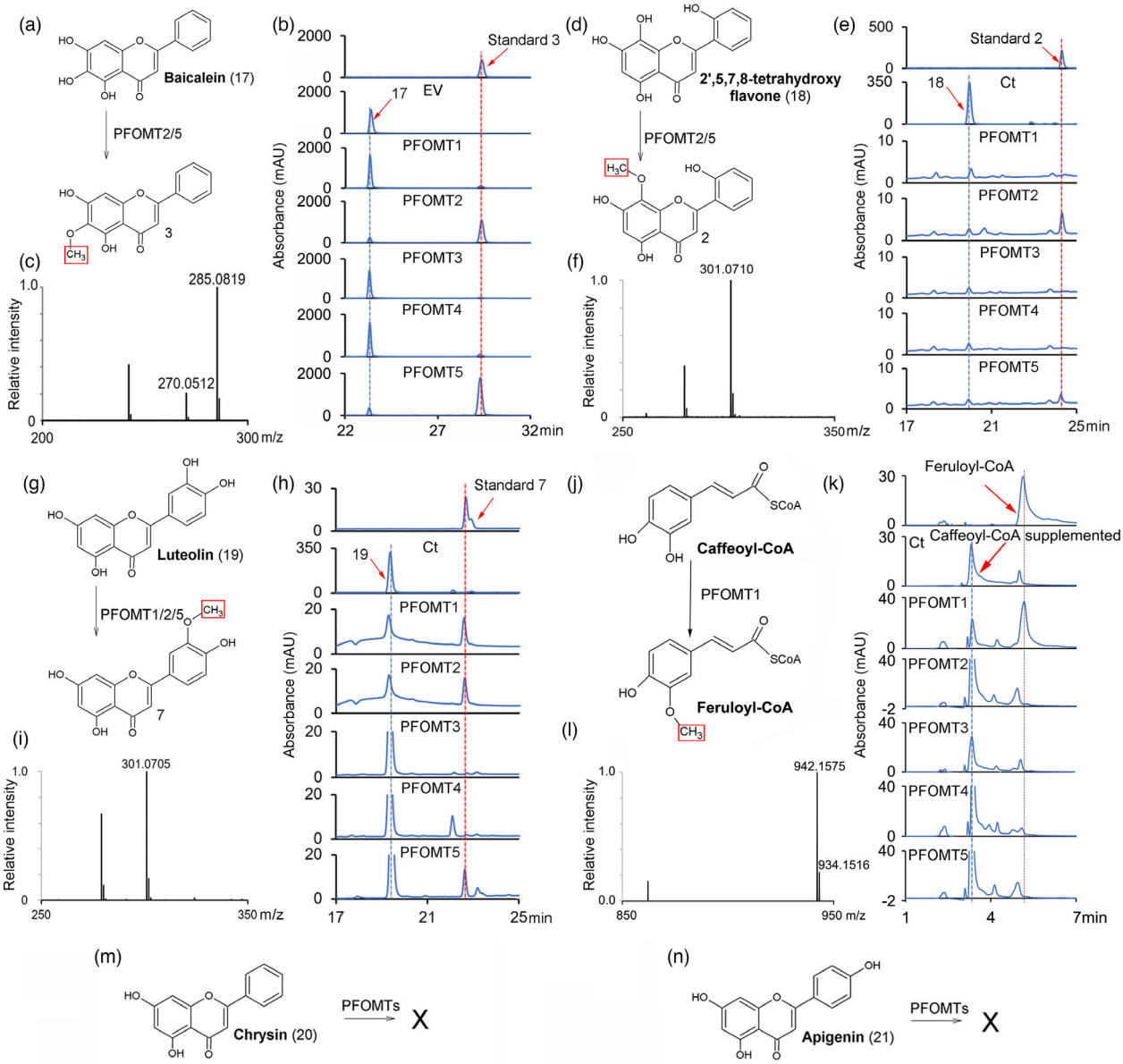


Figure 3 Enzymatic analysis of SbPFOMTs. (a) The reaction catalysed by SbPFOMT2 and SbPFOMT5 converts baicalein (17) to oroxylin A (3). (b) HPLC analysis of yeast samples fermented with baicalein *in vivo*; top, oroxylin A standard; EV, yeast carrying empty vector; PFOMT1-PFOMT5, yeast expressing corresponding PFOMT proteins. (c) MS patterns of the product of SbPFOMT 2 and SbPFOMT5, which was identical to the oroxylin A standard. (d) The reaction catalysed by SbPFOMT2 and SbPFOMT5 converts 2′,5,7,8-tetrahydroxyflavone (Compound18) to tenaxin II (Compound 2). (e) HPLC analysis of *in vitro* enzyme assay using 2′,5,7,8-tetrahydroxyflavone as a substrate; top, tenaxin II standard; Ct, control assay; assays with corresponding PFOMT1-PFOMT5 proteins. (f) MS pattern of the product, which was identical to the oroxylin A standard. (g) The reaction catalysed by SbPFOMT1, SbPFOMT2 and SbPFOMT5 converts luteolin (19) to chrysoeriol (7). (h) HPLC analysis of *in vitro* assays of PFOMT1-PFOMT5 using luteolin as a substrate; top, chrysoeriol standard; Ct, control assay; PFOMT1-PFOMT5 enzyme assays with corresponding proteins. (i) MS pattern of the product, which was identical to the chrysoeriol standard. (j) The reaction catalysed by SbPFOMT1 converts caffeoyl-CoA to feruloyl-CoA. (k) HPLC analysis of *in vitro* assays of PFOMT1-PFOMT5 using caffeoyl-CoA as a substrate; top, feruloyl-CoA standard; Ct, control assay;, PFOMT1-PFOMT5 enzyme assays with corresponding proteins. (l) MS pattern of the product, which was identical to the feruloyl-CoA standard. (m and n) The SbPFOMTs cannot use chrysin (Compound 20) or apigenin (Compound 21) as substrates.

None of the PFOMT enzymes had activity on flavones lacking adjacent hydroxyl groups on their aromatic rings, including the 4′-deoxyflavone, chrysin (20) and the 4′ hydroxyflavone, apigenin (21) (Figure 3m, n and Figures S3, S4).

In summary, SbPFOMT2 and 5 could efficiently decorate one of two adjacent hydroxyl groups of 4′-deoxyflavones at 6, 8 or 3′ positions on the aromatic rings of flavones. FPKM values showed that SbPFOMT1 is highly expressed in stem, but it has very low expression in roots, suggesting this enzyme is most likely involved in lignin biosynthesis. SbPFOMT2 and 5 are both highly expressed in roots and SbPFOMT5 could be induced significantly by MeJA (Zhao *et al.*, 2019). We propose that SbPFOMT5 as well as SbPFOMT2 can form mono-methoxyflavones oroxylin A (3), wogonin, tenaxin II (2) and chrysoeriol (7) with methoxy groups

on their C6, 8 and 3′-positions, respectively, although we know, *in vivo*, that SbPFOMT5 is the principal activity driving wogonin synthesis from norwogonin in *S. baicalensis* roots (Zhao *et al*., 2019). These results indicated that *S. baicalensis* employs different members of the PFOMT family for different purposes.

Isolation of type I OMTs from *S. baicalensis*

To identify the enzymes catalysing the methyl decorations of hydroxyl groups at other positions on 4′-deoxyflavones we searched for more *OMT* genes encoded by the *S. baicalensis* genome. This provided us with 10 genes encoding Type I OMTs. These predicted proteins were closely related, structurally, to ObFOMT (flavonoid OMT) from sweet basil, so we named them SbFOMTs (Table S2). Two of the *SbFOMT* genes had 99.7% identity at the nucleotide level and the encoded proteins showed 100% identity at the amino acid (aa) level, so we named them *SbFOMT3.1* and *SbFOMT3.2*. The *SbFOMT* genes were distributed randomly in the genome of *S. baicalensis*, with only *SbFOMT3.1* and *SbFOMT3.2* positioned relatively closely together on chromosome 1, suggesting them to be the product of a recent tandem-duplication. This was different from the *SbPFOMTs*, where four of the *SbPFOMT* genes that encode proteins that can transfer methyl groups to 4′-deoxyflavones, are located in tandem on chromosome 1, suggesting recent chromosome duplications have led to subfunctionalized PFOMT activities (Zhao *et al*., 2019).

We designed primers for the *FOMT* genes (Table S3), and nine of the genes were isolated successfully by RT-PCR of cDNA made from RNA from pooled tissues of *S. baicalensis*. The length of the ORFs of the *FOMT* genes ranged from 1026 to 1113 bp, encoding proteins predicted to range in size from 341 to 370 aa. The FOMT and PFOMT proteins were clearly separated, phylogenetically (Figure 4a). SbFOMT4 and SbFOMT7 were structurally most similar to the ObF6OMTs and ObF7OMTs from sweet basil (Berim and Gang, 2013), SbFOMT2, 5, 6 and 8 clustered with ObF8OMT1, an 8-OMT responsible for gardenin B and nevadensin synthesis in sweet basil (Berim and Gang, 2013). SbFOMT1, 3 and 9 comprised a *Scutellaria*-specific branch of Type I FOMTs.

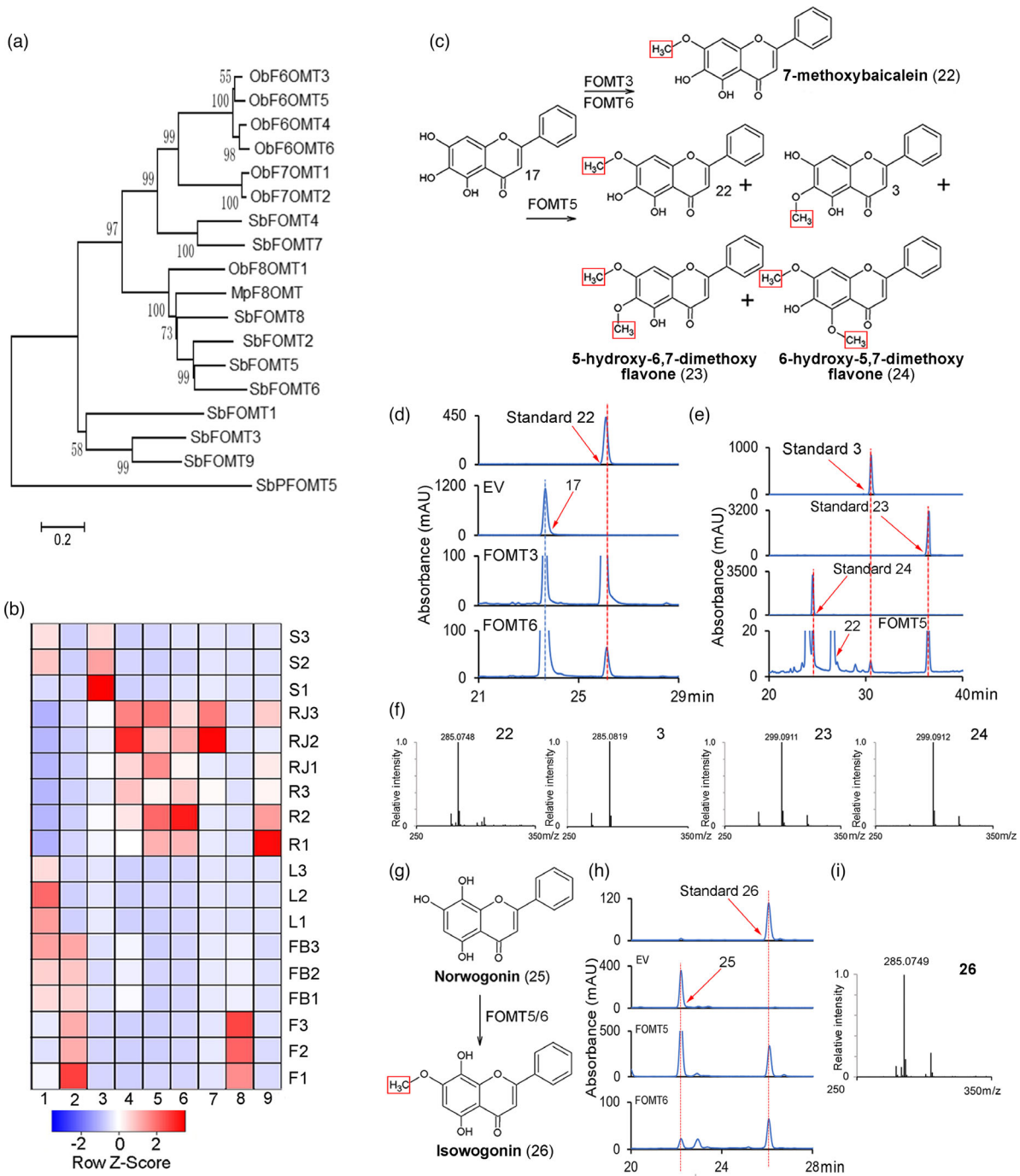
To evaluate expression patterns of the *SbFOMTs*, we explored the FPKM values from RNA-seq data from tissues of flower buds, flowers, leaves, stems, roots and JA-treated roots (Zhao *et al*., 2019). As shown in the heat-map (Figure 4b), *SbFOMT4*, *5, 6, 7* and *9* were highly expressed in both roots and JA-treated roots, indicating that the proteins encoded by these genes were good candidates for O-methylation of the 4′- deoxyflavones in roots. Transcripts of *SbFOMT2* and *SbFOMT8* accumulated in flower tissues, where the enzymes are probably involved in decorations of pigments and/or co-pigments in flowers. *SbFOMT1* was expressed specifically in leaves. *SbFOMT3* had the highest transcript levels in stems, followed by roots, leaves and flowers.

Enzyme assays of SbFOMTs with hydroxy 4′-deoxyflavones

To explore the activities of the SbFOMTs, we expressed cDNAs encoding the nine enzymes in yeast. We fed each strain with the main Scutellaria 4′-deoxyflavones: baicalein and norwogonin, respectively, and then grew the yeast overnight. Strains expressing SbFOMT1, 2, 4, 7, 8 and 9 showed no activity on either substrate (Figures S5 and S6). Strains expressing SbFOMT3 and SbFOMT6 converted baicalein to 7-methoxybaicalein, demonstrating that both enzymes have 7-OMT activity (Figure 4c, d, f). Interestingly, SbFOMT5-expressing yeast fed with baicalein, produced not only mono-methoxy 4′- deoxyflavones: 7-methoxybaicalein (22) and oroxylin A (3), but also di-methylated flavones: 5-hydroxy-6,7-dimethoxyflavone (23) and 6-hydroxy-5,7-dimethoxyflavone (24) (Figure 4c, e, f), indicating that SbFOMT5 can transfer methyl groups to the C5, 6, or 7-hydroxyl groups of baicalein. The molar ratio of the four compounds was 75.73: 1: 5.45: 7.32, showing that the major product of SbFOMT5 fed baicalin was 7-methoxybaicalein, and that 7-OMT was its major activity. Strains expressing either SbFOMT5 or SbFOMT6 converted norwogonin to isowogonin, indicating that both can transfer a methyl group to the C7 hydroxyl group of norwogonin (Figure 4g, h, i), but SbFOMT3 and other SbFOMTs had no activity on norwogonin (Figure S6).

We next compared the OMT activities of SbFOMT3, SbFOMT5 and SbFOMT6 with the major 4′-deoxyflavones from *S. baicalensis*, *in vitro*, by expressing SbFOMT3, SbFOMT5 and SbFOMT6 proteins in *E. coli* and purifying them (Figure S7). Enzyme assays were carried out by incubating the proteins with their substrates for 2 h. In accordance with our earlier experiments, FOMT3 and FOMT6 methylated the 7-hydroxyl group of baicalein (Figure S8). SbFOMT5 converted baicalein to both 7-methoxybaicalein and oroxylin A, with the molar ratio 4.5: 1, but di-methylated baicalein was not detected *in vitro* (Figure S8), perhaps because the reaction time of 2 h was inadequate to detect this more minor activity or because the *in vitro* conditions were not suitable

Figure 4 Isolation of SbFOMTs and their enzyme activities. (a) Phylogenetic tree of SbFOMT proteins. Maximum-likelihood (ML) method was used to construct the tree with 1000 replicate bootstrap support. The FOMT tree was rooted with SbPFOMT5. (b) Expression heat-map of the SbFOMT genes in *Scutellaria baicalensis*. Expression levels are shown as exponential values with base 10 (FPKM), and the scale is shown at the bottom. S, stem; RJ, MeJA-treated root; R, root; L, leaf; FB, flower bud; F, flower; the numbers following the letters indicate the number of independent replicates used. (c) The reactions catalysed by SbFOMT3, SbFOMT5 and SbFOMT6 using baicalein (Compound 17) as a substrate; Compound 22, 7-methoxybaicalein; Compound 3, oroxylin A; Compound 23, 5-hydroxy-6,7-dimethoxyflavone; Compound 24, 6-hydroxy-5,7-dimethoxyflavone. (d) HPLC analysis of SbFOMT3 and SbFOMT6-expressing yeast fermented with baicalein *in vivo*; top, 7-methoxybaicalein standard; EV, yeast carrying an empty vector; FOMT3 and FOMT6, yeast expressing corresponding FOMT3 and FOMT6 proteins respectively. (e) HPLC analysis of SbFOMT5-expressing yeast fermented with baicalein *in vivo*. Standard 3, 23 and 24, are standards of Oroxylin A, 5-hydroxy-6,7-dimethoxyflavone and 6-hydroxy-5,7-dimethoxyflavone respectively; FOMT5, yeast expressing FOMT5 protein. (f) MS patterns of the products, which were identical to Compounds 22, 3, 23, and 24 standards respectively. (g) The reaction catalysed by SbFOMT5 and SbFOMT6 (Combination 1, C1) that convert norwogonin (Compound 25) to isowogonin (Compound 26). (h) HPLC analysis of yeast expressing SbFOMT5 and SbFOMT6 (Combination 1) fermented with norwogonin *in vivo*. top, isowogonin standard; EV, yeast carrying empty vector; FOMT5-FOMT6, yeast expressing corresponding FOMT5-FOMT6 proteins. (i) MS patterns of the product, which was identical to the isowogonin standard. (j) Kinetic analyses of SbOMT3, SbOMT5 and SbOMT6 to baicalein and norwogonin. Each data set represents the mean ± SD from triplicate measurements. ND, not detectable.



(j)

Enzymme	Substrate	Km (μM)	Vmax (pkat mg^{-1}protein)	Vmax/Km
SbFOMT3	baicalein	0.343±0.047	828.383±33.595	2416.050
SbFOMT3	norwogonin	ND	ND	ND
SbFOMT5	baicalein	1.275±0.089	646.478±4.878	507.241
SbFOMT5	norwogonin	2.221±0.496	705.685±83.562	317.750
SbFOMT6	baicalein	2.787±0.268	225.454±8.206	80.905
SbFOMT6	norwogonin	1.667±0.041	271.088±2.767	162.664

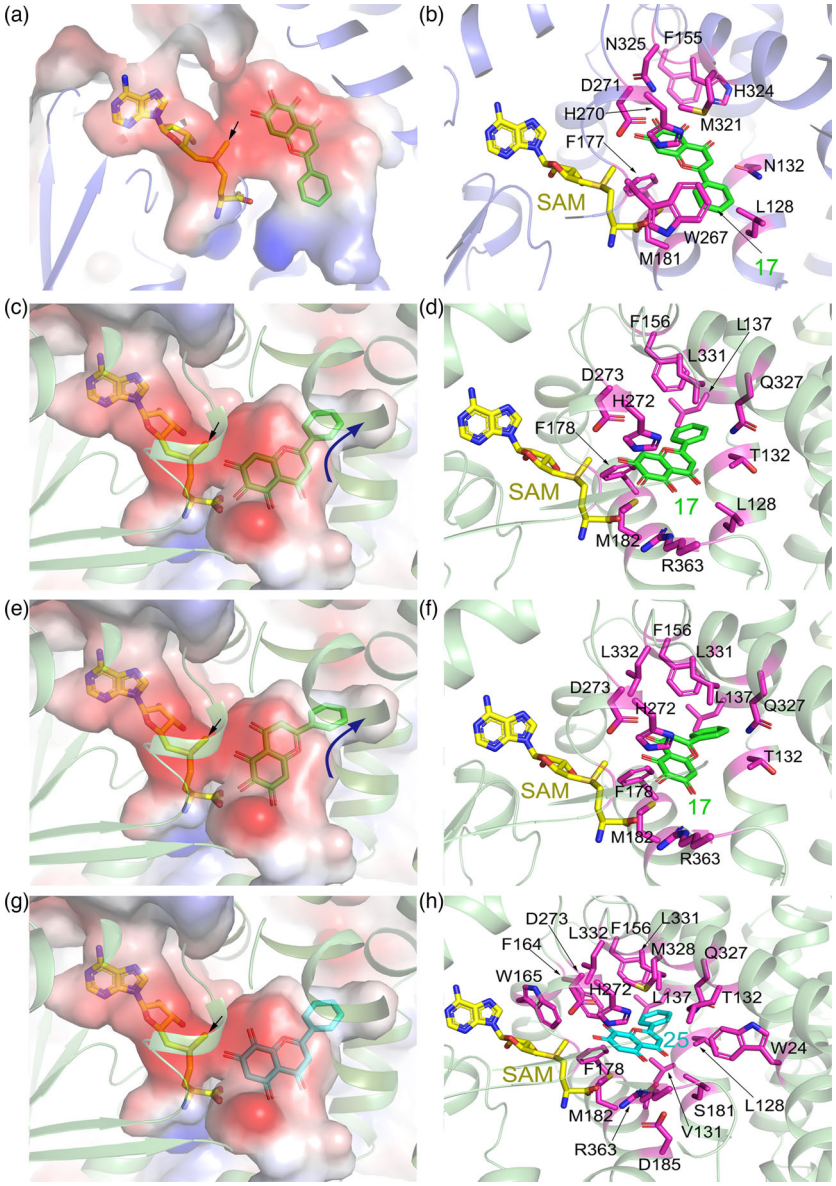


Figure 5 Protein modelling of SbFOMTs. The proteins were modelled using alfalfa isoflavone 7-O-methyltransferase as a template. Baicalein and norwogonin were docked into the active sites using Autodock Vina. SAM and flavone substrates are shown in yellow and green respectively. (a) SbFOMT3 substrate-binding pockets for baicalein, a black arrow indicates the methyl group for transfer. (b) Surrounding amino acids hosting baicalein in the active site of SbFOMT3. (c) Substrate binding pockets of SbFOMT5 for baicalein, a black arrow indicates the methyl to flavone transfer. A blue arrow indicates the extended hydrophobic hole for the B-ring of baicalein. (d) Amino acids surrounding baicalein in the active site of SbFOMT5. (e) The two substrate-binding pockets for baicalein in opposite orientations in SbFOMT5; a black arrow indicates the methyl group to transferred. A blue arrow indicates the extended hydrophobic hole for the B-ring of baicalein. (f) The amino acids surrounding baicalein docked in the opposite orientation in the active site of SbFOMT5. (g) SbFOMT5 substrate-binding pockets for norwogonin, a black arrow indicates the methyl group to be transferred. (h) Amino acids surrounding norwogonin in the active site of SbFOMT5.

for the additional 5- and 6-methyl transferase reactions to proceed. Both SbFOMT5 and SbFOMT6 also methylated the 7-hydroxyl group of norwogonin (Figure S9), confirming the results of the *in vivo* yeast experiments. Kinetic analysis showed that SbFOMT3 had the greatest catalytic efficiency with baicalein, as this enzyme has the lowest Km and highest Vmax/Km (Figure 4j). Although SbFOMT5 and SbFOMT6 can methylate baicalein and norwogonin, kinetic analysis showed that SbFOMT5 had a greater affinity for baicalein, whereas SbFOMT6 was more efficient at using norwogonin as its substrate.

Previous studies have shown that FOMTs transfer a methyl group to a specific position on the flavone molecule. However, SbFOMT5 appeared to be able to methylate hydroxyl groups at multiple positions on 4′-deoxyflavones. To explore the activity of SbFOMT5 further, we assayed the enzyme with three mono-hydroxy-4′-deoxyflavone substrates: 5-hydroxy-4′-deoxyflavone, 6-hydroxy-4′-deoxyflavone and 7-hydroxy-4′-deoxyflavone. Although we had only 7-methoxyflavone as a standard, LC-MS showed that each of the *in vitro* reactions with the different substrates produced a compound with an added methyl group with m/z 253 compared to their substrates with m/z 238 (Figure S10), confirming that SbFOMT5 can transfer a methyl group to each of the mono-hydroxy, 4′-deoxyflavones, *in vitro*. This experiment confirmed the novel activity of SbFOMT5 and suggested that this activity might operate in *S. baicalensis* roots.

The basis for the different regio-selectivities of SbFOMTs

To investigate the structural basis for the specificity of the SbFOMTs, we modelled SbFOMT3, SbFOMT5 and SbFOMT6 using alfalfa isoflavone 7-O-methyltransferase as a template (Zubieta *et al.*, 2001) and the substrates were docked into their active sites. Based on previous reports about OMTs of this type, we assumed that two SbFOMT monomers form a dimer through the interaction of their N termini, providing a back wall for the active site. Two pockets could be observed in SbFOMTs, one binding SAM and the other binding the substrate flavones (Figure 5). A channel lay between the two pockets, allowing transfer of the methyl group from SAM to the methyl-accepting

substrate (Figure 5a, c, e, g). A histidine amino acid (His-270 of SbFOMT3, His-272 of SbFOMT5 and His-261 of SbFOMT6) was located over the channel on the substrate-pocket side (Figure 5b, d, f, h), which could deprotonate the hydroxyl group of the substrate and work as the catalytic base for the reaction. The substrate pockets were slit-shaped and consequently suitable for the acceptance of planar flavone molecules.

The hydrophobic environment in the substrate pocket of SbFOMT3 is predicted to be formed by nonpolar amino acids; Leu-128, Met-181 and Trp-267 are required for hosting the B-ring of baicalein (Figure 5a, b). The oxygen atom of Asp-271 could form a hydrogen bond with the 6-OH of baicalein, maintaining a distance of 3.4 Å between the methyl group of SAM and the 7-hydroxyl group of baicalein, a perfect distance for the methyl transfer reaction. In contrast, with norwogonin, which has no 6-OH for hydrogen bonding with Asp-271, access to the 8-OH is probably blocked by a hydrophobic Phe-177 amino acid, making it very difficult to hold norwogonin in the pocket in a suitable orientation for the methyl transfer reaction. This could explain why SbFOMT3 is active only on the 7-OH of baicalein.

For SbFOMT5, nonpolar amino acids Leu-137, Phe-156 and Leu-331 are predicted to form a hydrophobic hole with extended room for the B-ring of flavones, which offers its substrates a flexible space (Figure 5c–f). Baicalein could be docked into the SbFOMT5 active site in two orientations. When the 4-OH of baicalein was closer to T-132, the 7-OH lay at a suitable distance (3.1 Å) to accept the methyl group from SAM. However, because there was extended room for the B-ring, baicalein had enough space to move towards Asp-273, providing the opportunity for the 6-OH to approach the methyl group of SAM for methyl transfer (Figure 5c, d). When baicalein was docked in the alternative orientation, the 5-OH lay at a suitable distance (3.4 Å) to accept the methyl group from SAM. Again, because of the extended space available for docking the B-ring, the substrate also had the space to move towards Asp-273, providing the opportunity for the 6-OH of baicalein to accept the methyl group from SAM (Figure 5e, f). Similarly, when 7-methoxybaicalein docked in the same orientation, both the 6-OH and 5-OH were available for methylation depending on the orientation of the substrate, producing 5-hydroxy-6,7-dimethoxyflavone (23) and 6-hydroxy-5,7-dimethoxyflavone respectively (24) (Figure S11a, b). These models provided plausible explanations for why SbFOMT5 could methylate the hydroxyl groups of baicalein at multiple positions. To test these hypotheses, we undertook mutagenesis of Leu-137, Phe-156 and Leu-331 of SbFOMT5. The hydrophobic amino acid Leu was mutated to Glu and Phe was mutated to Tyr, amino acid substitutions providing changes in polarity but similar R-group sizes to the original ones. We produced 3 mutants: M1 (SbOMT5 L137E), M2 (SbOMT5 L137E/F156Y) and M3 (SbOMT5 L137E/F156Y/L331E). Enzyme assays in yeast showed that M1 could produce only 7-methoxybaicalein when supplemented with baicalein, and the other three potential products: oroxylin A, 5-hydroxy-6,7-dimethoxyflavone, and 6-hydroxy-5,7-dimethoxyflavone, were not detected, when compared with yeast expressing unmutated SbOMT5 (Figure S12). In fermentation of M1 and M2, only tiny amounts of 7-methoxybaicalein were found (Figure S12). These results indicated that, replacement of Leu and Phe in the hydrophobic hole of SbFOMT5 with polar amino acids restricted movement of the B-ring, and eliminated the opportunity for the 6- or 5-hydroxyl groups to approach the methyl group from SAM in SbFOMT5, confirming our interpretations of the modelling experiments.

Norwogonin could be docked into SbFOMT5 in one orientation only (Figure 5g, h), and Asp-273 could hydrogen bond with the 8-OH, facilitating methylation of the 7-OH. This explained why SbFOMT5 is specific for methylation of the 7-OH group of norwogonin to form isowogonin.

The active site of SbFOMT6 is very spacious. Docking experiments did not identify potential binding states for the methylation of baicalein or norwogonin established by our enzymatic experiments (Figure S11c–f). Probably the isoflavone 7-OH methyltransferase model is unsuitable for docking analysis with SbFOMT6, and crystallography of the SbFOMT6 protein with its substrates would be necessary to determine the structural basis for the specificity of its activity.

Semi-biosynthesis of RMFs found in *Scutellaria baicalensis* roots

Based on the activities of the different OMTs from *S. baicalensis*, we attempted to synthesize those di/tri-methoxy 4′-deoxyflavones found naturally in Scutellaria, such as skullcapflavone I and tenaxin I, using the respective 4′-deoxyflavones as substrates in yeast fermentations.

In principle, skullcapflavone I could be produced from 2′,5,7,8-tetrahydroxy 4′-deoxyflavone (18) by methylation of its C7 and C8 hydroxyl positions. This substrate is similar to norwogonin in terms of its A ring. We knew that SbPFOMT5 could transfer a methyl group to the 8-hydroxyl group of compound 18 (Figure 3d). Although both SbFOMT5 and SbFOMT6 could methylate the 7-OH group of a 5-, 7- and 8-hydoxylated A ring (shown in Figure 4g), SbFOMT6 showed greater specificity for this reaction (Figure 4c, g). Based on this knowledge, we tested SbPFOMT5 plus SbFOMT6 (Combination 1 (C1)) for the ability to synthesize skullcapflavone I. We supplied compound 18 to the medium of yeast expressing SbPFOMT5 and SbFOMT6 (C1) and fermented the system overnight. LC-MS analysis showed that almost all the substrate was converted to skullcapflavone I, confirmed by the product having the same MS spectrum as its standard (Figure 6a–c).

Tenaxin I is a tri-methoxy 4′-deoxyflavone. Its precursor, 2′,5,6,7,8-pentahydroxyflavone (27), is methylated on its C6, C7 and C8 hydroxyl positions. We first incubated yeast expressing SbPFOMT5 with compound 27, and detected both mono-methoxy 4′-deoxyflavone and di-methoxy 4′-deoxyflavone by MS. Although we had no standard for mono-methoxy-2′,5,6,7,8-hydroxy-4′-deoxyflavone, we were able to determine that the dimethylation product was 6,8-di-methoxy-4′-deoxyflavone (28) by comparison to its standard. We then co-expressed SbPFOMT5 plus SbFOMT6 (C1) and SbPFOMT5 plus SbFOMT5 (Combination 2/C2) in yeast cells. Fermentation of compound 27 with the C1 enzymes produced only the products detected with SbPFOMT5 alone, demonstrating that SbFOMT6 could not function in this system (Figure 6d, e). In contrast, yeast expressing Combination 2 OMTs (SbPFOMT5 and SbFOMT5) produced tanaxin I as shown by LC-MS (Figure 6d–f).

Discussion

Huang-Qin is used traditionally to treat liver and lung ailments, so we screened both the lung and liver cancer cells initially to check the cytotoxicity of the root methoxylated flavones (RMFs). Our results demonstrate that improved pro-apoptotic activities are

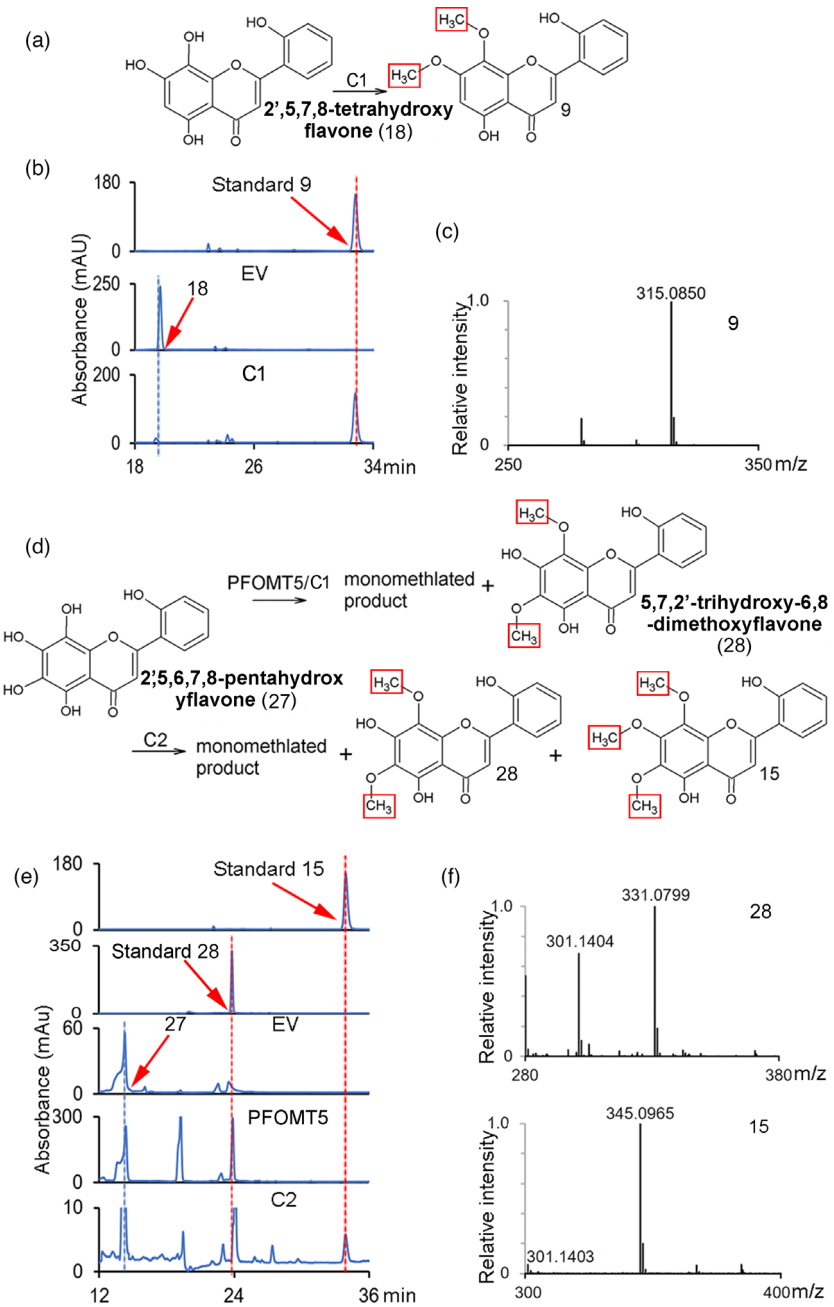


Figure 6 Production of skullcapflavone I and tenaxin I with SbPFOMT5 and SbFOMTs combinations. (a) The reaction catalysed by Combination 1 (SbPFOMT5 plus SbFOMT6) that converts 2′,5,7,8-tetrahydroxyflavone (Compound 18) to skullcapflavone I (Compound 9). (b) HPLC analysis of yeast expressing Combination 1 fermented with baicalein *in vivo*; top, standard skullcapflavone I; EV, yeast carrying empty vector; C1, yeast expressing C1 combination. (c) MS patterns of the product of C1, which is identical to standard skullcapflavone I. (d) The reactions catalysed by SbPFOMT5 and C2 (SbPFOMT5 plus SbFOMT5) using 2′,5,6,7,8-pentahydroxyflavone (27) as a substrate. (e) HPLC analysis of yeast expressing SbPFOMT5 and C2 fermented with 2′,5,6,7,8-pentahydroxyflavone *in vivo*; standards for compounds 15 and 28, standards for tanaxin I and 5,7,2′-trihydrxoxy-6,8 di-methoxyflavone; EV, yeast carrying an empty vector; C2, yeast expressing C2 enzyme combination. (f) MS profiles of the products, which were identical to the standards, tanaxin I (15) and 5,7,2′-trihydrxoxy-6,8 di-methoxyflavone (28).

conferred by more minor 4′-deoxyflavones found in Scutellaria roots including the RMFs, skullcapflavone I and tenaxin I (Figure 1f, g, j, k). Methylation has been proposed to improve the stability, absorption and bioavailability of phenylpropanoids (Ibrahim and Anzellotti, 2003), claims supported by extensive data comparing the efficacy of resveratrol with pterostilbene in the treatment of cancer (Kapetanovic *et al*., 2011; Rimando *et al*., 2002; Shin *et al*., 2020). To this, we can add that methylation improves the pro-apoptotic bioactivity of 4′-deoxyflavones to a degree that could be of clinical relevance.

Various RMFs with different numbers of methoxyl groups and diverse decoration positions have been reported in roots of *S. baicalensis*. To confirm which RMFs were present in the material we studied, roots of 2-month-old seedlings and 2-year grown plants were analysed by widely targeted metabolite analysis. All 43 RMFs detected have been reported in previous studies (Shang *et al*., 2010; Wang *et al*., 2018). The major mono-methoxy 4′-deoxyflavones found in roots of *S. baicalensis* were wogonin and orxylin A, the C8- and C6- methylated products of norwogonin and baicalein respectively. In addition, RMFs with methoxyl group numbers from two to five were detected (Figure 2c). Interestingly, di and trimethoxylated 4′-deoxyflavones were relatively abundant in roots of *S. baicalensis*.

We have previously reported two flavone biosynthetic pathways in *S. baicalensis*. One, producing 4′-deoxyflavones, which evolved recently in the genus *Scutellaria*, recruiting genes encoding specific isoforms of CLL-7, CHS-2, FNSII-2, F6H and F8H, which are highly expressed in roots, to produce chrysin and its derivatives, norwogonin and baicalein (Zhao *et al*., 2016, 2018). The other is the 'classic' flavone biosynthetic pathway that

uses naringenin to produce apigenin and its derivatives, and which operates in many Angiosperm plants. Based on the structures of the RMFs found in Scutellaria roots, we propose that the hydroxyl groups of 4′-deoxyflavones, norwogonin and baicalein, which are produced by the 4′-dexoxyflavone pathway and apigenin, from 'classic' 4′hydroxyflavone pathway, serve as the major precursors for RMF biosynthesis.

We previously identified and characterized five Type II OMTs (SbPFOMT1-SbPFOMT5) from *S. baicalensis*. PFOMT5 and PFOMT2 can methylate the 8-postion of norwogonin to make wognonin, as reported previously (Zhao *et al*., 2019), but here we showed that they can also transfer methyl groups to several other flavones with hydroxyl groups on adjacent carbon atoms such as 5,7,8,2′-tetraflavone, baicalein and luteolin, on their 8, 6 or 3′ positions, producing tenaxin II, oroxylin A and chrysoeriol respectively (Figure 3a–h). However, none of the SbPFOMTs showed activity on chrysin or apigenin, presumably because these substrates do not contain adjacent hydroxyl groups on their aromatic rings (Figure 3j,k).

A PFOMT Type II OMT from *Mesembryanthemum crystallumum* can methylate adjacent hydroxyl groups at the 3′ and the 6-positions of the flavonol, quercetagetin (Ibdah *et al*., 2003). Sweet basil ObPFOMT1 can methylate the 3′-OH of 4′ hydroxyflavones, luteolin and crisioliol, the 6-position of scutellarein, the 5-positon of 5,6-dihydroxy 4′ hydroxyflavone and the 8 position of 7,8- dihydroxy 4′ hydroxyflavones (Berim and Gang, 2013). The methylation steps producing oroxylin A and tenaxin II by SbPFOMT5 follow the same rule of methylating one of two adjacent hydroxyl groups on the aromatic rings of flavones as previously reported (Berim and Gang, 2013; Liu *et al*., 2020).

Based on genome and transcriptome searches, we identified 10 Type I OMT genes in the genome of *S. baicalensis* and successfully isolated nine of them. We have investigated the enzyme activities of the encoded proteins *in vivo* in yeast by supplying baicalein and norwogonin as substrates. These experiments showed that SbFOMT3 is a 7-OMT, specific for baicalein (Figure 4c, d), while SbFOMT6 is a 7-OMT that can use both baicalein and norwogonin as substrates (Figure 4c, d, g, h). These activities were confirmed by *in vitro* enzyme assays of proteins expressed in *E. coli* (Figures S8 and S9). SbFOMT5 is an unusual enzyme, as it produced four products in yeast when baicalein was used as substrate: 7-methoxybaicalein, oroxylin A, compound 23 (5-hydroxy-6,7-dimethoxy-4′-deoxyflavone) and compound 24 (6-hydroxy-7,5-dimethoxy-4′-deoxyflavone), with its major product being 7-methoxybaicalein (Figure 4c, e) suggesting that SbFOMT5 can methylate C5, C6, and C7-hydroxyl groups in baicalein. When we purified the SbFOMT5 enzyme, it produced only 7-methoxybaicalein and oroxylin A *in vitro* (Figure S8) possibly because the 2-hour reaction time was not long enough or the conditions *in vitro* were not optimal to produce dimethoxyflavones, as observed in yeast. When norwogonin was used as a substrate, SbFOMT5 produced only isowogonin both *in vivo* and *in vitro* (Figure 4g-i and Figure S9).

The activity of SbFOMT5 was distinct from the activity of any other FOMT previously described. FOMTs are usually active on hydroxyl groups at just one position on the flavone aromatic rings. For example mint MpOMT1, MpOMT2 and MpOMT3 transfer methyl groups to hydroxyl groups on the C7-, 8- and 3′-positions of a flavone molecule respectively (Willits *et al*., 2004). Sweet basil ObF8OMT-1 methylates only the 8-hydroxyl group, while ObFOMT1 and ObFOMT2 are specific for the 7-hydroxyl group. Exceptions to this rule of single site specificity are ObFOMT3, ObFOMT5 and ObFOMT6, since they can methylate both the C6-hydroxyl of the A ring and the C4′-hydroxyl of B ring (Berim *et al*., 2012). We also assayed activities on three 4′-deoxyflavones with single hydroxyl groups, 5-hydroxy-4′deoxyflavone, 6-hydroxy-4′-deoxyflavone and 7-hydroxy-4′-deoxyflavone. SbFOMT5 was able to convert these monohydroxy-4′-deoxyflavones to their methylated forms (Figure S10), confirming that SbFOMT5 can transfer methyl groups to C5, C6 or C7 hydroxyl positions on the 4′-deoxyflavone A-ring. The unique activity of SbFOMT5 for multiple acceptor sites is supported by its structure, which has a hydrophobic hole with more room for the B-ring of the flavones than other FOMTs (Figure 5c–h). Baicalein can be docked into the active site of SbFOMT5 in two orientations, facilitating methylation of its C7- or C5-OH groups. Because of the extended space for the B-ring in SbFOMT5, baicalein can move forward and around, offering additional opportunities for the methylation of the C6-OH of baicalein.

Based on our improved understanding of OMT specificity in *S. baicalensis*, we designed a method for bioproduction of two natural RMFs found in Scutellaria, skullcapflavone I and tenaxin I. We chose a combination of SbPFOMT5 and SbFOMTs for this purpose. Yeast expressing SbPFOMT5 and SbFOMT6 produced skullcapflavone I when supplied with the selected 4′-deoxyflavone precursor in the medium (Figure 6a–c) and tenaxin I when expressing SbPFOMT5 plus SbFOMT5 and supplied with its selected 4′-deoxyflavone precursor in the medium (Figure 6d–f).

Traditionally, decoctions of roots of *S. baicalensis* (Huang Qin) which are rich in 4′-deoxyflavones, are used for the treatment of liver and lung complaints, and have shown beneficial outcomes in combination therapies for COVID-19 infections. Over the last 15 years there has been increasing interest in the anticancer properties of such traditional preparations with baicalein and wogonin, in particular, showing cytotoxicity to a range of cancers including hepatic, breast, lung, cervical, ovarian and skin cancers, as well as leukaemia (EghbaliFeriz *et al*., 2018). However, there are problems with the adoption of plant extracts for chemotherapeutic purposes, for example the high concentrations required, the low bioavailability and poor solubility of baicalein. It has been reported that methylation of 4′-deoxyflavones improves their intestinal absorption and consequently their bioavailability (Lai *et al*., 2003; Li *et al*., 2012). Here we have shown that methylation can substantially improve the bioactivity of 4′-deoxyflavones against cancer cells as well as significantly reduce their IC50 values in inhibiting cancer cell growth specifically. Our work on defining the specificities of the OMTs in *S. baicalensis* therefore provides the tools to produce larger amounts of more bioactive anti-cancer 4′-deoxyflavones either by selection of natural variation in OMT activity in *S. baicalensis*, or by bioengineering of specific OMT activities in *S. baicalensis* root cultures or by using alternative production chassis such as yeast.

Methods

Enzyme assays

Saccharomyces cerevisiae BY4742 was used as the host strain for *in vivo* enzyme assays. Vector pAgl423 carrying SbFOMTs as well as a pAgl423 empty vector, were transformed into yeast using electrophoresis. Transformants were selected on a synthetic drop-out medium without histidine (SD-His). The recombinant strains were initially grown in SD-His liquid medium with glucose (20 g L^{-1}) at 28°C. Yeast cells were centrifuged and washed

with sterile water, then resuspended in the SD-His containing galactose (20 g L^{-1}) to induce expression of the target proteins. The co-factor SAM (*S*-Adenosyl methionine) and corresponding flavones were supplemented in the medium. After fermentation for 24 h, cells were harvested by centrifugation, freeze-dried and extracted with 70% MeOH for LC-MS analysis.

Plasmids with *OMTs* in pDest17 were used for the transformation of *E. coli* strain Rossetta (DE3). Cell cultures were set up in 5 mL LB medium with 100 mg L^{-1} ampicillin and incubated at 37 °C. Samples of these starter cultures were inoculated into 200 mL fresh LB medium and grown to an OD_{600} of 0.5. IPTG (isopropyl-1-thio-L-D-galactopyranoside) was added to a final concentration of 0.5 mM, and cultures were grown at 16 °C for 16 h to induce target proteins. Cells were harvested by centrifugation and resuspended in 5 mL buffer A (50 mM sodium phosphate, pH 7.8, containing 300 mM NaCl, 2 mM β-mercaptoethanol, 20% glycerol, 10 mM imidazole) and lysed by sonication. After centrifugation (12,000 ×*g*, 10 min, 4 °C), the supernatant was incubated with Ni-NTA agarose (1 mL) (Qiagen Gmbh, Hilden, Germany) with stirring (20 rmp) at 4 °C for 1 h for binding His6-tagged proteins. The agarose was washed with 30 mL of buffer B (50 mM sodium phosphate, pH 7.8, containing 300 mM NaCl, 20 mM imidazole, 15% glycerol 2 mM β-mercaptoethanol). Subsequently, the agarose was packed onto columns and bound proteins were eluted with buffer C (50 mM sodium phosphate, pH 7.8, containing 300 mM NaCl, 250 mM imidazole, 20% glycerol, 2 mM β-mercaptoethanol). Fractions of about 1 mL were collected and imidazole was removed by ultrafiltration. The concentrations of proteins were measured using Bradford's Solution (Bradford, 1976) and analysed further by SDS-polyacrylamide gel electrophoresis.

PFOMT enzymes were assayed in 100 mM Tris-HCl pH 7, 100 μM $MgCl_2$, 0.5 mM S-adenosyl methionine in 100 μL reactions. FOMT enzymes were assayed in 100 mM Tris-HCl pH 7.5, 10 μM $MgCl_2$, 0.5 mM SAM in 100 μL reactions. Reactions were incubated at 28 °C for 2 h. Flavone substrates were used at concentrations of 50 μM. The kinetic constants, Km and Vmax, were inferred using the nonlinear regression function integrated in the Origin 8 software. Each plot contained at least seven points, with flavone concentrations ranging from 0.08 to 10 μM. Products of the reactions were determined by comparing their retention time and MS/MS patterns (Figure S13) with the standards (Zhang, 2012).

Metabolite analyses

An Agilent 1260 Infinity II HPLC (high-performance liquid chromatography) system was used for metabolite analysis. Separation was carried out on a 100 × 2 mm 3 μ Luna C18 (2) column using 0.1% formic acid in water (A) versus 1:1 AcetoNitrile:MeOH + 0.1% formic acid (B) and run at 260 μl min^{-1} with the following gradient: 0–3 min, 20% B; 20 min, 50% B; 20–30 min, 50% B; 36 min, 30% B; 37 min, 20% B and 37–43 min 20% B. The column was maintained at 35°C and absorption was detected at 280 nm with a diode array detector (Agilent, USA). Metabolites were measured by comparing the area of the individual peaks to standard curves obtained from standard compounds.

Statistical analysis

All experiments were repeated using at least three biological replicates. Data are presented as means ± SEM unless otherwise stated. Student's *t*-test in Excel was used for the statistical analyses. To compare group differences, paired or unpaired, two-tailed Student's *t*-tests were used. *P* values < 0.05 were recognized as significant.

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Conflicts of interest

The authors declare no competing interest.

Authors' contributions

Q.Z. conceptualized and designed the project with some suggestions from CM. M.-Y.C. isolated the genes and characterized the enzymes. A.-R. L., J.L., M.-Y.C. and W.-Q. Q performed the apoptosis assays. J.-X. L modelled and analysed the OMTs. Y. K. assisted with the LC-MS analysis. All the authors analysed and interpreted the data. Q.Z. and C.M. wrote the paper and all authors corrected and approved the final version of the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Method S1. Extended Materials and Methods.
Figure S1. Scutellaria flavones induced early apoptosis in liver and lung cancer cells.
Figure S2. Effects of Scutellaria flavones on human cells.
Figure S4. HPLC analysis of *in vitro* enzyme assays with substrate chrysin (Chr); Ct, control assay; PFOMT1-PFOMT5, enzyme assays with corresponding proteins.
Figure S5. HPLC analysis of *in vitro* enzyme assays with substrate apigenin (Api); Ct, control assay; PFOMT1-PFOMT5, enzyme assays with corresponding proteins.
Figure S6. HPLC analysis of yeast expressing yeast SbFOMTs fermented with baicalein *in vivo*. EV, yeast carrying empty vector; FOMT1-FOMT9, yeast expressing corresponding proteins.
Figure S7. Purification of SbFOMTs. Proteins were analysed using SDS PAGE, stained with Coomassie blue. From left to right: protein markers, SbFOMT6, SbFOMT5 and SbFOMT3.

Figure S8. HPLC analysis of *in vitro* enzyme assays of SbFOMTs with baicalein. 22, 7-O-methylbaicalein standard; 3, oroxylin A standard; Ct, control assay; FOMT3, PFOMT5 and PFOMT6, enzyme assays with corresponding proteins.
Figure S9. HPLC analysis of *in vitro* enzyme assays of SbFOMT5 and SbFOMT6 with norwogonin. 26, isowogonin standard; Ct, control assay; PFOMT5 and PFOMT6, enzyme assays with corresponding proteins.
Figure S10. Enzyme assays of SbFOMT5 with momo-hydroxyflavnes.
Figure S11. Protein modelling of SbFOMTs, based on the 7-O-methyltransferase from alfalfa as a template. SAM and the flavone substrates are shown in yellow and green respectively.
Figure S12. *In vitro* enzyme assays of SbFOMT5 mutants.
Figure S13. MS/MS patterns of the products from enzyme assays.
Table S1. PMFs found from Scutellaira roots and their MS signal strength, baicalein and baicalin are offered as references.
Table S2. Genomic ID of the SbFOMTs isolated by this study.
Table S3. Primers used in this study, underlined sequences represent Gateway® primers.