Methyltransferases from *Ruta graveolens* L.:

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ABREVIATIONS

AdoHcy	*S*-adenosyl-L-homocysteine (SAH)
amp	ampicillin
AP	anchor primer
AS	anthranilate synthase
ATP	adenosine-5´-triphosphate
bp	base pares
BSA	bovine serum albumine
CCoAOMT	caffeoyl-CoA *O*-methyltransferase
cDNA	complementary DNA
CM	chorismate mutase
CoA	Coenzyme A
COMT	caffeic acid *O*-methyltransferase
CTP	cytidine-5´-triphosphate
Da	dalton
dCTP	desoxy-cytidintriphosphate
DEAE	diethylaminoethyl
DMA	1,3-dihydroxy-*N*-methylacridone
DMAPP	dimethylallyldiphosphate
DMS	dimethylsulfide
DMSO	dimethylsulfoxide
DMSP	3-dimethylsulfoniopropionate
DNA	desoxyribonucleic acid
dNTP	desoxy-nucleoside triphosphate
dpm	desintegration per minute
DTT	1,4-dithiothreitol
DXMT	3,7-dimethylxanthine methyltransferase (caffeine synthase)
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
g	gram
GAMT	guanidinoacetate *N*-methyltransferase
GC-MS	gas chromatography-mass specrometry
GNMT	glycine *N*-methyltransferase
GSP	gene specific primer
h	hour(s)
HBMT	betaine homocysteine *S*-methyltransferase
HIC	hydrophobic interaction chromatography
HMT	homocysteine *S*-methyltransferase
HNMT	histamine *N*-methyltransferase
IOMT	isoflavone *O*-methyltransferase
IPTG	isopropyl-ß-D-thiogalactoside
kan	kanamycin
kat	katal
kDa	kilodalton
Ki	inhibition constant
Km	Michaelis-Menten constant
l	liter
LC-MS	liquid chromatography-mass spectrometry

LSC	liquid scintillation counting
M	molar
MCS	multiple cloning site
min	minute(s)
MMT	methionine *S*-methyltransferase
MOPS	3-(*N*-morpholino) propane sulfuric acid
Mr	relative molecular mass
mRNA	messenger ribonucleic acid
MXMT	7-methylxanthine methyltransferases
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
nm	nanometer
NMT	*N*-methyltransferase
OD_{600}	optical density at 600 nm
OMT	*O*-methyltransferase
OOMT	orcinol *O*-methyltransferase
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine ammonia-lyase
PCR	polymerase chain reaction
PEG	polyethylenglycol
PEMT	phosphatidyl ethanolamine *N*-methyltransferase
Pmg	*Phytophthora megasperma* f. sp. *glycinea* (*P. sojae*)
RACE	rapid amplification of cDNA ends
Rf	retention factor
RLM-RACE	RNA ligase-mediated rapid amplification of cDNA ends
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
SAH	*S*-Adenosyl-L-homocysteine (SAH)
SAM	*S*-Adenosyl-L-methionine (SAM)
SAMT	salicylic acid carboxyl methyltransferase
SDS	sodium dodecylsulfate
SEC	size exclusion chromatography
SMM	*S*-methylmetionine
ssp.	subspecies
TLC	thin layer chromatography
TMB	1,3,5-trimethoxybenzene
TPMT	thiopurine methyltranferase
Tween 20	polyoxyethylene 20-sorbitane monolaurate
UTR	untranslated region
UV	ultraviolet
v/v	volume per volume
Vmax	maximum velocity of the reaction
w/v	weight per volume
X-gal	5-bromo 4-chloro 3-indolyl ß-D-galactopyranoside
XMT	xanthosine methyltransferase

Amino acids:

Amino acids	One letter designation	Three letters designation
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

Nucleotides:

Adenine	A
Cytosine	C
Guanine	G
Thymine	T
Uracil	U

1. INTRODUCTION

1.1 *Ruta graveolens* L.

1.1.1 General features

The common rue, *Ruta graveolens* L., also called "herb of grace", belongs to the Rutaceae family, order Sapindales, class Magnoliopsida, division Magnoliophyta. The 1700 species of the Rutaceae family includes herbs, shrubs and trees. The evergreen tropical and subtropical plants are distinguished by their common occurrence of spines and winged petioles. The main characteristics of Rutaceae are the schizogenous or lysogenous secretory cavities containing oil and the intrastaminal nectary disk situated between the stamens and the ovary. From about 160 genera, the most important are *Citrus*, *Choisya*, *Dictamnus*, *Fortunella*, *Melicope*, *Phellodendron*, *Poncirus*, *Ruta*, *Skimmia*, *Tetradium*, *Zanthoxylum*.

Citrus, which includes orange, lemon, grapefruit, lime and tangerine, originates from India and is now spread in most of the subtropical areas. It is cultivated for its berry fruits and has an important economical value.

Most important secondary products of the Rutaceae family are essential oils, alkaloids and coumarins.

Ruta graveolens (**Fig. 1.1**) is an ornamental, aromatic, culinary and medicinal plant. The rue shrubs are 0.6-0.9 m tall, robust, semiwoody and perennial. The stems are woody near the base and herbaceous near the tips. The leaves are pinnately divided, the leaflets sessile and oblong. The flowers are pale greenish-yellow in terminal, corymbose racemes. There are four type flowers with four petals, eight long stamens, four styles, distinct at the base and united upward into a single pistil. The fruit is a four-lobed roundish capsule, each lobe opening into two valves. In the middle of the inflorescence there are five-type flowers. The wild form is *Ruta graveolens ssp. divaricata* (TENORE) GAMS, which is restricted to

Figure 1.1 *Ruta graveolens* L. flower.

Adriatic surroundings. The cultivated form is *Ruta graveolens ssp. hortensis* MILLER (GAMS). The rue spread from around the Mediterranean Sea to Europe, North and South America and Southwest Asia. The preferred sites are warm, rocky areas with basic soils.

1.1.2 Content

*Ruta grave*olens L. contains more than 120 compounds of different classes of natural products such as acridone alkaloids, coumarins, essential oils, flavonoids and furoquinolines.

The essential oils, distributed mainly in the aerial parts accumulate in the specific secretory cavities of the plant and represent 0.2 - 0.7% of the dry aerial parts. The major constituents of the oil are undecan-2-one, nonan-2-one, decan-2-one and tridecan-2-one (De Feo et al., 2002).

There are over 40 coumarins and coumarin derivatives isolated from rue and described so far. The main constituent of the coumarins is the glucoside rutarin (0.9%) (Eilert, 1994). Other common furanocoumarins in rue are bergapten, psoralen, xanthotoxin, isopimpinellin, rutaretin.

The flavonoids are best represented in the common rue by rutin (2-5%). The leafs are rich in gossypetin, kaempherol and isorhamnetin, which contribute to the yellowish colour.

The alkaloids are divided into two distinct groups: furoquinoline and acridone. From the furoquinoline distinctive are skimmianine, gamma-fagarine, dictamnine, kokusagine. From the acridone alkaloids arboritin is common in the aerial parts of the plant while rutacridone and gravacridondiol occur in roots (Eilert, 1994).

1.2 Acridone alkaloids

About one hundred acridone alkaloids have been isolated from various species of the Rutaceae (Maier et al., 1993). Acridones were first isolated in Australia in 1948. They are yellow in colour and fluoresce a bright yellow under UV light, making detection in cell culture and on TLC plates easy. Robinson postulated in 1955 that acridones derive from anthranilic acid and acetate via a polyketo acid.

Ruta graveolens L. has been the object of numerous studies on the biosynthesis of secondary metabolites found in Rutaceae. Among these compounds are coumarins and furanocoumarins, which are synthesized via phenylalanine and furoquinoline and acridone alkaloids synthesized from anthranilic acid. Acridone alkaloids are a class of products restricted only to the Rutaceae family (Bohlmann and Eilert, 1994; Junghanns et al., 1998). These compounds have scientific value because of their antiviral (Yamamoto et al., 1989), and antiplasmodial activity (Queener et al., 1991) that has been shown to be achieved by DNA intercalation. However, this makes the compounds probably too toxic for curative use. In plants, they act as phytoalexines and phytoanticipines, which prevent predation and increase the resistance to microbial invasion (Junghanns et al., 1998).

In plants and cell cultures of *Ruta graveolens* L. acridone alkaloids are expressed and accumulated constitutively. In response to microbial attack, the accumulation of acridone epoxides are synthesized *de novo* and could be increased up to 100 fold within 72 hours in cell cultures *of Ruta graveolens*, while the content of acridones remains constant (Eilert et al., 1984, Eilert and Wolters, 1989).

1.2.1 Biosynthesis of acridone alkaloids

In Rutaceae plants, acidone alkaloids and epoxides as well as coumarins and furanocoumarins, are secondary metabolites derived from aromatic amino acids and consequently from the shikimate pathway. The shikimate pathway branches at chorismate. Chorismate is further channelled by chorismate mutase into the biosynthesis of phenylalanine, tyrosine and furanocoumarins; and by anthranilate synthase in tryptophan, acridone and furoquinoloine alkaloids (**Fig. 1.2**). Thus chorismate is a key intermediate, both chorismate mutase and anthranilate synthase competing for the same substrate.

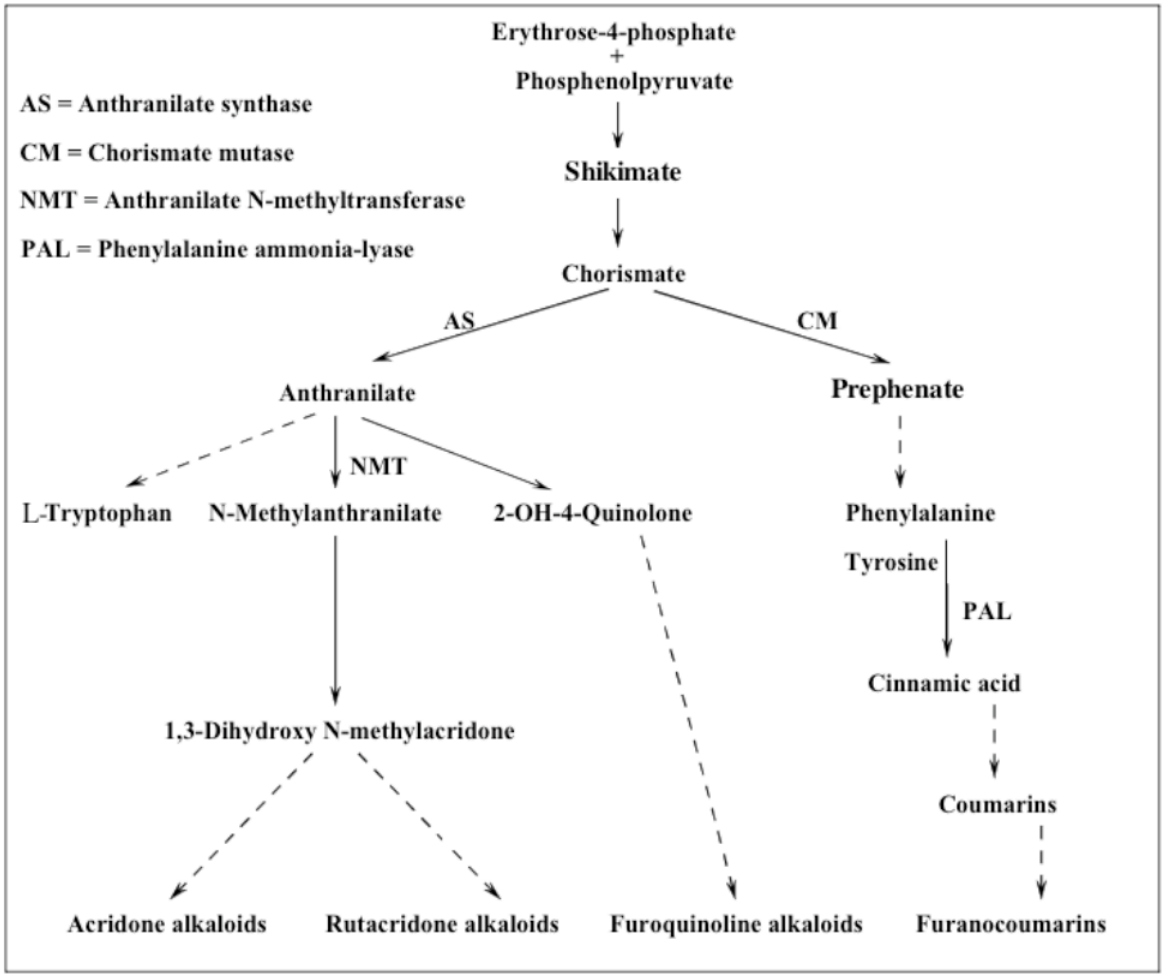

Figure 1.2 Biosynthetic pathways of chorismate derived secondary metabolites in *Ruta graveolens.*

1.2.2 Anthranilate synthase

Anthranilate synthase is a key regulatory enzyme for the biosynthesis of L-tryptophan in the primary metabolism and for the biosynthesis of acridone alkaloids and furoquinoline alkaloids in the secondary metabolism in Rutaceae (**Fig. 1.3**).

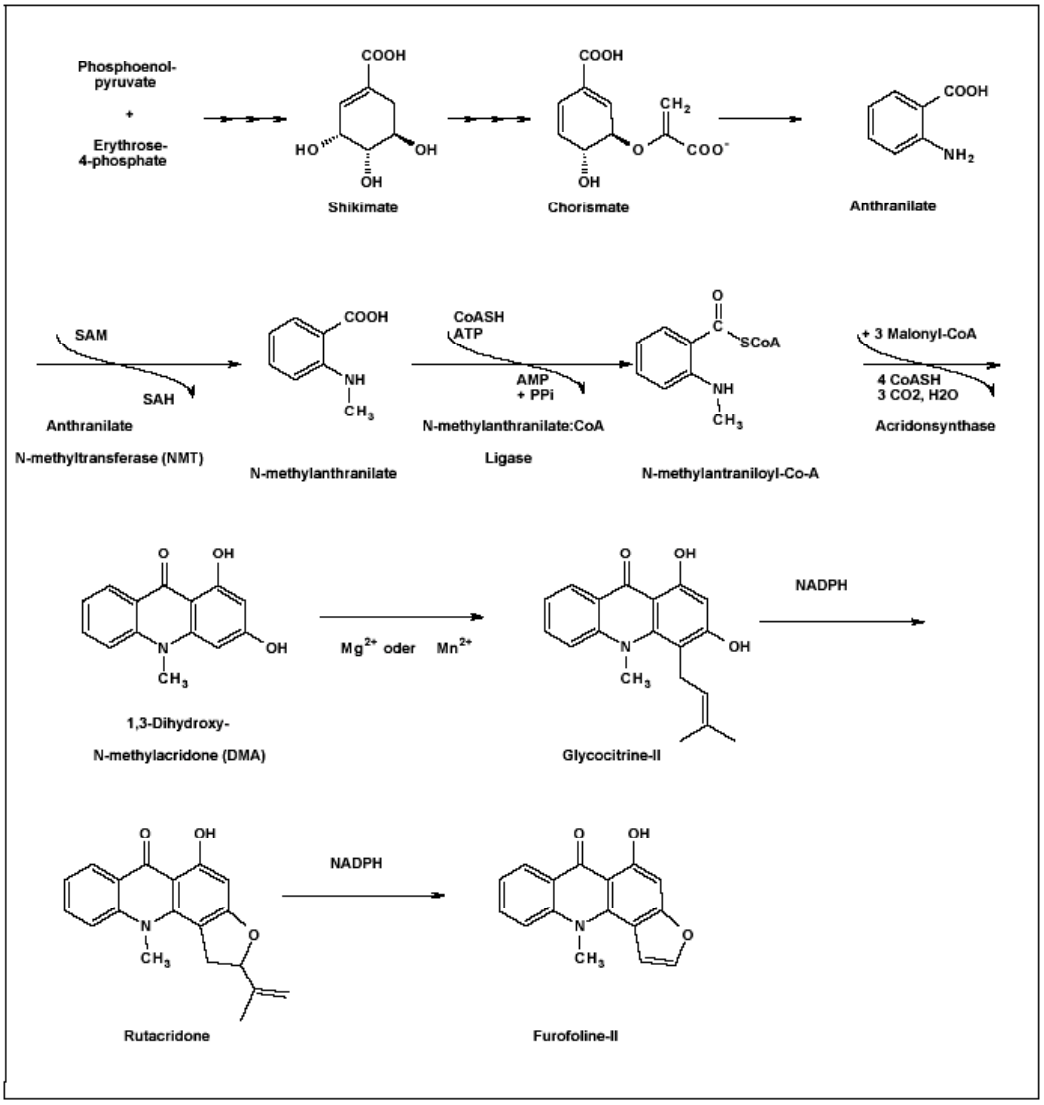

Figure 1.3 The biosynthesis of acridone alkaloids. Chorismate derived from the shikimate pathway is converted by anthranilate synthase into anthranilic acid. Anthranilate is further converted to *N*-methylanthranilate, the first pathway specific reaction to acridone alkaloids, by anthranilate *N*-methyltransferase. *N*-methylanthranilate, activated by CoASH, is converted to *N*-methylanthraniloyl CoA by the corresponding ligase and acridone synthase catalyzes the formation of 1,3-dihydroxy-*N*-methylacridone from *N*-methylanthraniloyl CoA and malonyl CoA. Further prenylation and cyclisation reactions lead to formation of complex rutacridones and furacridones.

Elicitor induced acridone alkaloid accumulation in *Ruta graveolens* was correlated with increased anthranilate synthase activity (Bohlmann and Eilert, 1994). Anthranilate synthase from *Ruta graveolens* was purified, cloned and expressed in *E. coli*. The purified *Ruta* enzyme consists of two non identical subunits α and β. Anthanilate synthase α catalyzes the conversion of chorismate to anthranilate with ammonia as amino donor via a two step reaction, while anthranilate synthase β contains a glutamine amidotransferase domain that donates ammonia from glutamine to anthranilate synthase α. *Ruta* expresses constitutively and equally two genes for anthranilate synthase α, one of which is inducible upon elicitation indicating that the anthranilate flux into the primary and secondary metabolism is regulated at the level of anthranilate synthase (Bohlmann et al., 1995).

1.2.3 Anthranilate *N*-methyltransferase

Methylation of anthranilic acid is the first pathway specific reaction in acridone alkaloid biosynthesis (**Fig. 1.3**). The first evidence which shows the existence of an enzyme responsible for this reaction was observed by feeding *Ruta* cell suspension cultures with labelled anthranilic acid (Baumert et al., 1983a). The formation of *N*-methylanthranilic acid proceed in the presence of *S*-adenosyl-L-methionine (SAM) as methyl group donor (Baumert et al., 1983b).

The enzyme was partially purified and characterized (Maier et al., 1994). The purified enzyme had a molecular weight of 62-70 kDa, a pH optimum of 7.8, was strongly inhibited by *S*-adenosyl-L-homocysteine and exhibited high substrate specificity. However, the purification failed to provide peptide sequences to enable the cloning of the enzyme. Therefore, the amino acid sequence of the enzyme has still not been elucidated.

1.2.4 *N*-methylanthranilate:CoA-ligase

N-Methylanthranilate is activated and converted to *N*-methylanthraniloyl CoA by the corresponding ligase (Baumert et al., 1982). The enzyme *N*-methylanthranilate:CoA-ligase has yet to be purified and characterized.

1.2.5 Acridone synthase

Acridone synthase catalyzes the formation of 1,3-dihydroxy-*N*-methyacridone from malonyl-CoA and *N*-methylanthraniloyl CoA. Acridone synthase from *Ruta* was purified to apparent homogeneity (Baumert et al., 1983). Partial fragments were used used to clone a full size cDNA (Junghanns et al., 1995), and the expression of the enzyme in *E. coli* proved the identity of acridone synthase. The enzyme has a molecular mass of 44 kDa and exhibited significant homology with other plant polyketide synthases (chalcone synthase and stilbene synthase) but yet very strict substrate specificity (Junghanns et al., 1995). Another acridone synthase, called acridone synthase II, was isolated from cell culture of *Ruta* sharing 94% identity with the one identified first (acridone synthase I). Both enzymes form homodimers (Lukacin et al., 1999). Acridone synthase transcript abundance, protein amount and activity were enhanced by treatment with fungal elicitor and suppressed by light irradiation (Junghanns et al., 1998). The distribution of acridone synthase observed by tissue print hybridization showed that the biosynthesis of acridones proceeds at all sites of deposition (Junghanns et al., 1998).

1.2.6 Rutacridone and furacridone

1,3-Dihydroxy-*N*-methylacridone is a key intermediate in the pathway, leading to more complex acridones. The rutacridone formation is accomplished through the prenylation of 1,3-dihydroxy-N-methylacridone with dimethylallylpyrophosphate by a Mn^{2+} (Mg^{2+}) dependent transferase, followed by a cyclisation forming a dihydrofuran with the help of a cytochrome P450-dependent enzyme (Maier et al., 1990). The reaction from rutacridone to furacridone implies a membrane associated enzyme, possibly another cytochrome P450-dependent enzyme (Maier et al., 1993).

1.3 Methyltransferases

S-Adenosyl-L-methionine (SAM)-dependent methyltransferases (EC 2.1.1) catalyze the methylation of many substrates ranging from arsenite to DNA with the release of adenosyl-L-homocysteine. Moreover, the atomic methylation target varies from oxygen, nitrogen, carbon, sulfur to halides (Attieh et al., 1995)

Methyltransferases are important enzymes for they involve in many biological functions such as biosynthesis, metabolism, detoxification, signal transduction, protein repair, chromatin regulation and gene silencing in all organisms from bacteria to mammals.

1.3.1 Plant methyltransferases

There are many plant metabolites which are useful for human health benefits. Among the diverse chemicals, alkaloids and terpenoids are non-phenolic methylated compounds, used by plants to protect themselves against predation. On the other hand, methylated phenylpronanoids and flavonoids play an important role in growth and development. Furthermore, intermediates of the phenylpropanoid pathway such as *p*-coumaric, caffeic, ferulic and sinapic acids act as antimicrobial compounds and regulators of seed germination.

S-Adenosyl-L-methionine-dependent methyltransferases play crucial roles in methylating these metabolites. For example, there are one or two methyltransferase steps involved in the biosynthesis of lignin. Methylation of plant flavonoids belonging to the chalcones, flavones, isoflavones and flavonols is also important because it modifies their solubility, intracellular compartmentation and increases their antimicrobial activity (Ibrahim et al., 1998, and literature citated therein). Finally, methyltransferases also play important roles in the biosynthesis of small volatile phenolic compounds involved in plant defence and pollinator attraction.

1.3.1.1 Plant *N*-methyltransferases

The transfer of a methyl group to amino substrate is catalysed by the small molecule *N*-methyltransferase (NMT) enzymes. NMTs participate in the biosynthesis of alkaloids, osmoprotectants and in regulating the plant resistance to low temperatures.

Alkaloids accumulate in 20% of all plant species, and some of these have pharmaceutical importance. Among this, of special interest is the biosynthesis of caffeine and nicotine, given their huge economical value and the great impact on human health.

The purine alkaloid caffeine is a present in high concentrations in coffee and tea is synthesized from xanthosine via three methylation steps performed by three *N*-methyltransferases: xanthosine methyltransferase (XMT), 7-methylxanthine methyltransferases (MXMT) and 3,7-dimethylxanthine methyltransferase (DXMT) or caffeine synthase. The first enzyme isolated and cloned was XMT (Mosli Waldhauser et al., 1997; Ogawa et al., 2001). Based on a PCR strategy implying gene specific primers created based on the XMT gene sequence, the other two enzymes were isolated, cloned and characterized (Uefuji et al., 2003). In tea leaves, the caffeine synthase was found to perform the last methylation and therefore to convert 7-methylxanthine to caffeine (Kato et al., 1999). Phylogenetic analysis of the *N*-methyltransferases involved in methylation of caffeine precursors placed them in an isolated cluster exhibiting more than 82% identity, and closely related to some carboxyl-methyltransferases rather than other *N*-methyltransferases (Uefuji et al., 2003). Suppression of MXMT activity in coffee plants reduced content of caffeine and theobromine to 30-50% indicating that the enzyme is involved in the major synthetic pathway and thus, the method can be applied to produce decaffeinated coffee plants (Ogita et al., 2004).

In tobacco plants putrescine *N*-methyltransferase catalyzes the first committed step in nicotine biosynthesis. Both putrescine *N*-methyltransferase and spermidine synthase compete for the same substrate. Putrescine *N*-methyltransferase transfers the methyl moiety of *S*-adenosylmethionine to putrescine forming *N*-methylputrescine, a precursor of nicotine, while spermidine synthase uses decarboxylated *S*-adenosylmethionine as aminopropyl donor, thus forming spermidine. Both proteins share a high sequence

homology, putrescine *N*-methyltransferases being more similar to spermidine synthase from mammals and *E. coli* than to other plant *O*- and *N*-methyltransferases. A phylogenetic tree based on the sequence similarity placed putrescine *N*-methyltransferase from tobacco together with different spermidine synthases in a separate cluster distinct from other methyltransferases, suggesting that putrescine *N*-methyltransferases might have evolved from spermidine synthase (Hibi et al., 1994).

After the complete genome of the yeast *Saccharomyces cerevisiae* was sequenced. Niewmierzycka and Clarke (1999) identified a number of methyltransferases with the help of conserved motifs. By disrupting the encoding genes they pointed out the function and importance of these proteins and used this technique also as a method to discover new enzymes. They assigned a new arginine *N*-methyltransferase that catalyzes the transfer of a methyl group to the δ-nitrogen atom of arginine residues. At that time, only an enzyme performing the ω-nitrogen methylation had been described. The newly described δ-*N*-methyltransferase had no significant similarity with other annotated methyltransferases except for guanidinoacetate *N*-methyltransferase which also methylates the δ-nitrogen atom of a guanidino derivative.

Ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) is methylated at the α-amino group of the N-terminal methionine of the small subunit and, in some species, at the ε-amino group of lysine 14 of the large subunit, in the post translation maturation process. In pea, both methylations are found to be performed by only one enzyme and probably in other organisms also by homologous enzymes (Ying et al., 1999). The sequences reported for spinach, pea and tobacco Rubisco methyltransferases encode 55 kDa polypeptides that do not share significant identity with any other reported proteins including diverse methyltransferases.

Coclaurine *N*-methyltransferase that catalyses the transfer of a methyl group from SAM to the amino group of the tetrahydrobenzylisoquinoline alkaloid coclaurine, was first purified from plant tissue of *Coptis japonica* and the gene was isolated based on the partial N-terminal amino acid sequence (Choi et al., 2001; Choi et al., 2002). The 41 kDa protein shows relatively high homology with a protein from *Arabidopsis* and cyclopropane-fatty acyl phospholipid synthase of *Mesorhizobium* and lower homology with

phosphoethanolamine NMT. The deduced amino acid sequence displays only the first conserved motif of methyltransferases with two mismatches located at the *N*-terminal end, the same as the hypothetical protein from *Arabidopsis* and the cyclopropane-fatty acyl phospholipid synthase.

On the other hand, β-alanine *N*-methyltransferase from *Limonium latifolium* shares similarity with other plant *O*-methyltransferases like caffeic acid OMT, chalcone OMT and isoflavone OMT from *Medicago sativa*. β-Alanine betaine is produced by species of the Plumbaginaceae adapted to diverse environmental stress factors including high salinity and hypoxia. β-Alanine *N*-methyltransferase is responsible for the synthesis of the osmoprotectant β-alanine betaine by methylating the non-protein amino acid β-alanine via *N*-methyl and *N,N*-dimethyl β-alanine (Rathinasabapathi et al., 2001). All three methylations are performed by only one enzyme, β-alanine *N*-methyltransferase, suggesting that transgenic plants can be easily obtained by expressing a single gene. β-alanine *N*-methyltransferase was purified from leaves of *L. latifolium* (Rathinasabapathi et al., 2001) and the complete cDNA was acquired based on the partial peptide sequences (Raman and Rathinasabapathi, 2003). Phylogenetic analyses of β-alanine *N*-methyltransferase indicate that it evolved from an ancestral *O*-methyltransferase.

1.3.1.2 Plant *O*-methyltransferases

O-methyltransferases are the most investigated plant methyltransferases. Plant *O*-methyltransferases are divided into two major classes (Joshi & Chiang, 1998). The low molecular weight (23-27 kDa subunits), Mg^{2+}-dependent OMTs which do not accept caffeic acid as substrate were grouped to class I. OMTs (38-43 kDa subunits) that methylate caffeic acid or caffeoyl aldehyde and caffeoyl alcohol (COMTs) independently of Mg^{2+} were designated class II. However, according to the methyl acceptor, the plant *O*-methyltransferases are divided into four classes, with several groups within each class (Ibrahim et al., 1998, and literature therein):

Class A: Methylation of phenylpropanoid compounds

Group 1: caffeic/5-hydroxyferulic acid

Group 2: CoA esters of caffeic/5-hydroxyferulic acid

Group 3: other phenylpropanoids, e.g. coumarins, furanocoumarins

Group 4: other phenolic compounds, including simple phenols, benzoic acids, phenolic esters, etc.

Group 5: polyketides and other acetate/malonate-derived compounds

Class B: Methylation of flavonoids

Group 1: flavonols and flavones

Group 2: chalcones and flavanones

Group 3: pterocarpanes and their isoflavone precursors

Group 4: flavanes and anthocyanins

Class C: Methylation of alkaloids

Group 1: benzylisoquinoline alkaloids

Group 2: morphine alkaloids

Group 3: carboxymethylation of secologanin, lysergic acid, etc.

Class D: Methylation of aliphatic acceptors and carboxymethylation of sugars

A formal structure of a plant *O*-methyltransferase name according with the rules recommended by the Commission of Plant Gene Nomenclature (CPGN) consists of: (a) the species acronym as used in SwissProt, (b) the name of the gene family, and (c) a member number. Methyltransferases genes within a substrate class are divided into "groups" considering the variation of the methyl acceptor molecules. Genes within a group receive a "member number" for each plant species in a sequential/chronological order when a GeneBank/EMBL accession number is provided. For example: Chram. OmtB1.1, is the proposed nomenclature for *Chrysosplenium americanum* flavonol *O*-methyltransferase gene, U16794 (example extracted from Ibrahim et al., 1998). For the induced proteins, the gene name is recommended to receive a suffix: (a) for microbial attack, (b) for environmental stress and (c) for biotic/abiotic elicitors.

1.3.1.2.1 Multifunctional *O*-methyltransferases

Most of the S-adenosylmethionine-dependent *O*-methyltransferases isolated from plants show preferences for aromatic substrates with narrow specificity concerning the pattern of ring substitution (Frick and Kutchan, 1999; Ounaroon et al., 2003) but multifunctional OMTs have also been reported (Chiron et al., 2000; Wein et al., 2002).

Class I of Mg^{2+} dependent plant *O*-methyltransferases (Joshi and Chang, 1998), were associated with methylation of a single substrate, caffeoyl-coenzyme A, and involved in lignin biosynthesis. This classification of "lignin-specific" OMTs on the basis of substrate specificities is not absolute, because an OMT accepting both caffeoyl-CoA and caffeic acid was reported from loblolly pine (Li et al., 1997). More recently, a class I OMT with broader substrate specificity, methylating both flavonoids and phenylpropanoid conjugates was reported from *Mesembryanthemum crystallinum* (Ibdah et al., 2003). For this reason, an *N*-terminal domain was proposed to determine the specificity of class I OMT (Vogt, 2004). Furthermore, a class I OMT from *Ammi majus* was shown to switch substrate specificity towards caffeic and 5-hydroxyferulic acid, when Mg^{2+} was replaced by Mn^{2+} or Co^{2+} in the assays (Lukacin et al., 2004).

Class II OMTs which are related to catechol OMT (Vidgren et al., 1994) appear to be even less discriminative in their substrate preferences. Several of these recombinant OMTs, functionally expressed in *E. coli*, were shown to methylate both phenylpropanoids and flavonoids (Gauthier et al., 1998; Schröder et al., 2002) or benzaldehydes (Kota et al., 2004). However, phylogenetic analysis revealed that the class II OMTs descend into two distinct groups: the first group includes mainly COMTs, preferring caffeic acid as substrate, while the second group designates enzymes with a more diverse range of specificities (Gang et al., 2002; Scalliet et al., 2002).

1.3.1.3 Plant *S*-methyltransferases

Sulfur plays an important role in plant growth and in the regulation of plant development being a macronutrient required for protein synthesis and a structural constituent of several coenzymes and prosthetic groups. Plants have an elaborate sulfur metabolism, and many of them are known to accumulate a variety of sulfur containing compounds. In spite of this

fact, there is not much information about the thiol methylation in plants. In microorganisms and animals, thiol methyltransferases catalyze a variety of detoxification reactions or take part in methionine synthesis.

Plant metabolism differs from that of other organisms by involving the non-protein amino acid S-methylmethionine (SMM). SMM is formed by methylation of methionine by methionine *S*-methyltransferase (MMT) using SAM as methyl donor and can be converted to methionine by donating a methyl group to homocysteine (Hcy) in a reaction catalysed by homocysteine *S*-methyltransferases (HMT). SMM is the first intermediate in the biosynthesis of the osmoprotectant 3-dimethylsulfonopropionate (DMSP) i.e. in salt-tolerant plant *Wollastonia biflora.* In plants that don't accumulate DMSP, the compound is converted into methionine or, by enzymatic hydrolysis, into homoserine and dimethylsulfide (DMS), a compound that may contribute to the aroma of flowers. When converted to methionine, SMM act as a source of sulfur in seeds. It is mainly synthesised in leaves and subsequently translocated to the grains where is recycled to methionine for use in protein synthesis (Bourgis et al., 1999). The SMM cycle in plants is tightly regulated and even if both tissues are able to perform the whole cycle, the flux is essentially from Met to SMM in leaves and from SMM to Met in seeds. Methionine *S*-methyltransferase is a large polypeptide of approximately 115 kDa with no similarity to small methyltransferases. It has been purified from leaves of the salt tolerant plant *Wollastonia biflora* (James et al., 1995) and from germinating barley (Pimenta et al., 1998). Homocysteine *S*-methyltransferase is less investigated than MMT, but activity was found in diverse plants, especially in seeds.

Two thiol methyltransferases described from *Brassica oleracea* (Attieh et al., 2000; Attieh et al., 2002) methylate the hydrolysis products of glucosinolates to volatile sulfur compounds that have putative anti-insect and anti-pathogenic roles. Both thiol methyltransferases are polypeptides of 25 kDa that show more than a 60% similarity with a methyl chloride transferase and two enzymes from *Arabidopsis thaliana* that have no assigned function. The conserved motifs LVPGCGGG, LKPDGEL and GPPY are present in the thiol methyltransferases as well as the chloride methyltransferase. They also possess

a 20-40% identity with partial sequences of some thiopurine *S*-methyltransferases from humans and microorganisms but no similarity to *O*- or *N*-methyltransferases.

1.3.2 Conserved motifs

The first alignment of methyltransferases was performed by Kagan and Clarke (Kagan and Clarke, 1994). They selected 84 sequences representing protein carboxyl methyltransferases, *O*-methyltransferases, *N*-methyltransferases, *S*-methyltransferases, porphyrin precursor methyltransferases, lipid methyltransferases and RNA methyltransferases from diverse organisms ranging from bacteria to humans. Three semi-conserved motifs were proposed as SAM-binding pocket (**Table 1.1**).

Joshi and Chiang (Joshi and Chiang, 1998), revised these conserved motifs in plant methyltransferases. They aligned 56 plant methyltransferases classified into ten distinct groups based on their amino acid sequence similarity and substrate preference including *myo*-inositol, caffeic acid and caffeoyl-CoA *O*-methyltransferases, 24-sterol *C*-methyltransferases, putrescine *N*-methyltransferase, L-isoaspartyl methyltransferases, uroporphyrinogen-III methyltransferases, scoulerine 9-*O*-methyltransferase and RbCL-*N*-methyltransferases. Their proposed conserved motifs I and II had some similarity with Kagan and Clarke's motifs I and II but motif III is entirely different (**Table 1.1**). Also the accuracy of the proposed conserved motifs is higher having a finding success rate of 98-100% with maximum 3 mismatches per motif.

Table 1.1 Comparision of methyltransferase consensus regions by Kagan and Clarke (1994) or Joshi and Chiang (1998).

Motif	Kagan & Clarke 1994	Joshi & Chiang 1998
I	(L/I/V)(V/L)(E/D)(V/I)G(C/G)G(P/T)	(V/I/L)(V/L)(D/K)(V/I)GGXX(G/A)
II	(G/P)(T/Q)(A/Y/F)DA(Y/V/I)(I/F)(L/V/C)	(V/I/F)(A/P/E)X(A/P/G)DAXXXK(W/Y/F)
III	LL(K/R)PGG(L/I/R)(I/L)(V/I/F/L)(V/L/I)	(A/P/G/S)(L/I/V)(A/P/G/S)XX(A/P/G/S)(K/R)(V/I)(E/I)(L/I/V)

Ibrahim et al. (1998) compared only plant *O*-methyltransferases in order to predict a consensus sequence for these proteins. They described five regions (I-V) comprising 36 amino acids rich in glycine and highly conserved (92-100%) close to the carboxy terminus of transferases methylating lignin precursors, flavonols, chalcones, furanocoumarins and benzylisoquinoline alkaloids (**Fig. 1.4).** Motif I, involved in SAM binding, is highly conserved in fungi and bacterial enzymes and partially conserved in the rat catechol *O*-methyltransferase, which also retains some amino acids of motif IV, involved in metal binding, in spite of the low overall amino acid identity. Motifs II and III were identified also as part of the SAM binding site and motif V plays an important role in catalysis (Zubieta et al., 2001). The function of motif IV, for Mg^{2+} binding was elucidated after the crystallisation of rat catechol *O*-methyltransferase. Yet the function of motif IV in methyltransferases that don't need to bind metal ions for activity is still not clear.

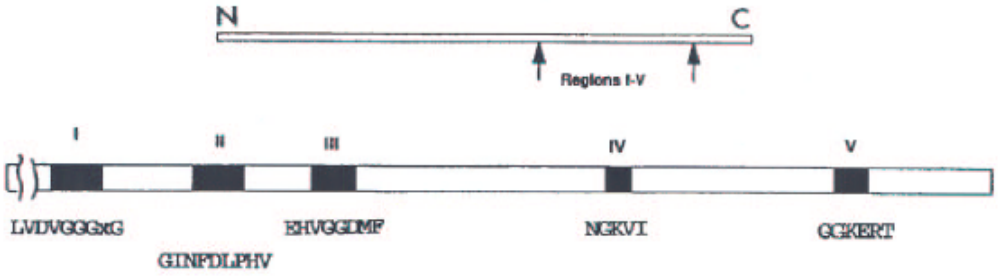

Figure 1.4 The relative position of the conserved motifs (I-V) in *O*-methyltransferase polypeptides (top) and the consensus sequences of these regions (bottom) after Ibrahim et al. (1997).

1.3.3 Active site

SAM is the second most widely used enzyme substrate after ATP. Evolution has independently achieved SAM-dependent methyltransferase activity at least five times, reflected in five analogous and structurally distinct enzyme families known to date (class I to V). SAM-dependent methyltransferases families are an example of functional convergence. All of the plant SAM-dependent methyltransferases along with most of the SAM-dependent methyltransferases structurally characterised belong to class I. The structures of these enzymes are very similar comprising a seven-stranded betasheet flanked by three alphahelices on each side that form an $\alpha\beta\alpha$ referred to as „class I"

methyltransferase structure. This structure resembles the nucleotide-binding motif, the Rossmann fold (Rossmann et al., 1973), found in NAD/NADP-dependent dehydrogenases.

The first structurally characterized SAM-dependent methyltransferase was catechol *O*-methyltransferase (COMT) from rat liver (Vidgren et al., 1994) which, coincidently, remains one of the best examples of a prototypical SAM-dependent methyltransferase, which fold with only two minor modifications to the core fold. COMT requires Mg^{2+} to position the two catechol hydroxyls for deprotonation by a lysine residue before attacking the SAM methyl group. The SAM binding site is located in the *N*-terminal part of the betasheet and is formed in part by residues from loops 1, 2 and 3 while the substrate-binding region is positioned in the *C*-terminal part of the betasheet.

The secondary and tertiary structures of catechol *O*-methyltransferase were compared with the secondary and tertiary structures of two DNA methyltransferases, M.*HhaI* and M.*Taq I*, which catalyse the transfer of the methyl group from SAM to carbon-5 of cytosine or nitrogen-6 of adenine. The protein folding of all three enzymes was comparable, while the cofactor binding site was almost identical and the functional residues were found in correponding regions. This was not expected on the basis of the primary structure, indicating that either all SAM-dependent methyltransferases evolved from a common ancestor, or they have converged to an extraordinary extend (Schluckebier et al., 1995).

The first two plant *O*-methyltransferase structures determined were chalcone and isoflavone *O*-methyltransferases. Both proteins are dimers and the dimerisation seems to be crucial for activity. SAM/AdoHcy is bound by equivalent residues in both methyltransferases through hydrogen bonds and van der Waals interactions. The reaction mechanism is independent off Mg^{2+} and proceeds via base-assisted deprotonation of the hydroxyl group by a conserved His residue, followed by a nucleophilic attack of the phenolate anion on the reactive methyl group of SAM. The catalytic His residue is held in a catalytically productive position by hydrogen bonds with a proximal Glu residue and positioned by another Glu or an Asp residue (Zubieta et al., 2001).

The same reaction mechanism is proposed for protein arginine *N*-methyltransferase from rat (Zhang and Cheng, 2003).

The catalytic mechanism described for caffeic acid/5-hydroxyferulic acid 3/5-O-methyltransferase from alfalfa (CAOM) is comparable with that described for chalcone and isoflavone *O*-methyltransferases (Zubieta et al., 2002). The enzyme is active only as a dimer. The dimer excludes solvent and forms the substrate-binding cavity. A catalytic His residue and Glu and Asp residues take part in the catalysis (**Fig. 1.5**). The difference is that the catalytic site is more spacious and the local environment around the reactive hydroxyl group depends more on the general chemical nature of the active site surface and less on precise positioning. This spacious active site allows a certain degree of substrate promiscuity that might be important for *in vivo* biological activity.

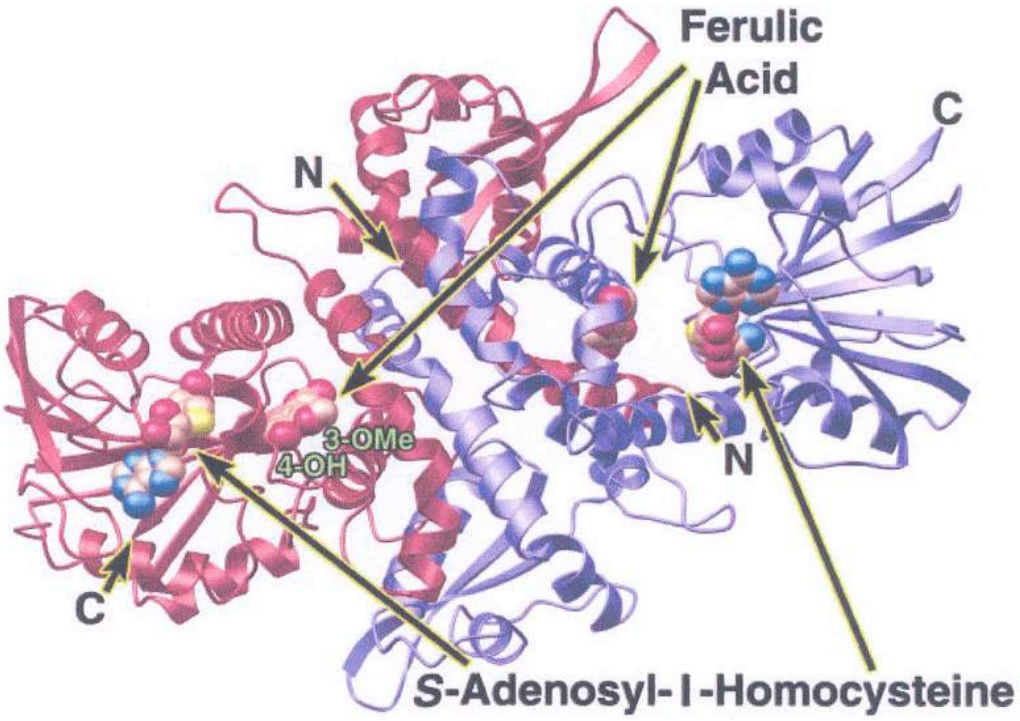

Figure 1.5 Ribbon diagram of the COMT dimer protein backbone from *Medicago sativa*. Ribbon diagram of the COMT dimer in complex with the reaction products ferulic acid and SAH. Monomer A is shown in red, and monomer B is shown in purple. The 3-methoxy and 4-hydroxyl sites of ferulic acid bound to the red monomer (left) are labeled. Ferulic acid and SAH are shown as color spheres. N and C represents the N- and C-terminus respectively.

Some *N*-methyltransferases like DNA N6-adenine, DNA N4-cytosine and protein N6-glutamine methyltransferases have an (D/N/S/)PP(Y/F) motif employed for nitrogens conjugated to a planar system (amide moiety or nucleotide base). Other class I *N*-methyltransferases including protein arginine, glycine and phenylethanolamine *N*-methyltransferases do not contain the DPPY motif. The reaction mechanism uses acid residues to neutralize the positive charge on the substrate amino group.

The structure of betaine homocysteine *S*-methyltransferase (HBMT) from both rat and human were elucidated. The enzyme belongs to the family of homocysteine *S*-methyltransferases that include methylmethionine and SAM homocysteine methyltransferases, methionine synthases and thiol/selenol methyltransferases, all enzymes depending on Zn^{2+} for activity. HBMT is a tetramer composed of identical subunits of 406 or 407 amino acids in human respectively in rat. The structure of HBMT suggests that the conserved motif DG(G/A) is involved in Hcy binding in all members of this enzyme family. The conserved Glu159 residue plays also a role in both binding of Hcy and stereospecificity. Considering the differences in the nature of methyl donor, there are also differences in the amino acid sequences. The structure of BHMT established additional markers for the Zn^{2+} binding site which was previously identified by two fingerprints GVNC and GGCC (Breska and Garrow, 1999). An additional conserved Pro248 was found to be important for the architecture of the Zn^{2+} binding site (Evans et al., 2002), and a Tyr160 was identified as a fourth ligand for the coordination of the Zn^{2+} (Gonzalez et al., 2004) along with the known three Cys residues (Cys217, Cys299 and Cys300).

1.3.4 Importance of methyltransferases in humans

Mammalian small molecule methyltransferases are primarily grouped into two categories according to their biological functions. The first group consists of enzymes which are involved in the biosynthesis and degradation of active amines, such as phenylethanolamine *N*-methyltransferase, hydroxyindole *O*-methyltransferase and catechol *O*-methyltransferase. The second category includes enzymes which participate in metabolic transformations, namely glycine *N*-methyltransferase and guanidinoacetate *N*-methyltransferase.

1.3.4.1 Human *O*-methyltransferases

The methylation of the meta- or para- hydroxyl group of catechols by catechol *O*-methyltransferase (COMT) in the central nervous system is of physiological significance because it affects the catecholamine neurotransmitters dopamine and noradrenaline as well as catechol-type compounds as levo-DOPA. Based on this reaction, a new Parkinson treatment was developed by combining COMT inhibitors and levo-DOPA derivatives.

In human, COMT is produced as both a soluble protein of 221 residues (s-COMT, 25 kDa) and a membrane-bound protein with an additional 50 residues at the *N*-terminus (mb-COMT of 30 kDa, Huh and Friedhoff, 1979). Both proteins are coded by a single gene but separate promoters to initiate their expression (Tenhunen et al., 1994). It is known that a microdeletion on chromosome 22q11 (containing COMT) leads to a lower expression of COMT mRNA, which is associated with schizophrenia in humans (Shifman et al., 2002). Moreover, in the absence of central nervous cardiovascular control, COMT has been shown to regulate blood pressure (Jordan et al., 2002).

Melatonin is an indoleamine synthesized from serotonine by sequential action of two enzymes: arylalkylamine *N*-acetyltransferase and hydroxyindole *O*-methyltransferase. The *O*-methyltransferase plays an important role as the final enzyme in melatonin synthesis. Melatonin was originally identified in the pineal gland. It is an endogenous mediator of photoperiodic information and a molecular component of the circadian timekeeping system (Arendt and Skene, 2005). Biosynthesis of melatonin was reported also in embryonic chicken retina (Ivanova and Iuvone, 2003). In human and mouse ovary, melatonin was associated with a reproductive regulatory activity at the follicular level (Itoh et al., 1999).

1.3.4.2 Human *N*-methyltransferases

Histamine is involved in allergies, regulates stomach acid production, and serves as a neurotransmiter in the central nervous system. Histamine *N*-methyltransferase (HNMT) is the predominant human enzyme for metabolising histamine in bronchial epithelium, and the only way to inactivate the neurotransmitter activity in the brain. HNMT activity in red blood cells exhibits a five fold individual variation, predominantly as a result of a genetic polymorphism. There are many identified mutations of HNMT which are associated with

human diseases. For example, genetically determined variation in HNMT activity might contribute to the pathophysiology of asthma (Yan et al., 2000). Moreover, high levels of HNMT in the frontal cortex is associated with the impaired cognitive function in Pick's disease. Generally, however, it is known that the change of Thr105 to Ile is associated with decreased enzyme activity, immunoreactive protein, and thermal stability of the protein (Horton et al., 2001).

The *N*-methylation of phosphatidylethanolamine by SAM-dependent phosphatidyl-ethanolamine *N*-methyltransferase (PEMT) is the only *de novo* pathway for choline biosynthesis. Choline and its derivatives are important intracellular compounds, for they are involved in cholinergic neurotransmission, transmembrane signalling, and lipid-cholesterol transport. The mammalian phosphatidylethanolamine *N*-methyltransferase is most active in the liver and kidney. It has been shown that when PEMT expression is inactive, mice are not able to reach normal concentrations of all choline metabolites even when provided with supplemental dietary choline, hence, exhibiting hepatic steatosis and apoptosis (Zhu et al., 2003).

Glycine *N*-methyltransferase (GNMT) is an abundant enzyme in the liver (1% of the total soluble protein) and catalyses the transfer of the methyl group of SAM to glycine forming *S*-adenosylhomocysteine and sarcosine. The enzyme is a homotetramer and the amino acid sequence was shown to be conserved in human, rat, rabbit, and pig. Glycine methylation by glycine *N*-methyltransferase is an alternate pathway to regulate the level of methionine and to preserve the ratio SAM/AdoHcy by linking *de novo* formation to the availability of dietary methionine (Ogawa et al., 1998). Leu49Pro and His176Asn mutations in the human GNMT gene are associated with mild liver disease which incapacitates normal methionine metabolism. Therefore, the level of both plasma methionine and plasma SAM increase (Luka et al., 2002). Additionally, down regulation of the GNMT is connected with hepatocellular carcinoma in human (Liu et al., 2003).

Guanidinoacetate *N*-methyltransferase (GAMT) catalyzes the second step in creatinine production, mutations leading to GAMT deficiency are responsible for a developmental delay during the first months of life associated with muscular hypotonia, poor head control, movement disorders, mental retardation, epilepsy and autistic behaviour (Leuzzi, 2002).

GAMT deficiency in GAMT-/- knockout mice was shown to have increased neonatal mortality, muscular hypotonia, decreased male fertility and reduction in body weight especially in females (Schmidt et al., 2004).

1.3.4.3 Human *S*-methyltransferases

Betaine homocysteine *S*-methyltransferases, methionine synthases and *S*-methylmethionine homocysteine *S*-methyltransferase catalyze the transfer of a methyl group to homocysteine to generate methionine. An elevated level of homocysteine is an independent risk factor for cardiovascular diseases (Clarke et al., 1991) but it could also play a role in the aetiology of Alzheimer's disease (Beyer et al., 2003). Moreover, an increasing level of methionine is required for tumour progression (Blackburn et al., 2004). Betaine homocysteine *S*-methyltransferases uses betaine and homocysteine to generate methionine and dimethylglycine as a side product. The reaction takes place mostly in the liver and kidney. Cobalamine-dependent methionine synthase and *S*-methylmethionine *S*-methyltransferase use the methyl group of methyltetrahydrofolate and *S*-methylmethionine of plant origin to generate methionine. All thiol methyltansferases involved in the biosynthesis of methionine require Zn^{2+} for activity; experiments made with Zn^{2+} depleted or mutated enzyme led to loss in enzyme activity (Matthews and Goulding, 1997).

In mammals and microorganisms, the biotransformation of different aliphatic sulfhydryl compounds can be done by *S*-methylation catalyzed by either thiopurine or thiol methyltranferase (TPMT and TMT). TPMT is a soluble enzyme involved in the methylation of aromatic and heterocyclic sulfhydryl compounds, whereas TMT is a microsomal enzyme involved in the methylation of aliphatic compounds. They are localized in different tissues, especially liver and kidney (Drummer et al., 1983). Both enzymes use SAM as methyl donor and are independent of metal ions. They are involved in the biotransformation and detoxification of endogenous and xenobiotic thiols and sulfhydryl compounds with high relevance in drug metabolization (Weinshilboum, 1992). The levels of TMT and TPMT are genetically regulated. This genetic polymorphism is responsible for the induced toxicity and variations in the therapeutic efficiency in patients treated with sulfhydryl drugs (Keith et al., 1983).

1.4 Plant volatile compounds

Many plants, particularly among the Rutaceae, emit volatile methabolites which likely have a physiological function. Ethylene was identified as early as 1934, but, for example, the scent of flowers has attracted attention only recently. Flower volatiles may function as attractants for the natural predators of herbivores, as airborne signal for the neighbouring plants to induce expression of defense-related genes or to deter herbivores (for review see Dudareva and Pichersky, 2000).

Volatiles from vegetative parts also have a variety of roles. The widespread non-floral volatile isoprene, emitted especially by trees, is involved in stabilizing the thylacoid membranes at high temperatures and thus helping the photosynthetic apparatus to recover from short episodes of temperatures in excess of 40^{o}C. The gene for isoprene synthase cloned eases further studies of the role and mode of action of this compound.

Moreover, leaves volatiles are thought to protect the plant against herbivores either directly or by attracting insects that protect the plant against insectivores. For example, some species of the Brazil nut family (*Lecytidiaceae*), which emit high levels of *S*-methylmethionine are less parasitized by wood-destructive beetles than other species of the same family which emit lower amounts of *S*-methylmethionine. An example of indirect defense is the case of the African tree *Leonardoxa africana*. The young leaves of this tree emit a high level of methylsalicylic acid - and it was observed that they are guarded by a species of ants, *Pethalomyrmex phylax,* that attack any phytophagous insect they encounter. The authors assume that the ants are attracted by methylsalycilate and use it either as a pheromone or as an antiseptic for their nests (Debout et al., 2003).

Interestingly, plant volatiles influence not only insects but also the neighbour plants thus activating their defence and reducing the herbivore's damage. Recent investigations show that plants of *Nicotiana attenuata* grown in the vicinity of artificially wounded *Artemisia tridentata*, with air but no soil contact, presented less herbivory damage than the controls grown next to intact plants (Preston et al., 2004). Similarly, *Phaseolus vulgaris* and *Vicia faba*, in contact with volatiles of injured plants had elevated expression of some genes involved in defence metabolism (Colazza et al., 2004).

1.4.1 Methyltransferases involved in methylation of volatile compounds

In general, plant volatiles are derived from three major classes of compounds: terpenoids, phenylpropanoids/benzenoids, and fatty acids that are often modified by methylation, oxidation, acylation, etc. Major progress in plant volatile research has been achieved in the last 15 years thanks to the adoption of simple, sensitive methods for headspace sampling and the accessibility of relatively inexpensive bench-top instruments for GC-MS. Some of the enzymes involved in the biochemistry of scent production are isolated and characterized but this field only starts to expand.

1.4.1.1 Methylation of carboxyl group

Volatile methyl esters such as methylbenzoate, methylcinnamate, methyljasmonate and methylsalicylate are some of the most common components of flower scent. The enzymes and the corresponding genes that catalyse the final steps in the formation of methyl benzoate (*S*-adenosyl-L-methionine:benzoic acid carboxylmethyltransferase, BAMT), methyljasmonate (*S*-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase, JMT) and methyl salicylate (*S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, SAMT) have been isolated and characterized (Ross et al., 1999; Dudareva et al., 2000; Seo et al., 2001). These enzymes form homodimers with the subunit molecular mass between 40-45 kDa that share about 40% sequence identity between them but not with other *S*-adenosy-L-methionine-dependent methyltransferases except a small group of *N*-methyltransferases involved in caffeine biosynthesis (Uefuji et al., 2003). In consequence, these enzymes form a new class of methyltransferases (designated SABATH) as proved also by the three-dimensional structure of SAMT from *Clarkia breweri* (Zubieta et al., 2003). Database searches using SAMT from *Clarkia* and JMT from *Arabidopsis* identified numerous putative proteins.

1.4.1.2 Methylation of hydroxyl group

Many plant volatiles contain methoxyl groups, and methylations are catalysed by related *S*-adenosyl-L-methionine dependent *O*-methyltransferases which share a similar SAM-binding domain and form homodimers from approx. 40 kDa subunits.

Among this, the (iso)eugenol OMT from *Clarkia breweri* and chavicol and eugenol OMTs from *Ocimum basilicum* that catalyse the last steps in the production of (iso)methyleugenol and methylchavicol, respectively, have been isolated and characterized (Wang et al., 1997; Wang and Pichersky, 1998; Gang et al., 2002). Interestingly, chavicol and eugenol methyltransferases from sweet basil share 90% similarity and are phylogenetically closer to flavone, isoflavone or other methyltransferases of unknown function than to eugenol methyltransferase from *Clarkia* (Gang et al., 2002). Thus, the eugenol methyltransferases evolved independently as an example of a special type of convergent evolution called repeated evolution.

Orcinol dimethylether and 1,3,5-trimethoxybenzene (TMB) are among the most common volatiles of many rose flowers. Orcinol dimethylether is produced from orcinol via two methylations catalysed by two very similar orcinol methyltransferases: OOMT1 and OOMT2 (Lavid et al., 2002; Scalliet et al., 2002) (**Fig. 1.6**). TMB forms a key fragrance component of *Rosa chinensis*, the ancestor of modern roses. TMB is generated from phloroglucinol through 3,5-dihydroxyanisole and 3,5-dimethoxyphenol via three methylations. Interestingly, both OOMT1 and OOMT2 of catalyzing the last two steps in the pathway to trimethoxybenzene (Lavid et al., 2002; Scalliet et al., 2002) (**Fig. 1.6**). The enzymes were cloned from rose petals and functionally expressed in bacterial cells. Phylogenetically, both enzymes are placed in a cluster of specialized enzymes and not related with COMT (Wu et al., 2004). The initial methylation of phloroglucinol, however is catalyzed by phloroglucinol *O*-methyltransferase which is unrelated to the other two enzymes and belongs to the COMT cluster (Wu et al., 2004).

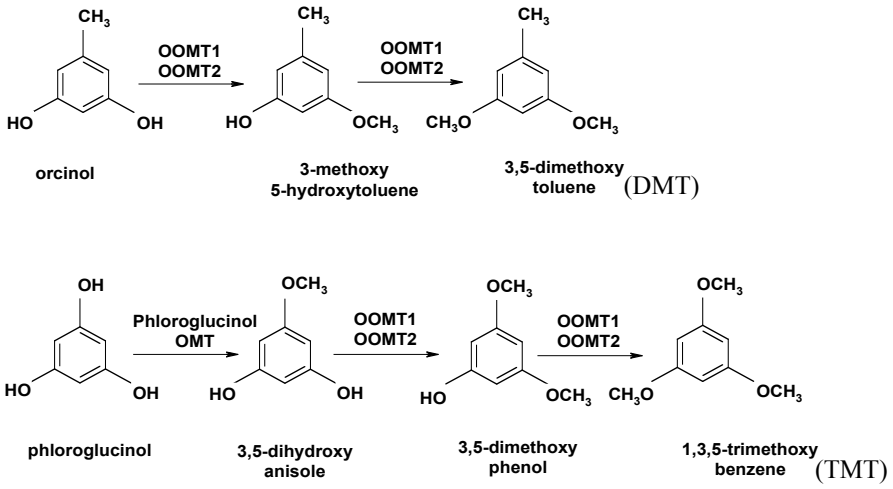

Figure 1.6 Proposed pathway for the biosynthesis of DMT and TMB in *Rosa*.

1.4.2 Volatile compounds in *Ruta graveolens*

The ethymology of *Ruta graveolens* (lat. "*Ruta*" for bitter and "*graveolens*" for strong smell) suggest already the aromatic quality of the plant species. *Ruta* is a traditional medicinal plant that, throughout the history has been used for everything from flavouring cheese to driving out evil spirits. Together with other aromatic plants (lavender, rosemary, sage, and mint) it forms the "vinegar of the four thieves" about which the tale says it was used by a family of perfumers to cover themselves and rob the homes of the victims during the Black Plague. The concoction is used even now as an insect-repellent, bactericide or as a food ingredient. The volatile oil from *Ruta graveolens* is traditionally used in many countries because of its antihelmintic, bacteriostatic and phytotoxic activities. Furthermore, the essential oils of aromatic plants attracted interest as potential herbicides and fungicides. Because modern agriculture and medicine require antimicrobials with less cross-resistance and minimal mammalian and environmental toxicity.

Considering its allelopatic, fungicidal and antibacterial activity, the composition of *R. graveolens* essential oil was investigated (De Feo et al., 2002; Oliva et al., 2003; Ivanova et al., 2004). The major groups of compounds identified are ketones, aliphatic acids,

alcohols, coumarins, mono- and sesquiterpenoides. The main constituents of the oil are 2-ketones represented by undecan-2one (46.8%), nonan-2-one (18.8%), decan-2-one (2.2%) and tridecan-2-one (2.5%) followed by terpenoids with α-pinene (1.3%), limonene (3.0%) and 1.8 cineole (2.9%). Other important compounds are: valeric acid (1.6%), octanoic acid (3.4%), nonal-2-ol (1.5%), methylsalicylate (3.9%) and xanthotoxin (0.8%) (De Feo et al., 2002). Guaiacol is also present in the plant, but not too much is known about other methoxylated phenols such as 3,5-dimethoxyphenol and their role.

1.5 Aims of this work

Acridone alkaloids are a class of compounds limited to the Rutaceae. It is postulated that they act as phytoalexins and phytoanticipins, thus increasing the plant resistance to pathogens, such as bacteria and fungi. This function makes acridone alkaloids and epoxides interesting for agricultural and medicinal use. However they are mutagenic and therefore their application as medicinal agents may be very limited.

The key reaction in acridone formation is catalyzed by acridone synthase (ACS) condensing *N*-methylanthraniloyl-CoA with three units of malonyl-CoA to yield 1,4-dihydroxy-*N*-methylacridone. Anthranilate *N*-methyltransferase catalysis the first pathway specific reaction in the biosynthesis of acridone and furoquinoline acridone alkaloids, directing anthranilic acid from the primary to the secondary metabolism. While anthranilate synthase and acridone synthase have been heterologously expressed and characterised and their mechanism of action explained, the other enzymes of this chain still need to be described.

The primary aim of this work was to purify the anthranilate *N*-methyltransferase in amounts high enough to allow microsequencing of the protein and then to isolate the gene encoding the polypeptide based on specific oligonucleotide primers. Suspension cell cultures of *Ruta graveolens* L. were used as plant material for purification of anthranilate *N*-methyltransferase. It was shown that they constitutively accumulate acridone alkaloids that could be enhanced by fungal elicitor treatment.

2. MATERIALS AND METHODS

2.1 Chemicals:

2-Mercaptoethanol	Roth (Karlsruhe)
Acrylamide	Promega (Mannheim)
Agarose NEEO Ultra-Quality	Roth (Karlsruhe)
Ammonium persulfate (APS)	Roth (Karlsruhe)
Ampicillin	Sigma (Deisenhofen)
Bacto-Agar	Difco Lab (Detroit, USA)
Bovine serum albumin (BSA)	Serva (Heidelberg)
Bromphenol blue	Serva (Heidelberg)
DNA for hybridisation (from hering sperm)	Sigma (Deisenhofen)
Desoxyribonucleotides (dNTPs)	MBI Fermentas (St. Leon-Rot)
Desoxycholate	Roth (Karlsruhe)
DMSO	Roth (Karlsruhe)
DTT (1,4-Dithiothreitol)	Roth (Karlsruhe)
EDTA	Roth (Karlsruhe)
Ethidium bromide	Roth (Karlsruhe)
Ficoll Type 400	Sigma (Deisenhofen)
Folin Ciocalteau Reagent	Merck (Darmstadt)
Formaldehyde	Sigma (Deisenhofen)
Formamide	Sigma (Deisenhofen)
GeneRuler 1 kp DNA-Ladder	MBI Fermentas (St. Leon-Rot)
GeneRuler 50 bp DNA-Ladder	MBI Fermentas (St. Leon-Rot)
GeneRuler 100 bp DNA-Ladder	MBI Fermentas (St. Leon-Rot)
Guanidinium thiocyanate	Roth (Karlsruhe)
Hybond N^+ (positive charged nylon membrane)	Amersham (Braunschweig)

Isopropyl-ß-D-thiogalactoside (IPTG)	Roth (Karlsruhe)
Kanamycin	Sigma (Deisenhofen)
Magnesium chloride	Merck (Darmstadt)
MOPS	Roth (Karlsruhe)
Peptone	Roth (Karlsruhe)
Phenol, saturated with 0,1 M Citrat-Puffer	Sigma (Deisenhofen)
Pmg elicitor	Lab's collection
Polyacrylamide	Promega (Mannheim)
Roti®-Phenol/Chloroform	Roth (Karlsruhe)
Rotiszint® eco plus	Roth (Karlsruhe)
Select Agar	GibcoBRL (Eggenstein)
Sodium ascorbate	Merck (Darmstadt)
Sodium sarcosinate	Sigma (Deisenhofen)
Tetracyclin	Roth (Karlsruhe)
N,N,N', N'-Tetramethylethylendiamine (TEMED)	Roth (Karlsruhe)
Whatman 3MM Chr blotting paper	Whatman (Banbury, UK)
X-Gal	Roth (Karlsruhe)
Yeast extract	Roth (Karlsruhe)

2.2 Radiochemicals

Substance	Specific activity	Company
[α-^{32}P]dCTP	> 3000Ci/ mmol	ICN (Meckenheim)
S-Adenosyl-L-[*methyl*-^{14}C]-methionine	52 mCi/mmol	Hartmann (Braunschweig)

2.3 Substrates

Thiols

1,4-Dithiothreitol	Roth (Karlsruhe)
2-Mercaptoethanol	Sigma-Aldrich (Steinheim)
L-Cysteine	Roth (Karlsruhe)
Homocysteine	Lab's collection

L-Methionine	Roth (Karlsruhe)
Thiopurine	Lab's collection
Methoxylated phenolics:	
3,5-Dimethoxyphenol	Fluka (Buchs, Switzerland)
Guaiacol	Sigma-Aldrich (Steinheim)
3-Methoxyphenol	Fluka (Buchs, Switzerland)
3,4-Dimethoxyphenol	Fluka (Buchs, Switzerland)
2,3-Dimethoxyphenol	Fluka (Buchs, Switzerland)
3,5-Dihydroxyanisole	Fluka (Buchs, Switzerland)
1,3,5-Trimethoxybenzene	Merck (Darmstadt)
Phenolis:	
Phenol	Roth (Karlsruhe)
Resorcinol	Sigma-Aldrich (Steinheim)
Phloroglucinol	Fluka (Buchs, Switzerland)
Catechol	Roth (Karlsruhe)
Toluenes:	
Orcinol	Sigma-Aldrich (Steinheim)
o-Cresol	Roth (Karlsruhe)
m-Cresol	Roth (Karlsruhe)
Shikimic acid	Lab's collection
Phenylpropanoids:	
Cinnamic acid	Roth (Karlsruhe)
Ferulic acid	Sigma-Aldrich (Steinheim)
Isoferulic acid	Roth (Karlsruhe)
p-Coumaric acid	Roth (Karlsruhe)
o-Coumaric acid	Fluka (Buchs, Switzerland)
m-Coumaric acid	Fluka (Buchs, Switzerland)
Caffeic acid	Roth (Karlsruhe)
Sinapic acid	Roth (Karlsruhe)
Coniferylalcohol	Fluka (Buchs, Switzerland)

Eugenol	Roth (Karlsruhe)
Phenylpropanoid esters:	
Caffeoyl-CoA	Lab's collection
Cinnamoyl-CoA	Lab's collection
Coumaroyl-CoA	Lab's collection
Feruloyl-CoA	Lab's collection
5-Hydroxyferuloyl-CoA	Lab's collection
Monoterpenes:	
Menthol	Roth (Karlsruhe)
Carvacrol	Roth (Karlsruhe)
Thymol	Roth (Karlsruhe)
Coumarins:	
Umbelliferone	Roth (Karlsruhe)
Aesculetin	Roth (Karlsruhe)
7-Methoxycoumarin	Fluka (Buchs, Switzerland)
Scopoletin	Roth (Karlsruhe)
Bergaptol	Extrasynthase (Geney, France)
Xanthotoxol	Roth (Karlsruhe)
Bergapten	Roth (Karlsruhe)
Xanthotoxin	Roth (Karlsruhe)
Isopimpinellin	Roth (Karlsruhe)
Stilbenes	
Resveratrol	Roth (Karlsruhe)
Flavonoids:	
Naringenin	Roth (Karlsruhe)
Eriodictyol	Roth (Karlsruhe)
Homoeriodictyol	Roth (Karlsruhe)
Hesperetin	Roth (Karlsruhe)
Pinocembrin	Sigma-Aldrich (Steinheim)

4'-Hydroxyflavanone	Lab's collection
7'-Hydroxyflavanone	Lab's collection
7'-Hydroxy-5'-methoxyflavanone	Lab's collection
Dihydrokaempherol	Roth (Karlsruhe)
Dihydroquercetin	Roth (Karlsruhe)
Dihydromyricetin	Roth (Karlsruhe)
Apigenin	Roth (Karlsruhe)
Luteolin	Roth (Karlsruhe)
Tricetin	Roth (Karlsruhe)
Kaempherol	Roth (Karlsruhe)
Quercitin	Roth (Karlsruhe)
Myricetin	Roth (Karlsruhe)
Isorhamnetin	Roth (Karlsruhe)
Isoflavones:	
Genistein	Roth (Karlsruhe)
Lignans:	
Matairesinol	Prof. Dr. M. Petersen
Hydroxymatairesinol	Prof. Dr. M. Petersen
Podophyllotoxin	Prof. Dr. M. Petersen
β-Peltatin	Prof. Dr. M. Petersen
Alkaloids/Alkaloid precursors:	
Anthranilic acid	Sigma-Aldrich (Steinheim)
Anthraniloyl-CoA	Lab's colection
Rutacridone	Dr. R. Lukacin
1,3-Dihydroxy-*N*-methylacridone	Dr. R. Lukacin
2,4-Quinolinol	Fluka (Buchs, Switzerland)

2.4 Enzymes

Calf Intestine Alkaline Phosphatase	MBI Fermentas (St. Leon-Rot)
Expand High Fidelity	Roche Diagnostics (Mannheim)
Lysozyme	Roth (Karlsruhe)
MMLV-Reverse Transcriptase	Promega (Mannheim)
Pfu DNA Polymerase	Promega (Mannheim)
Restriction enzymes	Boehringer (Mannheim)
	MBI Fermentas (St. Leon-Rot)
RNase A	Roth (Karlsruhe)
RNase H	Usb (Cleveland, USA)
Superscript II RT	Invitrogen (Leek, Niederlande)
T4-Ligase	Promega (Mannheim) und MBI Fermentas (St. Leon-Rot)
T4-Polynucleotidkinase	MBI Fermentas (St. Leon-Rot)
Taq DNA Polymerase	Promega (Mannheim) und MBI Fermentas (St. Leon-Rot)
Terminale Desoxynucleotide Transferase	Promega (Mannheim)

2.5 Kits

GeneRacer Kit	Invitrogen (Leek, Niederlande)
Nucleo Spin®/NucleoTrap®	Macherey-Nagel (Düren)
PCR Clean-up-Kit	Roche Diagnostics (Mannheim)
QiaexII Gel Extraction Kit	Qiagen (Hilden)
QuikChange® Multi Site-Directed Mutagenesis Kit	Stratagene (Heidelberg)
Rediprime II DNA Labeling System	Amersham Biosciences (Braunschweig)
Silver Stain Plus	Bio-Rad (München)
TOPO TA Cloning Kit	Invitrogen (Leek, Netherland)
Ultrafree-DA / DNA extraction from agarose gels	Millipore (Schwalbach)

2.6 Bacterial strains

2.6.1 *E. coli* cloning strains

Name	Genotype	Company
TOP 10	F⁻mcrA Δ(mrr-hsdRMS-mcrBC) θ80lac ZΔM15 ΔlacX74 recA1 deoR araD139 Δ(ara-leu)7697 galU galK rps (Str^r) endA1 nupG	Invitrogen (Leek, Netherland)
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proAB lacIqZDM15Tn10 (Tetr)]	Stratagene (Heidelberg)
XL10-Gold	Tet^R Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F´ proAB $lacl^q$ZΔM15 Tn10 (Tet^R) Amy Cam^R]	Stratagene (Heidelberg)
JM109	recA1 endA1 gyr A96 thi hsdR17 supE44 reA1 lambda- ?(lac-proAB) [F' traD36 proAB lacIqz M15	Stratagene (Heidelberg)

2.6.2 *E. coli* expression strains

Name	Genotyp	Company
M15[pREP4]	NalS StrS rifS lac- ara- gal- mtl- F- recA+ uvr+	Qiagen (Hilden)
BL21 Star	F- ompT $hsdS_B$ ($r_B^- r_B^-$) gal dcm (DE3)	Invitrogen (Leek, Netherland)

2.7 Vectors

2.7.1 *E. coli*- cloning vectors

pCR2.1-TOPO TOPO-TA Cloning Kit (Invitrogen, Leek, Netherland)
pCR4-TOPO Generacer Kit (Invitrogen, Leek, Netherland)
pTZ19r (MBI Fermentas, St Leon-Rot)

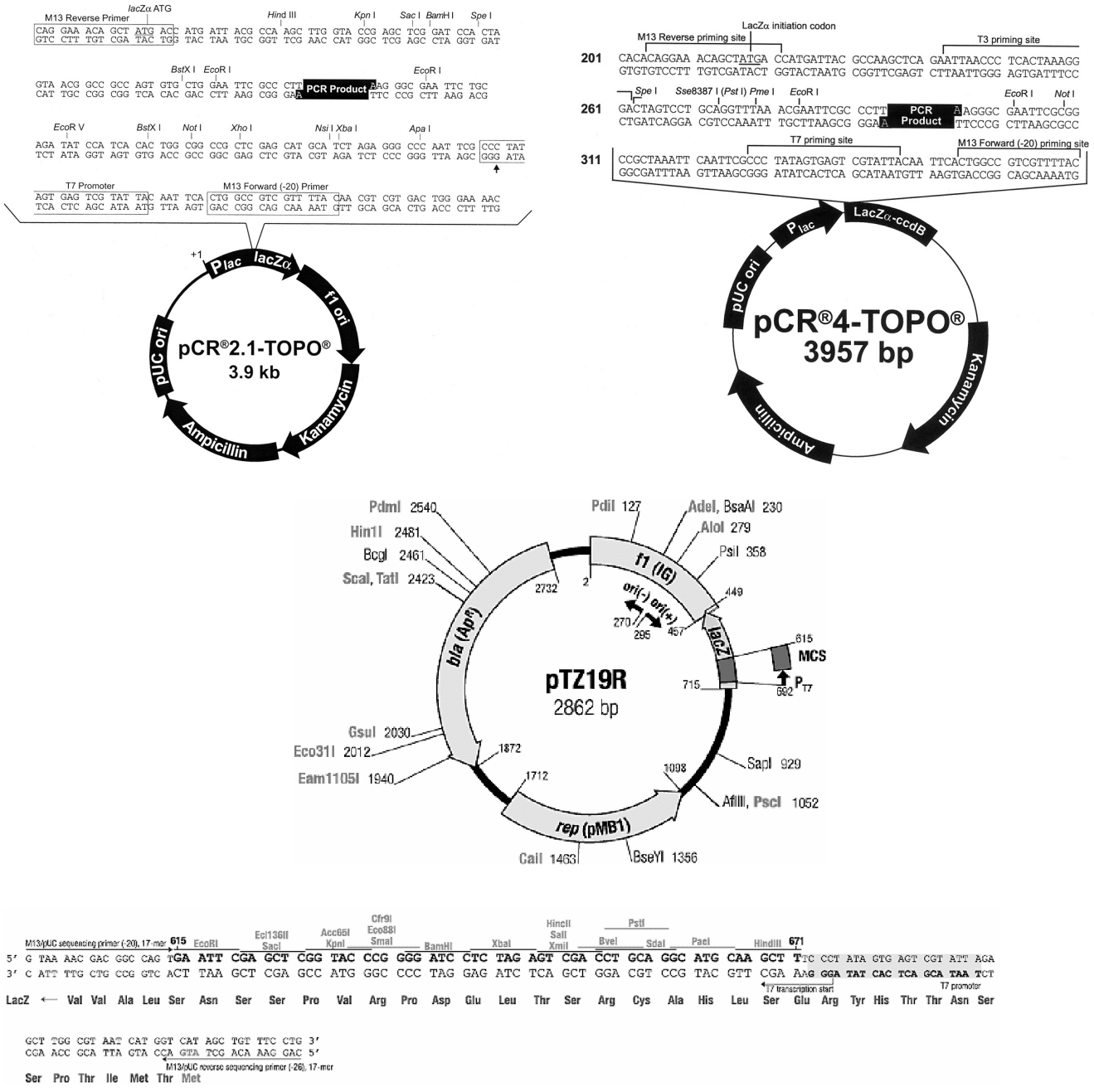

Figure 2.1: Cloning vectors: pCR2.1-TOPO (Invitrogen), pCR4-TOPO (Invitrogen) and pTZ19r (MBI, Fermentas).

2.7.2 *E. coli* expression vector

OMT genes were expressed in pQE-60 vector (Qiagen, Hilden). The vector contains a polyhistidine tag for easy nickel affinity cromatography purification of the expressed proteins.

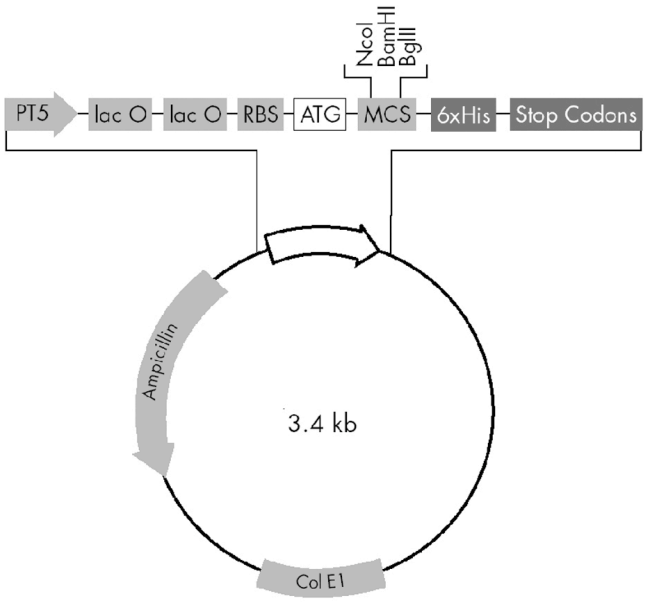

Figure 2.1: *E. coli* expression vector pQE-60

2.8 Buffers and solutions

2.8.1 Plant and cell culture

Polyclar extraction buffer:

100 mM Tris-HCl pH 7.5
1 mM EDTA
2 mM DTT
10% (v/v) Glycerol
10% (w/v) Polyclar AT

2.8.2 RNA isolation

Extraction buffer (Giuliano et al., 1993):

4.23 M Guanidiniumthiocyanate
0.225 M 3M Natriumacetate, pH 5.3
0.007 M Natriumsarcosinate 10%

2.8.3 Plasmid DNA isolation

GTE buffer:

50 mM Glucose
25 mM Tris-HCl (pH 8.0)
10 mM EDTA
autoclave
add 0.02 mg RNase/ml
store at 4°C

NaOH/SDS:

0.2 N NaOH
1% SDS

PAS:

29.5 ml acetic acid
KOH-plates till pH 4.8
fill up to 100 ml with H_2O

2.8.4 Dot-Blot

20x SSC:

3 M NaCl
300 mM Natriumcitrate
add 10 N NaOH to reach pH 7.0
fill up to 1l with H_2O

10x MOPS pH 7,0:

200 mM MOPS
50 mM Natriumacetate
10 mM EDTA
filter and store in a dark place

Denhardt`s Reagent 50x:

0.1% w/v Ficoll Typ 400
0.1% w/v Polyvinylpyrrolidone
0.1% w/v Bovine serum albumin (BSA)

2.8.5 SDS-PAGE

Separation gel:

1.5 M Tris-HCl pH 8.8

Stocking gel:

0.5 M Tris-HCl pH 6.8

5 x SDS sample buffer:

0.25 M Tris-HCl pH 6.8
8% (v/v) Glycerol
6% (w/v) SDS
0.02% (w/v) Bromphenol blue

10 x SDS running puffer:

30 g/l Tris-HCl pH 8.9
75.7 g/l Glycin
10 g/l SDS
pH 8.3

Coomassie staining solution:

40% (v/v) Methanol
10% (v/v) Acetic acid
0.1% (w/v) Coomassie Brilliant Blue R-250
in water

Coomassie destaining solution:

45% (v/v) Methanol
10% (v/v) Acetic acid
in water

Coomassie staining solution for protein sequencing:

0.5% (v/v) Acetic acid
10% (v/v) Methanol
0.15% (w/v) Coomassie Brilliant Blue R-250 diluted from 0.5 % (w/v) stock solution in water

Coomassie destaining solution for protein sequencing:

10% (v/v) Methanol
0.5% (v/v) Acetic acid
in water

2.8.6 FPLC

2.8.6.1 Purification of SAM:anthranilate *N*-methyltransferase

HIC elution:

Buffer A: 0.1 M Glycin-NaOH pH 8.9; 10% glycerol; 2 mM DTT; 1 mM EDTA
Buffer B: Buffer A + 1 M $(NH_4)_2SO_4$

SEC elution:

SEC buffer: 50 mM Tris-HCl, pH 7.5; 10% glycerol; 0.5 mM EDTA; 2 mM DTT

DEAE elution:

Buffer A: 50 mM Tris-HCl, pH 7.5; 10% glycerol; 0.5 mM EDTA; 2 mM DTT
Buffer B: Buffer A + 1 M NaCl

Affinity chromatograpy:

Buffer A: 50 mM Tris-HCl, pH 8.5; 20% glycerol; 2 mM EDTA; 2 mM DTT
Buffer B: Buffer A + 0.2 M NaCl
Buffer C: Buffer B + 2 mM SAM

Mono Q:

Buffer A: 50 mM Tris-HCl pH 7.0; 10% glycerol; 2 mM EDTA; 2 mM DTT
Buffer B: Buffer A + 1.2 M NaCl

2.8.6.2 Purification of 3,5-dimethoxyphenol *O*-methyltransferase

SEC elution:

SEC buffer: 0.1 M Tris-HCl pH 7.5

DEAE elution:

Buffer A : 0.1 M Tris-HCl pH 7.5
Buffer B : Buffer A + 1 M NaCl

Affinity chramatography:

Buffer A: 50 mM Tris-HCl, pH 8.5; 20% glycerol; 2 mM EDTA; 2 mM DTT
Buffer B: Buffer A + 0.2 M NaCl
Buffer C: Buffer B + 2 mM SAM

2.8.7 Other buffers and solutions

Lowry A:
2% Na_2CO_3
0.1 N NaOH
0.5% SDS

TE-Puffer, pH.0:
10mM Tris-HCl pH 8.0
1mM EDTA pH 8.0

TE-Puffer pH 7.5:
10mM Tris-HCl pH 7.5
1mM EDTA pH 7.5

50x TAE-Puffer:
242 g Tris-HCl
57.1 ml Acetic acid
100 ml 0.5 M EDTA pH 8.0
fill up to 1l with H_2O

2.9 Media

2.9.1 Plant cell culture

M20 media	20 ml Stock solution 1
	20 ml Stock solution 2
	20 ml Stock solution 3
	1 ml Vitamin stock solution
	30 g Saccharose
	fill up to 1l with H_2O, autoclave

Stock solution 1:

Macroelements:	NH_4NO_3	82.5 g
	KNO_3	95.0 g
	$MgSO_4$ x $7H_2O$	18.5 g
	KH_2PO_4	8.5 g
Microelements:	H_3BO_3	31 mg
	$MnSO_4$ x H_2O	1.12 mg
	$ZnSO_4$ x $7H_2O$	430 mg
	KJ	41.5 mg
	Na_2MoO_4 x $2H_2O$	12.5 mg
	$CuSO_4$ x $5H_2O$	1.25 mg
	$CoCl_2$ x $6H_2O$	1.25 mg
		fill up to 1l with H_2O, autoclave, store at 4°C
Stock solution 2:	$CaCl_2$ x $6H_2O$	22.0 mg
		fill up to 1l with H_2O, autoclave, store at 4°C
Stock solution 3:	Na_2EDTA	1866 mg
	$FeSO_4$ x $7H_2O$	1390 mg
		fill up to 1l with H_2O, autoclave, store at 4°C
Vitamins:	Nicotine amide	25 mg
	Pyridoxine-HCl	5.0 mg
	Thiamine-HCl	5.0 mg
	Calcium panthotenate	2.5 g
	myo-Inositol	4.0 g
		fill up to 1l with H_2O, autoclave, store at 4°C

The pH was adjusted at 5.6-5.8 with 0.1N NaOH.

Solid media were prepared by adding 0.8% (w/v) plant agar.

2.9.2 Bacterial media

LB (Luria Bertani)	10 g Bactotryptone, 5 g yeast extract, 10 g NaCl, 1 ml 1M $MgSO_4$, 4 ml glycerol 50% (v/v)
	fill up to 1l with H_2O, adjust the pH to 7.5, autoclave
LB-Agar plates	1.5% Bacto-agar
SOC	20 g Bactotryptone, 5 g yeast extract, 0.5 g NaCl, 20 mM glucose

2.10 Thin- layer chromatography

Plates:

Silica gel 60 F_{254}	Merck (Darmstadt)
Cellulose	Merck (Darmstadt)

Separation systems:

I: Toluene:acetic acid (4:1, v/v)
II: Toluene:ethylacetate (3:2, v/v)
III: n-Butanol:acetic acid:water (3:1:1, v/v/v)
IV: n-Propanol:formic acid:water (20:1:5, v/v/v)

2.11 Equipment

Biofuge pico/Biofuge 13	HERAEUS Instruments (Osterode)
Bio Imager FLA-2000	Fuji Photo Film (Tokyo, Japan)
	Software from Raytest (Straubenhardt)
Gene Quant	Pharmacia (Freiburg)
Julabo HC/F10 water bath	Julabo (Seelbach)
MiniCycler model PTC-150	Biozym (Hamburg)
Minifold I- SRc 96- Dot Blot	Schleicher & Schüll (Dassel)
1214 Rackbeta scintillation counter	PerkinElmer, Wellesley, USA)
Robocycler Gradient 96	Stratagene (Amsterdam, NL)
Sorvall®RC 5C Plus centrifuge	DuPont (Newton, USA)
Sorvall®RMC14	DuPont (Newton, USA)
Spectrophotometer UV-1602	Shimadzu (Kyoto, Japan)
Thermomixer 5436	Eppendorf (Hamburg)
Ultra Turrax® T25 basic	IKA Labortechnik (Staufen)
UP 200S Ultrasonication processor	Dr. Hielscher (Teltow)

2.12 Plant material preparation

2.12.1 Suspension culture

Ruta graveolens R-20 strain suspension culture was grown in the dark at 25°C and 110 rpm. The cells were propagated every seven days, 4 g wet weight of cells were transferred in 40 ml fresh M20 media in 250 ml Erlenmeyer flasks. Scaled up culture was used to produce large amounts of cells for protein purification. In this case, 40 g wet weight cells were inoculated in 400 ml M20 media in 2l Erlenmeyer flasks and propagated for ten days under the same conditions.

2.12.2 Elicitation

Scaled up suspension cultures (200 ml), nine-day-old, were elicitated with 1% sterilized yeast extract (Roth) for 16 hours. Seven-day-old small scale suspension culture (40 ml) was induced with 5 mg crude *Pmg* elicitor (*Phytophthora megasperma* f. sp. *glycinea*; stock solution 5 mg/ml) for 2 to 4 hours. Controls were treated with sterile distilled water.

2.12.3 Harvesting and storage

Induced and non-induced suspension cell cultures were harvested at the same time. The culture fluid was removed under vacuum. The cells were washed twice with distilled water and flash frozen in liquid nitrogen. Frozen cells were stored at –80°C .

2.12.4 Plant material

Ruta graveolens L. plants were harvested from the local Botanical Garden. The stems, emerging young leaves and flowers were collected separately in May 2004, immediately frozen and stored at –80°C until use.

2.13 Crude extract preparation

2.13.1 Plant crude extract

Frozen suspension culture material was ground in liquid nitrogen to a fine powder. The protein was extracted in double amounts (v/w) of polyclar extraction buffer. Aliquots of 10-100 ml were homogenized for 2 min at 13000 rpm (Ultra Turrax, IKA Labortechnik) and centrifuged for 10 min at 4^{o} C with 30000 g (Sorvall RC, 5C Centrifuge; Du Pont, Newton, USA). The supernatant was either flash frozen in liquid nitrogen and stored at $–80^{o}$C or used further for ammonium sulfate precipitation when protein purification was needed.

Alternatively frozen plant material was ground in liquid nitrogen in the presence of quartz sand. The protein was extracted in double amounts (v/w) of polyclar extraction buffer, centrifuged for 10 min at 4^{o} C with 30000 g (Sorvall RC, 5C Centrifuge; Du Pont, Newton, USA), then frozen in liquid nitrogen and stored at $–80^{o}$C.

2.13.2 Bacterial crude extract

Bacterial cells *E. coli* M15 transformed with pQE-60 vector harbouring the insert or transformed with empty vector for control reactions were grown in LB media with ampicillin (100 µg/ml) and kanamycin (25 µg/ml) selection. Overnight bacterial culture (4 ml) was inoculated in LB-amp/kan media (400 ml) and grown to OD_{600} 0.6-0.8. The incubation was carried out at 37°C with shaking at 220 rpm, and the expression was induced by adding IPTG to a final concentration of 1mM and incubated further for 3 h. The cells were harvested by centrifugation (30000 g for 5 min), frozen over night at –80 °C then disrupted by ultrasonication (0.1 M Tris-HCl buffer pH 7.5, 1 min), and the debris was removed by centrifugation (30000 g for 10 min.).

2.14 Molecular methods

2.14.1 Total RNA isolation (Giuliano et al., 1993)

Total RNA was isolated from *Ruta graveolens* R-20 cell culture.

Frozen ground plant cells (200 mg) were added to 500 µl ice cold extraction buffer mixed with 3.5 µl 2-mercaptoethanol (0.7%) and 400 µl citrate-saturated phenol. The samples were vortexed, 200 µl chloroform was added, mixed and incubated on ice for 20 min. The aqueous phase was collected by 20 min centrifugation at 13000 rpm and 4°C, mixed with the same volume of isopropanol and stored on ice for 40 min. The mixture was centrifuged for 30 min at 13000 rpm and 4°C and the resulted pellet was resuspended on ice in 500 µl TE buffer pH 7.5 (about 60 min). In order to precipitate the polysaccharides 100 µl pure ethanol was added, incubated on ice for 20 min and centrifuged for 10 min at 4°C with 10000 rpm. The supernatant was collected and total RNA was precipitated on ice for 60 min with 1/3 volume 8 M LiCl. After centrifugation (20 min, 4°C, 13000 rpm) the pellet was washed twice with 500 µl 80% ethanol, dried and resuspended in 50 µl sterile water. The concentration and purity of total plant RNA were determined by UV spectrophotometric analysis (260 and 280 nm) and the purity was proven by agarose gel separation.

2.14.2 Reverse transcription

The reverse transcription reaction was performed with MMLV-RT (Maloney-murine-leukaemia virus reverse transcriptase, Promega) when the cDNA was used as template for routine PCR reactions and with SuperScript™ II (Invitrogen) when the cDNA was used as template for expression. The reaction was carried out in the presence of oligo (dT)-adapter primer.

Total RNA (5 µg) and oligo (dT) adapter primer (1 µl, 25 pmol) were mixed and sterile distilled water was added up to 17.75 µl. The reaction was incubated for 5 min at 70°C then placed on ice. Five µl 5x MMLV reaction buffer and 1.25 µl 10 mM dNTPs were added, incubated for 2 min at 42°C followed by the addition of 1 µl MMLV-RT (200 u/µl) and incubated further at 42°C for 60 min.

When the reverse transcription was performed with SuperScript™ II, 1-5 µg RNA and 2.5 pmol primer in a final volume of 15.5 µl were employed. The mixture was denaturated for 10 min at 70°C thenplaced on ice and 2.5 µl 10x buffer, 2.5 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTPs and 2.5 µl 0.1 M DTT were added. After 1 min incubation at 42°C, 1 µl SuperScriptTM II was added and incubated further for 50 min at 42°C. The reaction was stopped by heating up to 70°C for 15 min and the RNA was digested with 1 µl RNase H at 37°C.

2.14.3 Polymerase chain reaction (PCR)

2.14.3.1 Oligonucleotides synthesis

All used oligonucleotides were prepared by MWG Biotech (Ebersberg).

2.14.3.2 Anthranilate *N*-methyltransferase (ANMT) PCR amplification

Polypeptides isolated from crude *Ruta* extracts with high ANMT specific activity were partially sequenced, and the iformation was used to construct degenerate oligonucleotide primers.

Peptide:	VLDCSVSG
Degenerated primer:	GTI CTI GAC/T TGC/T TCI GTI TCI GGI

The PCR reaction was done in 50 µl total volume containing 1x polymerase buffer, 0.2 mM dNTPs, 2 mM MgCl, 1 µM degenerated primer, 1 µM universal adapter primer and 2.5 U Taq-polymerase. The PCR program used was:

1 x	94°C	3 min	Denaturation
30 x	94°C	1 min	Denaturation
	40-55°C	1 min	Annealing
	72°C	2 min	Extension
1 x	72°C	5 min	Extension

2.14.3.3 OMT specific PCR

PCR reactions were carried out with degenerated primers created to bind to conserved regions of OMTs (Frick and Kutchan, 1999). The reactions succeeded in 1x polymerase buffer, in a final volume of 50 µl containing 0.2 mM dNTPs, 1.5-2.5 mM $MgCl_2$, 1 µM universe primer (OMT1), 1 µM reverse primer (OMT2 respectively OMT4) and 2.5 U Taq-polymerase. As template 0.5 µl cDNA was used:

OMT1: 5`- GTI GA(CT) GTI GGI GGI GGI ACI GGI GC -3`

OMT2: 5`-GGI GC(AG) TC(CT) TCI ATI AC(AG) TGI GG -3`

OMT4: 5`-CA(AG) TGI (GT)C(AG) TCI (GC)IC CA(AG) TC(AG) TG-3`

The PCR program used:

1 x	94°C	5 min	Denaturation
30 x	94°C	0,5 min	Denaturation
	40°C	1 min	Annealing
	72°C	1 min	Extension
1 x	72°C	5 min	Extension

PCR reaction products were visualized after agarose gel separation and the bands corresponding to 100 respectively 200 bp were cloned in pCR2.1 (TOPO TA Cloning Kit, Invitrogen). From each transformation 18-24 colonies were picked and analysed by digestion and sequencing.

2.14.4 RACE

Full length cDNA sequences were obtained using two different RACE (Rapid Amplification of cDNA Ends) methods: 3`-RACE and RLM-RACE.

2.14.4.1 3` RACE

In order to extend the 3` end, the employed cDNA was obtained by reverse transcription of total RNA with MMVL-RT in the presence of oligo (dT)-adapter primer. The adapter primer hybridises with the poly(A) RNA tail. The primer's 5`-end encloses a 20 bp adapter sequence that binds upstream AUAP (Abridged Universal Adapter Primer) in the following PCR ("nested" PCR). Gene specific sequences are selected as downstream primers.

The PCR reaction had a final volume of 50 µl including 0.2 mM dNTP, 1.5-2 mM $MgCl_2$, 2.5 U Taq-Polymerase and 0.4 µM of each primer in 1x polymerase buffer.

The PCR program used:

1x	3 min	94°C
30x	1 min	94°C
	1 min	53-64°C
	2 min	72°C
1x	7 min	72°C

Resulted PCR products were diluted 1:100 and used as template for the "nested" PCR performed under the same parameters.

A temperature gradient was requested to attain the optimal annealing temperature. The PCR products with the expected size were cloned (vector pCR2.1, TOPO TA Cloning Kit, Invitrogen) and the plasmid DNA isolated and sequenced.

2.14.4.2 RLM-RACE

RLM-RACE (**R**NA **L**igase-**M**ediated **R**apid **A**mplification of **c**DNA **e**nds) was performed with the GeneRacer™ Kit (Invitrogen). The method ensures the amplification of full length mRNA template only. Total RNA is treated with calf intestinal phosphatase to remove the 5` phosphates of incomplete transcripts but leaves the complete mRNA unaffected. The full length mRNA are extracted in phenol-chloroform, then treated with tobacco acid pyrophosphatase to remove the 5` cap structure. The product is again extracted in phenol-chloroform. This treatment leaves a 5` phosphate required for ligation to the 44 bp GeneRacer RNA oligo. After a new phenol-chloroform extraction the ligated mRNA is reverse-transcribed using SuperScript™ II and GeneRacer oligo dT primer. cDNA 5` ends are amplified in a two rounds cycle. In the first PCR round 0.5 µl cDNA are amplified using the GeneRacer 5` primer and a reverse gene specific primer (GSP) as described by manufacturer.

The PCR parameters were as follows:

1x	2 min	94°C
5x	30 sec	94°C
	2 min	72°C
25x	30 sec	94°C
	30 sec	60-68°C
	2 min	72°C
1x	7 min	72°C

The PCR product from the first round (1µl) was used as template for the second round, the "nested" PCR. This reaction was amplified with GeneRacer 5` nested primer and a nested GSP as described by manufacturer.

The "nested" PCR parameters were as follows:

1x	2 min	94°C
25x	30 sec	94°C
	30 sec	60-68°C
	2 min	72°C
1x	10 min	72°C

The resulted fragments were cloned in pCR4-TOPO vector (Invitrogen) and the plasmid DNA isolated and sequenced.

2.14.5 Plasmid DNA isolation

Bacterial culture harbouring the ligated plasmid was grown over night. Two ml culture was concentrated by centrifugation (1min at 13000 rpm) and resupended in 150 µl GTE buffer. The bacterial cells were disrupted by adding 200 µl NaOH/SDS solution, vortexed and

incubated on ice for 5 min. The proteins were precipitated with 150 µl PAS solution and briefly centrifuged. The mixture was incubated on ice for another 10 min, centrifuged (10 min, 13000 rpm) and the supernatant was treated with 1 ml 100% cold ethanol (-20^{o}C) and incubated at room temperature for 2 min. The precipitated plasmid DNA is colected by centrifugation (1 min, 13000 rpm), washed with 1 ml 70% ethanol, dried and resuspended in 20-50 µl TE buffer pH 8.

2.14.6 DNA sequence analysis

DNA was sequenced by Dr. Zauner (Philipps-Universität Marburg, Zellbiologie) and by MWG Biotech. DNA and protein sequence alignments with annotated genes were performed with WU-Blast 2 (http://dove.embl-heidelberg.de/Blast2/) und Megablast (http://www.ncbi.nlm.nih.gov/BLAST/) programmes and comparison alignments were executed with ClustalW programme (http://www.ebi.ac.uk.clustalw/).

2.14.7 Northern blotting

Crude *Pmg* elicitor was added to cell suspension cultures of *Ruta graveolens*, and the total RNA was isolated every 30 min for 8 hours following the addition. Control samples were treated in the same manner with sterile distilled water. The RNA was quantified by spectrophotometric measurements followed by a control agarose gel run with 4 µl total RNA read with the BioImager (Fuji Photo Film, Tokio). Total RNA (5 µg per time point) was denaturated for 15 min at 68°C in 30 µl 0.5 x Mops buffer pH 7.0 containing 50% formamide and 2.2 M formaldehyde. The reaction was placed on ice for 5 min, then 30 µl of ice cold 20x SSC was added and the samples were loaded on the nylon membrane prepared as follows.

The nylon membrane (Hybond-N^{+}, Amersham Biosciences, Braunschweig) was hydrated in sterile distilled water and equilibrated with 10x SSC. Whatman paper was also equilibrated with 10x SSC and the apparatus was assembled according to the manufacturer's instructions. The samples were transferred on the membranes by vacuum absorption and washed with 30 µl 10x SSC. The RNA was fixed on the membrane by baking for 2 h at 80°C, then the membrane was prehybridised for three hours at 68°C in 2x Denhardt´s reagent, 5x SSC, 0.1% SDS and 100 µg/ml herring sperm DNA.

The DNA sample was labeled with "Rediprime™ II-random prime labeling system" (Amersham Pharmacia Biotech, Braunschweig) and [α-^{32}P] dCTP (ICN, Meckenheim) according with the manufacturer's instructions. The labeled DNA was separated by PD10 column from the surplus nucleotides.

The ^{32}P labeled DNA probe was denaturated for 10 min at 95°C, immediately transferred on ice and added to the prehybridisation reaction. The hybridisation succeeded by further incubation at 68°C over night (16 h). In the end, the membrane was washed for 20 min under stringent conditions with 2x SSC, 0.1% SDS at room temperature followed by another 20 min at 68°C. When necessary the membrane was washed again for 20 min under milder conditions with 1x SSC and 0.1% at 68°C. The transcripts were quantified by exposing to BioImager (Fuji Photo Film, Tokio).

2.14.8 Mutagenesis

In order to clone the full length cDNA in pQE-60 expression vector (Qiagen, Hilden) an internal *Nco*I restriction sites had to be removed. The mutagenesis was performed with QuikChange "Multi Site Directed Mutagenesis Kit" (Stratagene, Heidelberg).

Mutagenesis primers were phosphorylated at the 5` end by forward reaction employing ATP. The phosphorylation reaction (20 µl) including 5 µl primer (200 ng/µl), 2 µl ATP (1 mM) and 10 U T4 polynucleotide kinase in 1x polynucleotide kinase buffer was incubated for 1h at 37°C.

The mutation was introduced by a PCR reaction of 25 µl comprising 100 ng of each phosphorilated primer, 50 ng cDNA template, 10 mM dNTPs and 25 U PfuTurbo DNA polymerase in 1x polymerase buffer.

The PCR programme used was:

1x	1 min	95°C
30x	1 min	95°C
	1 min	55°C
	8 min	65°C

The PCR product was digested with *Dpn*I to digest the methylated and hemimethylated products. The resulted mutated single stranded DNA was transformed into ultracompetent *E. coli* XL 10-Gold (QuikChange "Multi Site Directed Mutagenesis Kit", Stratagene, Heidelberg). The transformation was carried out as recommended by the manufacturer and the plasmidial DNA was subsequently isolated and sequenced.

2.14.9 Heterologous expression

Full length cDNA sequences resulted from PCR performed with special primers to provide a *Nco*I restriction site at the start codon and a *BamH*I restriction site after the original stop codon were employed. The reaction was catalysed by High Fidelity Taq polymerase (Roche Diagnostics, Mannheim) that ensures 3` and 5` end proofreading activity coupled with Taq polymerase activity.

The optimal PCR parameters were as follows:

1x	3 min	94°C
30x	1 min	94°C
	1 min	60°C
	2 min	72°C
1x	7 min	72°C

The PCR products were represented by single bands of about 1100 bp that were further cloned in pCR2.1 vector (TOPO TA Cloning Kit, Invitrogen). When necessary, internal *Nco*I restriction sites were removed by site-directed mutagenesis.

Additionally, a PCR fragment cloned in pCR2.1 vector was digested *Nco*I/*Xho*I at the start codon respectively after the original stop codon and cloned in a transformed pTZ19r vector (MBI Fermentas, St. Leon-Rot) that ensured a *Bam*HI restriction site.

2.14.10 Cloning into the expression vector pQE-60

The expression vector pQE-60 and the cDNA (R-27) cloned in pCR2.1 vector respectively pTZ19r (R-23), about 20 µg each, were digested *Nco*I/*Bam*HI over night as recommended by the manufacturer (MBI Fermentas, St. Leon-Rot). The digested fragments were separated on a agarose gel, cut out and extracted with NucleoTrap kit (Macherey&Nagel, Düren). Aliquots of the extracted fragments were loaded on an agarose gel for quantification using GeneRuller 100 bp DNA Ladder (MBI Fermentas, St. Leon-Rot). The vector/insert ratio was calculated after the following formula:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

The amount of vector in the ligation reaction was constant at 100 ng and the insert/vector ratio was 3/1. The ligation reaction had a final volume of 20 µl containing:

DNA fragment

PQE-60 vector

1x ligase buffer

5 U T4 DNA ligase

distilled water

After 1 h incubation at 22°C the reaction was ended by heating at 68°C for 10 min. Aliquots of the ligation product (4.5 µl) were incubated on ice for 30 min with 150 µl *E. coli* XL1 blue cell. The reaction was shock heated for 1 min at 42°C, immediately cooled down on ice for 2 min, then 500 µl LB medium was added and incubated for 1 h at 37°C without shaking. The reaction product was plated on LB-amp plates and incubated at 37°C over night. The plasmid DNA was isolated and sequenced.

2.14.11 Cloning into pET101/D-TOPO

When pET101/D-TOPO vector (Invitrogen) was used for expression, the cDNA fragments produced with High Fidelity Taq polymerase (Roche Diagnostics) were cloned directly in the expression vector. The ligation reaction contained PCR product and vector in a ratio of 3/1, salt solution (1 µl) and water up to a final volume of 6 µl. The reaction was incubated on ice for 20 min and the ligation product was cloned in TOP10 cells (Invitrogen). After the identity of the insert was confirmed by sequencing, the prepared plasmid DNA was cloned in the recommended expression cells, *E. coli* BL21 Star (Invitrogen).

2.14.12 Transformation of M15[pREP4] expression cells

The expression of the recombinant proteins was carried out in *E. coli* M15 strain harbouring a pREP4 plasmid. After the integrity of the cDNA insert cloned in pQE-60 was confirmed by sequencing, the plasmid DNA was isolated and used to transform the bacterial cells using the "1 min transformation" method. One µl of the prepared plasmid DNA was mixed with 5 µl M15 cells and incubated for 1 min at 43°C. The reaction was immediately placed on ice, 100 µl LB media was added and plated on LB-amp/kan plates.

2.14.13 Transformation of BL21 Star cells

One vial of *E. coli* BL21 Star cells was thawed on ice and 5 ng plasmid DNA was added and mixed gently. The reaction was incubated on ice for 30 min, shock heated for 30 sec at 42°C, placed again on ice and 250 µl room temperature SOC media was added. The cells were grown for 30 min at 37°C with 200 rpm and plated on LB-Amp plates. Plasmid DNA was prepared and the identity of the insert confirmed by sequencing. The sequenced clone was used for expression.

2.14.14 Induction of expression cells

The protein expression was induced with 1 mM IPTG. The vector pQE-60 combines a phage T5 promoter (recognised by *E. coli* RNA polymerase) with a double *lac* operator sequences. The promoter is regulated by *lac*I repressor present on pREP4 plasmid harboured by *E. coli* M15 cells. The transformed M15[pREP4] with pQE-60 construct was grown in LB media with ampicillin (100µg/ml, resistance to ampicillin gene is found on pQE-60 vector) and kanamycin selection (25 µg/ml, resistance to kanamycin gene is found on pREP4 plasmid).

2.15 Biochemical methods

2.15.1 Protein determination

Protein amounts were quantified according to Lowry et al., (1951). Bovine serum albumin was used as standard in concentration ranging from 5 to 40 µg.

2.15.2 SDS-PAGE

SDS-PAGE was carried out according to Laemmli (1970).

Separation gel (12.5%)

4.1 ml	30% Acrylamide solution
400 µl	10% SDS in water
2.5 ml	Separation gel buffer
3.0 ml	H_2O
16 µl	TEMED
50 µl	10% Ammoniumpersulfate in water (APS)

Stacking gel (5%)

375 µl	30% Acrylamide solution
100 µl	10% SDS in water
625 µl	Stacking gel buffer
1.4 ml	H_2O
5 µl	TEMED
15 µl	10% Ammoniumpersulfate in water (APS)

The electrophoresis was performed with a slab Gel Miniprotean II 2D Multicell equipment (Biorad, München). The protein samples 20 µl were mixed with 5 µl sample buffer and DTT (5 mM), denaturated for 5 min at 95°C and loaded on the gel. The separation succeeded in 1x running buffer at 200 V for 60 min. The proteins were stained with Coomassie Brilliant Blue R-250 for 20 min then the gel was destained for 20-40 min with the destaining solution. The separation of polypeptides for microsequencing was achieved in 1x running buffer at 90 V for 2 h.

The molecular weight was estimated with respect to SDS-7 marker (Sigma, Deisenhofen) containing the following proteins:

Bovine serum albumin	66 kDa
Ovalalbumin	45 kDa
Carbonic anhydrase	36 kDa
Glycerinaldehyde-3-phosphate dehydrogenase	29 kDa
Trypsinogen	24 kDa
Trypsin inhibitor	20 kDa
α-Lactalbumin	14,2 kDa

2.15.3 Silver staining

Sensitive silver staining was carried out with Silver Stain Plus (BioRad, München). The protein was loaded on a regular SDS-PAGE and separated for 1.5 h at 150 V. After electrophoresis, the gels were rinsed twice with 400 ml distilled water for 10 min then stained with the silver complex for 20 min. The staining reaction was stopped by placing the gels in 5% acetic acid for 15 min then rinsed with water for another 5 min. The gels stained with Coomassie Brilliant Blue were first completely destained with Coomassie destaining solution then restained with silver.

2.15.4 Anthranilate *N*-methyltransferase assay:

Anthranilate *N*-methyltransferase activity was measured in a 200 µl assay containing

50 µl Protein sample

0.3 mM Anthranilic acid

147 µl 0.1 M Gly-NaOH pH 8.9

37.5 µM *S*-Adenosyl-L-[*methyl*-^{14}C]-methionine (1.57 mCi/mmol)

The reaction was started by adding the SAM, then incubated for 30 min at 32^{o}C. The incubation was terminated with 20 µl 0.2 N HCl and the product was extracted in 400 µl ethyl acetate. Aliquots of the organic phase (200 µl) were mixed with 5 ml Rotiszint eco plus for hydrophobic samples (Roth, Karlsruhe) and quantified for five min by liquid scintillation counting (LSC) in a 1214 Rackbeta Counter (PerkinElmer, Wellesley, MA, USA).

2.15.5 Purification of anthranilate *N*-methyltransferase

2.15.5.1 Ammonium sulfate precipitation

Crude extract of *Ruta graveolens* R-20 strain was fractionated by ammonium sulfate precipitation. The extract was kept on ice and the calculated ammonium sulfate amount was added slowly. After the salt has dissolved, stirring was continued for another 30 min to allow complete equilibration between dissolved and aggregated proteins then the solution was centrifuged for 10 min at 30000 g. The protein precipitated at 35 to 60% saturation was dissolved in 1 ml SEC buffer and separated further by hydrophobic interaction chromatography.

2.15.5.2 Hydrophobic interaction chromatography

The column (Superformance 16 with a bed volume 36 ml, using Fractogel EMD Propyl 650(S) as material) was equilibrated with three to five column volumes of HIC buffer B containing 1 M ammonium sulfate at a flow rate of 2 ml/min. The samples were dissolved in the same buffer and loaded on the column via a superloop. The elution was achieved by linearily decreasing the salt concentration from 100 to 0% and the samples were tested for activity. The active samples were precipitated with ammonium sulfate to 80% saturation.

2.15.5.3 Size exclusion chromatography

The separation was performed with a Superformance column (600 x 16 mm) with a bed volume of 117 ml using Fractogel EMD Bio SEC (Merck, Darmstadt) as material. This is hydrophilic, macroporous, particulated polymethyl acrylate with particle size of 20-40 µm and pores diameter of 500-800 A.

The column was equilibrated with three to five times the column volume with SEC buffer at a flow of 1 ml/ min. The protein collected from the HIC separation was dissolved in 1 ml SEC buffer was loaded directly on the column and eluted with 1 ml/min. The eluted samples were tested for activity and the active samples were selected for the following purification step.

2.15.5.4 Anion exchange chromatography

The separation was performed with a Superformance SF column (156 x 26 mm) with a bed volume of 67.5 ml using as material Fractogel DEAE 650 (S) (Merck, Darmstadt) with particle size of 20-40 µm. The column was equilibrated with three to five volumes of DEAE buffer A at a flow rate of 1 ml/ min. The active fraction eluted from SEC were loaded via superloop on the anion exchange column and the unbound proteins were washed off with 20 ml of the same buffer. The bound proteins were eluted with a constant gradient of NaCl from 0 to 1 M in 40 min with a flow rate of 1 ml/ min. The samples were tested for activity and the active samples were examined for purity on SDS-PAGE stained with Coomassie Brilliant Blue.

2.15.5.5 Affinity chromatography

Adenosine agarose was prepared as described by James et al. (1995) and Kato et al. (1999). Adenosine 5'-monophosphate agarose (Sigma, A1271) was hydrated in water for 1 h at 22°C. The gel (volume 5 ml) was then washed with 0.5 M NaCl (50 ml), followed by water (50 ml), and equilibrated with 50 mM Tris-HCl, pH 8.5. It was dephosphorylated by shaking overnight (200 rpm, 25°C) in 5 ml of 100 mM Tris-HCl (pH 8.5) containing 1000 diethanolamine units of bovine alkaline phosphatase (Sigma. P0280). After transfer to the column, the gel was washed with 100 ml of 0.5 M NaCl and 100 ml of water, then equilibrated with buffer A. For storage, ethanol was added to a final concentration of 20% (v/v).

The active fractions eluted from DEAE was desalted through a PD10 column and equilibrated with affinity chromatography buffer A, then loaded on the column at a flow rate of 1ml/min. The column was washed with 2 ml buffer A, then with 2 ml buffer B containing 0.2 M NaCl and eluted with 10 ml buffer C containing 0.2 M NaCl and 2 mM SAM. The samples were desalted on PD10 column then tested for activity and examined on SDS-PAGE stained with Coomassie Brilliant Blue or/and silver staining.

2.12.5.6 Mono Q

Mono Q HR 5/5 column (Amersham Pharmacia Biotech, Freiburg) is designed for high performance ion exchange separation of proteins. The performance of this column is based on MonoBeads, a hydrophilic material with the narrowest particle size of any chromatographic support (10±0.3 µm).

The 1 ml column was hydrated for 60 min, washed with Mono Q buffer B for 30 min then with Mono Q buffer A for 2 h at 1 ml/min. The active samples from affinity chromatography were pooled, desalted on PD10 and loaded on Mono Q via superloop with 0.5 ml/min. The elution of the bound proteins was accomplished by linear increase of salt concentration from 0 to 100% buffer B containing 1.2 M NaCl at a flow rate of 1 ml/min. The collected samples (1 ml each) were assayed for activity and visualized on SDS-PAGE stained with Coomassie Brilliant Blue and silver staining.

2.15.6 Enzyme assays

The recombinant expressed methyltransferases were tested for activity with different substrates in the presence of *S*-adenosyl-L-[*methyl*-^{14}C]-methionine. The enzyme test consisted of:

250 µM Substrate

100 µl Bacterial crude extract

100 µl 0.1 M Tris pH 7.5

1 µl *S*-Adenosyl-L-[*methyl*-^{14}C]-methionine (10 µCi/ml)

The incubation was conducted for 1 h at 36°C and the reaction product was extracted in 400 µl ethyl acetate by vortexing and separation of phases by centrifugation (for 5 min, 13000 rpm). Aliquots of the organic phase (200 µl) were mixed with 5 ml Rotiszint eco plus and quantified by LSC in a 1214 Rackbeta Counter (PerkinElmer, Wellesley, MA, USA) as described for the anthranilate *N*-methyltransferase assay.

2.15.7 Purification of recombinant 3,5-dimethoxyphenol *O*-methyltransferase

2.15.7.1 Ammonium sulfate precipitation

The crude bacterial extract containing the OMT activity was fractionated by ammonium sulfate precipitation and the protein precipitated at 60 to 80% saturation was dissolved in 1 ml SEC buffer and separated further by size exclusion chromatography.

2.15.7.2 Size exclusion chromatography (SEC)

SEC was performed on a superose column as described for the purification of anthranilate *N*-methyltransferase.

For molecular mass comparition the column was calibrated with reference proteins (Gel Filtration LMW Calibration Kit; Amersham Biosciences, Braunschweig):

Protein	Molecular weight [kDa]
Alcohol dehydrogenase	150
Bovine serum albumin (BSA)	67
Ovalbumin	45
Carboanhydrase	25
Ribonuclease A	13,7

The molecular weight of the recombinant 3,5-dimethoxyphenol was calculated after elution from the calibrated size exclusion chromatography. The elution volume (Vo) was determined with Dextran Blue 2000 (Amersham Biosciences, Braunschweig). The eluted samples were assayed for activity and the molecular weight was calculated relating to the partition factor (K_{av}):

$$K_{av} = \frac{v_e - v_o}{v_t - v_o}$$

K_{av} = partition coefficient

V_e = the elution volume of each protein

V_o = the elution volume of Dextran Blue (35 ml)

V_t = total volume of the column (114 ml)

2.15.7.3 Anion exchange chromatography

The column, material and method used were as described previously for the purification of anthranilate *N*-methyltransferase. The samples were tested for activity and the active samples were examined for purity on SDS-PAGE stained with Coomassie Brilliant Blue.

2.15.7.4 Affinity chromatography

Affinity chromatography column, material and method were as described previously for the purification of anthranilic acid NMT. The samples were desalted on PD10 column then tested for activity and examined on SDS-PAGE stained with Coomassie Brilliant Blue.

2.15.8 Standard *O*-methyltransferase (OMT) assay

The OMT assays were routinely carried out at 36°C with 50 µl enzyme eluted from SEC (0.1 mg) or 10 µg apparently homogenous protein in 0.1 M Tris-HCl buffer pH 7.5 (200 µl total) containing 0.25 mM substrate. The incubation was started by the addition of 25.0 µM *S*-adenosyl-L-[*methyl*-^{14}C]methionine (4.72 µCi/µmol) and continued for 30 min. The reaction was stopped by addition of 0.2 N HCl (20µl), and the products were extracted in 400 µl ethyl acetate. Aliquots of the organic phase (200 µl) were mixed with 5 ml Rotiszint eco plus (Roth, Karlsruhe) and measured by liquid scintillation counting (LSC) in a 1214 Rackbeta counter (PerkinElmer, Wellesley, MA, USA).

2.15.9 Characterisation of 3,5-dimethoxyphenol OMT

The recombinant enzyme was used to examine the dependencies of conversion rate on protein amount, time, pH and temperature as well as the kinetic parameters. Apparent Km and Ki values were extrapolated from the Lineweaver-Burk reciprocal plots according to Segel (1976). For these studies, the enzyme eluted from SEC was employed.

2.15.9.1 Protein linearity

Protein linearity was determined from 10 to 400 µg. The reactions were carried out for 60 min at 32°C in the presence of 0.25 mM 3,5-dimethoxyphenol and 2 µM *S*-adenosyl-L-[*methyl*-^{14}C]-methionine.

2.15.9.2 Time

The turnover rates were determined in incubations at 32°C containing 0.1 mg SEC eluted protein in the presence of 0.25 mM 3.5-dimethoxyphenol and 2 µM *S*-adenosyl-L-[*methyl*-^{14}C]-methionine. The incubation were stopped at 0.5 / 1 / 2.5 / 5 / 7.5 / 10 / 12.5 / 15 / 30 / 45 / 60 and 90 min, respectively.

2.15.9.3 pH

The activity of the recombinant enzyme was measured in various buffers of different pH:

Buffer	pH
Glycinate	2-3.5
Natriumacetat	4.0-5.5
Bis-Tris-HCl	6.0-7.0
Kaliumphosphate	5.0-7.5
Tris-HCl	7.0-8.5
Natriumglycinate	8.5-10.0

The incubations were carried out for 30 min at 32°C with 0.1 mg protein eluted from SEC in the presence of 0.25 mM 3,5-dimethoxyphenol and 2 µM *S*-adenosyl-L-[*methyl*-^{14}C]-methionine.

2.15.9.4 Temperature

The assays were carried out in 0.1 M Tris-HCl buffer pH 7.5 for 30 min in the presence of 0.1 mg protein elute from SEC, 0.25 mM 3,5-dimethoxyphenol and 2 µM SAM in a final volume of 200 µl. The incubations were conducted at 20 / 25 / 30 / 32 / 33 / 34 / 35 / 36 / 38 / 40 / 42 and 47°C, respectively.

2.15.9.5 K_m SAM

The apparent K_m for SAM was determined under optimum conditions of pH, temperature, protein amount and incubation time. The concentration of 3,5-dimethoxyphenol was kept constant at 0.25 mM and SAM concentration was varied between 1.6 and 70 µM to determine the SAM concentration limits for which the reaction was linear. The incubations were conducted at 1.8 / 2 / 2.5 / 3.5 / 5 and 10 µM *S*-adenosyl-L-[*methyl*-^{14}C]-methionine (52 µCi/µmol), respectively.

2.15.9.6 Apparent K_m for the methoxylated phenolics

The apparent K_m values for methoxylated phenolics were determined under saturated concentration of SAM (25 µM) and optimum parameters of pH, temperature, protein amount and incubation time. In the case of 3,5-dimethoxyphenol, 3-methoxyphenol and guaiacol the incubations were conducted in the presence of 14 / 16 / 20 / 25 / 33 / 50 and 100 µM substrate, respectively. In the case of 3,4-dimethoxyphenol, 2,3-dimethoxyphenol and 2,3-dihydroxyanisole the incubations were conducted in the presence of 40 / 65 / 100 / 166 / 200 and 300 µM substrate, respectively.

2.15.9.7 Cations, PMSF and sulfhydryl reagents

The effect of the cations Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+} and Zn^{2+} was investigated at a final concentration of 2 mM. The inhibitory effect of the serine specific reagent PMSF (phenylmethylsulfonylfluoride) (5 mM) and the sulfhydryl reagents *p*-hydroxymercuribenzoate (*p*HMB) (0.1 mM) and *N*-ethylmaleimide (NEM) (0.5 mM) was investigated in the absence or in the presence of thiols DTT (5 mM) and 2-mercaptoethanol (50 mM). The assays were carried out under standard conditions with 0.1 mg protein eluted from SEC.

2.15.10 Thiol methyltransferase (TMT) assay

Standard *S*-methyltransferase assays were conducted with the apparent homogeneous enzyme or partially purified recombinant *R. graveolens* OMT (eluted from SEC), at 38°C in 0.1 M Tris-HCl buffer pH 7.5 (200 µl final volume) containing 5.0 mM (i.e. DTT, L-cysteine, 6-mercaptopurine, homocysteine, L-methionine) or 50.0 mM (2-mercaptoethanol) substrate in the presence of 3.0 mM $ZnCl_2$. The incubation was started by the addition of 25 µM *S*-adenosyl-L-[*methyl*-^{14}C]methionine (4.72 µCi/µmol) and continued for 30 min. The reaction was stopped, extracted and aliquots were measured by LSC as described for OMT assays. Alternatively, 100 µl of the extract were applied to silica gel plates, and the plates were developed in toluene:ethylacetate (3:2, v/v) for DTT or 2-mercaptoethanol as substrate ($R_{f\ DTT\text{-}monomethylthioether}$ 0.27). In case of L-methionine or homocysteine, aliquots (30 µl) of the aqueous incubation were applied to cellulose thin-layer plates and separated in n-butanol:acetic acid:water (3:1:1, v/v/v) or n-propanol:formic acid:water (20:1:5, v/v/v) (Bourgis et al., 1999) ($R_{f\ S\text{-}methylmethinone}$ 0.93; $R_{f\ SAM}$ 0.21). The radioactivity was located by a BioImager Analyzer FLA-2000 (Fujifilm, Japan).

2.16 Other methods

2.16.1 Mass spectrometry

Products were collected from 200 standard OMT or TMT assays employing 3,5-dimethoxyphenol (0.25 mM) and Zn^{2+}/DTT (0.01:0.1 mM), respectively, and subjected to LC-MS analysis. LS-MS analysis were carried out by Dr. Wilfried Schwab, Fachgebiet Biomolekulare Lebensmitteltechnologie der Technischen Universität München.

2.16.2 Radioactivity measurements

The radioactive substances were quantified by liquid scintillation countering (1214 Rackbeta; PerkinElmer, Wellesley, Massachusetts, USA) or by scanning with the BioImager FLA-2000 von Fuji Photo Film (Tokyo, Japan) using Raytest (Straubenhardt) software.

2.16.3 Protein microsequencing

Protein samples eluted from Mono Q chromatography that showed high specific activity for ANMT were separated by SDS-PAGE. The protein band visualized with Coomassie staining was cut out. Protein microsequencing was performed in the lab of Dr. Peter Hunziker, Abteilung Biochemie, Universität Zürich, Switzerland.

3. RESULTS

3.1 Purification of SAM:anthranilate N-methyltransferase

SAM dependent anthranilate *N*-methyltransferase (ANMT) activity was reported from extracts of *Ruta graveolens* cells (Baumert et al., 1983) as the first committed step in rutacridone alkaloid formation. The reaction is independent of cations, does not require EDTA, and the enzyme exhibits a remarkable stability in crude extract and partially purified fractions (Baumert et al., 1983). Apparently, *Ruta graveolens* cell culture line R-20 expresses a high level of ANMT activity (Baumert et al., 1983), the enzyme activity is inducible upon elicitation (Bohlmann and Eilert, 1994) and the enzyme was partially purified (Baumert et al., 1983). However, this purification ended with the enrichment of a 62 kDa protein which was subjected to partial microsequencing and failed to yield a methyltransferase. Since this methyltransferase catalyzes a branch-point reaction from primary to secondary metabolism, another approach was taken to purify and eventually clone the enzyme.

3.1.1 Ammonium sulfate precipitation

A 10 day-old suspension culture of *Ruta graveolens* (500 g wet weight) was elicited for 16 h, the cells were harvested by filtration and flash frozen in liquid nitrogen until use. The protein was extracted in 0.1 M Tris-HCl pH 7.5 by homogenization of the frozen cells and the resulting crude extract (4100 mg protein in 800 ml) was subjected to ammonium sulfate fractionation. The fraction precipitating from 35 to 60% ammonium sulfate saturation contained most of the activity and was used for further purification.

3.1.2 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) is based on the interactions between the hydrophobic patches on the surface of biomolecules and the hydrophobic ligands covalently attached to the gel matrix. The protein binding on HIC is modulated by the concentration of salt. Proteins in aqueous environment usually fold in a manner which buries their hydrophobic residues inside. In the presence of higher salt concentrations, these residues are partially exposed and can theninteract with a hydrophobic matrix. HIC is accomplished on a matrix that is substituted with hydrophobic alkane chains. The length of the chain essentially determines the separation characteristics and the recovery rate. A column substituted with propyl chains was used in these experiments.

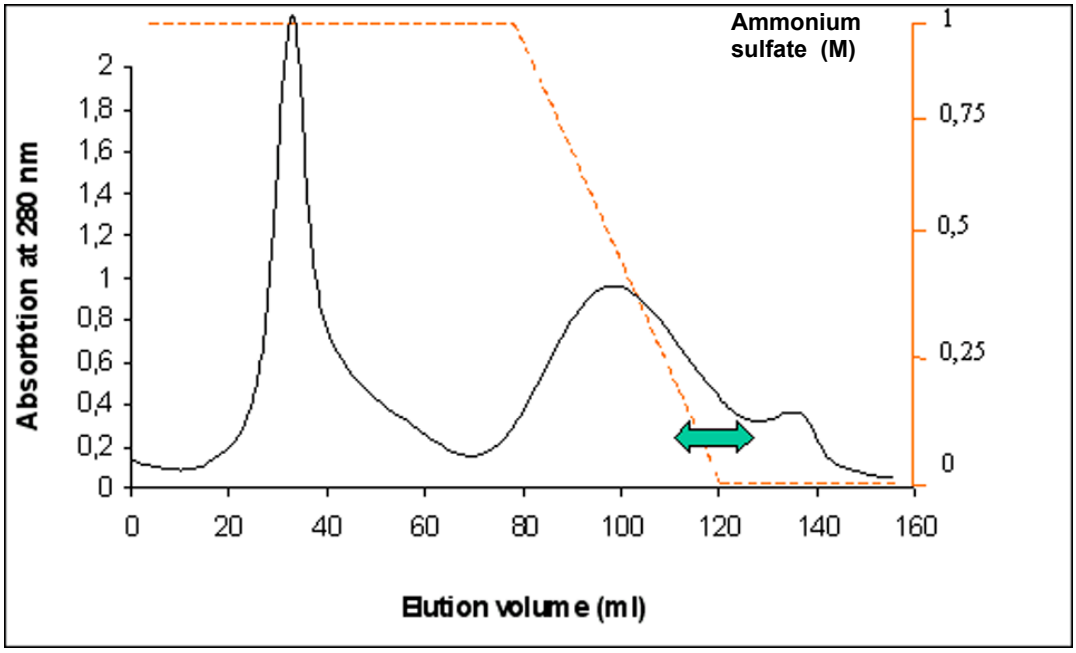

Figure 3.1 Separation of ANMT on a HIC column. The protein precipitated at 35-60% ammonium sulfate saturation was applied to the column in the presence of 1 M ammonium sulfate, and the elution succeeded by decreasing the salt concentration linearily (red line). ANMT active fractions are marked by the green arrow.

The protein precipitated at 35 to 60% ammonium sulfate saturation was dissolved in 200 ml HIC buffer B containing 1 M ammonium sulfate and applied in several runs to Fractogel Propyl (S) column (Merck, Darmstadt) which was preequilibrated with the same buffer. The bound proteins were eluted by gradually decreasing the ammonium sulfate concentration. Fractions eluted from 25 to 0% ammonium sulfate contained most of the activity. These fractions were pooled (400 mg protein, 125 ml) and used for the next step of purification.

3.1.3 Size exclusion chromatography

The method, also known as gel permeation, gel exclusion or molecular sieving is based on a matrix with defined pore size in the range of the diameter of the proteins to be separated. For globular proteins the diameter correlates with the molecular mass. Upon elution, smaller proteins distribute over the entire volume outside and inside of the matrix beads and elute gradually. Large proteins are excluded from the matrix volume and elute early. The Fractogel EMD BioSEC matrix allows fractionates molecules between 5 and 1000 kDa.

Pooled active fractions eluted from HIC were precipitated with 80% ammonium sulfate. The precipitated protein was dissolved in SEC buffer and aliquots of 1 ml were applied to the SEC column equilibrated in SEC buffer. The proteins were separated in the same buffer at a rate of 1 ml/min until the ground level of the absorption was reached. SEC chromatography has the advantage to desalt the enzyme solution from excess ammonium sulfate and thus the active fractions can be applied directly to the next column represented by an anion exchanger. The pooled active fractions eluted from SEC represented 69 mg (in 46 ml).

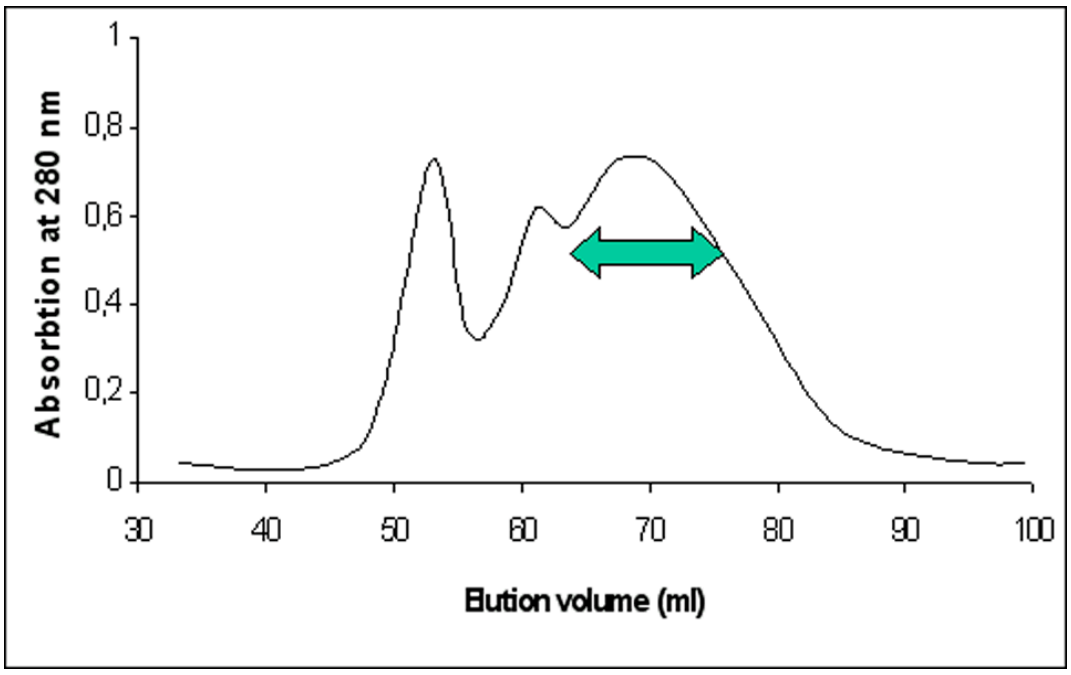

Figure 3.2 Separation of ANMT on Fractogel EMD SEC (S). The enzyme eluted from hydrophobic interaction chromatography (1 ml at a time) was loaded on the column and eluted in SEC buffer. ANMT active fractions are indicated by the green arrow.

3.1.4 Anion exchange chromatography

The pIs of most plant proteins are in the range of pH 4-6, the proteins are present in their anionic form in neutral or slightly basic buffers and can be thus separated on anion exchange. Increasing salt concentrations displace the proteins according to their partial charge. The column material consists of a methacrylate polymer to which diethylaminoethyl (DEAE) groups are attached. This groups are linked to the column material via flexible tentacles that have a high binding capacity and are proposed to preserve the proteins in their native state.

Active fractions eluted from SEC were applied through a superloop to the DEAE column. After washing, the bound proteins were eluted with a linear 0 to 1 M NaCl gradient at a flow rate of 1 ml/min. AANMT eluted between 0.45 and 0.55 M NaCl. The most active fractions were pooled (42 mg protein in 21 ml) and subjected to affinity chromatography.

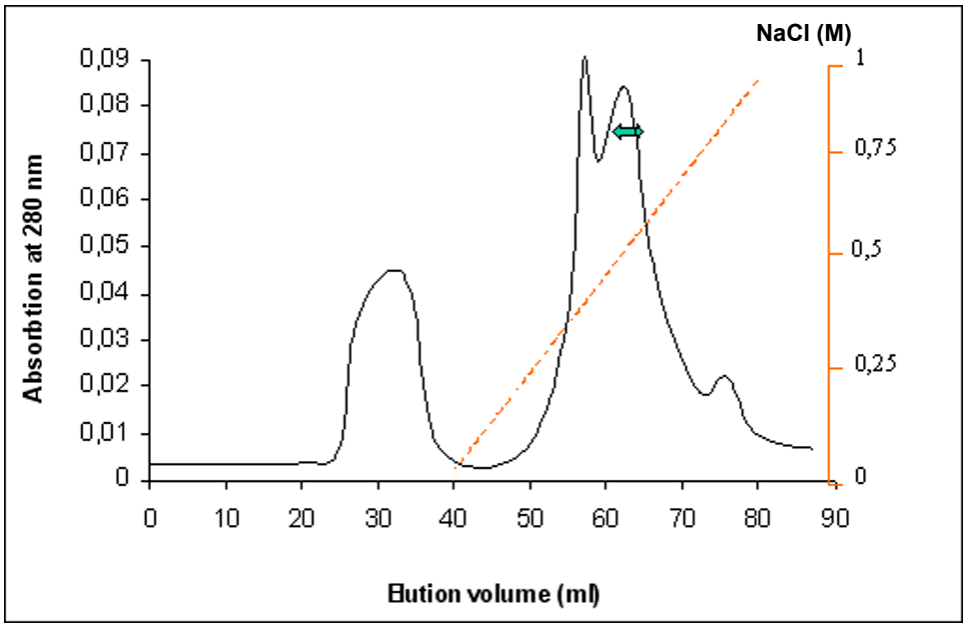

Figure 3.3 Separation of ANMT on Fractogel EMD DEAE (S). The enzyme eluted from SEC was loaded on the anion exchange column. The elution succeeded with a NaCl gradient (red line) from 0 to 1 M (right margin). The active fraction is indicated by the green arrow.

3.1.5 Affinity chromatography

Adenosine agarose has been developed and employed for the separation of nucleotide dependent enzymes, dehydrogenases as well as methyltransferases including plant methyltransferases (James et al., 1995; Kato et al., 1999). The commercial phosphorylated material was prepared for use by dephosphorylating with bovine phosphatase.

The enzyme eluted from DEAE chromatography was desalted on PD10 column in affinity chromatography buffer A and loaded on the 1 ml column equilibrated in the same buffer. The sample was first washed with 2 ml buffer A followed by 2 ml buffer B containing 0.2 M NaCl and eluted with 10 ml buffer C containing 0.2 M NaCl and 2 mM *S*-adenosyl-L-methionine at a flow rate of 1 ml/min. Fractions containing ANMT activity were separated on SDS-PAGE and the proteins were visualized with Coomassie Brilliant Blue or silver staining. After this purification step there were still some

contaminants left. The most active samples were selected for the next chromatographic step represented by Mono Q.

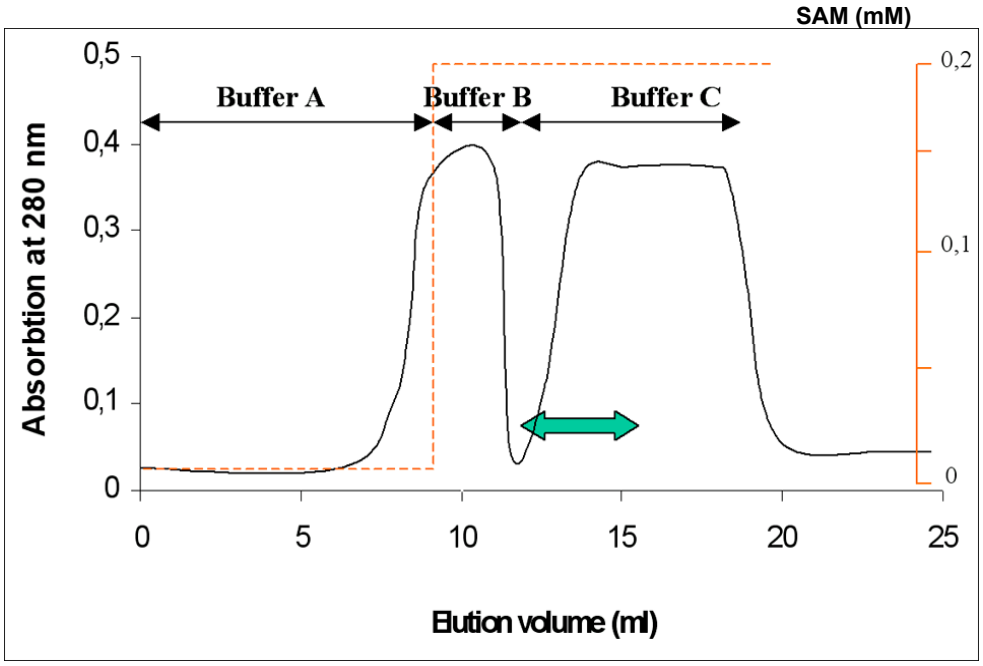

Figure 3.4 Separation of ANMT by adenosine-agarose chromatography. The enzyme fraction eluted from anion exchange chromatography was desalted and loaded on a 1 ml adenosine-agarose column equilibrated in buffer A. The column was washed subsequently with buffer A followed by buffer B, containing 0.2 M NaCl, and the enzyme was eluted in buffer C containing 2 mM SAM. The active fraction is indicated by the green arrow.

3.1.6 Mono Q

The pooled enzyme (10 ml) eluted from adenosine-agarose chromatography was desalted on a PD10 column in Mono Q buffer A and loaded via superloop on the Mono Q column (Amersham Pharmacia Biotech, Freiburg) equilibrated with the same buffer. The elution of the bound proteins was achieved in a gradient of buffer B in buffer A on a rate of 1 ml/min. The enzyme eluted at approximately 0.25 M salt (**Fig. 3.5**) in a small volume, which concentrated the activity from two Mono Q runs in about 5 ml (specific activity of 1.6 mKat/kg) (**Table 3.1).** SDS-PAGE of the active samples and staining with Coomassie Brilliant Blue R-250 showed two narrowly spaced bands in the range of 42-43 kDa (**Fig. 3.6**). Additional silver staining of the same gel confirmed the Coomassie staining and did not reveal any additional band.

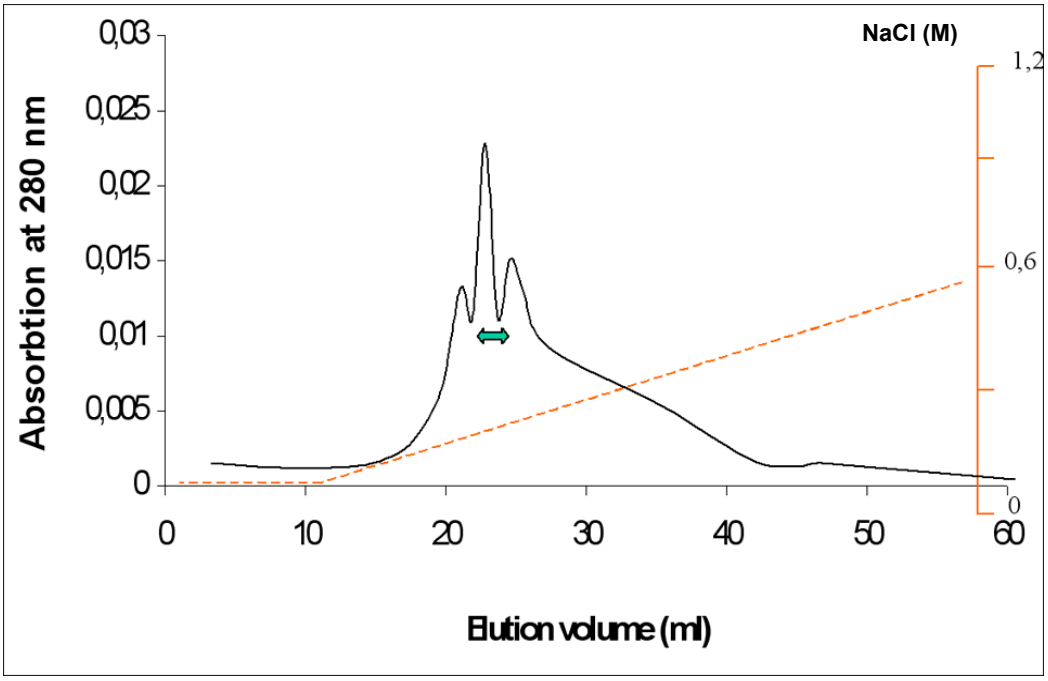

Figure 3.5 Separation of ANMT by Mono Q chromatography. The enzyme was eluted in a linear NaCl gradient at about 0.25 M.

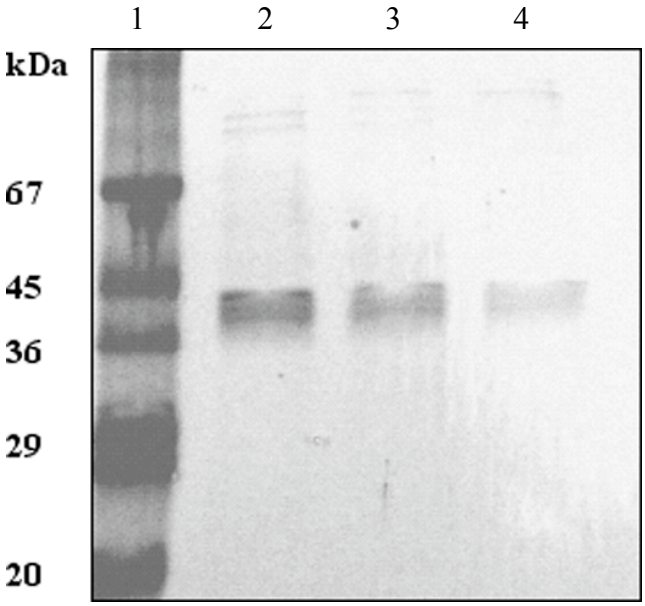

Figure 3.6: SDS-PAGE of ANMT fractions eluted from Mono Q chromatography and stained with Coomassie Brilliant Blue. Left lane: marker protein; lanes 2-4: protein from different active fractions corresponding to 200 µl (2-0.8 µg protein/lane, precipitated with tricloroacetic acid prior to SDS-PAGE).

Table 3.1 Purification of anthranilate *N*-methyltransferase from 500 g of *Ruta graveolens* R-20 cells.

Purification step	Protein	Specific activity	Apparent purification	Recovery
	mg	µkat/kg	-fold	%
Crude extract	4100	3.5	1	100
Fractogel Propyl (S)	400	12.3	3.5	34.3
Fractogel EMD BioSEC (S)	69	25.9	7.4	12.45
Fractogel EMD DEAE 650 (S)	42	44.9	12 .8	13.14
Adenosine agarose	ND	ND	ND	ND
Mono Q	0.04	1631.4	466.1	4.5

3.1.7 Protein microsequencing

Fractions eluted from Mono Q chromatography containing ANMT activity were collected, tricloroacetic acid and loaded on an SDS-PAGE gel. This electrophoresis was conducted at very low voltage (75-80V) to allow an efficient separation of proteins of similar molecular weight. The protein was visualized by mildly staining with 0.15% (w/v) Coomassie containing 0.5% (v/v) acetic acid and 10% (v/v) methanol (**Fig. 3.7**)

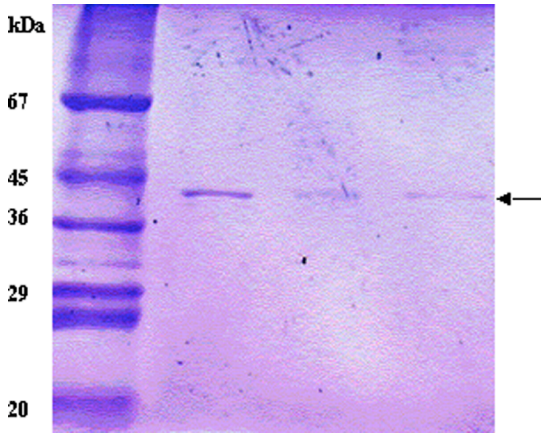

Figure 3.7 SDS-PAGE separation of ANMT fractions eluted from Mono Q chromatography. The band marked by an arrow was used for protein sequencing.

3.1.8 Partial microsequencing and oligonucleotide primers

Partial microsequencing of the proteins bands shown in **Fig. 3.7** proved the identity of the polypeptide in lanes 2-4. Five short peptide fragments were identified (**Fig. 3.8 A**) with no obvious relationship to annotated sequences. Unfortunately, the backtranslation revealed very degenerated coding sequences(**Fig. 3.8 B)**. The longest peptide (VLDCSVSG) was chosen to design PCR oligonucleotide primers. Low stringency PCRs were run with this primer (GTI CTI GAC/T TGC/T TCI GTI TCI GGI) and universal oligo-dT anchor primer that binds to the mRNA polyA tail. A fragment of 800 bp was amplified and cloned in TOPO TA pCR2.1 vector. The result was a peptide with 82% identity with sinapyl alcohol dehydrogenase from *Populus tremuloides*, and 80% identity with putative cinnamyl alcohol dehydrogenase from *Arabidopsis thaliana*, or 82% identity with putative cinnamyl alcohol dehydrogenase from *Fragaria x ananassa*. These results imply that either the purified enzyme was an alcohol dehydrogenase or the degenerated primer was inappropriate for ANMT amplification.

The enzyme purification was repeated another time with the aim to isolate more protein for another microsequecing. However, the activity was not retained by the hydrophobic chromatography column and the pattern of elution completely changed, which suggested that the column matrix could have been damaged. All attempts to restore the properties of the column failed and the experiment could not be reproduced.

A **MALDI peptide mass fingerprint:** no significant match.

NanoES, MS/MS: The following sequences were found:

1. **LYGLT(186)SK**
2. **(128)VMEGGLPS(174)R**
3. **(186)LSESHT(128)YK**
4. **(276)TELAL(332)K**
5. **VLD(174)SVSG(PAVSR)**

B L (Leu) and I (Ile) can not be distinguished, thus L can be also I.

(186) fits with W, VS, SV, DA, AD, GE, EG.

(128) is Q or K. Assuming that the tryptic cleavage is complete, Q is more likely.

(174) C (modified with acrylamide, ocurred during electrophoresis), SS may also be possible but less likely.

(276) could be EF, FE, LY or YL.

(332) unknown combination of more than two amino acids.

(PAVSR) could also be PADVK or PAADR, PA could also be AP with all three possibilities, most likely the sequence is PAVSR.

Figure 3.8 Microsequencing analysis report of putative ANMT. Five peptide sequences were inferred from the purified protein (bold letters) (A). The numbers in parenthesis indicate that different amino acids or amino acids combinations are possible (B).

3.2 OMT cloning by PCR amplification

3.2.1 OMT specific oligonucleotide primers

After the purification of ANMT had failed and specific oligonucleotide primers could not be constructed, a different approach was used to isolate methyltransferase clones. The approach was based on the assumption that the sequence motifs involved in SAM binding must be conserved and thus the corresponding degenerate oligonucleotide primers described in the literature might be effective also with the *Ruta* transcripts as template.

Primers designed for the amplification of class II OMTs (Frick and Kutchan, 1999) were used for RT-PCR at rather low stringency with total RNA from *Ruta graveolens* cells that have been treated for three hours with *Pmg* elicitor (Junghans et al., 1998).

Tabel 3.2 Oligonucleotide primers used to amplify OMT-specific fragments (Frick & Kutchan, 1999).

Name	Degenerate primer
OMT 1 (universe)	5´-GTI GAC/T GTI GGI GGI GGI ACI GGI GC-3´
OMT 2 (reverse)	5´-GGI GCA/G TCC/T TCI ATI ACA/G TGI GG-3´
OMT 4 (reverse)	5´-CAA/G TGI G/TCA/G TCI C/GIC CAA/G TCA/G TG-3´

These bases are alternatively used (/); Inosine (I).

A mixture of PCR products of either 107 bp (OMT1-OMT2 primer combination) or 214 bp (OMT1-OMT4 primer combination) were amplified from total RNA. Both fragments were subcloned, and sequencing predicted a close relationship to plant OMTs. The clones were designated R-23 and R-27.

3.2.2 Generation of full size R-23 cDNA

The 3`-end of the R-23 fragment was extended by 3`RACE employing the general "*Adapter Primer*" (AP) and a gene specific primer (GSP). The amplification succeeded with an annealing temperature gradient between 53-64°C and resulted in a cDNA fragments of which included the "stop" codon and 160 bp downstream into the untranslated region. Sequencing of the untranslated region did not succeed to give a clean DNA stretch. R-23 gene fragment revealed high similarity with caffeic acid *O*-methyltransferases.

Table 3.3 Oligonucleotide primers for the 3`-RACE of R-23

Gene fragment	Oligonucleotide	Sequence
R-23	R23 3' end	5′- GCT CCA GCA TTT CCT GGT GTT GAG C -3′
	AP	5′- GGC CAC GCG TCG ACT AGT ACT $T_{(16)}$ -3′

Extension of the sequence in 5` orientation was accomplished by RLM-RACE. The first step ("touch down PCR") was performed with „GeneRacer 5′-Primer" and a GSP inferred from the amplified fragments. The PCR products appeared as a thick smear between 300-1000 bp. The "nested" PCR amplified a distinct fragment of approximate 1100 bp which was fully extended toward the 5` end, including the "start" methionine codon and 25 bp upstream of the untranslated region.

Table 3.4 Oligonucleotides for the 5´-RACE

Gene fragment	Oligonucleotide	Sequence
R-23	GeneRacer 5' Primer	5´- CGA CTG GAG CAC GAG GAC ACT GA -3´
	GeneRacer 5' Nested Primer	5´- GGA CAC TGA CAT GGA CTG AAG GAG TA -3´
	16 Antisense 1 (Nested Primer)	5'- CAT TCG GCG ACG ATC ACT TTC C- 3'
	16 Antisense 2 (RT-PCR-Primer)	5'- CAA GAC TTG GGT CTG GGT TCG-3'

3.2.3 Sequence analysis of R-23 cDNA

The full size cDNA consisted of 1143 bp and contained an open reading frame (ORF) of 1098 bp, encoding a polypeptide of 366 amino acid residues. An Mr of 40066 was calculated for the translated protein. The ORF was flanked by 25 bp 5` and 17 bp 3` untranslated regions (UTR) without the poly(A) tail.

```
                                        R23-5'end final  CACC
                                      CGGCCGCGAATTCGCCCTTCAACTC   25

ATGGGTTCGACAAGCTCTG>
ATGGGTTCGACAAGCTCTGAAACTCAAATGACACCAACCCAAGTCTCGGATGACGAAGCC   85
M  G  S  T  S  S  E  T  Q  M  T  P  T  Q  V  S  D  D  E  A    20

ATCCTCTACGCGATGCAACTGACAAGTGCCTCGGTCTTGCCAATGGTTCTGAAATCAGCG  145
I  L  Y  A  M  Q  L  T  S  A  S  V  L  P  M  V  L  K  S  A    40

GTGGAGCTTGATCTCTTGGAGATCATTGCCAAAGCCGGGGATGGTGCTTTCATGTCCCCA  205
V  E  L  D  L  L  E  I  I  A  K  A  G  D  G  A  F  M  S  P    60

AAGGACATAGCTTCTCAGCTCCCCACGACGAACCCAGATGCCCATATCATGCTTGATCGG  265
K  D  I  A  S  Q  L  P  T  T  N  P  D  A  H  I  M  L  D  R    80

ATGCTGCGTCTTTTGGCTAGCTACTCAGTCCTCAATTGCTCTTTGAATACTCTTCCTGAT  325
M  L  R  L  L  A  S  Y  S  V  L  N  C  S  L  N  T  L  P  D   100

GGGAAAGTGGGGAGGCTCTACGGGCTTGCTCCCGTCTCTAAATTCCTCACGAGGAATGAA  385
G  K  V  G  R  L  Y  G  L  A  P  V  S  K  F  L  T  R  N  E   120

GATGGCGTTACACTATCTGATCTTTGTCTCATGAATCAAGATAAGGTTCTGATGGAGAGC  445
D  G  V  T  L  S  D  L  C  L  M  N  Q  D  K  V  L  M  E  S   140
```

```
TGGTACTACTTGAAAGACGCGGTGCTTGAAGGTGGGATTCCATTCAACAAAGCCCACGGG  505
W  Y  Y  L  K  D  A  V  L  E  G  G  I  P  F  N  K  A  H  G    160

ATGACGGCATTCGATTACCACGGCACAGATCCAAGATTCAACAAGATTTTCAACAATGGA  565
M  T  A  F  D  Y  H  G  T  D  P  R  F  N  K  I  F  N  N  G    180

ATGTCCTCTCACTCTACCATTACCATGAAGAAAATTCTTCAAACTTACAAAGGCTTCGAA  625
M  S  S  H  S  T  I  T  M  K  K  I  L  Q  T  Y  K  G  F  E    200
         OMT1  GTIGAYGTIGGIGGIGGIACIGGIGC>
GGCCTCAACTCAATTGTTGATGTTGGTGGCGGAATTGGCGCCACCCTTAACATGATTGTT  685
G  L  N  S  I  V  D  V  G  G  G  I  G  A  T  L  N  M  I  V    220
                                                R23 3′  GCT
TCCAAATATCCATCAATCAGAGGCATTAACTTCGACTTGCCACACGTTATTGAGGATGCT  745
S  K  Y  P  S  I  R  G  I  N  F  D  L  P  H  V  I  E  D  A    240
CCAGCATTTCCTGGTGTTGAG>
CCAGCATTTCCTGGTGTCGAGCATGTTGGAGGAGACATGTTTGTTAGTGTTCCGAAGGCA  805
P  A  F  P  G  V  E  H  V  G  G  D  M  F  V  S  V  P  K  A    260

GATGCCATTTTCATGAAGTGGATATGCCATGACTGGAGTGACGAGCATTGCGTGAAATTC  865
D  A  I  F  M  K  W  I  C  H  D  W  S  D  E  H  C  V  K  F    280
               16 antisens 1   <CCTTTCACTAGCAGCGGCTTAC
TTGAATAATTGTTACGAAGCACTTCCGGAAAACGGGAAAGTGATCGTCGCCGAATGCACT  925
L  N  N  C  Y  E  A  L  P  E  N  G  K  V  I  V  A  E  C  T    300
     <GCTTGGGTCTGGGTTCAGAAC  16 antisens 2
CTTCCGGCGAGCCCAGACCCAAGTCTTGAGAGCAGAGTAGTCATTCATATTGACTGCATC  985
L  P  A  S  P  D  P  S  L  E  S  R  V  V  I  H  I  D  C  I    320

ATGCTGGCTCATAATCACGGCGGGAAAGAGAGGACTGAACAAGAGTTCCAAGCCTTGTCG 1045
M  L  A  H  N  H  G  G  K  E  R  T  E  Q  E  F  Q  A  L  S    340

AAGGCAGCTGGATTCCAAGGCTTCCATGTTATGTGCTCTGCTTTTAATACTTACATTATG 1105
K  A  A  G  F  Q  G  F  H  V  M  C  S  A  F  N  T  Y  I  M    360
     R23rev  <GGTATTAAGGTAGCGAGATAGCC
GAATTTCTTAAAAACCCATAATTCCATCGCTCTATCGG 1143
E  F  L  K  N  P  *                    366
```

Figure 3.9 Sequences of R-23 cDNA and translated polypeptides. Nucleotides and amino acid residues are numberd in the right margin. The start and the stop codon are bold printed.

The translated *Ruta* polypeptide had high similarity with plant caffeic acid *O*-methyltransferases (**Table 3.5**) and contained the conserved motifs and the catalytic residues reported from crystallized alfalfa caffeic acid OMT. Moreover, a phylogenetic analysis placed the *Ruta* protein in the COMT cluster. Therefore, the *Ruta* polypeptide was preliminarily assigned as COMT-like protein.

Tabel 3.5 Identity of the R-23 translated polypeptide with annotated COMTs.

Plant species	Identity (%)	Reference
Prunus dulcis	79	Garcia-Mas et al., 1995; X83217
Rosa chinensis	78	Cock et al., 2002; AJ439740
Clarkia breweri	77	Wang and Pichersky, 1997; AF0060009
Populus kitakamiensis	77	Hayakawa et al., 1996 ; D49710
Ocimum basilicum (COMT1)	74	Wang et al., 1999; AF154917
Eucalyptus gunnii	78	Poeydomenge et al., 1994; X74814
Medicago sativa	76	Gowri et al., 1991; M63853
Ocimum basilicum (COMT2)	73	Wang et al., 1999; AF154918
Populus tremuloides	73	Wu and Chiang, 1997; U50522
Capsicum annuum	73	Lee et al., 1998; U83789
Catharanthus roseus	71	Schröder et al., 2002; AY028439

3.2.4 Heterologous expression

The first attempt to clone the R-23 cDNA into an expression vector used the vector pET101/D-TOPO but it failed for unxplained reasons. Another trial with the vector pQE-60 was successful and required the creation of an *Nco*I restriction site at the 3` of the gene and a *Bam*HI restriction site after the stop codon. The *Nco*I restriction site was generated by PCR amplifications with appropriate primers in pCR2.1 vector. The insert was cut out with *Nco*I/*Xho*I and cloned into a modified vector pTZ19r (kindly provided by Dr. Frank Wellmann), that contained an additional *Bam*HI restriction site downstream of the *Xho*I site. Finally, the pTZ19r construct was digested with *Nco*I/*Bam*HI and the resulting fragment was cloned for expression into the vector pQE-60.

The expression plasmid was used to transform *E. coli* M15 [pREP4] cells.

3.2.5 Functional identification

3.2.5.1 Expression

The expression was induced by adding IPTG to the transformant culture, and the incubation was continued for an additional three hours at 37°C. Uninduced transformant and/or untransformed bacteria served as controls.

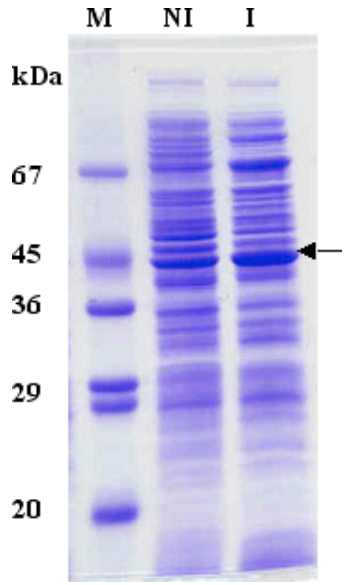

Figure 3.10 Expression of the *Ruta graveolens* R-23 protein in IPTG-induced M15[pREP4] transformants. Crude extracts (25 µg per lane) were separated by SDS-PAGE using 5% stacking gel and 12.5% separation gel and stained with Coomassie Brilliant Blue R-250. I, induced; NI: uninduced. Molecular weight markers (M) are indicated on the left margin.

3.2.5.2 Enzyme assays

Crude extracts of the transformed, IPTG-induced bacteria were evaluated for the ability to catalyze the methylation of caffeic acid, anthranilic acid and a wide variety of potential substrates (**Fig. 3.13**). However, in spite of the sequence similarity with other plant COMTs and the apparent expression of the polypeptide, no enzymatic activity could be assigned.

3.2.6 Generation of full size R-27 cDNA

The 3'-end of the R-27 fragment was extended by 3`RACE in two steps. The first PCR employed the general "*Adapter Primer*" (AP) and a gene specific primer (GSP) The second PCR used an additional „nested““*Abridged Universal Adapter Primer*" (AUAP) and another gene specific nested primer. The amplification of the 3` end succeeded with an annealing temperature gradient between 53-64°C and yielded a R-27 gene fragment of 657 bp containing the poly(A) tail.

Table 3.6 Oligonucleotide primers for the 3`-RACE of R-27

Gene fragment	Oligonucletide	Sequence
R-27	R27 3' end	5´- GCA GGG AAC TCA GGA CTT ACG -3´
	AP	5´- GGC CAC GCG TCG ACT AGT ACT $T_{(16)}$ -3´
	AUAP	5´- GGC CAC GCG TCG ACT AGT AC -3´
	3'RACE-R27-3	5´- CAA GGA GGA GAC ATG TTC GAC GCA ATT CC -3´

The 5` end of R-27 was identified using RLM-RACE. The first step ("touch down PCR") was performed with "GeneRacer 5´-Primer" and a GSP inferred from the amplified fragments. The PCR products of this first step could hardly be detected. A further "nested" PCR was required. In this step a distinct fragment of approximate 1100 bp was amplified which was fully extended toward the 5`-end, including the "start" methionine codon and 13 bp upstream of the untranslated region.

Table 3.7 Oligonucleotide primers for 5´-RACE

Gene fragment	Oligonucleotide	Sequence
R-27	GeneRacer 5´ Primer	5´- CGA CTG GAG CAC GAG GAC ACT GA -3´
	GeneRacer 5´ Nested Primer	5´- GGA CAC TGA CAT GGA CTG AAG GAG TA -3´
	R27-5'RACE	5'- GAG TGA CCT TGC ACC CAG ACA AGG-3'
	R27-5'nested 1	5'-GCT GAA GGC TCC TTC CAA GAC C-3'
	R27-5'nested 2	5'-CCA CTC TCG GAC GGT CCT CTG TTT GC-3'

3.2.7 Sequence analysis of R-27 cDNA

The full-size cDNA consisted of 1590 bp and contained an open reading frame (ORF) of 1122 bp, encoding a polypeptide of 374 amino acid residues. An Mr of 41559 was calculated for the translated protein. The ORF was flanked by 13 bp 5` and 455 bp 3`-untranslated regions (UTR) including a 20 bp poly(A) tail.

```
                                                  R27 5‘ final  CACC
                                                R27 5‘  GAAAGATCAAAAG
                                                        AGAAAGATCAAAG   13
ATGGCGATGGCTAATGG
ATGGCGATGGCTAATGG
ATGGCGATGGCTAATGGATCATCATCGTCACTGAGTGCTAGCTGGAGCTCTGCTGAGCTT   73
M  A  M  A  N  G  S  S  S  S  L  S  A  S  W  S  S  A  E  L    20

TTTAAAGCTCAAGGCCAGGTGTGGAATTGTGCACTTAATTGTGTGAATTCGCTGTCACTG  133
F  K  A  Q  G  Q  V  W  N  C  A  L  N  C  V  N  S  L  S  L    40

AAATGCGCCGTGGAGCTCGGCATACCGGATATAATTCACAGCCATGGCCAGCCCATGACC  193
K  C  A  V  E  L  G  I  P  D  I  I  H  S  H  G  Q  P  M  T    60

CTTTCACAGATATGCTCAGCACTCAACATCCAACCAAACAAGACCCAAGCCATGGAACGC  253
L  S  Q  I  C  S  A  L  N  I  Q  P  N  K  T  Q  A  M  E  R    80

GTCATGCGGATTCTTGTTCACTCCGGCTTCTTTTCTCTGAGCCGAGATGACGACCCTCAA  313
V  M  R  I  L  V  H  S  G  F  F  S  L  S  R  D  D  D  P  Q   100

GGGTATTACGGTCTCACGCCAGCTTCGAGACTCCTCCTAAAAGACGAGCCCTTCGGGGCG  373
G  Y  Y  G  L  T  P  A  S  R  L  L  L  K  D  E  P  F  G  A   120

GCCAAGTTTCTGCTCTTCATGACGCATCCCCACATGACAGATTCCTTGAACTCCTTGAGC  433
A  K  F  L  L  F  M  T  H  P  H  M  T  D  S  L  N  S  L  S   140

TCTTGGTTCCAGAACGATGTTCCCACCTCATTCGAGACGACGCATGGGAAAGGGTACTGG  493
```

```
S  W  F  Q  N  D  V  P  T  S  F  E  T  T  H  G  K  G  Y  W    160

GAGTTCGTAGCAGGTGAAGACAAGTTTAGCAAGATCTTTTATGATTGCATGGAGGCCGAC  553
E  F  V  A  G  E  D  K  F  S  K  I  F  Y  D  C  M  E  A  D    180

TCGGAGCTGATAGTAAGCGTTTTGATCAAGGATTATAAGGAGGTGTTCGAGGGGATTGGT  613
S  E  L  I  V  S  V  L  I  K  D  Y  K  E  V  F  E  G  I  G    200
OMT1  GTIGAYGTIGGIGGIGGIACIGGIGC>
TCCCTGGTCGATGTGGGAGGCGGCACCGGAGCTACGGCGCTGGCCCTGGCCAAGGCTTTT  673
S  L  V  D  V  G  G  G  T  G  A  T  A  L  A  L  A  K  A  F    220
                                      R27 3' end  GCAGGGAACT
CCTGAGATAAACTTCACTGTTTTCGATCAACCGCATGTTGTTCATAATTTGCAGGGGACT  733
P  E  I  N  F  T  V  F  D  Q  P  H  V  V  H  N  L  Q  G  T    240
CAGGACTTACG>   CAAGGAGGAGACATGTTCGACGCAATT>  3' RACE R27-3
CAGAACTTACGGTTTCAAGGAGGAGACATGTTCGACGCAATTCCCCCGGCAGATGCATTT  793
Q  N  L  R  F  Q  G  G  D  M  F  D  A  I  P  P  A  D  A  F    260
                  <GTRCTRACCISICTRCKIGTRAC  OMT6
CTACTCAAGTGGACTTCGCACGATTGGGGCGACGAAGACTGCGTGAAGATATTGAAGAAG  853
L  L  K  W  T  S  H  D  W  G  D  E  D  C  V  K  I  L  K  K    280

TGCAAAGAAGCAATACCAAGCAAAGGGAAGGTGATAATCATAGAGATCGCAATTAAAGAA  913
C  K  E  A  I  P  S  K  G  K  V  I  I  I  E  I  A  I  K  E    300

AACAGTAAAGAAGAAGCTGAAGAAGAAAAGGAAAAGGATGACTGCTCGAGTAAGACTGAG  973
N  S  K  E  E  A  E  E  E  K  E  K  D  D  C  S  S  K  T  E    320
                                    R27-5'nested 2  <CGTTTGTC
ACTGAGACGCAGCTGTGCTTCGACATGATGTGTATGCAGGCGTACAATTTCGGCAAACAG  1033
T  E  T  Q  L  C  F  D  M  M  C  M  Q  A  Y  N  F  G  K  Q    340
TCCTGGCAGGCTCTCACC     <CCAGAACCTTCCTCGGCCTAAGTCG  R27-5'nested 1
AGGACCGTCCGAGAGTGGGAGAAGGTCTTGGAAGGAGCCGGATTCAGCCATTACAAGATC  1093
R  T  V  R  E  W  E  K  V  L  E  G  A  G  F  S  H  Y  K  I    360
                   <CCAGTGAGTAACTTCGAATAGGG  R27-3' final
                            <GTAACTTCGAATAGGGACTAACCTAGGAAAC  Q27-3'
  <GGAACAGACCCACGTTCCAGTGAG  R27-5'RACE
ACTCCTTGTCTGGGTGCAAGGTCACTCATTGAAGCTTATCCCTGATTTTATCTCTGTGTT  1153
T  P  C  L  G  A  R  S  L  I  E  A  Y  P  *                   374

GGCTCCTGCGTAGAGAGACCTGCAAATCTCTTCTGCTCTTTTCATCATTTGTTCTGTGAA  1213
ATAAAACGAAGGAATTGCTTCGTCCCTCACGTTCTGTGAATGAATAAAATAAAAGCTGCC  1273
TGTTTCATCCTTATGAAGAGGCCACCGGGGATTTTACAATCTTGCTCCTTCTTCATTCCC  1333
CTCTGTGTATTTTTCTTCGCCTTCTTTGTGTTTGGTCATTTTCAGCAAACTCAAGTTTGT  1393
TATTCTTGATGCGAGCAGTTGATTAAGAATCCATTTGAGAGTTAGCATTTGTTAAAAGAA  1453
AAATTTGAGTGATATTATTGAGAGAAATCTTAGAAGAAACGTGTAATGTTTCTAATGTTG  1513
TTTATAACTGTTGGTTTGATCAGAGAAGATGAAGGAGCTTGTTGTCTTGAATGCTGCAAA  1573
AAAAAAAAAAAAAAAA                                              1590
```

Figure 3.11 Sequences of R-27 cDNA and translated polypeptide. Nucleotides and amino acid residues are numbered in the right margin. The start and the stop codon are bold printed.

Preliminary alignments of the full length *Ruta* sequence R-27 using the program WU-Blast2 (EMBL, Heidelberg) suggested a methyltransferase that presented a maximum 60% similarity with a methyltransferase from *Prunus dulcis* of unassigned function and chavicol and orcinol *O*-methyltransferase from *Rosa hybrida*, followed by a diverse list of OMTs for phenylpropanoids, flavonoids and alkaloids. The ambiguous role of this plant methyltransferases required further investigation on the substrate specificity.

3.2.8 Heterologous expression

The expression of R-27 cDNA was initially tried in vector pET 101/D-TOPO (Invitrogen, Netherland) and BL21 Star bacterial cells. Unfortunately, no enzyme expression could be detected.

Therefore, the open reading frame of R-27 cDNA including the stop codon was ligated into the vector pQE-60 and expressed in IPTG-induced *E. coli* M15 cells. This required the creation of an *Nco*I restriction site at the 3` of the gene and a *Bam*HI restriction site after the stop codon by PCR amplification. *Nco*I restriction sites in the ORF of R-27 cDNA had to be removed by *in vitro* PCR mutagenesis (QuikChange® Multi Site Directed Mutagenesis Kit; Stratagene) using mutagenesis primers without altering the amino acid sequence. The resulting expression construct was used to transform the *E. coli* M15 [pREP4] cells.

Table 3.8 Oligonucleotides for removing the internal *Nco*I restriction sites.

Gene	Oligonucleotide	Sequence
R-27	R27-mut1	5′-GTC ATG GGC TGG CCG TGA CTG TGA ATT ATA TCC -3′
	R27-mut2	5′-GCA TGA CGC GTT CCA TTG CTT GGG TCT TGT TTG G -3′

3.2.9 Functional identification

3.2.9.1 Expression

The expression was induced by adding IPTG and incubation for an additional three hours at 37^{o}C. Uninduced and/or untransformed bacteria served as controls.

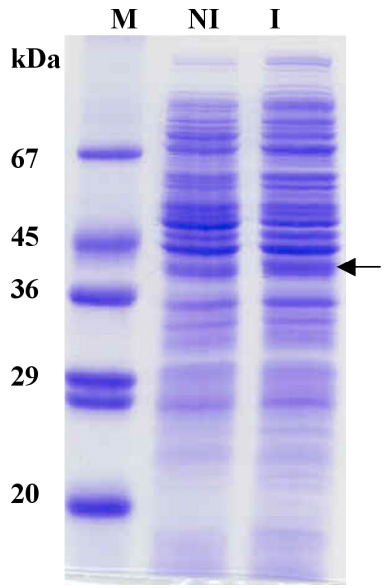

Figure 3.12 Expression of *Ruta graveolens* R-27 protein in IPTG-induced *E. coli* M15[pREP4] transformants. Crude extracts (25 µg per lane) were separated by SDS-PAGE using 5% stacking and 12.5% separating gel and stained with Coomassie Brilliant Blue R-250. I, extract from IPTG induced cells; NI: extract from uninduced cells. Molecular weight markers (M) are indicated in the left margin.

3.2.9.2 Substrate specificity

Because of the sequence relationship of the translated polypeptide to orcinol and chavicol OMTs, the crude extract from the induced transformants was evaluated for the ability to catalyze the methylation of a variety of low benzoic phenolics. Assays were conducted also with a variety of other phenolics, *e. g*. cinnamic acids, phenylpropanoid esters, coumarins, stilbenes, flavonoids, lignans, monoterpenes and acridone alkaloids (**Fig. 3.13**). Product formation was observed only with the methoxylated phenols 3,5-dimethoxyphenol, guaiacol, 3-methoxyphenol, 3,4-dimethoxyphenol, 2,3-dimethoxyphenol and 3,5-dihydroxyanisole. The preferred substrate was 3,5-dimethoxyphenol and the enzyme appeared more active towards *meta*-hydroxylated rather than *ortho*- or *para*-hydroxylated compounds. Extracts from *E. coli* transformed with the empty vector and fractionated with ammonium sulfate (60-80% saturation) did not yield any product. Anthranilic acid which was included in the assays because of a potential *N*-methyltransferase activity, was not accepted as substrate.

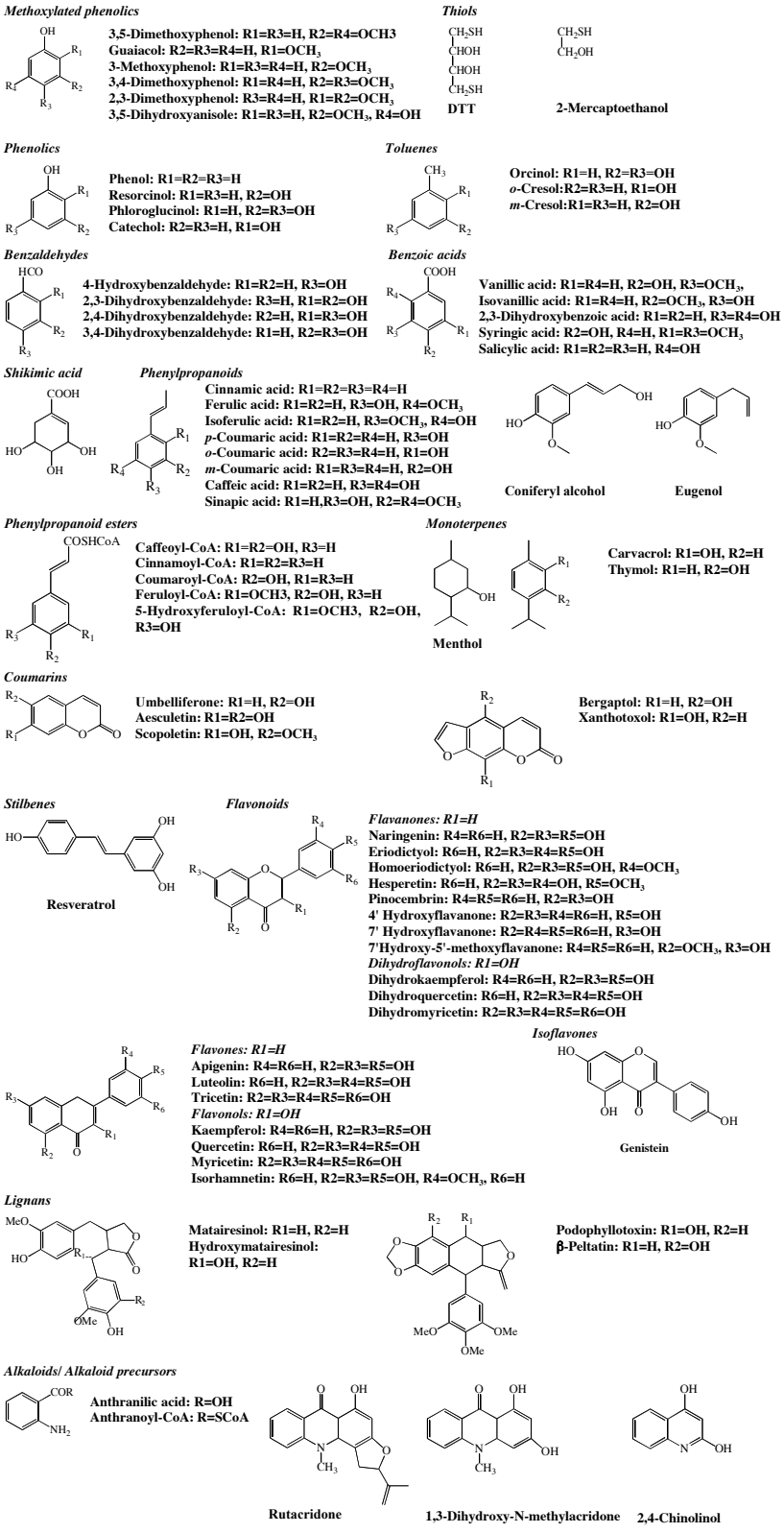

Figure 3.13 Compounds used as potential substrates in OMT assays with the enzymes expressed from R-23 or R-27 cDNA in *E. coli*.

3.2.10 *O*-methyltransferase reaction product identification

The conversion of the substrate 3,5-dimethoxyphenol was analyzed in furthe detail. The product of the reaction was identified as 1,3,5-trimethoxybenzene, initially by TLC and co-chromatography with an authentic reference sample (**Fig. 3.14**). The standards were visualized under UV irradiation (280 nm) and product was spotted by exposure to the BioImager Analyzer FLA-2000 (Fujifilm) (**Fig. 3.14**) based on S-adenosyl-L-[*methyl*-^{14}C]-methionine as cosubstrate (silica TLC developed in toluene:acetic acid 4:1, v:v; $R_{F\ 3,5\text{-dimethoxyphenol}}$ 0.46, $R_{F\ 1,3,5\text{-trimethoxybenzen}}$ 0.58).

Later, the product was isolated by semipreparative incubations (200 standard assays corresponding to 40 ml) and subjected to LC-MS analysis. The retention time and the fragmentation pattern in comparison to the authentic reference corroborated the identification as 1,3,5-trimethoxybenzene.

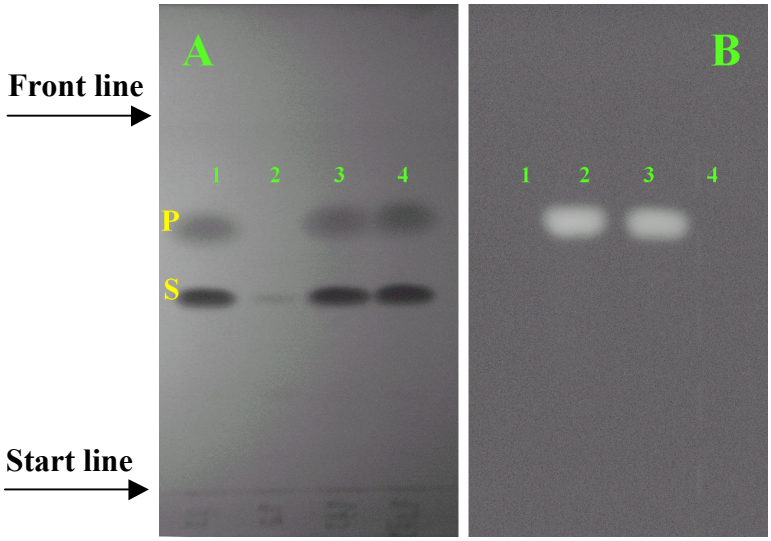

Figure 3.14 TLC separation of the product from OMT incubations with 3,5-dimethoxyphenol substrate. The assays were carried out with the recombinant pure enzyme (0.1 µg/ml) in the presence of S-adenosyl-L[*methyl*-^{14}C]-methionine (0.52 mCi/mmol). The incubations were extracted with ethylacetate, and the extracts were applied to silica F254 plates and developed in toluene:acetic acid (4:1, v/v). Authentic 3,5-dimethoxyphenol (S) and 1,3,5-trimethoxybenzene (P) were visualized under UV irradiation (254 nm) (A) and the enzymatic product was detected by autoradiography (B). Lane 1, references: 3,5-dimethoxyphenol (S) and 1,3,5-trimethoxybenzene (P); lane 2, extract from incubation of substrate; line 3, extract from incubation of substrate, and product; line 4, extract from the incubation of substrate and product with bacteria (1mg/ml) transformed with the empty vector.

3.2.11 Comparision of R27 with annotated plant OMT sequences

Alignments of *Ruta* 3,5-dimethoxyphenol O-methyltransferase (GenBank accession AY894417) with annotated methyltransferase using the program WU-Blast2 (EMBL, Heidelberg) did not reveal any significant sequence similarity to plant class I OMTs (caffeoyl CoA OMTs) or *S*- and *N*-methyltransferases. Alignment of the translated polypeptide with eight class II OMTs (catechol related OMTs) from data base accessions (**Fig. 3.15**) revealed 61-62% sequence similarity with chavicol and eugenol OMTs from *Ocimum basilicum* (ObCOMT, AAL30423; ObEOMT, AAL30424) and orcinol OMTs from *Rosa hybrida* and *Rosa chinensis* (RhOOMT, AAM23005; RcOOMT, CAD29458) at a 46-50% level of identity. Less identity was observed with a flavonoid OMT from *Chrysosplenium americanum* (CaCOMT, AAA86982) (41%) or pinosylvin OMT from *Pinus sylvestris* (PsPOMT, Chiron et al., 2000) and reticuline OMT from *Papaver somniferum* (PsROMT, AAQ01668) (36%), whereas the identity with caffeic acid OMT from *Medicago sativa* (MsCOMT, P28002) was in the range of 30% only. The five sequence elements conserved in SAM-dependent OMTs and involved in SAM and metal binding, were also recognized in the *Ruta* polypeptide R-27 (**Fig. 3.15,** regions I-V). These elements are universally present in plant class II OMTs (Ibrahim et al., 1998; Schröder et al., 2002). Nevertheless, two peculiar features distinguish this polypeptide from the plant class II OMTs reported so far: a serine-rich *N*-terminal region and a low complexity insert placed between Glu308 and Thr321. The evaluation by the Signal P program (Nielsen et al., 1997; Bendtsen et al., 2004) suggested a signal domain in the *N*-terminal 24 amino acids, although a particular function could not be assigned. The insertion of thirteen residues is separating motif IV and motif V which had been proposed as metal binding and catalytic regions, respectively, in catechol OMT (Vidgren et al., 1994). Finally, the phylogenetic analysis using the program CLUSTAL W, grouped the *R. graveolens* polypeptide closer to orcinol OMTs from roses (Lavid et al., 2002; Scalliet et al., 2002) than to class I COMT or COMT-related enzymes.

```
RgOMT           MAMANGSSSSLSASWS----SAELFKAQGQVWNCALNCVNSLSLKCAVELGIPDIIHSHG 56
RcOOMT          MERLN-SFRHLNQKWSNGEHSNELLHAQAHIWNHIFSFINSMSLKSAIQLGIPDIINKHG 59
RhOOMT          MERLN-SFKHLNQKWSNGEHSNELLHAQAHIWNHIFSFINSMSLKSAIQLGIPDIINKHG 59
ObEOMT          -----MALQKVDISLS----TEQLLQAQVHVWNHMYAFANSMSLKCAIQLGIPDILHKHG 51
ObCOMT          -----MALQNMDISLS----TEQLLQAQAHVWNHMYAFANSMSLKCAIQLGIPDILHKHD 51
PsROMT          -------MDT----------AEERLKGQAEIWEHMFAFVDSMALKCAVELGIPDIINSHG 43
MsCOMT          -----MGSTGETQITP---THISDEEANLFAMQLASASVLPMILKSALELDLLEIIAKAG 52
PsPOMT          -----MGSASESSEMN---AKIVNEDEWLLGMELGNFSCVPMAMKAAIELDVLQIIANAG 52
CaCOMT          ---------------------------MLFAMQLACASVLPMVLKSAIELDLLEIIR--G 31

RgOMT           QPMTLS--QICSALNIQPNKTQA----MERVMRILVHSGFFSLSR-DDDPQGYYGLTPAS 109
RcOOMT          YPMTLS--ELTSALPIHPTKSHS----VYRLMRILVHSGFFAKKKLSKTDEEGYTLTDAS 113
RhOOMT          -PMTLS--ELTSALPIHPTKSHS----VYRLMRILVHSGFFAKKKLSKTDEEGYTLTDAS 112
ObEOMT          RPMTLS--QLLQSIPINKEKTQC----FQRLMRALVNSNFFIEENNSNNQEVCYWLTPAS 105
ObCOMT          HPMTLS--QLLKAIPINKEKSQS----FQRLMRALVNSNFFIEEN-SNNQEVCYWLTPAS 104
PsROMT          RPVTIS--EIVDSLKTNTPSSSPNIDYLTRIMRLLVHKRLFTSELHQESNQLLYNLTRSS 101
MsCOMT          PGAQISPIEIASQLPTTNPDAPVMLDRMLRLLACYIILTCSVRTQQDGKVQRLYGLATVA 112
PsPOMT          NGVQLSPRQIVAHIPTTNPDAAITLDRILRVLASHSVLSCSVTTDENGKAERLYGLTPLC 112
CaCOMT          QDTCMSPTEIASHLPTTNPDAPAMVDRILRLLSCYSVVTCSVRSVDD---QRVYGLAPVC 88


RgOMT           RLLLKDEP-FGAAKFLLFMTHPHMTDSLNSLSSWFQND-VPASFETTHGKGYWEFVAGED 167
RhOOMT          QLLLKDHP-LSLTPFLTAMLDPVLTTPWNYLSTWFQNE-DPTPFDTAHGMTFWDYGNHQP 170
RcOOMT          QLLLKDHP-LSLTPYLTAMLDPVLTNPWNYLSTWFQND-DPTPFDTAHGMTFWDYGNHQP 171
ObEOMT          CLLLKEAP-LTVTPLVQVVLDPTFTNPWHHMSEWFTHEKHATQFEAANGCTFWEKLANEP 164
ObCOMT          RLLLKGAP-LTVAPLVQVVLDPTFTNPWHYMSEWFKHENHATQFEAANGCTFWEKLANKP 163
PsROMT          KWLLKDSK-FNLSPLVLWETNPILLKPWQYLGKCAQEK--SSPFERAHGCEIWDLALADP 158
MsCOMT          KYLVKNEDGVSISALNLMNQDKVLMESWYHLKDAVLDG--GIPFNKAYGMTAFEYHGTDP 170
PsPOMT          KYLVKNQDGVSLAPLVLMNQDKVLMESWYYLKDAVLDG--SQPFTKAHGMNAFEYPAMDQ 170
CaCOMT          KYLTKNQDGVSIAALCLMNQDKVLMESWYHLKDAVLDG--GIPFNKAYGMSSFEYHGTDP 146


RgOMT           KFSKIFYDCMEADSELIVSVLIKDYKEVFEGIG-SLVDVGGGTGATALALAKAFPEINFT 226
RhOOMT          SIAHLFNDAMASDARLVTSVIIDDCKGVFEGLE-SLVDVGGGTGTVAKAIADAFPHIECT 229
RcOOMT          SIAHLFNDAMASDARLVTSVIINDCKGVFEGLE-SLVDVGGGTGTLAKAIADAFPHIECT 230
ObEOMT          SKGRFFDEAMSCDSRLIAHVFTKDYKHVIEGIR-TLVDVGGGNGTMAKAIVEAMPTIKCT 223
ObCOMT          SMGRFFDEAMSCDSRLVAHVLTKDYKHVIDGIR-TLVDVGGGNGTMAKAIVEAVPTMKCT 222
PsROMT          KFNNFLNGAMQCSTTTIINEMLLEYKDGFSGIAGSLVDVGGGTGSIIAEIVKAHPHIQGI 218
MsCOMT          RFNKVFNKGMSDHSTITMKKILETYTG-FEGLK-SLVDVGGGTGAVINTIVSKYPTIKGI 228
PsPOMT          RFNRVFNRGMAEHSTMLMNKILDTYEG-FKEVQ-ELVDVGGGVGSTLNLIVSKYPHISGI 228
CaCOMT          RFNKVFNRGMSDHSTITMKKVFQTYQG-FQGLT-SLVDVGGGTGATLTMILSKYPTIRCI 204
                                                   I


RgOMT           VFDQPHVVHNLQGTQNLRFQGGDMFDAIPPADAFLLKWTLHDWGDEDCVKILKKCKEAIP 286
RhOOMT          VLDLPHVVADLQGSKNLKYTGGDMFEAVPPADTVLLKWILHDWNDEECIKILKRSRVAIT 289
RcOOMT          VLDLPHVVADLQGSKNLKYTGGDMFEAVPPADTVLLKWILHDWSDEECIKILERSRVAIT 290
ObCOMT          VLDLPHVVAGLESTDKLSYIGGDMFQSIPSADAILLKFIIHDWDDEEGLKILKRCKDAVG 282
ObEOMT          VIDLPHVVAGLESTDNLNYIGGDMFQSIPSADAILLKSIIHDWDDVEGLKILKKCKDAVV 283
PsROMT          NFDLPHVVATAAEFPGVKHVGGDMFVDIPEADAVIMKWILHDWSDEDCTIILKNCYRAIR 278
MsCOMT          NFDLPHVIEDAPSYPGVEHVGGDMFVSIPKADAVFMKWICHDWSDEHCLKFLKNCYEALP 288
PsPOMT          NFDMPHVVADAPHYPAVKHVGGDMFDSVPSGQAIFMKWILHDWSDDHCLRLLKNCHKALP 288
CaCOMT          NFDLPHVIEDAPEYPGIEHVGGDMFVSVPKGDAIFMKWICHDWSDEHCLKLLKNCYDALP 264
                  II              III


RgOMT           SK---GKVIIIEIAIKENSKEEAEEEKEKDDCSSKTETETQLCFDMMCMQAYNFGKQRTV 343
RhOOMT          SKDKKGKVIIIDMMMENQKGDEES-------------IETQLFFDMLMMALVR-GQERNE 335
RcOOMT          GKEKKGKVIIIDMMMENQKGDEES-------------IETQLFFDMLMMALVG-GKERNE 336
ObEOMT          MG---GKVIIIDVVVGVNHDIDEV-------------LEDQLHFDMAMMCYFN-AKERTM 326
ObCOMT          IG---GKVIIIDVVVGVNHDVDEV-------------LEDQLHFDMAMMSYFN-AKERTM 325
PsROMT          KKK-NGKVIIVDCVLRPDGNDLFD--------------KMGLIFDVLMMAHTTAGKERTE 323
MsCOMT          DN---GKVIVAECILPVAPDSSLA-------------TKGVVHIDVIMLAHNPGGKERTQ 332
PsPOMT          EK---GKVIVVDTILPVAAETSPY-------------ARQGFHIDLLMLAYNPGGKERTE 332
CaCOMT          NN---GKVILAECILPEVPDSSLA-------------TKGVVHIDVITVAHNPGGKERTE 308
                  IV                                                   V
```

```
RgOMT           REWEKVLEGAGFSHYKITPCLGARSLIEAYP----- 374
RhOOMT          KEWAKLFTDAGFSDYKITPILGLRSLIEVYP----- 366
RcOOMT          KEWAKLFTDAGFSDYKITPISGLRSLIEVYP----- 367
ObEOMT          SEWEKLIYDAGFKSYKLTPAFGVRSLIEAYP----- 357
ObCOMT          NEWEKLISAAGFTSYKLTPAFGVRSLIEAYP----- 356
PsROMT          AEWKILLNNAGFPRYNVIRTPAFPCIIEAFPE---- 355
MsCOMT          KEFEDLAKGAGFQG-FKVHCNAFNTYIMEFLKKV-- 365
PsPOMT          QEFRDLAKEVGFAGGVKPVCCVNGHWVMEFHK---- 364
CaCOMT          KEFEALAKAAGFQG-FQVFCNAFNTYIIEFSKKICN 343
```

Figure 3.15 CLUSTAL W alignment of the translated *Ruta* R-27 polypeptide with related plant *O*-methyltransferases. The regions designated I through V represent conserved motifs (Ibrahim et al., 1998; Ni and Hager, 1998; Frick and Kutchan, 1999; Schröder et al., 2002).

3.2.12 Purification of recombinant 3,5-dimethoxyphenol OMT

The recombinant 3,5-dimethoxyphenol OMT was biochemically characterized further after partial purification from the crude bacterial extract. The peptide was expressed without a His-tag, the purification was conducted by classical chromatographic methods. The enzyme was purified about 30-fold with 4.4% recovery from the crude extracts by ammonium sulfate precipitation and successive fractionation by size exclusion, anion exchange and affinity chromatographies. During the whole purification procedure the enzyme activity remained stable.

3.2.12.1 Ammonium sulfate precipitation

The transformed bacterial cells induced with IPTG for three hours were centrifuged. The resulting pellets (pellet weight: 3.5 g) were resuspended in 10 ml buffer 0.1 M Tris-HCl pH 7.5 then disrupted by ultrasonication and the crude extract with a specific activity of 0.4 µkat/kg was subjected to ammonium sulfate fractionation (60 to 80% saturation). After desalting on a PD 10 column (Amersham Biosciences, Braunschweig) the specific activity of the dissolved enzyme increased to 0.9 µkat/kg.

3.2.12.2 Size exclusion chromatography

The active fraction resulting from ammonium sulfate precipitation was dissolved in 0.1 M Tris-HCl buffer pH 7.5 (4 ml), and aliquots of 1 ml were loaded on a size exclusion column Fractogel EMD BioSEC (S) and eluted with the same buffer. Active fractions (24) were collected, pooled (average specific activity 1.9 µkat/kg) and flash-frozen in liquid nitrogen.

3.2.12.3 Anion exchange chromatography

The enzyme collected from size exclusion chromatography was loaded through a superloop on a Fractogel EMD DEAE 650 (S) column. The column was washed with 0.1 M Tris-HCl pH 7.5 and the bound proteins were eluted in the same buffer by a linear 1 M NaCl gradient ranging from 0 to 100%. The active fractions eluetd between 0.5 and 0.6 M NaCl. The most active fractions were pooled and desalted on a PD 10 column. The specific activity was increased to 4.3 µkat/kg and SDS-PAGE inspection of the active samples pointed out the enrichment of a 42 kDa band (**Fig. 3.16**).

3.2.12.4 Affinity chromatography

Pooled anion exchange fractions (6 ml) were loaded on a 1 ml adenosine-agarose affinity column via superloop. This column material has been successfully employed for the purification of different methyltransferases (James et al., 1995, Kato et al., 1999). The column material was first washed with the starting buffer and then with buffer containing 0.2 M NaCl at which the enzyme is just beginning to elute. The enzyme was eluted with buffer containing 0.2 M NaCl and 2 mM SAM. SDS-PAGE of the eluted fractions displayed only one band of 42 kDa (**Fig. 3.16**).

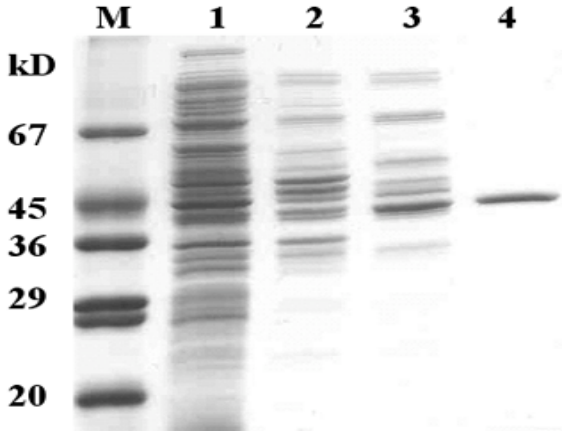

Figure 3.16 SDS-PAGE separation of the *R. graveolens* 3,5-dimethoxyphenol OMT expressed in *E. coli*. The crude extract (19.5 µg protein, lane 1) or the protein eluted from Fractogel EMD BioSEC (S) column (6.5 µg, lane 2) , Fractogel DEAE column (1.4 µg, lane 3), and adenosine-agarose (1.0 µg, lane 4) was separated on a 5% stacking and 12.5% separation gel, and staining of the gels was accomplished with Coomassie Brilliant Blue R-250. Molecular mass markers (lane M) are indicated in the left margin.

Table 3.9 Purification of recombinant *R. graveolens* OMT

Purification step	Protein	Specific activity	Apparent purification	Recovery
	mg	µkat/kg	-fold	%
Crude extract	206.6	0.4	1	100
Ammonium sulfate (0.6-0.8)	75.0	0.9	2.3	81.7
Fractogel EMD BioSEC (S)	15.4	1.9	4.8	35.4
Fractogel EMD DEAE 650 (S)	3.9	4.3	10.8	20.3
Adenosine-agarose	0.3	12.2	30.5	4.4

The enzyme was expressed in IPTG-induced *E. coli* M15, and the cells (14.0 g wet pellet) were extracted in 0.1 M Tris-HCl buffer pH 7.5. The OMT activity was measured with 3,5-dimethoxyphenol and S-adenosyl-[*methyl*-^{14}C]-methionine under standard assay conditions.

3.2.13 Molecular weight of the native protein

SDS-PAGE documented the apparent homogeneity of the purified OMT revealing only one protein band of about 42 kDa. Considering that most of the OMTs are dimers, comparative size exclusion chromatography of the recombinant enzyme was performed on a calibrated superose column. The results indicated a molecular mass of roughly 84 ± 5 kDa for the active dimethoxyphenol OMT suggesting a homodimeric composition. The relative molecular weight of 3,5-dimethoxyphenol OMT was calculated by extrapolating the regression line given by the elution of the reference proteins. The kit of reference proteins (Amersham Biosciences, Braunschweig) used for calibration contained proteins of low molecular weight covering the range from 67 to 13.7 kDa.

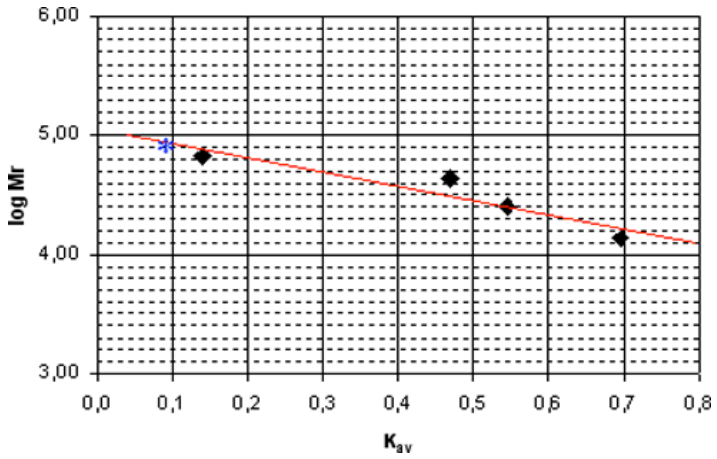

Figure 3.17 Relative molecular weigh of recombinant 3,5-dimethoxyphenol OMT. The enzyme fractionated by ammonium sulfate (60 to 80% saturation) was applied to the calibrated Fractogel EMD BioSEC column. The linear relation between the molecular weight logarithm and the K_{av} indicate a molecular weight of about 84 kDa (blue star). The reference proteins (solid square) are: BSA (67 kDa), ovalbumin (45 kDa), carboanhydrase (25 kDa) and ribonuclease A (13.7 kDa).

3.2.14 Catalytic parameters

The biochemical characterisation of the dimethoxyphenol OMT required the determination of optimal enzyme assay conditions. The recombinant enzyme partially purified through ammonium sulfate precipitation and SEC was used for these studies.

3.2.14.1 Protein amount

The protein amount was initially varied from 10 to 400 µg in 0.1 M Tris buffer pH 7.5, and the assays were run for 60 min at 37°C in 200 µl total volume. The turnover rate increased linearily with the protein amount up to 200 µg under these conditions. For further investigations, the protein amount was kept constant at 100 µg.

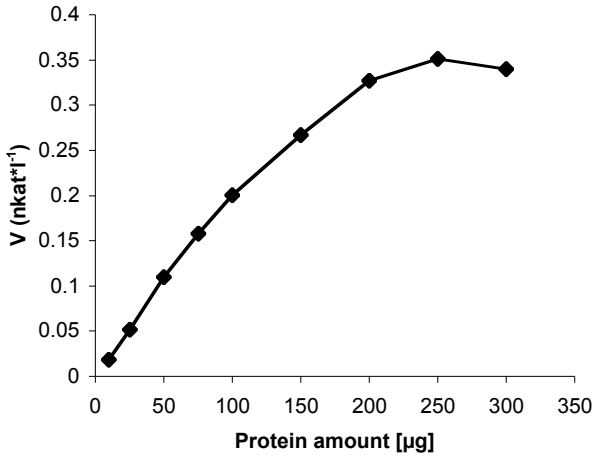

Figure 3.18 *Ruta* dimethoxyphenol OMT activity determined with varying amounts of enzyme.

3.2.14.2 Incubation time

The time of incubation was varied between 30 seconds and 90 minutes, and the amount of product increased steadily over the full time period. A reaction incubation time of 30 min was chosen for future experiments

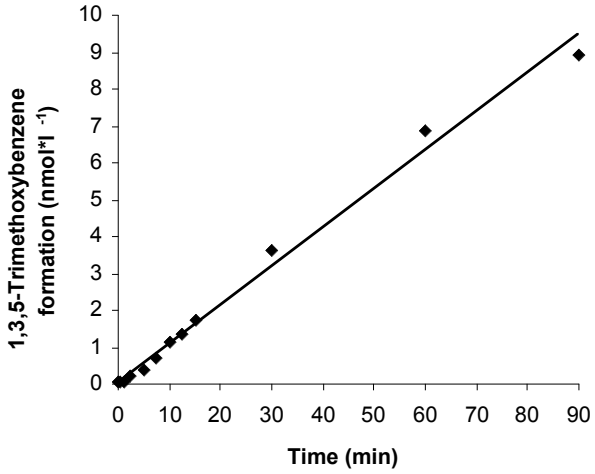

Figure 3.19 Dependency of *Ruta* dimethoxyphenol OMT activity of the incubation time.

3.2.14.3 Temperature

The recombinant enzyme showed a broad temperature optimum with maximal turnover at about 36°C.

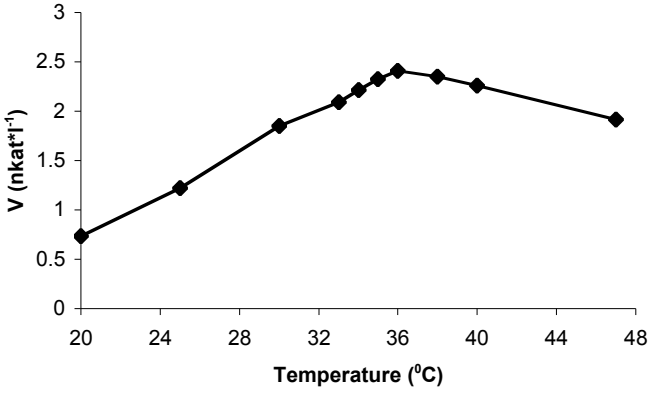

Figure 3.20 Temperature dependency of *Ruta* dimethoxyphenol OMT activity.

3.2.14.4 pH

Enzyme assays in various 0.1 M buffers covering the range from pH 2.0 to 10.0 revealed the highest rate of methylation at pH 7.5 in Tris-HCl. Half the maximal activity was found at pH 6.5 and pH 9.0 in potassium phosphate (Kpi) and Gly-NaOH buffers, respectively.

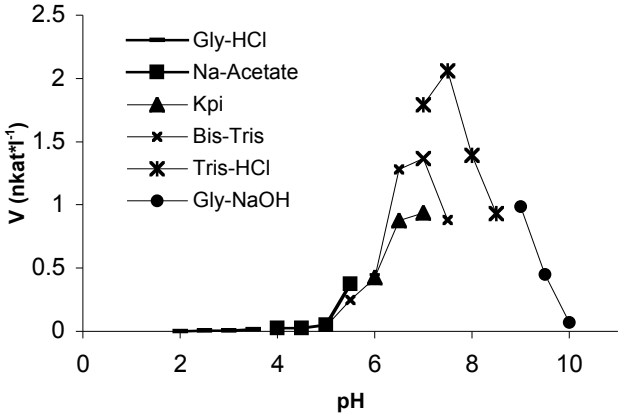

Figure 3.21 pH dependency of *Ruta* dimethoxyphenol OMT activity.

3.2.14.5 SAM saturation

Provisional assays aiming at a saturating SAM concentration were done with 3,5-dimethoxyphenol at 25 mM and the SAM concentration was varied between 1.6-75.0 µM. The conversion rate reached its maximal value beyond 25 µM SAM. The variable determination of substrate affinities, however requires more knowledge about the eventual affectes of metals or thiols

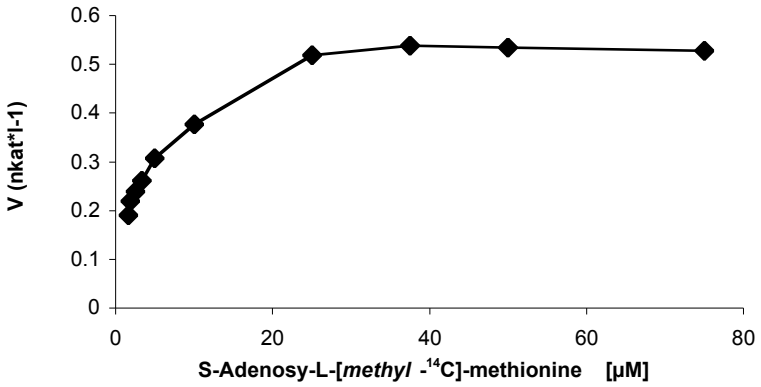

Figure 3.22 *Ruta* OMT activity at various SAM concentrations.

3.2.14.6 Metal dependency

The activity of recombinant 3,5-dimethoxyphenol *O*-methyltransferase obviously did not require divalent cations, but eventual inhibitory effects were examined under standard assay conditions (time, pH, temperature, substrate concentrations) in the presence of 2 mM of different ions. The addition of Cu^{2+}, Zn^{2+} or Hg^{2+} totally inhibited the OMT activity while Co^{2+} and Fe^{2+}, respectively, inhibited the activity by 77% or 78%. Other cations were even less inhibitory: Mn^{2+} 25%; Fe^{3+} 31%; Mg^{2+} 15%; Ca^{2+} 23%. The effect of Zn^{2+} was investigated further, and OMT inhibition of over 90%, 72% and 33%, respectively, was observed at 10 µM, 5 µM or 2 µM $ZnCl_2$ in comparison with control assays lacking zinc. The addition of 5 or 10 mM EDTA fully reversed the inhibitory effect of 2 mM $ZnCl_2$ on the OMT activity.

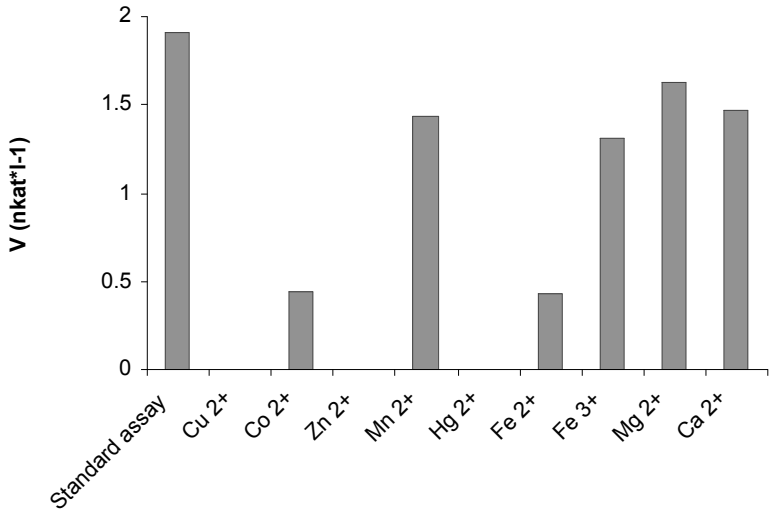

Figure 3.23 Effect of 2 mM metal concentrations on the 3,5-dimethoxyphenol OMT activity.

3.2.14.7 Effect of thiols and thiol reagents on OMT activity

Thiol blocking reagents are strong inhibitors of the methyltransferase reaction suggesting the presence of at least one essential Cys residue (James et al., 1995), whereas the addition of thiols (2-mercaptoethanol or dithiothreitol) commonly protects enzymes from oxidation. The effect of thiols on OMT activity was investigated in combination with thiol bloking reagents. The incubation mixtures lacking SAM were preincubated for 15 min with various compounds and the assays were started by the addition of SAM. The thiol blocking reagents phenylmethylsulfonyl fluoride (PMSF), *p*-hydroxymercuribenzoate (pHMB) or *N*-ethylmaleimide (NEM) completely inhibited the OMT activity, and this effect could be partly reversed by the addition of 5 mM DTT (**Table 3.10**). Preincubation of the assay with thiols, i.e. 5 mM DTT, surprisingly seemed to suppress the OMT activity by about 25% (**Table 3.10**). The addition of 2-mercaptoethanol also negatively affected the methylation activity, but required a relatively high concentration (50 mM).

Table 3.10 The effect of thiols and thiol blocking reagents on OMT activity.

Addition to the standard assay	Concentration	Relative OMT activity
	mM	%
None	-	100
1,4-Dithiothreitol (DTT)	5	76
2-Mercaptoethanol	50	80
Phenymethylsulfonyl fluoride (PMSF)	5	ND
PMSF + DTT [1]	5/5	1.3
p-Hydroxymercuribenzoate (*p*HMB)	0.1	ND
*p*HMB + DTT [1]	0.1/5	38
N-ethylmaleimide (NEM)	0.5	ND
NEM + DTT [1]	0.5/5	60

The recombinant dimethoxyphenol OMT, partially purified through ammonium sulfate fractionation and SEC chromatography, was preincubated for 15 min in the standard incubation mixture lacking the substrate with either one of the thiols or/and thiol reagents. The OMT reaction was started subsequently by the addition 3,5-dimethoxyphenol. The catalytic activity in the absence of thiols or thiol reagents was 12.2 µkat (100%). ND, undetectable. [1] Preincubation with thiol blocking reagent was followed by the addition of DTT and another 15 min period of preincubation prior to stating the OMT reaction with the substrate.

3.2.14.8 Substrate affinities

The Michaelis-Menten constant (Km) characterizes the enzyme turnover rate at half-maximal substrate saturation and is independent of the enzyme concentration. Kinetic OMT assays were conducted under pseudo-first order conditions for 30 min at 36^{o}C in 100 mM Tris-HCl buffer pH 7.5 in a total volume of 200 µl. Per incubation were used 100 µg partially purified enzyme (after SEC). To determine the apparent K_m for SAM, 3,5 dimethoxyphenol was kept constant at 0.25 mM (which is a saturating concentration as confirmed later) and the SAM was varied between 1.6-10 µM. K_m and V_{max} values were extrapolated from Lineweaver-Burk plots (**Fig. 3.24**). These assays revealed the apparent K_m for SAM at 2 µM. Control assays were carried out omitting the substrate or

the enzyme as well as using the denaturated enzyme (10 min at 100°C). No activity was recorded in these assays.

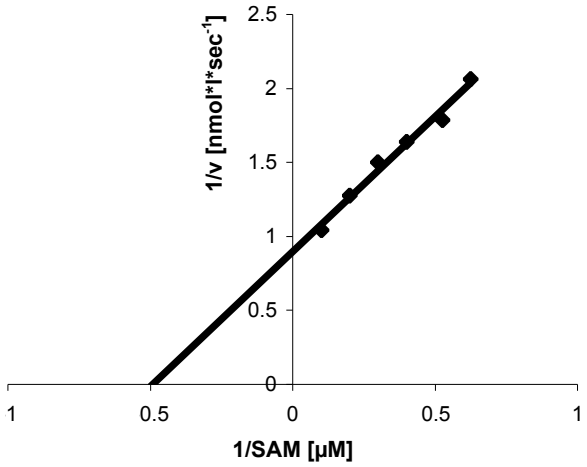

Figure 3.24 Lineweaver-Burk diagram for the determination of the apparent $K_{m\ SAM}$ (2 µM) of *Ruta* dimethoxyphenol OMT.

In order to determine the apparent K_m for 3,5-dimethoxyphenol 25 µM SAM (about 10-fold K_m) was used and 3,5-dimethoxyphenol was added over a range from 8 to 100 µM. Double reciprocal plots of the data revealed the apparent K_m at 20.4 µM (**Table 3.11**).

Higher substrate concentrations had to be employed in kinetic studies with other phenolics. In case of 3-methoxyphenol or guaiacol as substrate, concentrations between 8 to 100 µM were used and revealed K_m values at 44.6 µM and 66.6 µM, respectively (**Table 3.11**). The apparent K_m for 3,4-dimethoxyphenol, 2,3-dimethoxyphenol and 3,5-dihydroxyanisole was measured at 40 to 300 µM substrate concentrations. The apparent K_m values attained were 85.3 µM for 3,4-dimethoxyphenol; 275.8 µM for 2,3-dimethoxyphenol and 346.2 µM for 3,5-dihydroxyanisole (**Table 3.11**).

Table 3.11 Substrate affinities of recombinant dimethoxyphenol OMT from *R. graveolens*

Substrate	Apparent Km	Apparent V_{max}
	µM	nmol * s^{-1}
3,5-Dimethoxyphenol	20.4	7.1
3-Methoxyphenol	44.6	1.5
Guaiacol	66.6	0.9
3,4-Dimethoxyphenol	85.3	3.4
2,3-Dimethoxyphenol	275.8	2.3
3,5-Dihydroxyanisole	346.2	1.4
SAM	2.0	0.9

3.2.15 Thiol methyltransferase activity (TMT)

Enzyme incubations conducted with the recombinant 3,5-dimethoxyphenol OMT in the presence of 5 mM DTT as part of the stability and inhibition studies showed 24% less activity compared to the control standard assays. This result was unexpected and is contradictory to the literature which indicates a beneficial effect of DTT on activity (Fujioka, 1992; James et al., 1995). Therefore, DTT was assumed to act as a potential substrate and used in sets of activity assays in the absence and in the presence of different metal ions, because metal cofactors had been proposed for various methyltransferases including thiol methyltransferases (Matthews and Goulding, 1997). For these assays, the enzyme had to be partially purified at least from the crude bacterial extracts, because the non-transformed host strain of *E. coli* expressed some minor thiolmethyltransferase (TMT) activity that was completely removed by ammonium sulfate precipitation (60-80% saturation). When DTT was added to the incubation in stead of the phenolic substrate at 5 mM, a new product was formed although the activity was rather low (below 0.02 µkat/kg).

However, the activity increased significantly on the addition of zinc. The assays converted DTT to a new product that could be separated by thin-layer chromatography.

3.2.16 Thiol methyltransferase product identification

The recombinant dimethoxyphenol OMT revealed a specific activity with DTT as substrate in the presence of 1 mM Zn^{2+} of 1.9 µkat/kg which increased to 2.6 µkat/kg at 2 mM Zn^{2+} and reached a maximum of 3.7 µkat/kg when 4 mM Zn^{2+} were supplied (**Fig. 3.25**).

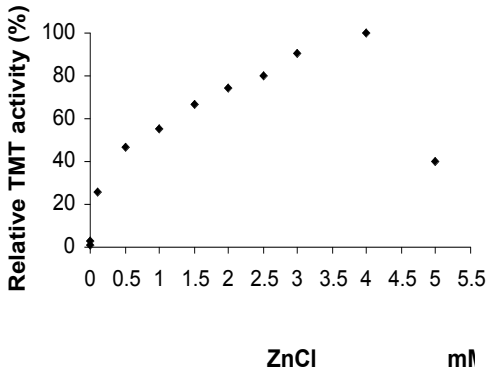

Figure 3.25 TMT activity of the recombinant *R. graveolens* OMT determined at 5 mM 1,4-dithiothreitol and 25 µM *S*-adenosyl-L-[*methyl*-^{14}C]methionine (7.9 µCi µmol^{-1}) as a function of increasing concentration of zinc chloride. The product was extracted from the incubation with ethylacetate and quantified by liquid scintillation counting. The highest conversion rate (3.7 µkat/kg) was set at 100%.

At a lesser extend, the TMT activity was also stimulated by addition of Fe^{2+} or Fe^{3+} (0.36 µkat/kg at a concentration of 2 mM).

The proper choice of Zn^{2+} concentration initially presented a problem, because the rate of turnover of DTT as a function of DTT concentration in the assay depended strongly on the Zn^{2+} concentration (**Fig. 3.26**): while at 10 µM Zn^{2+} maximal conversion rates were achieved at about 0.1 mM DTT, the stoichiometry approached 1:1 at concentrations exceeding 0.5 mM Zn^{2+}, suggesting an inhibition at elevated concentration as was observed previously for the 3,5-dimethoxyphenol OMT activity (**Table 3.10**). The data also suggested that excess DTT completely titrated the available Zn^{2+} and restored the methylating activity.

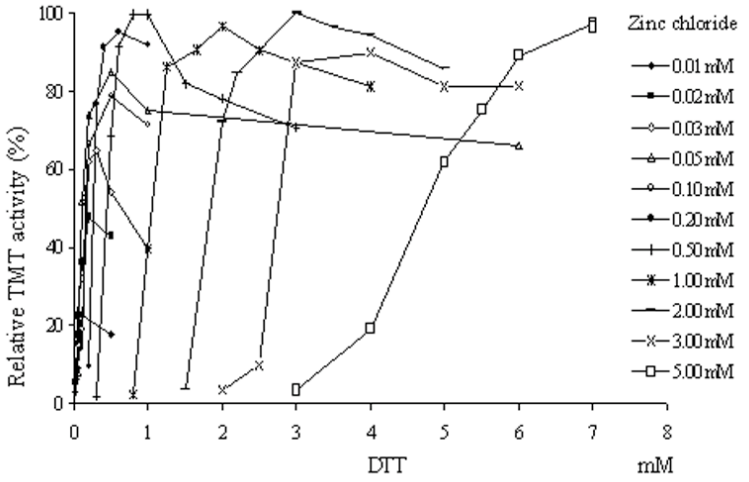

Figure 3.26 TMT activity of the recombinant *R. graveolens* OMT determined with 1,4-dithiothreitol and 25 µM *S*-adenosyl-L-[*methyl*-^{14}C]methionine (7.9 µCi µmol^{-1}) in the presence of various concentrations of zinc chloride. The product was extracted from the incubation with ethylacetate and quantified by liquid scintillation counting. Control thin-layer chromatography on silica gel revealed only one product corresponding to 1,4-dithiothreitol monomethylthioether. The highest conversion rate (3.7 µkat/kg) was set at 100%.

Semipreparative incubations were then conducted at 0.1 mM DTT with the pure recombinant *Ruta* enzyme in the presence of 10 µM $ZnCl_2$, and the product of 200 assays (40 ml) was collected for LC-MS analysis. The retention time, in comparison to an authentic sample, and the mass signal at m/z 167 ([M-H]$^-$) identified the product as dithiothreitol monomethylthioether. The reference sample was synthesized and unequivocally characterized by ^{1}H- and ^{13}C-NMR and mass spectrometry by Dr. Wilfried Schwab, Fachgebiet Biomolekulare Lebensmitteltechnologie der Technischen Universität München. Thus, the *R. graveolens* enzyme is capable of methylating also the pseudosubstrate DTT, exhibiting TMT activity.

3.2.17 Substrate specificity

From a few additional thiol compounds tested only 2-mercaptoethanol served as substrate with low affinity (**Fig 3.27**) whereas methionine, cysteine, 6-mercaptopurine, homocysteine or the fungicide zinc-ethylene-bis-dithiocarbamate were not accepted.

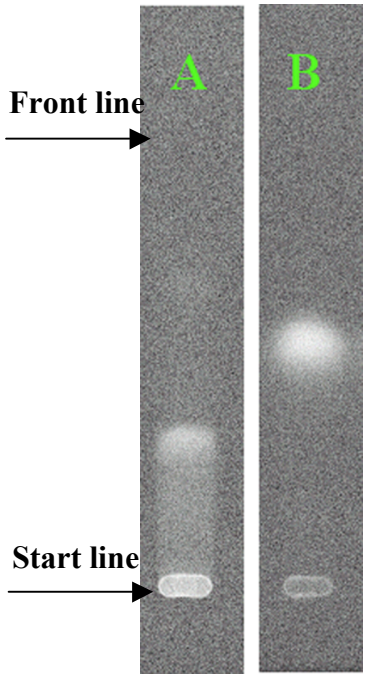

Figure 3.27 Radio-TLC determination of enzymatically formed methylated products when 1,4-dithiothreitol (A) and 2-mercaptoethanol (B) were incubated with the purified recombinant *Ruta* enzyme in the presence of Zn^{2+} and *S*-adenosyl-L-[*methyl*-^{14}C]methionine. The incubations were extracted with ethylacetate and the extracts were applied to silica F254 plates and developed in toluene:ethylacetate (3:2, v/v).

3.2.18 Biochemical characterisation for TMT activity

Ruta OMT was further characterized focusing on the thiolmethyltransferase (TMT) activity. The tests were carried out in the presence of 25 µM *S*-adenosyl-L-[*methyl*-^{14}C]methionine (4.72 µCi/µmol) using 50 µg of partially purified enzyme (after SEC) per incubation (100 µl total).

The pH optimum was evaluated in four buffer systems (pH between 5 and 10) and the temperature dependency of the activitz was determined from 25 to 46^{o}C in Tris-HCl pH 7.5. Under optimum pH and temperature conditions, the time linearity of the thiol methyltransferase reaction was evaluated over a time period from 1 to 90 minutes. The amount of product increased steadily over the full time period.

The pH and temperature dependencies of the turnover rates matched the pattern as determined for the OMT activity.

3.2.19 Competition of OMT and TMT activities

The bifunctionality of the recombinant methyltransferase cloned from *R. graveolens* and the fact that the OMT activity was apparently inhibited in the presence of Zn^{2+}/DTT raised the question of competitive binding of the phenolic and thiol substrates.

Reversible inhibition can be caused by competitive or non-competitive binding. Competitive inhibition requires that the enzyme binds at (or near) the same site either the substrate or the inhibitor but not both at the same time. Under these conditions the maximum velocity (V_{max}) is not affected, but the apparent K_m for a given substrate, i.e. 3,5-dimethoxyphenol, will increase to outcompete the inhibitor at the active site. Reciprocal plots of V versus the substrate concentration, at increasing concentrations must result in steeper graphs. Replotting of the inhibitor concentration versus the apparent K_m values will then allow to extrapolate the apparent inhibition constant K_i.

In the case of non competitive inhibition, both substrate and inhibitor bind randomly and independently at different sites of the enzyme. In this instance, V_{max} will decrease while the K_m remains unchanged, because at any concentration of the inhibitor a portion of the enzyme is blocked in an inactive enzyme-substrate-inhibitor complex.

Therefore, the inhibition of 3,5-dimethoxyphenol OMT activity by TMT was investigated in sets of experiments in the absence or in the presence of $ZnCl_2$/DTT-combinations of 2/20 µM, 5/50 µM and 10/100 µM, respectively (**Fig. 3.28**). These combinations were chosen because excess DTT chelates Zn^{2+} avoiding the metal-inhibition of enzyme activity, while the Zn-concentration is presumably sufficient to further binding of DTT to the enzyme by shifting the thiol-thiolate equilibrium to the thiolate anion. The assays were carried out at a saturating concentration of *S*-adenosyl-L-[*methyl*-^{14}C]methionine (11.54 µM; 52 µCi µmol/µmol) in 0.1 M Tris-HCl buffer pH 7.5 (0.1 ml total), and using 50 µg of the partially purified recombinant enzyme (eluted from size exclusion chromatography). The concentration of the phenolic substrate 3,5-dimethoxyphenol was varied from 14 to 100 µM. The apparent K_m recorded in the absence of Zn^{2+}/DTT was 20.4 µM and increased to 26.7 , 36.5 and 57.8 µM with the increase of Zn^{2+}/DTT concentration. Radio- labeled OMT and TMT products were

extracted in ethylacetate, separated on silica TLC-plates in toluene:acetic acid 4:1 (v/v) ($R_{f\ 1,3,5\text{-trimethoxybenzene}}$ 0.58; $R_{f\ monomethylthioether}$ 0.04) and evaluated with the BioImager Analyzer FLA-2000 (Fujifilm, Japan). A replot of the apparent K_m values against the inhibitor concentrations indicated an apparent K_i of 52.0 µM for DTT (**Fig. 3.28**) although merely the zinc-chelated proportion of DTT is the likely substrate. In any case, the shift in apparent K_m at a constant velocity suggested that 3,5-dimethoxyphenol and DTT compete for the same substrate binding site of the *R. graveolens* enzyme.

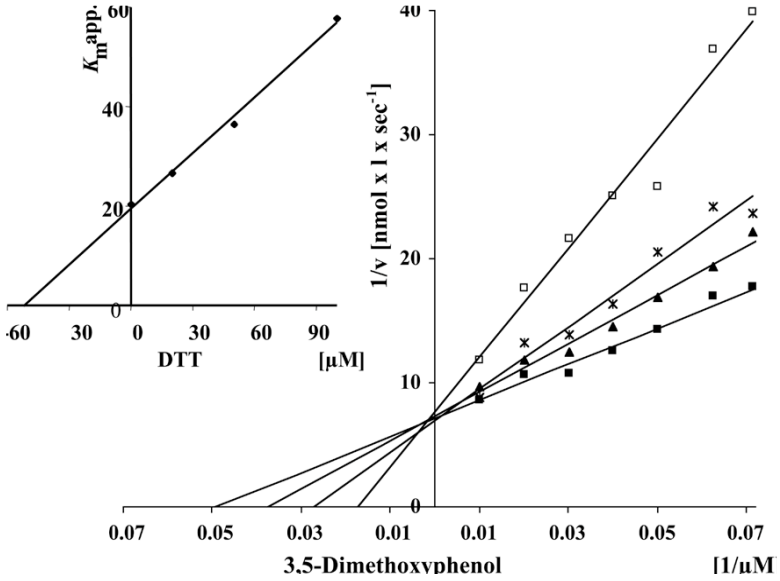

Figure 3.28 Kinetic analysis of the effect of various fixed $ZnCl_2$/DTT concentrations on the turnover rate of the recombinant *R. graveolens* OMT with 3,5-dimethoxyphenol as the variable substrate. The standard OMT assay was conducted in the absence of $ZnCl_2$/DTT (solid square) or in the presence of $ZnCl_2$/DTT fixed at concentrations of 2.0/20.0 µM (solid triangle), 5.0/50.0 µM (cross) or 10.0/100.0 µM (open square). The apparent K_m values inferred from the Lineweaver-Burk diagram plotted *vs*. the concentration of DTT (insert) revealed an apparent K_i of 52.0 µM.

3.2.20 Tissue distribution of OMT and TMT activities

Trimethoxybenzene is a volatile known from rose fragrance, and the physiological function of 3,5-dimethoxyphenol OMT in Ruta is likely connected with the scent production. The relative tissue distribution of the methyltransferase activities was examined in outdoor grown mature *R. graveolens* plants and compared to the expression

in suspension culture (**Fig. 3.29**).The 3,5-dimethoxyphenol OMT activity was predominantly present in stem extracts. However, the patterns of methylating activities for 2,3-dimethoxyphenol (primarily expressed in leaf extract), guaiacol and 3,5-dihydroxyanisole (leaf extracts) or 3,4-dimethoxyphenol (leaf and stem extracts), as well as the specific activities of the crude extracts related to guaiacol OMT (100%), suggested that more than one OMT must be responsible for these methylations (**Fig. 3.29**). This is also to be inferred for the TMT activities, because the specific activity of flower extracts for 2-mercaptoethanol exceeded that for DTT, and cell cultures showed almost equivalent activities with both substrates, whereas the OMT cloned from *R. graveolens* exhibited relatively low activity towards 2-mercaptoethanol.

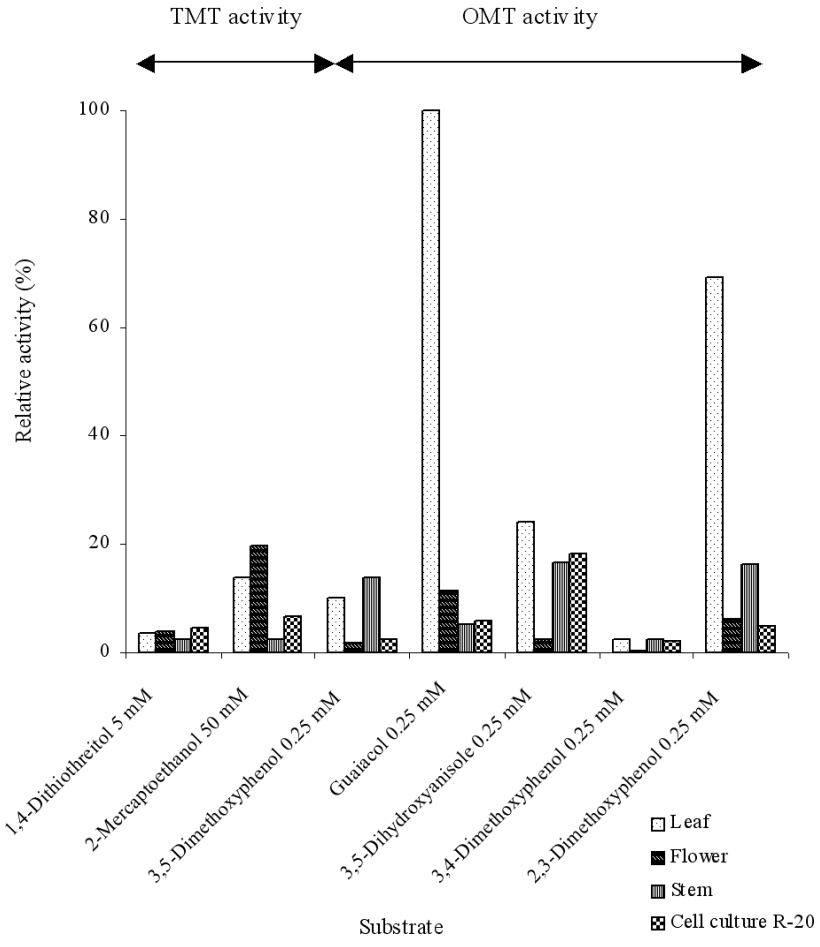

Figure 3.29 Relative OMT and TMT activities in crude extracts of different tissues of *R. graveolens* plants and cell cultures determined in standard assays (0.1 M Tris-HCl buffer pH 7.5 at 36°C and 38°C, respectively, in the presence of 25.0 µM labeled SAM) using various substrates. The highest conversion rate observed (8.4 µKat OMT activity with guaiacol in leaf extract) was arbitrarily set at 100%.

For comparison, the relative abundance of the 3,5-dimethoxyphenol OMT transcript in the outdoor grown plants was examined by RT-PCR. This experiment revealed the expression in all the *Ruta* tissues with high template activity in stem and developing flowers (**Fig. 3.30**). *R. graveolens* plants are known to develop an intensive scent, and it is conceivably that more than one OMT contribute to the formation of volatile methoxybenzenes, in addition to trimethoxybenzene, as documented by the preferred methylation of guaiacol or 2,3-dimethoxyphenol in the crude plant extracts.

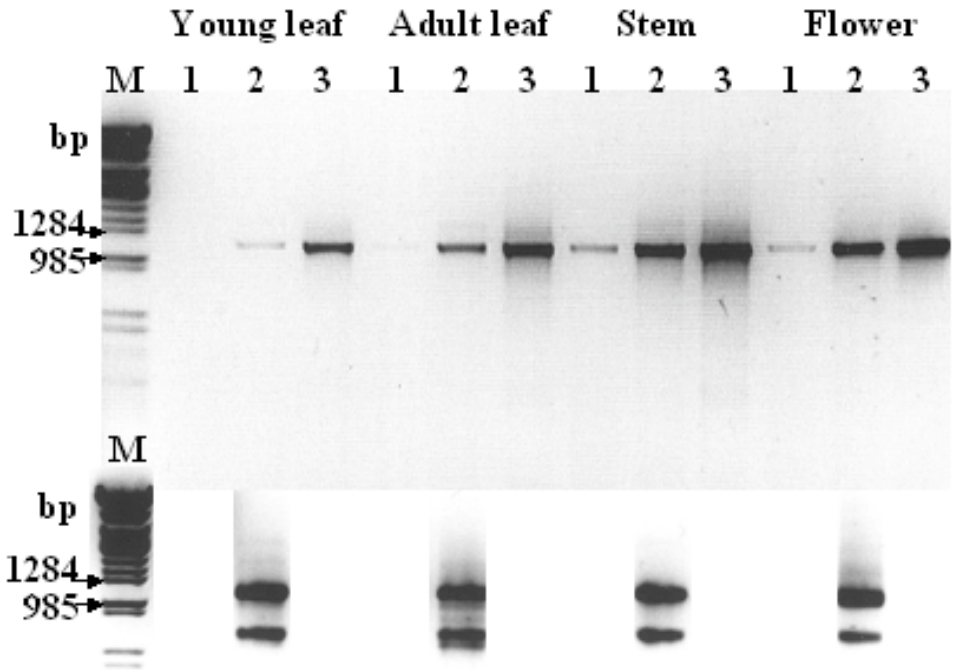

Figure 3.30 RT-PCR amplification of 1,3-dimethoxyphenol OMT from template of various *Ruta graveolens* tissues. The tissues (young leaf from the plant tip and adult leaf from a more basipetal region; top quarter stem segment; emerging flowers) were harvested from outdoor grown adult plants. Equivalent aliquots of cDNA generated from total RNA (about 5 µg per tissue) were heated for 2 minutes at 95°C and amplified subsequently with end-to-end oligonucleotide primers using (lane 1) 15, (lane 2) 30 or (lane 3) 35 cycles of 0.5 minutes at 95°C followed by 1 minute at 65°C and 2 minutes at 72°C. All samples were finally heated to 72°C for 2 minutes prior to separation on 1.5% agarose (OMT at 1122 bp). The total RNA contents of the various tissue extracts (triple the amount used for cDNA synthesis) are shown for comparison (bottom).

3.2.21 Northern blot analysis

Total RNA was isolated from *Pmg* elicited *Ruta graveolens* cells. Cells were harvested every 30 min up to 8 hours after elicitation. For each time point 4 µg total RNA was transferred on a Nylon membrane (Hybond-N^+, Amersham Biosciences, Braunschweig) and hybridized with the ^{32}P- labeled cDNAs probes spanning the ORF of R-23 or R-27. Individual blots were used for each hybridization. The abundance of *Ruta graveolens* COMT-like transcript (R-23) increased significantly to a transient maximum at 6 h (**Fig. 3.31**). The second polypeptide encoding 3,5-dimethoxyphenol OMT (R-27), exhibited hybridization signals of similar weak intesities at all time points suggesting the constitutive expression in the cells. Uninduced cells harvested under equivalent conditions served as control.

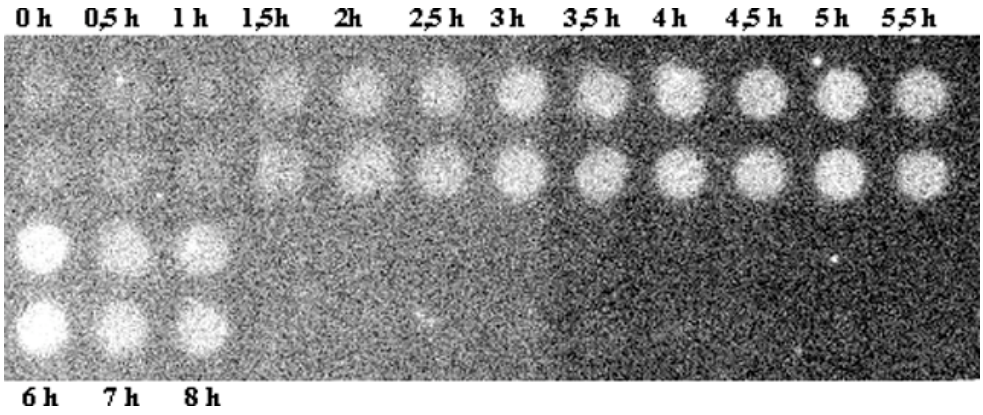

Figure 3.31 Northern dot blot hybridization of COMT-like transcript from elicitor-treated *Ruta graveolens* cells. Total RNA (4 µg per dot) was isolated at different time points (0-8 h) following the induction and employed for Northern hybridisation using ^{32}P-labeled R-23 cDNA as a probe. The dot blots were arranged in parallel duplicates.

4. DISCUSSION

The *S*-adenosyl-L-methionine-dependent methylation of small molecules is commonly observed in all kinds of organisms. Prominent examples are the DNA-methylation in bacteria, used to regulate the transcriptional activity or the stability to restriction enzymes, as well as the methylations in mammals involved in the biosynthesis or the degradation of bioactive amines including dopamine. Much less is known about the physiological function of plant methyltransferases, although OMTs have been shown to increase the environmental persistence of phenolics and the toxicity of phytoalexins.

4.1 Acridone alkaloids

4.1.1 Purification of anthranilate *N*-methyltransferase from *Ruta graveolens*

Anthranilic acid is a common metabolite in plants as an intermediate in the biosynthesis of tryptophan. In addition, a number of alkaloids, mostly found in Rutaceae, are synthesized from this compound. Small amounts of *N*-methylanthranilate are found in many essential oils and a simple anthranilic acid-derived alkaloid, damascenine, is found in the seeds of *Nigella damascena* of the Ranunculaceae. In Rutaceae, the transfer of a methyl group from *S*-adenosyl-L-methionine dependent methylation of anthranilate to *N*-methylanthranilate, the first committed reaction to acridone alkaloids, channels anthranilate into the secondary metabolism.

Anthranilate *N*-methyltransferase activity is present in *Ruta graveolens* cell-line R-20 that also produces acridone alkaloids (Baumert et al., 1983). Partial purification of the enzyme based on a twelve-step-procedure of gel filtration, ion exchange and hydrophobic interaction chromatographies has already been reported (Maier et al., 1994). The protein was partially purified 370-fold from 350 g not induced lyophilized cells and provisionally characterized. It required an alkaline pH (7.8) for optimal activity, exhibited a remarkable

substrate specificity for anthranilate (although other substances were not tested), was inhibited by *S*-adenosyl-L-homocysteine and a molecular weight of 62 kDa on SDS-PAGE and 70 kDa on gel filtration (Maier et al., 1994) was determined. The activity was independent of Mg^{2+} and could be inhibited by Fe^{2+}, Cu^{2+} and Zn^{2+} (Maier et al., 1994).

Anthranilate *N*-methyltransferase is inducible upon elicitation since the activity was shown to increase at least ten times when rutacridone producing *Ruta* cells were treated with a 1% homogenate of *Rodotorula rubra* micelia (Bohlmann and Eilert, 1994).

Anthranilate *N*-methyltransferase (ANMT) was purified from 500 g of *Ruta graveolens* cell-line R-20 elicited suspension culture. In stead of *Rodotorula rubra*, commercial yeast extract was used in our study for elicitation and the cells were not liophilized prior to enzyme extraction. The cells harvested were directly frozen in liquid nitrogen and stored at -80^{o}C until further use to protect the activity. The crude extract was prepared with DTT, Polyclar AT, EDTA and glycerol. The addition of DTT as a reducing agent inhibits possible oxidation of protein sulfhydryl groups, Polyclar AT absorbs the phenolic compounds which can then be removed by centrifugation, the heavy metal chelating agent EDTA prevents activation of proteases or inhibition by divalents cations and the water activity modifying agent glycerol stabilizes the enzyme activity against denaturation or subtle conformational changes.

The precipitation of anthranilate NMT from crude extract of lyophilized cells of uninduced R-20 *Ruta* cells had been reported on 0-50% ammonium sulfate saturation with a recovery of 91.7% (Maier et al., 1994). In our case, proteins were precipitated at 35-60% ammonium sulfate saturation and subjected to chromatographic purification. The fraction precipitated at 0-35% ammonium sulfate was discarded because it contains material that is generally particulate and preaggregated or very-high-molecular-weight protein (Scopes, 1987).

The purification was based on a six-step-protocol representing a combination of hydrophobic interaction, size exclusion, anion exchange and adenosine-agarose affinity chromatographies. The main improvement of the purification procedure was the use of adenosine-agarose affinity chromatography, which had been successfully employed for the

purification of diverse SAM-dependent plant methyltransferases, i.e. methionine *S*-methyltransferse from *Wollastonia biflora*, (James et al., 1995) or purine *N*-methyltransferase (caffeine synthesis) from *Camellia sinensis* (Kato et al., 1999).

Anthranilate NMT was purified ~ 450 fold with a recovery of 4.5%. Separation on an SDS-PAGE followed by staining with Coomassie or silver showed a major band around 42 kDa. This is in accordance with the molecular weight of most plant methyltransferases (Ibrahim et al., 1998; Kato et al., 1999; Choi et al., 2002; Raman and Rathinasabapathi, 2003). The 42 kDa band often appeared as a double band which may be interpreted as isoforms or that the protein was incompletely denaturated. A protein not fully denaturated would run probably faster than its denaturated form. This is known for several microsomal proteins. Another possibility is that the double band resulted from differently posttranslational modified forms of the same protein. Similar observations were reported on purification of phloroglucinol *O*-methyltransferases from *Rosa chinensis* (Wu et al., 2004). In this instance, MS analysis proved that all three bands represent the same protein and the authors suggested that it may exist in several posttranslational modified versions (Wu et al., 2004). However, a molecular weight of 42 kDa did not agree with the earlier purification report that proposed the enrichment of a 62 kDa protein on SDS-PAGE and 70 kDa on calibrated size exclusion chromatography (Maier et al., 1994). It must be emphasized that various methyltransferases form homodimers and thus an Mr of 70 kDa of the native protein could represent the dimer form.

4.1.2 Partial sequence of the purified polypeptide

Three different fractions eluted from Mono Q as the last chromatographic step and possessing anthranilate NMT activity were sequenced and the results confirmed the protein homogeneity. However, none of the peptide fragments had any similarity with annotated methyltransferases or other listed proteins. This finding was not surprising because the few known plant *N*-methyltransferases cloned so far differ greatly in their primary structures with a sequence identity of only about 5-15% (Choi et al., 2002). Yet, a major problem was encountered on designing degenerate oligonucleotide primers for PCR amplification as in case of cloning the enzyme beta-alanine *N*-methyltransferase from *Limonium latifolium* (Raman and Rathinasabapathi, 2003). The peptide fragment sequences were

short and highly degenerated making the creation of several primers impossible. Only one sequence of moderate degeneracy was chosen to desigh a forward oligonucleotide primer for amplification. Adapter primers that bind to the poly(A) tail were used as reverse primers. The cDNA fragment amplified (~800 bp) was compared with data-base accessions and, unfortunately, showed no similarity with OMT sequences but rather some similarity with plant alcohol dehydrogenases. Yet, none of the other peptide sequences identified by partial microsequencing of the purified polypeptide was encoded in the amplified fragment suggesting that the amplified fragment did not correlate to the purified protein band. Failure to isolate full-length cDNA by use of degenerate oligonucleotide primers designed from the *N*-terminal sequence was also reported for coclaurine *N*-methyltransferase from *Coptis japonica* (Choi et al., 2002). Furthermore, it was previously reported that one of the copurified proteins on the purification of carboxyl OMT was a cinnamyl alcohol dehydrogenase (Ross et al., 1999). These enzymes have a conserved sequence identity of 60-80%. As none of our microsequenced peptides had any similarity with alcohol dehydrogenases, the possibility that the purified band is an alcohol dehydrogenase appeared extremely small. Therefore, another protein microsequencing was required in order to produce less degenerated peptides.

Another approach was taken to purify the enzyme. This time, however, the pattern of elution from hydrophobic chromatography was completely different suggesting that the matrix material was damaged. As no more column material was available, the purification could not be reproduced.

4.2 PCR amplification of methyltransferase- specific cDNA

4.2.1 The choice of degenerated primers

After the attempts to clone the anthranilate *N*-methyltransferase on the basis of microsequenced peptide fragments had failed, a different approach was taken which relied on the use of degenerate oligonucleotide primers originally designed for cloning of OMTs involved in the biosynthesis of isoquinoline alkaloids (Frick and Kutchan, 1999). Such procedure is feasible because of the structural unity and sequence identity of plant methyltransferases. Five distinct motifs are highly conserved in the sequences of plant methyltransferases, especially *O*-methyltransferases. Plant *N*-methyltransferases are much more diverse (Hibi et al., 1994; Ying et al., 1999; Uefuji et al., 2003), although some of them show extended homology with OMTs (Raman and Rathinasabapathi, 2003) and thus preserve all the five conserved regions or at least parts of them.

The knowledge of conserved motifs allowed to identify new genes including *N*-methyltransferases in the genome of *Saccharomyces cerevisiae* (Niewmierzycka and Clarke, 1999). However, as long as the genomes of most plants, including Ruta sp., have not been sequenced, the cloning strategy for plant methyltransferases is based primarily on RT-PCR with primers inferred from the conserved motifs. The oligonucleotides suggested by Dumas et al. (1992) and improved by Frick and Kutchan (1999) were designed to bind motifs I, II and IV and used up to now for cloning OMTs; these primers might also be useful for NMT amplification provided that motifs I, II and IV are conserved also in these enzymes. The protocol is simple but might lead to multiple methyltransferase fragments. The chances to amplify a particular methyltransferase sequence can be improved, if the transcript abundance can be induced by elicitor treatment of plant cells. In non-induced cells the expression of transcripts for the biosynthesis of secondary metabolites might be very low requiring more sophisticated cloning protocols.

4.2.2 Cloning of *Ruta* methyltransferases

In order to raise the abundance of transcripts realted to acridone biosynthesis, the Ruta cultures were treated with fungal elicitor. Acridone epoxide is known to accumulate after 12 h of elicitation and peaks after 24 h to 72 h. This process is preceded by a rapid transient increase of ANMT activity with a maximum after 10-20 h (Eilert and Wolters, 1989) while the specific abundance is unknown. More information is available about the accumulation of acridone synthase. The activity starts after 6 h and reaches its maximum after 24 h, with the highest transcript level after 6 h elicitation (Junghanns et al., 1995; Junghanns et al., 1998). In this work, total RNA was isolated from *Ruta* cells after 2-4 h of elicitation and used as template for RT-PCR. The PCR method used a very low annealing temperature (40°C) to allow the binding of the degenerated primers.

Two fragments were amplified which revealed some similarity with plant methyltransferases and full length cDNAs named R-23 and R-27 were generated by RACE and RLM-RACE. Clone R-23 showed extended identity (79%) with plant COMTs, while R-27 shared a maximum of 50% identity with orcinol and chavicol OMTs from rose, and less with methyltransferases of very diverse functions.

4.2.3 Sequence analysis of clone R-23

Databank investigations of R-23 (COMT-like) sequence revealed a high similarity with plant caffeic acid *O*-methyltransferases and all the conserved motifs could be identified. The five regions, comprising 36 amino acids, rich in glycine, and grouped in the last third of the sequence, were characterized (Ibrahim et al. 1998) as being highly conserved (92-100%) among plant *O*-methyltransferases. *Ruta* COMT-like enzyme shows 97% identity within the described motifs (**Fig. 4.1**). Since the crystal structure of catechol *O*-methyltransferase from rat liver was elucidated (Vidgren et al., 1994) followed by three plant *O*-methyltransferases (Zubieta et al., 2001; Zubieta et al., 2002; Zubieta et al., 2003) additional catalytic and substrate binding essential amino acids that could not be predicted from the alignments were identified by crystallization. Therefore, the SAM/SAH recognition requires the interaction with several highly conserved Lys-Asp residues and the interaction with hydrophobic and aromatic residues. For the catalytic mechanism the

general base is represented by a His residue, and two supplementary Glu residues contribute to the architecture of the catalytic site (Zubieta et al., 2001; Zubieta et al., 2002; Zubieta et al., 2003).

The structurally characterized plant *O*-methyltransferases form homodimers which is critical for enzyme activity. Amino acids essential for dimer interaction are found close to the *N*-terminus and intimately contribute to substrate binding (Zubieta et al., 2001; Zubieta et al., 2002; Zubieta et al., 2003). The alignment of R-23 with the structurally characterized COMT from *Medicago sativa* displayed 100% preservation of the amino acids essential for catalytic activity, dimerisation and SAM/SAH binding, and an overall identity of 76% (**Fig. 4.1**). The results suggest that R-23 is very likely a caffeic acid methyltransferase.

Most COMTs described in the literature play an important role in the lignin biosynthesis and are not inducible upon elicitation. Exceptions are the alfalfa COMT respectively two COMTs isoforms from tobacco (Gowri et al., 1991; Pellegrini et al., 1993). The alfalfa COMT shares high similarity with other COMTs (more than 75%), while the tobacco enzymes have the highest level of similarity of 52%. The transcription of the gene encoding COMT was strongly activated in elicited alfalfa cell cultures but this was followed by a small increase in enzyme activity (Ni et al., 1996). This suggests that the pattern of gene expression is functionally determined at the posttranscriptional level (Ni et al., 1996). In addition, the COMTs from tobacco are hardly detectable in healthy tobacco leaves and are highly induced by tobacco mosaic virus (TMV) infection (Legrand et al., 1978). Both enzymes are encoded by the same gene and are differentiated by posttranslational modification (Pellegrini et al., 1993). Northern blot analysis showed that *Ruta*-COMT like transcript accumulation is increased by elicitation with *Pmg*. The *Ruta* enzyme shares a higher similarity with alfalfa COMT (76%) than with the tobacco enzymes (53%), and it is conceivable that the increase in transcript level may not necessarily lead to an increase of protein activity. This issue must remain open until the appropriate substrate for the expressed protein is identified.

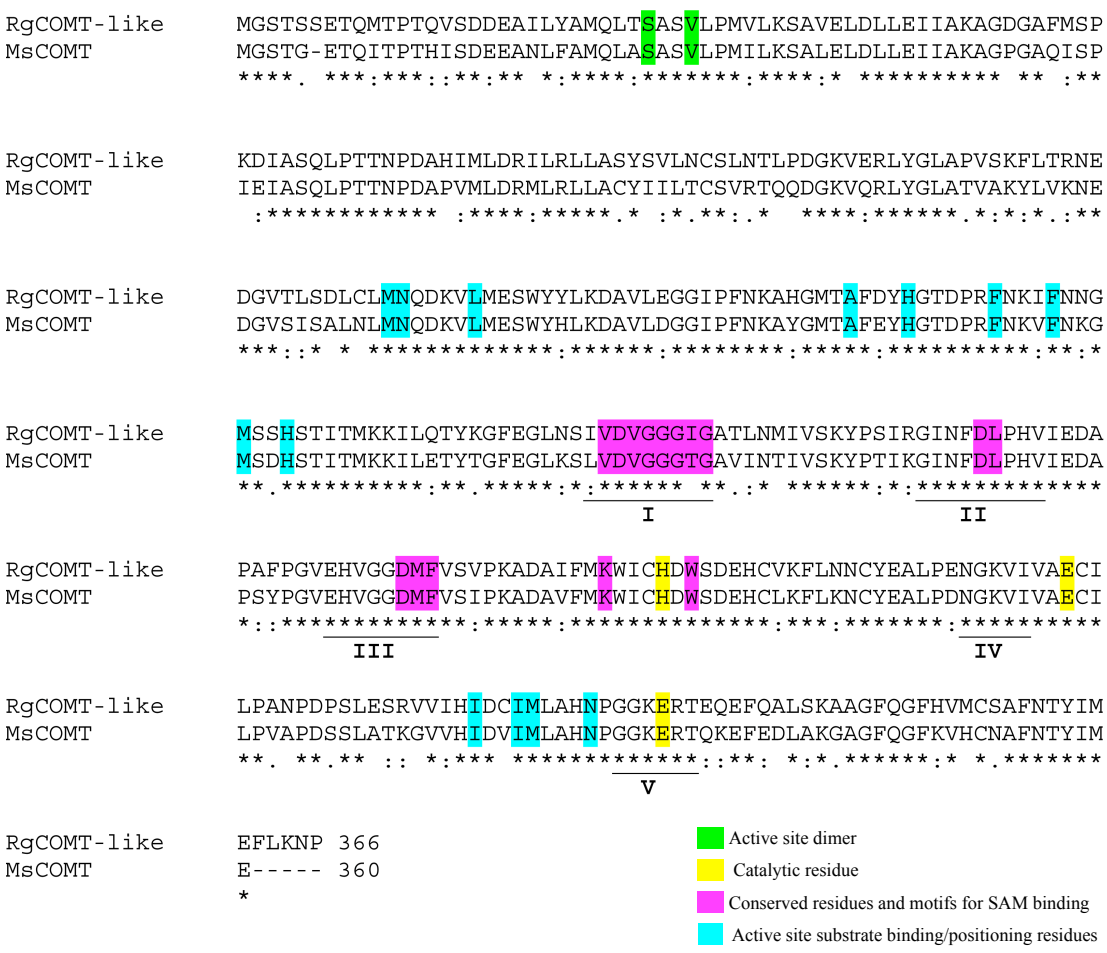

Figure 4.1 Clustal W alignment of *Ruta* COMT-like polypeptide with COMT from *Medicago sativa*. Asteriks (*) represent identical amino acids, double-dots (:) designate conserved substitution, one point (.) means that semi-conserved substitution is observed. The function of the colored amino acids was clarified in *Medicago sativa* COMT (Zubieta et al., 2002)

4.2.5 Sequence analysis of clone R-27

The second cDNA clone isolated from *Ruta* suspension culture, R-27, had a more intriguing amino acid composition. The overall identity of 50% with orcinol *O*-methyltransferases from roses or eugenol and chavicol OMTs from basil classified the clone, although the COMT-specific motifs were less conserved (72%). Even the catalytic site could only partly be recognized based on simple sequence alignment. Thus His269 and Glu297 in COMT from *Medicago sativa* correspond to His267 and a Glu295 in *Ruta* sequence but no correspondent could be found for the third catalytically essential residue Glu329. This can be due to the fact that *Ruta* enzyme carries an insert of 13 amino acids (Glu308 to Thr321) that does not match with any of the compared sequences in database accessions. The insert contains four glutamic and three aspartic acid residues besides two lysine residues which conceivably contribute to the surface charge and modify folding of the enzyme polypeptide. The enzyme clearly prove the substrate specificity of the OMT for methoxyphenols, different from eugenol or chavicol, and also orcinol was not accepted. The divergent substrate specificity can not be explained at present due in part to the fact that most of the conserved sequence elements are involved in SAM-binding. Furthermore, the *Ruta* polypeptide bears a possible signal domain of 24 amino acids in the *N*-terminal distinguished by the presence of eight serine residues in a stretch of 17 amino acids. The precise effect of these unusual features on the substrate specificity remains to be established. In addition, it should be mentioned that a simple change of an amino acid can drastically modify the substrate specificity. In basil, the exchange of Phe260Ser in chavicol OMT respectively Ser261Phe in eugenol OMT, completely switched the activities of both enzymes (Gang et al., 2002). This result supported the previous finding that an Asn134Ala mutation in isoeugenol OMT from *Clarkia breweri* generated a COMT (Wang and Pichersky, 1999).

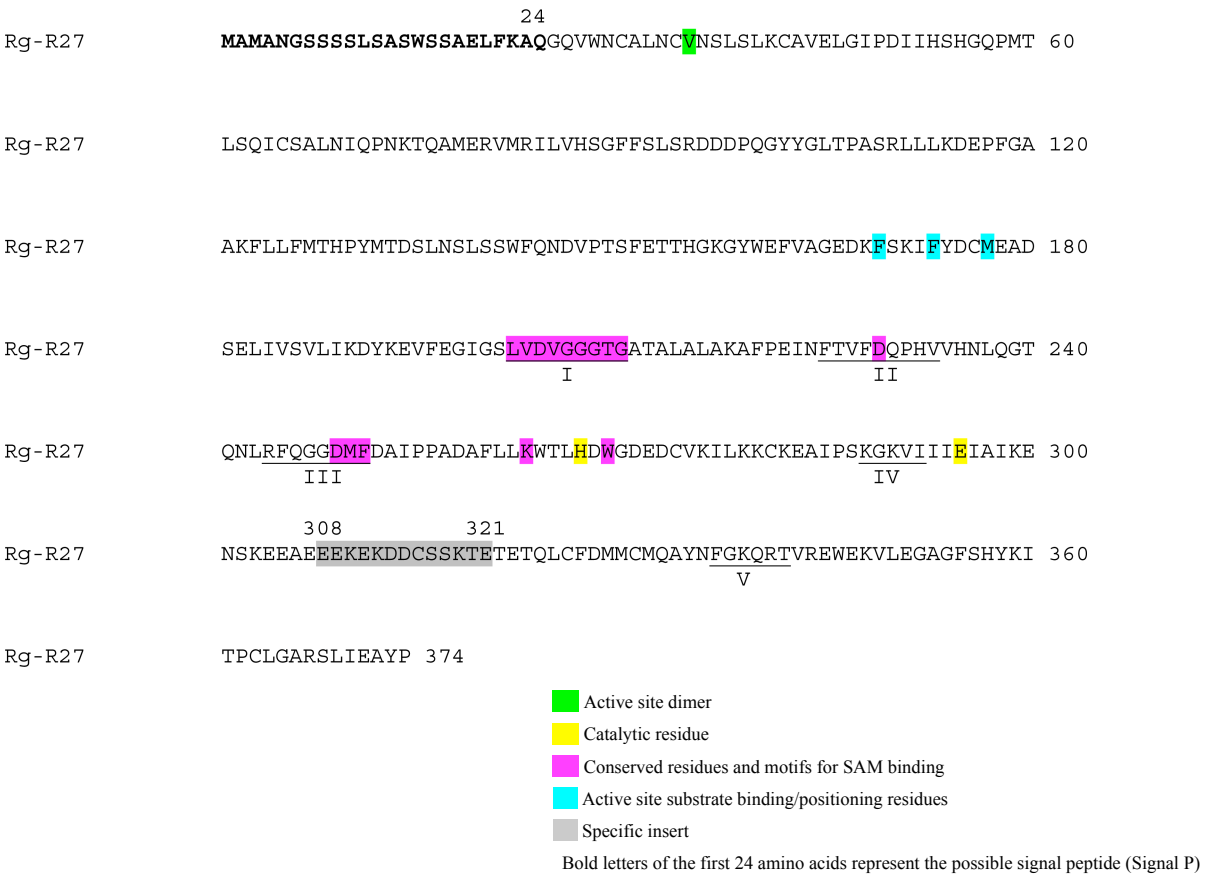

Figure 4.2 Conserved amino acids in *Ruta* 3,5-dimethoxyphenol *O*-methyltransferase (coloured) corresponding to functionally amino acids assigned in *Medicago sativa* COMT (Zubieta et al., 2002).

Phylogenetic analysis of *Ruta* R-23 and R-27 polypeptides placed them in two different clusters: COMT-like protein (R-23) in the big cluster of COMTs (class I) and R-27 in the cluster with methyltransferases that use a wide range of substrates (class II) (Gang et al., 2002) (**Fig. 4.3**).

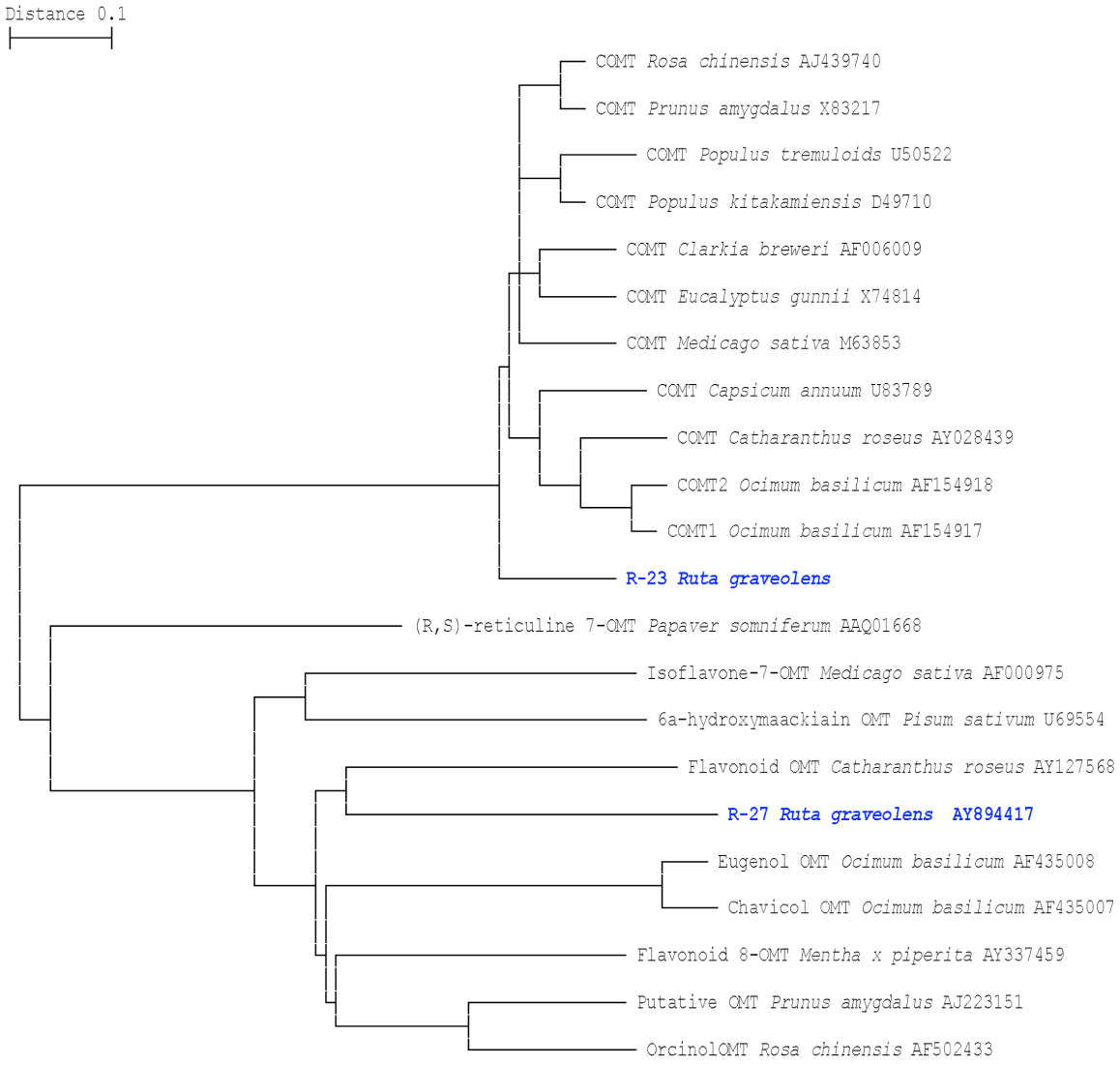

Figure 4.3 Relationship tree of *Ruta* OMTs with other related OMTs. Related OMT data were obtained from Genbank with accession numbers given after each name. The alignment was realized with the program Treecon (Van der Peer and De Wachter, 1994).

Class I methyltransferases covers COMTs and also multifunctional OMTs that have activity with phenylpropanoid and flavonoid substrates (Gauthier et al., 1998), or alkaloid precursors (Frick and Kutchan, 1999) or even enzymes that have no significant activity against caffeate or ferulate and evolved only recently from COMTs. Numerous enzymes that do not have an assigned function but show high amino acid similarity with COMTs are classified as putative or COMT-like enzymes. These enzymes might be involved in

secondary metabolism of phenolics other than those related to lignification. The *in vitro* determination of substrate specificities is the first step to assign a physiological function to the enzyme.

4.2.6 Functional expression and substrate specificity of clones R-23 and R-27

Expression of the enzyme from the expression vector was successful as had been observed before with a coumarin-specific OMT, and the expression was accomplished in both instances without a His-tag. As could have been assumed by the sequence similarity to orcinol OMT the R-27 OMT showed specificity for methoxylated phenols, and the activity was selective for the pattern of methoxylation. The enzyme expressed from clone R-23 was assayed in paralell but failed to show any activity. This result was not expected considering the high similarita with other plant COMTs, althought overall similarity is insufficient to predict the substrate specificity (Schröder et al., 2002).

4.2.7 Biochemical characterization of 3,5-dimethoxyphenol OMT

The LC-MS-indentification of 1,3,5-trimethoxybenzene as the product of the reaction from the R-27 encoded enzyme corroborated the functional assignment. Methoxylated benzenes, particularly 1,3,5-trimethoxybenzene and toluenes, besides mono- and sesquiterpenes, are the predominant components of the scent of roses. Recently, because of its economical value, the biochemistry of rose volatiles has been the subject of genomic approaches (Guterman et al., 2002). Most of the European rose species emit orcinol dimethyl ether (DMT) as a main volatile, while the Chinese rose, the ancestor of many modern roses, has rich emission of 1,3,5-trimethoxybenzene (TMB). The biosynthesis of DMT is catalyzed by two very similar orcinol *O*-methyltransferases, OOMT1 and OOMT2, capable of methylating orcinol to 3-methoxy 5-hydroxy toluene and this further to DMT (Lavid et al., 2002). The biosynthesis of TMB involves three methylationreactions: phloroglucinol to 3,5-dihydroxyanisole, 3,5-dihydroxyanisole to 3,5-dimethoxyphenol and 3,5-dimethoxyphenol to TMB. OOMT1 and OOMT2 also catalyze the last two methylations in the TMB pathway (Scalliet et al., 2002), whereas the methylation of phloroglucinol is performed by a different enzyme only distantly related to the other two (Wu et al., 2004).

The dimethoxyphenol OMT cloned from *Ruta* clearly differs from the rose orcinol OMTs at the level of the primary structure as well as in substrate specificity. The, *Ruta* OMT efficiently conversed 3,5-dimethoxyphenol to TMB while 3,5-dihydroxyanisole was a rather poor substrate and orcinol was not at all accepted. Irrespective of the fact that 1,3,5-trimethoxybenzene has not yet been reported from *Ruta*, it is unlikely that these volatiles are restricted to roses. However, a related compound, namely 1,2,3-trimethoxybenzene is mentioned as constituent of the volatile of *Ruta* (Hegnauer, 1998). The enzyme also methylates efficiently guaiacol, which is a common compound of the plant. The enzyme is constitutively expressed in all plant tissues suggesting that it might fulfill an allelopathic function.

Although the recombinant enzyme was purified to homogeneity, the biochemical characterization of the enzyme was carried out with partially purified enzyme after ammonium sulfate precipitation and gel filtration. The reason to use partially purified enzyme was the low recovery after the last purification step.

The biochemical properties of recombinant *Ruta* 3,5-dimethoxyphenol OMT meet those generally described for class II methyltransferases with a homodimeric composition (Ibrahim et al., 1998; Zubieta et al., 2001; Gang et al., 2002; Zubieta et al., 2002), a pH optimum at 7.5, and independancy of cations.

The affinity to 3,5-dimethoxyphenol (Km 20.4 μM) was in range with the substrate affinities of orcinol OMT1 and OMT2 from roses(Lavid et al., 2002), and in the range of other plant *O*-methyltransferases with their preferred substrates (Ibrahim et al., 1998). This is also a common feature of plant *O*-methyltransferases.

4.2.8 The effect of thiols and thiol methyltransferase activity

The addition of DTT seemed to suppress the OMT activity by 20-30%, which is a rather unexpected effect considering the respective literature (Fujioka, 1992) and raised the idea of athioltransferase side activity of the OMT. Thiol methyltransferases are well characterized from biotransformations and from detoxification of xenobiotic and endogenous thiols and sulhydryl compounds. Much less is known about thiolmethylations in plants mainly related to formation of *S*-methylmethionine. The non-protein amino acid *S*-methylmethionine is formed by transferring a methyl group of SAM to methionine. The reaction is very common in flowering plants. The enzyme catalyzing the formation of SMM (*S*-adenosyl-L-methionine:L-methionine *S*-methyltransferase, MMT) is a polypeptide of 115 kDa without similarity with plant small molecule methyltransferases (Mudd and Datko, 1990; James et al., 1995; Pimenta et al., 1998).

Recently, a new class of plant thiolmethyltransferases involved in the biosynthesis of sulfur volatiles has been described from *Brassica oleracea* (Attieh et al., 2000; Attieh et al., 2002). The *Brassica* enzymes were capable of methylating HS^-, thiocyanate and some organic thiols including thiophenol and thiosalicylic acid, yielding volatile products that were proposed to have function in the defence gainst microorganism. The enzymes share no similarity with known *O*- and *N*-methyltransferases, but are related to a methyl chloride transferase (Ni and Hager, 1998, 1999) and to partial sequences of thiopurine *S*-methyltransferase from humans and microorganisms. The sequences contain three conserved motifs involved in the binding of SAM, their molecular weights range from 26 to 31 kDa and they are active as monomers which differs from class II methyltransferases.

4.2.9 The role of Zn^{2+}

One of the intriguing results of our study was the thiol methyltransferase (TMT) activity observed with DTT or 2-mercaptoethanol which must be considered as surrogate substrates. The methyltransferase activity with DTT was considerably enhanced in the presence of Zn^{2+}, which might affect the enzyme or/and the substrate. Zn appears to inhibit the enzyme as the OMT activity was significantly suppressed. It is conceivable that the metal binds to the conserved metal binding site (motif IV) and might even promote the dissociation of the enzyme subunits. However, the the complexation of DTT with Zn has been reported in the literature (Matthews and Goulding, 1997; Krezel et al., 2001; Machuqueiro and Darbre, 2003), shifting the thiol-thiolate equilibrium towards the thiolate.

The catalytical role of zinc include the activation of water for nucleophilic attack and the electrophilic activation of the aldehyde carbonyl group in alcohol dehydrogenase. In this case, the metal ion is tetra- or penta-coordinated to His, Asp, Glu and Cys (Gonzalez et al., 2004). A new role emerged in the last years for the enzyme-bound zinc as activator of thiols for nucleophilic attack as proposed for Ada protein, prenyltransferases and betaine homocysteine *S*-methyltransferase (Fu et al., 1996; Lin et al., 2001; Gonzalez et al., 2003). The ligands are represented mostly by Cys and His residues but Tyr residues were also shown to play an important role in coordination of Zn^{2+} in betaine homocysteine *S*-methyltransferase (Gonzalez et al., 2004). The Lewis acid zinc coordinates to the thiol substrates shifting the thiol-thiolate equilibrium at neutral pH to the thiolate therefore activating the thiol for methyltransfer (Matthews and Goulding, 1997; Krezel et al., 2001; Machuqueiro and Darbre, 2003). Studies on the function of zinc in modulating the reactivity of thiolates to methyl transfer show that dissociated thiolate anion was the reacting species (Machuqueiro and Darbre, 2003). Besides, the thioether product did not remain coordinated to zinc (Matthews and Goulding, 1997). Maximal TMT activity was observed at a Zn:DTT ratio of 1:10 up to 0.1 mM DTT, whereas the addition of Zn^{2+} in excess at any DTT concentration was inhibitiory. This effect was likely due to to enzyme binding, which is supprted by titration experiments using increasing concentration of DTT to complex Zn^{2+}.

The bifunctionality of the enzyme with dimethoxyphenol or thiol substrates was further suppoted by the demosntration of competitive binding. This finding is surprising, although competitive zinc-DTT inhibition was reported also with ribonucleotides in rho transcription termination assays determining the poly(dC)-ribo$(C)_{10}$-dependent ATPase activity (Weber et al., 2003).

4.3 Conclusions and future prospects

The attempt to isolate *S*-adenosyl-L-methionine-dependent anthranilate *N*-methyltransferase by purification or via PCR amplification finnaly resulted in the amplification of two cDNAs supposed to encode O-methyltransferases.

The clone R-23 encoded a polypeptide with high similarity with annotated plant caffeic acid *O*-methyltransferases. However, no substrate was identified for the recombinantly expressed enzyme. Unfortunately, the phenolic components of *Ruta graveolens* essential oil have not been studied excessively, and it is possible that the enzyme encoded by R-23 accepts one as yet unidentified phenolic metabolite.

from cell cultures of L. was purified to a single band of 42 kDa on SDS-PAGE. The short peptides obtained from protein microsequencing showed no relationship to annotated polypeptides and, under low stringency conditions PCRs, did not succeed to amplify the desired polypeptide fragment. A different method, based on degenerated oligonucleotide primers designed from the conserved structure of SAM-binding sites in plant class II methyltransferases was used in the attempt to clone the cDNA of AANMT. This method successfully amplified the full open reading frame of two new genes (R-23 and R-27) that show similarity with plant *O*-methyltransferases (OMTs).

R-27 cDNA encodes a novel methoxybenzene OMT which is related to plant class II OMTs and shows the highest homology to orcinol OMTs from roses. This suggests that the enzyme participates in the formation of *Ruta* volatiles. The capacity to methylate also DTT to the monomethylthioether is a unique feature of this OMT and must be considered as a surrogate activity. Nevertheless, these findings may indicate a broader substrate specificity

and physiological function of plant class II OMTs than anticipated from standard *in vitro* assays.

The spectrum of methoxyphenols and methoxybenzes in *Ruta graveolens* remains to be investigated, including their potential ecological relevance. One idea to test is the involvement of these volatiles in plant defence because investigation of the relative abundance of the transcript revealed the expression in all plant tissues with high template activity in stem and developing flowers. Finally, the crystallization of the enzyme in the presence of the substrates would reveal the mechanism of binding and mutational studies might define the conditions of substrate specificity.

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Zusammenfassung

Die gemeine Weinraute, *Ruta graveolens* L., ist eine aromatische Heilpflanze. Sie enthält Acridonalkaloide, Furochuinoline, Cumarine und zahlreiche leicht flüchtige Ölbestandteile mit antimikrobieller bzw. allelopathischer Wirkung.

Ausgangspunkt der vorliegenden Arbeit war die Acridonalkaloid-Biosynthese in *Ruta graveolens* L., die allgemein nur in der Familie der Rutaceae gefunden wird. Der erste Schritt der Acridonsynthese ist die *N*-Methylierung von Athranilat, die durch das Enzym Athranilat *N*-Methyltransferase katalysiert wird. Athranilsäure wird durch diese Reaktion dem Primärmetabolismus entzogen.

Die für Antranilat *N*-Methyltransferase kodierende cDNA sollte aus *R. graveolens* Zellkulturen kloniert und das rekombinant exprimierte Enzym charakterisiert werden. Eine oft verwendete Methode zur Klonierung von unbekannten eukaryotischen Proteinen ist die Isolierung des entsprechenden Proteins aus nativem Gewebe und die anschließende Mikrosequenzierung des Peptids. Basierend auf der Peptidsequenz werden dann spezifische Oligonukleotid-Primer für die PCR-Amplifizierung abgeleitet.

In dieser Arbeit wurde das Enzym aus 500 g Nassgewicht *R. graveolens* Suspensionszellen der Zelllinie R-20 gereinigt und in sechs Schritten ~450-fach angereichert. Die abschließende Gelelektrophorese zeigte eine Polypeptid-Bande, die ein apparentes Moleculargewicht von etwa 42-43 kDa hatte. Aktivitätstests mit [*Methyl*-^{14}C]-SAM belegten eine hohe spezifische Athranilat-*N*-Methyltransferase-Aktivität.

Die Mikrosequenzierung des Proteins, durchgeführt von Dr. Peter Hunziker (Abteilung Biochemie, Universität Zürich), erbrachte jedoch nur fünf kurze Peptidfragmente, die keine Homologie zu bekannten Methyltransferase-Sequenzen zeigten. Degenerierte Oligonukleotid-Primer, die auf der Basis der Peptidfragmente synthetisiert worden waren, lieferten ein PCR-Amplifikat ohne Homologie zu Methyltransferasen.

Da die Reinigung erfolglos blieb, wurden degenerierte Oligonukleotid-Primer zur PCR eigesetzt, die aus konservierten Motiven (SAM-Bindstelle) anderer *O*-Methyltransferasen (OMT) entwickelt worden waren. Mit dieser Methode wurden schließlich zwei vollständige cDNAs, R-23 und R-27, isoliert. R-23 codiert für ein 366 Aminosäuren unfassendes Protein mit einem berechneten Molekulargewicht von 40 kDa. R-27 codiert für ein 41,6 kDa Protein mit 374 Resten. Die Gene wurden jeweils in pQE-60-Vektor kloniert und in *E. coli* M-15 exprimiert. Das R-23 Protein wies eine Ähnlichkeit von 79% mit Kaffeesäure *O*-Methyltransferasen auf und enthielt alle konservierten für die Methylierung wichtigen Aminosäuren. Das R-27 Protein wies ebenfalls alle wichtigen konservierten Methyltransferase-Sequenzen auf und war in seiner

Sequenz zu etwa 50% identisch mit einer putativen nicht näher charakterisierten Methyltransferase aus *Prunus dulcis* bzw. mit Orcinol/Chavicol *O*-Methyltransferase aus *Rosa hybrida*. Beide Proteine wurden in *E. coli*-Zellextrakt auf ihre Substratspezifität und ihre katalytische Methylierungsfunktion getestet. Das Enzym, das von R-23 kodiert wird, methylierte keine der untersuchten Substrate, auch nicht Kaffeesäure. R-27 hingegen methylierte 3,5-Dimethoxyphenol und andere methoxylierte Phenole.

Das R-27 Protein wurde in vier Chromatographie-Schritten aufgereinigt und genauer charakterisiert. Das Enzym wies eine sehr enge Substratspezifität auf. Die höchste Affinität zeigte es gegenüber 3,5-Dimethoxyphenol (K_m = 20,1 μM) bei einem pH-Wert von 7,5. Aber auch 3-Methoxyphenol, Guaiacol, 3,4-Dimethoxyphenol und 3,5-Dihydroxyanisol wurden methyliert. Die Reaktion war unabhängig von Kationen. Das Temperaturoptimum lag bei 36˚C. Das gereinigte Protein zeigte im Coomassie-gefärbten SDS-Gel eine charakteristische Bande bei 42 kDa. Das Molekulargewicht, das durch kalibrierte Größenausschlusschromatographie ermittelt wurde, war jedoch 84 kDa. Dies lässt auf einen homodimeren nativen Zustand des Enzyms schließen.

Das Reaktionsprodukt der 3,5-Dimethoxyphenol-Methylierung 1,3,5-Trimethoxybenzol ist ein wichtiger Bestandteil des Rosenduftes. Es liegt deshalb nahe zu postulieren, dass das Produkt der in dieser Arbeit isolierten Methyltransferase R-27 *in vivo* ein Bestandteil der flüchtigen Aromen in *R. graveolens* darstellt. Das Protein wurde aufgrund seiner Substratspezifität als 3,5-Dimethoxyphenol *O*-methyltransferase benannt.

Es konnte gezeigt werden, dass in Anwesenheit von Zn^{2+} das Protein jedoch auch effizient DTT methylieren konnte. Das Produkt wurde durch LC-MS als Monomethylthioether identifiziert. 3,5-Dimethoxyphenol *O*-Methyltransferase hat also neben der OMT-Aktivität eine zusätzliche Thiolmethyltransferase (TMT)-Aktivität. Kinetische Experimente zur 3,5-Dimethoxyphenol-Methylierung in Anwesenheit von variierenden Zn^{2+}/DTT-Konzentrationen zeigten eine kompetitive DTT-Bindung mit einer Affinität von K_i = 52,0 μM. Dieses Ergebnis legte nahe, dass die OMT- und TMT-Aktivität im gleichen katalytischen Zentrum des Enzyms stattfindet. Allgemein zeigen die Resultate, dass die Substratspezifität der pflanzlichen *O*-Methyltransferasen II und deren physiologische Funktion *in vivo* wahrscheinlich breiter ist als angenommen.

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