Partial purification, biochemical characterization, kinetic analysis, and amino acid sequencing information of a flavonol 3-*O*-methyltransferase from the leaves of *Serratula tinctoria*.

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ABSTRACT

Partial purification, biochemical characterization, kinetic analysis, and amino acid sequencing information of a flavonol 3-*O*-methyltransferase from the leaves of *Serratula tinctoria*.

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Serratula tinctoria L. (Dyer`s savory, Asteraceae) accumulates small amounts of 3-methylquercetin as an intermediate. 3-Methylquercetin is known as a specific inhibitor of viral RNA replication (Castrillo *et al.*, 1986; Castrillo and Carrasco, 1987), an anti-inflammatory and antiviral agent (Middleton and Kandaswami, 1993), and a phytoanticipin in tobacco plants in response to wounding and insect herbivory (Roda *et al.*, 2003). The flavonol 3-*O*-methyltransferase (3-OMT) was partially purified from *Serratula* leaf tissues using ammonium sulfate precipitation, Superose 12, Mono Q, and adenosine-affinity columns. Result of the purification showed an increase of 194-folds of enzyme specific activity. It catalyzes the methylation reaction of quercetin at position 3, yielding 3-methylquercetin. This enzyme exhibited substrate specificity towards flavonols, which classifies it as a member of the Group II OMT. It has a *pH* optimum of 7.6, a *pI* of 6.0, and an apparent molecular mass of 31 kDa, which is an unusual small protein size in contrast to the 40 to 45 kDa reported for members of this group thus far. Its K_m values for quercetin and AdoMet were 12 and 45 μM, respectively. It has no requirement for Mg^{2+}, and was severely inhibited by *p*-chloromercuribenzoate (*p*CMB), an indication of SH groups requirement for catalytic activity. Kinetic analyses indicated that the enzyme followed an ordered bi-bi reaction mechanism, with AdoMet as the first binding substrate and AdoHcy being released last. Results of LC-MS/MS of the protein yielded several peptides, which showed sequence homology to a number of Group II plant OMTs in the MASCOT database.

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I would also like to thank my supervisor, Dr. Ragai Ibrahim for his patient guidance and sharing of extensive knowledge on the subject of flavonoids. His high expectations of me to give my best regarding my work have always motivated me to become a better scientist. He taught me the importance of persistence in research, which is something one cannot do without. As one of his students, I have learned and gained many valuable skills and lessons from him that will serve me well in my research career.

Finally, to Dr. Y. Fukushi for synthesizing the 3-methylquercetin used in this project, Dr. M. Difalco at McGill University and Genome Québec Innovation Centre for carrying out the LC-MS/MS analysis of the 3-OMT protein, and Dominique Anzellotti for teaching me FPLC, HPLC, and calculation of kinetics.

DEDICATION

I would like to dedicate this work to my parents for all the sacrifices they made moving to Canada to give their children a better education and life.

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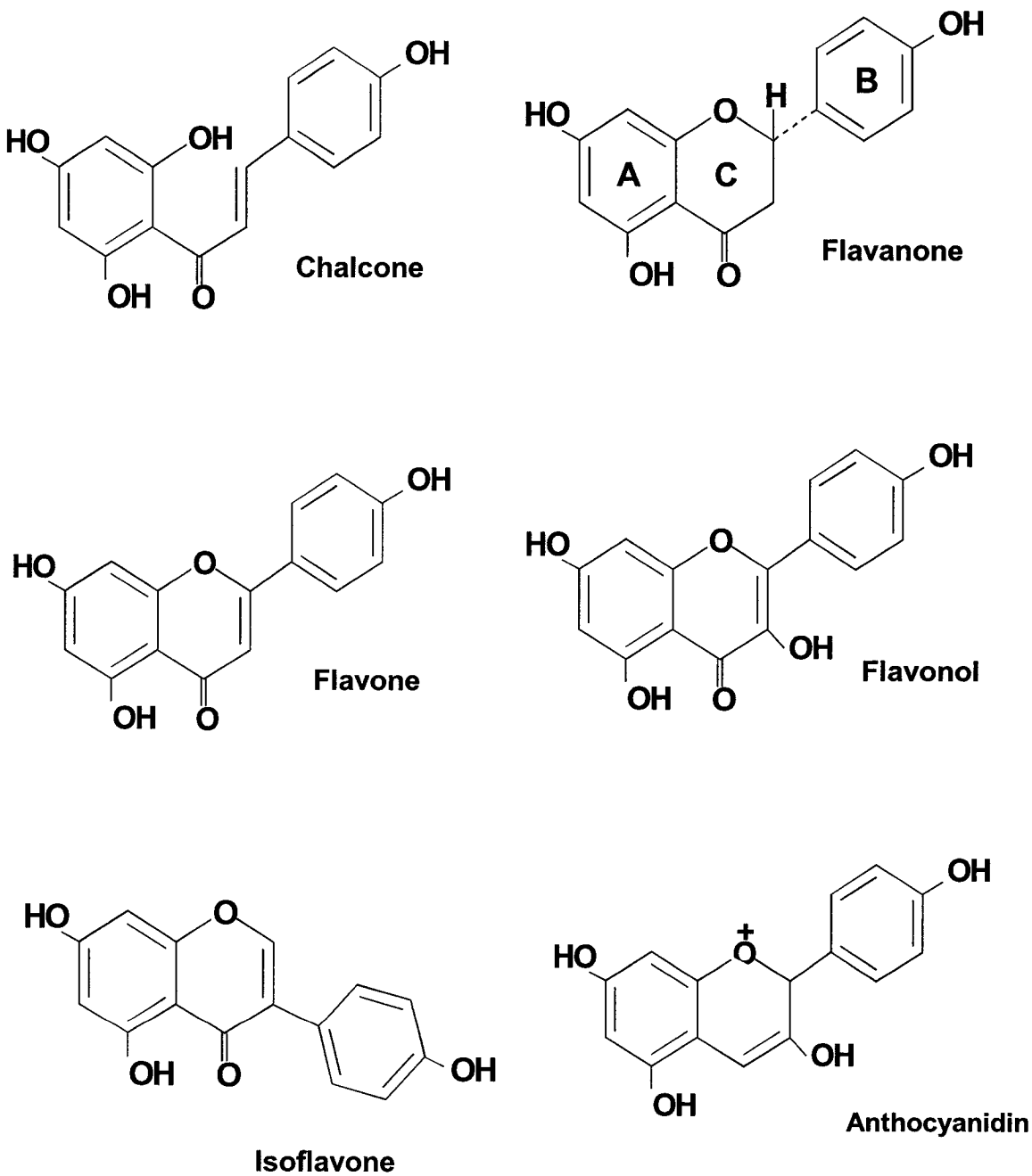
C. INTRODUCTION

Plants synthesize an enormous variety of organic compounds that are collectively known as secondary metabolites, of which flavonoids constitute one of the major groups. Based on the oxidation level of ring C, flavonoid compounds are subdivided into several classes namely, chalcones, flavanones, flavones, flavonols, isoflavones, and anthocyanins (Fig. 1). Flavonoid compounds have several functions, as light shields, flower pigments, phytoalexins/phytoanticipins, as well as determinants of pollen germination and pollen functionality depending on the plant species, to mention a few (Bohm, 1998 and references therein). Due to their ubiquitous occurrence in nature and the wide range of important roles they play in plant growth and development, the biosynthesis of flavonoids has been studied extensively.

Flavonoids undergo a number of enzymatic substitution reactions that are catalyzed by substrate- and position-oriented enzymes. These include hydroxylation, glycosylation, sulfation, methylation, acylation, and prenylation that lead to the modification of their ring systems and structural biodiversity (Ibrahim and Anzellotti, 2003 and references therein). Among these substitutions, *O*-methylation is of particular interest since it reduces the toxicity of flavonoids by decreasing the chemical reactivity of their phenolic hydroxyl groups, thus rendering these compounds more lipophilic and facilitating their transport into membranes (Luckner, 1990), as well as enhancing their antimicrobial activity (French *et al.*, 1991).

O-Methylation of flavonoids is catalyzed by a family of *O*-methyltranferases (OMTs, EC 2.1.1.6). This reaction involves the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to a target hydroxyl group on the flavonoid ring system resulting in the formation of the corresponding methyl ether derivative and *S*-adenosyl-L-homocysteine (AdoHcy) as products. The latter reaction product often acts as a competitive inhibitor of the enzyme reaction.

Figure 1 **The major classes of flavonoids**



Joshi and Chiang (1998) proposed to categorize OMTs into two groups, Group I OMTs are Mg^{2+}-dependent, accept caffeoyl CoA as substrate, and exhibit a molecular mass range of 27 to 29kDa. OMTs of Group II have no requirement for Mg^{2+}, utilize caffeic and 5-hydroxyferulic acids as well as flavonoids and alkaloids as substrates, and possess a molecular mass range between 40 and 45 kDa. Enzymes of the latter group exhibit substrate- and position-selectivity. *O*-Methylation by Group II enzymes commonly involves aglycones and partially methylated compounds as in the case of the stepwise sequential methylation of flavonols in *Chrysosplenium americanum* (De Luca and Ibrahim, 1985a). In some cases, they also utilize flavonol gluco/glycosides, such as the tetra- and pentamethylated flavonol glucosides 2'/5'-OMTs in *C. americanum* (Khouri *et al.*, 1986), vitexin 2" *O*-rhamnoside 7-OMT in *Avena sativa* (Knogge and Weissenbock, 1984), and the cyanidin- and delphinidin 3-*O*-(*p*-coumaroyl)-rutinoside-5-*O*-glucosides 3′- and 3′/5′-OMTs, respectively in *Petunia hybrida* (Jonsson *et al.*, 1982). Recently, based on amino acid sequence comparisons and structural studies, a new OMT classifying scheme separated OMTs into three subfamilies (Zubieta *et al.*, 2003). The first group comprises OMTs that utilize small molecules such as phenylpropanoids, alkaloids, and flavonoids as substrates. The second involves OMTs that are metal-dependent with a substrate preference for caffeoyl and 5-hydroxyferuloyl CoAs. The third group involves OMTs that convert carboxylic acids to their respective methyl ether derivatives.

Methylated flavonoids can act as phytoalexins/phytoanticipins in response to pathogen attack in stress conditions, thus exhibiting antiviral and antimicrobial activities. Phytoalexins are defined as `low molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms′ (Paxton, 1980, 1981). Whereas phytoanticipins are `low molecular weight, antimicrobial compounds that are present in plants before challenge by microorganisms or are

produced after infection solely from preexisting constituents (VanEtten *et al.*, 1994). For example, Christensen *et al.* (1998) isolated a flavonoid 7-OMT from fungus-infected barley leaves that methylates apigenin to its methyl ether derivative, genkwanin, which is only found in stressed tissues and is suspected to act as phytoalexin in the plant stress response. Similarly, a narigenin 7-OMT isolated from uv-irradiated rice leaves is also involved in the biosynthesis of the plant phytoalexin, sakuranetin (Rakwal *et al.*, 2000). He *et al.* (1998) also isolated a cDNA clone encoding isoflavone 7-OMT from alfafa cell cultures that methylates daidzein at position 4' *in vivo* thus producing formononetin, an intermediate in the biosynthesis of the phytoalexin medicarpin. A study of the major metabolites of cudweed, *Gnaphalium affine*, which include several polymethylated flavones, 5-hydroxy-3,6,7,8,4'-pentamethoxyflavone, 5-hydroxy-3,6,7,8-tetramethoxyflavone, and 5,6-dihydroxy-3,7-dimethoxyflavone provided support for methylated flavonoids as potent antifeedants against the common cutworm (*Spodoptera litura*) (Morimoto *et al.*, 2003).

Recently, 3-methylquercetin, a flavonoid compound that rarely accumulates in plants, was reported to act as a selective inhibitor of cAMP- and cGMP-phosphodiesterases (PDE) (Ko *et al.*, 2003), and as a phytoanticipin that accumulates in response to wounding and insect herbivory in tobacco (Roda *et al.*, 2003). This compound is also known for its roles as an antiinflammatory and antiviral agent (Middleton and Kandaswami, 1993; Malhotra *et al.*, 1996), and as a specific inhibitor of viral RNA replication (Castrillo and Carrasco, 1987). Although its precursor, quercetin, is commonly found in many plants, the accumulation of its 3-monomethyl ether is rare under normal condition, since it acts as an intermediate in the sequential methylation of quercetin. Therefore, it was considered important to isolate the gene that encodes the quercetin 3-OMT and study its expression in response to plant stress as well as the constitutive synthesis of 3-methylquercetin in economically valuable crops. To date,

flavonoid OMT cDNA clones that encode the methylation of stilbenes, chalcones, flavones, isoflavones, and flavonols have been isolated, to the exception of a cDNA clone encoding a flavonol 3-OMT that remains to be isolated and characterized (Ibrahim and Muzac, 2000 and references therein).

D.1. AIM OF THE WORK

Serratula tinctoria (Asteracea) accumulates mainly 3,3'-dimethylquercetin and small amounts of 3-methylquercetin as an intermediate. 3-Methylquercetin acts as an intermediate in the biosynthesis of 3,3'-dimethylquercetin in *S. tinctoria* (Dedaldechamps and Ibrahim unpublished), 3,7,4'-trimethylquercetin in apple cell culture (Macheix and Ibrahim, 1984) and spinach leaves (Thresh and Ibrahim, 1985), polymethylated flavonols in *C. americanum* (Ibrahim *et al*, 1987) and orange peel (Brunet and Ibrahim, 1980). Given the important roles 3-methylquercetin plays in plants and its potential uses in human welfare as an antiviral agent (Middleton and Kandaswami, 1993), the cloning of the gene encoding flavonol 3-OMT would be desirable for its manipulation in plants by genetic metabolite engineering,

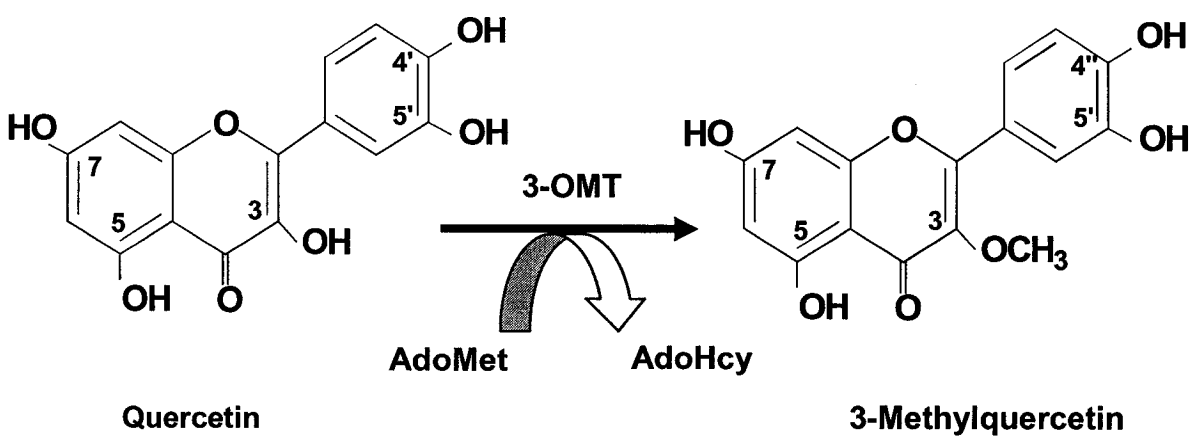
The goal of this project was to purify, biochemically characterize, and ultimately microsequence flavonol 3-*O*-methyltransferase (3-OMT) purified from the leaves of *S. tinctoria*. This enzyme catalyzes the 3-*O*-methylation of quercetin to yield 3-methylquercetin as the flavonoid product (Fig. 2). The enzyme protein was purified using standard chromatographic methods, Western blot analysis, and radiolabelled AdoMet for enzyme assays to monitor the purity of the protein. The antibody used in the blot analysis was raised against a flavonol 3'-OMT from *Arabidopsis thaliana* (data not published) that cross-reacts with the 3-OMT from *S. tinctoria* possibly due to the presence of common epitopes in both OMTs. The partially purified 3-OMT protein was

subjected to LC-MS/MS to obtain its amino acid sequence that matched the plant OMTs listed in the database for the cDNA library screening of the flavonol 3-OMT gene.

The choice of *S. tinctoria* as a source of the 3-OMT was dictated by the fact that this plant expresses only the flavonol 3- and 3'-OMTs, apart from the OMT involved in lignin biosynthesis (Dedaldechamps and Ibrahim unpublished). The availability of seeds and production of abundant leaf tissues also makes it the preferred plant for this project. Previous studies on 3-OMT were carried out with *C. americanum* (De Luca *et al.*, 1982; De Luca and Ibrahim, 1985a,b), which contains six flavonol OMTs that have similar physico-chemical properties, thereby rendering the purification of any of them a difficult task. In addition, *C. americanum* has disappeared from its native habitat due to urbanization and the lack of germinable seeds.

Considering the scarcity of published work on the flavonol 3-OMT and the abundant literature on the antiinflammatory and antiviral properties of 3-methylquercetin render it an interesting enzyme to study with the ultimate goal of its use in metabolic engineering. It was also considered of interest to compare the results obtained from the biochemical studies of *S. tinctoria* 3-OMT with those previously reported from *C. americanum* (De Luca *et al.*, 1982; De Luca and Ibrahim, 1985a,b). Results of the LC-MS/MS of the partially purified protein would be used for designing specific nucleotide probes or PCR primers for the screening of a *S. tinctoria* cDNA library in order to isolate a full-length gene encoding the 3-OMT.

Figure 2 **3-*O*-Methylation of quercetin**



Quercetin **3-Methylquercetin**

D.2. LITERATURE REVIEW

Flavonoids constitute one of the most important groups of plant secondary metabolites. These compounds exhibit numerous biological activities related to plant growth and development, and they play multiple roles in the defense response to pathogen attack and wounding stress. The ubiquitous occurrence of flavonoids in plants, each with its own unique flavonoid profile, suggests a biosynthetic pathway that may be ancestral, and is well adapted through evolution that contribute to its ability to match the needs of different plants given the appropriate signals.

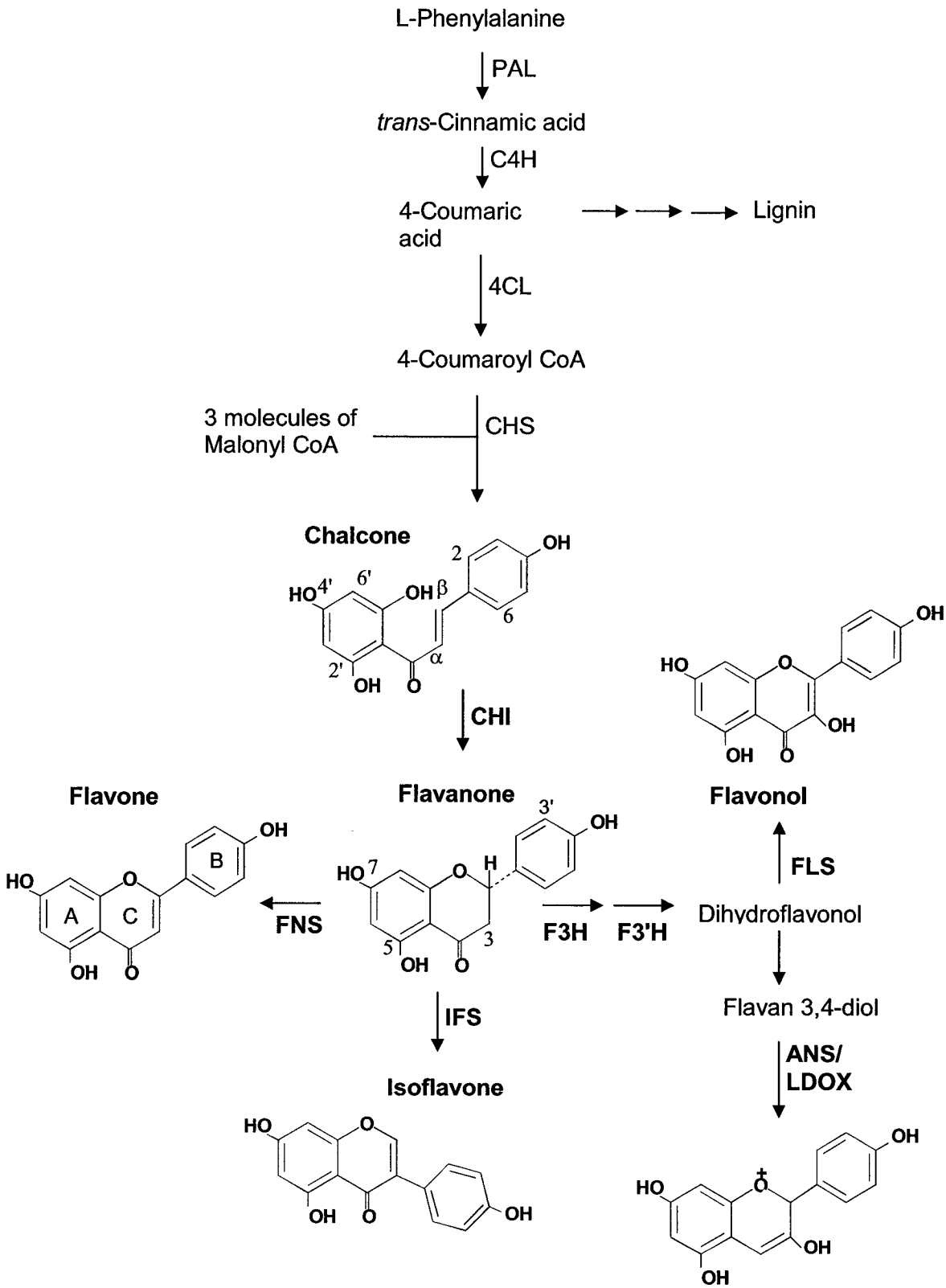
This review includes a general summary of the major steps involved in the phenylpropanoid and flavonoid biosynthetic pathways. Among the multiple substitution reactions flavonoids undergo, the review will focus mainly on the *O*-methylation reaction. Detailed discussions will be focused on the *O*-methylation reaction mechanism and classification schemes of OMTs. These discussions include characteristics of the phenylpropanoid, caffeoyl CoA, and flavonoid plant OMTs, as well as researches that involve the manipulation of their enzyme activities and/or gene expressions. Finally, the review will end with a summary of the functions of *O*-methylated flavonoids in nature, as well as their potential uses in human welfare and in disease therapies.

D.2.1. Biosynthesis of flavonoids

Flavonoids are synthesized in the later steps of the phenylpropanoid pathway. Their basic skeleton consists of two aromatic rings, A and B that are connected by a heterocyclic ring C. The B-ring and its three-carbon side chain are derived from phenylalanine *via* the shikimate pathway, whereas ring A results from the condensation of three acetate units derived from three malonyl CoA molecules. The pathway of flavonoid biosynthesis (Fig. 3) starts with the aromatic amino acid L-phenylalanine that is first converted to *trans*-cinnamic acid, then to 4-coumaric acid by phenylalanine

ammonia lyase (PAL, EC 4.3.1.5) and cinnamate 4-hydroxylase (C4H, EC 1.14.13.11), respectively. 4-Coumaric acid serves as a branch point, where it can either be converted to caffeic acid, the precursor of different types of monolignols involved in lignin synthesis or subsequently esterified with coenzyme A by 4-coumarate CoA ligase (4CL, EC 6.2.1.12) yielding 4-coumaroyl CoA. The condensation of 4-coumaroyl CoA and three malonyl CoA molecules is catalyzed by chalcone synthase (CHS, EC 2.3.1.74), resulting in the formation of the first stable flavonoid intermediate, a chalcone, the precursor of all flavonoid compounds. Chalcone can be converted to the flavanone, naringenin by chalcone isomerase (CHI, EC 5.5.1.6), which acts as the branch-point intermediate towards the formation of other classes of flavonoids, including flavones, flavonols, and isoflavones through various enzymatic reactions. Flavanones can be hydroxylated at position 3 by flavanone 3-hydroxylase (F3H, EC 1.14.11.9) leading to the synthesis of dihydroflavonols. Desaturation of flavanones by flavone synthase (FNS) yields flavones. On the other hand, desaturation of flavanones by flavonol synthase (FLS) results in the formation of flavonols, a major class of flavonoids that is responsible for protecting plants against uv radiation. Reduction of dihydroflavonols by dihydroflavonol reductase (DFR) produces leucoanthocyanidins, the precursor of all anthocyanidins, a main component of plant flower pigments. The last possible route for flavanones in this pathway is 2-hydroxylation, followed by dehydration and aryl-migration by isoflavonoid synthase (IFS) leading to the formation of isoflavones. The latter class of flavonoids is commonly found in leguminous species and is known for their involvement in plant defense against pathogens. More details of these enzymes were recently reviewed by Forkmann and Heller (1999).

Figure 3 Flavonoid biosynthesis pathway



D.2.2. Substitution reactions of flavonoids

Flavonoid compounds undergo a number of substitution reactions that result in the wide spectrum of substituted flavonoids in nature. Given the abundance of these compounds in plants and the versatility of their metabolism in response to the various stresses that plants encounter in their environment, significant amount of research has focused on the enzymes catalyzing these substitution reactions. These reactions include hydroxylation, glycosylation, methylation, acylation, prenylation, and sulfation (see review Ibrahim and Varin, 1993). Among these substitutions, *O*-methylations have been studied most extensively due to their common occurrence in flavonoid metabolism, and the many roles their reaction products play. To mention only a few, these include being precursors of monolignols, which are the building blocks of plant cell wall and play an important role in disease response (Dixon *et al.*, 2001), precursors/intermediates/final products in the biosynthesis of phytoalexins (e.g. medicarpin and pisatin) (He and Dixon, 1996; Preisig *et al.*, 1991). These functions of methylated flavonoids render them desirable compounds for metabolic engineering in increasing plant disease resistance.against microorganism attacks.

D.2.3. *O*-Methylations

O-Methylation occurs in all classes of flavonoids, as well as in other groups of plant secondary metabolites, such as alkaloids. This reaction involves the transfer of a methyl group from AdoMet to the hydroxyl group of substrates, resulting in the formation of the methyl ether derivative and AdoHcy as reaction products. The methylated reaction products has higher lipophilicity that facilitates their transport into membrane and their intracellular compartmentation, as well as increasing their antimicrobial/antiviral activities (French *et al.*, 1991). This group of enzymes shares high amino acid sequence similarity, and exhibits both substrate- and position-specificity. Plant OMTs that utilize

the same class of substrates also exhibit several common biochemical characteristics, such as molecular mass and the lack/requirement of divalent cations for their catalytic activities. Flavonoid OMTs catalyze methylations on various hydroxyl groups located on both rings A and B of various flavonoids. In the case of flavonols, position 3 on ring C may also be methylated.

D.2.4. Classification of OMTs

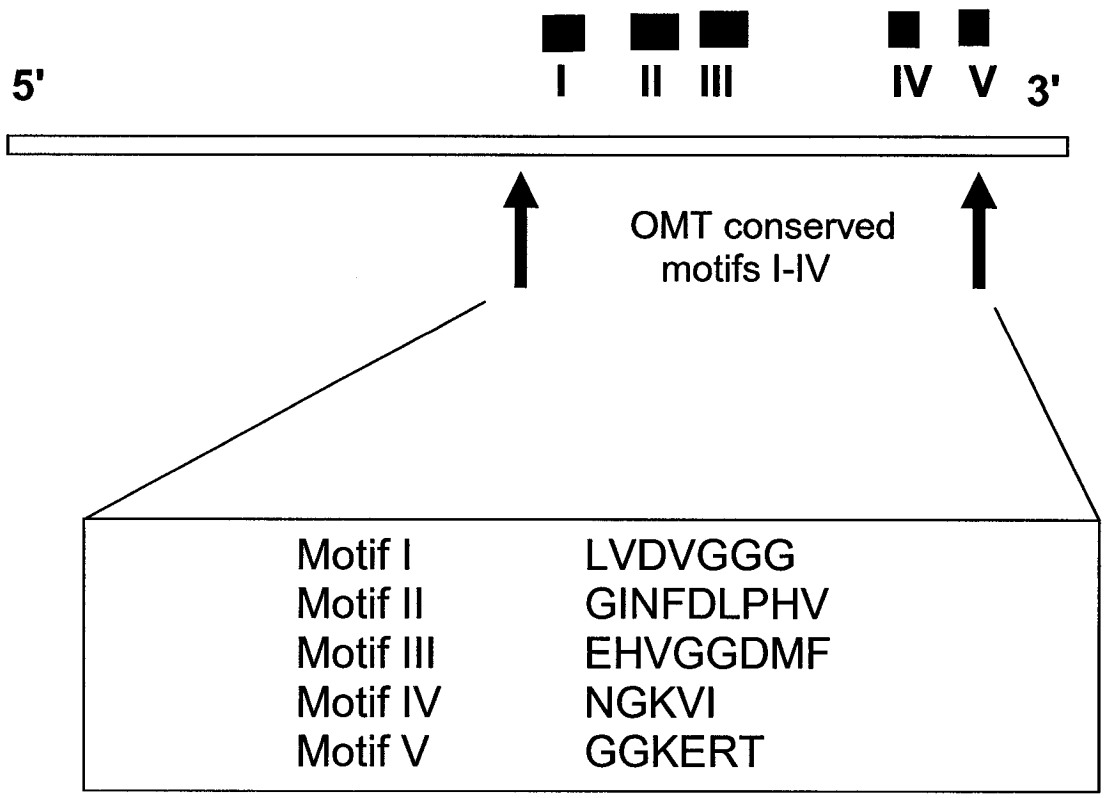
OMT cDNA clones have been isolated for all flavonoid classes except anthocyanins. These cloned OMTs methylate positions 3 of stilbenes, 2' of chalcones and retrochalcones, 7 of flavanones, flavones, and isoflavones, 3' of flavonols, 3'/5 'of partially methylated flavonols, and 3 of pterocarpans (Ibrahim and Muzac, 2000 and references therein). OMTs also methylate phenylpropanoids (e.g. caffeic/5-hydroxyferulic acids, caffeoyl CoA and 5-hydroxyferuloyl CoA). They are the subject of intensive research and their cDNA clones have been isolated from both angiosperms and gymnosperms including parsley (Schmitt *et al.*, 1991), tobacco (Jaeck *et al.*, 1996), alfalfa (Gowri *et al.*, 1991), poplar (Dumas *et al.*, 1998), aspen (Li *et al.*, 2000), Scots pine (Chiron *et al.*, 2000), and Loblolly pine (Li *et al.*, 1997). The voluminous amount of plant OMT sequences present in the database that target different classes of substrates (e.g. phenylpropanoids, flavonoids, and alkaloids), and different positions on the phenolic rings have rendered searches on the organization of these sequence information a necessity. The aim of classifying plant OMTs is to facilitate their taxonomic organization, identify potential OMT candidates, and predict the appropriate substrates of a gene product.

So far, all the plant OMTs that have been characterized are AdoMet-dependent. It is, therefore, the common goal among all proposed OMT classification schemes to identify the AdoMet-binding site, since this domain would be conserved in all OMT

sequences. Based on sequence analyses, several OMT common motifs have been identified by various groups of researchers (Kagan and Clarke, 1994; Ibrahim, 1997; Ibrahim *et al.*, 1998; Joshi and Chiang, 1998; Zubieta *et al.*, 2001; Zubieta *et al.*, 2002; Zubieta *et al*, 2003). Of the many proposals reported, three of them were recognized as being the most accepted and well represented in classifying OMT cDNA clones with the widest applications of the different classes of OMTs and the least mismatches in their proposed common OMT domains.

The first proposal is by Ibrahim *et al.* (1998) that is based on a comparative analysis of amino acid sequences of thirty-six characterized and putative OMTs obtained from the sequence databases (EMBL, GenBank, SwissProt). These OMTs include seventeen clones involved in lignin methylation, six in flavonoid methylation, six from organisms other than plants, and the remaining seven are involved in other types of methylation or as potential OMT candidates. Phylogenetic analyses placed all plant OMT sequences in one monophyletic group. However, the caffeoyl CoA cDNA clones were not included in this group, rather they were recognized to be phylogenetically closer to the non-plant OMTs due to its short gene sequence. Ibrahim *et al.* (1998) suggested that all plant OMTs might have diverged from a common ancestral gene that led to the evolution of the various functional OMTs presently found in plants. They also identified five conserved motifs (I, II, III, IV, and V) located near the carboxy terminal end of the OMT protein sequence (Fig. 4). These regions are highly conserved (92-100%) in the majority of the thirty-six plant OMT sequences. Motifs I to V consist of thirty-six amino acid, rich in glycine residues, it was suggested by Ibrahim *et al.* (1998) that motif I is responsible for the AdoMet binding domain, and IV for the OMT metal binding domain.

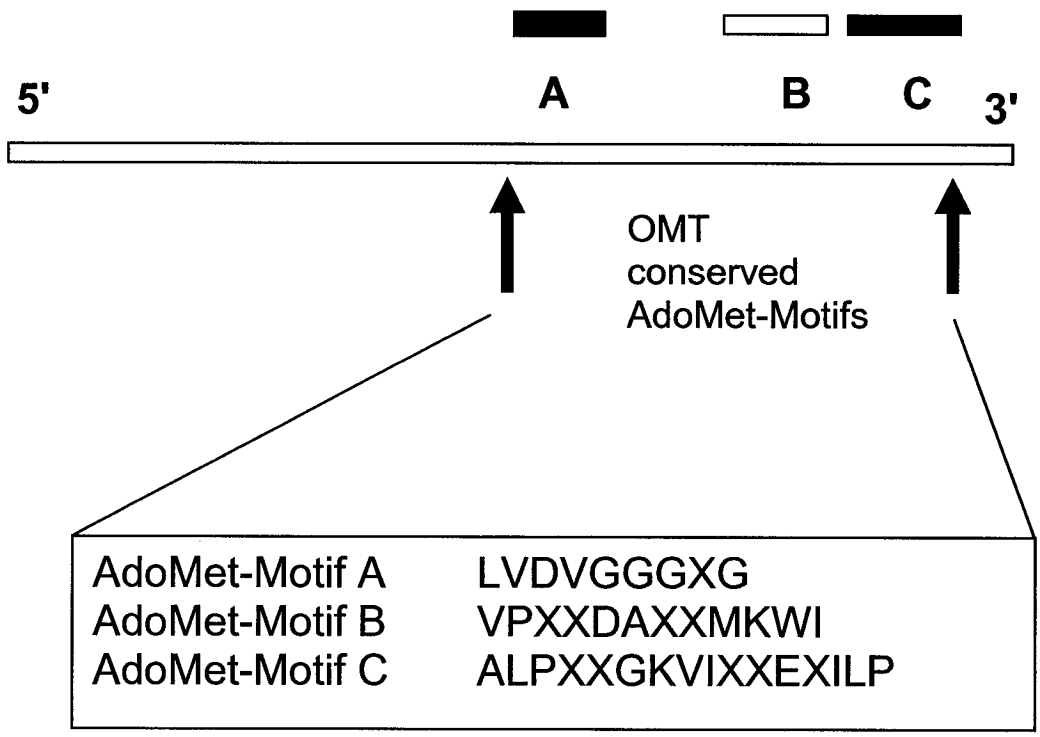
Figure 4 **Five conserved OMT motifs (adaptation from Ibrahim *et al.*, 1998)**



Following the proposal of Ibrahim *et al.* (1998), Joshi and Chiang (1998) reported their classification scheme of OMTs. Based on sequence alignment and phylogenetic analysis of fifty-six plant OMT clones isolated from various species, the OMTs are clustered into two groups, the PI-OMT I and PI-OMT II groups. Each group contains genes that are taxonomically related and shares the same preference in the type of methyl acceptors. The first group (PI-OMT I) constitutes OMTs that utilize only a pair of substrates, caffeoyl and 5-hydroxyferuloyl CoAs (CCoAOMTs). Enzymes from this group share an overall 90% sequence similarity with protein sizes similar to those of mammalian catechol OMTs (ca. 27 kDa) and require the divalent cation, Mg^{2+} for activity. The other group (PI-OMT II) consists of OMTs that accept a wide range of substrates, including phenylpropanoids and flavonoids. PI-OMT II enzymes exhibit a higher molecular weight of ca. 40 kDa with no Mg^{2+} requirement for activity, and share approximately 80% sequence similarity. Sequence alignments of all the genes revealed three signature motifs (A, B, and C) as the potential candidates for the AdoMet binding domain of these enzymes (Fig. 5). These motifs are 98-100% conserved in the fifty-six OMTs examined. In addition to the three putative AdoMet-binding motifs, these authors also designated five additional conserved regions (motifs D, E, F, G, and H) within 75% of the PI-OMT I protein sequences, which may serve as CCoAOMT signatures in plants. Interestingly, within the fifty-six OMT clones, a novel, multifunctional OMT from Loblolly pine (AEOMT) (Li *et al.*, 1997) was identified as a member of the PI-OMT II group in the phylogeny analysis. Substrate specificity studies of this enzyme indicated that it accepts caffeic/5-hydroxyferulic acids as well as 5-hydroxyferuloyl/caffeoyl CoA esters as substrates.

Recently, a new OMT classification scheme proposed by Zubieta *et al.* (2003), based on amino acid sequence alignments and structural studies of OMT enzyme

Figure 5 **Three putative AdoMet-binding motifs (adaptation from Joshi and Chiang, 1998)**



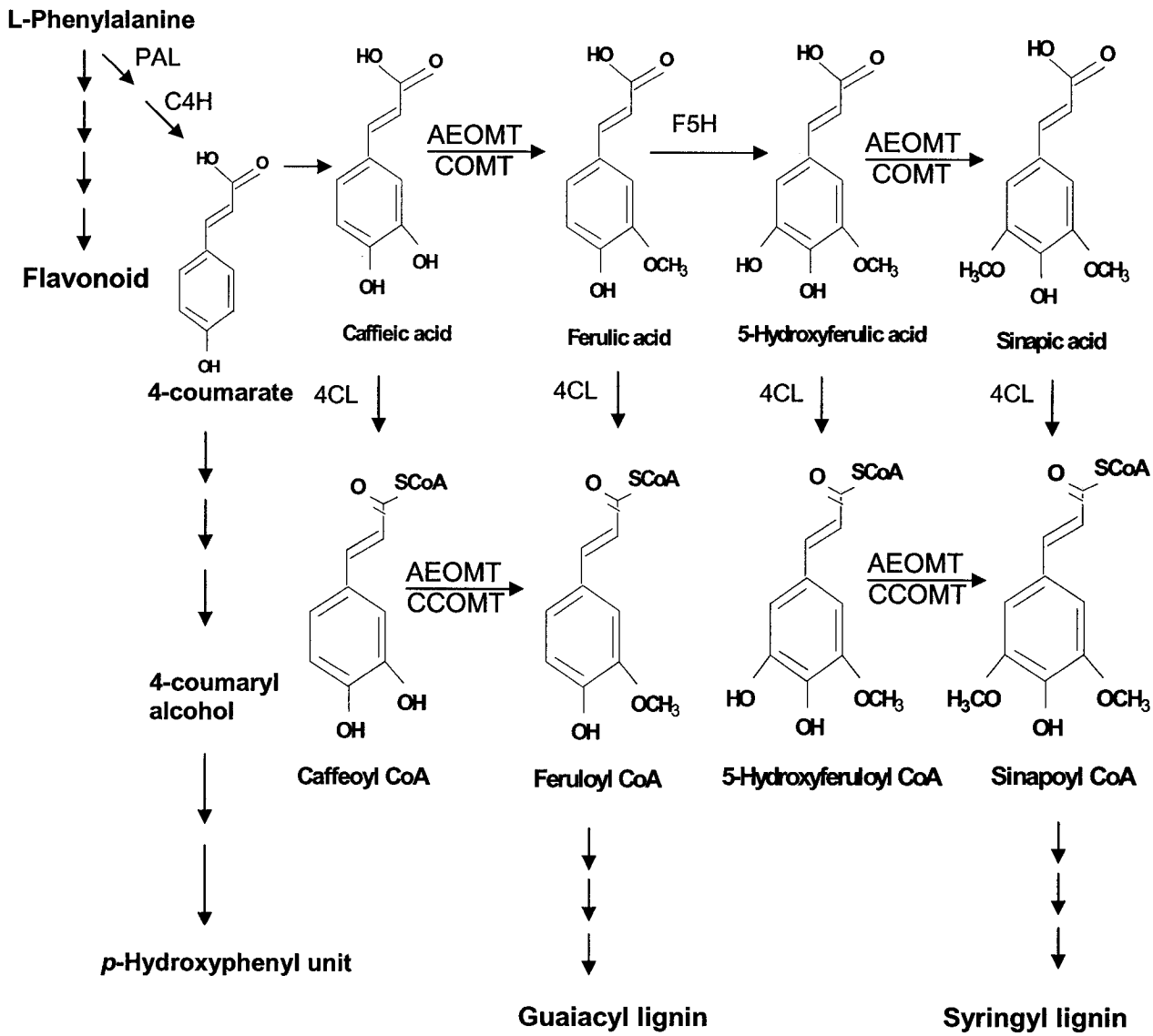
complexes (Zubieta *et al.*, 2001; Zubieta *et al.*, 2002; Zubieta *et al.*, 2003), categorized plant OMTs into three subfamilies. Enzymes belonging to the first OMT family that methylate the hydroxyl groups of phenylpropanoids and flavonoids are the largest among the three groups. OMTs in this family utilize a histidine-based active site for methionine deprotonation, and their substrate specificity is based on a preset active site on the enzyme (Zubieta *et al.*, 2003). The structure of these OMTs allows the methionine residue to be positioned in a way that isolates the targeted hydroxyl group on the flavonoid substrate. OMTs that utilize caffeoyl and 5-hydroxyferuroyl CoAs as substrates (CCoAOMTs) with a Mg^{2+}-dependence are classified into the second family. The metal requirement of CCoAOMTs correlates with the presence of an octahedral metal binding site within their active sites. Surprisingly, CCoAOMTs are reported most closely related to the mammalian catechol OMTs in their primary, secondary, and tertiary structures likely due to the proximity of their protein sizes (Zubieta *et al.*, 2003). The third family consists of enzymes that convert carboxylic acids to their methyl ether derivatives. Transmethylation by these OMTs occurs at physiological pH, thus requiring the proper positioning of the substrate within the active site, and no dependence on general base or metal mediated protonation (Zubieta *et al.*, 2003).

D.2.5.1. Phenylpropanoid OMTs

Phenylpropanoid OMTs (COMTs) utilize caffeic and 5-hydroxyferulic acids as substrates. Methylation occurs at positions 3 of caffeic acid and position 5 for 5-hydroxyferulic acid, producing ferulic and sinapic acids, respectively. Ferulic and sinapic acids are the precursors of guaiacyl and syringyl residues, respectively (Fig. 6) (see review Dixon *et al.*, 2001). Both residues are utilized in the biosynthesis of monolignols in lignifying tissues in plants. "Lignin is a complex phenylpropanoid polymer, deposited mainly as a secondary wall of plant cells to provide the rigid structure of the tracheary

elements for support against the negative pressure generated from transpiration. It also functions as a defense mechanism in response to wounding and pathogen attacks" (Boudet *et al.*, 1995). The syringyl/guaiacyl ratio in lignin composition is closely related to its forage digestibility by ruminant animals (Casler, 1987) and degradability as a pulping material (Whetten and Sederoff, 1991). It is, therefore, of great interest to study the enzymes involved in the biosynthesis of lignin precursors for the genetic manipulation of better quantity and/or quality of the lignin polymer. Methylations of caffeic/5-hydroxyferulic acids were originally thought to be carried by a single OMT, contrary to this theory, De Carolis and Ibrahim (1989) reported, for the first time, the purification of two isoforms of COMT (I and II) from cabbage leaves. COMT I and II exhibit different ferulic:sinapic ratios suggesting the preference of each isoform to one or another of their substrates. COMT cDNA clone was first isolated from aspen and is considered as the type-member of this group (Bugos *et al.*, 1991). Subsequently, COMTs have been isolated from alfalfa, poplar, and several other species, including both angiosperms and gymnosperms (Ibrahim and Muzac, 2000 and references therein). Biochemical characterizations of COMTs demonstrate similar protein size (ca 40 kDa) and no requirement for Mg^{2+} in the assays for enzyme activity. Sequence analyses at the amino acid level demonstrated high similarity (80-95%) in all reported COMTs that contained most of the five conserved OMT regions (Ibrahim *et al.*, 1998). The only exceptions are those stress-inducible COMTs, which shared an overall 50% sequence similarity with the type member of COMT isolated from aspen (Ibrahim and Muzac, 2000). Recently, four cDNA OMT clones were isolated from elicitor treated cell suspension cultures of meadow rue (*Thalictrum tuberosum*) involved in both benzyl isoquinoline alkaloid and phenylpropanoid biosynthesis (Frick and Kutchan, 1999). These four OMTs utilize a wide range of substrates including simple catechols as well as

Figure 6 **The general lignin biosynthesis pathway proposed by Dixon *et al.*, 2001**



phenylpropanoids, tetrahydrobenzylisoquinoline, protoberberine and tetrahydrophenethylisoquinoline alkaloids.

D.2.5.2. Caffeoyl CoA OMTs

CCoAOMTs methylates positions 3 of caffeoyl CoA and 5 of 5-hydroxyferuloyl CoA to give rise to feruloyl CoA and sinapoyl CoA, respectively. Both products serve as the precursors of the lignin monomers guaiacyl (G) and syringyl (S) residues (Fig. 6). The first CCoAOMT cDNA clone was isolated from parsley cell cultures (Schmitt *et al.*, 1991). Later, it was found that the CCoAOMT isolated from *Zinnia* accepts also the 5-hydroxyferuloyl CoA as a substrate to the same degree as caffeoyl CoA (Ye *et al.*, 1994). To date, cDNA clones encoding CCoAOMT have been isolated from *Arabidopsis*, hybrid poplar, alfafa, tobacco, *Stellaria*, aspen, grape, and Zinnia (Joshi and Chiang, 1998 and references therein). This group of enzymes has a molecular weight of ca. 33 kDa and requires Mg^{2+} for catalytic activity. The hypothesis that CCoAOMT is involved in an alternative pathway of lignin biosynthesis that excludes COMT was first proposed by Ye *et al.* (1994), since CCoAOMT expression is found closely related to lignifying tissues in Zinnia (see review by Dixon *et al.*, 2001). The same pattern of expression was later shown in a number of other species, including forsythia, tobacco, alfalfa, soybean, and tomato (Zhong *et al.*, 1998). A study by Day and colleagues (2001), which compared CCoAOMT activity to that of COMT during flax stem development, showed that the activity patterns of the two enzymes were similar during this growth stage, which suggested the involvement of both enzymes in lignin synthesis. Antisense studies of CCoAOMT in transgenic tobacco plants resulted in a dramatic decrease in lignin content and an alteration of lignin composition, in which the guaiacyl content was significantly reduced compared to the control (Zhong *et al.*, 1998). Transgenic plants with reduced COMT activity alone exhibited only decreases in the

syringl lignin levels, but no reduction in the overall lignin content. In addition, activities of CCoAOMT are detected in elicitor- treated carrot and parsley cell cultures, which suggests their involvement in the plant defense mechanism, (Matern *et al.*, 1988; Kuhnl *et al.*, 1989; Pakusch *et al.*, 1989). Pakusch *et al.* (1989) proposed that the involvement of CCoAOMT in plant defense response might be through the reinforcement of cell walls under conditions that triggered the disease resistance response. Recently, Li *et al.* (1999) reported the isolation of a cDNA clone encoding a multifunctional CCoAOMT (AEOMT) from Loblolly pine that utilizes both caffeoyl/5-hydroxyferuloyl CoAs as well as caffeic/5-hydroxyferulic acids as substrates (Fig. 6). Its preferred substrate is caffeoyl CoA followed by 5-hydroxyferuloyl CoA with a 3.2-folds lower activity. The calculated molecular mass of this CCoAOMT is 29 kDa, which corresponds with the usual size of CCoAOMT proteins. Interestingly, Chiron *et al.* (2000) also reported the isolation of a stress-induced multifunctional pinosylvin (stilbene) OMT from Scots pine, which accepts caffeic acid, 5-hydroxyferulic acid, caffeoyl CoA, astrigenin, resveratrol, catechol, quercetin, and luteolin as substrates. This wide range of substrate utilization positions this enzyme in a novel category from those previously classified OMTs. The isolations of the multifunctional CCoAOMTs from Scots pine and Loblolly pine provided support that although OMTs usually exhibit substrate- and position-specificity, it is not true for the CCoAOMTs.

D.2.5.3. Flavonoid OMTs

Flavonoid methylation occurs on all available hydroxyl groups, including positions 5, 6, 7, and 8 (ring A), 2', 3', 4', and 5' (ring B), as well as position 3 (ring C) (Ibrahim and Anzellotti, 2003 and references therein). Flavonoid OMT activity was first reported in tobacco cell suspension culture (Tsang and Ibrahim, 1979), catalysing the *O*-methylation of three cinnamic acids (caffeic/5-hydroxyferulic, and 3,4,5-trihydroxycinnamic acids),

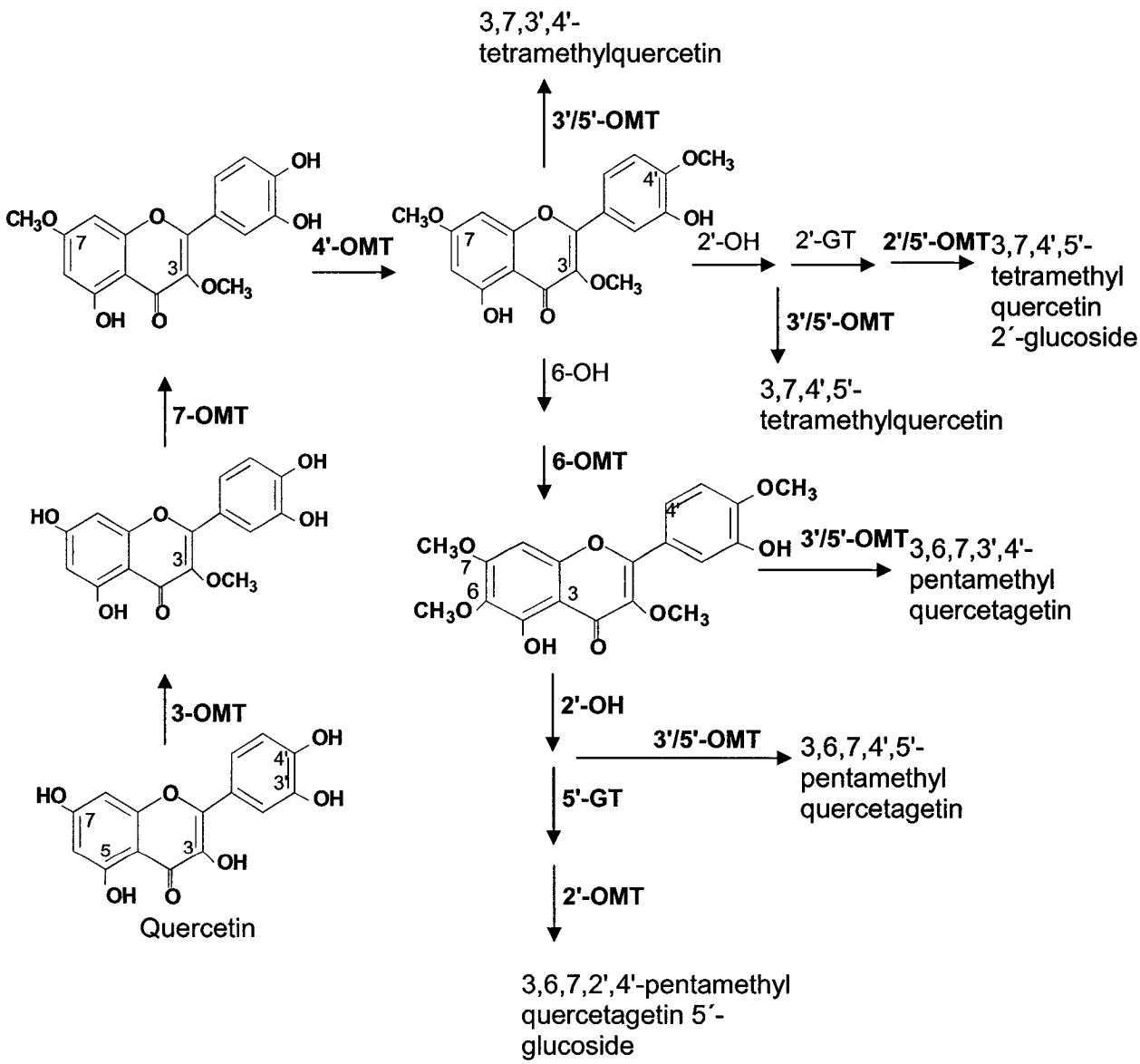
two coumarins (daphnetin and esculetin), and two flavonoids (quercetin and luteolin), albeit with different extents. Two forms of this OMT were detected, OMT I catalyses the *meta* methylation of caffeic acid, and OMT II mediates the *para* methylation of quercetin. Both forms were isolated, partially purified, and biochemically characterized. OMT I exhibited a molecular mass of 74 kDa, a pH optimum of 7.3 and a p*I* value of 6.1. OMT II has a molecular mass of 70 kDa with an optimum pH of 8.3 and a p*I* value of 6.3. Of the many classes of flavonoids, flavonol and isoflavone methylations have been studied most extensively due to the common occurrence of these types of methylations and the important roles their reaction products play in plants. However, there have been two reports of chalcone 2'-OMT cDNA clones isolated from alfalfa (Maxwell *et al.*, 1993) and licorice (Haga *et al.*, 1997). The chalcone 2'-OMT methylates the 2'-hydroxyl group of isoliquiritigenin (2',4',4-trihydroxychalcone) to form 4',4-dihydroxy-2'-methoxychalcone, a potent *nod*-gene-inducing flavonoid derivative released from alfalfa roots. The isoliquiritigenin 2'-OMT is elicitor-inducible in alfalfa cell suspension culture, and exhibits sequence similarity to other plant OMTs. Expression of this OMT takes place mainly in alfalfa roots, and is encoded by a small gene family.

D.2.5.3.1. Biochemically characterized flavonoid OMTs from plant tissues

Sequential methylation of flavonols was first reported in *C. americanum*, where polymethylated quercetin derivatives accumulate as major metabolites (Collins *et al.*, 1981). The 3-OMT enzyme activity, which is responsible for the first committed step of stepwise methylation of quercetin in *C. americanum* was also reported in Calamondin orange peel and root tissues, as well as tobacco pith cell culture (Brunet and Ibrahim, 1980; De Luca *et al.*, 1982). Interestingly, the 3-OMTs of Calamondin orange and tobacco also utilize galangin (3,5,7-trihydroxyflavone) as a substrate. Flavonol OMTs that methylate positions 3, 6, 7, and 4' in the biosynthesis of polymethylated flavonols

have all been partially purified and characterized biochemically by Ibrahim and co-workers (De Luca and Ibrahim, 1985a,b; Khouri *et al.*, 1988) (Fig. 7). Substrates utilized by these OMTs include flavonol aglycones and partially methylated derivatives. In addition, it was found that polymethylated flavonol glycosides can also act as substrates for OMTs as in the case of the 2'/5'-OMT from *C. americanum* (Khouri *et al.*, 1986) (Fig. 7), exhibiting substrate and position specificities. These two enzymes are responsible for the two final steps in the biosynthesis of polymethylated quercetin derivatives in *C. americanum*. The flavonol aglycones quercetin and kaempferol are also substrates of an 8-OMT from *Lotus corniculatus L.* (Jay *et al.*, 1985). This enzyme also utilizes the monomethyl derivative of quercetin, isorhamnetin (3'-methylquercetin) as a substrate. The physical properties of these OMTs include a molecular mass between 55 to 57 kDa, a pH optimum of 7.9-9.0, and a p*I* of 4.0-5.8. Kinetic analysis of most OMTs revealed an ordered bi-bi mechanism, where AdoMet is the first substrate to bind followed by the flavonol substrate, with the reverse order of product release. The only OMT that did not follow this order of substrate binding was the flavonol 8-OMT, where the enzyme binds first to the flavonoid substrate followed by AdoMet, and the products are released in the reverse order. Among these OMTs, only the 6- and 8-OMTs exhibited a requirement for Mg^{2+} ions for enzyme activity. Although none of these enzyme proteins was purified to near homogeneity or sequenced, their biochemical characteristics provided information on the types of substrates accepted as well as the identification of the class of plant OMT for which they belong.

Figure 7 **Methylation substitution patterns of quercetin in *C. americanum* (adaptaion from Khouri *et al.*, 1988 and Gautheir *et al.*, 1996)**



D.2.5.3.2. Characterization of flavonoid OMT cDNA clones

Recent advances in molecular techniques, especially the construction of cDNA libraries and genome sequencing resulted in a rapid increase of isolated plant OMT clones, although the majority of these clones were designated as putative OMTs based on their protein sequence similarity with those in the database. This method of identification of putative OMT candidates allows for quick narrowing down of interesting clones from a library screen. But, it does not provide any further information or classification with regard to the actual function or the group of the encoded gene product belonging to in the multi-branched OMT family. The biochemical characterization of four flavonol OMT clones mentioned below revealed a discrepancy between their predicted and actual gene functions.

The first report is the cDNA clone encoding a flavonol 3'/5'-OMT isolated from *C. americanum* (Gauthier *et al.*, 1996). Sequence analysis of this gene identified all of the five OMT signature motifs (Ibrahim *et al.* 1998). When it was aligned with other plant OMTs in the database, including the COMTs, catechol OMT, scoulerine 9-OMT, and isoliquiritigenin 2'-OMT, it showed a range of 67-85% sequence similarity, having the highest score to the COMT from alfalfa (M63853). Substrate specificity analysis of the 3'/5'-OMT indicated this enzyme to be a flavonol OMT involved in the later steps of the biosynthesis of polymethylated flavonols. It exhibits strict substrate specificity for the partially methylated flavonols only, including 3,7,4'-triOMeQ, 2'-OH-3,6,7,4'-tetraOMeQg, 2'-OH-3,7,4'-triOMeQ, and 3,6,7,4'-tetraOMeQg as substrates (Fig. 7). No activity was detected with the caffeic/5-hydroxyferulic acids. Based on sequence similarity, it would seem that the 3'/5'-OMT could be a COMT, however, results of biochemical characterization demonstrated that this was not the case. The calculated molecular mass of this enzyme is ca. 38 kDa, which is in agreement with those reported for OMTs belonging to PI-OMT II (Joshi and Chiang, 1998). Southern analysis revealed that

multiple related sequences are present in the genome, which is not surprising since *C. americanum* contains at least six flavonol OMTs, and plant OMTs are known to share high sequence similarities, especially those utilizing the same class of substrates.

Following the isolation of the flavonol 3'/5'-OMT, two other cDNA OMT clones (OMT 1 and OMT 2) involved in the methylation of flavonoid and phenylpropanoid compounds were isolated from *C. americanum* (Gauthier *et al.*, 1998). OMT 1 catalyzes the 3'-*O*-methylation of both luteolin (a flavone) and quercetin (a flavonol), as well as the 3/5-*O*-methylation of caffeic/5-hydroxyferulic acids. OMT 2 exhibits the same substrate preference as OMT 1 with respect to phenylpropanoid substrates but is less efficient with the flavonoids. Sequence alignments showed an overall 99% amino acid sequence identity between the two OMTs. Comparing the sequences of either OMT 1 or OMT 2 to that of the aspen bispecific COMT showed 85% sequence similarity. Likewise, there is a 90% sequence similarity among the OMT 1, OMT 2, and the 3'/5'-OMT isolated from *C. americanum* (Gauthier *et al.*, 1996). Based on sequence alignments, OMT 1 and OMT 2 are both considered as COMTs, but the fact that they can also utilize flavonoids efficiently as substrates contradicted this assigned classification.

The calculated molecular masses of OMTs 1 and 2 are approximately 38 kDa, with p*I* values of 5.8 and 6.0, respectively. These values correspond well with most enzymes in the PI-OMT II group (Joshi and Chiang, 1998). Comparison of the open reading frames of these clones identified a difference of only three amino acid residues, the variation in these amino acids was proposed to be related to the substrate selectivity of these enzymes since both enzymes accept the same types of substrates. However, the authors did note that that there were few variations between the OMT 1, OMT 2 and the 3'/5'-OMT within their AdoMet- (signature I) and metal binding (signature IV) sites. It was proposed by Gauthier *et al.* (1998) that these variations, which are mostly near the N-terminal, might be the key to identifying residues that are responsible for enzyme

substrate preference. Support of this hypothesis that a few amino acid residues determine an OMT substrate preference was provided by Wang and Pichersky (1999). These authors reported the identification of seven specific residues located near the N-terminal end that are involved in the substrate selectivity and methylation regiospecificity of a phenylpropane, (iso)eugenol OMT (IEMT) and a COMT in *Clarkia breweri*. These two OMTs share 83% sequence identity, both function as homodimers with a molecular mass of ca. 40 kDa, an optimal pH of 7.5 for their enzyme activities and do not require any metal ion as a cofactor. However, they do exhibit strict substrate preferences and methylation positions. The IEMT methylates isoeugenol and eugenol (both phenylpropenes) at the *para* position, whereas the COMT utilizes caffeic and 5-hydroxyferulic acids as substrates that are methylated at the *meta* position. When the corresponding residues of COMT replaced the seven amino acids in IEMT, the hybrid protein exhibited only COMT enzyme activity. Similarly, when the seven residues of COMT were substituted with that of IEMT, the hybrid protein showed only IEMT activity. These results suggest that new OMTs with different substrate specificities could evolve from an already existing OMT by mutation of a few amino acids, which was a hypothesis first proposed by Gauthier *et al.* (1998). Support for this proposal came from the result of phylogenetic analysis, which suggested that the IEMT was recently evolved from COMT. If this were the case, then the majority of plant OMTs should share high similarity in their gene sequences and protein structures. Investigation of the structural similarity between different plant OMTs was carried out by Seguin *et al.* (1998), using a polyclonal antibody raised against the recombinant protein of a flavonol 3'/5'-OMT clone from *C. americanum*. This antibody exhibited cross reactivity with the 3-OMT in *S. tinctoria*, a plant species that synthesizes 3-methylquercetin and 3,3'-dimethyquercetin (Andary *et al.*, 1996), as well as OMT 1 and OMT 2 (Gauthier *et al.*, 1998). These results indicated a high structural similarity between the flavonol OMTs in these plants.

A cDNA clone isolated from *A. thaliana* that was originally predicted to encode a COMT based on its sequence similarity and identity to other COMTs (Zhang *et al.*, 1997) was found to encode a flavonol 3′-OMT after carrying out biochemical characterization of its recombinant protein (Muzac *et al.*, 2000). It was surprising to find that the gene encoding a flavonol 3′-OMT shares 88% amino acid sequence similarity and 80% homology to the aspen bispecific OMT, which is the type member of the COMT group (Bugos *et al.*, 1991). Results from the biochemical analyses of the gene product identifies it as a flavonol OMT, exhibiting strict substrate specificity and position selectivity for quercetin (Muzac *et al.*, 2000). Considering the examples mentioned above, it should be taken into account that the assigned putative function of an OMT gene based on its sequence similarity or identity to other OMTs could sometimes be misleading. Therefore, it is useful to carry out biochemical characterization of the gene product in order to confirm its true identity.

The OMTs mentioned above catalyzes a single methylation reaction. Recently, a flavonol OMT performing two sequential methylations at the 3′- and 5′-positions was isolated from *Catharanthus roseus* (Cacace *et al.* 2003). Using conventional methods, the enzyme protein was purified to near homogeneity and subjected to microsequencing in order to obtain peptide sequence information. Two cDNA clones were isolated from the *C. roseus* library (CrOMTs 2 and 4). The calculated molecular mass of the two gene products were both ca. 39 kDa. Sequence comparison of the two clones to other plant OMTs in the database showed an approximately 48-53% sequence identity to the OMTs obtained from *Prunus amygdalus* (AJ223151) and *Prunus armeniaca* (U82011), and 35% identity to other COMTs. Phylogenetic analysis revealed that the CrOMTs 2 and 4 formed a distinct clade within the main cluster, which comprised phenylpropanoids, flavonoids, and alkaloids OMTs. Enzyme activity of CrOMT2 was detected with the flavonol aglycones myricetin and dihydromyricetin, but not with phenylpropanoids as

substrates. CrOMT4 was enzymatically inactive against all the flavonol and phenylpropanoid substrates tested. Based on such substrate preference, the authors proposed that the CrOMT2 requires a minimum of two hydroxyl groups on ring B for catalytic activity. Evidence that CrOMT2 catalyzes two sequential methylations on the same substrate was derived from TLC analysis of the reaction products. Two product peaks were detected on the chromatogram, and identity of the second peak was confirmed by mass-spectral analysis, which identified the peak as syringetin (3',5'-dimethylmyricetin), indicating that CrOMT2 performs two successive methylations at positions 3' and 5' of myricetin. This is the first report to date of an OMT capable of transferring two methyl groups onto the same substrate, carrying out sequential methylations, thus representing a new category of plant OMTs. The only other reports of OMTs carrying out sequential methylations are those involved in the biosynthesis of rose scent utilizing benzenoid derivatives as substrates (Lavid *et al.*, 2002; Scalliet *et al.*, 2002).

Interestingly, an OMT from ice plant that is irradiation inducible was found to utilize both caffeoyl-CoA and flavonoids as substrates (Ibdah *et al.*, 2003). This is the first report of an OMT that exhibits a substrate preference for both Group I and II OMT members. The molecular mass of the recombinant OMT protein is ca. 26.6 kDa and requires the presence of Mg^{2+} for its catalytic activity. Its protein size, and cation dependence are the usual characteristics of Group I OMTs (Joshi and Chiang, 1998). Its sequence also showed a high degree of similarity to members of Group I OMT (Ibdah *et al.*, 2003), thus it was not expected to accept flavonoids as its substrates. In addition, substrate specificity analysis reveals this multifunctional OMT also methylates caffeoylglucose, a compound never before reported to be utilized by any plant OMT (Ibdah *et al.*, 2003). Phylogenetic analysis positions this enzyme in a separate clade between Group I and Group II enzymes. The authors of this paper propose this enzyme

from ice plant constitutes a novel subclass within the CCoAOMT group due to its diverse substrate preference.

D.2.5.3.3. Flavonoid OMTs involved in phytoalexin biosynthesis

Many isoflavone OMTs (IOMTs) are involved in the biosynthesis of phytoalexins, such as medicarpin and pisatin. Activity of IOMT was first reported in soybean cell cultures methylating texasin (6,7-dihydroxy-4'-methoxyisolfavone) (Poulton *et al.*, 1977). Unfortunately, due to unavailability of a reference compound, the exact position of methylation could not be determined. Later, the partial purification and biochemical characterization of a 5-IOMT from the roots of yellow lupin (*Lupinus luteus*) was carried out by Khouri *et al.* (1988). Roots of this plant accumulate many 5-*O*-methylated isoflavone derivatives, such as 5-methylgenistein, 5-methyllupiwighteone, and 5-methylderrone, to mention only a few. The 5-position of flavonoids is considered to be the least reactive due to its chelation with the carbonyl group of the heterocyclic ring C (Khouri *et al.*, 1988), hence the researchers considered it important to characterize the enzyme responsible for this methylation. The 5-IOMT has a molecular mass of 55 kDa, no requirement for Mg^{2+}, and an apparent p*I* of 5.0. In *L. luteus*, the 5-IOMT exhibits both strict position- and subsrate-specifcities.

A pterocarpan OMT was purified to near homogeneity from $CuCl_2$-stressed pea seedling (Preisig *et al.*, 1989). The purified protein has a molecular mass of 43 kDa, and carries out the methylation of the pterocarpan, (+)-6a-hydroxymaackiain at position 3 to give rise to its 3-methyl derivative, which is the terminal step in the biosynthesis of pisatin, a phytoalexin synthesized by pea in response to microbial infection and metal stress. The fact that the enzyme activity is inducible by $CuCl_2$ treatment supports the involvement of (+)-6a-hydroxymaackiain 3-OMT in the plant stress response. Furthermore, the amount of phenylalanine ammonia-lyase mRNA detected in the tissues

also exhibited a similar increasing pattern as the (+)-6a-hydroxymaackiain 3-OMT levels, demonstrating a coordinated transcriptional induction in both the early and terminal steps in the biosynthetic pathway of pisatin. Isolation of the cDNA clone of (+)-6a-hydroxymaackiain 3-OMT from fungal-infected pea tissue revealed a gene product with a *ca.* 40 kDa (Wu *et al.*, 1997) that exhibits the same substrate specificity as the purified protein (Preisig *et al.*, 1989).

An IOMT involved in the biosynthesis of medicarpin, a phytoalexin commonly found in leguminous species, was first purified to near homogeneity from alfalfa cell cultures (He and Dixon, 1996). This enzyme methylates daidzein (7,4'-dihydroxyisoflavone) leading to the formation of formononetin (7-hydroxy-4'-methoxyisoflavone) and it is elicitor-induced. This IOMT exhibited a preference for the 7-hydroxyl group of daidzein *in vitro* that produces isoformononetin; whereas *in vivo* it methylates position 4' yielding formononetin. These authors proposed that IOMT might be part of an in vivo metabolic channel that favors the 4'-*O*-methylation over 7-*O*-methylation, where the reaction product from the isoflavone synthase reaction is channeled through to form formononetin or beyond to 2'-hydroxyformononetin. IOMT has a molecular mass of 41 kDa, and four internal peptides were obtained from its microsequence. These four peptides have approximately 55% sequence identity to four regions of the (+)-6a-hydroxymaackiain 3-OMT from pea (Wu *et al.*, 1997). None of the latter sequences exhibited any sequence identity to the COMTs or chalcone 2'-OMT (Wu *et al.*, 1997). Interestingly, the IOMT also methylates the 5-hydroxyl group of genistein. Based on the internal amino acid sequence information of the purified alfalfa IOMT, three full-length IOMT cDNA clones were isolated (He *et al.*, 1998). A search in the gene database revealed related sequences of IOMT present in chickpea and cowpea, two other species that are also known to produce methylated isoflavones. The recombinant IOMT (7-IOMT) has a molecular mass of 41 kDa, which corresponds to that

of the purified protein (He and Dixon, 1996), and methylates daidzein as well as the pterocarpan (+)-6a-hydroxymaackiain. The fact that 7-IOMT activity is elicitor-induced suggests that the enzyme may be responsible for the 4'-*O*-methylation of daidzein *in vivo* under stress condition (He and Dixon, 2000). Evidence of this finding was derived from elicitor-induced 7-IOMT activity in 7-IOMT overexpressed alfalfa resulted in an accumulation of formononetin. These plants showed an enhanced disease resistance against fungal elicitation (He and Dixon, 2000). Recently, radiolabeling and isotope dilution studies confirmed the involvement of 2,7,4'-trihydroxyisoflavanone as an intermediate in the biosynthesis of formononetin (Liu and Dixon, 2001), and proposed that the 7-IOMT is also responsible for the 4'-*O*-methylation of 2,7,4'-trihydroxyisoflavanone in alfalfa. Further support of this finding was derived from the three-dimensional crystal structure of 7-IOMT, which incorporates the 2,7,4'-trihydroxyisoflavanone into its active site on a computer-generated illustration (Zubieta *et al.*, 2001). In contrast, Akashi *et al.* (2003) isolated a cDNA clone (HI4'OMT) from *Glycyrrhiza echinata* that methylates 2,7,4'-trihydroxyisoflavanone at the 4'-hydroxyl group, but exhibiting no 7-IOMT activity. HI4'OMT-related sequences were identified in the databases of several leguminous species, including *Lotus japonicus* (Accession no. AV407445) (Asamizu *et al.*, 2000), *Medicago truncatula* (Accession no. TC28631), and *Glycine max* (Accession no. TC101829). Similar to the 7-IOMT from alfalfa, the HI4'OMT also exhibits amino acid sequence identity (83%) to the (+)-6a-hydroxymaackiain 3-OMT (Wu *et al.*, 1997).

Although the majority of phytoalexins occurring in plants are of isoflavonoid origin, other flavonoids such as flavanones, and flavones may also act as phytoalexins. Several of these flavonoid phytoalexins, or their precursors, are the reaction products of *O*-methylation. Examples of OMTs involved in the biosynthesis of these phytoalexins include the apigenin 7-OMT (Christensen *et al.*, 1998), narigenin 7-OMT (Rakwal *et al.*,

2000), and kaempferol 4`-OMT (Curir *et al.*, 2003), producing genkwanin (5,4'-dihydroxy-7-methoxyflavone), sakurenetin (5,4'-dihydroxy-7-methoxyflavanone), and kaempferide, respectively. The apigenin 7-OMT was isolated from fungus-infected barley leaves. The recombinant protein of this enzyme has a molecular mass of 43 kDa and is found only in pathogen-infected tissues. Sequence alignment of the apigenin 7-OMT revealed an overall 39% sequence identity to (+)-6a-hydroxymaackiain 3-OMT (Wu *et al.*, 1997) and 72% to one of the four peptides obtained from 7-IOMT in alfalfa (He *et al.*, 1998). Likewise, naringenin 7-OMT was purified from uv-irradiated rice leaves (*Oryza sativa*) with a molecular mass of 41 kDa and no requirement of Mg^{2+} for activity. Activity of narigenin 7-OMT is induced in a time-dependent manner with treatments of jasmonic acid and copper chloride. Information obtained from its N-terminal amino acid sequencing exhibited sequence homology to the COMT from maize (Rakwal *et al.*, 2000). Investigation of enzyme substrate specificity revealed that the flavanone naringenin 7-OMT utilizes naringenin, apigenin, and luteolin, where the latter compound is its most preferred substrate. However, the fact that the induction of naringenin 7-OMT activity upon uv irradiation corresponded to the accumulation of sakuranetin in the tissue provided evidence of this enzyme's role in the biosynthesis of sakuranetin. In contrast to the previous two plant OMTs, the kaempferol 4´-OMT can be purified from both fungal stressed and healthy tissues of *Dianthus caryophyllus L.* (Curir *et al.*, 2003). The enzyme, with a molecular mass of ca. 45 kDa, specifically methylates the 4´-position of flavones, flavanones, and isoflavones, with kaempferol as its most preferred substrate. It has the usual flavonoid OMT characteristics, such as no Mg^{2+} requirement and inhibition of catalytic activity by SH-group reagents. However, the 4´-OMT does not follow an ordered bi-bi but a ping-pong reaction mechanism, where AdoHcy is released prior to the binding of its flavonoid substrate, it is the first plant OMT reported to display this mechanism.

D.2.6. Functions of *O*-methylated flavonoids in plants

Plant OMTs utilize a wide range of substrates including flavonoids, phenylpropanoids, and alkaloids (Ibrahim and Anzellotti, 2003 and references therein). Many of the methylated flavonoids are involved in the biosynthesis of phytoalexins or phytoanticipins. These methylated compounds can act as intermediates or end products that exhibit antiviral or antimicrobial activities. For example, the phytoanticipin 3-*O*-methylquercetin, a flavonol derivative that accumulates in response to wounding and insect herbivory in tobacco (Roda *et al.*, 2003). Sakuranetin (7-methylnaringenin) is a flavanone phytoalexin that is *de novo* synthesized in rice leaves post uv-irradiation (Kodama *et al.*, 1992). Formononetin (2,7-dihydroxy-4′-methoxyisoflavanone or 4′-methyldaidzein), is an essential intermediate in the biosynthesis of the isoflavone phytoalexins, medicarpin, vestitol, and maackiain, defense compounds that are commonly found in leguminous plants (Akashi *et al.*, 2000). *O*-Methylation of phenylpropanoids (e.g. caffeic/5-hydroxyferulic acids) and their CoA esters (e.g. caffeoyl/5-hydroxyferuloyl CoAs) leads to the synthesis of monolignol precursors (guaiacyl and syringyl units) used for lignin production. Lignin functions for structural support and as a secondary defense mechanism against chemical, physical, and biological stresses (Boudet *et al.*, 1995).

Methylated phenolics serve as components of floral scent used to attract pollinators (Bohm 1998). Dudareva *et al.* (2000) reported that methyl benzoate is synthesized in snapdragon as the most abundant scent compound found in the flowers that is responsible for the attraction of bumblebees for pollination. Wang *et al.* (1997) also demonstrated that in *Clarkia breweri* both methylisoeugenol and methyleugenol contribute to floral scent. Furthermore, *O*-methylated carbohydrates, especially inositols, frequently accumulate in higher plants in response to salinity, drought or cold stresses (Adams *et al.*, 1992). These compounds accumulate in the cytoplasm for the

intracellular osmotic adjustment in plants during stress conditions (Popp and Smirnoff, 1995).

D.2.7. Potential uses of methylated compounds for human welfare

Flavonoids, especially those *O*-methylated exhibiting antibiotic activities are well-documented and have stimulated numerous studies to assess their potential pharmaceutical value in the treatment of human diseases, as well as enhancement of the immune system. Soybean isoflavonoids, formononetin (7-hydroxy-4′-methoxyisoflavone) and biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) are reported to have important biological activities, mainly as estrogenic and anticarcinogenic compounds (Sarkar and Li, 2003). They have been implicated in the growth inhibition of human breast cancer cells (Peterson and Barnes, 1991). It has also been reported that women consuming elevated levels of isoflavonoid-containing diets exhibit a lower incidence of breast cancer than those with reduced intake of such compounds (Aldercreutz *et al.*, 1988; Aldercreutz *et al.*, 1990; Lee *et al.*, 1991; Messina and Messina, 1991: Sarkar and Li, 2003). However, more clinical studies of these compounds are required for adequate evaluation of their effects.

On the other hand, methylated flavonols, flavones and flavanones exhibit inhibitory activities towards virus replication and bacterial synthesis. It was reported that 3-methylquercetin is a potent and selective inhibitor of *Poliovirus* RNA replication (Castrillo *et al.*, 1986), in addition to possessing antipasmodic (Karamenderes and Apaydin, 2003) and antiinflammartory activities (Ko *et al.*, 2003). Recent studies on mono- and polymethylated flavonols revealed that these compounds are effective anti-inflammatory (Williams *et al.*, 1999) and antioxidant agents (Sang *et al.*, 2002; Spencer, 2003), vasorelaxants (Guerrero *et al.*, 2002), and with cytotoxic activity against certain cancer cell lines (KA3IT and NIH3T3) (Gedara *et al.*, 2003). Given the numerous

biological activities these compounds exhibit, their large-scale production will help in the determination of their medicinal and pharmaceutical potentials, as well as facilitate the development of more effective drugs.

D.3. MATERIALS AND METHODS

D.3.1. Plant material and growth conditions

Serratula tinctoria (Dyer's Savory, Asteracea) seeds were kindly provided by the Botanical Garden of the University of Gottingen, Germany. Plants were cultivated in sterile potting soils at 23°C under a 16-h light period and controlled conditions of temperature, humidity, and light. Plants were watered with 20:20:20 (N-P-K, 250 mg/L) solution as required.

D.3.2. Chemicals

Flavonoid compounds were from our laboratory collection. 3MeQ was synthesized by Dr. Y. Fukushi, Hokkaido University, Japan. S-Adenosyl-*L*-[$^{14}CH_3$] methionine (55mCi/mmol) and S-Adenosyl-*L*-[C^3H_3] methionine (80Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO); unlabeled AdoMet, AMP agarose (adenosine 5'-monophosphate, cross-linked 4% beaded agarose with C-8 attachment and a nine-atom spacer), betaine, KCl, *p*CMB (*p*-chloromercuribenzoate), PMSF (phenylmethylsulfonyl fluoride), and PVPP (polyvinylpolypyrrolidone) were purchased from Sigma (Oakville, ON). AdoHcy and PB-74 (Polybuffer-74) were purchased from Amersham Biosciences (Baie d'Urfé, QC). Protein quantification reagent, 40% acrylamide/bis solution (37.5:1), SDS (sodium dodecyl sulfate), glycine, and non-fat dry milk were from Bio-Rad (Mississauga, ON). Chromatography-grade cellulose powder was purchased from Avicel (Philadelphia, PA). Anhydrous ammonium sulfate was purchased from EM science (Darmstadt, Germany). DIECA (diethylquinoethyldithiocarbamate) and EDTA were from Aldrich (Oakville, ON). CIAP (calf intestine alkaline phosphatase) and 10X reaction buffer were obtained from MBI Fermentas (Burlington, ONT). All chromatographic column supports were prepared

according to manufacturers' instructions for subsequent use with enzyme purification. All other chemicals were of analytical grade unless otherwise specified.

D.3.3. Buffers

The following buffers were used: **A**, 0.2 M sodium phosphate, pH 7.6 containing 14 mM β-mercaptoethanol, 10% glycerol (v/v), 5 mM EDTA (w/v), 10% PVPP (w/w), 0.2% DIECA (w/v) and 0.5% PMSF (w/v); **B**, 50 mM Tris-HCl, pH 7.6 containing 14 mM β-mercaptoethanol, 10% glycerol (v/v), and 0.3 M NaCl; **C**, 50 mM Tris-HCl, pH 7.6 containing 14 mM β-mercaptoethanol, 10% glycerol (v/v), and 50 mM NaCl; **D**, 20 mM Tris-HCl, pH 7.6 containing 14 mM β-mercaptoethanol, 10% glycerol (v/v), and 1 mM EDTA (w/v); **E**, 50 mM Tris-HCl, pH 7.1 containing 14 mM β-mercaptoethanol, and 10% glycerol (v/v); **F**, PB-74 (1:15, v/v), pH 4.0 containing 14 mM β-mercaptoethanol, 10% glycerol (v/v), 5% betaine (w/v), and 10 mM KCl. All FPLC buffers were filtered with MF-Millipore membrane filters (0.45 μM pore size), degassed, and stored at 4°C.

D.3.4. Preparation of adenosine-agarose affinity gel

Preparation of the ligand was carried out according to Rakwal *et al.* (2000) with modifications in components of the buffer used to equilibrate the adenosine-agarose column (buffer D) and pH of the 10X calf intestinal alkaline phosphatase reaction buffer (pH 7.5, 0.1 M Tris-HCl containing 0.1 M $MgCl_2$).

D.3.5. Protein extraction

All steps were carried out at 4°C unless otherwise stated. Leaf tissues were harvested from 3-month old plants (20g), ground to a fine powder in liquid nitrogen before being homogenized with buffer A (1:5, w/v). The homogenate was filtered

through Miracloth and the filtrate was centrifuged at 12,000*g* for 20 min. The supernatant was stirred with Dowex resin (10%, w/v) that had previously been equilibrated with the extraction buffer for 1 hour, in order to remove phenolics, and filtered. Solid ammonium sulfate was added to the filtrate and the protein which precipitated between 30 to 70% saturation was collected by centrifugation at 12,000*g* for 10 min. The protein pellet was re-suspended in buffer B and used immediately for enzyme purification. The dissolved protein was desalted on PD-10 columns that were previously equilibrated with buffer B.

D.3.6. Gel-filtration

The Amersham Biosciences FPLC (fast protein liquid chromatography) system was used for the purification of the flavonol 3-OMT. The first step of protein purification was carried out using Superose 12 (prep grade) HR 16/50 column (500 X 16 mm i.d.) that had previously been equilibrated with buffer B. The desalted protein sample was (*ca.* 40 mg) loaded onto the Superose 12 column and eluted with the same buffer at a flow rate of 1 mL/min (0.5 MPa) and 2-mL fractions were collected and assayed for enzyme activity using Q, 3MeQ, and 5-hydroxyferulic acid (5-HF) as substrates in order to detect 3-OMT, 3MeQ 3′-OMT, and 5-HF OMT activities.

D.3.6.1. Anion-exchange chromatography

The Superose 12 fractions containing the 3-OMT activity were pooled and their buffer was exchanged against buffer C on a PD-10 column before loading onto a Mono Q HR 5/5 column (50 X 5 mm i.d.) pre-equilibrated with buffer C. The column was run at a flow rate of 0.5 mL/min. (2.5 MPa). Bound proteins were eluted with a linear 50-500 mM NaCl gradient in buffer C. Four-mL and 2-mL fractions were collected for the unbound and bound proteins, respectively. All fractions were assayed for the Q 3-OMT,

3MeQ 3′-OMT, and 5-HF OMT activities. Mono Q fractions containing the Q 3-OMT activity were collected and concentrated with an Amicon stirred cell (Model 8050) and their buffer exchanged with buffer D on a pre-equilibrated PD-10 column.

D.3.6.2. Ligand-affinity column

The concentrated Mono Q protein fractions were loaded on an adenosine-agarose affinity column (130 X 7 mm i.d.) previously equilibrated with buffer D. The unbound protein was eluted at a flow rate of 0.05 mL/min and the column was washed with 50 mL of buffer D prior to elution of the bound proteins at a flow rate of 0.6 mL/min. The bound protein was eluted with 15 mL of 4 mM AdoMet in buffer D containing 0.2 M KCl. Bound fractions (2.0 mL) were collected and assayed for Q 3-OMT activity. Active fractions were dialyzed overnight in buffer D containing 0.2 M KCl for the determination of specific activity before being subjected to amino acid sequencing.

D.3.7. Molecular mass determination

The molecular mass of Q 3-OMT was estimated by gel filtration on a calibrated Superose 12 column. The reference proteins used were bovine serum albumin (67.0 kDa) and chymotrypsinogen A (25.0 kDa).

D.3.8. Determination of *pI*

The *pI* value of the Q 3-OMT was determined from its elution pattern on a chromatofocusing Mono P HR 5/20 column (200 X 5 mm i.d.). Concentrated Mono Q protein fractions were desalted against buffer E on a PD-10 column and loaded onto a Mono P column pre-equilibrated with buffer E. The column was washed with 50 mL of the same buffer. Column was washed with 4 mL of buffer E prior to the elution of the

bound proteins using a descending pH gradient (pH 7-4) and buffer F at a flow rate of 0.5 mL/min, and 2-mL fractions were collected and assayed for the Q 3-OMT activity.

D.3.9. *O*-Methyltransferase assay

The enzyme assay was carried out in a final volume of 100 μL containing 10 μM quercetin (dissolved in 50% DMSO), 25 nCi [^{3}H] or [^{14}C] AdoMet (80Ci/mmol or 55mCi/mmol, respectively) as the co-substrate, and 10-100 μg enzyme protein in buffer C. The assay mixture was incubated for 30 min at 30°C, and the reaction was terminated by the addition of 10 μL of 6 N HCl. The labeled *O*-methylated products were extracted with 500 μL of a benzene-ethyl acetate mixture (1:1, v/v), and shaken for 2 min. The organic phase containing the methylated products was separated from the aqueous layer by a 3 min centrifugation. Aliquots (250 μL) of the organic phase were transferred to plastic scintillation vials (Beckman Bio-Vials) containing 2.5 mL scintillation fluid (5 g PPO/L of toluene) and were counted for radioactivity using an LKB 1217 Rackbeta Liquid Scintillation counter. Enzyme assays for the calculation of Q 3-OMT specific activity in the purification table (Table 4) and substrate specificity (Table 8) were carried out in triplicates using proteins from the same extraction. Assay value with a difference greater than 15% to the average in each set was omitted and redone. The final substrate concentrations tested in the substrate specificity assays were 5, 10, 50, and 100 μM. Control assay was carried out using the same condition but without the flavonoid susbtrate in the reaction.

D.3.10. Identity of the enzyme reaction products

Products of the enzyme assay reaction using [^{14}C] AdoMet were collected and lyophilized to dryness and re-dissolved in 20 μL of MeOH to be chromatographyed on

cellulose TLC plates in ethyl acetate:acetic acid:water (1:3:7, v/v/v). The identity of the methylated products was confirmed by co-chromatography with reference compounds, visualized under uv light, and autoradiographyed on X-ray film. Several enzyme assays were carried out using unlabeled AdoMet in order to collect the methylated product for HPLC analysis. The non-radioactive reaction product was lyophilized to dryness, dissolved in 150 μL HPLC grade MeOH and filtered through 0.20 μm Millipore syringe-driven filters prior to injection onto an analytical Waters Symmetry RP C18 silica column (5 μm, 4.6 X 250 mm i.d.). Both reference compounds (Q and 3MeQ) and the reaction product were eluted using a linear gradient method with the starting conditions at MeOH-H_2O-HOAc (44:55:1, v/v/v) for 2 min, followed by an ascending to MeOH-H_2O-HOAc (70:29:1, v/v/v) over a period of 25 min. The gradient was kept at this plateau for 10 min before re-equilibrating back to the starting conditions over a period of 25 min. The identity of the reaction product was confirmed by comparing the retention time and elution profile of the product to those of the reference compounds.

D.3.11. SDS-polyacrylamide gel electrophoresis and Western Blotting

The purity of the active protein fractions from each chromatography column was monitored by the use of SDS-PAGE analysis according to the method of Laemmli (1970), using molecular weight markers (Bio-Rad) ranging from 14.4 to 97.4 kDa for calibration under denaturing conditions. After electrophoresis, proteins were stained with Coomassie Brilliant Blue (R-250). The gels were submitted to a semidry electrophoretic transfer onto nitrocellulose membranes, and probed with the IgG-purified Q 3'-OMT (1:500 dilution) and horseradish peroxidase-linked donkey anti-rabbit IgGs (Amersham Biosciences), or the secondary antibody labeled with alkaline phosphatase (1:3000 dilution). Peroxidase activity was revealed in a chemiluminescent reaction

detected by a 30-min exposure to blue light-sensitive autoradiography film. Determination of alkaline phosphatase activity was carried out using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates.

D.3.12. Protein determination

Protein concentrations were determined using the Bio-Rad protein assay based on the method of Bradford (1976) with bovine serum albumin as the standard protein according to the manufacturer's specifications.

D.3.13. Kinetic analysis

Kinetic analyses were performed using the Mono Q fraction Q 3-OMT protein. Product formation was linear with respect to the assay time and the amount of protein used. Assay of each graph point was repeated five times within the same set of experiment. Values that have a difference greater than 15% to the norm in each set was omitted and redone with proteins from the same extraction. Standard deviations of the intercept and slope replots used in the determination of Q 3-OMT kinetic parameters were calculated according to Zar (1984).

Kinetic studies of substrate interactions between Q and AdoMet were carried out using 10 μg of protein, and varying concentrations of AdoMet (5, 10, 15, 20, 25, and 30 μM) in the presence of Q (at a final concentrations of 1, 3, and 4 μM) (Table 1). The inverse was also performed with different concentrations of Q (0.125, 0.25, 0.5, 1, and 2 μM) in the presence of fixed, constant concentrations of AdoMet (35, 45, and 60 μM) (Table 1). Results of the substrate interaction studies are presented as Lineweaver-Burk plots (Segel, 1975) fitted by linear regression. The replots of the intercept and slope values obtained from the Lineweaver-Burk plot were used for the calculation of substrate

K_m values, the K_i for AdoMet, V_{max} of Q, catalytic efficiency of Q 3-OMT, and enzyme turnover ratio. Similarly, substrate interactions of kaempferol (K) and myricetin (M) with AdoMet were carried out for the determination of their respective K_m and V_{max} values. Studies of product inhibition are carried out only for Q, under unsaturating conditions (Table 2). The order of substrate binding, and the K_{ip} and K_{iq} values of 3MeQ and AdoHcy, respectively, were both determined from the Lineweaver-Burk plot patterns of the product inhibition studies.

D.3.14. MS/MS analyses of the partially purified Q 3-OMT protein

The protein fraction containig Q 3-OMT activity from the adenosine-agarose chromatography step was analyzed by SDS-PAGE using 12% polyacrylamide gel. The protein band which migrated at *ca.* 31 kDa was excised, dehydrated with acetonitrile and washed with 100 mM $(NH_4)_2CO_3$ for two 10 min cycles before the addition of an equal volume of acetonitrile. The destained gel slices were treated for 30 min with 10 mM DTT to reduce the cysteine residues, and for 20 min with 55 mM iodoacetamide to alkylate the sulfhydryl cysteine side chains. After a second round of $(NH_4)_2CO_3$ and acetonitrile washes, the slices were dehydrated with acetonitrile at 37°C. The gel slices were subsequently incubated with trypsin (6 ng/μL in 50 mM $(NH_4)_2CO_3$) for 5 hours at 37°C and the peptides extracted first using 1% formic acid / 2% acetonitrile, followed by another extraction with 25% acetonitrile. The final composition of the 45 μL extraction volume was composed of 0.5% formic acid / 9% acetonitrile. For the purpose of sample analysis by MALDI Q-Tof (Matrix Assisted Laser Desorption Ionization Quadrupole time of flight) Ultima Mass Spectrometry (Waters-Micromass), 4 μL of the resulting peptide extraction was mixed with 1 μL of an α-cyano-4-hydroxycinnamic acid solution in 0.1%

Table 1 **Concentrations of substrates (Q, AdoMet, K, and M) in substrate interaction studies with 10 μg of protein in the enzyme assay**

A

[Q] (μM)	[AdoMet] (μM)					
1	5	10	15	20	25	30
3	5	10	15	20	25	30
4	5	10	15	20	25	30

B

[AdoMet] (μM)	[Q] (μM)				
35	0.12	0.25	0.50	1.0	2.0
45	0.12	0.25	0.50	1.0	2.0
60	0.12	0.25	0.50	1.0	2.0

C

[K] (μM)	[AdoMet] (μM)				
0.50	5	10	20	30	40
1	5	10	20	30	40
2	5	10	20	30	40

D

[M] (μM)	[AdoMet] (μM)		
0.5	5	30	40
1.5	5	30	40
2	5	30	40

Table 2 **Concentrations of substrates (Q and AdoMet) and reaction products (AdoHcy and 3MeQ) in product inhibition studies with 10 μg of protein in the enzyme assay under unsaturated condition**

AdoMet/AdoHcy

[AdoHcy] (μM)	[AdoMet] (μM)					
0	5	10	15	20	25	30
2	5	10	15	20	25	30
10	5	10	15	20	25	30
20	5	10	15	20	25	30

AdoMet/3MeQ

[3MeQ] (μM)	[AdoMet] (μM)				
0	10	15	20	25	30
20	10	15	20	25	30
30	10	15	20	25	30

Q/AdoHcy

[AdoHcy] (μM)	[Q] (μM)			
0	0.125	0.25	0.5	1
2	0.125	0.25	0.5	1
10	0.125	0.25	0.5	1
20	0.125	0.25	0.5	1

Q/3MeQ

[3MeQ] (μM)	[Q] (μM)		
0	0.25	2	4
20	0.25	2	4
55	0.25	2	4

trifluroroacetic acid:50% acetontrile. The peptide masses obtained were submitted to MASCOT (Matrix Science) software for database mass-fingerprint search analysis against NCBI non-redundant protein database. Another portion (30 μL) of the peptide extraction was injected into LC-MS/MS system (Liquid chromatography-electrospray ionization-quadrupole-time of flight) at a flow rate 0.1 mL/min, positive ion mode. The result obtained from LC-MS/MS was analyzed with MASCOT to identify the protein. McGill University and Genome Québec Innovation Centre carried out all peptide treatments involved in the MS/MS analysis of the protein.

D.4. RESULTS

D.4.1. *pH* optima for the Q 3-, 3MeQ 3'-, and 5-HF OMT activities in *S. tinctoria*

Assay buffers containing 14 mM β-mercaptoethanol and 10% glycerol (v/v) adjusted between *pH* 6.0 to 9.0 in increments of 0.5 units were used to determine the *pH* optimum for the assays of Q 3-OMT, 3MeQ 3'-OMT, and 5-HF OMT using Superose 12 enzyme protein. Table 3 and Fig. 8 show that the highest enzyme activity of Q 3-OMT was obtained at *pH* 7.5, whereas the 3MeQ 3'-OMT and 5-HF OMT activities were highest at *pH* 7.0 and 7.5, respectively. An increase or a decrease of 0.5 *pH* unit resulted in a drop in Q 3-OMT activity by 18% or 38%, respectively. Using Fig. 8 as a guide, the optimum *pH* for the 3-OMT enzyme activity was estimated to be approximately *pH* 7.6.

D.4.2. Purification of Q 3-OMT

S. tinctoria accumulates 3,3′-dimethylquercetin as the major flavonoid, thus expressing the gene encoding the flavonol 3-OMT and monomethylated flavonol 3′-OMT (Dedaldechamps and Ibrahim unpublished). The former OMT catalyzes the *O*-methylation of the hydroxyl group at position 3 of Q, yielding 3MeQ as the intermediate flavonol. The 3-OMT, an AdoMet-dependent enzyme, was partially purified in a three-step purification procedure, using gel filtration on Superose 12, anion exchange on Mono Q, and affinity chromatography on adenosine-agarose column. The purification data obtained utilizing these chromatographic columns are summarized in Table 4.

Table 3 ***pH*** **optima of Q 3-OMT, 3MeQ 3'-OMT, and 5-HF OMT activities in** ***S. tinctoria***

	OMT specific activity (pkat/g)		
Buffer *pH*	**Q 3-OMT**	**3MeQ 3'-OMT**	**5-HF OMT**
6.0 (Bis-Tris)	0.297	0.0312	0.303
6.5 (Bis-Tris)	0.823	0.0944	0.576
7.0 (Bis-Tris propane)	1.40	0.0758	0.637
7.5 (Tris-HCl)	2.26	0.0950	0.436
8.0 (Tris-HCl)	1.86	0.0921	0.162
8.5 (Bicine)	0.866	0.0129	0.258
9.0 (Bicine)	0.426	0.0184	0.0291

Figure 8 ***pH*** **optima of Q 3-OMT, 3MeQ 3'-OMT, and 5-HF OMT activities in *S. tinctoria***

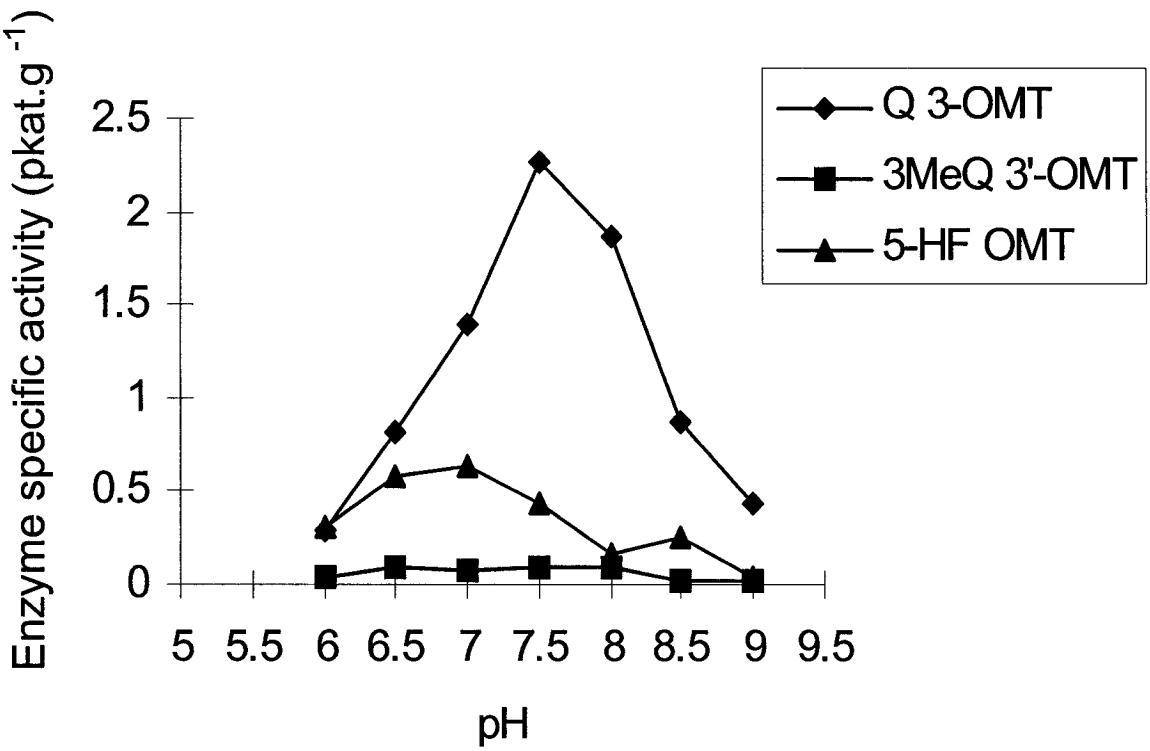


Table 4 **Purification data of Q 3-OMT from *S. tinctoria* leaves**

Purification steps	Total protein (mg)	Specific Activity (pkat/g)	Total Activity (pkat)	Purification (*x*-fold)	Recovery (%)
$(NH_4)_2SO_4$	56	0.071	0.004	-	100
Superose 12	20	0.81	0.016	11.5	35.7
Mono Q	4	3.26	0.013	46	7.2
Adenosine-agarose	0.12	13.8	0.0017	194	0.21

D.4.2.1 Superose 12 chromatography

Gel-filtration chromatography on a Superose-12 column resulted in one broad activity peak that contains the Q 3-, 3MeQ 3'-, and 5-HF OMT enzyme activities (Fig. 9). This protein peak was eluted between fractions 22 to 28. The dominant enzyme activity in this peak was the Q 3-OMT, which was 5- and 13-folds higher than that of 3MeQ 3'-OMT and 5-HF OMT, respectively. Purification of the ammonium sulfate pellet on a Superose-12 column yielded an 11.5-fold increase of Q 3-OMT specific activity and a recovery of 35.7%.

D.4.2.2. Mono Q chromatography

Separation of the Q 3-, 3MeQ 3'- and 5-HF OMT activities were achieved by Mono Q fractionation. The three OMT enzyme activities eluted in the unbound fractions 1, 2 and 3 of the first run (Table 5). These fractions were separately pooled and reloaded onto the Mono Q column. Proteins eluted in the unbound fractions 1 and 3 of the second run exhibited the 3MeQ 3'-OMT and Q 3-OMT activities, respectively (Fig. 10). In contrast, the 5-HF OMT activity was detected in the bound fraction that eluted at a concentration of 194 mM NaCl (Fig. 10). Proteins collected from these fractions were loaded on separate lanes of a denaturing gel for subsequent Western blot analysis using the antibody IgG-purified Q 3'-OMT. Results of this experiment provided evidence of these OMTs exhibiting different molecular masses from each other (Fig. 11A-C). The band at 66 kDa in all lanes was likely due to the presence of human keratin, in which the rabbit antibody cross-reacted to it. It appears in all the different purification steps of the 3-OMT protein (Fig. 14). The faint band in Fig. 11C that migrated at *ca.* 31 kDa was likely the result of an overflow of the Q 3-OMT protein from the adjacent lane (Fig. 11B). The Mono Q purification step resulted in an increase of 46-fold enzyme activity of the Q 3-OMT and 7.2% of protein recovery.

Figure 9 **Superose 12 enzyme activity elution patterns**

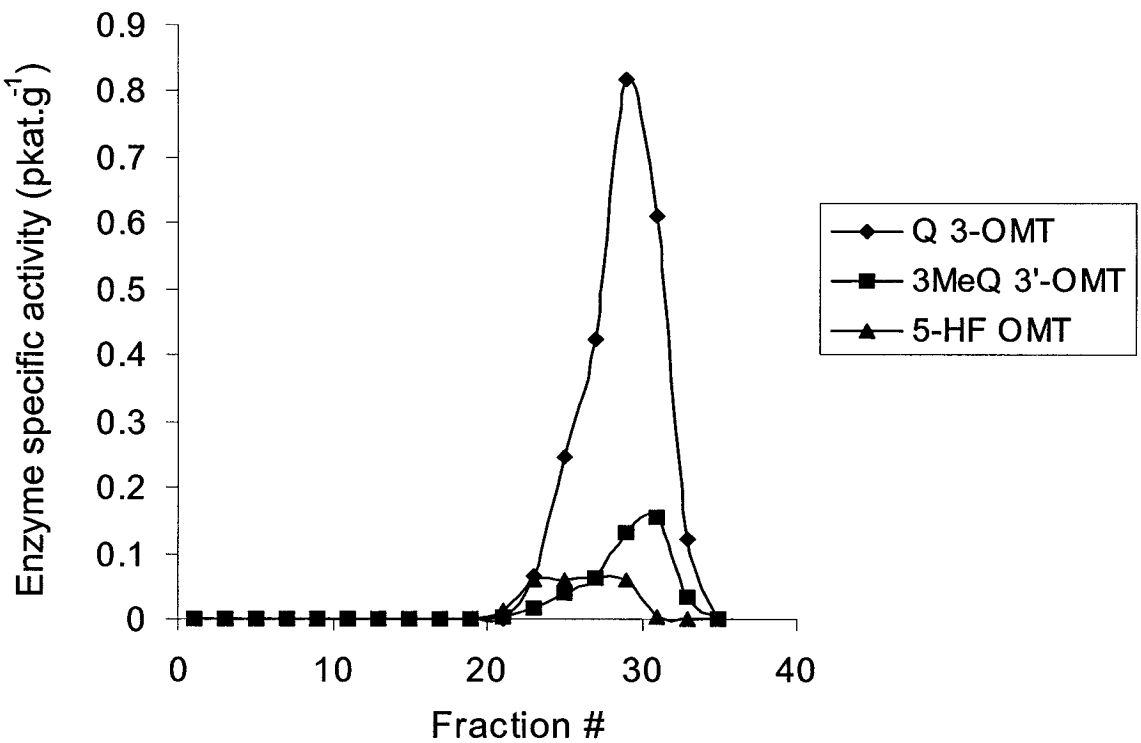


Table 5 **Enzyme activities of Q 3-OMT, 3MeQ 3'-OMT, and 5-HF OMT in the unbound fractions of first Mono Q run**

	OMT Specific activity (pkat/g)		
Unbound fraction #	**Q 3-OMT**	**3MeQ 3'-OMT**	**5-HF OMT**
1	0.297	0.0312	0.303
2	0.823	0.0944	0.576
3	1.40	0.0758	0.637

Figure 10 **Mono Q enzyme activity elution patterns**

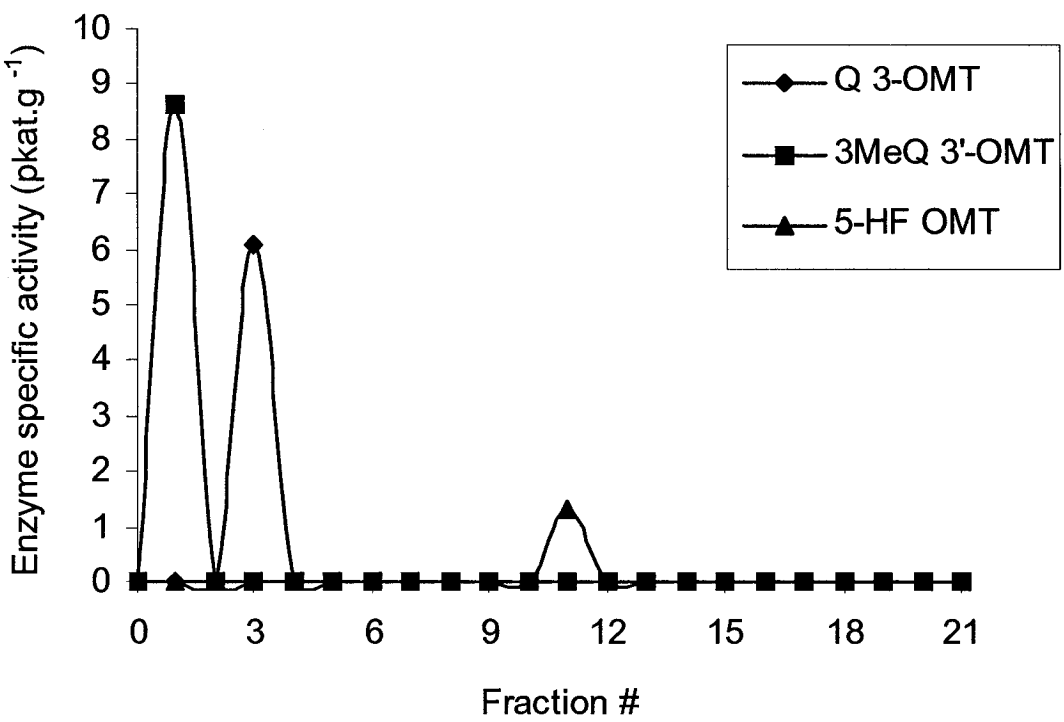
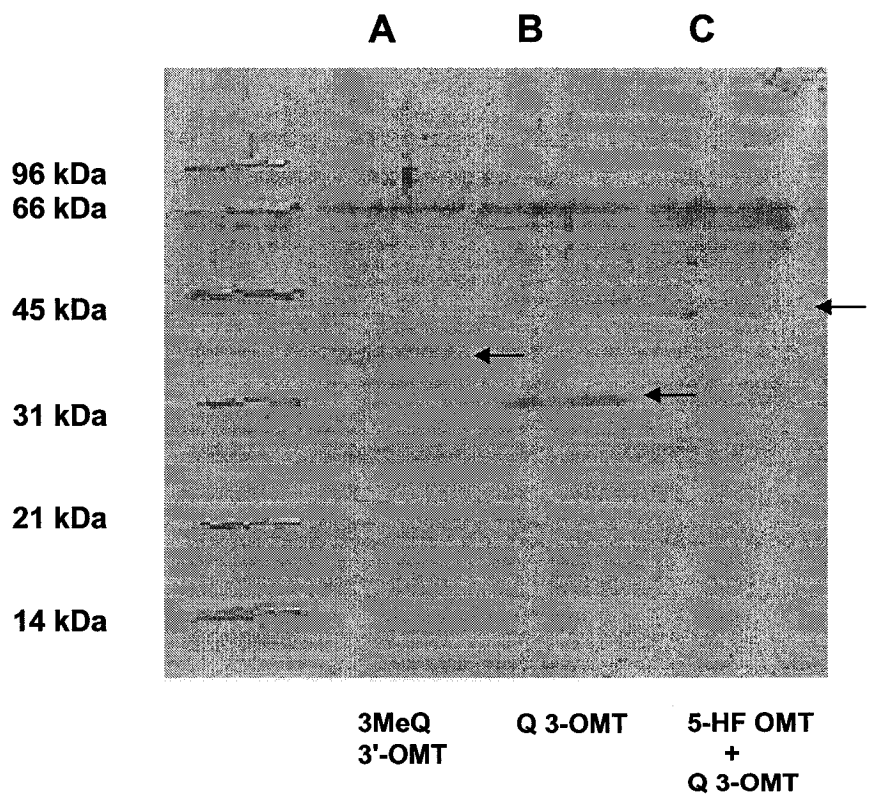


Figure 11 **Western blot of Mono Q fractions containing the Q 3-, 3MeQ 3'-, and 5-HF OMT activities**



D.4.2.3. Adenosine-agarose chromatography

The final stage of the Q 3-OMT purification was carried out on an adenosine-agarose column, using an affinity ligand resin that resembled the structure of the reaction co-substrate, AdoMet. The 3-OMT protein was eluted in the bound fraction (Fig. 12). Proteins in this active adenosine-agarose fraction exhibited exclusively the Q 3-OMT activity when assayed against quercetin, 3-methylquerceitn, and 5-hydroxyferulic acid. This purification step yielded a 194-fold increase of Q 3-OMT activity, albeit with a very low recovery, 0.21% (Table 4). The Coomassie blue stain of this fraction revealed five protein bands (Fig. 13), however, only one band was recognized in the Western blot with a molecular mass of *c.a.* 31 kDa (Fig. 14). This result provided further support that the Q 3-OMT is a protein with a molecular mass of 31 kDa in *S. tinctoria* leaves.

D.4.3. Molecular mass and isoelectric point of the Q 3-OMT

The estimated native molecular mass of Q 3-OMT using gel filtration chromatography was estimated to be 35 kDa with a standard deviation of 2.1 (Fig. 9). This value was slightly higher when compared to that obtained after SDS-PAGE (31 kDa) (Fig. 13). This difference may be due to the conformational changes of the protein between the two states, since protein in its denatured condition is linearlized, which gives a protein size that is slightly smaller than that of gel filtration column. However, results of coomassie blue stained gel and Western blot (Fig. 14) both indicated that the Q 3-OMT is a 31 kDa protein. Gel filtration profile of Q 3-OMT also provided support of Q 3-OMT is a monomer. The isoelectric point of 3-OMT was determined by chromatofocusing on a Mono P column, based on its activity profile eluted with a pH gradient. The apparent isoelectric point of Q 3-OMT was estimated to be 6.0 (Fig. 15).

Figure 12 **Adenosine-agarose elution pattern of Q 3-OMT activity**

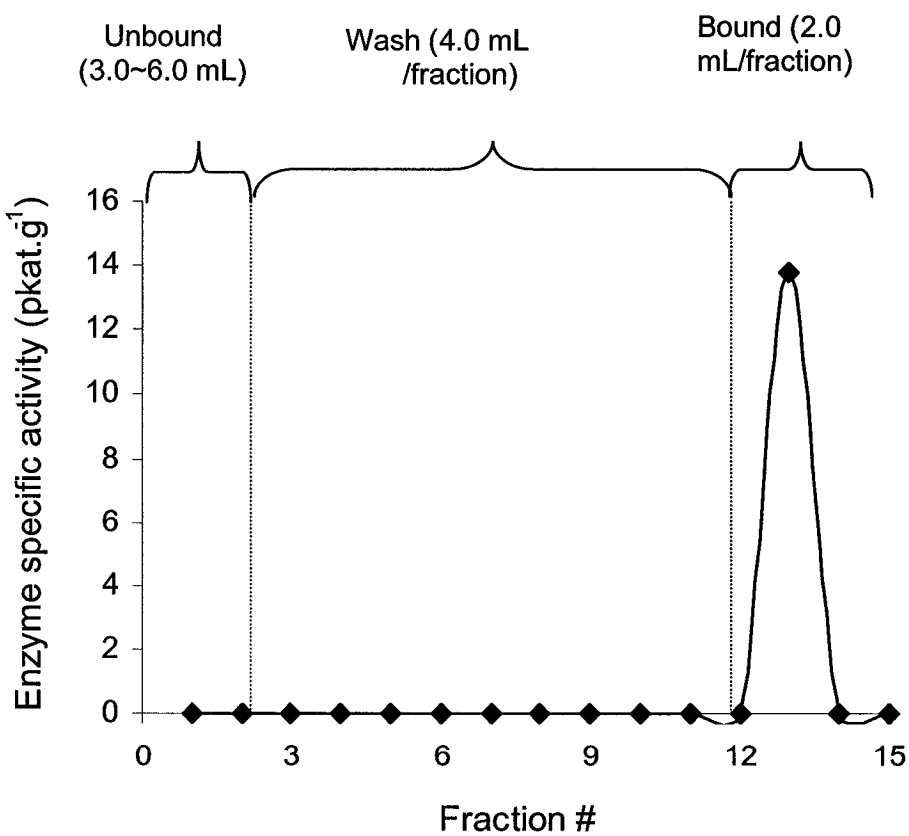


Figure 13 **Coomassie blue stain of Adenosine-agarose Q 3-OMT fraction**

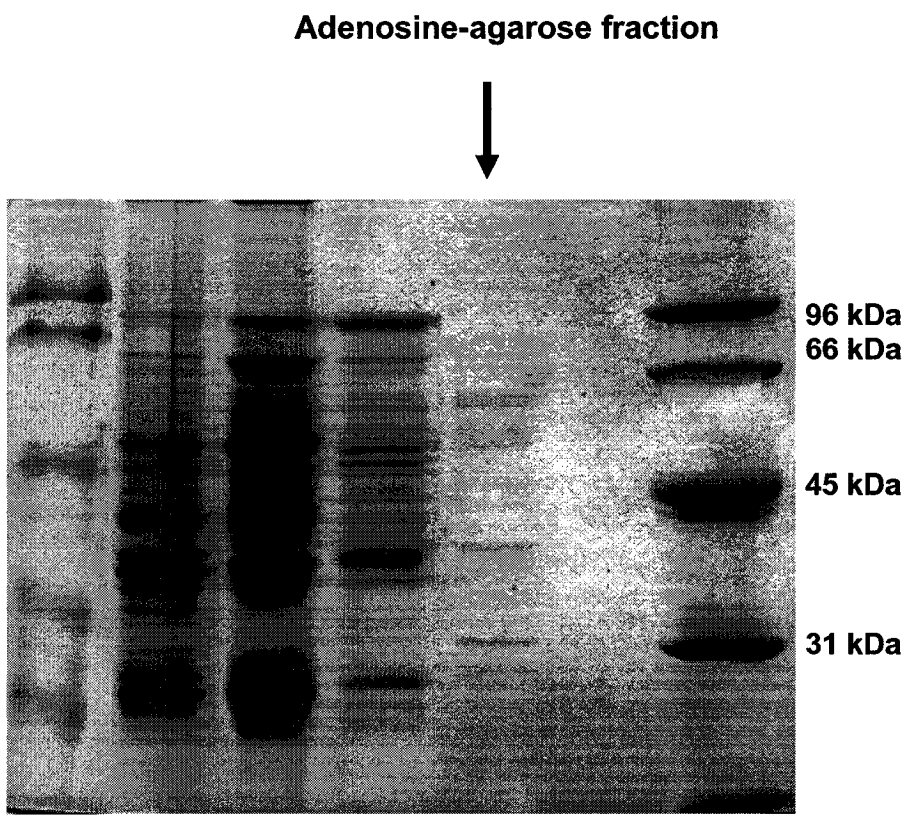
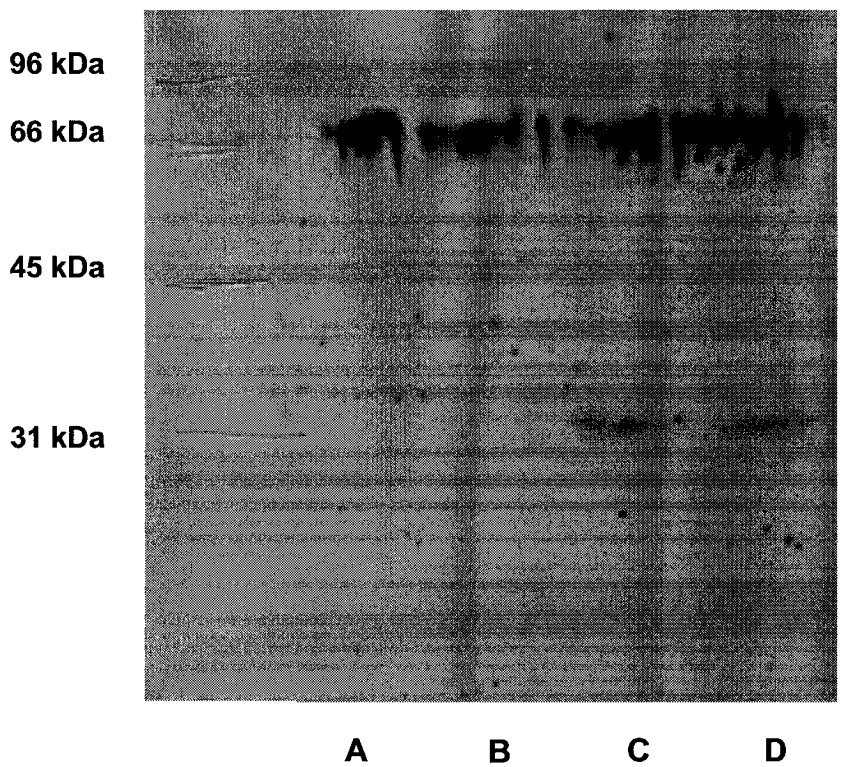
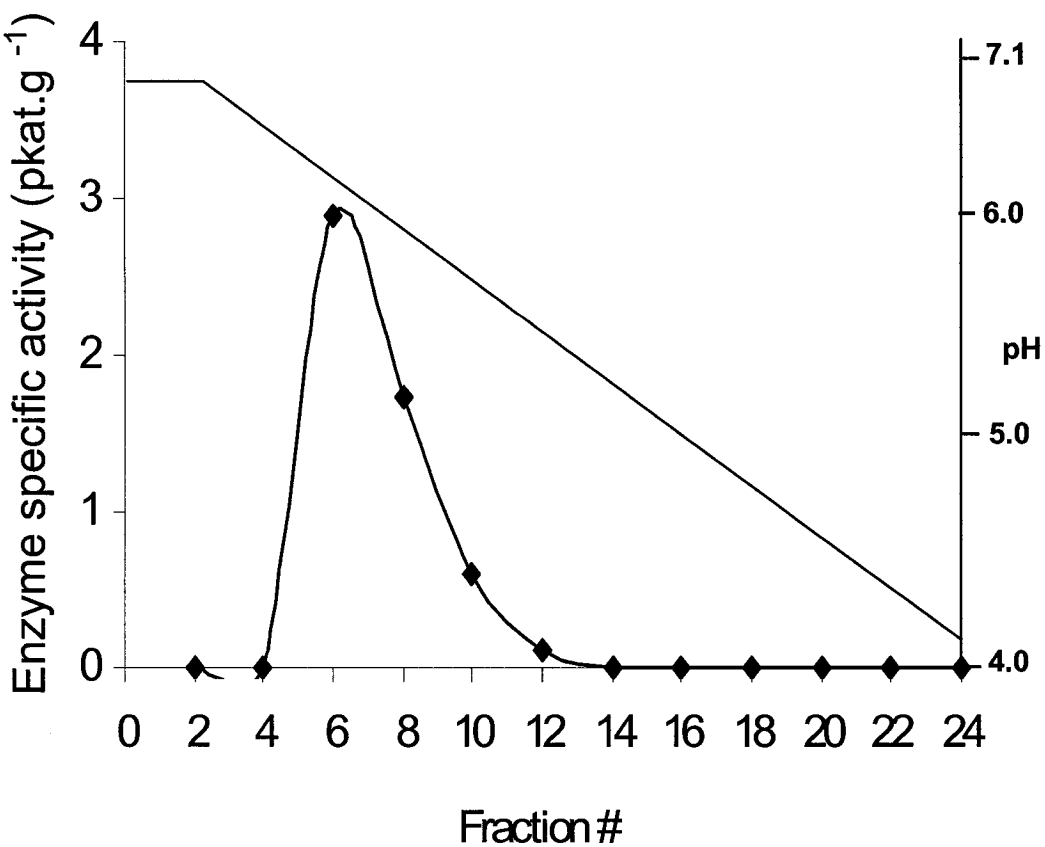


Figure 14 Western blot of Q 3-OMT at different purification steps



Lane A = Crude (after ammonium sulfate precipitation)
Lane B = Superose 12 fraction
Lane C = Mono Q fraction
Lane D = Adenosine-agarose fraction

Figure 15 **Mono P elution pattern of the Q 3-OMT activity**



D.4.4. Effects of divalent cations on the Q 3-OMT activity

The effect of divalent cations at a final concentration of 5 mM (the same concentration used by Rakwal *et al.*, (2000)) were tested on enzyme activity (Table 6). These included $BaCl_2$, $CaCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$. The results of these assays demonstrate that none of the salts increased the enzyme activity by more than 10%, and in some cases the Q 3-OMT enzyme activity was completely lost. The loss of enzyme activity might be due to competitive binding of active sites on the OMT protein between the cation and substrate, since the substrate specificity of flavonol OMTs is based on a preset active site that is specific to the hydrogen bond donors and acceptors (Zubieta *et al.*, 2003).

D.4.5. Effects of *p*CMB and EDTA on Q 3-OMT activity

Investigation of the effect of the SH-group inhibitor, *p*CMB on 3-OMT activity (Table 7) showed a complete inhibition of activity (100%) in the presence of 1 mM *p*CMB, and by 90% in presence of 0.1 mM of the inhibitor. However, the addition of 140 mM β-mercaptoethanol (10-folds higher than the usual amount added in the buffer) in the 0.1 mM inhibitor assay led to a recovery of 16% of the Q 3-OMT enzyme activity, indicating the requirement of disulfide bonds for catalytic activity. The addition of 1 mM EDTA, a metal chelator, resulted in an increase of 17% in enzyme activity, indicating the lack of metal requirement for 3-OMT enzyme activity and the removal of activity inhibition caused by the presence of metal ions on the enzyme.

D.4.6. Substrate specificity of the Q 3-OMT

The Q 3-OMT accepts quercetin as its substrate with the highest specific enzyme activity, yielding 3MeQ as the flavonol product. The identity of the reaction product was confirmed both by TLC (Fig. 16) and HPLC (Fig. 17). However, the enzyme also utilizes

Table 6 Effect of divalent cations on Q 3-OMT activity

Divalent salt (5 mM)	Relative activity[a] (%)
None	100
$BaCl_2$	104
$CaCl_2$	8
$CuCl_2$	0
$MgCl_2$	107
$MnCl_2$	75
$ZnCl_2$	0

[a] Relative to the Q 3-OMT activity with Q as substrate using the Mono Q protein fraction (3.26 pkat g^{-1})

Table 7 **Effects of the SH-group inhibitor, *p*CMB, and metal chelator, EDTA, on Q 3-OMT activity**

*P*CMB (mM)	Relative activity[a] (%)
1	0
1 + 140 mM β-mercaptoethanol	0
0.5	0
0.5 + 140 mM β-mercaptoethanol	8
0.1	2
0.1 + 140 mM β-mercaptoethanol	16
EDTA (mM)	
10	0
1	17

[a] Relative to the Q 3-OMT activity with Q as substrate using the Mono Q protein fraction (3.26 pkat g^{-1})

other flavonol aglycones as well as a number of partially methylated quercetin derivatives as substrates (Table 8). Kaempferol, K, (3,5,7,4'- tetrahydroxyflavone), a flavonol aglycone with one less B-ring hydroxyl group than Q exhibited a similar activity (93% of control), but with a higher V_{max} value than Q. On the other hand, galangin (3,5,7-trihydroxyflavone), a flavonol with no B-ring hydroxyl groups, was a less efficient methyl acceptor with 60% activity of Q. Myricetin, M, (3,5,7,3',4',5'-hexahydroxyflavone), a flavonol aglycone with a trihydroxy B-ring resulted in 46% of Q. Interestingly, isorhamnetin (3'-methylquercetin) and tamarixetin (4'-methylquercetin) were good methyl acceptors, with enzyme activities of 88 and 85%, respectively. These results suggested that modifications of the B-ring substititution pattern have a minor effect on the potential of these compounds as substrates for the flavonol 3-OMT. Rhamnetin (7-methylquercetin) exhibited the lowest enzyme activity (13%) among the flavonol compounds tested. Conversely, flavonols with a hydroxyl group located at positions 6 or 8, also exhibited lower methyl acceptor abilities (Table 8). In addition to these flavonols, several other flavonoid compounds were assayed against the Q 3-OMT. These include 3-methylquercetin, two flavones (luteolin and apigenin), one flavanone (naringenin), two phenylpropanoids (5-hydroxyferulic acid and caffeic acid), and one phenylpropanoid CoA ester (caffeoyl CoA) with final concentrations ranging from 10 to 100 μM. None of these compounds served as methyl acceptors at a final concentration of 10 μM, except for naringenin with a 5% activity at a final concentration of 100 μM (Table 8).

Table 8 Substrate specificity of 3-OMT

Compounds[c]	Relative activity[a] (%)	K_m Value (μM)	V_{max} (pkat/mg)	V_{max}/K_m
Control[a]				
Quercetin (Q)[b]	100	12	61	5.1
Flavonol aglycone:				
Galangin	60			
Kaempferol (K)[b]	93	13	169	13.0
Myricetin (M)[b]	46	7	15	2.1
Monomethylated quercetin:				
3-Methylquercetin	0			
Rhamnetin	13			
Isorhamnetin	88			
Tamarixetin	85			
6-OH or 8-OH substituted flavonol aglycones:				
Quercetagetin	39			
Gossypetin	38			
Herbacetin	33			
Flavonoid:				
Luteolin	0			
Apegenin	0			
Naringenin	5			
Phenylpropanoid:				
5-Hydroxyferulic acid	0			
Caffeic acid	0			
Phenylpropanoid CoA ester:				
Caffeoyl CoA	0			

[a] Relative to the 3-OMT activity against Q with the Mono Q protein fraction (3.26 pkat g^{-1}).
[b] V_{max} and K_m values were determined for these compounds only.
[c] See Appendix 1 for the chemical structures of flavonoid compounds listed in the table.

D.4.7. Identification of Q 3-OMT reaction product by TLC

The identity of the assay product of Q 3-OMT against Q was revealed by TLC by the characteristics of the radioactive spot (Fig. 16). The reaction product was co-spotted with three references compounds, Q, 3MeQ, and 3,4'-dimethylquercetin on the TLC plate for comparison of their R_f values. The R_f value of the radioactive spot generated by the Q 3-OMT reaction product is exact to that of 3MeQ (Table 9), identifying the reaction product is 3MeQ. Exposure of the TLC plate under the UV light (λ_{max} 366 nm) revealed the reaction product as a dark brownish/purple spot in contrast to the bright yellow color of the substrate. This provided further support that the reaction product is 3MeQ, since methyl substitution at position 3 of the flavonol aglycone, Q, results in a color change from yellow to dark brownish/purple fluorescence in UV light (Markham, 1982). In addition, the presence of a single radioactive spot illustrates the enzyme catalyzes a single methylation reaction.

D.4.8. Identification of Q 3-OMT reaction product by HPLC

The identity of the reaction product of Q 3-OMT against Q was further confirmed by HPLC analysis. The HPLC profile of the reaction product was compared to that of the reference compounds, Q and 3MeQ (Fig. 17A). The methylated reaction product (Fig. 17B) exhibited a retention time (R_t) of 22.3 min. with $\lambda_{max}^{MeOH+HOAc}$ values of 254, 360, which compared well with the values obtained for the reference compound 3MeQ. In Fig. 17B, a small peak was observed immediately before the reaction product peak with a R_t of 19.8 min. and $\lambda_{max}^{MeOH+HOAc}$ 254, 370. These values correspond to those of the reference compound Q, identifying this small peak as the remaining substrate, Q.

Figure 16 **The reaction product of Q 3-OMT against Q**

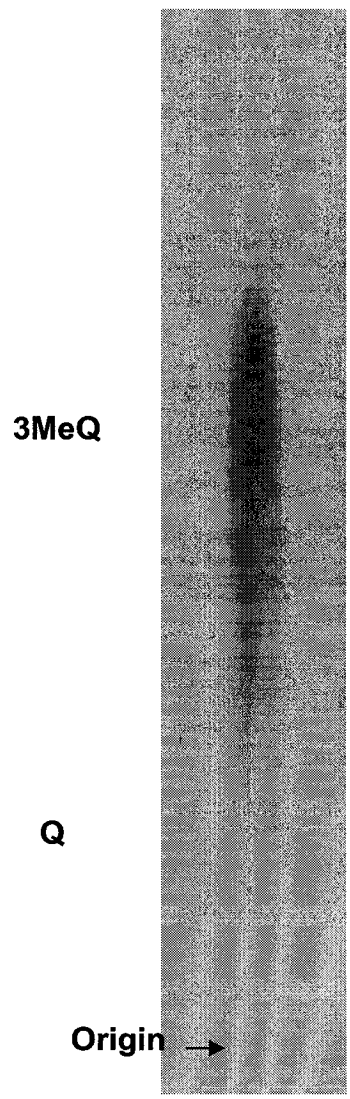
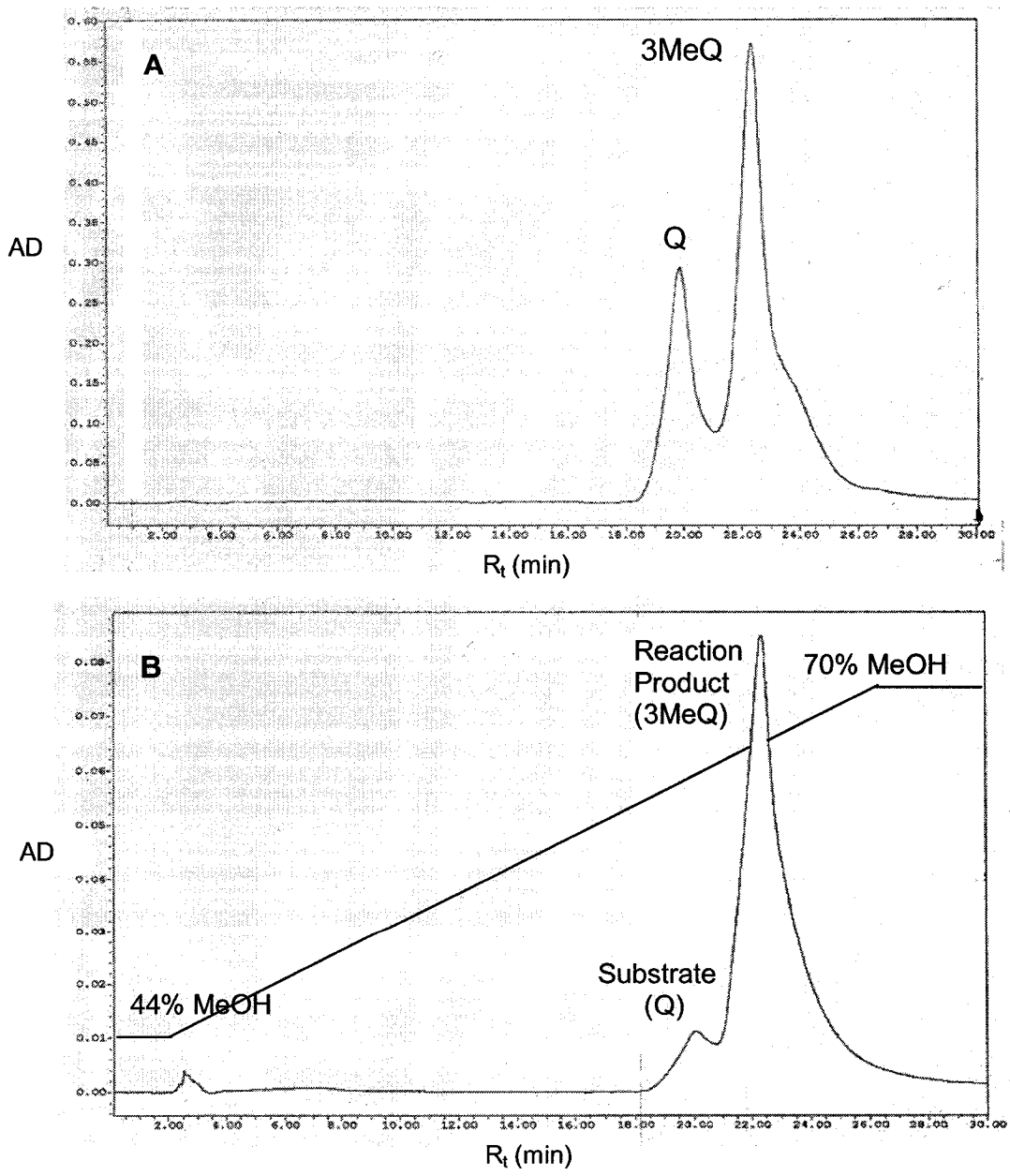


Table 9 **(A) R_f values of reference compounds: Q, 3-methylquercetin, 3,4'-dimethylquercetin (B) R_f value of reaction products against Q**

(A)	**Reference compounds**	**R_f value**
	Quercetin	0.43
	3-methylquercetin	0.8
	3,4´-dimethylquercetin	0.87
(B)	Reaction product	
	with Q as susbstrate	0.8

Figure 17 **(A) HPLC profile of the reference compounds, Q and 3MeQ, (B) HPLC profile of the reaction product of Q 3-OMT against Q**



D.4.9. Substrate interaction kinetics of Q 3-OMT against Q

Both Lineweaver-Burk plots of substrate interactions (Fig. 18A and B) of AdoMet versus Q and Q versus AdoMet showed intersecting patterns. Using the equation obtained from the respective replots of the intercept and slope values, the calculated K_m values of the substrate and co-substrate, quercetin and AdoMet were 12 μM and 45 μM, respectively. The V_{max} value of quercetin was 61 pkat mg^{-1}. All kinetic parameter values, including the turnover rate and enzyme efficiency, are summarized in Table 10.

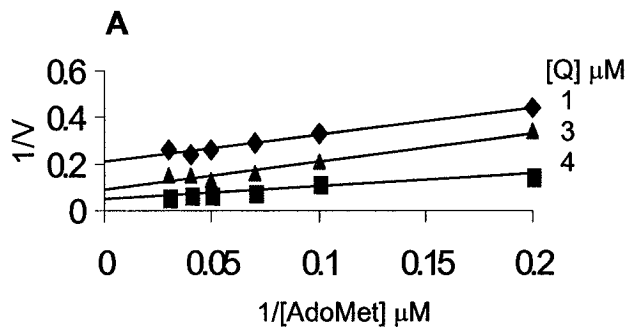
D.4.9.1 Substrate interaction kinetics of 3-OMT against K and M

Substrate kinetics studies of K and M produced an intersecting pattern in their Lineweaver-Burk plots (Fig. 19A and B). The calculated K_m values of K and M were 13 μM and 7 μM, respectively, and their respective V_{max} values were 169 and 15 pkat mg^{-1}. Compared to the values of Q, the 3-OMT has the highest V_{max} value towards K, which was not expected since *S. tinctoria* was not known to synthesize or accumulate monomethylkaempferol.

D.4.9.2 Product inhibition studies of Q 3-OMT against Q

Lineweaver-Burk plots of product inhibition results-data exhibited competitive inhibition patterns between AdoMet and AdoHcy (Fig. 20A). Non-competitive, mixed-inhibition patterns were observed between Q and 3MeQ, Q and AdoHcy, as well as AdoMet and 3MeQ (Fig. 20B to D). The exclusive competitive inhibition among the four combinations of substrates and products was observed between AdoMet and AdoHcy. The pattern of the product inhibition studies of Q 3-OMT fits the profile of an ordered bi-bi reaction mechanism. Where the two compounds that illustrated competitive interactions are the first and last compounds in this mechanism. Thus, it was proposed

Figure 18 **Lineweaver-Burk plots of substrate interactions: (A) AdoMet versus Q and (B) Q versus AdoMet**



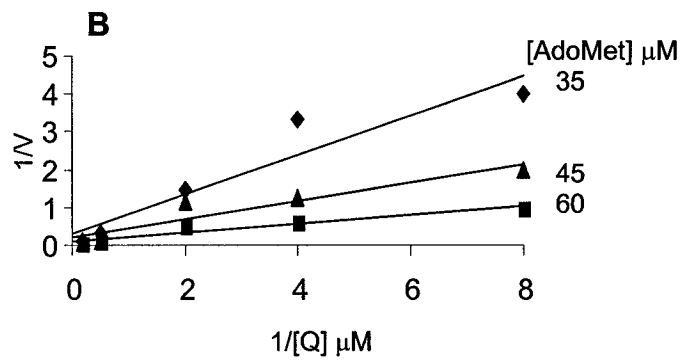


Figure 19 **Lineweaver-Burk plots of substrate interaction kinetics of: (A) AdoMet versus K and (B) AdoMet versus M**

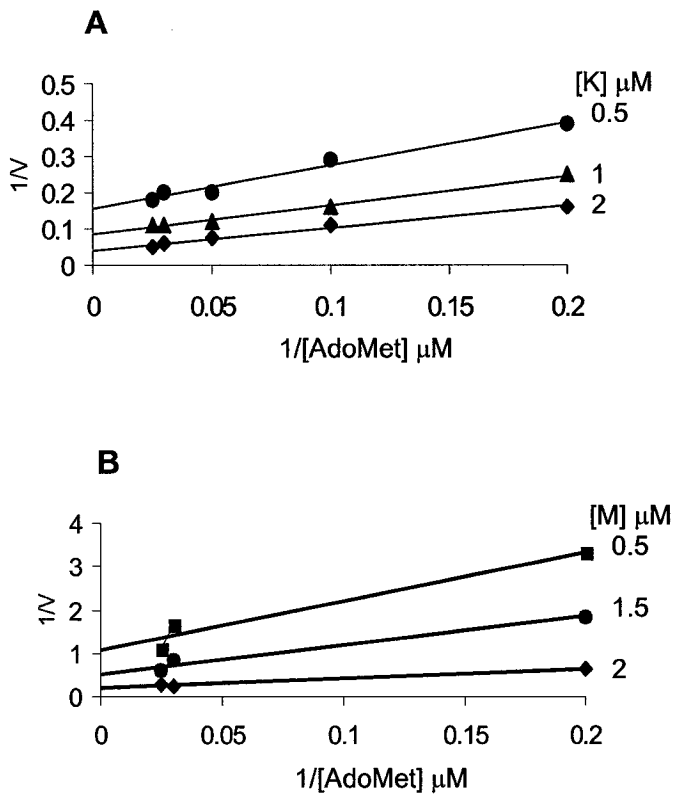
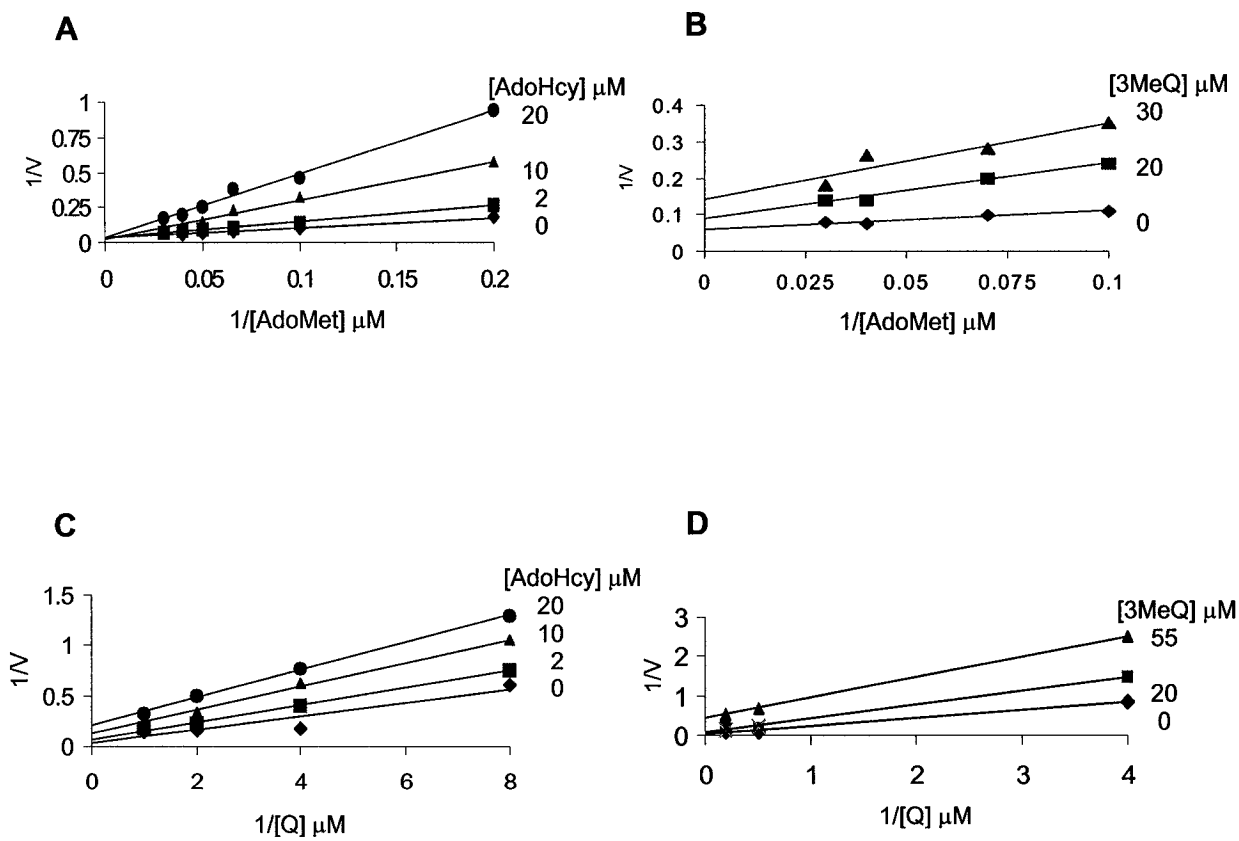


Figure 20 **Lineweaver-Burk plots of product inhibition studies: (A) AdoMet versus AdoHcy, (B) AdoMet versus 3MeQ, (C) Q versus AdoHcy, and (D) Q versus 3MeQ**



that AdoMet is the first substrate to bind since the enzyme was shown to bind the adenosine-agarose affinity column, and AdoHcy is the last product to be released from the enzyme. The non-competitive, mixed-inhibition interactions between Q, 3MeQ, and AdoHcy suggested the involvement of both direct as well as indirect competitiveness in the reaction, which places Q as the second substrate to bind, and 3MeQ as the first product released (Fig. 21). The kinetic values obtained from the replots of the Lineweaver-Burk intercept and slope values are listed in Table 10.

D.4.10. LC-MS/MS of the Q 3-OMT

The analysis of the partially purified Q 3-OMT protein by Matrix-Assisted Laser Desorption Ionization (MALDI-Qtof) mass spectrometry yielded several polypeptides, six of which were assigned highest MASCOT score. These peptide fragments range from 7 to 12-amino acids in length, exhibiting high homology to a number of Group II OMTs in the MASCOT database (Table 11). These OMTs include four catechol OMTs from *Thalictrum tuberosum* (Accession number AF064693.1 to AF064696.1), two catechol OMTs from *Nicotiana tabacum* (S36403, S36404), two COMTs from poplar (Q43047, Q41086), two unknown function OMTs from *A. thaliana* (NM_124796.2, NP_200227), one COMT from *Catharanthus roseus* (Q8W013), one flavonol OMT from *C. americanum* (Q42653), and one COMT from *Ocimum basilicum* (Q9XGV9). Among these peptides, peptides 3 and 4 exhibited 100% and 83% identity to the analogous regions of the *C. americanum* querceitn 3′/5′-OMT. However, none of these peptides aligned within any of the previously reported five OMT conserved motifs (Ibrahim *et al.*, 1998). Rather, they were mostly distributed within the N-terminal half of the protein sequence, with the exception of peptide 6, which aligned near the C-terminal (Fig. 22).

Figure 21 **Q 3-OMT kinetic reaction mechanism**

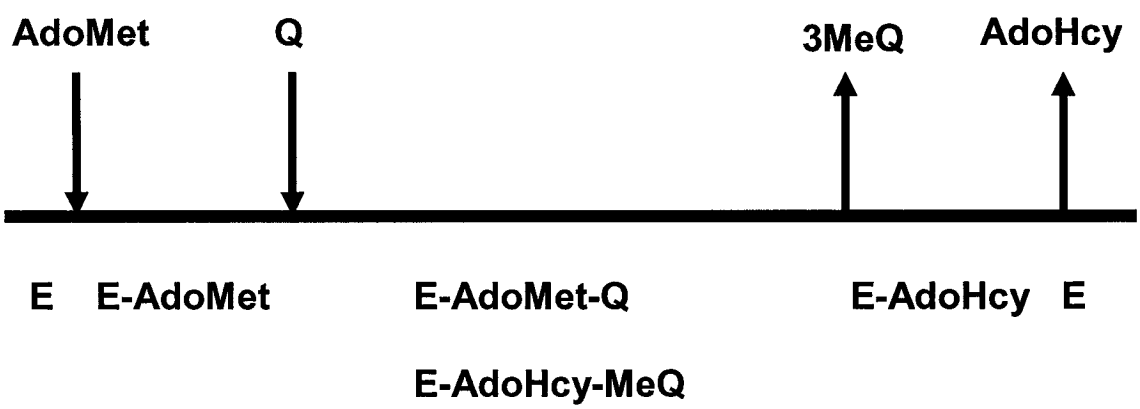


Table 10 Kinetic parameters of the flavonol Q 3-OMT against Q as substrate

Substrate A	Substrate B	Parameter value		SD[b]
AdoMet	Q	K_a	45 μM	0.001
		K_{ia}	2.2 μM	—
		K_a/K_{ia}	20	—
		K_b	12 μM	0.259
		K_{ip}[a]	21 μM	—
		K_{iq}[a]	3.3 μM	—
		$V_{max(Q)}$	61 pkat mg^{-1}	0.008
		K_{cat}/K_m	15.8 μM^{-1} s^{-1}	—

[a] K_{ip} and K_{ip} represent the inhibition constants for 3-MeQ and AdoHcy, respectively.
[b] SD, standard deviation calculated according to Zar (1984).

Table 11 **Q 3-OMT peptides obtained from LC-MS/MS**

Q 3-OMT peptide
1. TTMMHRLK
2. ICRLLER
3. VLMESWYHLK
4. GMSDHSTMSMKK
5. GVDIILAALPK
6. VIALIHK

Figure 22 **Global amino acid sequence alignment of the Q 3-OMT peptides with the flavonol 3'/5'-OMT from *C. americanum* (Accession number Q42653)**

MLFAMQLACASVLPMVLKSAIELDLLEIIRGQDTCMSPT

EIASHLPTTNPDA**PAMVDRIL**RLLSCYSVVT

CSVRSVDDQRVYGLAP**VCKYKNQ**DGVSIAALCLMNQDK

VLMESWYHLKDAVLDGGIPFNKAYGMSSFEGTDPRFNK

VFNR**GMSDHSTITMKK**VFQTYQGFQGLTSLVDVGGGTGAT

LTMILSKYPTIRCINFDLPHVIEDAPEYPGIEHVGGDMFV

SVPKGDAIFMKWICHDWSDEHCLKLLKNCYDALPNNGKVI

LAECILPEVPDSSLATKGVVHID**VITVAHN**PGGKERTEKE

FEALAKAAGFQGFQVFCNAFNTYEIEFSKK ICN

E. DISCUSSION

The flavonol 3-OMT was isolated and characterized from *S. tinctoria* catalyzes the 3-*O*-methylation of quercetin, a flavonol aglycone that is commonly occurring in plants, giving rise to its 3-methyl derivative. 3-Methylquercetin is known for its roles as a specific inhibitor of viral RNA replication (Castrillo and Carrasco, 1987), as an anti-inflammatory and antiviral agent (Middleton and Kandaswami, 1993; Malhotra *et al.*, 1996), and as a phytoanticipin in tobacco plants in response to wounding and insect herbivory (Roda *et al.*, 2003). These important properties of 3-methylquercetin prompted us to purify and characterize the enzyme that is responsible for its biosynthesis, with the aim of obtaining useful peptide information to be utilized in the cloning of its gene and expressing the gene product in transgenic plants. Experssional and wounding studies of these plants could provide information regarding the antiviral activities of the 3-OMT and what role it may have in plant defense response. Furthermore, it would also be interesting to see wheather by inclduing these plant as part of a diet for viral infected mice could improve their health condition.

S. tinctoria synthesizes 3-methylquercetin as an intermediate in the biosynthesis of 3,3´-dimethylquercetin, the major flavonol that accumulates in the plant. The flavonol 3-OMT has been partially purified using a three-step purification process, including a gel filtration (Superose 12), an anion exchanger (Mono Q), and an affinity chromatography (Adenosine-agarose) resulting in a 194-fold increase in its specific activity with a final recovery of 0.12%. The fact that 3-OMT is found in the supernatant of tissue homogenates suggests that the enzyme is a cytosolic protein like those previously reported plant OMTs. Chromatography on the anion exchanger, Mono Q, was the key step that separated the 3-OMT activity from those of 3MeQ 3'- and 5-HFA OMTs, thus demonstrating that these three OMTs have distinct ionic properties.

Substrate specificity studies of the flavonol 3-OMT revealed an exclusive substrate acceptance of flavonols. Its most preferred substrate appears to be kaempferol as shown in Table 8, where it has the highest V_{max}/K_m ratio. However, since there has been no report of methylated kaempferol accumulation in *S. tinctoria*, kaempferol was therefore not considered as the most preferred substrate of *S. tinctoria* flavonol 3-OMT. Rather, based on the comparison of all the enzyme specific activities listed in Table 8, it was quercetin that was the most perferred substrate of flavonol 3-OMT. In addition, the 3-OMT also accepted a number of the monomethyl derivatives of quercetin as its substrates. Of all the substrates used by the 3-OMT, only the reaction product with quercetin was identified through TLC and HPLC analyses (Fig. 16 and 17) because of the availability of the reference sample, 3-MeQ. The chromatographic and UV spectral properties determined for the reaction product are uniquely characteristics of a 3-methylated quercetin (Harborne, 1967; Markham, 1982).

The ability of 3-OMT to utilize kaempferol, isorhamnetin, tamaraxin, galangin, myricetin, and rhamnetin (arranged in descending order of activity) as substrates may be due to the presence of a free hydroxyl group at position 3 that is available on all these compounds as indicated by the lack of activity with flavones and flavanones. Assays of 3-OMT with quercetagetin and gossypetin both exhibited a 3-fold decrease of enzyme activity as compared to that of Q, possibly due to the presence of 6- or 8-OH. Based on the different compound structures tested in substrate specificity studies, it was concluded that modifications at A-ring drastically decreases enzyme activity, whereas changes of substitution pattern in B-ring has little effect on the ability of the 3-OMT to utilize the compound as a substrate. The fact that caffeoyl CoA was not a methyl acceptor confirms the enzyme is a member of Group II OMT, in spite of its relatively low molecular mass.

The general physico-chemical properties of the flavonol 3-OMT are similar to those of other flavonol OMTs (Forkmann and Heller, 1999). However, the *S. tinctoria* 3-OMT has a slightly higher *pI* value (6.0 vs. 4.8) and a smaller molecular mass (31 kDa vs. 57 kDa) than those previously reported from *C. americanum* 3-OMT (De Luca and Ibrahim, 1985a). The higher *pI* value of *S. tinctoria* 3-OMT indicates a lower abundance of acidic residues, whereas the higher molecular mass value reported for the *C. americanum* 3-OMT may due to the non-specific binding of other proteins.

The low molecular mass of 3-OMT renders it the smallest flavonol OMT reported to date among the Group II OMTs (Forkmann and Heller, 1998). Its size resembles those of Group I OMTs, which accept caffeoyl-CoA as the preferred substrate (Joshi and Chiang, 1998). However, the fact that the 3-OMT does not utilize either caffeoyl CoA or caffeic acid, as substrates, eliminates the possibility of it being a Group I OMT or a multifunctional OMT, respectively. Hence, it may be considered as a distinct member of Group II OMT. Further confirmation of the actual molecular mass of this novel enzyme will have to await the cloning of its gene and characterization of the gene product.

Kinetic analysis of the flavonol 3-OMT revealed that this enzyme exhibits a low K_m value for Q, reflecting its low concentration *in vivo*. This is in contrast to the relatively high K_m value for AdoMet, which is commonly utilized as methyl donor in several other metabolic pathways in the plant. The low K_i values for the two reaction products, 3-methylquercetin and AdoHcy, suggest their roles as inhibitors of this enzyme, regulating the two sequential methylations of 3,3′-dimethylquercetin biosynthesis. The kinetics of *S. tinctoria* 3-OMT follows the same pattern as that of *C. americanum*, which is a sequential substrate binding mechanism also known as the ordered bi-bi (Segel, 1975), with AdoMet as the first substrate to bind, followed by quercetin, and the release of products in the reverse order (Fig. 21). The elution pattern of 3-OMT in the adenosine-agarose affinity column further supports this reaction order, since the ligand resembles

the structure of the co-substrate AdoMet. The ordered bi-bi reaction mechanism has been found in plant OMTs that have been characterized to date (De Luca and Ibrahim, 1985b; Jay *et al.*, 1985; Khouri *et al.*, 1988; Khouri *et al.*, 1988; Christensen *et al.*, 1998; Wang and Pichersky, 1998), with the only exception of a recently cloned *4′*-OMT from *D. caryophyllus*, which follows a ping-pong bi-bi mechanism (Curir *et al.*, 2003). Ping-pong bi-bi mechanism is when one substrate binds to the enzyme followed by the release of its corresponding product before the second substrate binds to the enzyme. Comparison of the kinetic replot patterns of *S. tinctoria* 3-OMT and *Chrysosplenium* 3-OMTs demonstrated the same trend in substrate interactions and product inhibition with the exception of AdoHcy and Q. *S. tinctoria* 3-OMT exhibited a non-competitive inhibition between AdoHcy and Q, but the *Chrysosplenium* 3-OMT showed an uncompetitive pattern between the two compounds.

Sequencing of the partially purified protein resulted in several peptide fragments that matched a number of Group II OMT members in the MASCOT database. The majority of these OMT clones are found in plants under stressed conditions, which is not unexpected since 3-methylquercetin functions in tobacco in response to wounding (Roda *et al.*, 2003). Importantly, none of the peptide fragments aligned with the five known conserved regions of OMT (Ibrahim *et al.*, 2000), thus suggesting the advantage of these peptide sequences. Four of the six fragments are from regions that are not conserved in *C. americanum* 3′/5′-OMT and are likely to be highly specific. The peptide sequences obtained from this study are considered as a valuable tool for the design of appropriate probes for cDNA library screenings.

The substrate- and position-specific flavonol OMT derives its novelty from being structurally and functionally a member of Goup II OMTs, in spite of its low molecular mass that is reminescent of Group I members. Cloning and characterization of this gene would allow its introduction into transgenic plants for the constitutive expression of this

enzyme protein. In addition, sequence alignments of the *S. tinctoria* 3-OMT with those involved in plant stress responses could identify conserved regions shared by these OMTs that may assist the identification of potential stress-inducible OMTs in the database.

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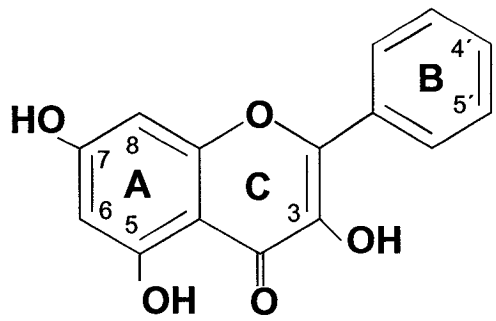
F. APPENDIX 1

LIST OF GENERIC NAMES



3,5,7-trihydroxyflavone

Compound name	Generic name
Galangin	as shown above (3,5,7-trihydroxyflavone)
Kaempferol (K)	3,5,7,4′-tetrahydroxyflavone
Herbacetin	3,5,7,8,4′-pentahydroxyflavone
Quercetin (Q)	3,5,7,3′,4′-pentahydroxyflavone
Myricetin (M)	3,5,7,3′,4′,5′-hexahydroxyflavone
3-Methylquercetin	3-*O*-methyl-5,7,3′,4′-tetrahydroxyflavone
Isorhamnetin	3′-*O*-methyl-3,5,7,4′-tetrahydroxyflavone
Tamarixetin	4′-*O*-methyl-3,5,7,3′-tetrahydroxyflavone
Rhamnetin	7-*O*-methyl-3,5,3′,4′-tetrahydroxyflavone
Quercetagetin	3,5,6,7,3′,4′-hexahydroxyflavone
Gossypetin	3,5,7,8,3′,4′-hexahydroxyflavone