The *Catharanthus roseus* 16-hydroxytabersonine \(O\)-methyltransferase involved in vindoline biosynthesis

by

Dylan Levac, B.Sc.

A Thesis

Submitted to the Department of Biological Sciences

In partial fulfillment of the requirements

For the degree of

Masters of Science

February 25, 2008

Brock University

St. Catharines, Ontario

© Dylan Levac, 2008
Acknowledgements

First and foremost I would like to thank Dr. Vincenzo De Luca for the opportunity to learn under his mentorship. His endless knowledge in the field, enthusiasm for his work and the lab, personal experience, but above all love for research fostered a perfect environment for me to mature as an academic researcher.

Secondly, I would like to thank Dr. Jun Murata. I am the researcher I am today because of his patience, and unparalleled enthusiasm through my training. The thirst for knowledge, vision, and passion for research he brought to the lab every day made working under his supervision a spectacular experience, and a joy even through the hardest times.

I would also like to thank my colleagues and co-workers. Particularly I would like to thank Pierre Laflamme, Dawn Hall, and Jonathon Roepke for the many stimulating conversations and personal perspectives that have modified how I approach not only research, but life. I would also like to thank Jian-Xin Chen, Maggie Wu, Ashok Gosh, and Kim Gosh.

I would especially like to thank my examination committee of Dr. Malcom Campbell, Dr. Tomas Hudlicky, Dr. Alan Castle, and Dr. Rick Cheel. I recognize it takes significant time and effort to evaluate a thesis based on its quality, cohesiveness, how scientifically sound it is. I would like to especially thank Dr. Malcom Campbell for insightful comments, criticisms, and helpful suggestions on how to improve my writing, all of which I’m sure will make me a better professional.

Of course I could never forget to thank my close friends in the Chemistry department without whom I would most definitely be the token biologist. The
stimulating conversations and constant insight has truly allowed me to bridge biology and chemistry.

Lastly I would like to thank my family and friends for all their continued love and support. Particularly I would like to thank my mother and father, as well as Brandi Rae.
Abstract

Madagascar periwinkle (*Catharanthus roseus*) produces the well known and remarkably complex dimeric anticancer alkaloids vinblastine and vincristine that are derived by coupling vindoline and catharanthine monomers. This thesis describes the novel application of carborundum abrasion (CA) technique as a tool for large scale isolation of leaf epidermis enriched proteins. This technique was used to facilitate the purification to apparent homogeneity of 16-hydroxytabersonine-16-0-methyltransferase (16OMT) that catalyses the second step in the 6 step pathway that converts tabersonine into vindoline. This versatile tool was also used to harvest leaf epidermis enriched mRNAs that facilitated the molecular cloning of the *16OMT*. Functional expression and biochemical characterization of recombinant 16OMT enzyme showed that it had a very narrow substrate specificity and high affinity for 16-hydroxytabersonine, since other closely related monoterpene indole alkaloids (MIAs) did not act as substrates. In addition to allowing the cloning of this gene, CA technique clearly showed that 16OMT is predominantly expressed in *Catharanthus* leaf epidermis, in contrast to several other OMTs that appear to be expressed in other *Catharanthus* tissues. The results provide compelling evidence that most of the pathway for vindoline biosynthesis including the O-methylation of 16-hydroxytabersonine occurs exclusively in leaf epidermis, with subsequent steps occurring in other leaf cell types.

Small molecule O-methyltransferases (OMTs) (E.C. 2.1.1.6.x) catalyze the transfer of the reactive methyl group of *S*-adenosyl-L-methionine (SAM) to free hydroxyl groups of acceptor molecules. Plant OMTs, unlike their monomeric mammalian homologues, exist as functional homodimers. While the biological advantages for dimer
formation with plant OMTs remain to be established, studies with OMTs from the benzylisoquinoline producing plant, *Thalictrum tuberosum*, showed that co-expression of 2 recombinant OMTs produced novel substrate specificities not found when each rOMT was expressed individually (Frick, Kutchan, 1999). These results suggest that OMTs can form heterodimers that confer novel substrate specificities not possible with the homodimer alone. The present study describes a 16OMT model based strategy attempting to modify the substrate specificity by site-specific mutagenesis. Our failure to generate altered substrate acceptance profiles in our 16OMT mutants has lead us to study the biochemical properties of homodimers and heterodimers. Experimental evidence is provided to show that active sites found on OMT dimers function independently and that bifunctional heterodimeric OMTs may be formed *in vivo* to produce a broader and more diverse range of natural products in plants.
Chapter 1. Literature Review – S-Adenosyl-L-Methionine (SAM) dependent O-methyltransferases: Their function and relevance in plant metabolism.

1.1.1 S-adenosyl-L-methionine dependant methyltransferases

1.1.2 Evolution of secondary biosynthetic pathway genes

1.1.3 Common amino acid signatures and proposed classification of OMTs

1.1.4 Plant OMTs occur as dimers in order to be enzymatically active

1.1.5 Classification of plant OMTs

1.2.1 Phenolic O-methyltransferases

1.2.2 Lignin: Monolignol biosynthesis

1.2.3 Lignin: Maize bm mutants and COMT/CCOMT antisense transgenics

1.2.4 Flavonoid methylation

1.2.5 Flavonoid methylation: UV-B protectants

1.2.6 Flavonoid methylation: Phytoalexins

1.2.7 Isoflavonoid methylation: Phytoalexins
Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2.8 Flavonoid methylation: Anthocyanins</td>
<td>21</td>
</tr>
<tr>
<td>1.2.9 Isoflavonoid methylation: Structural analysis</td>
<td>22</td>
</tr>
<tr>
<td>1.2.10 Isoflavonoid methylation: Catalytic mechanism</td>
<td>24</td>
</tr>
<tr>
<td>1.3.1 Methylation of plant hormone and hormone like molecules</td>
<td>24</td>
</tr>
<tr>
<td>1.3.2 SABATH Methyltransferases</td>
<td>25</td>
</tr>
<tr>
<td>1.3.3 Salicylic Acid Methyltransferase (SAMT)</td>
<td>25</td>
</tr>
<tr>
<td>1.3.4 Benzoic Acid Methyltransferase (BAMT)</td>
<td>28</td>
</tr>
<tr>
<td>1.3.5 Jasmonic Acid Methyltransferase (JAMT)</td>
<td>28</td>
</tr>
<tr>
<td>1.4.1 Methylation of plant alkaloids</td>
<td>29</td>
</tr>
<tr>
<td>1.4.2 O-methylation of benzoisoquinoline alkaloids</td>
<td>30</td>
</tr>
<tr>
<td>1.4.3 O-methylation of benzoisoquinoline alkaloids: Berberine biosynthesis</td>
<td>32</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Abstract</td>
<td>36</td>
</tr>
<tr>
<td>2.2 Introduction</td>
<td>37</td>
</tr>
<tr>
<td>2.3 Materials and Methods</td>
<td>40</td>
</tr>
<tr>
<td>2.3.1 Plant Material</td>
<td></td>
</tr>
</tbody>
</table>
### Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3.2 A large scale CA extraction method to harvest leaf epidermis protein: purification of 16OMT and protein sequencing.</td>
<td>40</td>
</tr>
<tr>
<td>2.3.3 Small scale CA extraction of leaf epidermis enriched protein compared to whole leaf extraction for estimating the 16OMT activity in the epidermis.</td>
<td>43</td>
</tr>
<tr>
<td>2.3.4 Extraction of whole leaves for comparative enzyme assays or for comparative purification of CA-based extraction.</td>
<td>43</td>
</tr>
<tr>
<td>2.3.5 CA extraction to harvest leaf epidermis enriched mRNA.</td>
<td>44</td>
</tr>
<tr>
<td>2.3.6 Construction and random sequencing of leaf epidermis-specific cDNA library.</td>
<td>45</td>
</tr>
<tr>
<td>2.3.7 Identification of potential 16OMT Expressed Sequences.</td>
<td>45</td>
</tr>
<tr>
<td>2.3.8 Molecular cloning of 16OMT.</td>
<td>46</td>
</tr>
<tr>
<td>2.3.9 Expression, refolding and purification of r16OMT.</td>
<td>47</td>
</tr>
<tr>
<td>2.3.10 Enzyme kinetic analysis of r16OMT.</td>
<td>48</td>
</tr>
<tr>
<td>2.3.11 Preparation of cDNA from laser capture microdissected cells.</td>
<td>48</td>
</tr>
<tr>
<td>2.3.12 Real time PCR for quantitation of 16OMT in different cell types obtained by laser capture microdissection.</td>
<td>48</td>
</tr>
<tr>
<td>2.3.13 Expression of 16OMT, TDC and DAT enzyme activity in light and dark grown seedlings.</td>
<td>50</td>
</tr>
<tr>
<td>2.3.14 Protein determinations.</td>
<td>50</td>
</tr>
</tbody>
</table>
### Table of Contents

#### Results

2.4.1 MIA pathway enzyme activity profiling throughout seedling development, in leaves, flowers, stems and roots of *Catharanthus roseus*.  

2.4.2 Purification of *Catharanthus roseus* 16OMT to homogeneity and molecular cloning from a cDNA library prepared from leaf epidermis enriched mRNA.  

2.4.3 Expression and functional characterization of recombinant 16OMT (r16OMT)  

2.4.4 Recombinant 16OMT is a highly specific O-methyltransferase.  

2.4.5 Kinetic analysis of the recombinant 16OMT.  

2.4.6 Leaf epidermal cells are enriched 900-fold in 16OMT activity compared to whole leaves and 6-fold in 16OMT transcripts compared to LCM captured whole leaves.  

#### Discussion

2.5.1 Carborundum abrasion technique is a cost effective, robust method to study leaf epidermis biology.  

2.5.2 Properties of r16OMT  

2.5.3 The leaf epidermis expression of 16OMT activity in *Catharanthus*

3.1 Abstract

3.2 Introduction

3.3 Materials and Methods

3.3.1 Cloning, expression and purification of recombinant C. roseus 16OMT from inclusion bodies.

3.3.2 16OMT assays

3.3.3 Comparative enzyme kinetic analysis between 16OMT and 16OMT mutants.

3.3.4 Relationship tree

3.3.5 Homology modeling and substrate positioning within the 16OMT active site.

3.3.6 Site directed mutagenesis of 16OMT and mutant Identification.

3.3.7 Titration of functional r16OMT with inactive H260A r16OMT mutant.

3.3.8 Use of His tag purification and protease treatment to isolate purified r16OMT.

3.3.9 Preparation of cDNA from laser capture microdissected cells.

3.3.10 Cell-type expression profiling of selected OMTs compared to 16OMT.
# Table of Contents

## 3.4 Results

3.4.1 Phylogenetic analysis: 16OMT is most closely related to two *Catharanthus* class II OMTs.  
3.4.2 The identification of 16OMT active site residues based on the crystal structure of *Ms71OMT*.  
3.4.3 Site-directed mutagenesis to identify residues essential for catalysis and substrate binding.  
3.4.4 Functional expression and biochemical analysis of r16OMT mutants.  
3.4.5 Heterodimers of r16OMTi protein with active r16OMT are biochemically active.  
3.4.6 His-tag affinity purified r16OMT:r16OMTi heterooligomers are not enzymatically active.  
3.4.7 Expression analysis of *C. roseus* OMTs.

## 3.5 Discussion

3.5.1 Evolutionary origin of the *Catharanthus* 16OMT  
3.5.2 Mutagenesis of the 16OMT  
3.5.3 *O*-methyltransferase heterodimerization as a method for developing new chemistries *in vivo*.

## 3.6 Conclusion

References  
Appendix I
<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td>Purification of 16OMT compared to FOMT using Carborundum abrasion technique compared to extraction of enzymes from whole leaves.</td>
<td>53</td>
</tr>
<tr>
<td>2.2</td>
<td>Kinetic data for 16-hyrdoxytabersonine, S-adenosyl-L-methionine as substrates and S-adenosyl-L-homocysteine as an inhibitor for r16OMT.</td>
<td>62</td>
</tr>
<tr>
<td>Chapter 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.1</td>
<td>Specific activities of OMT reaction products with different mutants proteins with 16-hydroxytabersonine and quercetin as substrates</td>
<td>92</td>
</tr>
<tr>
<td>3.2</td>
<td>Kinetic data for 16-hyrdoxytabersonine, S-adenosyl-L-methionine as substrates and for r16OMT (A) and C20F site directed mutant (B).</td>
<td>93</td>
</tr>
<tr>
<td>3.3</td>
<td>Purification of r16OMT polypeptide lacking N-terminal poly histidine tag, preparation of r16OMT homodimers lacking N-terminal poly histidine tag, and 1:1 r16OMT: H260A herodimers for biochemical analysis.</td>
<td>97</td>
</tr>
<tr>
<td>Appendix I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>Comparative % amino acid identities between Catharanthus OMTs.</td>
<td>116</td>
</tr>
</tbody>
</table>
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 1. 1.1</td>
<td>Monolignol biosynthesis</td>
<td>10</td>
</tr>
<tr>
<td>1.2</td>
<td>Reticuline biosynthesis</td>
<td>33</td>
</tr>
<tr>
<td>Chapter 2. 2.1</td>
<td>The biosynthesis of Vindoline from tryptamine and secologanin.</td>
<td>39</td>
</tr>
<tr>
<td>2.2</td>
<td>Distribution of 16OMT enzyme activities in <em>Catharanthus roseus</em> seedlings, organs and cells.</td>
<td>51</td>
</tr>
<tr>
<td>2.3</td>
<td>Alignment of peptide sequences 1 to 7 in relation to the putative open reading frame of 16OMT and identification of a unique PCR product that is preferentially expressed in leaf epidermal cells compared to whole leaf.</td>
<td>56</td>
</tr>
<tr>
<td>2.4</td>
<td>SDS-PAGE gel and audioradiogram demonstrating solubilized and renatured r16OMT inclusion bodies have 16-hydroxytabersonine O-methyltransferase biochemical activity.</td>
<td>58</td>
</tr>
<tr>
<td>2.5</td>
<td>List of substrates assayed for against r6OMT for its methyl acceptor.</td>
<td>60</td>
</tr>
<tr>
<td>2.6</td>
<td>Quantitation of 16OMT, FOMT, NMT and DAT enzyme activities in whole leaf and CA enriched leaf epidermis extracts. Quantitation of 16OMT mRNA transcript levels in LCM dissected leaf tissues by Real Time PCR.</td>
<td>63</td>
</tr>
<tr>
<td>Chapter 3. 3.1</td>
<td>Phylogenic tree of plant O-methyltransferases that have been partially or completely biochemically characterized.</td>
<td>84</td>
</tr>
<tr>
<td>Figure</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>3.2</td>
<td>Molecular models of the 16OMT.</td>
<td>87</td>
</tr>
<tr>
<td>3.3</td>
<td>Clustal W multiple sequence alignment of <em>Catharanthus</em> methyltransferases functionally annotated and established to have <em>in vivo</em> functions.</td>
<td>89</td>
</tr>
<tr>
<td>3.4</td>
<td>SDS-PAGE gels of protein extracts and audioradiograms of 16OMT or FOMT enzyme assays demonstrating 16OMT activity, or lack of biochemical activity among r16OMT and site directed mutants.</td>
<td>91</td>
</tr>
<tr>
<td>3.5</td>
<td>Linear relationship of r16OMT:r16OMTi titration</td>
<td>95</td>
</tr>
<tr>
<td>3.6</td>
<td>Cellular and tissue <em>Catharanthus</em> OMT expression profiles.</td>
<td>99</td>
</tr>
</tbody>
</table>

*Appendix I*

<table>
<thead>
<tr>
<th>S1</th>
<th>Carborundum abrasion technique for extraction of leaf epidermis enriched proteins.</th>
<th>117</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2</td>
<td>SDS-PAGE combined with silver staining of the gel and enzyme activity profile of 16OMT eluting from a MonoQ anion exchange column.</td>
<td>118</td>
</tr>
<tr>
<td>S3</td>
<td>ClustalW multiple sequence peptide alignment of all published functionally annotated or putative O-methyltransferases.</td>
<td>119</td>
</tr>
<tr>
<td>S4</td>
<td>Description of the expressed sequence tags obtained from extensive sequencing of a cDNA library produced from epidermis enriched mRNA isolated by CA of <em>Catharanthus</em> leaves.</td>
<td>120</td>
</tr>
</tbody>
</table>
Chapter 1

Literature Review

S-Adenosyl-L-Methionine (SAM) dependent O-methyltransferases: Their function and relevance in plant metabolism.

Dylan Levac and Vincenzo De Luca
S-Adenosyl-L-Methionine (SAM) dependant O-methyltransferase; Their function and relevance in plant metabolism.

Dylan Levac and Vincenzo De Luca

1.1.1 - S-adenosyl-L-methionine dependent methyltransferases

Enzymatic methylation is a reaction all organisms utilize in varied biological processes (Ibrahim, et al. 2001). In plants, methylation of small molecules occurs in metabolic pathways essential for plant survival (primary metabolism), as well as in metabolic pathways that were traditionally considered non-essential to the plant (secondary metabolism). We now know that most secondary metabolic pathways do in fact produce small molecules essential to plant defense, pollination, communication, and seed dispersal, all elements essential to plant survival and reproduction.

Methylation reactions are catalyzed by diverse classes of small molecule S-Adenosyl-L-Methionine (SAM) dependant methyltransferases (MTs) (Ibrahim, et al. 2001). Methylation of small molecules in plants dramatically increases the diversity of metabolites that appear to be important for plant development, defense, reproduction and for intra and/or inter plant communication (Gang, 2005). In the case of secondary metabolites, SAM dependent MTs methylate a variety of reactive centers that modify all three major classes of secondary metabolites (phenolics, terpenes, and alkaloids). The wide variety of methylated small molecules ensures a diverse library of bioactive products that appear to have wide variety of biological roles that remain to be understood. Some methylated small molecules are nutritionally important (ie. \( \alpha \)-tocopherol,
isoflavones), while other are commercial medicines (i.e. morphine, vincristine, vinblastine) or provide esthetic value (i.e. anthocyanins) to human populations.

Plant small molecule O-methyltransferases (OMTs) (E.C. 2.1.1.6.x) catalyze the enzymatic transfer a reactive methyl group from SAM, to a hydroxyl, or reactive oxyanion of the target substrate. This reaction typically occurs via a general S$_{n}$2 acid/base reaction mechanism facilitated by a basic catalytic residue (i.e. histidine, lysine), but there are variants of SAM MTs that use the ionized character of their target substrate at cellular pH rather than basic catalytic residues to facilitate transmethylation (Class III MTs). This review will focus on plant OMTs that are involved in primary and secondary plant metabolism, their essential roles in biosynthetic pathways, and their role in establishing biosynthetic pathway order and their evolution together with structure-function relationships.

1.1.2 - Evolution of secondary biosynthetic pathway genes

There is a vast library of structurally characterized secondary metabolites (>50 000), and an even larger predicted array of yet to be discovered compounds. Although plants possess this massive library of small molecules, the enzymes responsible for their biosynthesis perform limited chemical modifications. It is a consequence of enzyme substrate specificities, substrate stereospecificities, and enzyme promiscuity that have permitted a few reaction types to yield the diversified library of plant secondary metabolites found in modern plants (Gang, 2005).

When discussing evolution of biosynthetic pathways, we must first define what constitutes a new or unique gene. Genes are considered distinct from ancestral progenitor
genes when their gene products perform the same catalytic modification on a new substrate, or when they perform a new chemical reaction on the same substrate (Pichersky, Gang, 2000). It would then follow that if a new gene product performs new chemistry on a new substrate it would also be considered distinct from the ancestral gene. However, the appearance of a new substrate preference together with new enzyme chemistry is less likely to occur (Gang, 2005).

It is generally accepted that new OMTs arise from a given ancestor by gene duplication followed by mutation (Wang, Pichersky, 1999, Gang, 2005). The ancestral gene is duplicated in the plant genome to yield a copy that is free to mutate and potentially acquire a new biochemical function at no detrimental cost to the plant (Pichersky, Gang, 2000).

A well known example of gene evolution by gene duplication is that of the isoeugenol OMT (IEMT). Comparing the amino acid sequences of 13 OMTs, it was shown that the *Clarkia breweri* IEMT gene was undoubtedly derived from the *C.breweri* caffeic acid OMT (COMT) gene (Wang, Pichersky, 1999). Phylogenetic analysis determined that this evolution likely occurred by gene duplication of COMT followed by mutation. Detailed kinetic analysis using recombinant wild type IEMT and COMT enzymes, as well as recombinant site-specific mutagenized IEMT and COMT gene products established that only 7 amino acid differences caused the change in substrate specificity (Wang, Pichersky, 1999).

While gene duplication events followed by mutation are probably the most well understood mechanisms of new gene evolution, other forms of gene evolution may also occur in the diversification of plant biosynthetic pathways (Pichersky, Gang, 2000).
Repeated or convergent evolution could also yield new functional gene products, as exemplified by the terpene synthases (Pichersky, Gang, 2000), but examples of this type of OMT gene evolution have yet to be reported.

1.1.3 - Common amino acid signatures and proposed classification of OMTs

SAM-dependent OMTs almost always possess a highly conserved nucleotide binding domain, more commonly referred to as the SAM binding domain that is characterized by a highly conserved α/β Rossmann fold. The α/β Rossmann fold consists of at least 3 β-sheets and at least 2 α-helices in alternating order (Rao, Rossmann, 1973) and is localized to the C-terminal 1/3 of the protein (Ibrahim, 1998). The only reported example of a SAM dependent OMT not possessing a discernable SAM binding domain is *Vitis vinifera* Caffeoyl CoA 3-O-methyltransferase (Busam, et al. 1997). For all other OMTs the reported SAM binding consensus sequence annotated as LVDGGGxG is glycine rich (Ibrahim, 1998).

In addition to the highly conserved SAM binding domain, OMTs possess metal binding domains within the consensus amino acid sequence, NGKVI (Vidgren, et al. 1994, Ibrahim, et al. 1998). It should be noted, however, that some OMTs that do not require divalent metal cations for catalytic activity still show predicted metal binding domains in their protein sequence (Joshi, Chiang, 1998). While the function of these metal binding domains in non-metal requiring OMTs is currently unclear, it is possible that the domain is vestigial and is simply a remnant of the ancestral gene that required divalent cations for catalytic function.
1.1.4 - Plant OMTs occur as dimers in order to be enzymatically active

Plant OMTs are unique compared to those of animals since they almost always occur as homodimers through a common N-terminal dimerization domain (Zubieta, et al. 2001). For example the isoflavone OMT (IOMT) from alfalfa, and Salicylic acid OMT (SAMT) from Clarkia both possess N-terminal dimerization domains (Zubieta, et al. 2001, Zubieta, et al. 2003). Varying between OMT classes is the size of the domain in general and its extent of involvement in the complementary monomer’s active site. The crystal structure of IOMT suggests that the N-terminal dimerization domain participates in the catalytic site of the opposing monomer (Zubieta, et al 2001), whereas the N-terminal dimerization domains of SAMT and other Class III MTs do not (Zubieta, et al 2003). There appears to be a relationship between the size of the dimerization domain and its participation in the active site. For example, the dimerization domain of IOMTs representing 30% of the polypeptide chain, participates in the active site (Zubieta, et al. 2001), while SAMT as well as other Class III MTs have a simple dimerization domain comprising only 7% of the polypeptide that do not participate in the active site (Zubieta, et al. 2003).

1.1.5 - Classification of Plant OMTs

Several classification systems have been proposed for SAM-dependent OMTs. Initially OMTs were classified based on substrate preferences (Ibrahim, et al. 1998). Phenylpropanoid, flavonoid and alkaloid methyltransferases were organized into A, B and C classes, respectively. Class A OMTs were organized into subclasses 1 through 5 representing enzymes acting on caffeic and 5-hydroxyferulic acids, caffeoyl and 5-
hydroxyferuoyl-CoA esters, coumarins/furanocoumarins, phenols/benzoic acids/phenolic esters, and polyketides, respectively. Class B MTs were organized into enzymes acting on flavonols/flavones, chalcones/flavanones, pterocarps, and flavans/anthocyanin subclasses, respectively. Class C MTs were organized into enzymes acting on benzoisoquinoline alkaloid, morphinan alkaloid, and secologanin/lysergic acid subclasses, respectively (Ibrahim, et al. 1998). Unfortunately this classification system did not consider that the substrate specificity of OMTs did not necessarily represent their phylogenetic origin, the OMT active site structural relationships, or their catalytic activity properties (D’Auria, et al. 2003). More recently OMTs have been classified as class I, class II and class III OMTs (Joshi, Chiang, 1998, D’Auria, et al. 2003). Class I OMTs have subunit masses of approximately 30 kDa and are active only in the presence of divalent cations. While Class I OMTs represent the majority of the enzymes that O-methylate CoA esters (ie. caffeoyl-CoA, 5-hydroxyferuoyl-CoA), some Class I OMTs also methylate flavonoids (Joshi, Chiang, 1998). Class II OMTs have subunit masses of approximately 40 kDa, and show no requirement for divalent cations for catalytic activity. Class II MTs typically methylate a broad range of substrates, including monolignol precursors (caffeic acid, caffeoyl alcohol, caffeoylaldehyde), flavonoids, and alkaloids (Joshi, Chiang, 1998). Class III OMTs, also named the SABATH methyltransferases after the first three enzymes identified from this new class (Salicylic Acid OMT, Benzoic Acid OMT, Theobromine synthase) have subunit masses of 40 kDa, do not require divalent cations, and O-methylate the oxyanion of carboxylic acid functional groups (Ross, et al. 1999, Murffit, et al. 2000, Ogawa, et al. 2001, Uefugi, et al. 2003). This classification system, although simple, is more effective in classifying
methyltransferases according to their structural similarities, catalytic requirements, and phylogenetic origins.

1.2.1 - Phenolic O-methyltransferases

Phenolics, one of the largest groups of plant small molecules, are identified by their aromatic six member ring, and by the presence of at least one hydroxyl functional group. These compounds (ie. p-coumaric, caffeic, ferulic, sinapic acids) have a large range of biological functions including deterrent roles against herbivores, fungi and bacteria, or as allelopathic agents in modulating plant-plant interactions (Siqueria, et al. 1991). Some volatile phenols are important attractants for pollinators or fruit dispersers, and act as signaling molecules for pathogen attack (eg. salicylic acid) (Gang, 2005). Other phenolics serve a more structural role (eg. lignin) or protection against ultraviolet radiation (eg. flavonoids, anthocyanins) (Ibrahim, et al. 1998).

1.2.2 - Lignin: Monolignol biosynthesis

Lignin is one of the most abundant organic molecules on the planet, and is second in abundance only to cellulose. Using atmospheric O\textsubscript{2} and \(\delta^{13}\text{C}\) it has been estimated that approximately 30% of total fixed carbon is directed to lignin biosynthesis (Battle, et al. 2000). Lignin’s hydrophobic nature and its intrinsic strength makes it an intrinsic component of plant vasculature that prevents these tissues from collapsing under the negative pressure produced by transpiration. These properties also ensure structural stability of plants for upward growth. In terms of plant defense the structural strength of lignin deters herbivores and protects plants against pathogen invasion (Guo, et al. 2001).
Lignin is a complex, branched polymer formed from basic phenolics (sinapyl, coumaryl, and coniferyl alcohols) that are referred to as monolignols (Figure 1.1) (Ralph, et al., 2004). The exact structure of lignin is still unknown due to its complexity and the difficulties involved in isolating the pure polymer. Moreover, the mechanism of monolignol polymerization is a highly debated topic (Lewis, 2005, Ralph, et al. 2004). Some researchers believe the linking of monolignol subunits is achieved by a mechanism analogous to non-catalyzed organic reactions (Ralph, et al., 2004). Conversely other in vitro research has demonstrated that lignin polymerization occurs via a far more ordered mechanism mediated by so called dirigent proteins (DPs) purified from Forsythia suspense (Laurence, et al. 1997). DPs, contrary to enzymes, are not biological catalysts but are hypothetically stereo-selective guides for monolignol coupling. It must be noted that to date any reproduction of this original DP purification, or purification of any homologous DPs from other plants has yet to been reported.

Lignin biosynthesis is of great economic interest, in particular to both animal and human food crop industries, as well as in pulp and paper production. The major monolignol subunits affecting food digestibility are the methoxylated monomers, known as guaiacyl (G) and syringyl (S) subunits (Campbell, Sederoff, 1996). The S/G composition of lignin varies significantly from one plant species to another, within one plant species and even within a single plant depending on its tissue type, and developmental stage (Campbell, Sederoff, 1996). In terms of nutrition, a high S/G ratio will have a negative effect on lignin digestibility and as a consequence there is interest in developing legumes with
Figure 1.1 Proposed routes of monolignol biosynthesis modified from Parvathi et al. 2001. Green arrows indicate proposed route for G monolignol unit biosynthesis. Red arrows indicate proposed route for S monolignol unit biosynthesis.
decreased syringyl monolignol lignin to improve the nutritional use of food crops (Campbell, Sederoff, 1996).

In terms of the pulp and paper industry, lignin must be removed from cellulose for paper production. Plants containing higher levels of condensed lignin (ie. low S/G ratio and greater p-hydroxyphenyl unit content) are poor raw materials for paper production (Chiang, Funaoka, 1990). For example, softwood gymnosperms have high guaiacyl monolignol content, as well as some p-hydroxyphenyl (H) monolignols, and consequently are less suitable for paper production. Lignin of this composition is more difficult to remove by hydrolysis than in angiosperms that produce less condensed lignin (Chiang, Funaoka, 1990).

The differences between condensed and non-condensed monolignols are their methoxylation states. Two particular OMTs, caffeic acid 3-O-methyltransferase (COMT), and caffeoyl CoA 3-O-methyltransferase (CCOMT) control O-methylation of monolignol precursors. COMT catalyzing the conversion of caffeic acid to ferulic acid was first reported in the early 1960s from crude protein extracts of apple and pampas grass tissues (Finkle, Nelson, RF., 1963, Finkle, Masri, 1964). The COMTs from ginko (Shimada, 1972), poplar (Higuchi, et al. 1977), and tobacco (Hermann, et al. 1987) were purified by gel filtration and demonstrated to have molecular masses between 60 – 86 kDa. These purified COMTs were also shown to not require divalent cations for catalytic function.

Research of the 1970s and early 1980s revealed that lignin biosynthesis involved the combined participation of hydroxylases and OMTs that controlled the production of lignin precursors such as p-coumaric acid and its acid derivatives (ferulic acid, and
These intermediates were thought to be reduced to monolignols through their CoA derivatives (Humphreys, Chapple, 2002) (Figure 1.1).

The first reported isolation of a cDNA encoding COMT (Ptomtl), whose gene product was capable of O-methylating caffeic acid and 5-hydroxyferulic acid, was cloned from Populus tremuloides (Bugos, et al. 1991). Ptomtl enzyme preferentially accepted 5-hydroxyferulic acid 2.5 times more efficiently than caffeic acid, thus supporting the model for lignin biosynthesis where monolignols were derivatized through their acid intermediates. More recent kinetic analyses of alfalfa COMT enzyme suggest that caffeoyl aldehyde (Km 6.9 µM) and 5-hydroxyconiferaldehyde (Km 1.8 µM) have a 10 and 3 times lower Km than caffeic and ferulic acid (Parvathi, et al. 2001). While this remains to be proven, the preference of COMT for the aldehyde intermediates suggests an alternative, more favoured route for monolignol biosynthesis through caffeoyl aldehyde and 5-hydroxyconiferaldehyde (Figure 1.1).

While COMT enzyme substrate saturation kinetics suggest that lignin biosynthesis proceeds through aldehyde intermediates, further pioneering studies reported the discovery of caffeoyl-Co-enzyme-A OMTs (CCoAOMTs) (Kneusel, et al. 1989, Schmitt, et al., 1991, Ye, et al. 1994, Ye, et al. 1995) that led to the hypothesis that monolignol biosynthesis occurs though CoA ester intermediates. The first CCoAOMT gene was cloned and its enzyme functionally characterized was from parsley (Schmitt, et al, 1991). In general, CCOMT requires divalent cations (Mg$^{2+}$, Zn$^{2+}$, Ca$^{2+}$) for enzyme activity and the subunit Mr of this class of varies between 27 – 28 kDa (Kneusel, et al. 1989, Ye, et al. 1994, Ferrer, et al. 2005). Substrate specificities of recombinant alfalfa CCOMT showed its strict substrate preferences for caffeoyl-CoA (Km 4.3 µM), and 5-
hydroxyferuloyl-CoA (Km 6.2 μM) whereas other monolignol intermediates were not accepted (Parvathi, et al. 2001). The ability of CCoAOMTs to methylate the CoA ester intermediates of monolignols led to the development of the initial alternative pathway for monolignol biosynthesis (Ye, et al. 1994) (Fig 1.1).

The current proposed model for monolignol biosynthesis follows a grid pattern using coniferaldehyde as a common intermediate for both G and S monolignol biosynthesis (Parvathi, et al. 2001) (Figure 1.1). Unfortunately this theory is based largely on combined enzymatic data from widely varying plant species and is primarily a representation of in vitro kinetic data rather than in vivo biological reality.

1.2.3 - Lignin: Maize bm mutants and COMT/CCOMT antisense transgenics

Maize brown-midrib (bm) mutants, first reported nearly 40 years ago (Kuc, et al. 1968), are characterized by a red-brown midrib, reduced lignin content, and are more easily digested compared to normal varieties. However, the improved digestibility of low lignin lines is offset by low crop yields compared to normal lignin producing lines (Cherney, et al. 1991). Of the four maize bm lines known, bm3 has been identified as a mutation of COMT (Vignols, et al. 1995). Two independent bm3 mutant alleles have been characterized, where bm3-1 corresponds to an insertion near the highly conserved intron of COMT and bm3-2 is characterized by a partial deletion of COMT (Vignols, et al. 1995). Both mutations lack COMT activity. Maize bm3 mutants contain 12% less lignin (Grand, et al. 1985) together with increased p-hydroxyphenyl content in the lignin as well as increased levels of soluble monolignols (p-coumaric acid, ferulic acid, 5-hydroxyferulic acid) (Chabbert, et al. 1994).
hydroxyferuloyl-CoA (Km 6.2 µM) whereas other monolignol intermediates were not accepted (Parvathi, et al. 2001). The ability of CCoAOMTs to methylate the CoA ester intermediates of monolignols led to the development of the initial alternative pathway for monolignol biosynthesis (Ye, et al. 1994) (Fig 1.1).

The current proposed model for monolignol biosynthesis follows a grid pattern using coniferaldehyde as a common intermediate for both G and S monolignol biosynthesis (Parvathi, et al. 2001) (Figure 1.1). Unfortunately this theory is based largely on combined enzymatic data from widely varying plant species and is primarily a representation of in vitro kinetic data rather than in vivo biological reality.

1.2.3 - Lignin: Maize bm mutants and COMT/CCOMT antisense transgenics

Maize brown-midrib (bm) mutants, first reported nearly 40 years ago (Kuc, et al. 1968), are characterized by a red-brown midrib, reduced lignin content, and are more easily digested compared to normal varieties. However, the improved digestibility of low lignin lines is offset by low crop yields compared to normal lignin producing lines (Cherney, et al. 1991). Of the four maize bm lines known, bm3 has been identified as a mutation of COMT (Vignols, et al. 1995). Two independent bm3 mutant alleles have been characterized, where bm3-1 corresponds to an insertion near the highly conserved intron of COMT and bm3-2 is characterized by a partial deletion of COMT (Vignols, et al. 1995). Both mutations lack COMT activity. Maize bm3 mutants contain 12% less lignin (Grand, et al. 1985) together with increased p-hydroxyphenyl content in the lignin as well as increased levels of soluble monolignols (p-coumaric acid, ferulic acid, 5-hydroxyferulic acid) (Chabbert, et al. 1994).
The significant role played by COMT in general monolignol biosynthesis has triggered metabolic engineering efforts using antisense technology to modulate lignin content in transgenic poplar. Poplar lines expressing antisense poplar COMT had very low levels of COMT mRNA and COMT enzyme activity that was only 5% of the levels found in untransformed control lines (van Doorssealere, et al. 1995). Stable transgenic lines accumulated 56% less S monolignols while G monolignol content slightly increased compared to control lines, but total lignin levels remained unchanged (van Doorssealere, et al. 1995). These results suggest an alternative route for G monolignol biosynthesis through CCOMT enzyme, and illustrate the absolute requirement for COMT enzyme in S monolignol biosynthesis (Figure 1.1). Over expression of COMT in woody plants, particularly angiosperms used in the pulp and paper industry, could be of economic benefit.

Transgenic tobacco expressing antisense tobacco CCoAOMT produced 53 – 67% less total lignin (Zong, et al. 1998). While total lignin content was decreased on average by 58%, the ratios of G and S monolignol remained unchanged compared to control lines (Zong, et al. 1998). This demonstrated that CCoAOMT enzyme contributed significantly to the overall monolignol subunit pool drawn upon for lignin biosynthesis. The results suggest that CCoAOMT enzyme contribution is significant enough that the alternative COMT pathway to G and S monolignols might not compensate for the CCoAOMT deficit (Figure 1.1).

Enzyme kinetics, substrate specificities, maize bm mutants, and antisense transgenics were all used to develop the current model of monolignol biosynthesis (Fig 1.1). The essential roles of COMT and CCOMT enzymes in the production of G and S
monolignol precursor pools suggest that modulation of \textit{COMT} and \textit{CCOMT} gene expression, and consequently their products, in transgenic lines could be used to produce improved plants for the production of pulp and paper or improved food crops that are more easily digested.

1.2.4 - Flavonoid methylation

Flavonoids, one of the largest classes of phenolic compounds, include the anthocyanins, flavones, flavonols, and isoflavones. Flavonoids serve a variety of plant functions. Anthocyanins attract insects through flower pigmentation (Ibrahim, 1998). Flavonoids in general protect against UV-B radiation damage, act as signaling molecules (Long, 1988) and appear to control plant growth by regulating auxin transport (Jacobs, 1988).

Methylation of flavonoids alters their solubility and subcellular localization. While glycosylation of flavonoids in many cases targets them to the vacuole (Harborne, Williams, 2000), methoxylation of flavonoids increases their lipophilic nature and these partition to more hydrophobic environments like the waxy cuticle outside of the leaf epidermis (Harborne, Williams, 2000). Additionally, methoxylation of flavonoids shifts their UV absorption spectrums to shorter wavelengths. This property, coupled with their waxy cuticle localization, makes them great UV-B protectants. In terms of their potential commercial uses, methoxylated flavonoids are candidates for sunscreen additives (Li, et al. 1993). Similarly, methoxylated anthocyanins modulate the colours of flowers. For example, the pale blue/lavender of petunidin (Colijn, et al. 1981), and the deep red color
of wines are derived from methoxylated anthocyanins and are commercially relevant for floral, aesthetic and cultural industries.

In *Catharanthus roseus*, two flavonol OMT genes have been cloned (*CrOMT2, CrOMT6*), and their products partially characterized (Cacace, et al. 2003, Schröder, et al. 2004). *CrOMT2* enzyme is a bifunctional flavonol OMT responsible for two sequential methylations at the 3’ and 5’ hydroxyl positions of the flavonol B ring of myricetin (Cacace, et al. 2003). In contrast, *CrOMT6* preferentially accepted the flavanone homoeriodictyol or the flavonol isorhamnetin at its 4’ hydroxyl position (Schröder, et al. 2004). The Mr of *CrOMT2* monomer is 39 kDa while *CrOMT6* has a monomer molecular mass of 41 kDa. Since neither enzyme showed an apparent requirement for divalent cations for catalytic activity, they belong to Class II OMTs according to the accepted classification system. This is characteristic of almost all characterized flavonoid OMTs. The only exceptions to this pattern are an OMT which accepts both phenylpropanoids and flavonoids (*PFOMT*), that was identified from *Mesembryanthemum crystallinum* (Ibdah, et al. 2003), and SOMT-9 from *Glycine max* (Kim, et al. 2005). Both belong to Class I MTs. These recently discovered genes could encode a new class of small molecule OMTs that will emerge in the future as more research is done, but at this time they remain exceptions to current methyltransferase classification.

*PFOMT* has a predicted molecular weight of 29 kDa and requires Mg\(^{+2}\) for catalytic activity (Ibdah, et al. 2003). SOMT-9 has a molecular mass of 27 kDa and shows a conserved metal-binding amino acid consensus sequence, although requirement of divalent cations for catalytic function was not reported in the enzyme characterization.
(Kim, et al. 2005). Until the discovery of these two coding sequences, all Type I methyltransferases preferentially accepted phenolic CoA esters as substrate. The discovery of this apparent new class of OMTs underlines the importance of proper biochemical characterization of enzymes with complete kinetic analyses, and demonstration of their actual biochemical functions, and when possible, actual in vivo biological functions. Functional classification based solely on amino acid, or nucleotide sequence is insufficient.

1.2.5 - Flavonoid methylation: UV-B protectants

Chalcone synthase (CHS) mutants of *Arabidopsis thaliana* lack flavonoids and are reported to be more sensitive to UV-B radiation compared to wild-type *Arabidopsis* (Li, et al. 1993). CHS mutants lack flavonoids because CHS is responsible for the condensation of 4-coumaroyl CoA with malonyl-CoA, the first major committed step in flavonoid biosynthesis (Winkel-Shirley, 2001). It has been speculated that the effects on growth in the mutant lines most likely relates to the lack of UV-B damage protection flavonoids confers to plants.

The UV-B damage protection conferred by methoxylated flavones and the induction of their biosynthesis under high light conditions has been reported from *Gnaphalium vira-vira* (Cuadra, Harborne, 1996). *Gnaphalium* possesses two methoxylated flavones on the leaf surface araneol (5,7-dihydroxy-3,6,8-trimethoxyflavone), 7-O-methylaraneol (5-hydroxy-3,6,7,8-tetramethoxyflavone). UV-B irradiation of *Gnaphalium* for extended periods of time triggers the increased accumulation of 7-O-methylaraneol and reduces levels of araneol. This result
demonstrates that in response to prolonged UV-B exposure *Gnaphalium* further methylates highly methylated flavones (Cuadra, Harborne, 1996).

1.2.6 - Flavonoid methylation: Phytoalexins

Methylated flavonoids have been shown to possess more potent anti-fungal activity than their non-methylated counterparts (VanEtten, et al. 1989). Structure-activity relationships of flavonoid phytoalexins show a strong positive correlation between flavonoid lipophilicity and their anti-fungal activity (Laks, Pruner, 1989). It is thought that the methoxylated derivatives are better suited to cross phospholipids membranes and cell walls. Consequently the methoxylated flavonoids reach their site of action easier and yield anti-fungal activities at lower concentrations (Laks, Pruner, 1989, VanEtten, et al. 1989).

When fungal pathogens attack plants, plants respond by activating multicomponent defense pathways that include the production of low molecular weight phytoalexins (Baker, et al. 1997). A well characterized example of phytoalexin production during a plant-pathogen interaction has been demonstrated between *Blumeria graminis* (*bgh*) and barley (*Hordeum vulgare*). Infection of barley by *bgh* stimulates the pathways for phenylpropanoid and flavonoid biosynthesis. Phenylalanine ammonia lyase (*PAL*), chalcone synthase (*CHS*) and caffeic acid OMT (*COMT*) gene expression is quickly up regulated, and their gene product catalytic activities increase to promote flavonoid biosynthesis (Clark, et al. 1994, Gregersen, 1994, Christensen, et al. 1997, Shiraishi, 1995). Flavonoid 7-O-methyltransferase (*Fl-OMT*) mRNA transcripts and protein levels have been reported to increase in *H. vulgare* in response to fungal attack.
(Christensen, et al. 1997, Christensen, et al. 1998). Induction of this Class II OMT (Christensen, et al. 1998) was accompanied by the accumulation of 7-O-methyl apigenin (Cushnie, Lamb, 2005), which has significant antifungal activity suggesting a defensive role for F1-OMT during pathogen attack by bgh.

1.2.7 - Isoflavonoid methylation: Phytoalexins

Isoflavones have been the subject of intense research for decades because of their numerous heath benefits, as well as their participation in plant disease resistance (Dixon, Steele, 1999). Isoflavones appear to play critical roles in the constitutive and inducible defenses of legumes against various diseases (Lamb, Dixon, 1997, Dixon, 2001). For example, the methoxylated isoflavone, pisatin, is a potent antimicrobial compound compared to its non-methoxylated derivative (Deavours, et al. 2006). It has been suggested that 3-O-methylation of 6α-hydroxymaackiain is a critical step in the development of disease resistance (Deavours, et al. 2006). In peas (Pisum sativum), 6α-hydroxymaackianin OMT (PsHMM) is responsible for 3-O-methylation of 6a-hydroxymaackianin (Preisig, et al. 1989). PsHMM is a dimer composed of two 43 kDa subunits that does not require divalent cations for activity, and the enzyme is highly selective for (+)6a-hydroxymaackianin, although other substrates and their enantiomers were accepted at lower levels [(−)6a-hydroxymaackianin (+)maackiain, (−)maackiain, (+)medicarpin, (−) medicarpin] (Preisig, et al. 1989). It is interesting that the (+) enantiomer of 6a-hydroxymaackianin is preferentially accepted in vitro since only the (−) enantiomers of pterocarpan accumulate to any appreciable extent in the plant upon fungal elicitation (Dewick, 1988).
Medicago sativa (alfalfa), like P. sativum, also accumulates isoflavone phytoalexin in response to disease (He, Dixon, 2000). In M. sativa the 4’ O-methylated isoflavones (Dixon, 1999) are particularly important disease resistance factors. Early efforts to identify the 4’IOMT responsible for medicarpin biosynthesis, the major isoflavonoid phytoalexin of alfalfa, were only successful in identifying a 7-IOMT that yielded 7-O-methylated isoflavones (isoformononetin, 7-O-methylgenistein) in in vitro enzyme assays. These methylated isoflavones are not major soluble phenolics in either alfalfa or of other legumes (He, Dixon, 1996, He, et al. 1998); however, studies with alfalfa cell cultures elicited with yeast elicitor triggered a 200-fold increase in 7-IOMT activity that was correlated with the accumulation of the 4’-O-methylated isoflavone phytoalexin, medicarpin (Dalkin, et al. 1990, Kessmann, et al. 1990, Edwards, Dixon, 1991). Two further attempts to purify a 4’ IOMT from elicited alfalfa cell cultures only yielded 2 separate 7-IOMT activities (Edwards, Dixon, 1991, He, Dixon, 1996). The first 7-IOMT preferentially accepted daidzen (Km 20 μM), and did not require divalent cations for catalytic function (Edwards, Dixon, 1991) while the second preferentially accepted 6,7,4-trihydroxyisoflavone, but also accepted daidzein, and genistein at lower levels (He, Dixon, 1996). The failure to isolate a 4’-IOMT was attributed to the possibility that 7-IOMT could catalyze a 4’IOMT activity in association with other isoflavonoid pathway enzymes as part of a multienzyme complex’. 

Supporting the hypothesis of a multienzyme complex, work using transformed alfalfa expressing 7-IOMT accumulated higher levels of 4’-O-methylated isoflavones (He, Dixon, 2000). Additionally, co-localization studies (Dixon, 1999, He, et al. 1998) together with the X-ray crystal structure of the 7-IOMT enzyme, which showed that the
active site of the enzyme could accommodate 2,4,7-trihydroxyisoflavanone in orientations favorable for both 4' or 7' O-methylation (Zubieta, et al. 2001), were used to suggest that 7-IOMT was in fact a 4'IOMT in vivo. Recently a 4'-OMT (HI4'OMT) was cloned from both Lotus japonicus (accession number AB091686), and Glycyrrhiza echinata (accession number AB091684) (Akashi, et al. 2003). These enzymes, with subunit Mr of 44 kDa, catalyzed the highly specific 4'-O-methylation of 2,7,4-trihydroxyisoflavanone (Akashi, et al. 2003). So while there is still some controversy that 7-IOMT is in fact a 4'IOMT in vivo, or if it works in co-ordination with a multienzyme complex, it is undeniable that the 4'IOMT isolated by Akashi et al. (2003) is involved in medicarpin biosynthesis.

1.2.8 - Flavonoid methylation: Anthocyanins

Flavonoid, carotenoid, and betalain-derived pigments are important to aesthetic and cultural industries (Brugliera, et al. 1999). Methylated anthocyanins, for example, impart blue/violet colors and are responsible for pigmentation of various economically important flowers, as well as wines. Four independent Petunia hybrida OMTs (Mt1/Mf1, Mt2/Mf2) are responsible for anthocyanin methylation, and are named according to their control line (Jonsson, et al. 1984). The major anthocyanin components of P.hybrida change depending on the cultivar’s Mt and Mf alleles (Jonsson, et al. 1983). If any Mt gene is dominant, only 3'-O-methylated anthocyanins (ie. peonidin, petunidin) will accumulate. Conversely, when any Mf gene is dominant, 3'-5'-dimethoxylated anthocyanin malvidin will accumulate (Jonsson, et al. 1983).

Biochemical characterization of the four P.hybrida OMTs revealed comparable biochemical characteristics. Each of the P.hybrida anthocyanin OMTs belonged to class
II OMTs since they had a pH optima of 7.7, had subunit Mr's between 45-50 kDa and did not require Mg\(^{2+}\) for activity (Jonsson, et al. 1984). Each isoenzyme only differed slightly in their affinity constants (Kms) for the substrates, petunidin and cyanidin (Jonsson, et al. 1984).

Although anthocyanin methylation is important in modulation of flower and fruit pigmentation, most breeding or transformation studies have focused primarily on manipulating the major committing steps to anthocyanin biosynthesis such as CHS or dihydroflavonol 4-reductase (DFR)) rather than on OMTs (Tanaka, et al. 1988). Transgenic plants with decreased CHS and/or DFR diversified the available cultivars of purple *P. hybrida* to lines with patterned white flowers (van der Krol, et al. 1988), and red *Gerbera* to lines with uniform pink flowers (Elomaa, et al. 1993). While this has not been widely adopted for commercial manipulation of flower or fruit pigments, transformation technologies could be used for rapid generation of useful phenotypes. For example, genetic engineering a blue pigmented *Vitis labrusca* grapes to eliminate anthocyanin 5'-O-glucosyltransferase (GT) could generate a more visually pleasing red wine that is preferred by consumers, while at the same time maintaining the cold tolerant character of *V. labrusca*, an important property for growing grapes in the temperate zones like the Niagara region.

1.2.9 - Isoflavonoid methylation: Structural analysis

The X-ray crystal structure of IOMT revealed a highly conserved C-terminal SAM binding domain, consisting of the \(\alpha/\beta\) Rossmann fold, as well as novel N-terminal dimerization for plant methyltransferases (Zubieta, et al. 2001). The crystal structure of
IOMT suggested that dimerization was essential for catalytic activity as parts of both subunits participate in the formation of each active site (Zubieta, et al. 2001). This observation could be extended to other class II OMTs since they behave as dimers as determined by gel filtration chromatography (Sato, et al. 1994). The X-ray crystal structure studies suggested that approximately 30% of the protein participates in the dimerization interface of IOMT, significantly more than other structurally characterized OMTs (ie. chalcone OMT, SAMT) (Zubieta, et al. 2001, Zubieta, et al. 2003). In addition to a significant dimerization interface, several amino acids in the N-terminal dimerization domain appear to contribute to the catalytic pocket of the complementary subunit, since M29, T32 and T33 of IOMT insert into the complementary subunit’s catalytic site, and form the back wall of the substrate binding pocket (Zubieta, et al. 2001).

The binding of SAM/S-adenosylhomocysteine (SAH) appears to be highly conserved among OMTs in a SAM binding domain (Zubieta, et al. 2001) involving a network of hydrogen bonds interacting with the ribose hydroxyls and carboxylic acid group of SAM, as well as Van der Waal forces bracketing the adenine ring to bind and orient SAM within the active site (Zubieta, et al. 2001, Zubieta, et al. 2003). The binding of substrate by IOMT involves highly conserved thioethers of M168 and M311 that bracket aromatic phenol and orient the target hydroxyl towards a catalytic histidine residue (Zubieta, et al. 2001). This interesting structural feature appears to be conserved among phenolic OMTs (Zubieta, et al. 2001, Zubieta, et al. 2003) and 16OMT (Levac, et al. 2008). While the crystal structure showed that IOMT could accommodate the isoflavone substrate in either orientation to direct 7’ or 4’ O-methylation, enzyme assays
showed that IOMT had a 9-fold greater preference for O-methylating position 7 than position 4' of the substrate.

1.2.10 - Isoflavonoid methylation: Catalytic mechanism

O-methylation catalyzed by class I and class II OMT enzymes proceeds by an S\textsubscript{n2} reaction mechanism utilizing base-assisted deprotonation of the target hydroxyl by the enzyme's catalytic His residue, followed by transmethylation of the reactive oxyanion intermediate by SAM (Zubieta, et al. 2001). His257 is the IOMT catalytic residue, and its basic properties are potentiated by Glu318 which hydrogen bonds to His257 \( \delta \) nitrogen (Zubieta, et al. 2001). His257 deprotonates the target hydroxyl generating a reactive oxyanion intermediate which is quickly quenched by the transfer of SAMs positive methyl group along its thermodynamically favored path. This yields the trans-methylated isoflavone (Zubieta, et al. 2001).

1.3.1 - Methylation of plant hormone and hormone like molecules

Plants, like all other multicellular organisms, require signal molecules that regulate development, growth, maturation, reproduction, and defense (Taiz, Zeiger, 2002). In animals, some hormones are derived from amino acids while others are steroid derivatives. Plants on the other hand have an entirely different array of signaling molecules (auxins, gibberellins, cytokinins, ethylene, abscisic acid, jasmonic acid, salicylic acid) (Taiz, Zeiger, 2002).

Methylated hormones and hormone like molecules are responsible for floral scents and flavors of plants. Methylsalicylate, methylbenzoate, methyljasmonate, and
methylcinnimate are important floral scents attracting pollinators and are aesthetically pleasing (Murffit, 2000). Methylesters are of major interest to agricultural, floral, cultural and food industries.

1.3.2 - SABATH Methyltransferases

Since the cloning of the first three members of the SABATH family, indole acetic acid (IAA), jasmonic acid (JA), and two gibberellin MTs have been cloned and characterized (Qin, et al. 2005, Seo, et al. 2001, Varbanova, et al. 2007). SABATH MTs methylate a variety of plant signaling molecules, some of which are important for floral scent and plant-plant, plant-insect communication (Murffit, et al. 2000, Seo, et al. 2001, Zubieta, et al. 2003, Qin, et al. 2005, Varbanova, et al. 2007). SABATH (class III) MTs differ dramatically from class I and class II MTs in that they do not possess a catalytic residue, their two active sites apparently have no contributed residues from the complementary subunit (Zubieta, et al. 2003), and they could represent an evolutionary intermediate from ancient MTs that functioned as monomers to what we observe as functional homodimers in plants today.

1.3.3 - Salicylic Acid Methyltransferase (SAMT)

Salicylic acid (SA) is a signal molecule involved in systemic acquired resistance (SAR) of plants (Ryals, et al 1996). The methylester of SA is also important in systemic acquired resistance (Methylsalicylate; MeSA) and quickly accumulates in damaged tissues along with SA (Seskar, et al. 1998, Shulaev, et al. 1997). MeSA is also participates to attract pollinators in moth-pollinated plants (Knudsen, Tollsten, 1993).
MeSA is also artificially added to candies, foods, and medicines for its desirable flavor and scent attributes (Cauthen, Hester, 1989, Das Gupta, 1974, Howrie, et al. 1985).

MeSA is far more lipophilic than its carboxylic acid precursor and under atmospheric conditions, it is easily volatilized making MeSA a suitable intra- and inter-plant signal molecule (Shulaev, 1997). In this context, the involvement of the SAMT enzyme in floral scent development was partially purified along with benzoic acid MT from Clarkia breweri (Dudereva, 1998). The cDNA clone of SAMT encodes a 359 amino acid 40 kDa protein, and the bioactive form elutes as an 80 kDa protein in gel filtration chromatography (Ross, et al. 1999). This suggests that the enzyme exists as a homodimer, an observation that was later confirmed when the SAMT structure was elucidated by X-ray crystallography (Zubieta, et al. 2003).

The substrate preference for SAMT is highly specific for salicylic acid; although, it is also able to methylate benzoic acid at a slightly lower efficiency (~69%). Michaelis–Menten kinetic analysis of SAMT revealed affinity constants (Km) for salicylic acid (24 μM) and SAM (9 μM), typical of class III MTs (Zubieta, Ross, et al. 1999).

Interestingly, His-tagged recombinant protein showed higher affinity for salicylic acid (Km 1.7 μM), and 4 times higher turnover rates than the untagged recombinant protein, suggesting the His-tag alters the protein structure thereby affecting SA binding and trans-methylation (Ross, et al. 1999).

Crystal structures of SAMT in complex with SA and SAH were reported. These revealed a highly conserved SAM binding domain, consistent with other SAM-dependant MTs (Zubieta, et al. 2003). SAMT co-substrate (SAH) binding involves hydrogen bonding with SAH ribose hydroxyls from Asp-98, as well as SAH carboxylic acid from
Asn-65, and the SAH amino tail through a water bridge from Asp-57. Additionally Van der Waals forces bracket the adenine ring of SAH (Zubieta, et al. 2003). The binding of SA, like other class II MTs, involves highly conserved methionines responsible for orienting the SA appropriately for methylation (Zubieta, et al. 2003). The thioether residues (Met130, Met-308) interact through pi-stacking to properly hold the benzene ring of SA in place (Zubieta, et al. 2003). The carboxylate moiety of SA is bound by intramolecular hydrogen bonds, as well as hydrogen bonds to Trp-151 and Gln-25 (Zubieta, et al. 2003). These bonds constrain the carboxylate moiety of SA and orient it towards the reactive methyl group of SAM facilitating trans-methylation. The remainder of the binding pocket provides the hydrophobic environment to promote deprotonation of the SA carboxylic acid (Zubieta, et al. 2003).

Structurally characterized SAM OMTs (IOMT, ChOMT, COMT) use $S_n2$ reaction mechanisms facilitated by lysine or histidine catalytic residues to methylate target substrates (Zubieta, et al. 2001, Zubieta, et al. 2002). Phylogenetic alignments and structural elucidation of SAMT suggest that it, along with other Class II MTs, does not possess lysine or histidine catalytic residues (Zubieta, et al. 2003). SAMT appears to use the SA carboxylate anion form to facilitate transmethylation. Positioning of the negatively charged anion within the proximity of the positively charged methyl of SAM is sufficient to transmethylate SA (Zubieta, et al. 2003, Takusagawa, et al. 1998). Class III MTs therefore utilize an $S_n1$ mechanism to transmethylate their substrates.
1.3.4 - Benzoic Acid Methyltransferase (BAMT)

Methyl benzoate (MeBA), like MeSA, methyl jasmonate (MeJA) and methyl cinnamate are important components of floral scent and of the aesthetic industry (Murffit, et al. 2000). In addition to floral scent, MeBA has been implicated in plant resistance to pathogen attack (Shulaev, et al. 1997).

*BAMT* cloned from *Antirrhinum majus* (Snapdragon), encodes a 49 kDa monomer that behaves as an active 100 kDa dimeric protein as determined by gel filtration (Murffit, et al. 2000). Further kinetic analysis revealed that BAMT enzyme has low affinity constants for its BA (Km =1.6 μM) and SAM (Km=78 μM) (Murffit, et al. 2000) similar to those found for SAMT. Additionally it should be noted that enzymatic methylation of BA by BAMT was potentiated two fold by monovalent K⁺ and NH₄⁺ cations (Murffit, et al. 2000). This is of interest as all previously reported SABATH methyltransferases did not require either monovalent or divalent cations.

1.3.5 - Jasmonic Acid Methyltransferase (JAMT)

Methyl Jasmonate (MeJA) is the methyl ester of jasmonic acid (JA) and was first isolated from *Jasminum grandiflorum* (Demole, et al. 1962). Like SA, JA has been shown to be involved in systemic acquired resistance (Hamberg, Gardner, 1992). MeJA levels quickly increase upon wounding (Parc, Tumlinson, 1999) within the plant and also in unwounded nearby plants (Arimura, et al. 2001).

The cDNA clone corresponding to *JAMT* was cloned from *Arabidopsis thaliana* and corresponded to a 389 amino acid, 49 kDa protein (Seo, et al. 2001). Biochemical analysis of Arabidopsis JAMT demonstrated it required monovalent cations (K⁺), and
that this enzyme had a significantly lower optimum temperature (20°C) compared to other class III MTs. Michaelis–Menten kinetic analysis of JAMT determined affinity constants for BA (Km=38.5 μM) and SAM (Km=6.2 μM) that were slightly higher than other class III MTs (Seo, et al. 2001). JAMT transcript was not detectable in young seedlings but rather the gene is expressed systemically in mature plants and is quickly upregulated upon flower maturation or wounding (Seo, et al. 2001). These observations further support JA and MeJA as regulators of flower development and plant disease.

1.4.1 - Methylation of plant alkaloids

Over 12 000 nitrogen-containing small molecules have been identified and characterized both structurally and functionally from plants (Facchini, 2001). In plant biology and biochemistry, alkaloids are defined as small molecular weight molecules (<900 Da) that contain nitrogen. Plant alkaloids have a wide range of physiological effects and serve a variety of biological functions in humans and other organisms. For example, plant alkaloids can act as central nervous system stimulants (eg. cocaine, nicotine, and caffeine), analgesics (eg. morphine) and potent hallucinogens (eg. lysergic acid). Other alkaloids act as antimicrobials (eg. berberine), antineoplastic agents (eg. vincristine, vinblastine) or as cardiovascular hypotensives (eg. reserpine) or cardio stimulants (eg. digoxin). Plant alkaloids are generally derivatized from amino acids, and their in planta functions, in terms of antimicrobials or antibiotics, mostly reflect their medicinal applications (Facchini, 2001).
1.4.2 - *O*-methylation of benzoisoquinoline alkaloids

Benzoisoquinoline alkaloids (BIA) are important contributors to plant defense (eg. berberine), and serve extremely important medical applications (eg. morphine, codeine). The biosynthetic pathways of berberine and morphine share the common intermediate, (S)-reticuline which is derived, like all other BIAs, from the condensation of L-dopamine, and 4-hydroxyphenylacetaldehyde into (S)-norcoclaurine (Facchini, 2001). The biosynthesis of (S)-reticuline from this precursor involves four biosynthetic steps and two of these involve OMTs (Facchini, 2001).

The first biosynthetic step in the conversion of (S)-norcoclaurine to (S)-reticuline is the 6'-*O*-methylation of norcoclaurine (Sato, et al. 1994). Purified 6-OMT enzyme from *Coptis japonica* cell cultures had a native Mr of 95 kDa as determined by gel filtration chromatography and an apparent subunit Mr of 40 kDa, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Sato, et al. 1994). The 6-OMT was highly specific for norcoclaurine, but would accept both R- and S-enantiomers, as well as both R and S enantiomers of norlaudanosoline, a further oxidized form of norcoclaurine (Sato, et al. 1994). Berberine inhibited the activity of 6-OMT, but concentrations (10 mM) far above those present *in vivo* were required. The 6-OMT affinity constants for SAM (Km=3.95 mM) and (S)-norlaudanosoline (Km=2.23 mM) (Sato, et al. 1994) were significantly higher than those of other OMTs that have been described.

The fourth step in the conversion of (S)-norcoclaurine to (S)-reticuline is the 4'-*O*-methylation of (S)-3'-hydroxy-N-methylcoclaurine. The 4' OMT responsible for this reaction was purified from *Berberis koetineuna* cell culture, and copurified with 6' OMT
Similar results were also reported by other researchers (Sato, et al. 1994). In spite of this, the biochemical characterization of 4’OMTs was possible since substrate specificities of these enzymes did not overlap (Morishige, et al. 2000). The 4’ OMT purified from Berberis accepted (S)-3’-hydroxy-N-methylcoclaurine, (R,S)-laudanosoline and (R,S)-7-O-methylnorlaudanosoline equally well; whereas, (S)-3’-hydroxy-coclaurine was a poor substrate (10% as active relative to (S)-3’-hydroxy-N-methylcoclaurine) (Frenzel, Zenk, 1990). The 4’OMT showed high affinity for (S)-3’-hydroxy-N-methylcoclaurine (Km=4.5 μM), and for SAM (Km=30 μM) (Morishige, et al. 2000).

The 6- and 4’-OMTs were cloned from a Coptus japonica cell culture cDNA library. Each recombinant enzyme was highly specific for their respective substrates and neither required divalent cations for enzyme activity (Morishige, et al. 2000). These properties, together with their subunit Mr (~40kDa), classify these as Type II MTs.

The biosynthesis of reticuline from norcoclaurine requires two O-methylations, one N-methylation, and one hydroxylation, but almost any order could occur theoretically. The substrate specificities of the 4’OMT and 6-OMT suggested that the biosynthesis of reticuline from corcoclaurine occurs in a linear order beginning with 6-O-methylation, and finishing with 4-O-methylation (Facchini, 2001).

It is possible to hypothesize enzyme order in biosynthetic pathways making use of enzyme substrate specificities. For reticuline biosynthesis, r6-OMT was reported to preferentially accept (R,S)-norcoclaurine, but also (R,S)-laudanosoline and (R,S)-norlaudanosoline at significantly lower levels, suggesting that the 6-OMT precedes both N-methylation and hydroxylations (Figure 1.2) (Morishige, et al. 2000). Similarly, the
4’OMT preferentially accepts (R,S)-laudanosoline (N-methylated derivative) over (R,S)-norlaudanosoline and (R,S)-6-O-methylnorlaudanosoline (non N-methylated) (Morishige, et al. 2000). Although these particular compounds are not be part of the BIA pathway per se, they do report substrate preference and suggest pathway order in the sense that the N-methylation and hydroxylation precede 4’O-methylation. On this basis, order of the BIA pathway was hypothesized. The first step in reticuline biosynthesis from coclaurine was 6-O-methylation while the 4-O-methylation occurs last (Morishige, et al. 2000).

1.4.3 - O-methylation of benzoisoquinoline alkaloids: Berberine biosynthesis

OMTs are involved in BIA biosynthesis from Thalictrum tuberosum cell cultures under MeJa induction (Frick, Kutchan, 1999). Work conducted with T.tuberosum focused on isolating OMTs involved in berberine biosynthesis. Berberine biosynthesis was shown to be strictly regulated by four methyltransferases (3 OMTs, 1 N-methyltransferase) (Rueffer, et al. 1983, Sato, et al. 1994, Frenzel, Zenk, 1990, Galneder, Zenk, 1990). Four unique OMTs were cloned from T.buberosum. Only two showed biochemical activity in vitro (OMTII.1, OMTII.2) (Frick, Kutchan, 1999). These encoded OMTs differ by only a single amino acid residue, but show highly promiscuous, and different substrate preference profiles (Frick, Kutchan, 1999), reinforcing the idea that functional annotation based on sequence identity is insufficient.

OMT heterodimers formed through heterologous expressions of OMTs demonstrate variations in methylated substrate profiles compared to non-heterologous expressions (Frick, Kutchan, 1999), leading support to the possibility that in planta when
Figure 1.2 Biosynthetic pathway of (S)Reticuline from (S)-Norcoclaurine. Modified from Facchini, 2001.
multiple methyltransferases are expressed within a single cell type heterodimers form, enabling new substrate specificities, and opening entirely new biosynthetic pathways (Frick, Kutchan, 1999). The X-ray crystal structures of IOMT and ChOMT revealed the participation of the N-terminal domain of each OMT monomer in the catalytic site backwall of the complementary monomer (Zubieta, et al. 2001). The structural analysis of IOMT illustrated that substrate discrimination is determined at the deep backwall of the catalytic pocket, which has a significant contribution from the complementary subunit in class II MTs (Zubieta, et al. 2001). Therefore if a heterodimer were formed, the backwall residues would be changed and substrate preference could also be altered.
Chapter 2.

Application of carborundum abrasion for investigating leaf epidermis: Molecular cloning of *Catharanthus roseus* 16-hydroxytabersonine-16-O-methyltransferase.

This manuscript was published in *The Plant Journal* (2008) 53: 225-236

**Contribution:** With the exception of conducting Real Time PCR experiments, their Microsoft Excel analysis, and some preliminary carborundum abrasion ground work, all work detailed in this chapter was performed by Dylan Levac.
Application of carborundum abrasion for investigating leaf epidermis: Molecular cloning of *Catharanthus roseus* 16-hydroxytabersonine-16-O-methyltransferase.

Dylan Levac, Jun Murata, Won S. Kim and Vincenzo De Luca

Department of Biological Sciences, Brock University, 500 Glenridge Ave., St. Catharines, ON, L2S 3A1, Canada

2.1 – Abstract

The Madagascar periwinkle (*Catharanthus roseus*) produces the well known and remarkably complex anticancer dimeric alkaloids vinblastine and vincristine that are derived from the coupling of vindoline and catharanthine monomers. This study describes the novel application of carborundum abrasion (CA) technique for large scale isolation of leaf epidermis-enriched proteins to purify 16-hydroxytabersonine-16-O-methyltransferase (16OMT) that catalyses the 2nd of 6 steps in the conversion of tabersonine into vindoline to apparent homogeneity and to clone the gene. Functional expression and biochemical characterization of recombinant 16OMT showed its very narrow substrate specificity and high affinity for 16-hydroxytabersonine. In addition to allowing the cloning of this gene, CA technique clearly showed that 16OMT is predominantly expressed in *Catharanthus* leaf epidermis. The results provide compelling evidence that most of the pathway for vindoline biosynthesis, including the O-methylation of 16-hydroxytabersonine, occurs exclusively in leaf epidermis, with subsequent steps occurring in other leaf cell types.
2.2 – Introduction

_Catharanthus roseus_ (Madagascar periwinkle) makes a variety of biologically active monoterpenic indole alkaloids (MIAs) that include the antihypertensive corynanthe alkaloid, ajmalicine (Tikhomiroff and Jolicoeur, 2002), and that can accumulate to 0.57 mg g⁻¹ dry weight in _Catharanthus_ hairy root cultures (Vázquez-Flota, et al., 1994). In contrast the dimeric bisindole alkaloids, vinblastine (VBL) and vincristine (VCR), both used to treat Hodgkin’s disease and childhood lymphomas, accumulate up to 46.6 μg g⁻¹ dry weight and trace amounts, respectively, in above ground parts of _Catharanthus roseus_ (Choi, et al., 2002). The economic and medical importance of VBL and VCR, as well as their chemical complexity, has prompted extensive research efforts to facilitate their production from the plant since their discovery over 45 years ago. In fact, the recent development of the new dimeric MIA drug known as Vinflunine (Javlor) (Fahy, et al., 1997) produced by Pierre Fabre Medicament still requires the plant to supply the MIA precursors, vindoline and catharanthine for its production. Vinflunine is in Phase III clinical trials due to its increased effectiveness, lower toxicity usefulness for the treatment of a broader range of human cancers.

VBL and VCR are produced as a result of a condensation of the Iboga alkaloid catharanthine, and the aspidosperma alkaloid vindoline. While catharanthine accumulating cell suspension cultures have been successfully produced (Moreno, et al., 1995), cultures that make vindoline have never been reported. The complex regulation of vindoline biosynthesis, including the requirement for light and the involvement of several cell types in its production (St. Pierre, et al. 1999), could not be reproduced in cell culture
systems. More recently it has been suggested that the many of the genes responsible for MIA biosynthesis up to 16-methoxytabersonine may in fact be expressed and localized to the leaf epidermis of *Catharanthus* (Figure 2.1, Murata and De Luca, 2005), whereas the last 4 steps required to make vindoline may occur in specialized idioblast and laticifer cell types within the leaf (St. Pierre et al., 1999).

It is well known that most plant MIAs are derived from tryptamine and secologanin to produce the central intermediate, strictosidine, from which is derived tabersonine through a number of uncharacterized enzymatic steps (Figure 2.1). The enzyme tabersonine 16-hydroxylase converts tabersonine into 16-hydroxytabersonine and the second of six steps involved in the conversion of tabersonine into vindoline is catalyzed by 16OMT (Figure 2.1). Significant efforts to use homology based methods (Cacacea et al., 2003, Schröder et al., 2004) have failed to clone the 16OMT, but several full length flavonoid-O-methyltransferases (FOMTs) were successfully obtained, including a flavonoid/anthocyanin-OMT that can perform sequential 3', 5' O-methylation of flavonoids and anthocyanins that accumulate in *Catharanthus roseus*. While additional efforts to clone this gene involved the successful purification to homogeneity of 16OMT from *Catharanthus roseus* cell cultures, the approach only succeeded in cloning yet another flavonoid 4'-OMT that was functionally characterized (Schröder et al., 2004). Both of these 16OMT cloning efforts used plant cell cultures that were activated for MIA biosynthesis as a source of enzymes. Although such induced cell cultures may be a cost effective and convenient source of enzymes, these results suggest that they are not always suitable for selective purification of enzymes normally expressed in plant leaves.
Tryptophan $\xrightarrow{TDC}$ Tryptamine + Strictosidine

Secologanin

Multiple steps

16-Hydroxytabersonine

16-Methoxytabersonine

16-OMT

$^{14}$CH$_3$-AdoMet

AdoCys

Deacetylvindoline

Vindoline

Figure 2.1. The biosynthesis of Vindoline from tryptamine and secologanin. Tryptamine is derived from tryptophan via the action of Tryptophan decarboxylase (TDC) whereas strictosidine synthase (STR1) converts tryptamine and secologanin into the central intermediate strictosidine. Strictosidine is converted into 16 methoxytabersonine through a series of uncharacterized reactions together with tabersonine 16-hydroxylase (T16H) and 16-hydroxytabersonine-16-O-methyltransferase (16-OMT). The enzyme assay for T16H uses [14-CH$_3$]-S-adenosyl-L-methionine ([14-CH$_3$]-AdoMet) and radiolabelled 16- methoxytabersonine can be detected. The biosynthesis of Vindoline involves 3 more enzymatic steps to form deacetylvindoline that is converted to vindoline by the action of deacetylvindoline-4-O-acetyltransferase.
The present study describes the convenient use of carborundum abrasion (CA) technique (Murata and De Luca, 2005) as a tool for large scale isolation of leaf epidermis enriched proteins and for purification of 16OMT to apparent homogeneity. This versatile tool was also used to harvest leaf epidermis enriched mRNAs that facilitated the molecular cloning of the 16OMT. Additionally, the functional expression and biochemical characterization of the recombinant 16OMT is reported, as well as its preferred localization and expression in the leaf epidermis compared to other OMTs that are expressed in other Catharanthus tissues.

2.3 - Materials and Methods

2.3.1 - Plant Material

Madagascar Periwinkle (Catharanthus roseus (L.) G. Don, Little Delicata) was used throughout the study. Seeds were germinated in moist artificial soil (Sun Grow Horticulture Canada Ltd.) and grown under controlled greenhouse conditions (25°C, 57% humidity and 16 hour photoperiod). The plants were watered once a week, and once every three months they were provided with all purpose fertilizer (Miracle Grow, 12% nitrogen, 4% phosphate, 8% potassium) (Scotts Canada Ltd.).

2.3.2 - A large scale CA extraction method to harvest leaf epidermis protein: purification of 16OMT and protein sequencing.

Young leaves (128 g, 1.5 cm long) harvested near the apical meristem of shoots were abraded with carborundum number F (Fisher Scientific) using a vortex to selectively extract the leaf epidermis (refer to appendix, Fig. S1) using a protocol
modified from Murata et al., 2005. The length and intensities of vortex treatment were optimized to ensure underlying cell layers were largely left undisturbed by monitoring for absence of idioblast and laticifer localized DAT enzyme activity. The optimized protocol used a 1:1 w/w fresh weight of young leaves to carborundum together with a 1:12 w/v fresh weight of young leaves to protein extraction buffer (100 mM Tris–HCl pH 7.5, 13 mM β-mercaptoethanol) at 4°C to perform CA. Leaf epidermal proteins were extracted at maximum vortex intensity over 10 minutes. To keep extraction buffer temperature cool, the extract was placed on ice for 2 min after the first 5 minutes of CA.

During CA, the extract was vigorously shaken approximately every 30 seconds to suspend any sedimented carborundum. To ensure that all leaf material was abraded as evenly as possible the angle at which the vessel rested on the vortex during abrasion was approximately 45 degrees. The same extraction buffer was used up to 4 times by removing abraded leaf material and carborundum by filtration through a nylon mesh and by adding fresh leaf material and fresh carborundum (1:1 w/w ratio), as well as fresh extraction buffer to compensation for the loss in volume.

The combined extract was filtered sequentially through a 20 mm nylon mesh, then by suction through 0.45 µm Filtropur® vacuum filtration unit (Sarstedt) and the filtrate was centrifuged at 500 g for 5 min at 4°C to remove remaining carborundum particles. Depending on the volume, extracts were concentrated to 2.5 ml using Centricon Plus-70®, 10 kDa and Amicon Ultra®, 10 kDa centrifugation concentrators (Fisher Scientific) and desalted by PD-10 column chromatography (GE Healthcare, Piscataway, NJ).

The concentrated protein extract (5 mL) that was submitted to Sephadex G150 (Sigma-Aldrich) column chromatography [125 mL bed volume, equilibrated in Buffer A
(100 mM Tris–HCl pH 7.5, 13 mM β-mercaptoethanol), flow rate of 0.3 mL/min. Eluted fractions were collected in 4 ml volumes, fractions were assayed for 16OMT and FOMT to harvest peak 16OMT fractions that were concentrated by Amicon Ultra® 10 kDa centrifugation concentrator (Fisher Scientific).

The concentrated sample (2 mL) was incubated with 1 ml activated adenosineagarose affinity resin [prepared from 5’-AMP-agarose (Sigma-Aldrich) by dephosphorylation with bovine alkaline phosphatase (James et al., 1995)] overnight at 4°C with constant gentle mixing. The mixture was centrifuged at 10,000 g for 10 seconds in a benchtop microcentrifuge. The resin washed 3 times with 2.5 mL Buffer A and bound proteins were eluted 3 times with 2.5 ml Buffer A containing 500 μM SAM (Sigma-Aldrich). After desalting, active fractions were pooled and applied to a Mono QTM Anion exchange column (2 mL bed volume (GE Healthcare, Piscataway, NJ) equilibrated in Buffer A at 4°C and after washing the column, 16OMT activity was eluted in 0.5 ml fractions using a 20 ml gradient 0 to 50% gradient of 2 M NaCl in Buffer A. Active fractions were concentrated, subjected to SDS-PAGE and stained with colloidal Coomassie blue (Invitrogen Co., Burlington, ON, Canada). A 40 kDa protein band (Fig. S2) which positively correlated with 16OMT activity was excised from the gel and forwarded for sequence analysis, performed by Harvard Microchemistry Facility (Harvard University) by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Finnigan LCQ DECA XP Plus quadrupole ion trap mass spectrometer. Seven peptide sequences were obtained from this protein: (Peptide 1: WILHDWNDEDCVK; Peptide 2: GIVLTMDDPAELK; Peptide 3: MVLHDWNDEDCVK; Peptide 4: NEDGTAFETAHGK; Peptide 5: IPPAHVVFLK;
Peptide 6: EAGFSSYK, Peptide 7: CTVFDLPHVANLESK).

2.3.3 - Small scale CA extraction of leaf epidermis enriched protein compared to whole leaf extraction for estimating the 16OMT activity in the epidermis.

Young leaves (2 grams fresh weight, 1.5 cm long) were harvested and processed as described for the large scale method with the following exceptions; Fresh leaves, carborundum and 24 ml of extraction buffer were placed in a 50 ml PP tube (Sarstedt) and shaken vigorously by hand for 1 minute. The extraction buffer was then filtered through P5 filter paper (Fisher Scientific) to remove residual carborundum. The filtrate was further filtered using a Luer-Lok™ syringe and 25mm syringe filter (pore size 0.22 μm) (Fisher Scientific). The second filtrate was concentrated to 2.5 ml using Amicon Ultra centrifugal filter units (10 kDa molecular cut off) (Sigma Aldrich). Concentrated epidermis enriched protein extract was desalted using PD-10 desalting columns (GE Healthcare) and then used for enzyme assay.

2.3.4 - Extraction of whole leaves for comparative enzyme assays or for comparative purification to CA-based extraction.

Whole young leaves (2.0 to 6 grams fresh weight) were homogenized in 3 to 9 ml protein extraction buffer using a mortar and pestle. The sample was centrifuged at 500 g for 5 min at 4°C to pellet cell debris and the supernatant was processed as described for leaf epidermal extracts for direct assay of crude extracts or for protein purification. The protein concentration of extracts was determined using a Protein assay kit (Bio-Rad, Hercules, CA, USA).
2.3.5 - CA extraction to harvest leaf epidermis enriched mRNA.

Young *C. roseus* leaves (2.5 grams FW) were harvested and combined with 8 ml Trizol® reagent, and 2.5 g carborundum in a conical 50 ml tube (Starstedt AG & Co., Montreal, Québec, Canada) (Figure S1). The tube containing leaf material, carborundum and Trizol was vortexed at maximum intensity for 1 min.; ensuring most of the leaves in the tube were well abraded.

The tube was then placed at room temperature for 5 minutes to allow extraction buffer to settle to the bottom. Abraded leaves were removed with forceps, and the process was repeated with another 2.5 g of fresh leaf tissue. The extract was centrifuged at 2600 g for 5 min, the supernatant (5 ml) was harvested and mixed by vortex sequentially with 1 ml 5 M NaCl, followed with 3 ml chloroform. The mixture was centrifuged at 2600 g for 30 minutes and aliquots of the aqueous phase were transferred to sterile 1.5 ml tubes together with 0.9 volumes isopropanol with vortex mixing and the mixture was incubated at -20°C for 1 hour or overnight in the presence of linear acrylamide to maximize the amount of mRNA being isolated. Samples were centrifuged at 21,000 g for 30 minutes, the supernatant was removed, and the precipitated pellet was washed with 70% ethanol, followed by centrifugation at 21,000 g for 5 minutes. The pellet, containing mRNA was resuspended in 20-50 μl RNase-free water. RNA quality and quantity was measured by spectrophotometry and ca. 50 μg of total RNA was be obtained from 5 g FW of leaves extracted by CA.
2.3.6 - Construction and random sequencing of leaf epidermis-specific cDNA library.

*Catranthus* leaf epidermis-specific cDNA library was constructed using a SMART cDNA library construction kit (Clontech, Mountain View, CA) according to the manufacturer’s instruction. The whole cDNA was amplified by PCR prior to the packaging to Gigapack III gold packaging extract (Stratagene, La Jolla, CA). The primary library (1.0 x 10^6 pfu) was directly converted to plasmids by *in vivo* excision, and the obtained colonies of *E. coli* were randomly picked for single path sequencing using primers from 5’ end of the inserts. The sequencing reactions were performed using Templiphi DNA sequencing template preparation kit (GE Healthcare, Piscataway, NJ), and the resulting DNA templates were sequenced using ABI Prism Big Dye terminator sequencing kits (Applied Biosystems, Foster City, CA) and an ABI 3730 genetic analyzer (Applied Biosystems).

2.3.7 - Identification of potential 16OMT Expressed Sequences.

The sequence files with ABI format were analyzed using the BLASTX algorithm (Altschul *et al.*, 1997). Multiple clones with the overlapping areas of identical sequences were clustered and classified as ‘Clustered’, while sequences that appeared only once in the EST were classified as ‘Singletons’. The threshold of the sequence similarity was set as the *E-values* at 10^-6 and lower, and the sequences that did not show significant homology were named ‘No hits’. The sequences were archived in FIESTA software package (http://bioinfo.pbi.nrc.ca/napgen.beta//login.html) at Plant Biotechnology Institute of the National Research Council of Canada (NRC/PBI). The functional
categorization was first done automatically by the putative annotations, followed by manual inspection to verify the annotation.

2.3.8 - Molecular cloning of 16OMT.

Gene-specific forward primers were designed to the mRNA domains of CrOMT7 (AY343492) corresponding to peptide 4. The associated forward primer corresponding to peptide 4 was 5’ CTGCTTTTGAAACAGCTCATGG 3’ (F1) and the reverse primer designed to the mRNA domain of CrOMT6 (AY343490) corresponding to the N-terminus of peptide 1 was 5’ CAGTCATGGAGAATCCACTT 3’ (R1). PCR with F1, R1 and 2 μl of epidermal enriched cDNA in phage λ was run for 25 cycles. The amplified reaction was diluted 1000-fold and 2 μl used as template for a second PCR amplification with F1 and R1 to yield a novel 361 bp putative OMT sequence. The PCR product was sequenced (Robarts Research Institute, London, ON, Canada) and specific primers corresponding to the novel putative OMT were then designed to amplify the 5’ and 3’ ends by RACE using phage λ primers flanking the cDNA insert. This sequence was used to produce a contig with Vector NTI V 6.0.0.0 (InforMax Inc., Calgary, AB). The full-length gene (1065 bp, encoding the full length ORF) was amplified using forward (5’CACCATGGATGTTCAATCTGAGGA 3’) and reverse (5’TCAAGGATAAACCTCAATGAGACTCC 3’) primers that were compatible to the Gateway® pENTR directional vectors (Invitrogen Co.) and directionally ligated into pENTR and subsequently mobilized to pDEST-17 of Gateway’s ® expression system (Invitrogen Co.), which harbors six histidine residues at the N-terminus. This was done according manufacturer’s specifications. pDEST-17 vectors containing full-length
amplified product-His fusion was transformed into *E. coli* (DE3)pLysS cells.

**2.3.9 - Expression, refolding and purification of r16OMT.**

A 3 mL culture in 2 x YT media containing 50 μg/ml ampicillin was inoculated, grown overnight at 37°C to produce a saturated culture. 500 μl saturated culture was then used to inoculate 50 ml 2 x YT media containing 50 μg/ml ampicillin. Cultures were allowed to grow at 37°C to OD600 = 0.8, induced with 2 mM final IPTG concentration and grown at 16°C for 24hr. The culture was then centrifuged at 5000 rpm for 10 min to harvest cells, supernatant was removed and cell pellets were stored at -20°C until they were processed for extraction.

Cell pellets were resuspended in 5 ml 100 mM Tris-HCl, pH 7.5, 13 mM β-mercaptoethanol) and lysed by sonication. After centrifugation at 21000 g (4°C) for 20 min, the pellet with inclusion bodies was washed 3x in 10 mL Wash Buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1 M urea per gram pellet weight, 0.1 % Triton X-100, after each wash inclusion bodies were re-pelleted by centrifugation at 21000 g for 10 min at 4°C. This was followed by a single wash step with Wash Buffer to remove residual Triton X-100. Purified inclusion bodies were then solubilized in solubilization buffer [100 mM Tris-HCl, pH 7.5, 8 M urea, 1 mM phenylmethylsulfonyl fluoride to a final ≤10 mg/ml protein concentration (Kopito, 2000) . Protein inclusion bodies were solubilized in a beaker with gentle stirring for one hour at room temperature and solubilized proteins were dialyzed (100 mM Tris, pH 7.5) overnight at 4°C using a 30 ml Slide-A-Lyzer Dialysis Cassette (10 MWCO, Fisher Scientific) according to the manufacturer’s instructions. Refolded proteins were used directly for enzyme assays.
2.3.10 - Enzyme kinetic analysis of r16OMT.

*E. coli* expressing recombinant tabersonine-16-hydroxylase: NADPH cytochrome P450 reductase protein (Schröder et al., 1999) was used to biotransform tabersonine into 16 hydroxytabersonine used in r16OMT assays. For Km determinations, triplicate r16OMT enzyme assays were performed with 2, 6, 10, 14 and 18 μM 16-hydroxytabersonine at constant 2.08 μM (0.025 μCi) (Methyl-\(^{14}\)C)SAM and with 1, 2, 5, 10 and 20 μM (Methyl-14C)SAM at constant 0.1 mM 16-hydroxytabersonine. For Ki determinations, triplicate r16OMT enzyme assays contained 1, 2, 5, 10 and 20 μM (Methyl-14C)SAM at constant 30 μM 16-hydroxytabersonine and changing AdoCys concentrations (0x, 0.05x and 0.3x SAM Km). All assays were performed in triplicate at 35°C for 30 min at pH 7.5 (100mM Tris-Cl, 0.1% β-mercaptoethanol).

2.3.11 - Preparation of cDNA from laser capture microdissected cells.

cDNA was prepared from RNA amplified after being isolated from epidermal, mesophyll, idioblast, laticifer, vascular and representative whole leaf cells isolated by laser capture microdissection as previously described (Murata et al., 2005).

2.3.12 - Real time PCR for quantitation of 16OMT in different cell types obtained by laser capture microdissection.

β-Actin and 16OMT sequences were aligned using Clone Manager Professional Suite (version 7.11, Scientific & Educational software, Cary, NC, USA) to determine possible regions for the best primer candidates. Initial primer design was done with the software PRIMER 3 (Whitehead Institute, MIT, Cambridge, MA, USA) and further examined through Integrated DNA Technology (IDT, Coralville, IA, USA) web based
software (http://scitools.idtdna.com/scitools/Applications/OligoAnalyzer/) to avoid hairpin, hetero or self-dimer structures. The following primer pairs (Actin-F-GGAGCTGAGAGATTCCGTTG; Actin-R-GAATTCTGAGCTTCCATC and 16OMT-F-CCTTACCCCATCAAGTCGAA; 16OMT-R-ACAGGGTCAGCCATGGTAAG) were used to generate 73 and 81 bp PCR products, respectively. The real-time quantitative PCR reaction was optimized in a 20 μl of volume containing with 150 nM of each primer, QuantiTect® SYBR Green PCR Master mix (Qiagen, Mississauga, ON, Canada) and 2 μl of cDNA (corresponding to approximately 216 ng cDNA). Real-time PCR conditions were as follow: 95 °C for 15 min, then 45 cycles of 95 °C for 10 s, 57 °C for 15 s and 72 °C for 30 s. All real-time PCR experiments were run in triplicate for each biological replicate of cDNA produced from mRNA isolated from whole leaf and from cells from the leaf epidermis, mesophyll, idioblasts, laticifers and vasculature. The average threshold cycle (Ct) and relative quantities were calculated using SDS software v2.1 (Applied Biosystems). A Ct value from each sample was calculated from the amplification curves by selecting the optimal ΔRn (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. β-Actin was used as an internal standard to calculate the relative fold difference based on the comparative Ct method. To determine relative fold differences for each sample in each experiment, the Ct values for 16OMT genes was normalized to the Ct values for β-Actin and was calculated relative to a calibrator (laticifer cells) using the formula $2^{-\Delta\Delta Ct}$.
2.3.13 - Expression of 16OMT, TDC and DAT enzyme activity in light and dark grown seedlings.

*Catharanthus roseus* seeds were germinated and grown for different times in the absence and presence of light (De Luca *et al*., 1988). Enzyme assays for TDC, DAT (De Luca *et al*., 1988) and 16OMT (Fahn *et al*., 1986; Murata *et al*., 2005) were performed as previously described.

2.3.14 - Protein determinations. The protein concentration of extracts was determined using a Protein assay kit (Bio-Rad, Hercules, CA, USA).

2.4 – Results

2.4.1 - MIA pathway enzyme activity profiling throughout seedling development, in leaves, flowers, stems and roots of *Catharanthus roseus*.

Seedlings were prepared, harvested and assayed for tryptophan decarboxylase (TDC), 16OMT and deacetylvindoline 4-0-acetyltransferase (DAT) activity, as previously reported (De Luca *et al*., 1988). TDC enzyme activity rose in 4 day old dark grown seedlings, it increased until 8 days of growth and decreased thereafter (Figure 2.2A). While light treatment did not affect the TDC profile, DAT activity was increased several fold by light treatment compared to dark grown control seedlings (Figure 2.2A, 2.2B), confirming previous results describing the light induction of DAT activity in etiolated seedlings (De Luca *et al*., 1988; St. Pierre *et al*., 1998). When seedling extracts were assayed for 16OMT activity, it first appeared in 2 day old dark grown seedlings and
Figure 2.2 Distribution of 16OMT enzyme activities in Catharanthus roseus seedlings, organs and cells. A and B) Regulated appearance of TDC, 16OMT and DAT enzyme activities during 10 days of dark (A) and light (B) growth of germinating seedlings. C) Comparison of 16OMT activites in leaves of different ages from the youngest 1st leaf pair to the oldest 3rd leaf pair. D) Comparison of 16OMT and FOMT enzyme activities in different plant organs.
increased to a maximum after 8 days of growth (Figure 2.2A). Unlike the effect of light on DAT induction, 16OMT activity was only increased 30% compared to dark grown controls (Figure 2.2A, 2.2B), displaying a very similar intermediate expression pattern that was also observed with a N-methyltransferase responsible for the 3rd to last step in the vindoline biosynthesis (Dethier and De Luca, 1993; De Luca et al., 1988; De Luca et al., 1987a,b).

The distribution of 16OMT enzyme activities were determined in the 1st, 2nd and 3rd pair of leaves from the leaf apical meristem that represent various stages of leaf development. The specific activity of 16OMT was highest in crude extracts of the youngest leaf pair and this activity diminished with developmental age (Figure 2.2C). The distribution of 16OMT and FOMT enzyme activities were also determined in crude extracts of leaves, flowers, stems and roots (Figure 2.2D). While 16OMT enzyme activities were highest in flowers and leaves, respectively, those of FOMT were highest in flowers and roots. The relative 16OMT and FOMT enzyme activities in different Catharanthus tissues may reflect the different biochemical roles played by these enzymes in each tissue as well as their relative tissue-specific distribution.

2.4.2 - Purification of Catharanthus roseus 16OMT to homogeneity and molecular cloning from a cDNA library prepared from leaf epidermis enriched mRNA.

Young Catharanthus leaves (128 g) were extracted by CA technique (Figure S1) to selectively extract approximately 4 mg of crude leaf epidermis enriched protein compared to the much higher levels of protein (640 mg) that would be obtained from extraction of 128 g of whole leaves (Table 2.1). After concentrating the extract by
<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total protein (mg)</th>
<th>Total activity (pkatal)</th>
<th>Specific Activity (pkat mg(^{-1}))</th>
<th>16OMT /FOMT</th>
<th>16OMT Purification (fold)</th>
<th>16OMT Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CA</strong></td>
<td>3.9</td>
<td>3.96</td>
<td>0.78</td>
<td>1.1</td>
<td>6.8</td>
<td>100</td>
</tr>
<tr>
<td>Concentrated crude extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex G150</td>
<td>1.1</td>
<td>4.4</td>
<td>ND</td>
<td>4.0</td>
<td>N/A</td>
<td>3.6</td>
</tr>
<tr>
<td>Adenosine Agarose</td>
<td>0.03</td>
<td>2.41</td>
<td>ND</td>
<td>80.3</td>
<td>N/A</td>
<td>73.0</td>
</tr>
<tr>
<td>MonoQ Anion Exchange</td>
<td>0.0004</td>
<td>5.56</td>
<td>ND</td>
<td>1.41 E(^4)</td>
<td>N/A</td>
<td>1.29 E(^4)</td>
</tr>
<tr>
<td><strong>Whole Leaf</strong></td>
<td>29.8</td>
<td>0.89</td>
<td>1.19</td>
<td>0.03</td>
<td>0.84</td>
<td>100</td>
</tr>
<tr>
<td>Concentrated crude extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex G150</td>
<td>5.4</td>
<td>1.08</td>
<td>0.16</td>
<td>0.20</td>
<td>6</td>
<td>6.7</td>
</tr>
<tr>
<td>Adenosine Agarose</td>
<td>2.01</td>
<td>3.02</td>
<td>0.12</td>
<td>1.5</td>
<td>27</td>
<td>50</td>
</tr>
<tr>
<td>MonoQ Anion Exchange</td>
<td>0.0012</td>
<td>0.26</td>
<td>ND</td>
<td>216.2</td>
<td>N/A</td>
<td>7.2 E(^3)</td>
</tr>
</tbody>
</table>

Table 2.1. Purification of 16OMT compared to FOMT using Carborundum abrasion technique compared to extraction of enzymes from whole leaves*
*CA epidermis 16OMT was purified from 128g of FW C.roseus leaf while whole leaf 16OMT was purified from 6g FW C.roseus leaf. ND, Not Detected and NA, Not applicable.
ultrafiltration, it was subjected to Sephadex G150 size exclusion chromatography as described in Materials and Methods. Active fractions with 16OMT activity were not active against FOMT substrate and were further purified by adenosine agarose affinity chromatography, and MonoQ anion exchange column chromatography (Table 2.1). In contrast whole leaf extracts contained residual FOMT activity after size exclusion and affinity chromatography, but it completely disappeared in the MonoQ anion exchange chromatography step (Table 2.1). The MonoQ step completed the purification of CA extracted protein to produce a 1.29x104-fold enrichment of 16OMT compared to crude extracts [Figure S2, see SDS PAGE protein profile of whole leaf crude extracts (W) and from Carborundum leaf epidermis enriched extracts (CA)] to yielded a total of 4 μg of a purified 40 kDa protein (Table 2.1, Figure S2) as determined by SDS-PAGE and silver staining (Figure S2, Fractions 21-23). In contrast purification of this enzyme from whole leaves produced a partially purified preparation containing several proteins that did not include the predominant 40 kDa protein purified from CA leaf epidermis enriched extracts. Fractions containing the purified 16OMT were pooled, concentrated and submitted to SDS-PAGE to harvest a 40 kDa protein that was submitted for sequencing.

Seven peptides with 100% sequence identity to different published Catharanthus OMTs were obtained and protein BLAST analysis of the sequenced peptides demonstrated that two peptides (Peptide 1: WILHDWNDEDCVK; Peptide 2: GIVLTMMLDPAELK) had 100% sequence identity to CrOMT6 (AAR02420), four peptides (Peptide 3: MVLHDWNDEDCVK; Peptide 4: NEDGTAFETAHGK; Peptide 5: IPPAHVVFVLK; Peptide 6: EAGFSSYK) had 100% sequence identity to CrOMT7
(AAR02421), and one peptide (Peptide 7: CTVFDLPHVANLESK) had 100% sequence identity to CROMT2 (AAM09497), CrOMT5 (AAR02417) and CrOMT6.

The high amino acid sequence identities between different Catharanthus OMTs made it difficult to design 16OMT gene-specific cloning primers (Figure S3) and this may explain one reason for the failure of previous efforts to clone this gene (Cacacea et al., 2003; Schröder et al., 2004). This issue was solved by designing 3 different sets of primers based on these peptides in CrOMT6 alone (primers F1 and R1), CrOMT7 alone (primers F2 and R2) and a mixed set based on both CrOMT6 and CrOMT7 (Primers F3 and R3) (Figure 2.3). Both the CrOMT6 and CrOMT7 sets of primers only produced PCR products in preparations from whole leaves but not in epidermis enriched preparations (Figure 2.2, inset). In contrast, PCR with a gene-specific forward primer based on CrOMT7 (Figure S2, Peptide 4) and the reverse primer based on CrOMT6 (Figure S2, Peptide 1) produced a unique 361 base pair product (Figure 2.3, inset) from cDNA libraries derived from mRNA isolated from whole leaves and from CA leaf epidermis enriched extracts that corresponded to a putative 16OMT gene. Identical PCR products were obtained from either cDNA library as shown by DNA sequencing and they were 87% identical to a putative uncharacterized CrOMT5 and 83% identical to CrOMT6, that appears to catalyze the 4′-O-methylation of the flavanone, eriotitytol, of the flavonols isorhamnetin, quercetin and kaempferol and of the flavone, chrysoseryl (Schröder et al., 2004). New unique sequence specific primers based on this novel sequence were designed to amplify the remainder of the clone by 5′ and 3′ RACE using a leaf epidermis enriched cDNA library (see Materials and Methods) to produce a 1323 bp
Figure 2.3: Alignment of peptide sequences 1 to 7 in relation to the putative open reading frame of 16OMT and identification of a unique PCR product that is preferentially expressed in leaf epidermal cells compared to whole leaf. The following primer sets CrOMT6 (515-735 bp) (F2: 5' CCATGGCTAATGACTCTG 3'; R2: 5' GTCTCCTCCAACAAACTC 3'); CrOMT7 (348-716 bp) (F1: 5' TCCAATGCTAGATCCACTTC 3', R1: 5' CCACCAACAAACTCCAAGT 3'), and CrOMT7/CrOMT6 (425-786 bp of 16OMT clone) (F3: 5' CTGCTTTTGAACAGCTCATGG 3'; R3: 5' CAGTCATGGAATCCACTT 3') were used to produce the PCR products shown in the inset using cDNA libraries derived from mRNA isolated from CA leaf epidermal cells and from whole leaf.
fragment encoding a 1068 bp ORF for a putative 355 amino acid OMT with a theoretical mass of 39.8 kDa (Figure S3) (Genbank accession # Ef444544). This novel protein with significant sequence identity to CrOMT6 (64%) and CrOMT5 (66%) (Table S1) possessed a highly conserved C-terminal S-adenosyl-L-methionine (SAM) catalytic binding domain characterized by a central $\alpha/\beta$ Rossman fold as well as an N-terminal domain responsible for dimerization and catalytic site back wall formation required for substrate recognition (Zubieta et al., 2001).

2.4.3 - Expression and functional characterization of recombinant 16OMT (r16OMT)

Biotransformation experiments with E. coli transformed with r16OMT containing vector, completely converted 16-hydroxytabersonine that was supplied exogenously into the expected 16-O-methylated derivative, whereas induction with IPTG prevented this conversion from occurring. Control E. coli cultures expressing the empty vector were unable to catalyze the biotransformation since only authentic 16 hydroxytabersonine was recovered.

The ability of E. coli cultures expressing r16OMT to produce the 16-O-methylated product prompted efforts to directly characterize the enzyme. Cell free extracts from whole Catharanthus roseus leaves (Figure 2.4A, Lane 1 & 2.4B, Lane 2) as well as from E. coli transformed with r16OMT containing vector (Figure 2.4A, Lane 2 & 2.4B, Lane 2) catalyzed the enzymatic O-methylation of 16-hydroxytabersonine in the presence of SAM methyl group donor. In contrast IPTG treated soluble (Figure 2.4A, Lane 3 & 2.4B, Lane 3) and inclusion body (Figure 2.4A, Lane 4 & 2.4B, Lane 4) extracts from E. coli transformed with r16OMT were not active, nor were extracts from untransformed E. coli.
Figure 2.4: A) SDS PAGE profile of protein extracts stained with colloidal Comassie Blue. M: Standard protein molecular weight markers; Lane 1: Whole leaf; Lane 2: Soluble proteins from E.coli expressing 16OMT before adding IPTG; Lane 3: Soluble proteins from E.coli expressing 16OMT after adding IPTG; Lane 4: Inclusion body proteins found in the pellet after centrifugation from E.coli expressing 16OMT after adding IPTG; Lane 5: Soluble proteins from E.coli expressing empty vector before adding IPTG; Lane 6: Soluble proteins from E.coli expressing empty vector after adding IPTG.

B) Enzyme activity assays for 16OMT visualized by thin layer chromatography of radioactive reaction products and autoradiography. Each of the native protein extracts (1 to 6) was assayed for 16OMT as well as inclusion bodies after solubilization (4*) as described in Materials and Methods. A section of the autoradiogram for assays 1 to 6 is represented to show the production of 16[14C]-methoxytabersonine in the presence of S-Adenosyl-L-methionine, 16-hydroxytabersonine (+S) and with r16OMT or with boiled r16OMY (B), or with r16OMT in the absence of alkaloid substrate (-S).
cells before (Figure 2.4A, Lane 5 & 2.4B, Lane 5) or after (Figure 2.4A, Lane 6 & 2.4B, Lane 6) IPTG induction. Inclusion bodies were harvested, purified, dissolved and renatured by dialysis (see Materials and Methods) to produce approximately 250-400 mg of highly purified r16OMT (Figure 2.4B, Lane 4*) from a 50 ml E. coli culture.

2.4.4 - Recombinant 16OMT is a highly specific O-methyltransferase

The substrate specificity of r16OMT was assayed for its ability to use a range of different substrates including MIAs, flavonoids and aromatic amines. When assayed between pH 5.0–9.0, 16-hydroxytabersonine, but not tabersonine, (Figure 2.5) was converted to the 16-O-methylated product with a pH optimum of 7.0 to 7.5. Aromatic and indole amines like 3-hydroxytyramine, 4-hydroxytyramine, and 5-hydroxytryptamine (5HT) were also assayed at different pHs, but no reaction product could be produced. In addition, r16OMT activity was assayed in vitro against various alkaloids (2,3-dihydro-3-hydroxytabersonine, lochnericine, hörhammericine) and their respective 16-hydroxylated products (Figure 2.5), but none were accepted as substrates for O-methylation. Finally r16OMT activity was assayed in vitro against quercetin, kaempferol and caffeic acid and none were accepted as substrates. The results show that in spite of its high similarity to other functionally characterized FOMTs (76 – 78% nucleotide sequence identity, 60 to 65% amino acid identity (Figure S3 and Table S1), 16OMT is a highly specific enzyme for its alkaloid substrate.

2.4.5 - Kinetic analysis of the recombinant 16OMT

Substrate saturation kinetics revealed that r16OMT displayed a high affinity for
Figure 2.5: Substrate specificity of r16OMT for its methyl acceptor. The only methyl acceptor for r16OMT was 16-hydroxytabersonine [Specific Activity: 6.95 ± 0.8 pkat/mg protein (n=6 replicates)]. The substrates, 16-hydroxy-2,3 dihydrotabersonine, 16-hydroxylochnericine, 16-hydroxyhorhammericine were produced by biotransformation of 2,3 dihydrotabersonine, lochnericine, and hörhammericine using E. coli expressing a recombinant tabersonine-16-hydroxylase: NADPH Cytochrome P450 reductase fusion protein (Schroeder et al., 1999).
16-hydroxytabersonine and SAM, with Km values of 2.6 μM, and 17 μM, respectively (Table 2.2). In addition S-adenosyl-L-homocysteine (AdoCys), a well known competitive inhibitor of OMTs, had an inhibitory constant (K_i) of 600 nM (Table 2.2) whereas 16-methoxytabersonine was not an inhibitor at the concentrations tested (5 to 50 μM).

2.4.6 - Leaf epidermal cells are enriched 900-fold in 16OMT activity compared to whole leaves and 6-fold in 16OMT transcripts compared to LCM captured whole leaves.

Crude epidermis-enriched and total leaf extracts were prepared by CA technique using the small scale protocol described in Materials and Methods. Extracts were desalted to remove small molecular weight molecules and these were assayed for different OMT activities using 16-hydroxytabersonine and quercetin as substrates, as well as for NMT and DAT activities that are not expressed in leaf epidermis (Murata, De Luca, 2005).

Inspection of 16OMT reaction products showed that CA treatment was required to obtain leaf epidermis enriched 16OMT extracts, since simply dipping leaves in extraction buffer without carborundum treatment did not produce any activity. Extractions were performed in triplicate to show that the specific activities of whole leaf protein extracts were 0.03 and 0.04 pkat mg^{-1} protein for 16-hydroxytabersonine and quercetin, respectively (Figure 2.6A). In contrast leaf epidermis enriched extracts had 300-fold (9.43 pkat mg^{-1}) and 30-fold (1.37 pkat mg^{-1}) higher 16OMT and FOMT activity, respectively, compared to those of whole leaves (Fig. 2.6A). To control for extraction of cells within the leaf mesophyll, the leaf epidermis enriched extracts were
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km (µM)</th>
<th>Vmax (µM s(^{-1}))</th>
<th>Kcat (s(^{-1}))</th>
<th>Kcat/Km (M(^{-1}) s(^{-1}))</th>
<th>Ki (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-hydroxytabersonine</td>
<td>2.6 ± 0.06</td>
<td>0.59 ± 0.08</td>
<td>6154.4</td>
<td>2.4 x 10(^9)</td>
<td>-</td>
</tr>
<tr>
<td>S-adenosyl-L-methionine</td>
<td>21.7 ± 11.59</td>
<td>0.79 ± 0.47</td>
<td>8324.5</td>
<td>3.8 x 10(^8)</td>
<td>-</td>
</tr>
<tr>
<td>S-adenosyl-L-homocysteine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.6 ± 0.5</td>
</tr>
</tbody>
</table>

Table 2.2 Kinetic data for 16-hydroxytabersonine, S-adenosyl-L-methionine as substrates and S-adenosyl-L-homocysteine as an inhibitor for r16OMT. Mean values from three independent experiments are shown.
Figure 2.6: A) Quantitation of 16OMT, FOMT, NMT and DAT enzyme activities in leaf epidermis enriched extracts (CA) compared to whole leaf extracts (WL). The specific activities were obtained in triplicate for each enzyme and are denoted on the graph: CA (16OMT, 9.43 ± 0.74; FOMT, 1.37 ± 0.122; NMT, 0.00; DAT, 0.00) and WL (16OMT, 0.03 ± 0.0015; FOMT, 0.04 ± 0.0060; NMT, 0.06 ± 0.0050; DAT, 0.05 ± 0.0080). B) Real time PCR quantitation of 16OMT in different cell types: leaf epidermis (E); palisade mesophyll cells (M); palisade assisted idioblast cells (I); cross-connected laticifer cells (L); vascular cells (V). Actin is used to calibrate the reaction.
shown to have no NMT or DAT activity compared with those of whole leaves (Figure 2.6A).

The levels of 16OMT transcripts were quantitated by Real Time PCR in tissues obtained by laser capture microdissection (whole leaves, epidermal cells, palisade mesophyll cells, palisade-assisted idioblast cells, cross-connected laticifer cells and vascular cells). As described previously (Murata, J., De Luca, V., 2005), the amount of RNA isolated from extraction of LCM-dissected cells was not sufficient for direct RT-PCR analysis, it was amplified by T7-based RNA amplification prior to PCR as previously described (Nakazono et al., 2003) to produce good cDNA useful for detecting expression of various genes in different cell types. Real time PCR analysis revealed that the leaf epidermis of Catharanthus roseus detected at least 6 times more 16OMT transcript than in whole leaves or in other any of the other cell types (Figure 2.6B). Together the very high leaf epidermis enrichment ratio of 16OMT activity and the preferential detection of 16OMT transcript in LCM captured leaf epidermal cells strongly suggests that this reaction occurs in leaf epidermis in contrast to the later stages in vindoline biosynthesis (NMT and DAT) (Figure 2.6A, B).

Further RT-PCR analysis showed that all CrOMTs, with the exception of CrOMT5, could be detected in all LCM captured cells. It is interesting to note that CrOMT6 whose gene product catalyzes the 4’-O-methylation of flavonoids (Schröder et al., 2004) showed a strong signal in Catharanthus roots, whereas CrOMT5, of unknown biochemical function, was strongly detected in flowers and stems, respectively. This result also points to the flowers as a promising tissue to elucidate the function of CrOMT 5, since may have an obvious biological role in flower biochemistry.
2.5 – Discussion

2.5.1 - Carborundum abrasion technique is a cost effective, robust method to study leaf epidermis biology.

Carborundum abrasion technique has been used successfully to differentially extract indole alkaloids, enzymes active in MIA biosynthesis, and mRNA from the leaf epidermis of *Catharanthus* (Murata, J., De Luca, V., 2005). Combined use of this technique with enzyme assays suggested that leaf epidermis preferentially expresses *16OMT* compared to other leaf cell-types (Figure 2.2; Murata, J., De Luca, V., 2005). These results were used to suggest that leaf epidermal cells are biosynthetically competent to produce tryptamine and secologannin precursors that are converted via many enzymatic transformations to make 16-methoxytabersonine (Murata, J., De Luca, V., 2005) while the remainder of the pathway leading to vindoline biosynthesis appears to occur within specialized cells in the leaf mesophyll (St Pierre et al., 1999).

The present study shows that CA technique could be scaled up to useful levels (Figure S1) for purifying leaf epidermis localized proteins like *16OMT* to homogeneity (Table 2.1). Using the scaled up approach, the specific activity of *16OMT* was 36 times higher in concentrated crude CA extracts compared to those of whole leaf extracts (Table 2.1). This enrichment of *16OMT* by CA extraction was essential to purify this enzyme to homogeneity (Figure S2) and to obtain peptide sequences that led to the successful cloning (Figure 2.3; Figure S3) of *16OMT* from a cDNA library produced from leaf epidermis enriched mRNA. In contrast, previous efforts using plant cell suspension cultures only led to the isolation of a number of novel contaminating OMTs (*CrOMTs*-2, -4, -5, -6 and -7) (Cacacea et al., 2003; Schröder et al., 2004).
The usefulness of CA technique as a tool to enrich for leaf epidermis localized biological processes has also recently been validated through random sequencing of cDNA libraries produced from leaf epidermis enriched mRNA (Murata et al, in preparation). The sequencing of close to 10,000 random clones from this library produced 4 independent sequences identical to various parts of 16OMT (Figure S4), whereas this clone was not represented in EST sequencing of cDNA libraries from mRNA isolated from whole leaves or from root tips (Murata, J., De Luca, V., 2006). This study clearly shows that CA technique is a cost effective, robust means by which to enrich for epidermal components of plant tissues and this can be selectively used to dissect different metabolic pathways, to purify the proteins involved or to generate useful cDNA libraries that represent the biological activities of leaf epidermis. It is clear that combining CA technique with traditional protein purification or with modern proteomics tools improves significantly our capability to study epidermis localized biological processes. The versatility of epidermis enriched extracts for investigating cellular specialization is surprisingly similar to studies performed with isolated glandular trichomes for investigating their highly specialized gene expression and chemistry (Lange et al., 2000; Gang et al., 2001; Wagner et al., 2004; Fridman et al., 2005). Clearly this approach might be of great use to study the comparative biology of Catharanthus leaf epidermis during growth and development or it could be used to compare the leaf epidermis biology between plant species.
2.5.2 - Properties of r16OMT

The r16OMT showed very high substrate specificity for 16-hydroxytabersonine since slight modifications of this substrate eliminated enzyme activity (Figure 2.5). The enzyme did not accept selected aromatic amines or the flavonoid, quercetin that is a substrate for other related Catharanthus OMTs (Cacacea et al., 2003; Schröder et al., 2004). The inability of the enzyme to O-methylate 16-hydroxy-2,3 dihydrotabersonine does suggest that this reaction occurs prior to further substitution of 16-hydroxytabersonine (Figure 2.1) and supports its order in the pathway of vindoline biosynthesis. Both the r16OMT and the enzyme from leaf extracts showed characteristic features of OMTs (Koch et al., 2003) such as strong inhibition by low concentrations of S-adenosyl-L-homocysteine, but neither was affected by divalent cations (Mn2+,Mg2+,Zn2+,Ca2+,Cu2+) that modulate the activities of some OMTs (Lin et al., 2006; Schubert et al., 2000). Together, these data suggest that I6OMT is the gene responsible for in vivo 16OMT activity in Catharanthus.

2.5.3 - The leaf epidermis expression of 16OMT activity in Catharanthus

The enrichment of 16OMT activity (Figure 2.6A) and of 16OMT (Figure 2.6B) transcript in leaf epidermis compared to that of FOMT (Fig. 2.6A) and the greater FOMT activity found in most Catharanthus organs tested (leaf, flower, stem, and root) (Figure 2.2D) provides very strong evidence for preferential expression of 16OMT within leaf epidermal cells that have been shown to be specialized for much of the MIA pathway (Murata, J., De Luca, V., 2005; St Pierre et al., 1999). While combined use of carborundum abrasion and laser capture microdissection techniques has enabled targeted
dissection of specialized biochemical function, the expression profiles of all currently
published CrOMTs were very similar in most Catharanthus tissue types, with the
exception of CrOMT5 that was preferentially expressed in flowers and stems and
CrOMT6 that was preferentially expressed in roots. In addition, neither idioblast nor
mesophyll cells appear to express at high levels any of the tested OMT transcripts.

During seedling development 16OMT activity is present under both dark and light
conditions, although temporally it appears approximately at the same time as the 2,3-
dihydro-3-hydroxytabersonine N-methyltransferase (De Luca et al., 1987a,b; De Luca et
al., 1988). Additionally the 16OMT responds to light in a similar mode as the
downstream N-methyltransferase where both enzyme activities are increased slightly
when etiolated seedlings are treated with light. In contrast both deacetylvindoline-4-
hydroxylase (Vázquez Flota and De Luca, 1998) and DAT (Fig. 2B; (De Luca et al.,
1988; St. Pierre et al., 1998) activities were induced many fold when etiolated seedlings
were submitted to light treatment.

2.6 – Conclusions

Using Catharanthus roseus as a model system, a simple, general and highly
useful method for harvesting proteins and mRNA by carborundum abrasion technique
was reported. This method was essential for the successful molecular cloning, functional
characterization and expression profiling of 16OMT (Figure 2.1) that is responsible for
the leaf epidermis localized 5th to last step in vindoline biosynthesis. This versatile
method could be applied for studying the changing biochemistry of specialized leaf
epidermis during growth and development, as has already been done with harvested
glandular trichomes (Lange et al., 2000; Gang et al., 2002; Wagner et al., 2004; Fridman et al., 2005). The specialized nature of the leaf epidermis suggests that CA combined with methods for studying the complement of genes expressed in this cell type should yield remarkable new information as illustrated in this study.
Chapter 3.

Molecular Modeling, Structural Characterization, Site Directed Mutagenesis and Analysis of Oligomeric Activity of 16OMT mutants: New strategy in the development of new cellular chemistries
Molecular Modeling, Structural Characterization, Site Directed Mutagenesis and
Analysis of Oligomeric Activity of 16OMT mutants: New strategy in the
development of new cellular chemistries

Dylan Levac, Heather Gordon, Vincenzo De Luca
Department of Biological Sciences, Brock University, 500 Glenridge Ave., St.
Catharines, ON, L2S 3A1, Canada

3.1 – Abstract

Small molecule O-methyltransferases (OMTs) (E.C. 2.1.1.6.x) catalyze the
transfer of the reactive methyl group of S-adenosyl-L-methionine (SAM) to free hydroxyl
groups of acceptor molecules. Plant OMTs, unlike their monomeric mammalian
homologues, exist primarily as functional homodimers. While the biological advantages
for dimer formation with plant OMTs remain to be established, studies with OMTs from
the benzylisoquinoline producing plant, *Thalictrum tuberosum*, showed that co-
expression of 2 recombinant OMTs produced novel substrate specificities not found
when each rOMT was expressed individually (Frick, Kutchan, 1999). These results
suggest that OMTs can form heterodimers that confer novel substrate specificities not
possible with the homodimer alone. The present study describes a molecular model of the
16OMT based on the known X-ray structure of isoflavone OMT (Zubieta, et al. 2001).
Based on this model, site specific mutagenesis has been used in an attempt to modify the
substrate specificity and to inactivate 16OMT. Specific mutants were produced that could
be used to study the biochemical properties of homodimers and heterodimers.
Experimental evidence is provided to show that active sites found on OMT dimers
function independently, and that bifunctional heterodimeric OMTs may be formed in vivo to produce a broader and more diverse range of natural products in plants.

3.2 - Introduction

Small molecule O-methyltransferases (OMTs) (E.C. 2.1.1.6.x) are involved in both primary and secondary plant metabolism. They catalyze the enzymatic transfer of the reactive methyl group from S-adenosyl-L-methionine (SAM) to a hydroxyl, or reactive oxyanion of their substrate, by means of a general acid/base mechanism facilitated by a basic catalytic residue (ie. histidine, lysine). There are also variants of SAM-dependant methyltransferases (ie. Class III methyltransferases) that use the ionization character of their substrate at cellular pH rather than catalytic residues to facilitate this reaction.

Plant small molecule OMTs are distinct from their mammalian homologues in that the plant OMTs are functional homodimers; whereas, the mammalian catechol OMTs function as monomers (Zubieta, et al. 2001, Vidgren, et al 1994, Männistöö, Kaakkola, 1999). While it has yet to be established if there is any evolutionary advantage in forming dimers in plants, one report has shown that heterologous co-expression of two recombinant benzoisoquinoline alkaloid OMTs (rBAOMT) in E.coli produced novel methylated products compared to bacterial cultures expressing either enzyme alone (Frick, Kutchan, 1999). These results were used to suggest that the two OMTs could form heterodimers that were responsible for producing the new substrate specificity observed.

The X-ray crystal structure analyses of the class II isoflavone O-methyltransferase (IOMT) revealed a highly conserved C-terminal SAM binding domain, consisting of a typical nucleotide binding motif known as an α/β Rossmann fold (Rao, Rossmann,1973),
as well as an N-terminal dimerization domain that is unique to plant methyltransferases (Zubieta, et al. 2001). The three dimensional structure of IOMT, together with its behaviour on gel filtration columns, suggested that it functions in vitro as a homodimer. The dimerization domain of IOMT involves approximately 30% of the N-terminal end of the protein, which contributes amino acid residues that appear to be important for substrate binding within the catalytic site of the complementary monomer (Zubieta, et al. 2001). The crystal structure of IOMT showed that Met29, Thr32 and Thr33 of IOMT insert into the complementary subunit’s catalytic site and form the back wall of the substrate binding pocket (Zubieta, et al. 2001). The hypothetical heterodimer described by Frick and Kutchan (1999) also belongs to class II OMTs, and its novel substrate specificity could be produced if the dimerization domains between the two rBAOMTs altered the composition of the back wall of a catalytic pocket to accommodate a broader range of substrates.

The Madagascar periwinkle (Catharanthus roseus) produces the well-known and remarkably complex anticancer dimeric alkaloids vinblastine and vincristine that are derived from the coupling of vindoline and catharanthine monomers. Previous studies have described the molecular cloning and functional characterization of 16-hydroxytabersonine-16-O-methyltransferase (16OMT) that catalyses the second of six steps in the conversion of tabersonine into vindoline (Levac, D., et al, 2008). Functional expression and biochemical characterization of recombinant 16OMT showed its narrow substrate preference and high affinity for 16-hydroxytabersonine (Km=2.6μM).

The present study uses molecular modeling, site-directed mutagenesis (SDM), and classical biochemical techniques to identify residues important for substrate binding.
The 16OMT enzyme model was used to suggest amino acid substitutions or deletion by SDM of 16OMT to change the enzyme’s substrate specificity to accept flavonoids like the closely related CrOMT2 and CrOMT6 enzymes of Catharanthus roseus (Levac, et al. 2007, Cacace, et al. 2003, Schröder, et al. 2004). This study also uses r16OMT and inactive form of r16OMTi (H260A) to determine if active heterodimers can be produced. The results suggest that modeling studies and phylogenetic alignments may not be sufficient to identify amino acid residues to modify to alter substrate specificity of the r16OMT. Experimental evidence also suggests that heterooligomeric r16OMT:r16OMTi could be produced and that the active sites of the oligomer could function independently of each other.

3.3 - Materials and Methods

3.3.1 - Cloning, expression and purification of recombinant C. roseus 16OMT from inclusion bodies

The 16OMT responsible for O-methylation of 16-hydroxytabersonine was identified and cloned from an epidermis enriched phage λ cDNA library as reported in Chapter 2. Briefly, the full-length ORF was amplified by PCR from an epidermis enriched phage λ cDNA library using forward (5’CACCATGGATGTTCAATCTGAGGA 3’) and reverse (5’TCAAGGATAAACC TCAATGAGACTCC 3’) primers that were compatible to the Gateway® pENTR directional vectors (Invitrogen Co., http://www.invitrogen.com/). The PCR product was directionally ligated into pENTR and subsequently mobilized to pDEST-17 (Invitrogen Co., http://www.invitrogen.com/), according to the manufacturer’s specifications. The
pDEST-17 vectors harboring the full-length 16OMT ORF-His fusion were transformed into *E. coli* (DE3)pLysS cells as described in Chapter 2.

Three mL saturated pDEST-17 16OMT *E. coli* cultures were generated using 2 x YT media containing 50 µg/mL ampicillin, and grown overnight at 37°C. Five hundred µL of the saturated culture was then used to inoculate 50 ml 2 x YT media containing 50 µg/mL ampicillin. Cultures were allowed to grow at 37°C to OD 0.8, then induced with 2 mM final concentration IPTG and grown at room temperature overnight to promote inclusion body formation. The culture was then centrifuged at 5000 rpm for 10 min to harvest cells, the supernatant was removed and cell pellets were stored at -20°C until they were processed for inclusion body purification and extraction.

Cell pellets were resuspended in 5 ml 100 mM Tris-HCl, pH 7.5, 13 mM β-mercaptoethanol and lysed by sonication. After centrifugation at 21000 g (4°C) for 20 min the pellet containing inclusion bodies was washed 3x in 10 mL Wash Buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1 M urea per gram pellet weight and 0.1 % Triton X-100. After each wash inclusion bodies were re-pelleted by centrifugation at 21000 g for 10 min at 4°C as described in Chapter 2. This was followed by a single wash step with Wash Buffer to remove residual Triton X-100. Purified inclusion bodies were dissolved in solubilization buffer (100 mM Tris-HCl, pH 7.5, 8 M urea, 1 mM phenylmethylsulfonylfloride) to a final ≤10 mg/mL protein concentration (Kopito, R., 2000) and solubilized in a beaker with gentle stirring for at least one hour at room temperature. Solubilized proteins were dialyzed (100 mM Tris, pH 7.5) overnight at 4°C using a 30 ml Slide-A-Lyzer Dialysis Cassette (10 MWCO, Fisher Scientific) according to the manufacturer’s instructions. Refolded proteins were used directly for enzyme
assays. The protein concentration of extracts was determined using a Protein assay kit (Bio-Rad, Hercules, CA, USA).

3.3.2 - 16OMT assays

The substrate required for 16OMT assays was produced by biotransformation of tabersonine into 16 hydroxytabersonine using *E. coli* expressing recombinant tabersonine-16-hydroxylase: NADPH cytochrome P450 reductase protein (Schröder, *et al.*, 1999). Induced bacterial cells were incubated at 37°C with 500 mg of tabersonine and after 16 h of incubation approximately 400 mg of 16 hydroxytabersonine could be produced. After purification this substrate was used in r16OMT enzyme assays. Enzyme assays were performed in a final volume of 100 μL using 4 μg of solubilized and refolded r16OMT protein, or the corresponding 16OMT mutants in the presence of substrate (0.1 mM 16-hydroxytabersonine, kaempferol or quercetin) and co-substrate (2.08 μM (0.025 μCi) (methyl-¹⁴C)SAM). Enzyme assays were performed at 35°C for one hour, pH 7.5 (100mM Tris-Cl, 0.1% β-mercaptoethanol) and stopped using 20 μl 10 M NaOH for alkaloid enzyme assays, or 20 μl 10% H₂SO₄ for flavonoid enzyme assays. Radioactive reaction products were separated from unreacted (methyl-¹⁴C)SAM co-substrate by extraction of the reaction mixture with 400 μl ethyl acetate. Fifty μL of ethyl acetate containing reaction product was dissolved in 5 ml ScintiSafe™ Econo 2 scintillation cocktail (Fisher Scientific) and radioactive product was measured with an LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Fullerton, CA, USA). The remaining 350 μL ethyl acetate containing enzyme reaction products were taken to dryness by vacuum centrifugation in an SPD SpeedVac (Thermo Savant, Holbrook, NY, USA).
Each sample was dissolved in 20 μL methanol, 5 μL of which was applied to TLC for analyses. Alkaloid products were applied to Polygram® SIL G/UV<sub>254</sub> TLC plates (Fisher Scientific) and developed in a 50:50 hexane:ethyl acetate solvent. Flavonoid reaction products were applied to Polygram® Polyamid-6 TLC plates (Fisher scientific) and developed in a solvent system that will resolve polymethylated flavonols (4:3:3 benzene:methylethyl ketone:methanol). Radioactive products were detected by exposing TLCs to a Storage Phosphor Screen (Amersham Biosciences) for 16 hr and visualized using a Phosphorimager FLA-3000 (Fujifilm, Tokyo, Japan) and MULTI GAUGE ver. 3.0 (Fujifilm).

3.3.3 - Comparative enzyme kinetic analysis between r16OMT and r16OMT mutants.

For Km determinations, triplicate enzyme assays were performed with 2, 6, 10, 14 and 18 μM 16-hydroxytabersonine at constant 2.08 μM (0.025 μCi) (methyl-<sup>14</sup>C)SAM and with 1, 2, 5, 10 and 20 μM (methyl-<sup>14</sup>C)SAM at constant 0.1 mM 16-hydroxytabersonine.

3.3.4 - Relationship tree

Sequences were aligned using CLUSTAL W (Thompson, et al. 1994). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar, et al. 2004). Cladistic analysis was performed using UPGMA clustering method, and bootstrap values were determined by 10000 replicates using the heuristic search algorithm. All branch points with less than 50% bootstrap support were considered inclusive and collapsed to produce a condensed relationship tree.
3.3.5 - Homology modeling and substrate positioning within the 160MT active site

The *C. roseus* 160MT amino acid sequence was submitted to The PSIPRED Protein Structure Prediction Server mGenTHREADER fold recognition software to identify suitable crystal structures for modeling 160MT. Only crystal structures having an overall mGenTHREADER score and percent identity of >0.95 and >40% respectively were considered suitable. With those constraints in mind, only the Ms7IOMT (U97125) (Zubieta, et al. 2001) appeared to be suitable to perform the 160MT modeling study. Swiss Model First Approach (Schwede, et al. 2004) was used to generate an initial model of the 160MT based on Ms7IOMT in complex with S-adenosyl-homocysteine (SAH) and isoformononetin (PDB: 1FP2).

The resulting model was refined using Quanta (Molecular Simulations Inc., San Diego, CA) to add protons, C-terminal carboxylic acid and N-terminal amino groups that are absent in original models. Following this, SAH and isoformononetin were replaced with SAM and 16-hydroxytabersonine, respectively within the active site of the 160MT model using Quanta. The backbone of 16-hydroxytabersonine was overlaid with isoformononetin using the indole group 16-hydroxytabersonine as an anchor, similar to the A/C ring of isoformononetin. The 16-hydroxyl position of 16-hydroxytabersonine, corresponding to 7'-hydroxyl of isoformononetin, was positioned next to the catalytic residue (H260) of 160MT.

The model was then energy minimized twice using Chemistry at HARvard Macromolecular Mechanics (CHARMM) program (Brooks, et al. 1983). The first energy minimization was done constraining all the peptide atoms in place and allowing the
substrate and co-substrate molecules to move to their lowest possible potential energy locations. The second energy minimization was performed by fixing only peptide backbone atoms in place in order to allow substrate, co-substrate and protein side chains to move to locations of lowest possible potential energy.

3.3.6 - Site directed mutagenesis of 16OMT and mutant identification

Single (C20F, H260A, Del316-317) and double (C20F and Del316-317) mutants of 16OMT were generated according to the manufacturer’s instructions using the QuickChange® Multi Site-Directed Mutagenesis Kit (Stratagene). Mutations were performed on pDEST-17 Gateway® expression vector harboring 16OMT. Oligonucleotide primers compatible with the QuickChange® kit were designed using the web based QuickChange® Primer Design Program (http://www.stratagene.com/qcprimerdesign). Primers were designed to mutate cysteine 20 to phenylalanine (C20F: 5’
CTCAATATGGGAGCCAATCCTTCTTTCACTTCTGCTTC 3’), histidine 260 to alanine (H260A: 5’
ATCTTTCTCAAGTGATTTCGCTGACTGGAACGATGAAGATTG 3’), and to delete phenylalanine 316 and alanine 317 (Del316-317: 5’ CTATGGATATG GCAATGCTTGTAATGCTAAAGAAAGATGTGAG 3’).

Mutant clones were immediately transformed according to the manufacturer’s protocols into XL10-Gold ultracompetent E. coli cells (Stratagene). Transformants were identified by plating E. coli on ampicillin selection plates. Positive transformants were used to inoculate 3 ml LB liquid cultures containing 50 µg/ml ampicillin and grown
overnight at 37°C to generate respective saturated bacterial cultures. Saturated cultures were pelleted by centrifugation at 3000 g for 20 minutes and recombinant plasmids were purified from the resultant cell pellet using the QIAprep spin miniprep plasmid purification kit (Qiagen Inc.) according to manufacturer's instructions. The identity of mutant clones was verified by sequencing (Robarts Research Institute, London, Ontario, Canada). Identified mutants were retransformed into E. coli (DE3)pLysS for expression according to the protocol in 3.2.1.

3.3.7 - Titration of functional 16OMT with inactive H260A 16OMT mutant

Both r16OMT and H260A r16OMT (r16OMTi) mutant proteins were produced independently and harvested as inclusion bodies that were washed, and solubilized to a final concentration of 2 mg protein/ml urea as detailed in section 3.2.1. The solubilized denatured protein solutions were mixed in ratios of 1:0, 1:0.12, 1:0.75, 1:1, 0.75:1, 0.5:1, and 0:1 of r16OMT/r16OMTi mutant protein in a final volume of 25 ml. Denatured protein solutions were gently mixed in a 50 ml beaker over a 1 hr period. To renature the proteins, each urea soluble protein mixture was dialyzed (100 mM Tris-HCl, pH 7.5) overnight at 4°C using a 30 ml Slide-A-Lyzer Dialysis Cassette (10 MWCO, Fisher Scientific) according to the manufacturer's instructions and the dialysates was used directly for 16OMT enzyme activity assays. The protein concentration of extracts was determined using a Protein assay kit (Bio-Rad, Hercules, CA, USA).
3.3.8 - Use of His tag purification and protease treatment to isolate purified r16OMT.

To generate r16OMT whose histidine tag had been cleaved, inclusion bodies of r16OMT were solubilized, cleaned and renatured as described previously in 3.2.1. Immediately after dialysis the protein was concentrated 3-fold by centrifugation using Amicon Ultra concentrators (Fisher Scientific). The concentrated dialysate containing functional r16OMT enzyme harboring an N-terminal poly-histidine tag tethered by a TEV domain was then treated with AcTEV™ protease (Invitrogen; according to a 10 fold scaled up version of manufacturers instructions) in order to remove the poly-histidine tag. The cleaved, poly-histidine tag lacking r16OMT was purified away from histidine tag harboring r16OMT by Ni-NTA column chromatography by harvesting non binding fractions. Non-binding fractions were then acetone precipitated for preparation of r16OMT:r16OMTi heterooligomers.

Both histidine-tag-lacking r16OMT homodimers and r16OMT:r16OMTi heterooligomers were generated as described in section 3.2.7. The dialysate was then concentrated 3-fold using Amicon Ultra concentrators (Fisher Scientific). Ten mL of the concentrated sample was applied to 1 mL of his-tag resin in a 15 ml conical tube (Sarstedt) to allow binding of H260A homodimers or 16OMT:H260A heterodimers by constant gentle mixing overnight at 4°C. The next morning Ni-NTA resin was allowed to sediment by gravity, and the resin was then pelleted by centrifugation in a microcentrifuge with a 10 second pulse of maximum speed. The supernatant was collected for analysis and the remaining Ni-NTA resin was washed 3 times with 10 mL of 100 mM Tris-HCl pH 7.5, 14mM mercaptoethanol, 20 mM imidazole and the supernatants were pooled for enzymatic analysis. His-tagged proteins bound to Ni-NTA
resin were released with 10 mL of 100 mM Tris-HCl pH 7.5, 14mM mercaptoethanol, and 500 mM imidazole. The supernatant (10 mL), the wash (30 mL) and eluted (10 mL) fractions were concentrated to 5 ml using Amicon Ultra concentrators (Fisher Scientific). The concentrated wash (2.5 mL) and eluted fractions (2.5 mL) were further desalted using PD-10 columns. All fractions including the supernatant were assayed for 16OMT activity.

3.3.9 - Preparation of cDNA from laser capture microdissected cells

cDNA was prepared from RNA amplified after being isolated from epidermal, mesophyll, idioblast, laticifer, vascular and representative whole leaf cells isolated by laser capture microdissection as previously described (Murata, De Luca, 2005).

3.3.10 - Cell-type expression profiling of selected OMTs compared to 16OMT

One μL cDNA prepared from RNA isolated from laser captured epidermal, mesophyll, idioblast, laticifer, vascular and representative whole leaf cells was used as template for PCR amplification using gene specific primers for TDC (A23291), T16H (AJ238612), DAT (AF053307), 16OMT, CrOMT1 (AY028439), CrOMT2 (AY127568), CrOMT4 (AY127569), CrOMT5 (AY343488), CrOMT6 (AY343490), CrOMT7 (AY343492). Due to high sequence identity between OMTs, the right primer was designed using the 3' UTR of each respective gene as template. PCR amplification was optimized for Tm and run over 30 cycles. The PCR products were analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide.
3.4 - Results

3.4.1 - Phylogenetic analysis: 16OMT is most closely related to two *Catharanthus roseus* class II OMTs

Cladistic analysis of 56 biochemically characterized OMTs with highly divergent substrate specificities could be resolved into independent phyla (Figure 3.1) of all three currently known OMT classes (Class I, II and III) (Joshi, Chiang, 1998, Zubieta, et al. 2003). Such analyses combined with substrate specificity studies have suggested that particular biochemical functions of OMTs evolved by gene duplication followed by mutation (Gang, 2005). For example, the caffeic acid OMT (*COMT*) from *Medicago sativa* (Accession number CAB65279.1) appears to be more closely related to *Stylosanthes humilis* COMT (Accession number 2119166A), than it is to *M. sativa* chalcone OMT (*ChOMT*) (Accession number AAB48059), or another putative *M. sativa* OMT (Accession number CAB6279) (Gang, 2005). Cladistic analysis of OMTs (Figure 3.1) shows that 16OMT is most closely related to two *Catharanthus roseus* class II OMTs; a myricetin OMT that performs 3' and 5' sequential O-methylations (*CrOMT2*) (Cacace, et al. 2003) and a flavone/flavonol 4' OMT (*CrOMT6*) (Schröder, et al. 2004).

These results suggest that 16OMT, *CrOMT2* and *CrOMT6* probably evolved by gene duplication from a common ancestral phenylpropanoid OMT, followed by mutations to alter substrate preference to yield the highly substrate-specific 16OMT involved in the late stages of vindoline biosynthesis.
Figure 3.1 Phylogenetic tree of plant O-methyltransferases that have been biochemically characterized. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar et al., 2004)
3.4.2 - Identification of 16OMT active site residues based on the crystal structure of Ms7IOMT

The phylogenetic relationship between 16OMT to _C. roseus_ flavonoid OMTs (_CrOMT2, CrOMT6_) prompted modeling studies of the 16OMT active site with the _M. sativa_ isoflavone-7-O-methyltransferase (Ms7IOMT) whose crystal structure is known (Zubieta, et al. 2001). In addition, the program known as mGenTHREADER (McGuffin, Jones, 2003, Jones, 1999) showed that the Ms7IOMT crystal structure could be used to model the active site of 16OMT because of its 41.2% sequence identity to 16OMT and an overall mGenTHREADER score of 0.952.

Modeling the 16OMT after Ms7IOMT using SWISS-MODEL (Schwede, et al. 2003) automated protein homology-modeling server yielded a primary model that represented the entire 16OMT sequence in its predicted tertiary structure with the exception of the first five amino acid residues (M1 – S5). This initial model was further refined using Quanta and CHARMM (see Materials and Methods) and the model’s root mean square deviation (R.M.S.D) was determined to be 1.02 Å over the entire protein sequence (E6 – P352 for 16OMT) when comparing the 16OMT peptide backbone atom locations to those of Ms7IOMT. When comparing the locations of the peptide backbone atoms of 16OMT and Ms7IOMT within the α/β Rossmann fold domain (SAM binding domain) of both models (E184 – D340 for 16OMT) the R.M.S.D was determined to be only 0.66 Å. These values measure the fidelity of the 16OMT model as it is derived from the Ms7IOMT crystal structure to produce a good approximation of the native tertiary structure of 16OMT. The quaternary structure of the 16OMT dimer was generated using Swiss-PDB Deep Viewer, and the same transformation matrix required
to reconstitute the functional unit of Ms7IOMT model from its monomer crystal structure (1FP2).

The 16OMT model revealed that 32% of each subunit (F8 to P121) was involved in the dimerization complex and that the N-terminal residues Gln12 to Ile16 insert into the back wall of the catalytic site of the complementary monomer, as also shown for Ms7IOMT (Zubieta, et al. 2001). Further inspection of this model also suggested that W16 and F144 in the back wall might provide a hydrophobic sink for binding the terpene moiety of 16-hydroxytabersonine and promote a favorable interaction within the active site (Figure 3.2). In addition, the highly conserved methionine residues (M170 and M312) could orient the aromatic ring of the indole moiety towards the catalytic residue (H260) and SAM to promote transmethylation of 16-hydroxytabersonine (Figure 3.2 E). The methionine residues are homologous to M168 and M311 of Ms7IOMT that orient to the A ring of 2,4,7-trihydroxyisoflavanone (Zubieta, et al. 2001) as predicted by a Clustal W amino acid alignment (Figure 3.3) and mGenTHREADER iterative alignment algorithm. These residues might stabilize 16-hydroxytabersonine within the active site by means of pi-pi stacking between d-orbitals of the methionine sulfur atom and the pi orbitals of the aromatic ring of the indole moiety of 16-hydroxytabersonine (Figure 2 E). Interestingly there is only one predicted hydrogen bond that occurs between the catalytic residue H260 and the target hydroxyl group of 16-hydroxytabersonine. The lack of more stabilizing hydrogen bonds within the active site do not appear to be detrimental to the positioning of the reactive hydroxyl group of 16-hydroxytabersonine that is located 2.46 Å from the primary basic nitrogen of H260 and 3.16 Å from the reactive methyl group of SAM.
Figure 3.2. Molecular models of the 16OMT. Amino acid alignment was performed by mGenTHREADER iterative alignment algorithm. The 16OMT model was visualized using Swiss PDV Deep Viewer (GSK), Final images visualized in SPDV were refined using PovRay. A-C show overall enzyme structure by doing a 180 degree rotation about space filled model. D. Close up of one of the active sites showing its constriction, and sequestration of the substrate (red) deep within the enzyme. E. 16OMT active site showing important residues (grey carbon bonds), 16-hydroxtyabersonine and SAM (black carbon bonds).
These distances are sufficiently close for deprotonation of the 16-hydroxyl by H260 and then subsequently transmethylated by SAM.

The binding of SAM is accomplished by the α/β Rossmann fold of 16OMT, a characteristic nucleotide binding domain common to almost all SAM-dependant OMTs (Zubieta et al. 2001, Ibrahim, et al. 1998). Compared to Ms7IOMT, 16OMT also has a similar network of hydrophobic interactions and hydrogen bonds that stabilize SAM within the active site in a favorable orientation for transmethylation of 16-hydroxytabersonine. More specifically a hydrogen bond is predicted to occur between D222 and one of the ribose hydroxyls of SAM and 2 hydrogen bonds with G19 and K251 are predicted to form in association with the terminal carboxylic acid group of SAM.

3.4.3 Site-directed mutagenesis to identify residues essential for catalysis and substrate binding

Modeling studies combined with Clustal W alignment of 16OMT, CrOMT2 and CrOMT6 amino acid sequences (Figure 3.3) prompted the testing of a C20F mutant and an F316, A317 deletion for altering the substrate specificity of this enzyme. The C20F modification was also tested since both CrOMT2 and CrOMT6 (Cacace, et al. 2003, Schröder, et al. 2004), contain F instead of C at this amino acid position, where this potential active site residue is located at F27 in Ms7IOMT. The deleted F316, A317 mutant (Del 316-317) was selected for testing since these amino acids could be part of a random coil-coil motif adjacent to a predicted flavonoid binding site in Ms7IOMT whose removal might stabilize the flavonoid substrate within the active site of 16OMT. The H260A mutation was tested to knockout this catalytic residue and to yield an inactive
Figure 3.3 Clustal W multiple sequence alignment of *Catharanthus* methyltransferases functionally annotated and established to have *in vivo* functions. CrOMT 2 (AAM97497, 3'-5'-flavonoid OMT), CrOMT6 (AAR02419, 4'-flavonoid OMT), 16OMT (Ef444544).
protein. All mutants were sequenced to show that the appropriate codons had been modified or deleted to generate each of the putative mutant proteins.

3.4.4 Functional expression and biochemical analysis of r16OMT mutants

Recombinant 16OMT C20F, Del 316-317, H260A as well as C20F/Del 316-317 double mutants were expressed in DE3 pLySs E.coli cells to produce inclusion bodies containing denatured protein as shown previously for r16OMT (Levac, et al 2008). Inclusion bodies for each protein were harvested, solubilized, renatured by dialysis (Figure 3.4B; see Materials and Methods) and soluble proteins were assayed directly for enzyme activity with 16-hydroxytabersonine, quercetin, and kaempferol as methyl acceptors in the presence of SAM (Levac, et al, 2008).

The renatured rC20F16OMT was enzymatically active and it showed the same high substrate specificity for 16-hydroxytabersonine as shown previously for r16OMT (Table 3.1). Kinetic analysis for this mutant showed that the Km for both 16-hydroxytabersonine (Km=3.4 ± 1.6 μM) and SAM (Km=10.5 ± 4.1 μM) were comparable to those of authentic r16OMT (Table 3.2). With this new information and inspection of the model the results could be explained if the C20 residue was too far removed from 16-hydroxytabersonine to interact with it, even if C20 was replaced with a large hydrophobic F20 residue.

Remarkably neither the rDel 316-317 nor the rC20F/Del 316-317 16OMT mutants had catalytic activity against 16-hydroxytabersonine or quercetin suggesting that deletion of residues 316 and 317 abolished enzyme activity. These unexpected results suggest that F316 and A317 may in fact alter the rotation of all amino acid residues about
Figure 3.4. A) SDS PAGE profile of protein extracts stained with colloidal Comassie Blue. M: Standard protein molecular weight markers; Lane 1: Whole leaf; Lane 2: Soluble proteins from E. Coli expressing 16OMT before adding IPTG; Lane 3: Soluble proteins from E. Coli expressing 16OMT after adding ; Lane 4: Inclusion body proteins found in the pellet after centrifugation from E. Coli expressing 16OMT after adding IPTG; Lane 5: empty vector 6 empty vector. B) SDS PAGE profile of solubilized and renatured inclusion body protein extracts stained with colloidal Comassie Blue. M: Standard protein molecular weight markers; Lane 1: r16OMT; Lane 2: rH260A; Lane 3: 1:1 mixture r16OMT:rH260A ; Lane 4: rC20F; Lane 5: rDel316-317.
<table>
<thead>
<tr>
<th>Extract</th>
<th>Specific Activity (pkat mg(^{-1}))</th>
<th>16-hydroxytabersonine</th>
<th>Quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole leaf protein</td>
<td>0.03</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Whole leaf protein (boiled)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>r16OMT</td>
<td>32</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>r16OMT (boiled)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>rC20F mutant</td>
<td>28</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>rC20F mutant (boiled)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>rDel316-317 mutant</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>r16OMTi mutant</td>
<td>0</td>
<td>0</td>
<td>na</td>
</tr>
<tr>
<td>r16OMT/r16OMTi (1:1)</td>
<td>14</td>
<td>na</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1 Specific activities of OMT reaction products when assayed with different mutant proteins with 16-hydroxytabersonine and quercetin as substrates. na represents not assayed.
### A.

<table>
<thead>
<tr>
<th></th>
<th>Km (μM)</th>
<th>Vmax (μM s⁻¹)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/Km (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-hydroxytabersonine</td>
<td>2.6 ± 0.06</td>
<td>0.59 ± 0.08</td>
<td>6154.4</td>
<td>2.4 x 10⁷</td>
</tr>
<tr>
<td>S-adenosyl-L-methionine</td>
<td>21.7 ± 11.59</td>
<td>0.79 ± 0.47</td>
<td>8324.5</td>
<td>3.8 x 10⁸</td>
</tr>
</tbody>
</table>

### B.

<table>
<thead>
<tr>
<th></th>
<th>Km (μM)</th>
<th>Vmax (μM s⁻¹)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/Km (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-hydroxytabersonine</td>
<td>3.4 ± 1.6</td>
<td>0.77 ± 0.17</td>
<td>8113.8</td>
<td>2.4 x 10⁷</td>
</tr>
<tr>
<td>S-adenosyl-L-methionine</td>
<td>10.5 ± 4.1</td>
<td>0.58 ± 0.33</td>
<td>6111.7</td>
<td>5.8 x 10⁸</td>
</tr>
</tbody>
</table>

Table 3.2 Kinetic data for r16OMT (A) and rC20F (B) mutant enzymes for 16-hydroxytabersonine and S-adenosyl-L-methionine as substrates.
the key α-helix central axis, thus affecting amino acid residue E320 and increase the H-bonding distance with the ε-nitrogen of the catalytic H260 residue. This could lead to a reduction in the basic nature of this catalytic residue and possibly abolish all catalytic activity of the 16OMT.

The renatured rH260A mutant (r16OMTi) was completely inactive (Figure 3.4B and Table 3.1 respectively) demonstrating that H260 is in fact the catalytic residue and partially validating the accuracy of the 16OMT molecular model.

3.4.5 Heterooligomers of r16OMTi inactive protein with active r16OMT are biochemically active.

When recombinant 16OMT and r16OMTi mutant protein were harvested as inclusion bodies they contained relatively pure denatured proteins (Figure 3.4B). Inclusion bodies of r16OMT or r16OMTi were solubilized in urea (2 mg protein/ml) and were mixed in ratios between 1:0 to 0:1, r16OMT: r16OMTi. Each solubilized protein mixture was then dialyzed and renatured proteins were assayed for 16OMT specific activity using 16-hydroxytabersonine substrate and SAM as co-substrates. When the r16OMTi mutant was renatured with active 16OMT the putative heterooligomer was enzymatically active against 16-hydroxytabersonine. There was a strong ($R^2 = 0.93$) positive linear correlation between increasing the ratio of r16OMT and the specific activity obtained (Figure 3.5). Assuming that a heterodimer or heterooligomer is produced, the increasing r16OMT:r16OMTi ratios to overall enzyme specific activity suggested that the function each 16OMT active site could function independently of the other (Figure 3.5).
Figure 3.5 Effect of titrating functional r16OMT solubilized inclusion bodies with solubilized r16OMTi catalytic residue knock out inclusion bodies. Linear regression was performed by Microsoft Excel, and line equation as well as $R^2$ is represented.
3.4.6 Catalytically active His-tag affinity purified r16OMT:r16OMTi heterooligomers could not be purified.

In order to show that r16OMT:r16OMTi heterooligomers were enzymatically active, the poly-histidine tag was removed from r16OMT in order to create r16OMT:r16OMTi heterooligomers that could be purified by using the poly-histidine tagged r16OMTi subunit. This would allow the separation of heterooligomers from active homodimers by histidine affinity chromatography. Upon completion of overnight dialysis, renatured proteins were shown to be active by enzyme assay (specific activity 18.41 pkat mg\(^{-1}\) protein) (Table 3.3). Next, r16OMT enzyme had its N-terminal poly-histidine tag cleaved using AcTEV protease and the N-terminal tag lacking enzyme purified by negative selection using Ni-NTA column chromatography was still biochemically active (specific activity 16.33 pkat mg\(^{-1}\) protein). Purified poly-histidine tag lacking r16OMT was concentrated by acetone precipitation, and resolubilized as described 3.3.1. Each protein (poly-histidine lacking r16OMT and r16OMTi) was resolubilized individually or in 1:1 ratio and renatured to generate various oligomers as described in 3.3.1.

While enzyme activity was observed in all r16OMT containing fractions until TEV domain cleavage, acetone precipitation and renaturation of various oligomers produced inactive proteins (Table 3.2). These problems made it impossible to perform these experiments, unless a renaturation protocol could be developed to produce active enzyme after acetone precipitation.
<table>
<thead>
<tr>
<th></th>
<th>Specific Activity (pkat mg(^{-1}))</th>
<th>16OMT</th>
<th>16OMT:H260A mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solubilized and dialyzed</strong></td>
<td>Input</td>
<td>18.41</td>
<td>-</td>
</tr>
<tr>
<td><strong>inclusion bodies</strong></td>
<td>AcTEV treated</td>
<td>16.33</td>
<td>-</td>
</tr>
<tr>
<td><strong>Polyhistidine tag purification</strong></td>
<td>Unbound</td>
<td>11.49</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Wash 1</td>
<td>3.03</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Wash 2</td>
<td>3.93</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Wash 3</td>
<td>3.09</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Elute</td>
<td>7.95</td>
<td>-</td>
</tr>
<tr>
<td><strong>Solubilized and dialyzed acetone precipitated protein</strong></td>
<td>Input</td>
<td>N/D</td>
<td>N/D</td>
</tr>
</tbody>
</table>

Table 3.3 Purification of r16OMT polypeptide lacking N-terminal poly histidine tag, preparation of r16OMT homodimers lacking N-terminal poly histidine tag, and 1:1 r16OMT:r16OMT\(i\) heterodimers for biochemical analysis.
3.4.7 Expression analysis of C.roseus OMTs

The expression profiles of CrOMTs 1, 2, 4, 5, 6 and 16OMT were compared in cDNA samples produced from mRNA obtained from whole leaves and individual cell types harvested by LCM (Figure 3.6). These profiles were compared to cDNA samples produced from mRNA harvested by regular RNA extraction of flowers, stems and roots (Figure 3.6). The results show that all CrOMTs, with the exception of CrOMT5, are variably expressed in the epidermis and laticifer of C.roseus leaf tissue (Figure 3.6). The expression profiles suggest that flavonoids (CrOMT 2, 6) and 16-hydroxytabersonine (16OMT) could be produced throughout the whole plant (Chapter 2). While these expression profiles suggest that several OMTs are co-expressed in the same cell type (leaf epidermis), it remains to be shown if they could form heterooligomers with novel substrate specificity.

3.5 - Discussion

3.5.1 – The evolutionary origin of the Catharanthus 16OMT

Previous studies have suggested that plant OMTs involved in floral sent production appear to have evolved by gene duplication followed by mutation (Gang, D., 2005). The phylogenetic analysis of 56 biochemically characterized plant OMTs with broadly different substrate specificities (Figure 3.1) suggests that gene duplication followed by mutation to yield new gene function has been an important evolutionary tool for biochemical diversification. One interesting result of this cladistic analysis is that although the majority of the aligned plant OMTs clade according to substrate class preference, the biochemically characterized C.roseus OMTs cluster together regardless of
Figure 3.6 Expression profile of 16OMT in relation to CrOMT 1, 2, 4, 5, 6, 7 in mRNA extracted from Whole leaves, CA enriched leaf epidermis, mesophyll cells, idioblast cells, vascular cells, flowers, stems and roots. The primer sets used for the RT-PCR reactions were designed to the 3'UTRs of CrOMT1,2,4,5,6 and 7 that were unique to each gene: CrOMT 1 (Forward: 5'AGGTGATACTTGCTGAATGTCT 3'; Reverse: 5' AAAAGACAGGTAGTTGGGTG 3'); CrOMT 2: (Forward: 5' GAGGAA AAGTGATCTCTAGA 3'; Reverse: 5' TATGGGTGTAATACCACACATGG 3'); CrOMT4: (Forward: 5' GGTGATCGTCATAGACATTGTA 3'; Reverse: 5'AGCTAG TTAAGTACCCAAGC 3'); CrOMT 5 (Forward: 5' GACAGC AAGAAAGAAG ACTAT 3'; Reverse: 5'AGGAAAATCTTTGTGTGTGTCTC 3'); CrOMT 6: (Forward: 5'GCTAAA GAAAGAACTGAGGAAG 3'; Reverse: 5' ACCTTACCTCAATAAGC AAGTG 3'); CrOMT 7 (Forward: 5'TTAAGACAGAGATAG CAATGGA 3'; Reverse: 5' GCAAATGATGATCATCCCT TAGT GAT 3'; Reverse: 5'CGTAACAAATGGGTACAAA 3'). Actin is used as an internal loading control.
substrate class. The three biochemically characterized *C. roseus* OMTs (16OMT, CrOMT2, and CrOMT6) have varied substrate preference (alkaloid vs flavonols), which suggests that the *C. roseus* 16OMT may have evolved from a phenylpropanoid ancestral gene common to CrOMT2 and CrOMT6. It will be interesting to see if this common relationship between *Catharanthus* OMTs will also be found when the biochemical roles of CrOMT4, CrOMT5 and CrOMT7 are discovered. This information can only be obtained by following biochemical characterization of OMTs to properly hypothesize their *in vivo* functions.

### 3.5.2 - Mutagenesis of the 16OMT

Site-directed mutagenesis of the 16OMT revealed that even combined use of modeling based on the known crystal structure of IOMT and amino acid alignments of the 16OMT to closely related CrOMT2 and CrOMT6 flavonol OMTs to identify substrate binding residues can be insufficient in efforts to engineer new substrate preferences in well characterized enzymes. The failure of this approach to change substrate preference of 16OMT from 16-hydroxytabersonine to flavonols highlights the complexity involved in biological systems, substrate binding in enzymes and the chemistries enzymes perform. It is somewhat surprising that our approach, which took advantage of molecular models based on crystal structures failed, while efforts using simple amino acid alignments have been previously successful (Wang, Pichersky, 1999). The contribution of non-catalytic site residues to overall enzyme structure and catalytic site topology cannot be ignored when considering possible reasons for this outcome. Unfortunately these complexities were not considered in our engineering effort. For this
reason, when attempting to alter enzyme function in enzymes that show even as much as 62-65% sequence homology, as is the case between the 16OMT and CrOMT2 and CrOMT6, sophisticated algorithms (Dwyer, et al. 2004) in the design of active enzymes from proteins that are previously inactive in terms of biological catalysis would be beneficial. Unfortunately such programs are not freely available, even to educational institutions.

Our SDM study shows that C20 is not absolutely required for 16OMT substrate binding. Both the C20F mutant’s catalytic efficiency with 16-hydroxytabersonine and SAM (kcat/Km = \(2.4 \times 10^9\) M\(^{-1}\) s\(^{-1}\) and kcat/Km = \(5.8 \times 10^8\) M\(^{-1}\) s\(^{-1}\) respectively) are comparable to r16OMT (kcat/Km = \(2.4 \times 10^9\) M\(^{-1}\) s\(^{-1}\) and kcat/Km = \(3.8 \times 10^8\) M\(^{-1}\) s\(^{-1}\) respectively), as well as C20F’s affinity constants for 16-hydroxytabersonine and SAM (Km = 3.4 \(\mu\)M and Km = 10.5 \(\mu\)M respectively) are comparable to r16OMT (Km = 2.6 \(\mu\)M and Km = 21.7 \(\mu\)M respectively).

The results obtained for the Del 316-317 mutant were not expected, since the model predicted that the deleted residues were part of a random coil motif and would not adversely affect the overall tertiary structure of the enzyme. However, it is possible that \(\alpha\)-helix C-terminal to the deleted residues predicted by mGenTHREADER is really extended to include the F316 and A317 residues. In this case the Del 316-317 mutation would cause a 200° rotation of all downstream residues about the \(\alpha\)-helix central axis. This effect would be detrimental to overall enzyme activity as the downstream E320 residue potentates the basic nature of 16OMT catalytic residue H260. A 200° rotation about this \(\alpha\)-helix axis would place E320 out of H-bonding distance with the \(\varepsilon\)-nitrogen of
H260 leading to a reduction in the basic nature of the catalytic residue and possibly abolish all catalytic activity of the 16OMT.

3.5.3 - *O*-methyltransferase heterodimerization as a method for developing new chemistries *in vivo*.

The r16OMT was renatured with varying amounts of the inactive r16OMTi in order to form heterooligomers. The strong positive linear correlation between increasing functional r16OMT content and enzyme specific activity (Figure 3.5), suggested that the heterooligomers containing a single functional active site could still be active in spite of the presence of inactive r16OMTi and the two active sites can function independently of each other.

When initially conducting the titration experiment two possible outcomes were considered: i) the heterodimer (r16OMT/r16OMTi) was completely inactive due to a dependence of one active site’s function on the activity of the second, an exponential relationship would be observed when plotting increasing r16OMT:r16OMTi ratios to overall r16OMT specific activity and ii) if the two active sites could function independently of each others, then a linear relationship would be expected when comparing increasing r16OMT:r16OMTi ratios to overall r16OMT specific activity. This would be comparable to a dilution effect as functional r16OMT would be in essence “diluted” with inactive protein.

To date it has not been possible to biochemically purify the heterodimer for more detailed biochemical analysis. From our experimental approach this most likely reflects the long, and often harsh refining steps taken to generate non-tagged functional r16OMT to mix with tagged inactive, histidine tagged r16OMTi mutant to generate
heterooligomers. Shortening the process by producing non-tagged functional r16OMT initially, rather than cleaving the affinity tag, would greatly facilitate the process, assuming this untagged r16OMT is also localized to inclusion bodies after recombinant expression. Otherwise generating functional recombinant r16OMT with any other affinity tag other than poly-histidine would be necessary to facilitate both, purification of the r16OMT protein from E.coli, and purification to homogeneity of r16OMT:r16OMTi heterodimers.

These preliminary results, although insufficient to establish heterooligomer function in vivo, give new insight into the development of new chemistries within plant cell types expressing multiple OMTs. In this report we demonstrated that there are multiple OMTs expressed in the leaf epidermis and idioblast cells of C.roseus (Figure 3.6). This co-expression of multiple OMTs within single cell types enables a situation, like previously reported (Frick, Kutchan, 1999), where heterodimers could possibly form, yielding enzymes with novel substrate specificities and consequently new methylated products, and in the end radiating the small molecule chemistries of these particular cell types.

3.6 – Conclusion

For plants, the biochemical advantage of having OMTs with functionally independent catalytic sites is obvious. In the condition of an OMT heterodimer or heterooligomer there is a distinct possibility that only a single active site will be suited to accept a particular metabolite available within the heterodimer OMT cell type. Therefore in terms of developing new chemistries for the purpose of producing new phytoalexins,
plant volatiles or signal molecules, functionally independent active sites for plant OMTs would promote this particular strategy of radiating chemistries, and small molecules in plants. It appears that in addition to the evolutionary strategies undertaken by plants to develop new gene products for small molecule biosynthetic pathways (Pichersky, Gang, 2000, Gang, 2005), enzyme heterooligomer formation may be yet another level plants use to generate their vast library of small molecules used by plants. What remains to be established is if the in vitro work we have reported here, and the heterologous expression of enzymes with novel substrate specificities reported previously (Frick, Kutchan, 1999), are representative of in vivo reality. More work to define if our heterooligomers are heterodimers needs to be performed. In addition, pure heterooligomers are required for biochemical analysis, although ultimately the identification of an in vivo OMT heterodimer or heterooligomer would be the only proof to establish if this work at all represents reality, or is simply an artifact.
References


Chiang VL., Funaoka M., (1990) "The difference between guaiacyl and guaiacyl-syringyl lignins in their responses to kraft delignification." Holzforschung 44: 309


Hamberg, M., Gardner, H.W., (1992) "Oxylipin pathway to jasmonates: biochemistry and biological significance." Biochimica et Biophysica Acta 1165:1


Pare, P.W., Tumlinson, J.H., (1999) "Plant Volatiles as a Defense against Insect Herbivores." Plant Physiology 121:325


Shimada, M., Fushiki, H., Higuchi, T., (1972) "O-methyltransferase activity from japanese black pine." Phytochemistry 11: 2657


Appendix I
<table>
<thead>
<tr>
<th></th>
<th>16OMT*</th>
<th>CrOMT1</th>
<th>CrOMT2</th>
<th>CrOMT4</th>
<th>CrOMT5</th>
<th>CrOMT6</th>
<th>CrOMT7</th>
</tr>
</thead>
<tbody>
<tr>
<td>16OMT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrOMT1</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrOMT2</td>
<td>62</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrOMT4</td>
<td>60</td>
<td>31</td>
<td>74</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrOMT5</td>
<td>66</td>
<td>28</td>
<td>64</td>
<td>59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrOMT6</td>
<td>65</td>
<td>29</td>
<td>61</td>
<td>58</td>
<td>63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrOMT7</td>
<td>62</td>
<td>30</td>
<td>63</td>
<td>57</td>
<td>61</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>SMT</td>
<td>30</td>
<td>54</td>
<td>28</td>
<td>29</td>
<td>26</td>
<td>30</td>
<td>29</td>
</tr>
</tbody>
</table>

*16OMT (Ef444544), CrOMT1 (AAK20170), CrOMT2 (AAM97497), CrOMT4 (AAM97498), CrOMT5 (AAR02418), CrOMT6 (AAR02419), CrOMT7 (AAR02422).

Table S1. Comparative % amino acid identities between Catharanthus OMTs.
Figure S1: Carborundum abrasion technique for extraction of leaf epidermis enriched protein proteins. The container with leaves and extraction buffer was tilted at an angle for vortexing in order to generate a swirling motion of the carborundum containing buffer and to generate the impacts of the carborundum particles onto the leaf surfaces.
Figure S2: SDS-PAGE combined with silver staining of the gel and enzyme activity profile of 16OMT eluting from a MonoQ anion exchange column. Note that 16OMT activity co-elutes with a 40 KDa protein (at 0.52-0.53 M NaCl) that was later harvested to obtain peptide sequences. The SDS PAGE profiles of whole leaf crude extracts (W) and epidermis enriched crude extracts obtained by carborundum abrasion (CA) are shown for comparative purposes.
Figure S3: Clustal W multiple sequence peptide alignment of all published Catharanthus functionally annotated or putative O-methyltransferases CrOMT1 (AAK20170), CrOMT2 (AAM97497), CrOMT4 (AAM97498), CrOMT5 (AAR02418), CrOMT6 (AAR02419), CrOMT7 (AAR02422), 16OMT (Ef444544). The 8 peptides (1 to 8) obtained by by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry are indented in the figure and the 2 peptides used to design the primers used to amplify the unique PCR product [CrOMT7/CrOMT6 (425-786 bp)] highlighted in Figure 2 are highlighted in red with red arrows indicating where the PCR product was generated. Highlighted in green are the 2 amino acid differences found between peptides 1 and 3.
Figure S4: Description of the expressed sequence tags obtained from extensive sequencing of a cDNA library produced from epidermis enriched mRNA isolated by CA of Catharanthus leaves. Full length 16OMT (16OMT ORF; Accession: Ef444544); ESTs: 160_E05 [16OMT (CROLF1NG_EX2_160_E05_26AUG2006_039; Accession:EF444545)]; 035_A12 [16OMT (CROLF1NG_EX2_035_A12_14APR2006_096; Accession: Ef444546)]; 051_H10 [16OMT (CROLF1NG_EX2_051_H10_23JUN2006_066; Accession: Ef444547)]; 018_A04 [16OMT (CROLF1NG_EX2_018_A04_09APR2006_032; Accession: Ef444548)]