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Bio-fermentation of modified flavonoids: an example of in vivo diversification of secondary metabolites

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This work is dedicated to the late Professor Jeffrey B. Harborne (The University of Reading, UK) in honor of his comprehensive research on flavonoids

Abstract

A bio-fermentation technique was used for the in vivo diversification of flavonoid structures based on expression in *Escherichia coli* of six *O*-methyltransferases (*OMT*s) from *Mentha x piperita* and one *O*-glucosyltransferase (*GT*) each from *Arabidopsis thaliana* and *Allium cepa*. Enzymes were shown to be regio-specific in in vitro experiments and modified a broad range of flavonoid substrates at various positions. Using the flavonol quercetin as a model substrate, we show that the product spectrum produced with the in vivo approach is identical to that found in vitro. Additionally, using mixed cultures of *E. coli* expressing different classes of modifying genes (*OMT*s and *GT*s), the production of polymethylated flavonoid glucosides was observed. This report demonstrates the potential to increase the structural diversity of plant secondary metabolites using a multi-enzyme, bio-fermentation approach.

Keywords: *Mentha x piperita*; Labiatae; *Arabidopsis thaliana*; Cruciferae; Plant secondary metabolites; Natural products; Drug discovery; Flavonoids; *O*-Methyltransferases; *O*-Glucosyltransferases

1. Introduction

Over 100 years after the discovery of the natural product salicylic acid and its chemical modification to the drug aspirin®, there is still a strong interest in finding efficient ways to increase the number of potential pharmaceuticals derived from natural sources. Half of the best selling pharmaceuticals are based on natural product chemistry (Cordell, 2000; O'Neil and Lewis, 1993), demonstrating the unique resource that plant secondary metabolites provide for drug discovery. A variety of synthetic chemistry approaches have been developed in the last century to enhance activity and to reduce undesirable side effects of these compounds (Sneader, 1985).

The enormous structural diversity of plant secondary metabolites is often obtained by derivatization of specific lead structures through simple reactions such as hydroxylation, glycosylation, methylation, acylation, prenylation, sulfation and many others (Buckingham, 2000). Hundreds of secondary metabolite modifying enzymes (e.g. oxidases, acyltransferases, methyltransferases, glycosyltransferases, sulfotransferases, etc.) have been cloned and characterized (Ibrahim and Varin, 1993; Facchini, 1999), providing a large and unique toolbox for the specific design of modified natural products. For industrial uses, enzymes that act on a wide range of structures are of great interest because a relatively small number of these enzymes could create a multitude of modified compounds. However, very few of these natural product-modifying enzymes have been extensively studied for substrate specificity (Frick and Kutchan, 1999; Christensen et al., 1998) or are known to be active in bio-fermentation assays using recombinant *Escherichia coli* cultures expressing these enzymes (Kutchan, 1989; Facchini and De Luca, 1995; Arend et al., 2001; Albrecht et al., 2000).

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To investigate the potential uses of modifying enzymes in the synthesis of new structures derived from plant natural products, we studied substrate specificity and in vivo activity of two different but well known classes of plant secondary metabolite-modifying enzymes: flavonoid *O*-glucosyltransferases (GTs) and flavonoid *O*-methyltransferases (OMTs). Flavonoid OMTs catalyze the *S*-adenosyl-L-methionine-dependent methyl group transfer to OH-groups of a flavonoid acceptor molecule, whereas flavonoid GTs catalyze the UDP-glucose-dependent glucosylation of OH-groups on a flavonoid acceptor molecule.

Flavonoids represent a very interesting group of plant secondary metabolites that are ubiquitous in vascular plants. They are known to have a wide range of beneficial health effects (antioxidant, antitumor, anti-inflammatory, etc.) and specific pharmacological effects (antituberculosis, antimalarial, antimicrobial, antiviral, etc) as shown in a multitude of in vitro assays (Harborne and Williams, 2000; Di Carlo et al., 1999) and epidemiological studies (Hollmann and Arts, 2000). Flavonoids such as baicalein from *Scutellaria baicalensis georgi*, and structures related to flavonoids such as the silimarins from *Silybum marianum* (milk thistle), are widely used in traditional medicine and have well-established products on the market (e.g. Legalon®SIL, Madaus/Germany). Based on their backbone structures, flavonoids are grouped into major classes (Fig. 1), which include flavonols (**1–4**, **12–15**, **28–32**, **34–37**), flavones (**5–11**), flavanones (**16, 18–20**), flavanonols (**17**), isoflavones (**21–24, 33**), chalcones (**25**) and anthocyanins. From these main classes an incredible variety of structures are derived by modification of the backbone, with approximately 6500 different naturally occurring flavonoids identified so far (Harborne and Baxter, 1999).

GTs are involved in many different pathways including flavonoid biosynthesis, where they often catalyze the final step of biogenesis. Plant-derived glucosides are difficult to synthesize, so an alternative approach for their synthesis would be valuable (Arend et al., 2001). Methylation of flavonoids has an impact on solubility, intracellular compartmentation and anti-microbial activity. Because chemical methylation is in most cases easy and inexpensive, the advantage of using enzymes is in their regiospecificity and methylation efficiency, both of which are known to vary for different hydroxyl (OH)-groups on the flavonoid backbone structure (Brunet and Ibrahim, 1980). Previous studies have described GTs and OMTs with both high and low substrate- and regiospecificity (Ibrahim and Varin, 1993; Vogt and Jones, 2000; Frick et al., 2001). Some are even active on more than one class of secondary metabolites (Frick and Kutchan, 1999; Ford et al., 1998; Luque et al., 2002).

For our studies six different *OMT* genes were isolated from *Mentha x piperita* (peppermint) and one *GT* was isolated from *Arabidopsis thaliana*. These genes were expressed in *E. coli*, the enzymes purified, and the substrate specificity of each investigated. The enzymes showed very low substrate specificity and were active on a wide range of flavonoids and flavonoid-precursors. When flavonoid substrates were fed to *E. coli* cultures expressing the specific enzymes, the products formed were the same as those formed using purified enzymes. When mixed recombinant *E. coli* cultures were used, variably substituted compounds were produced. Using quercetin **1** as a model substrate, we describe here the in vivo combinatorial generation of quercetin derivatives using *E. coli* cultures expressing flavonoid-modifying enzymes. This technique holds promise for industrial-scale generation of additional classes of modified secondary metabolites.

2. Results and discussion

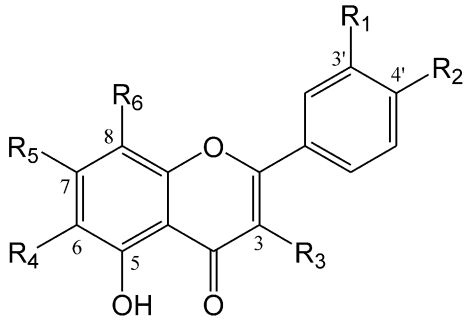
2.1. Cloning and sequence analysis of peppermint OMTs

Peppermint produces a large number of poly-*O*-methylated flavonoid aglycones, which are stored in peltate glandular trichomes—specialized anatomical structures located on the leaf surfaces (Voirin et al., 1993). The 1300 expressed sequence tags (ESTs) from a glandular trichome cDNA library (Lange et al., 2000) yielded 60 sequences that were homologous to *OMT*s. Using the information from these ESTs, 33 full-length peppermint *OMT*s (*MpOMT*s) were cloned and sequenced. Sequence homology comparisons identified five major classes of clones (labeled *MpOMT*1 through *MpOMT*5) that were between 26 and 62% identical to each other at the nucleotide level, but within each class the members were over 94% identical to each other at the nucleotide level. Since the variation within each class was likely due to allelic variation, two *MpOMT1* clones and one clone from each of the other *MpOMT* classes were chosen for further analysis.

2.2. Amino acid sequence and phylogenetic analysis

The deduced amino acid sequences of *Mp*OMT1 (38 kD), *Mp*OMT2 (40.8 kD), *Mp*OMT3 (40.3 kD), *Mp*OMT4 (37.8 kD) and *Mp*OMT5 (40.7 kD) were remarkably different from each other, since they displayed 31% (*Mp*OMT2), 28% (*Mp*OMT3), 54% (*Mp*OMT4) and 34% (*Mp*OMT5) sequence identity, respectively, compared to *Mp*OMT1A (*Mp*OMT1B was 94% identical to *Mp*OMT1A). The putative molecular weights obtained were representative of OMTs that do not require the addition of Mg^{2+} ions for enzyme activity, and the deduced amino acid sequences displayed five characteristic motifs that have been associated with plant OMTs (Ibrahim, 1997; Ibrahim et al.,

Flavonols, Flavones and Flavonol Glycosides



1 Quercetin: R_1=OH, R_2=OH, R_3=OH, R_4=H, R_5=OH, R_6=H

2 Kaempferol: R_1=H, R_2=OH, R_3=OH, R_4=H, R_5=OH, R_6=H

3 Rhamnetin: R_1=OH, R_2=OH, R_3=OH, R_4=H, R_5=OCH_3, R_6=H

4 Isorhamnetin: R_1=OCH_3, R_2=OH, R_3=OH, R_4=H, R_5=OH, R_6=H

5 Luteolin: R_1=OH, R_2=OH, R_3=H, R_4=H, R_5=OH, R_6=H

6 Apigenin: R_1=H, R_2=OH, R_3=H, R_4=H, R_5=OH, R_6=H

7 6-hydroxy-apigenin: R_1=H, R_2=OH, R_3=H, R_4=OH, R_5=OH, R_6=H

8 7,8,3′4′-tetrahydroxy-flavone: R_1=OH, R_2=OH, R_3=H, R_4=H, R_5=OH, R_6=OH, (H in C-5)

9 7,8,4′-trihydroxy-flavone: R_1=H, R_2=OH, R_3=H, R_4=H, R_5=OH, R_6=OH, (H in C-5)

10 Eupatorin: R_1=OH, R_2=OCH_3, R_3=H, R_4=OCH_3, R_5=OCH_3, R_6=H

11 8-hydroxy-flavone 7-methyl ether: R_1=H, R_2=H, R_3=H, R_4=H, R_5=OCH_3, R_6=OH, (H in C-5)

12 Quercitrin: R_1=OH, R_2=OH, R_3=rhamnosyl, R_4=H, R_5=OH, R_6=H

13 Isoquercitrin: R_1=OH, R_2=OH, R_3=glucosyl, R_4=H, R_5=OH, R_6=H

14 Rutin: R_1=OH, R_2=OH, R_3=rutinosyl, R_4=H, R_5=OH, R_6=H

15 3-hydroxy-flavone 7,2′,4′-trimethyl ether: R_1=H, R_2=OCH_3, R_3=OH, R_4=H, R_5=OCH_3, R_6=H, (OCH_3 in C-2′)

28 Quercetin 4′-methyl ether: R_1=OH, R_2=OCH_3, R_3=OH, R_4=H, R_5=OH, R_6=H

29 Quercetin 3′,4′-dimethyl ether: R_1=OCH_3, R_2=OCH_3, R_3=OH, R_4=H, R_5=OH, R_6=H

30 Quercetin 7,3′-dimethyl ether: R_1=OCH_3, R_2=OH, R_3=OH, R_4=H, R_5=OCH_3, R_6=H

31 Quercetin 7,4′-dimethyl ether: R_1=OH, R_2=OCH_3, R_3=OH, R_4=H, R_5=OCH_3, R_6=H

32 Quercetin 7,3′,4′-trimethyl ether: R_1=OCH_3, R_2=OCH_3, R_3=OH, R_4=H, R_5=OCH_3, R_6=H

34 Quercetin 7-glucoside: R_1=OH, R_2=OH, R_3=OH, R_4=H, R_5=glucosyl, R_6=H

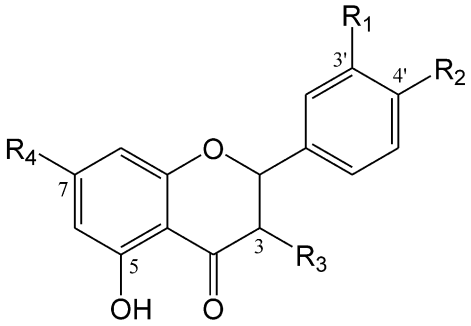
35 Quercetin 3,7-diglucoside: R_1=OH, R_2=OH, R_3=glucosyl, R_4=H, R_5=glucosyl, R_6=H

36 Quercetin 3′-methyl ether 7-glucoside: R_1=OCH_3, R_2=OH, R_3=OH, R_4=H, R_5=glucosyl, R_6=H

37 Quercetin 4′-methyl ether 7-glucoside: R_1=OH, R_2=OCH_3, R_3=OH, R_4=H, R_5=glucosyl, R_6=H

38 Quercetin 3′-methyl ether 3-glucoside: R_1=OCH_3, R_2=OH, R_3=glucosyl, R_4=H, R_5=OH, R_6=H

Flavanones, Flavanonols, and Flavanone Glycosides



16 Naringenin: R_1=H, R_2=OH, R_3=H, R_4=OH

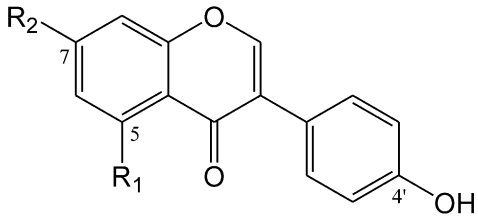
17 Taxifolin: R_1=OH, R_2=OH, R_3=OH, R_4=OH

18 Hesperetin: R_1=OH, R_2=OCH_3, R_3=H, R_4=OH

19 Naringin: R_1=H, R_2=OH, R_3=H, R_4=neohesperidosyl

20 Naringenin 7-glucoside: R_1=H, R_2=OH, R_3=H, R_4=glucosyl

Isoflavones and Isoflavone Glycosides



21 Genistein: R_1=H, R_2=OH

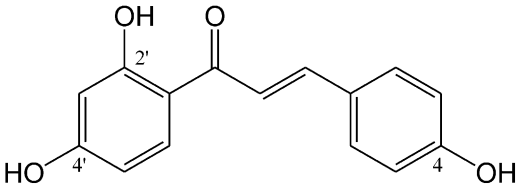
22 Daidzein: R_1=OH, R_2=OH

23 Genistin: R_1=OH, R_2=glucosyl

24 Daidzin: R_1=OH, R_2=glucosyl

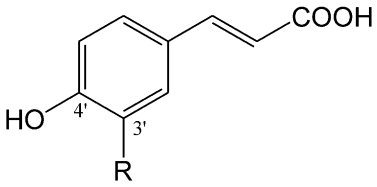
33 Genistein 7-methyl ether: R_1=H, R_2=OCH_3

Chalcones



25 Isoliquiritigenin

Phenylcarbonic Acids



26 Caffeic acid: R=OH

27 Ferulic acid: R=OCH_3

Fig. 1. Flavonoid and flavonoid-related structures.

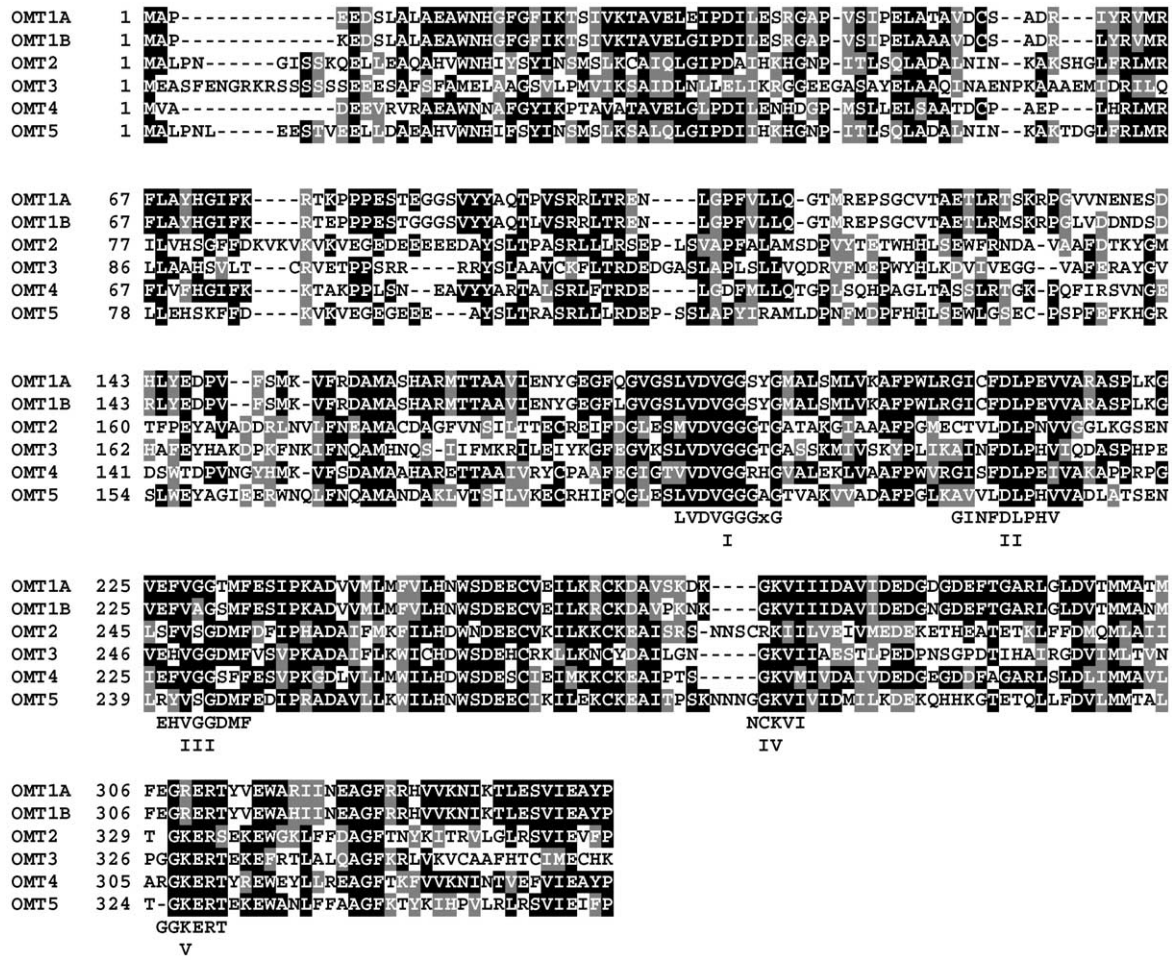


Fig. 2. Multiple sequence alignment of *Mp*OMTs. The six representative *Mp*OMT amino acid sequences were compared using ClustalW, and the boxshading was performed with BOXSHADE 3.21. Black background represents identities, gray background represents similarities, and dashes represent gaps. The consensus sequences of five plant OMT motifs, labeled I through V, are shown below the sequence alignment.

1998) (Fig. 2). X-Ray diffraction studies have demonstrated that motifs I and IV are involved in *S*-adenosyl-L-methionine and metal binding (Vidgren et al., 1994). These two motifs are also the most highly conserved sequences among all plant OMTs.

Phylogenetic analysis showed that *Mp*OMT3 was distinct from the other four peppermint OMTs (Fig. 3), by placing it with phenol ring-modifying genes involved with *O*-methylation of phenolics like catechol and caffeic acid **26**. In contrast, the other OMTs were closely associated with enzymes modifying the A/C flavonoid ring system and to OMTs involved in isoquinoline alkaloid biosynthesis.

2.3. Substrate specificity of the peppermint OMTs

For characterization of the protein products of the *MpOMT* genes, the coding sequences of the six clones were expressed in *E. coli* and the encoded enzymes purified and assayed with in vitro OMT assays. The identities of the reaction products were verified by HPLC retention times, UV-spectra and liquid chromatography-mass spectrometry (LC-MS).

The regiospecificity of the *Mp*OMTs was investigated with various hydroxylated flavonoid (**1**–**14**, **16**–**18**, **21**) and flavonoid-related substrates **26**, **27** (Table 1). Five active *Mp*OMTs were identified which showed very low substrate specificity but high regiospecificity. Regiospecificity was deduced by the positions of the available OH-groups on the substrates utilized by each of the enzymes as follows: *Mp*OMT1A and *Mp*OMT1B, both C-7; *Mp*OMT2, C-8; *Mp*OMT3, C-3′ and *Mp*OMT4, C-4′. These enzymes were able to use either nonmethylated (**1**, **2**, **5**–**9, 16, 17, 21**) or monomethylated (**3**, **4**, **11, 18**) flavonoid substrates, suggesting that the sequence of methylation is not critical. Each gene family appears to methylate the flavonoid backbone at a single position known to be methylated in peppermint (Voirin et al., 1999), strongly suggesting involvement in peppermint flavonoid metabolism.

None of the enzymes showed activity with the investigated flavonoid glycosides (**12**–**14**), suggesting that

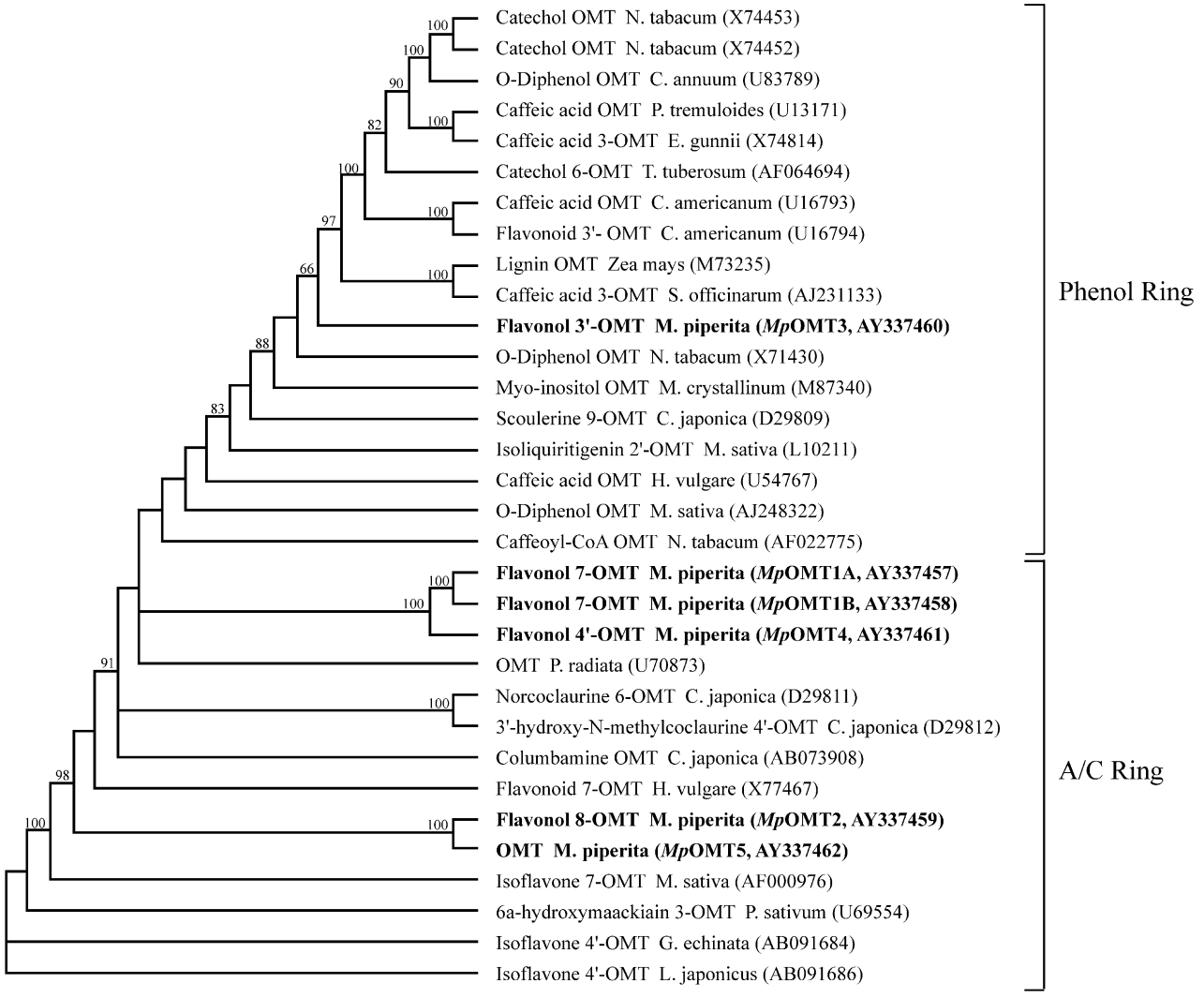


Fig. 3. Phylogenetic tree of plant *O*-methyltransferase amino acid sequences. In addition to sequences of the five classes of peppermint OMT discussed in this paper (in bold), 26 plant OMT sequences were obtained from GenBank™. The phylogenetic tree was constructed using the program PAUP through the Accelrys GCG® Wisconsin Package® programs PAUPSearch and PAUPDisplay. The numbers at the branch points are bootstrap values that indicate the percentage values for obtaining this particular branching in 1000 repetitions of the analysis; only values above 65% are shown.

they are specific for flavonoid aglycones, which is consistent with most known flavonoid OMTs (Ibrahim and Varin, 1993). There was also no activity observed with the investigated phenylcarbonic acids **26**, **27** (Table 1). One of the enzymes identified as a 7-OMT (*Mp*OMT1A) was even active with the isoflavone substrate genistein **21**, a flavonoid almost exclusively restricted to legumes such as soybean. In contrast, an isoflavone OMT isolated from alfalfa showed specificity only for isoflavones and isoflavone-related structures (He et al., 1998).

*Mp*OMT1B, from the same class as *Mp*OMT1A, showed the same substrate spectrum as *Mp*OMT1A (with the exception of not using genistein **21** as a substrate, data not shown), suggesting that the observed regiospecificity is related to the different OMT classes found in peppermint.

*Mp*OMT5 showed no activity with any of the offered substrates. It is possible that *Mp*OMT5 is not an OMT involved in flavonoid biosynthesis or is very substrate specific and the correct substrate was not offered.

2.4. Cloning and sequence analysis of an Arabidopsis 7-O-glucosyltransferase

Arabidopsis has been shown to produce mainly 3- and 7-*O*-glycosides of quercetin **1** and kaempferol **2** (Graham, 1998; Veit and Pauli, 1999), but flavonoid GTs have not previously been characterized from *Arabidopsis*. We have cloned an *Arabidopsis GT* (*UGT73B2*, *Arabidopsis* gene *At4g34135*) with homology to a known 7-*O*-glucosyltransferase from *Scutellaria baicalensis* (Fig. 4) (GenBank™ Accession No. AB031274; Hirotani et al., 2000). The amino acid sequence of

Table 1
Substrate specificity of different flavonoid *O*-methyltransferases isolated from peppermint (*Mentha×piperita*)[a]

Substrates	OH-groups available	*OMT1A*	*OMT2*	*OMT3*	*OMT4*
Flavonols and flavonol glycosides					
1 Quercetin	3,5,7,3′,4′	58	–	63	5
2 Kaempferol	3,5,7,4′	58	–	–	28
3 Rhamnetin	3,5,3′,4′	–	–	35	3
4 Isorhamnetin	3,5,7,4′	36	–	–	100[e]
12 Quercitrin	5,7,3′,4′	–	–	–	–
13 Isoquercitrin	5,7,3′,4′	–	–	–	–
14 Rutin	5,7,3′,4′	–	–	–	–
Flavones					
5 Luteolin	5,7,3′,4′	100[b]	–	100[d]	7
6 Apigenin	5,7,4′	79	–	–	38
7 6-OH-Apigenin	5,6,7,4′	29	–	–	22
8 7,8,3′,4′-OH-flavone	7,8,3′,4′	10	9	30	8
9 7,8,4′-OH-flavone	7,8,4′	17	76	–	15
10 Eupatorin	5,3′	–	–	4	–
11 8-OH-7-MeO-flavone	8	–	100[c]	–	–
Flavanones and flavanonols					
16 Naringenin	5,7,4′	15	–	–	18
17 Taxifolin	3,5,7,3′,4′	3	–	24	17
18 Hesperetin	5,7,3′	1	–	7	–
Isoflavones					
21 Genistein	5,7,4′	9	–	–	–
Phenylcarbonic acids					
26 Caffeic acid	3′,4′	–	–	–	–
27 Ferulic acid	4′	–	–	–	–

[a] Products were identified by HPLC retention times, UV spectra, LC-MS and, for the products of quercetin, by ^{1}H NMR. Activities for each enzyme are presented as percent activity. Dash (–) means that no product was detected.
[b] 100% activity OMT1A = 22.8 pkat/mg protein.
[c] 100% activity OMT2 = 86.2 pkat/mg protein.
[d] 100% activity OMT3 = 7.9 pkat/mg protein.
[e] 100% activity OMT4 = 6.0 pkat/mg protein.

UGT73B2 contains a region homologous to the plant secondary product GT consensus sequence (PSPG box), which is thought to be involved in nucleotide-diphosphate-sugar binding (Vogt and Jones, 2000). UGT73B2 also displays 100% identity to two smaller, highly conserved regions of the PSPG sequence (Fig. 4, underlined).

2.5. Substrate specificity of purified UGT73B2

For protein characterization, the coding region of the *UGT73B2* gene was expressed in *E. coli* and substrate specificity investigated with different flavonoid substrates

```
MGSDHHHRKLHVMFFPFMAYGHMIPTLDMAKLFSSRGAKSTILTTSLNSKILQKPIDTFK  60
NLNPGLEIDIQIFNFPCVELGLPEGCENVDFFTSNNNDDKNEMIVKFFFSTRFFKDQLEK 120
LLGTTRPDCLIADMFFPWATEAAGKFNVPRLVFHGTGYFSLCAGYCIGVHKPQKRVASSS 180
EPFVIPELPGNIVITEEQIIDGDGESDMGKFMTEVRESEVKSSGVVLNSFYELEHDYADF 240
YKSCVQKRAWHIGPLSVYNRGFEEKAERGKKANIDEAECLKWLDSKKPNSVIYVSFGSVA 300
FFKNEQLFEIAAGLEASGTSFIWVVRKTKDDREEWLPEGFEERVKGKGMIIRGWAPQVLI 360
                                                  WAPQVLA

LDHQATGGFVTHCGWNSLLEGVAAGLPMVTWPVGAEQFYNEKLVTQVLRTGVSVGASKHM 420
  HPAVGCFVTHCGWNSTLESISAGVPMVAWPFFADQ

KVMMGDFISREKVDKAVREVLAGEAAEERRRRAKKLAAMAKAAVEEGGSSFNDLNSFMEE 480
FSS                                                             483
```

Fig. 4. Amino acid sequence of UGT73B2. The PSPG consensus sequence is shown in bold. Two highly conserved regions of this consensus, which are completely conserved in UGT73B2, are underlined.

(**1, 2**, **4**, **5**, **6**, **12**–**25**). Products were identified by HPLC retention times, UV-spectra and LC-MS. The summarized results in Table 2 show that UGT73B2 uses a wide range of flavonoid substrates including flavonols (**1**, **2**, **4**, **15**), flavones (**5**, **6**), flavanones (**16**, **18**), flavanonols (**17**), isoflavones (**21**, **22**), flavonol glycosides (**12**–**14**), and chalcones (**25**). Although the predominant activity was specific for the C-7 position (Table 2), a 20-fold lower activity could also be identified for the C-3 position. The isoflavone aglycones genistein **21** and daidzein **22** were also accepted as substrates with formation of a single product from each. Because isoflavones are almost exclusively restricted to legumes, and are thus considered to be unnatural substrates for *Arabidopsis* GTs, this result emphasizes the low substrate specificity of the UGT73B2 enzyme.

2.6. In vivo combinatorial biochemistry

E. coli cultures transformed with flavonoid-modifying genes were induced with isopropyl-1-thio-β-D-galactoside (IPTG) and the culture medium was supplemented with quercetin **1** as a model substrate for the enzymes. Non-recombinant *E. coli* lines used as controls exhibited

Table 2
Substrate specificity of a flavonoid *O*-glucosyltransferase isolated from *Arabidopsis thaliana*[a]

Substrates	OH-groups available	Activity	
		1° Prod	2° Prod
Flavonols and flavonol Glycosides			
1 Quercetin	3,5,7,3′,4′	67	3
2 Kaempferol	3,5,7,4′	100[b]	6
4 Isorhamnetin	3,5,7,4′	94	6
12 Quercitrin	5,7,3′,4′	43	–
13 Isoquercitrin	5,7,3′,4′	28	–
14 Rutin	5,7,3′,4′	11	–
15 3-OH 7,2′,4′-MeO-Flavone	3	3	–
Flavones			
5 Luteolin	5,7,3′,4′	44	–
6 Apigenin	5,7,4′	44	–
Flavanones, flavanonols and flavanone glycosides			
16 Naringenin	5,7,4′	56	–
17 Taxifolin	3,5,7,3′,4′	6	–
18 Hesperetin	5,7,3′	12	–
19 Naringin	5,4′	–	–
20 Naringenin 7-glucoside	5,4′	–	–
Isoflavones and isoflavone glycosides			
21 Genistein	5,7,4′	56	–
22 Daidzein	7,4′	50	–
23 Genistin	5,4′	–	–
24 Daidzin	4′	–	–
Chalcones			
25 Isoliquiritigenin		22	–

[a] Products were identified by HPLC retention times, UV spectra, LC-MS and, for genistin, by ^{1}H NMR. Enzyme activities are presented as percent activity. Dash (–) means that no product was detected.
[b] 100% activity UGT73B2 = 4.9 pkat/mg protein.

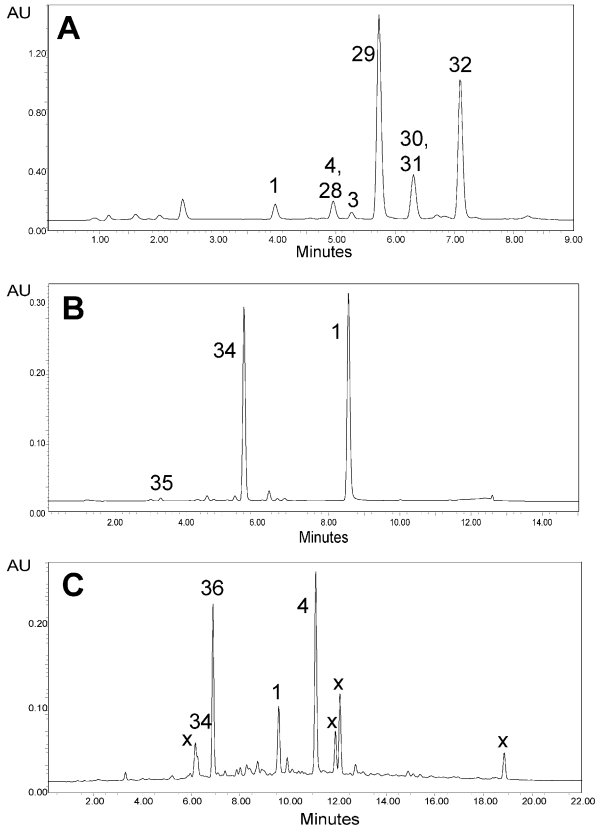


Fig. 5. HPLC chromatograms recorded at 354 nm of modified flavonoids produced using an in vivo culture technique with recombinant *E. coli* expressing flavonoid modifying enzymes and quercetin **1** (3,5,7,3′,4′-pentahydroxyflavone) as substrate. (A) Production of quercetin 3′-methyl ether (isorhamnetin **4**), quercetin 4′-methyl ether **28** (**4** and **28** eluting together in one peak), quercetin 7-methyl ether (rhamnetin **3**), quercetin 3′,4′-dimethyl ether **29**, quercetin 7,3′,-dimethyl ether **30**, quercetin 7,4′-dimethyl ether **31** (**30** and **31** eluting together in one peak), and quercetin 7,3′,4′-trimethyl ether **32** by *Mp*OMT1A, *Mp*OMT3 and *Mp*OMT4. (B) Production of quercetin 3,7-*O*-diglucoside **35** and quercetin 7-*O*-glucoside **34** by UGT73B2. (C) Production of quercetin 7-*O*-glucoside **34**, quercetin 3-methyl ether 7-*O*-glucoside **36** and quercetin 3′-methyl ether (isorhamnetin **4**) using a combination of *Mp*OMT3 and UGT73B2. Peaks labeled as x were not identified as flavonoids.

a linear uptake of quercetin **1** up to concentrations of 1 mM in the medium without any backbone modification.

Three methyltransferases isolated from peppermint were used for the in vivo studies: *Mp*OMT1A (7-OMT), *Mp*OMT3 (3′-OMT) and *Mp*OMT4 (4′-OMT). When cultures expressing each of the individual *MpOMT* genes were supplemented with exogenous quercetin **1**, the expected monomethylated products were generated. More than 95% of the methylated products were found in the cell pellet. For product identification 5–10 mg of the pure compounds were analyzed by ^{1}H NMR (data not shown). Comparing the proton spectra with authentic standards, the product from *Mp*OMT1A could be identified as quercetin 7-methyl ether (rhamnetin **3**), the product from *Mp*OMT3 as quercetin 3′-methyl ether (isorhamnetin **4**) and from *Mp*OMT4, quercetin 4′-methyl ether **28**, which is completely consistent with the in vitro studies (Table 1). In mixed-culture experiments using any two *Mp*OMTs, the two expected monomethylated products and the single dimethylated product were produced (data not shown). Mixed-cultures with a combination of all three *Mp*OMTs resulted in the production of three mono- (**3**, **4**, **28**, Fig. 5A), three di- (**29**–**31**, Fig. 5A), and one trimethylated (**32**, Fig. 5A) products. LC-MS analysis clearly showed the expected molecular ions for the corresponding products (data not shown). In addition to quercetin **1**, genistein **21** was also used as a substrate for *Mp*OMT1A in the in vivo assay and was converted into genistein 7-methyl ether **33** (data not shown).

In in vivo glucosyltransferase assays using quercetin **1** as a substrate, UGT73B2 produced a major product which could be identified by HPLC retention times, UV spectrum and LC-MS as quercetin 7-*O*-glucoside **34** (Fig. 5B), along with a minor product, quercetin 3,7-*O*-diglucoside **35** (Fig. 5B). Experiments with UGT73B2 using the isoflavone genistein **21** as the substrate resulted

in a product identified as genistin **23** (genistein 7-*O*-glucoside) by ^{1}H NMR. Over 90% of the glucosylated products accumulated in the supernatant.

Up to 50% of the flavonoid substrates were found to be degraded by *E. coli* cultures (either non-recombinant or recombinant) after a 15 h incubation, and 25–40% was converted to the methylated or glycosylated products by the recombinant *E. coli* cultures. In 500 ml cultures supplemented with 150 mg quercetin **1**, it was possible to produce up to 61 mg of methylated product and up to 78 mg of glucosylated product. For all substrates utilized in the in vivo culturing technique, the same products were produced as those observed in in vitro assays, confirming the feasibility of this approach for bulk production of substituted flavonoids.

In combinatorial assays using both *Mp*OMT3 and UGT73B2, a methylated flavonoid glucoside (quercetin 3-methyl ether 7-*O*-glucoside **36**, Fig. 5C) could be identified in addition to the products derived from the single activities of the enzymes (isorhamnetin **4** and quercetin 7-*O*-glucoside **34**, Fig. 5C). Similar results were obtained for *Mp*OMT4 in combination with UGT73B2, with the production of quercetin 4′-methyl ether 7-*O*-glucoside **37**. Recombinant *E. coli* strains expressing UGT73G1, an onion flavonoid GT isolated and partially characterized in our workgroup (Kramer et al., 2003), was also found to be active in the in vivo culture technique. In combination with *Mp*OMT3, the formation of quercetin 3′-methyl ether 3-*O*-glucoside **38** was observed besides the products derived from the single activity of both enzymes (e.g. isorhamnetin **4** and isoquercitrin **13**). These examples demonstrate the feasibility of combinatorial generation of flavonoid structural diversity by fermentation. In vivo, simultaneous expression of different enzyme classes yielded the predicted modified flavonoids based on their in vitro activities.

3. Conclusions

The in vivo culture technique presented here demonstrates the potential for using enzymes for generating plant natural product diversity. Two different classes of natural product-modifying enzymes (GTs and OMTs) showing low flavonoid substrate specificity but high regiospecificity could be used to manufacture a broad range of precisely substituted flavonoids by administering the model flavonoid quercetin **1** directly to *E. coli* lines expressing flavonoid *OMT* and *GT* genes. Both methylated and glucosylated flavonoids were produced in individual transformed cultures, whereas mixed cultures expressing specific *OMT* and/or *GT* genes were able to produce polymethylated flavonoids and/or their related glucosides. This is the first report describing a multiple enzyme, in vivo approach for modification of flavonoids. Using different substrates that are found to be active with the recombinant enzymes (Tables 1 and 2) novel flavonoids could be produced very easily.

The applicability of this method for modification of other secondary metabolites will need to be determined empirically, and conditions for optimization of substrate uptake, substrate effects on bacterial cultures, host selection, codon usage, enzyme solubility, and final location of modified products (e.g. intra- or extracellular) may differ for various combinations of substrates and recombinant enzymes. However, the evidence presented here with two different classes of flavonoid-modifying enzymes indicates the potential of this technique.

Secondary metabolite-modifying enzymes with low substrate specificity are also found for other secondary metabolite classes, suggesting that this approach is broadly applicable and not limited to flavonoids. Frick and Kutchan (1999) characterized a norcoclaurine 6-OMT from *Thalictrum tuberosum* that *O*-methylated 24 different substrates including catechol, caffeic acid **26** and phenethylisoquinoline. These studies suggest that other enzymes characterized for a limited number of substrates may actually be active for a larger range of substrates than reported (Ibrahim et al., 1998). This unrecognized broader substrate specificity provides an enormous source of variability and a completely new platform for designing new, and/or generating large quantities of, pharmacologically active compounds derived from natural products. Using these genes singly, in combination, in succession and with chemical modification, an enormous number of structures can be produced, including those not known to exist in nature. The availability of an increasing number of gene sequences and the possibility of designing enzymes for special needs, promises a great opportunity for drug design in the near future. The in vivo culture technique provides an attractive method to produce preparative or industrial amounts of novel products.

4. Experimental

4.1. Materials and reagents

Mentha x piperita (Burpee seeds, Philadelphia, PA) and *Arabidopsis* (Lehle Seeds, Round Rock, TX) were grown under greenhouse conditions, with a day/night cycle of 14/10 h. *E. coli* BL21 (DE3) pLysS cells were purchased from Novagen (Madison, WI). Flavonoid standards were purchased from Indofine (Somerville, NJ) and Sigma-Aldrich (St. Louis, MO) and dissolved in DMSO. MeCN was ordered from Fisher Scientific (Springfield, NJ) and TFA from Pierce Biotechnology (Rockford, Il). All other reagents were from Sigma-Aldrich unless otherwise noted. H_2O was purified with a Milli-Q Water System (Millipore, Bedford, MA).

4.2. RNA purification

Peppermint leaf tissue was frozen in liquid nitrogen, ground in a mortar and pestle to a fine powder, and RNA was isolated as previously described (Lagrimini et al., 1987). Poly A+ RNA was isolated using the PolyATract® System (Promega, Madison, WI).

4.3. Cloning peppermint O-methyltransferases

60 peppermint ESTs with homology to *OMT*s (Lange et al., 2000) were downloaded from GenBank™. Using Sequencher (Gene Codes, Ann Arbor, MI) the ESTs were grouped into contigs with a minimum match of 95%. With these parameters, 11 contigs and 18 individual sequences were obtained. Based on their homology to flavonoid or isoflavonoid *OMT*s, as opposed to caffeic acid or catechol *OMT*s, 11 of the 18 individual sequences were chosen for further study, along with the 11 contigs. Based on these 22 sequences, 5′- and 3′-RACE PCR (rapid amplification of cDNA ends polymerase chain reaction) primers were designed using Primer3 (Rozen and Skaletsky, 1998) and were ordered from Sigma-Genosys (The Woodlands, TX). RACE PCR was carried out on peppermint leaf cDNA using the SMART™ RACE cDNA Amplification Kit (BD Biosciences-Clontech, Palo Alto, CA). RACE PCR products were gel purified with the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA), cloned into pCR®2.1-TOPO® (Invitrogen, Carlsbad, CA), and multiple individual clones for each primer were sequenced using dye terminator chemistry on ABI3700 DNA sequencers (Applied Biosystems, Foster City, CA). The EST sequence contigs were then disassembled, and all of the sequences (ESTs and RACE PCR clone sequences) were reassembled into contigs using Sequencher. The resulting contigs were analyzed for homology to *OMT* genes and the presence of a recognizable start or stop codon. Based on this analysis, five unique start and stop codons were identified, and primers were designed to anneal to these sequences (F1-5′-CTCGAGATGGCACCGGAAGAAGATTC-3′; F2-5′-CTCGAGATGGTGGCGGATGAAGAAGT-3′; F3-5′-CTCGAGATGGCATTGCCTAATGGCATAT-3′; F4 5′-CTCGAGATGGAAGCAAGCTTCGAAAATG-3′; F5-5′-CATATGGCATTGCCTAATTTAGAAGAGT-C-3′; R1-5′-CTCGAGTCAKGGATAGGCCTCRAT-GACCG-3′; R2-5′-CTCGAGTTATGGAAAAACCT-CAATGACGG-3′; R3-5′-CTCGAGTCAAGGATAG-GCCTCAATGACA-3′; R4-5′-CTCGAGTCATTTAT-GGCATTCCATGATAC-3′; R5-5′-CATATGTTATG-GGAAAATCTCAATCACAGATC-3′). *Xho*I or *Nde*I cloning sites were engineered into the primers for subcloning into pET19b (Novagen, Madison, WI). Using the Advantage 2® Kit (BD Biosciences-Clontech, Palo Alto, CA), *MpOMT* coding regions were PCR amplified from peppermint cDNA using all combinations of the five forward primers and five reverse primers. Successful amplification products (from primers F1/R1, F1/R3, F2/R3, F3/R2, F4/R4, and F5/R5) were cloned into pCR®2.1-TOPO® and 33 individual clones were fully sequenced. Based on homology, five classes of *MpOMT*s were identified (labeled *MpOMT1* through *MpOMT5*), and two *MpOMT1* clones and one clone each from the remaining four classes were chosen for further analysis. Coding regions of the six *MpOMT* genes were cut out with either *Xho*I or *Nde*I/*Xho*I and cloned into pET19b.

The six representative *Mp*OMT amino acid sequences were compared using ClustalW, and boxshading was performed with BOXSHADE 3.21. The phylogenetic tree was constructed using the program PAUP through the Accelrys GCG® Wisconsin Package® programs PAUPSearch and PAUPDisplay.

4.4. Cloning an Arabidopsis glucosyltransferase

RACE PCR primers were designed and ordered to verify the cDNA ends of a putative glucosyltransferase gene from *Arabidopsis* (GenBank™ Accession Number AL161584; Vogt and Jones, 2000). RACE PCR products were cloned and sequenced as described for the *OMTs*. Based on the RACE PCR sequence, primers were designed to anneal to the start and stop codon of the coding sequence (F-5′-CATATGGGTAGT-GATCATCATCATCGAAAG-3′; R-5′-AACCCGGG-TTATGAACTAAACTCTTCCATGAAGCTGTTTA-3′). *Nde*I and *Xho*I sites were added before the start codon and after the stop codon, respectively. The coding sequence for *UGT73B2* was amplified by PCR, cloned into pCR®2.1-TOPO®, several clones were fully sequenced and a clone without PCR errors was selected. Due to an internal *Nde*I site, the *UGT73B2* coding sequence was cloned into pET15b in two pieces: the 5′-end as an *Nde*I-*Bgl*II fragment and the 3′-end as a *Bgl*II-*Xho*I fragment. Both fragments were cloned into pET15b cut with *Nde*I and *Xho*I.

4.5. GenBank™ accession numbers

The nucleotide sequences reported in this paper have been submitted to the GenBank™ database under the following accession numbers: *MpOMT1A*-AY337457; *MpOMT1B*-AY337458; *MpOMT2*-AY337459; *MpOMT3*-AY337460; *MpOMT4*-AY337461; *MpOMT5*-AY337462 and *UGT73B2*-AY339370.

4.6. Enzyme extraction and purification

The *OMT* and *GT* pET plasmids were transformed into *E. coli* BL21(DE3)pLysS cells for enzyme production and purification. Bacterial cultures were inoculated from

overnight cultures and grown in 300 ml Luria-Bertani medium (Sambrook et al., 1989) with ampicillin. Cultures were grown for 3 h at room temp ($A_{600}=0.4$–1), induced with 1 mM IPTG and grown for another 3 h at room temp. Cells were pelleted by centrifugation for 10 min at 10,000 *g*, resuspended in 10 ml cold 20 mM Tris (pH 8), pelleted again and stored overnight at −80 °C. *E. coli* culture pellets were resuspended in 2.5 ml Bugbuster reagent (Novagen, Madison, WI) per gram of cells. Benzonase nuclease (25 Units ml^{-1}) was added and the lysates were incubated for 10 min at room temp with gentle shaking. The lysate was centrifuged (10 min, 10,000 *g*) and the soluble fraction desalted via PD-10 columns (Pharmacia, Peapack, NJ). The desalted extract was purified using HisBind columns, (Novagen, Madison, WI) followed by another desalting with PD-10 columns. The purified extracts (3.5 ml) were concentrated to ca. 1.5 ml, brought to a final concentration of 1% Tween20 and used immediately for enzyme assays or stored at −80 °C until further analysis.

4.7. Enzyme assays for substrate specificity studies

Apparent flavonoid OMT activity was measured by HPLC detection of the methylated products. The OMT standard enzyme assay (total volume 120 μl) contained 20 mM Tris-HCl (pH 7.9), 2 mM DTT, 1% Tween20, 160 μM flavonoid substrate and 500 μM *S*-adenosyl-L-methionine (SAM). The reaction was started by adding 5 to 10 μl of enzyme (2 to 5 μg protein) extract to the reaction mixture. Enzyme assays were incubated at 37 °C for 10–90 min in the linear range of product formation. The reaction was terminated by addition of 10 μl of 6 N HCl and 80 μl MeOH and centrifuged for 10 min at 12,000 *g*. The supernatant was used for HPLC analysis. Apparent activity of UGT73B2 was assayed by measuring the formation of glucosylated flavonoid products with HPLC. The reaction mixture (total volume 120 μl) consisted of 10 mM KH_2PO_4 (pH 6.8), 2 mM DTT, 5 mM $MgCl_2$, 1% Tween20, 160 μM flavonoid substrate and 500 μM UDP-glucose. The reaction was started by adding 30–50 μl enzyme (4–10 μg protein) solution. Enzyme assays were incubated at 37 °C for 15 to 45 min in the linear range of product formation. Termination of the reaction and preparation for HPLC analysis was done in the same way as described for the OMT assay.

4.8. In vivo culture technique

Bacterial cultures (3 ml) of *E. coli* BL21(DE3)pLysS expressing flavonoid *OMT* or *GT* genes were grown in LB medium at 37 °C overnight and used to inoculate 50–100 ml of LB medium. Cultures were incubated for 2–3 h ($A_{600}=0.5$) at 37 °C, induced with IPTG to a final concentration of 1 mM and grown for another 2-3 h. Flavonoid substrates (50–300 μM) were added directly into the medium, and the cultures were grown for another 15 h at room temp. For experiments using mixed bacterial cultures, *OMT* expressing cells were grown and induced as above, quercetin **1** added as the flavonoid substrate and cells incubated for another 3 h. OMT cultures (25 ml) were mixed with previously induced 75 ml GT cultures and 100 mM UDP-glucose added. Mixed cultures were grown overnight at room temp. Cells were collected by centrifugation and extracted for 5 min in MeOH-HCl (v/v, 10:1) in an ultrasonic bath. The flavonoid products extracted from the cells were up to 95% pure. Insoluble particles were removed by centrifugation and the extract was dried under nitrogen. The bacterial medium was acidified with 6 N HCl to pH 1, extracted with EtOAc, dried, and dissolved in MeOH. The dried extracts were dissolved in MeOH for HPLC analysis.

4.9. HPLC analysis

Flavonoid analysis was carried out on a Waters (Milford, MA) 2690 series system equipped with a column oven and a 996 diode-array detector. The procedure employed a C_{18}, fully-endcapped, reversed phase column (Xterra RP_{18}, 150×4.6 mm i.d., 5 μm particle size) operated at 1.5 ml min^{-1} at 50 °C. Methylated flavonoids were eluted with a gradient of 25–71% B in 8 min (solvent A: H_2O, 0.1% TFA; solvent B: MeCN). Flavonoid glucosides were eluted with a gradient of 10–55% B in 10 min and methylated/glucosylated flavonoid mixtures were eluted with a gradient of 10–85% B in 20 min. For separation of semi-preparative amounts of methylated flavonoid mixtures, a C_{18}, fully-endcapped, reversed phase column (Xterra RP_{18}, 150×7.8 mm i.d., 10 μm particle size) operated at 4.4 ml min^{-1} at 50 °C with a gradient of 25–65% B in 7 min. The HPLC system was equipped with a C_{18} Nova-pack Sentry guard column (3.9×20 mm i.d.).

4.10. Mass spectrometry

LC-MS analyses of the flavonoid glucosides were carried out on an Agilent HP1100 HPLC connected to a Finnigan LCQ Classic Mass Spectrometer equipped with a electrospray ionization (ESI) source. The procedure employed a Phenomenex Synergi 4μ MAX RP 150 mm×4.6 mm column, operated at a flow rate of 0.4 ml min^{-1} at 25 °C. Flavonoid glucosides were eluted from the HPLC using a linear gradient of 20–40% B in 22 min (solvent A: 0.1% sodium formate, 0.1% formic acid, MeOH; solvent B: 0.1% sodium formate, 0.1% formic acid, H_2O, w/v/v). Negative ion mass spectra were acquired from 200 to 2000 a.m.u. at a scan rate of one scan/s. The ESI capillary voltage was −43.6 V and

the spray needle voltage 0.76 kV. Helium was used as damping gas, nitrogen as sheath gas (60 psi) and the capillary temperature was held at 250 °C.

4.11. NMR spectrometry

NMR analysis of the methylated flavonoid products was carried out on a Varian INOVA 500 spectrometer (499.66 MHz for ^{1}H and 125.65 for ^{13}C) using a 3 mm inverse detection probe at 25 °C. The samples were dissolved in DMSO-d_6 (D, 99.96%, Cambridge Isotope Laboratories). All spectra were referenced to the ^{13}C and residual protic resonances of DMSO-d_6 at 39.5 and 2.50 ppm, respectively. Quercetin **1**, isorhamnetin **4** and rhamnetin **3** were used as standards for signal comparison.

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