# Structure-function relationships of wheat flavone *O*-methyltransferase: Homology modeling, site-directed mutagenesis and regulation

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**A Thesis**

**in**

**The Department**

**of**

**Biology**

**Presented in Partial Fulfillment of the Requirements**

**for the Degree of Master of Science (Biology) at**

**Concordia University**

**Montreal, Quebec, Canada**

**February 2011**

**CONCORDIA UNIVERSITY**

**SCHOOL OF GRADUATE STUDIES**

This is to certify that the thesis prepared

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Entitled: **Structure-function relationships of wheat flavone *O*-methyltransferase: Homology modeling, site-directed mutagenesis and regulation**

and submitted in partial fulfillment of the requirements for the degree of

**MASTER OF SCIENCE (Biology)**

complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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# Abstract

## Structure-function relationships of wheat flavone *O*-methyltransferase: Homology modeling, site-directed mutagenesis and regulation

**Francesca Kanapathy-Sinnaiaha**

Wheat *(Triticum aestivum) O*-methyltransferase (TaOMT2) catalyzes the sequential methylation of the flavone, tricetin (5,7,3',4',5'-pentahydroxyflavone) to its 3'-methyl-(selgin), 3',5'-dimethyl-(tricin) and 3',4',5'-trimethyl ether derivatives, although tricin is the major product of this reaction. The novelty of TaOMT2 to perform three sequential methylations of tricetin as a substrate, the chemopreventive properties of its major product, tricin, and the compelling interest in the protein's structure-function relationships, prompted us to further investigate this novel protein at the biochemical, molecular and structural levels. A 3-D model of this protein was constructed using the crystal structure of the highly homologous *Medicago sativa* caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (MsCOMT) as a template with the aim of proposing a mechanism for multiple methyl transfer reactions in wheat. Homology modeling experiments in which each of the substrates tricetin, selgin and tricin, was docked into the model revealed a number of amino acid residues putatively involved in substrate binding and catalysis. Results suggest that substrate binding is mediated by an extensive network of H-bonds and van der Waals interactions. Mutational analysis of structurally-guided active site residues identified those involved in binding and catalysis. A possible reaction mechanism is discussed.

The biological significance of this methylation reaction was also investigated by analyzing its expression, enzyme activity patterns at different wheat developmental

stages, in response to cold acclimation and to different abiotic stresses such as salt and drought. Results show that TaOMT2 predominantly accumulates in wheat influorescences compared to leaves, coinciding with the increased methyltransferase activity in the influorescence tissues. The effect of abiotic stresses on wheat reveals that TaOMT2 accumulates in cold-acclimated winter wheat leaves. In contrast, TaOMT2 activity with tricetin as a substrate shows a tendency to decrease during cold acclimation. Other abiotic stresses, such as salt and drought have no effects on TaOMT2 accumulation in wheat leaves, but a slight decrease in activity. The importance of tricetin methylation during developmental stages and during abiotic stresses is discussed.

# Acknowledgements

I wish to express my deepest gratitude to Dr. Ragai Ibrahim whose expertise, patient guidance and vast knowledge added considerably to my graduate experience. His inspiration and his easiness in explaining things clearly and simply helped me to appreciate this field of study. His promptness, sharpness and the talent to observe things in different perspectives were the qualities I admired a lot.

I would like to thank Dr. Fathey Sarhan who gave me a wonderful opportunity to work in his lab at UQAM. His scientific intuition and smartness has made him a constant oasis of ideas that inspired a lot of new projects. I thank him for the trust he had in me to pursue some of these projects.

I am very greatful to Dr. Jian Min-Zhou for helping me with the *TaOMT2* project. Since the beginning of my graduate studies, he has been very supportive. He shared his knowledge and trained me in different lab skills. His humble character and his vast knowledge were among the qualities I admired the most in him.

I would like to thank Dr. Yoongho Lim for the modeling work, Dr. Zahra Agharbaoui for providing samples of wheat leaf exposed to salt and drought stresses.

I am indebted to all fellow graduate students and colleagues in the Biology departments at Concordia and UQAM. I'd like to thank Amira Moheb. Her perseverence, her hard work and her ability to quickly adapt fields outside her background inspired me a lot. I'm also indebted to Khalil Kane. His advice, his training in lab skills helped me during my graduate studies. His generosity in sharing his knowledge and experience were among his best qualities.

My thanks go to Amadou Diallo, Melanie Grondin, Wilfrid Saron for their valuable advice and help; Ming Sun, Yero Seydi, Ada Harold for their wonderful friendship and support.

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# C LIST OF ABBREVIATIONS

| | |
|---|---|
| TMeT | Trimethyltricetin |
| 4CL | 4-Coumarate: CoA ligase |
| AEOMT | Acid/ester *O*-methyltransferase |
| C4H | Cinnamate-4-hydroxylase |
| CCOMT | Caffeoyl CoA *O*-methyltransferase |
| CHS | Chalcone synthase |
| CoA | Coenzyme A |
| COMT | Caffeic acid/5-hydroxyferulic acid *O*-methyltransferase |
| DFR | Dihydroflavonol reductase |
| F3H | Flavanone 3-hydroxylase |
| FNS | Flavone synthase |
| FLS | Flavonol synthase |
| OMT | *O*-Methyltransferase |
| IFS | Isoflavonoid synthase |
| PAL | Phenylalanine ammonia-lyase |
| pI | Isoelectric point |
| SAM | *S*-Adenosyl-L-methionine |
| SDS-PAGE | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis |

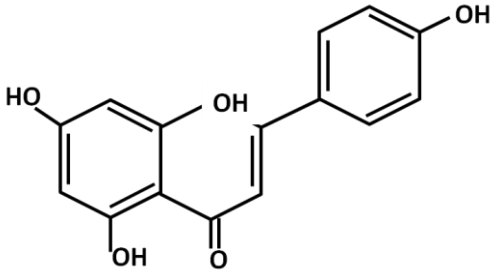
## D INTRODUCTION

During evolution, plants acquired the ability to synthesize a large variety of small molecular-mass organic compounds collectively known as secondary metabolites. These can be classified into several groups, of which flavonoids constitute one of the major classes. Flavonoids consist of two aromatic ring systems, A and B, which are connected by a heterocyclic ring C (Figure 1). They exhibit a variety of functions; being involved in plant-microorganism interactions; act as shields against UV radiation, as flower pigments that attract pollinators, to mention only a few (Bohm, 1998 and refs therein).

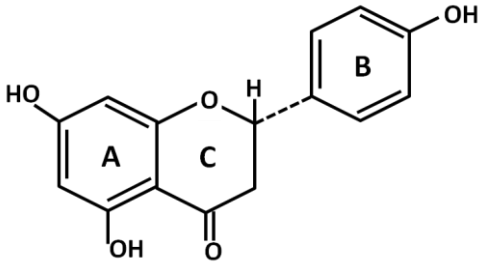
Based on the oxidation level of the C-ring, flavonoid compounds are classified into several groups, most important of which are the chalcones, flavanones, flavones, flavonols, isoflavones and anthocyanidins (Figure 1). In addition to their wide occurrence, flavonoids are also recognized for their health promoting properties. Several studies revealed their functional roles as antioxidants, radical scavengers, antiviral and anti-inflammatory agents as well their potential anticarcinogenic activities (Middleton *et al.*, 1994 and refs therein).

Enzymatic and chemical substitution reactions contribute to the structural and functional diversity of flavonoid compounds. These include glycosylation, acylation, hydroxylation, methylation and prenylation that take place mostly on the phenolic rings (Ibrahim and Anzellotti, 2003 and refs therein). Enzymatic *O*-methylation, which is catalyzed by a large family of *O*-methyltransferases (OMTs) plays an important role in reducing the toxicity and chemical reactivity of their phenolic hydroxyl groups and increasing their lipophilicity; and hence modulates their compartmentation and antimicrobial activity (Middleton *et al.*, 1994 and refs therein).

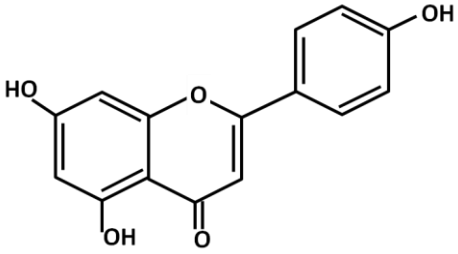
**Figure 1 The major classes of flavonoids**



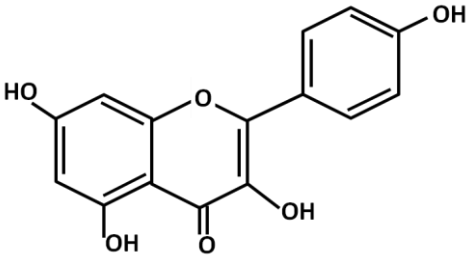

**Chalcone**



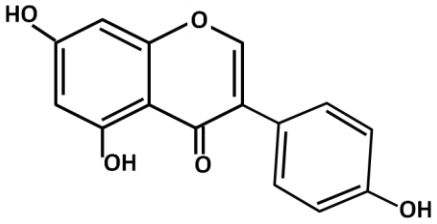

**Flavanone**



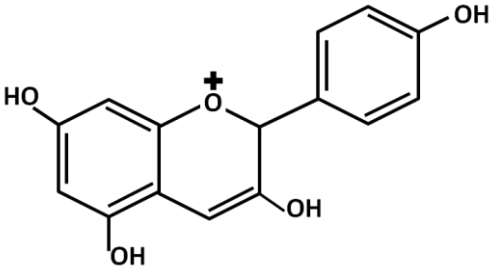

**Flavone**




**Flavonol**




**Isoflavone**




**Anthocyanidin**

*O*-Methylation of flavonoids is catalyzed by a family of *S*-Adenosyl-L-methionine (SAM)-dependant methyltransferases (OMTs), which catalyzes the transfer of the methyl group of SAM to an appropriate acceptor molecule with the concomitant formation of the corresponding methyl ether derivative and *S*-Adenosyl-L-homocysteine (SAH), as products. The latter reaction product often acts as a competitive inhibitor of the enzyme reaction.

Phenolic metabolites participate in the plant's protection against various abiotic stresses, e.g. cold which is a predominant factor that affects various crop plants in Canada. Winter survival and crop productivity are influenced by many different winter stresses, such as freezing temperature, length of the freezing period, ice encasement and flooding (Fowler et al., 1999). Wheat (*Triticum aestivum* L.), a prevalent crop in Canada, has developed characteristics to circumvent these stresses through a mechanism known as 'cold acclimation' (CA) which includes the expression of certain cold-induced genes that function to stabilize membranes against freeze-induced injury (Thomashow et al., 1999).

In a recent study to identify the genes involved in cold acclimation and associated stresses, a large-scale EST sequencing approach was conducted by the Functional Genomics Abiotic Stress (FGAS) project, through the joint efforts of Professors F. Sarhan (Université du Québec à Montréal) and P. Gulick (Concordia University). Using the published *Arabidopsis thaliana* *O*-methyltransferase 1 (AtOMT1) sequence (Muzac et al., 2000), Zhou et al., have searched for an OMT-like clone in the FGAS cDNA database. Of the several OMT-like clones obtained, one full-length clone exhibited the

highest score and contained all of the plant OMT signatures (Ibrahim et al., 1998). The clone was isolated and expressed in *E.coli*, and its gene product (TaOMT2) was used for further analysis (Accession number ABB03907). It was shown that the recombinant protein had a pronounced preference towards tricetin (5,7,3',4',5'-pentahydroxyflavone) as substrate, being converted to its monomethyl- (selgin, 5,7,4',5'-tetrahydroxy-3'-methoxyflavone), dimethyl- (tricin, 5,7,4'-trihydroxy-3',5'-dimethoxyflavone) and trimethyl- (TMeT, 5,7-dihydroxy-3',4',5'-trimethoxyflavone) ether derivatives, with tricin being the major enzyme reaction product (Zhou et al., 2006) (Figure 2). This enzyme was then selected for further study.

In contrast with mammalian methyltransferases, plant OMTs exhibit attenuated substrate specificities. The sequential methylation of flavonols reported in *Chrysosplenium americanum* (Saxifragaceae) (Ibrahim et al., 1987) and of the volatile phenolic derivatives in rose petals (Lavid et al., 2002; Wu et al., 2003) were catalyzed, in a stepwise manner, by a number of substrate-specific and position-oriented OMTs. Examples of multiple methylations catalyzed by single enzymes were reported for the mammalian phosphatidylethanolamine (Walkey et al., 1996), plant phosphoethanolamine (Charron et al., 2002) and viral histone *N*-methyltransferase (Qian et al., 2006). The novelty of TaOMT2 lies in the fact that it is capable of catalyzing a sequence of three methylations of tricetin resulting in three different products.

In contrast with the widespread occurrence of flavonoids in plants, the pentahydroxy flavone tricetin and its dimethyl ether derivative, tricin are reported to occur sporadically; and mostly in unrelated families (Wollenweber and Dörr, 2002).

Tricetin was reported to occur together with other flavonoid aglycones in the Myrtaceae pollen (Campos, et al. 2002) and is considered as a marker of eucalyptus honey (Martos et al., 2000). Tricin has been reported to possess several potential health beneficial effects. The presence of tricin in cereal grains, especially the husks and bran, was shown to inhibit the growth of human malignant breast tumour cells and colon cancer cells (Hudson et al., 2000; Cai et al., 2004). The fact that TaOMT2 performs multiple methylations of the same substrate, with tricin being the major reaction product, prompted us to investigate its molecular structure in relation to the site(s) of substrate binding and the methyl transfer reactions.

## E.1. AIM OF THE WORK

This work derives from the recent isolation and characterization of a novel flavone *O*-methyltransferase cDNA clone (*TaOMT2*) from a wheat cDNA library (Zhou et al., 2006). The novelty of TaOMT2 to perform three sequential methylations of tricetin as a substrate, the chemopreventive properties of its major product, tricin, and the compelling interest in the protein's structure-function relationships, prompted us to further investigate this novel protein at the biochemical, molecular and structural levels. The aims of the present work are summarized as follows:

### *E.1.1 To investigate the molecular structure of TaOMT2*

Since several attempts to crystallize TaOMT2 resulted in crystals with unsatisfactory diffraction, we resorted, therefore, to study its 3-D structure by the 'homology modeling' approach, using the crystal structure of *Medicago sativa* caffeic

acid/5-hydroxyferulic acid OMT (MsCOMT; Zubieta et al., 2002) as a template. Both proteins share 63% sequence identity. A 3-D structure of TaOMT2 was obtained through structural homology modeling by courtesy of Prof. Y. Lim (Konkuk U, Rep. of Korea). Modeling experiments in which each of the substrates, tricetin, selgin and tricin, was docked into the model revealed a number of amino acid residues putatively involved in substrate binding and catalysis. These were selected for site-directed mutagenesis and the derived proteins were assayed for enzyme activity in order to evaluate their structural and functional significance. These analyses will help to elucidate a putative reaction mechanism of TaOMT2 for the sequential methylation of tricetin.

***E.1.2. To study the activity and expression of TaOMT2 in wheat leaves and influorescences.***

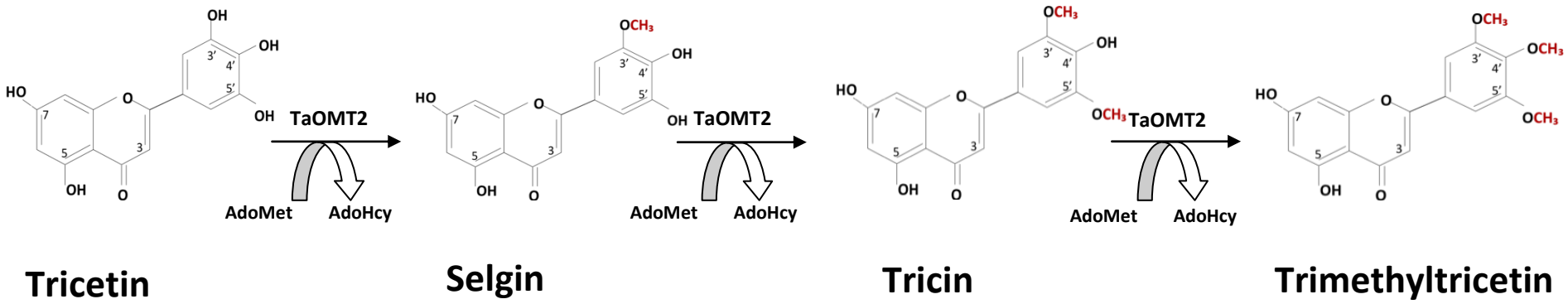
Since it was recently shown that tricin accumulates predominantly in wheat influorescences (Amira Moheb, personal communication), and that TaOMT2 synthesizes tricin as the major product of tricetin methylation, it was preponderant, therefore, to investigate its presence in influorescence tissues.

***E.1.3 To study the regulation of wheat methyltransferases, and specifically TaOMT2 in relation to cold acclimation and other abiotic stresses.***

Given the importance of methyltransferases in the biosynthesis of lignin and other phenolic compounds (Charron et al., 2002), it was considered important to study the regulation of OMTs, and specifically TaOMT2, in relation to cold acclimation and other abiotic stresses, such as salinity and drought. The aim of this study was to determine the

activity of total OMTs acting on the endogenous phenolic compounds, including tricetin, during abiotic stresses. In order to achieve this goal, we resorted to immunoblotting of TaOMT2 and enzyme activity assays. Considering the scarcity of published work on TaOMT2, and the evidence of the potential beneficial properties of tricin on mammalian cells, it renders it an interesting enzyme to study with the ultimate goal of its use in metabolic engineering.

**Figure 2** ***O*-Methylation of tricetin by TaOMT2**



## E.2. LITERATURE REVIEW

Flavonoid compounds constitute one of the major groups of plant secondary metabolites. Even though termed “secondary metabolites”, flavonoids play important roles in plant growth and development, its interaction with the environment, in defense mechanisms against pathogen and insect attacks, and a variety of wounding and abiotic stresses. Recent advances have contributed an added impulse with the discovery of their potential benefits to humans as bases for pharmaceuticals and nutraceuticals (reviewed in Tapas et al., 2008).

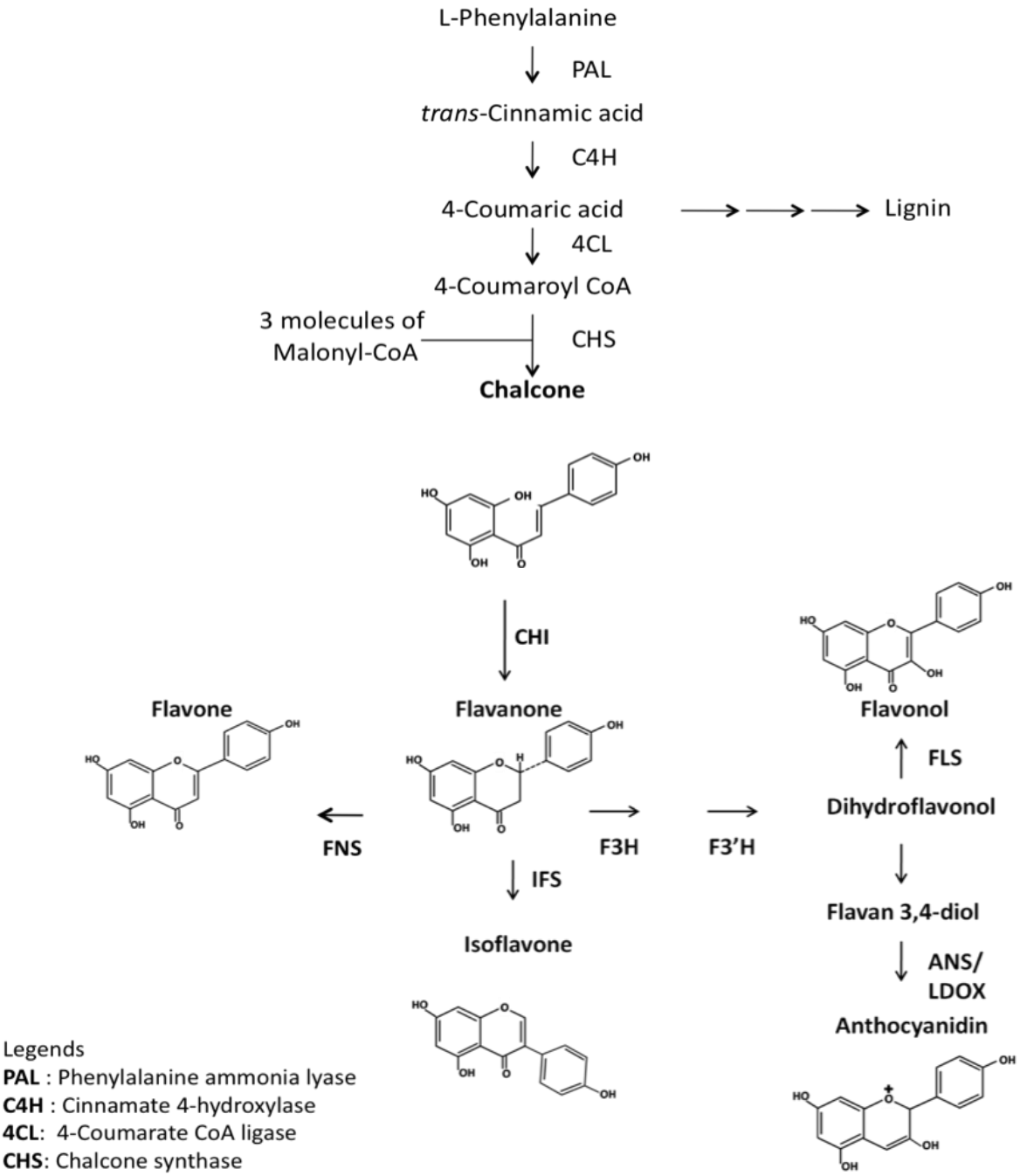
This review will encompass a summary of the major steps of phenylpropanoid and flavonoid biosynthetic pathways. Various substitution reactions of flavonoid compounds contribute to their structural and functional diversity. In this review, a special emphasis will be given to *O*-methylation reaction mechanisms. These discussions include phenylpropanoid- and flavone *O*-methyltransferases, and their implications in genetic manipulations. The review will highlight the importance of methylated phenolic compounds, their implications in health-promoting properties and their potential uses in human welfare and disease therapies. Finally, the review will end with a summary of the effects of cold acclimation and abiotic stresses such as salt and drought stresses on plants.

### E.2.1. Biosynthesis of flavonoids

Flavonoids consist of two aromatic rings, A and B that are connected by the heterocyclic ring-C. Both aromatic rings derive from two different pathways: ring A, from the head-to-tail condensation of three acetate units (derived from the malonate/acetate pathway), whereas ring B and the attached 3-C side chain are derived from L-phenylalanine *via* the shikimate pathway. The pathway of flavonoid biosynthesis was described in detail by Forkmann and Heller (1999). Briefly, it starts with the conversion of the aromatic amino acid L-phenylalanine to *trans*-cinnamic acid by phenylalanine ammonia lyase (PAL, EC 4.3.1.5), which is then hydroxylated at the 4-position by cinnamate 4-hydroxylase (C4H, EC 1.14.13.11). 4-Hydroxycoumaric acid is then converted to 4-coumaroyl CoA by 4-coumarate CoA ligase (4CL, EC 6.2.1.12). Chalcone-synthase (CHS, EC 2.3.1.74) catalyzes the stepwise condensation of 4-coumaroyl CoA with three molecules of malonyl CoA to yield a chalcone, the central intermediate in the pathway and the precursor of all flavonoids. Chalcone isomerase (CHI, EC 1.14.11.9) catalyses the closure of the heterocyclic ring C, with the formation of the flavanone, naringenin. The latter serves as the branch-point intermediate for all other classes of flavonoids, including flavones, flavonols and isoflavones. Flavanones can be hydroxylated at position 3 by flavanone 3-hydroxylase (F3H, EC 1.14.11.9) leading to the formation of dihydroflavonols, flavonols and anthocyanidins. Flavones are synthesized by flavone synthase (FNS) by the removal of H-atoms from positions 2 and 3 of flavanones. Desaturation of 3-hydroxy flavanones by flavonol synthase (FLS, EC 1.14.11.23) yields flavonols. Reduction of dihydroflavonols by dihydroflavonol reductase (DFR, EC 1.1.1.219) results in the formation of leucoanthocyanidins, the precursors of all

anthocyanidins responsible for the diverse vibrant colors of flowers. Flavanones can also be subjected to 2-hydroxylation, followed by dehydration and aryl migration of the B-ring to C-3 (Figure 1), catalyzed by isoflavonoid synthase (IFS, E.C. 1.14.13.86), thus resulting in the formation of isoflavones. These reactions are depicted in figure 3.

**Figure 3** **Flavonoid biosynthetic pathway**




Legends
**PAL** : Phenylalanine ammonia lyase
**C4H** : Cinnamate 4-hydroxylase
**4CL**: 4-Coumarate CoA ligase
**CHS**: Chalcone synthase
**CHI**: Chalcone isomerase
**F3H**: Flavanone 3-hydroxylase
**FNS**: Flavone synthase
**FLS**: Flavonol synthase
**DFR**: Dihydroflavonol reductase
**IFS**: Isoflavonoid synthase

### E.2.2. Substitution reactions of flavonoids

Enzymatic substitutions are common reactions that occur on phenolic compounds; they have been reviewed by Ibrahim and Anzellotti (2003). Modifications of flavonoids may include hydroxylation, glycosylation, (iso)prenylation, acylation, and/or methylation. These reactions are catalyzed by substrate-specific and position-oriented enzymes, and contribute to the enormous variety of flavonoid compounds and, hence, to their wide spectrum of functional roles in plants. The occurrence of the phenolic compounds and their versatility of functions involved in the response to various stresses, are somehow dependent on the various modification reactions of flavonoids catalyzed by these enzymes. Even though, these enzymes are not mutually exclusive in function, they may act in a sequential manner, with one modification determining future substitution events.

### E.2.3. *O*-Methylation

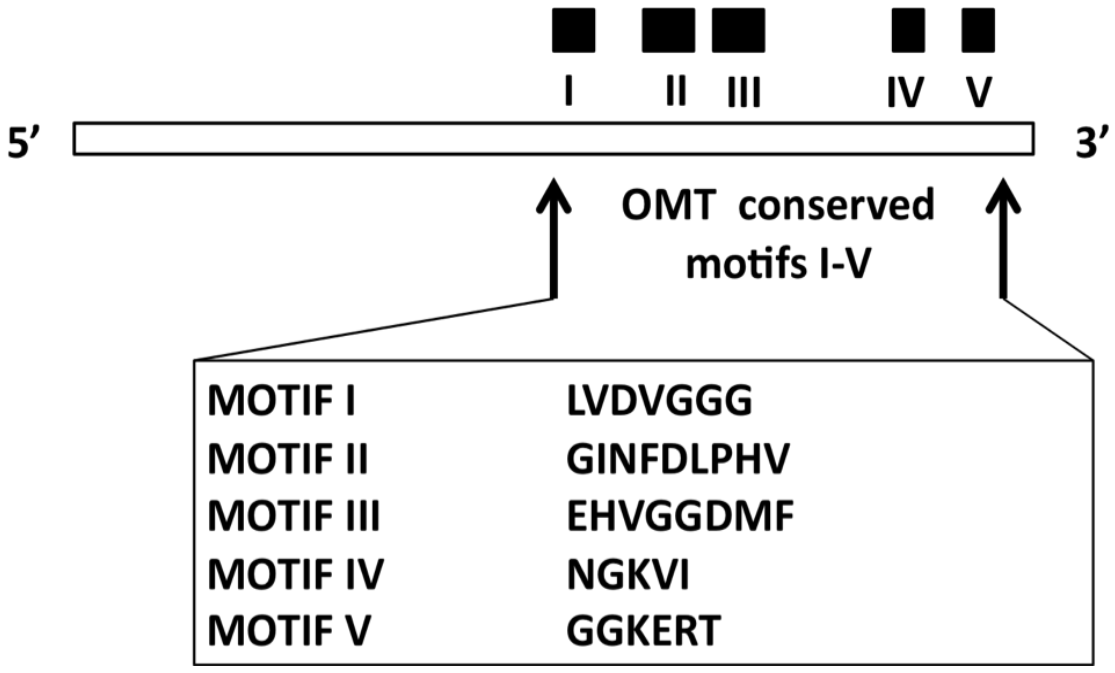
Among the various substitution reactions that occur on flavonoids, *O*-methylations has attracted special attention due to its ubiquity in flavonoid biosynthesis and the various roles methylated products play in plant growth and development. *O*-Methylation of flavonoids is essential for reducing toxicity of their reactive hydroxyl groups. In addition, it increases lipophilicity, thus helping the methylated products to pass through cell membranes, thus enabling internal compartmentation. Although the chemical mechanisms of methyl group transfer reactions are identical, and their enzymes share high amino acid sequence similarity, OMTs are both substrate- and position- specific enzymes. In fact, although phenylpropanoids and flavonoids share some structural similarity, where the phenolic B-ring and its 3-carbon side chain of flavonoids are derived from phenylpropanoids, *O*-methylation of both groups of compounds is catalyzed by distinct *O*-methylatransferases. Moreover, position-specific methylation has been well demonstrated in the biosynthesis of polymethylated flavonols in *Chrysosplenium americanum* (Ibrahim et al., 1987). A recent account of the various OMT cDNA clones involved in methylation of phenolic compounds has been reported (Akashi et al., 2003, Lam et al., 2007, Lavid et al., 2002, Scalliet et al., 2002).

### E.2.4. Classification of *O*-Methyltransferases (OMTs)

The extensive plant OMT sequence database has made a profound impact and a valuable contribution to the biology research community. Several cDNA clones have been isolated from both angiosperms and gymnosperms: parsley (Schmitt et al., 1991), tobacco (Jaeck et al., 1996), alfalfa (Gowri et al., 1991), to mention only a few. Subsequently, organization of these sequence information became a necessity. The combination of molecular biology and conventional biochemical approaches provided a powerful approach to classify the proteins encoded by these sequences. Several research groups embarked in classifying OMTs. Among several proposals, three were recognized as being the most accepted and well represented in classifying OMT cDNAs.

The first proposal, by Ibrahim et al. (1998), was based on thirty-six cDNA clones belonging to several angiosperm families, both monocotyledons and dicotyledons and two conifer species, as well as representatives of bacterial, fungal, bovine, rat and human sequences. These OMTs included clones involved in lignin precursors methylation, flavonoid methylation and other types of methylation. Ibrahim et al. (1998) suggested that all plant OMTs have diverged from a common ancestral gene that led to the evolution of the various functional OMTs. They also reported that five regions (I-V) near the carboxy terminal end of the OMT protein sequence, comprising 36 amino acid residues and rich in glycine, were highly conserved (92-100%) among the majority of plants. Region I is thought to be involved in SAM binding and region IV, in metal binding.

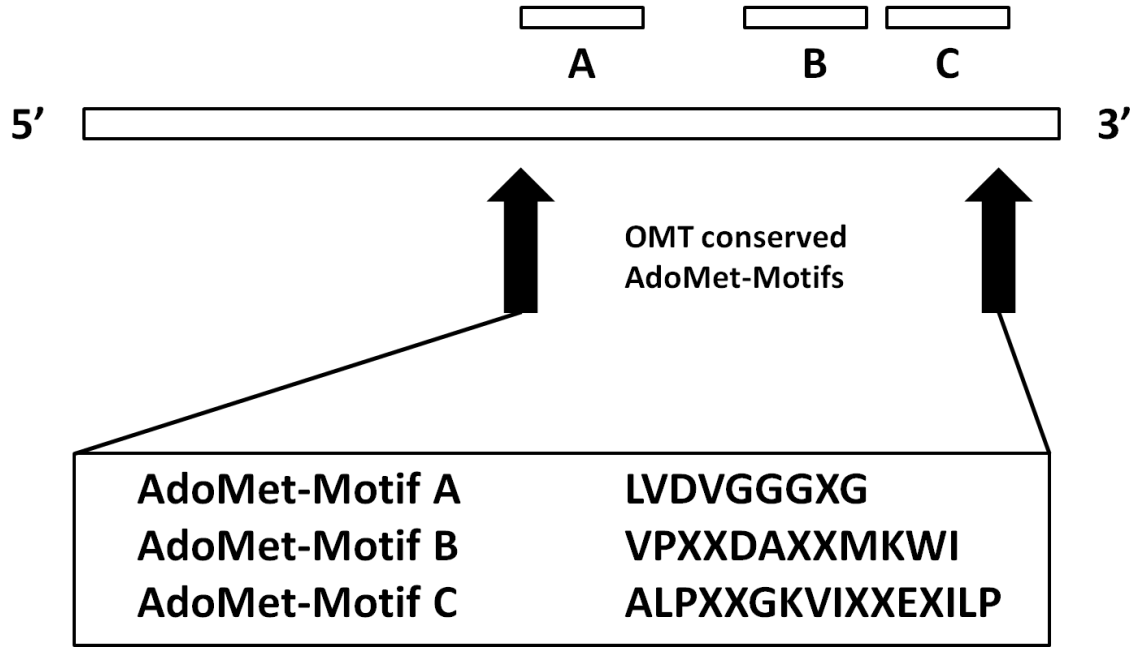
**Figure 4 Five conserved OMT motifs (adapted from Ibrahim *et al.*, 1998)**



The second proposal by Joshi and Chiang (1998) was based on sequence alignment and phylogenetic analysis of fifty-six plant OMT clones representing various species. The OMTs were classified into two groups, the PIOMT I and PIOMT II, according to their distinct substrate preferences and amino acid sequence homology. The first group contains OMTs that methylate caffeoyl and 5-hydroxyferuloyl CoAs (CCoAOMTs). These enzymes require $Mg^{2+}$ for activity; they exhibit a smaller molecular mass similar to the mammalian catechol OMTs (ca. 27 kDa). The PIOMT II group constituted OMTs that have wide range of substrate preferences, including phenylpropanoids, flavonoids and alkaloids. In contrast to the first group, they do not require any cofactors for enzymatic activity, but exhibit a higher molecular mass of ca. 40 kDa. Sequence alignments of the 56 genes revealed three signature motifs (A, B and C), a putative SAM binding motif and five additional motifs (D, E, F, G and H) that may serve as CCoAOMT signatures (Figure 5).

The third proposal reported by Zubieta et al. (2003) was based on the amino acid sequence and structural studies of several OMTs that were categorized into three subfamilies. The first subfamily consisted of phenylpropanoid and flavonoid OMTs, where methyl transfer is catalyzed by a histidine-based active site for methionine deprotonation (Zubieta et al., 2003). The second subfamily consisted of OMTs methylating caffeoyl and 5-hydroxyferuoyl CoAs with an $Mg^{2+}$ requirement. The third group comprised enzymes that convert carboxylic acids to their methyl ether derivatives (Zubieta et al., 2003).

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



### E.2.5. Phenylpropanoid OMTs

Lignin is a complex of phenylpropanoid polymer, deposited as a secondary wall on plant cells that provide an impervious, rigid structure to the tracheary elements for support against the negative pressure generated from transpiration, and acts as a defense barrier against wounding and pathogen attack (Boudet et al., 1995). The most common monolignols (building blocks) of lignins are guaiacyl and syringyl polymers that originate from ferulic acid and sinapic acid, respectively. Methylation of caffeic acid and 5-hydroxyferulic acid at positions 3 and 5, respectively, produce ferulic acid and sinapic acid respectively (reviewed in Dixon et al., 2001).

The fact that phenylpropanoids are precursors of the monolignols and that the guaiacyl-syringyl ratio is related to lignin degradability as a pulping material, and grass digestibility by ruminants (Whetten and Sederoff, 1991), prompted a number of studies that focused on phenylpropanoid OMTs. Genetic manipulation of these enzymes could lead to a better yield and quality of the lignin polymer.

A number of COMT cDNA clones were isolated from different plant species; both from angiosperms and gymnosperm (reviewed by Lam et al 2007). Comparison of these proteins revealed that they were similar in size (ca. 40-45 kDa), $Mg^{2+}$ -independent and highly similar in amino acid sequence (80-95%).

Due to the similarity in structure of caffeic acid and 5-hydroxyferulic-acid, it was believed that their methylation was catalyzed by the same OMT. However, De Carolis and Ibrahim (1989) purified two isoforms of COMT (COMT I and COMT II) from

cabbage leaves and demonstrated that each isoform had highest affinity for its own substrate, thus resulting in different ferulic/sinapic acid ratios. Unfortunately, no cloning of the corresponding genes was performed at the time.

### E.2.6. Caffeoyl CoA OMTs

Feruloyl CoA and Sinapoyl CoA are the precursors of the respective lignin monomers, guaiacyl (G) and syringyl (S) residues. These two precursors derive from the methylations, catalyzed by caffeoyl CoA OMT (CCoAOMT) and 5-hydroxyferuloyl CoA OMT (5HFCoAOMT) at positions 3 and 5 respectively.

The first CCoAOMT cDNA was isolated from parsley cell cultures treated with a crude fungal elicitor (Schmitt et al., 1991). This class of enzymes has a molecular weight of 33 kDa and $Mg^{2+}$ requirement for activity (Schmitt et al., 1991). Even though CCoAOMT is specific to caffeoyl CoA; it was demonstrated by Ye et al. (1994) that it can also accept 5-hydroxyferulic acid to the same extent. Moreover, the fact that CCoAOMT mRNA and enzyme activity were specifically associated with the process of lignification during tracheary element formation from *Zinnia* cultured cells, suggested that CCoAOMT group of enzymes may act as an alternative pathway for lignin biosynthesis (Ye et al., 1994; Zhong et al., 1998). This was explained by the fact that the central branch point for lignin synthesis starts from *p*-coumaroyl CoA, instead of p-coumaric acid, via its CoA ligase. It was also demonstrated that AEOMT (acid/ester *O*-methyltransferase) cDNA from developing secondary xylem of loblolly pine encodes a multifunctional OMT with both caffeic acid OMT (COMT) and CCoAOMT activities, catalyzing efficient methylation of caffeic acid, caffeoyl CoA, 5-hydroxyferulic acid and

5-hydroxyferuloyl CoA (Li et al., 1999). Chiron et al (2000) demonstrated the promiscuity of an OMT isolated from Scots pine capable of methylating a wide range of substrates: caffeic acid, caffeoyl CoA, 5-hydroxyferulic acid, astrigenin, resveratrol, catechol, quercetin and luteolin.

CCoAOMT was also shown to be involved in the plant defense mechanisms. In fact, Matern et al. (1998) showed that the activity of CCoAOMT in cell suspension culture of *Vitis vinifera* L. increased upon treatment with fungal elicitors. Other studies have demonstrated the increased activity of CCoAOMT in response to elicitors in cell suspension cultures (Kneusel et al., 1989; Kühnl et al., 1989; Schmitt et al., 1991; Ni et al., 1996; Busam et al., 1997), thus demonstrating the importance of this class of enzymes in lignin synthesis in response to elicitors.

### E.2.7. Flavonoid OMTs

Flavonoid methylation occurs on all available hydroxyl groups on ring A, B and C (reviewed in Ibrahim and Anzellotti, 2003). The first report of flavonoid OMT activity was in tobacco cell suspension (Tsang and Ibrahim, 1979). The preferred substrates at different extents were two hydroxycinnamic acids (caffeic/5-hydroxyferulic acid), two coumarins (daphentin and esculetin) and two flavonoids (quercetin and luteolin). The two forms of OMTs isolated, partially purified and biochemically characterized from the tobacco cell cultures were OMT1, catalyzing the *meta* methylation of caffeic acid and OMT II, catalyzing the *para* methylation of quercetin. OMT I exhibits a molecular

weight of 74kDa (presumably a dimer), a pH optimum of 7.3 and a *pI* value of 6.1. OMT II instead exhibits a molecular mass of 70 kDa, a pH optimum of 8.3 and a *pI* value of 6.3; thus indicating two distinct enzymes.

In rice, a functionally characterized OMT was reported by Rakwal et al., (2000). The flavonoid 7-OMT was involved in biosynthesis of the phytoalexin, sakuranetin. Following that, Kim et al. (2006) reported the characterization of another flavonoid OMT in rice: ROMT-9, which transfers the methyl group to the 3'-OH group of quercetin. ROMT-9 is the first flavonoid 3'-OMT cloned in rice.

Another flavonol OMT was characterized from *Catharanthus roseus*. A CrOMT2 was identified as a flavonoid OMT that was expressed in dark-grown cell cultures and co-purified with 16-hydrotabersonine (HT) OMT, involved in indole alkaloid biosynthesis. Surprisingly, substrate preference studies indicated that while the protein was inactive with 16-HT, it catalyzed two sequential methylations at the 3'- and 5' positions of the B-ring in myricetin (a flavonol) and dihydromyricetin (a dihydroflavonol) (Schröder et al., 2003). A cDNA clone encoding the gene for a 3'/5'-*O*-methyltransferase of partially methylated flavonols was isolated in *Crysosplenium americanum* (Gauthier et al., 1996)

Moreover, there had been two reports of chalcone 2'-OMT cDNA clones isolated from alfalfa (Maxwell et al., 1993) and licorice (Haga et al., 1997). This OMT performs the methylation of the 2'-OH of isoliquiritigenin (2', 4', 4-trihydroxychalcone) to form 4', 4-dihydroxy-2'-methoxychalcone, a potent nod-gene inducing flavonoid derivative released from alfalfa roots (reviewed in Lam et al., 2007).

### E.2.8. Functions of *O*-methylated flavonoids in plants

The biodiversity of phenolic compounds and their specific OMTs resulted in a wide array of methylated products encompassing a diversity of functions related to plant growth, reproduction and its interaction with the environment; examples of which abound. Polymethylated flavones and flavonols act as phytoalexins against certain microorganisms, during pathogen attack (Iwashina, 2003 and refs therein). Six compounds were isolated from the aerial parts of *Helicrysum nitens* (Compositae) as fully methylated flavones and flavonols, 5,7-dimethoxyflavone, baicalein trimethyl ether, galangin trimethyl ether, 5,6,7,8-tetramethoxyflavone, 3,5,6,7-tetramethoxyflavone, 3,5,6,7,8-pentamethoxyflavone which inhibited the growth of *Cladosporium cucumerinum* (Tomas-Barberan et al., 1988a, 1988b). In addition, the fully methylated flavones, nobiletin and tangeretin, isolated from leaves of *Citrus spp.*, displayed antifungal activity against *Deuterofoma tracheiphila* which is responsible for the highly destructive citrus disease known as Malsecco (Piattelli and Impellizzeri, 1981; Pinkas et al., 1968). Plants synthesize and accumulate phytoalexins not only during exposure to microorganisms but also to physical and chemical stress (Deverall, 1982). Sakuranetin (5, 4'-dihydroxy-7-methoxyflavanone) accumulates in uv-irradiated (Kodama et al., 1992), and in $CuCl_2$- and jasmonic acid-treated rice leaves (Rakwal et al., 1996). Furthermore, naringenin (5, 7, 4'-trihydroxyflavanone)-specific 7-OMT which yields sakuranetin, was found to accumulate in uv-irradiated rice leaves (Rakwal et al., 2000). Other examples underlining the function of methylated compounds during chemical and physical stresses were reported in tobacco, where seven methylated flavonols were

identified in the glandular trichomes after elicitation with methyl jasomonate, herbivore attack and uv-C exposure, being secreted on the leaf surface (Roda et al., 2003).

### E.2.9. Potential uses of methylated flavonoids for human welfare

Dietary flavonoids have many potential biological properties that can be used as chemopreventive and anti-inflammatory agents to mention a few (Middleton et al., 2000). One of the limiting factors is their poor bioavailability, due to glucuronidation and/or sulfation of mono- or polyhydroxylated flavonoids in the intestinal/hepatic barrier (Otake et al., 2002). Recent studies have shown the methylated flavones, 5, 7-dimethoxyflavone and 3', 4'-dimethoxyflavone, were metabolically stable in the human liver and, therefore, more bioavailable compared to the non-methylated flavones, galangin (3, 5, 7-trihydroxyflavone; Wen and Walle, 2006). In addition, methylated flavonoids have been reported to be involved in the inhibition of chemically induced cancer initiation and promotion. In fact, 3', 4'-dimethoxyflavone and 5,7-dimethoxyflavone possess a greater potential to inhibit enzymes involved in the bioactivation of the chemical cancer inducer, benzo-α-pyrene (BaP) than the non-nmethylated stilbene, resveratrol (Tsuji et al., 2006). The antiproliferative effects of polymethylated flavonoids were also demonstrated: 5, 7,4’-trimethoxyflavone was eight times more potent than the non-methylated derivative, apigenin (Walle, 2007). Tricin, a 5,7,4'-trihydroxy-3',5'-dimethoxyflavone, was shown to interfere with intestinal carcinogenesis in $Apc^{min}$ mice by inhibiting cyclooxygenase enzymes (Cai et al., 2005).

In addition, several examples of methylated flavonoids were reported to act as antimicrobial and antifungal agents. Four 3-methylated flavonols, ayanin, casticin,

chrysosplenol-D and 5,7,4'-trihydroxy-3,8-dimethoxyflavone were reported to possess antifungal activities in *Psiadia trinervia* (Wang et al 1989).

### E.2.10. Cold acclimation in wheat

Cold stress is one of the limiting environmental factors in crop productivity (Fowler et al., 1999). Plants have the ability to sense changes in the environment that signal the up-coming of winter and as a result, they exhibit an increase in freezing tolerance (Thomashow, 1999). Exposure to sublethal, non-freezing temperatures allows plants to acquire freezing and chilling tolerance, a process called cold acclimation (Thomashow, 1999; Chinnuswamy, 2006). This process induces a myriad of morphological, physiological and biochemical changes that will prevent damage induced by cell dehydration resulting from ice formation (Steponkus, 1984, Uemura et al., 1997), formation of reactive oxygen species and protein denaturation (McKersie et al., 1997).

Several studies have shown that a large number of genes are being altered during the process of cold acclimation (Thomashow, 1999). The genes could be classified into three groups. The first group includes genes encoding structural proteins involved in protecting the cells during low temperature stress. Plants perceive cold stress by changes in membrane fluidity and protein conformation. Cold stress-induced rigidification of the plasma membrane may lead to actin cytoskeleton rearrangement (Chinnuswamy, 2006). The second group represents genes that regulate gene expression and signal transduction pathways, such as transcription factors, protein kinases and enzymes involved in phosphoinositide metabolism. ICE (inducer of CBF expression), a transcription factor present in the cell under normal conditions, is activated by cold stress and subsequently

activates CBF (C-repeat binding factors) genes coding for other transcription factors. Downstream to this cascade, COR (cold responsive) genes (Wang et al., 1994) are transduced. The third group represents genes encoding enzymes involved in the biosynthesis of osmoprotectants, membrane lipids and those involved in antioxidative response. LEA (late embryogenesis abundant) proteins, hydrophilic simple proteins are capable of forming amphipathic alpha helices (Thomashow et al., 1999). Like COR-15, it was hypothesized that LEA and dehydrin polypeptide protect the cell membrane by decreasing the propensity of the cell to form hexagonal II phase transition, known to damage membranes during dehydration, by altering intrinsic curvitures of the inner membrane through their α-helices (Stepnokus et al., 1998). The Arabidopsis *FAD 8* gene which encodes a fatty acid desaturase (Gibson et al., 1994) contributes to freezing tolerance by altering lipid composition. To overcome the effects of reactive oxygen species, antioxidative mechanisms are enhanced (McKersie et al., 1997). Denaturation of proteins, induced by low temperatures, is corrected by the up-regulation of genes expressing molecular chaperones (Guy et al., 1999).

In the advent to study the role of methyltransferases during cold acclimation, Charron et al. (2002) analysed a gene product obtained from a cDNA library prepared from cold acclimated winter wheat. Their study revealed that this gene encodes an *N*-methyltransferase that is capable of catalyzing the three-step methylation of phosphoethanolamine to form phosphocholine, an important metabolite involved in low temperature stress (Harwood, 1999). Another study conducted by N'Dong et al. (2002) showed a cold regulated *O*-methyltransferase isolated from rye capable of methylating 7,8-dihydroxycoumarin (daphnetin) to 7-hydroxy-8-methoxycoumarin.

### E.2.11. Salt and drought stresses

Salinity and drought are among the major limiting factors restricting growth and development in plants. In particular, salinity can severely limit crop productivity (Boyer, 1982). Salt stress involves osmotic stress, by limiting absorption of water from the soil (ionic stress) producing detrimental effects on seed germination and plant growth (Mer et al., 2000). Protective mechanisms have evolved to circumvent the effects of these stresses. One of these is the synthesis and accumulation of low molecular weight metabolites, known as compatible solutes. Their main role is to increase the ability of cells to retain water without affecting normal metabolism (Hamilton & Heckathorn, 2001). Amino acids, sugars, quaternary ammonium compounds may accumulate as compatible solutes. Betaines, quaternary ammonium compounds (Rhodes & Hanson, 1993) and prolines (Serrano, 1995) are the most common nitrogen-containing compatible compounds. Sakomoto and Murata (2000) have shown that betaine stabilizes the quaternary structure of proteins and membranes during salinity and drought stresses. Soluble sugars also contribute to the regulation of ROS signaling as well as osmotic adjustments during abiotic stresses (Ristic et al., 1993).

Genes involved in signaling cascades and in transcriptional control, such as mitogen-activated protein- (MAP) (Shou et al., 2004) and salt overly sensitive (SOS) (Qiu et al., 2002) kinases, phospholipases (Thiery et al., 2004) have been extensively studied. Salt or drought stresses can cause denaturation of proteins. Heat shock proteins, molecular chaperones were shown to be involved during abiotic stresses (Wang et al., 2003).

## E.3. MATERIALS AND METHODS

### E.3.1. Plant material and growth conditions

Wheat (*Triticum aestivum* L. cv. Claire (winter) and cv. Bounty (spring)) grains were germinated in a soil-vermiculite mixture (1:1, w/w) for 7 days. One batch was transferred to environmental chambers at 4$^{o}$C (cold acclimated), and another batch at 20$^{o}$C (control plants) for various periods of time. Leaves of different developmental stages and floral spikes were used for enzyme activity assays and western blot analyses. Wheat leaves exposed to salt and drought stresses were obtained by the courtesy of Dr. Zahra A.

### E.3.2. Chemicals

Most flavonoid compounds were from our library collection, except tricetin was purchased from Indofine Chemical Company (Hillsborough, NJ). *S*-adenosyl-L-[$^{3}$H]-methionine (SAM; 60Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO), and unlabeled SAM and phenylmethylsulphonyl fluoride (PMSF) were from Sigma (Oakville, ON). Protein quantification reagents, 40 % acrylamide/bis solution (37:5:1), sodium dodecyl sulfate (SDS), glycine and non-fat dry milk were purchased from Bio-Rad (Mississauga, ON). All other chemicals were of analytical reagent grade, unless otherwise specified.

### E.3.3. Buffers

The following buffers were used: A, (extraction buffer) 50 mM Tris-HCl, pH 7.6 containing 14 mM ß-mercaptoethanol, 7 mM PMSF; B, (wash buffer) 50 mM $NaH_2PO_4$, pH 7.8 containing 300 mM NaCl, 20 mM Imidazole; C, (elution buffer) 50 mM $NaH_2PO_4$, pH 7.9 containing 300 mM NaCl, 250 mM imidazole; D, (protein assay buffer), 50 mM Tris-HCl (pH: 7.6), 300 mM NaCl; E, (assay buffer) as in D buffer but containing 10% glycerol (v/v); F, (blocking buffer), PBS containing 0.05% Tween 20 and 5% skim milk.

### E.3.4. Plasmid construction

DNA manipulations were performed using standard protocols (Sambrook et al., 2000). After sequence confirmation, the open reading frame (ORF) of TaOMT2 (Accession number ABB03907) cDNA was amplified and subcloned into the expression vector pET200/D-TOPO for in vitro protein expression. The primers used for PCR were:

TaOMT2F: 5′-CACCATGGGGTCGATCGCCGCCGGC;

TaOMT2B: 5′-CTACTTAGTGAACTCGATGGC.

To reinforce the reliability of TaOMT2 cDNA ORF sequence, AccuPrime*Pfx* DNA Polymerase (Invitrogen, Carlsbad, CA) was used in the PCR following the manufacturer's manual. PCR was performed on PTC DNA 200 Thermal Cycler (GMI, Ramsey, MI): 94 °C (2 min), followed by 35 cycles at 94 °C (30 s), 52 °C (30 s), 68 °C (1 min), and a further extension at 68 °C (8 min). The PCR product was resolved by electrophoresis on a 1% agarose gel. After electrophoresis, the expected DNA band was recovered with DNA Gel Extraction Kit (Qiagen, Mississauga, ON), and cloned into

pET200/D-TOPO expression vector (Invitrogen). The sequence and orientation of TaOMT2 ORF in the expression vector were confirmed by sequencing before chemically transformed into *E. coli* BL21 (DE3) cells (EMD, Darmstadt, Germany) for protein production. This construct was prepared by Dr. Jian-Min Zhou, and constituted the starting point for site-directed mutagenesis.

**E.3.5. Site-directed mutagenesis**

Variants of wild type TaOMT2 were engineered using the QuickChange site directed mutagenesis kit (Stratagene, CA) and the wild type cDNA in vector pET200/D-TOPO as template together with various mutagenic primers which (Table 1) introduced the desired amino acid change. PCR was performed on PTC DNA 200 Thermal Cycler (GMI, Ramsey, MI): 94 °C (2 min), followed by 35 cycles at 94 °C (30 s), 52 °C (30 s), 68 °C (1 min), and a further extension at 68 °C (8 min). The PCR product was resolved by electrophoresis on a 1% agarose gel to confirm the presence of the band in question. The PCR product was then digested with Dpn-1 restriction enzyme to cut the parental strands of the vector, therefore leaving only the mutated strands. The vector was then introduced in TOP10 *E.coli* cells to obtain more of the vectors containing the mutated insert. The sequence and orientation of the mutagenized plasmid DNA were confirmed by sequencing to ensure that no unexpected mutations were present before chemically transformed into *E. coli* BL21 (DE3) cells (EMD, Darmstadt, Germany) for protein production.

## Table 1. Construction of TaOMT2 variants

| Mutants | Primer sequence |
|---|---|
| D263IF | AAGTGGATCCTCCACattGGAGCGACGAGCAC |
| D263IR | TGCTCGTCGCTCCAaatGTGGAGGATCCACTTC |
| D263EF | ATCCTCCACcGAATGGAGCGACGAG |
| D263ER | TCGTCGCTCCAtTCGTGGAGGATC |
| W259AF | CATCCTCATGAAGgcGATCCTCCACGAC |
| W259AR | TCGTGGAGGATCgcCTTCATGAGGATGG |
| W259YF | CCATCCTCATGAAGTatATCCTCCACGACTGG |
| W259YR | CAGTCGTGGAGGATatACTTCATGAGGATGGC |
| E322IF | ACAACCCGGGTGGCAGGattTAGGTACGAGAGGGAGTTC |
| E322IR | AACTCCCTCTCGTACCTaatCCTGCCACCCGGGTTGTG |
| H262FF | ATGAAGTGGATCCTCCtgGACTGGAGCGACGAGC |
| H262FR | CTCGTCGCTCCAGTCcaGGAGGATCCACTTCATG |
| G305SF | GAGGCGACGCCTAAGGCGCAGagcGTGTTCCATGTCGACATGATC |
| G305SR | GATCATGTCGACATGGAACACgctCTGCGCCTTAGGCGTCGCCTC |
| N124QF | TCGCGCTCATGcagCAGGACAAGGTC |
| N124QR | ACCTTGTCCTGctgCATGAGCGCGAG |

**Legends: F, forward; R, reverse; mutated codons are underlined with lowercase letters indicating a base change from the wild-type sequence.**

### E.3.6. Heterologous expression of TaOMT2 and variants in *E.Coli*.

In order to characterize the expressed protein, a single colony was incubated in 5 mL of Luria–Bertani (LB) medium containing 100 μg $mL^{-1}$ kanamycin and grown overnight at 37 °C. The innoculum was then added to 100 mL of LB medium containing kanamycin (100 μg $mL^{-1)}$ and grown at 37 °C until the $OD_{600}$ reached 0.8, followed by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The culture was incubated at 37 °C for an additional 4 h, then harvested by centrifugation (3000×*g*, 10 min), and stored at −80 °C for further protein purification.

### E.3.7. Extraction and purification of His-tagged TaOMT2 and variants

For protein extraction, the cell pellet from 100 mL culture was resuspended in a mixture of 4 mL Bugbuster Protein Extraction Reagent, 100 U Benzonase Nuclease and 10 KU rLysozyme (EMD, Darmstadt, Germany), and agitated at 500 rpm on an orbital shaker for 20 min at ambient temperature. The suspension was centrifuged at 15,000×*g* for 20 min, and the supernatant was used for immobilized metal affinity chromatography (Ni-NTA, Qiagen) following the manufacturer's instructions. The affinity-purified protein fraction was passed through a prepacked PD10 column (Amersham, Bai-d'Urfé, QC) for desalting, and the highly purified fraction was stored at 4 °C until used.

### E.3.8. SDS-polyacrylamide gel electrophoresis and protein determination.

The quality of extracted proteins were determined by SDS-PAGE analysis according to the method of Laemmli (1970). After electrophoresis, proteins were stained with Coomassie Brilliant Blue (R-250). Quantification of proteins was done by

measuring the absorbance at 280; the extinction coefficient for TaOMT2 was obtained from Dr. J.A. Kornblatt.

### E.3.9. Kinetic analysis of TaOMT2

Enzyme assays were performed using 2 µg of the affinity-purified protein with a saturating (ca. 1 mM) concentration of SAM containing 25nCi of radioactivity, and varied concentrations (5 µM to 500 µM) of the phenolic substrates. Assays were performed in triplicates and were repeated twice. Enzymatic rates measured for tricetin and 5-hydroxyferulic acid (5HF) methyltransferase activity were converted from DPM to pkat/min then fitted to the Michaelis Menten equation with Grafit v4.0 (Erythacus). With this software, Lineweaver plots were constructed from non-linear regression fitting of the rate data, and kinetic constants such as apparent Vmax and Km for tricetin and 5HF were obtained from the fitted parameters. Product inhibition studies were carried out with variying concentration of TMeT (0, 10, 50, 100, 500 µM) in the presence of tricetin (at a final concentration of 10, 50, 100, 500 µM). Micahelis-Menten plots and the kinetic parameters were derived using GraphPad Prism 5 software.

### E.3.10. Enzyme activity assays with TaOMT2 variants and identification of reaction products

Enzyme assays were performed to assess their relative activity compared to the wild type by using 4-6 µg of affinity purified proteins with 1 mM of SAM containing 25nCi of radioactivity and 50 µM of tricetin. Assays were performed in triplicates and were repeated twice. Mutants with 40% or more of wild-type enzyme activity were

selected for kinetic analysis using the same protocol as for the wild type (see E3.9.). Semipreparative incubations were also carried out using unlabeled SAM and the methylated products were prepared for HPLC analyses (E.3.9). HPLC analysis was carried out with a Millennium HPLC System (Waters, Milford, MA). Separation of the enzyme reaction products was performed on a Waters YMC-Pack Pro C18 column (150×4.6 mm I.D., S-5 µM, 12 nm), using a linear gradient consisting of 40–90% MeOH in 1% acetic acid for 30 min. This condition was maintained for 10 min. before returning to the initial conditions. The flow rate was 1.0 mL min−1. Reference standards were used to compare the corresponding retention times (Zhou et al., 2006).

**E.3.11.Extraction of wheat leaf proteins and quantification**

All steps were carried out at 4$^{o}$C, unless otherwise stated. Wheat leaves were ground to a fine powder with dry ice, before being homogenized with 0.1 M of extraction buffer A. After centrifugation (14,000x*g*) for 30 min, the supernatant was used immediately for enzyme assays. The quantity of extracted proteins was determined by SDS-PAGE analysis. After electrophoresis, proteins were stained with Coomassie Brilliant Blue (R-250). Protein concentration was determined using Quantity-one software from Bio-Rad with bovine serum albumine as standard protein.

**E.3.12.*O*-Methyltransferase enzyme assays**

OMT activity was assayed using tricetin as substrate, SAM containing 0.025 µCi of [$^{3}$H] label as the methyl donor and up to 100 µg of protein in a total volume of 100 µl. The reaction was started by addition of the enzyme, incubated at 30°C for 30 min and

stopped by the addition of 10µl 6M HCl. The methylated products were extracted with ethyl acetate, and then counted for radioactivity using a toluene-based scintillation fluid. Control incubations were performed in the absence of added substrate, or with boiled enzyme for background correction, and all assays were conducted in duplicates.

### E.3.13. Protein immunoblots

Western blot analysis was performed to evaluate TaOMT2 levels at different stages of development, abiotic stresses (salinity and drought) and periods of cold acclimation. Protein samples were resolved by 12% SDS-PAGE, then electro-transferred onto polyvinylidene fluoride (PVDF) membranes. The latter were incubated in the blocking buffer F at 4 $^{o}$C overnight, and then incubated with rabbit anti-wheat TaOMT2 serum at 1:250 dilution in blocking buffer F for one hr. The membranes were washed with PBST (PBS with 0.05% Tween 20) and incubated with peroxidase-conjugated goat anti-rabbit polyclonal antibodies (Bio-Rad) at 1:10,000 dilutions in PBST for one hr. The signals obtained were detected with a chemiluminescent reaction reagent (ECL, Amersham) according to the manufacturer's protocol.

## E.4. RESULTS

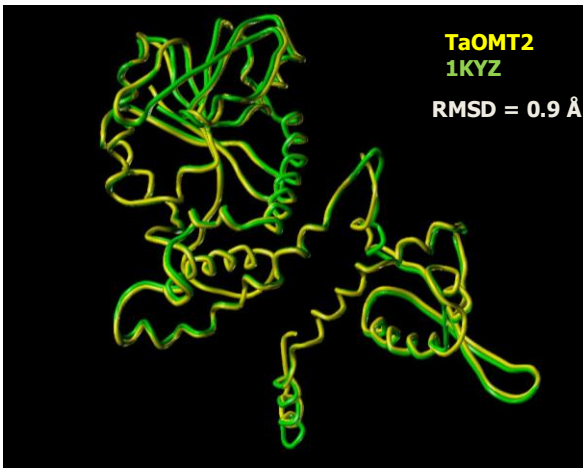
### E.4.1 Structure of TaOMT2

The gene product of *TaOMT2* encodes a 356 amino-acid polypeptide with a calculated molecular mass of 38.5 kDa and an isoelectric point of 5.71 (Zhou et al., 2006). The molecular mass obtained is representative of Class II OMTs (Joshi et al., 1998). Its ability to sequentially methylate three different substrates: tricetin, selgin and tricin (Figure 2), raised the question as to whether the enzyme contains one substrate binding pocket and one catalytic site, or multiple binding and sub-catalytic sites for these substrates. In order to answer this question, a 3-D structure of the protein was produced by homology modeling; since attempts to crystallize TaOMT2 were unsuccessful. In this study (conducted by Dr. Y. Lim, Division of Bioscience and Biotechnology, Konkuk University, Korea), the crystal structure of MsCOMT (PDB, 1KYZ; Zubieta et al., 2002) was used as a template, since TaOMT2 and MsCOMT catalyze methylations of structurally similar substrates and they share 63% sequence identity. The backbones of both proteins were superimposed with a root mean square deviation (RMSD) value of 0.9 Å, suggesting a high similarity of their secondary structures (Figure 6) and the relative location of their putative active sites. In addition, all the amino acid residues neighboring SAM and substrate binding sites are conserved, except for one residue where Val309 in TaOMT2 is replaced with Ile316 in MsCOMT, a conservative replacement as well (Figure 6). Unlike other OMTs, TaOMT2 mediates the transfer of SAM methyl groups to tricetin B-ring hydroxyl groups in a sequential manner, resulting in three different methyl ether derivatives (Figure 2) and *S*-adenosyl-L-homocysteine (SAH) as products

(Zhou et al., 2006). Based on docking of the individual substrates (tricetin, selgin and tricin) into the model, several H-bonds were observed between the ligands' hydroxyl groups and the functional groups of the neighboring residues. As shown in Table 2, these H-bonds lie within 1.60 Å to 2.71 Å from the ligands and the functional groups of neighbouring residues and within 4.92 Å to 5.72 Å from the SAH-S catalytic site. Asp263 plays an important role in binding of the 3'-OH group of tricetin 4', 5'-OH of selgin and 4'-OH group of tricin (Figure 7). The γ-carboxyl groups of Glu290 and Glu322 form H-bonds with the 4' and 5'-OH groups of tricetin (Figure 8).

In almost all methyltransferases, the transmethylation reaction is catalyzed by a general acid/base mechanism using a histidine residue, as reported for MsCOMT (Zubieta et al., 2004) and isoflavone OMT (Zubieta et al., 2002), among others. The fact that histidine in MsCOMT acts as the deprotonating residue of the substrate hydroxyl groups (Zubieta et al., 2002), suggests that His262 is also involved in the deprotonation reaction of TaOMT2 in collaboration with its neighbouring residue, Asp263. In fact, when tricetin is docked into the active site pocket of the proposed model, it is held by a network of H-bonds extending from Asp263 to its 3'-OH group; Glu290 and Asn317 to its 4'-OH and His262, Glu322 to its 5'-OH groups, as well as to ring-A 5-O by Asn124 (Figure 8 and Table 2). Methyl transfer in TaOMT2 is proposed to be catalyzed by a nucleophilic attack of the resulting phenolate anion on the reactive methyl group of SAM. The proton elimination process is likely to take place through a His262-Asp263 proton-relay system, which is favored by the proximity of both residues within the active site.

**Figure 6 Superimposition and amino acid sequence alignments of Triticum aestivum flavone *O*-methyltransferase (TaOMT2) and Medicago sativa caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (MsCOMT).***




*** Figures provided by Prof. Y. Lim (Bio/molecular informatics Centre, Konkuk University, S. Korea) and Dr. Jian Min Zhou (Concordia Universtiy)**

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TaOMT2  MGS-------IAAGADEDACMYALQLVSSSILPMTLKNAIELGLLETLMAAG-------G 46
MsCOMT  MGSTGETQITPTHISDEEANLFAMQLASASVLPMILKSALELDLLEIIAKAGP------G 54

TaOMT2  KFLTPAEVAAKLPSA-ANP-EAPDMVDRMLRLLASYNVVSCRTEDGKDGRLSRRYGAAPV 104
MsCOMT  AQISPIEIASQLPTT--NP-DAPVMLDRMLRLLACYIILTCSVRTQQDGKVQRLYGLATV 111
                                *
TaOMT2  CKYLTPNEDGVSMSALALMNQDKVLMESWYYLKDAVLDGGIP-FNKAYGMSAFEYHGTDP 163
MsCOMT  AKYLVKNEDGVSISALNLMNQDKVLMESWYHLKDAVLDGGIP-FNKAYGMTAFEYHGTDP 170

TaOMT2  RFNRVFNEGMKNHSIIITKKLLESYKGFEG-LGTLVDVGGGVGATVAAITAHYPTIKGIN 222
MsCOMT  RFNKVFNKGMSDHSTITMKKILETYTGFEG-LKSLVDVGGGTGAVINTIVSKYPTIKGIN 229
                                        *  **
TaOMT2  FDLPHVISEAPPFPGVTHVGGDMFQKVP-SGDAILMKWILHDWSDEHCATLLKNCYDALP 281
MsCOMT  FDLPHVIEDAPSYPGVEHVGGDMFVSIP-KADAVFMKWICHDWSDEHCLKFLKNCYEALP 288
                  *                *                    *
TaOMT2  AHG---KVVLVECILPVNPEATPKAQGVFHVDMIMLAHNPGGRERYEREFEALAKGAGFA 338
MsCOMT  DNG---KVIVAECILPVAPDSSLATKGVVHIDVIMLAHNPGGKERTQKEFEDLAKGAGFQ 345

TaOMT2  AMKTTYXYANAWAIEFTK----356
MsCOMT  GFKVHCNAFNTYIMEFLKKV--365
```

**α-Helices (magenta) and ß-sheets (blue) depict the residues that form the secondary structures of both proteins. Green stars indicate the putative residues involved in substrate binding, and the magenta star indicates that involved in catalysis. The putative residues involved in substrate preference of both OMTs are boxed.**

**Table 2. Modeling data for amino acids involved in ligand binding***

| Ligand | Reaction product | H-bond (Å) | Distance from active site (Å) |
|---|---|---|---|
| Tricetin (3',4',5'OH) | 3'OH→3'OMe | Asp263 γCOO⁻ - 3'OH (1.92)<br>Trp259 bb*CO - 3'OH(2.36)<br>Glu290 γCOO⁻ - 4'OH (1.76)<br>Glu322 γCOO⁻ - 5'OH (2.11)<br>Asn124 ßCNH$^{3+}$ - 5O (2.25) | 4.92 |
| Selgin (3'OMe-4',5'OH) | 5'OH→5'OMe | Gly305 bbCO - 7OH (2.10)<br>Asn124 ßCNH$^{3+}$ - 4O (1.87)<br>Asn124 ßCO - 5OH (2.00)<br>Trp259 bbCO - 4'OH (2.61)<br>Asp263 γCOO⁻ - 5'OH (2.30)<br>Asp263 γCOO⁻ - 4'OH (2.05) | 5.72 |
| Tricin (3',5'OMe-4'OH) | 4'OH→4'OMe | Gly305 bbCO - 7OH (1.92)<br>Asn124 ßCNH$^{3+}$ - 4O (1.60)<br>Asn124 ßCO - 5OH (2.13)<br>Asp263 γCOO⁻ - 4'OH (1.76) | 6.56 |

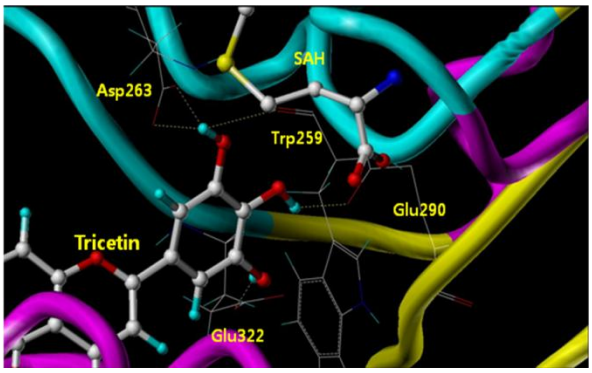
*bb=Backbone

*Table provided by Dr. Y. Lim (Bio/molecular informatics Centre, Konkuk University, S. Korea)

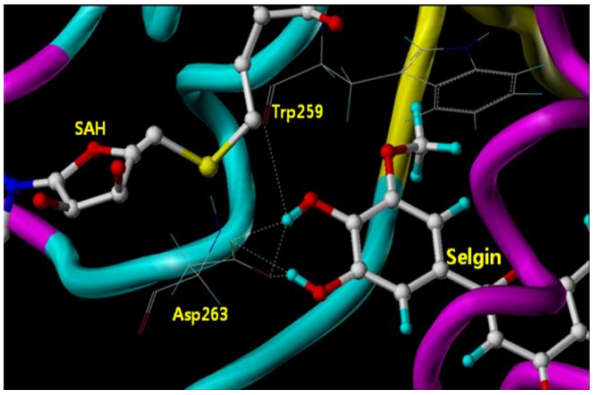
**Figure 7 Homology modeling of TaOMT2 with the following substrates docked in: A, tricetin; B, selgin;C, tricin**

**Figures provided by Prof. Y. Lim (Bio/molecular informatics Centre, Konkuk University, S. Korea)**

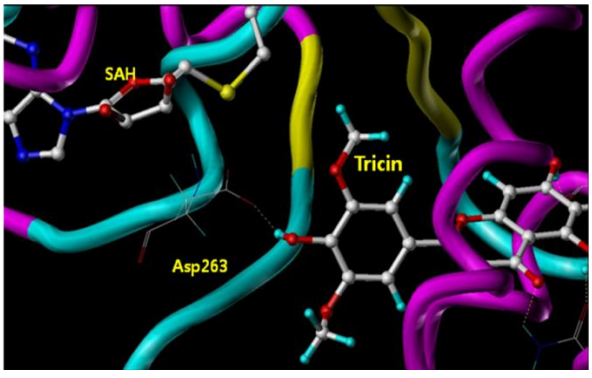
(A)




(B)

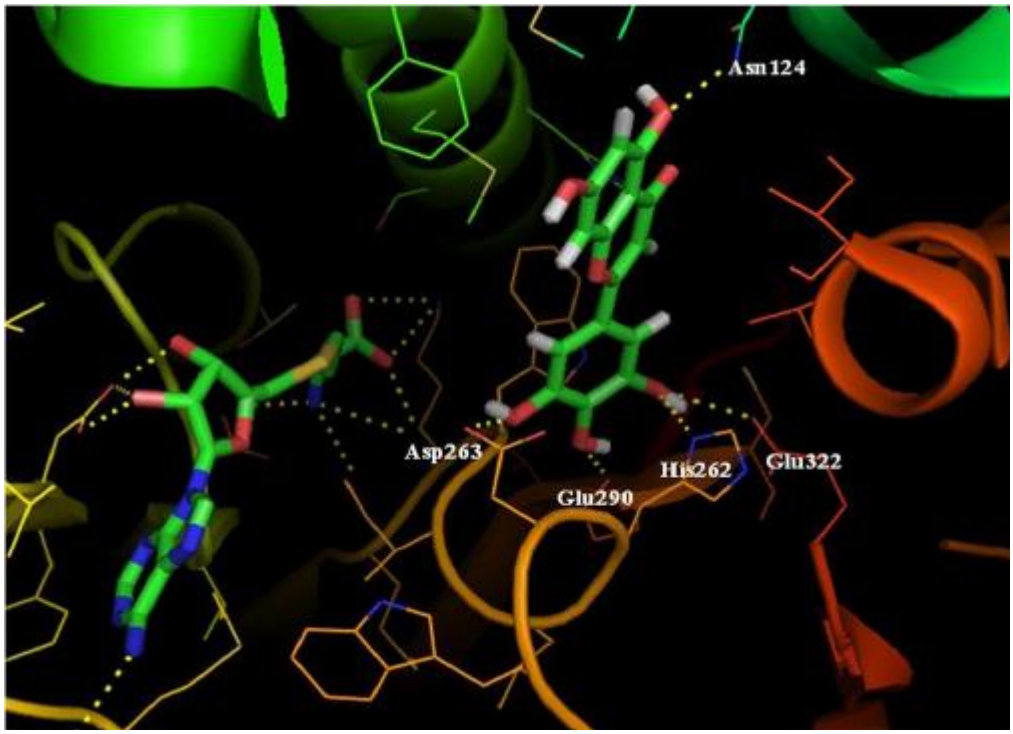



(C)



## Figure 8 Homology modeling of TaOMT2: catalytic site

**Figure provided by Prof. Y. Lim (Bio/molecular informatics Centre, Konkuk University, S. Korea)**



### E.4.2 Purification of wild type TaOMT2 and its mutants

In order to confirm the importance of the putative residues involved in substrate binding and catalysis (Table 2, Figure 8), they were subjected to site-directed mutagenesis. In order to study the catalytic behavior of recombinant TaOMT2 and its mutants *in vitro*, the cDNA clone harboring a His-tag at its N-terminal was expressed in *E.coli* BL21 (DE3) cells and the recombinant proteins were purified to near homogeneity by Ni-NTA affinity chromatography, as verified by SDS-PAGE. The apparent molecular mass corresponds to the combined M*r* of TaOMT2 subunit (38.5 kDa) and His-tag (3.0 kDa). Figure 9 demonstrates the protein purity observed after the last purification step. These results indicate typical purities for wild type and mutant preparations as well. No bands were seen on the SDS-PAGE for H262F.

### E.4.3 Substrate interaction kinetics of TaOMT2 against tricetin, selgin, tricin and trimethyltricetin (TMeT)

Activity assays were performed with tricetin, selgin, tricin and TMeT to assess the percentage of relative activity. Due to unavailability of selgin in sufficient amount, 5HF was used as a substitute, since it shares B-ring substitution and the 3-C side chain of selgin. Figure 10 shows the relative activity of TaOMT2 with these substrates. TaOMT2 exhibited high relative activity with tricetin and 5HF, with 100% and 93% respectively; but the protein had no significant activity with either tricin or TMeT (Table 4). The apparent Km values for tricetin and 5HF were 54.79 µM (±11.6 µM) and 181.56 µM (±38.0µM) respectively and their respective Vmax values were 137 pKat/mg (±5.85 pKat/mg) and 135 pkat/mg (±9.96 pKat/mg). All kinetic parameter values and graphs

obtained from the Michaelis Menten and Lineweaver-Burk plots (Figure 11) are summarized in Table 4.

### E.4.4 Product inhibition studies

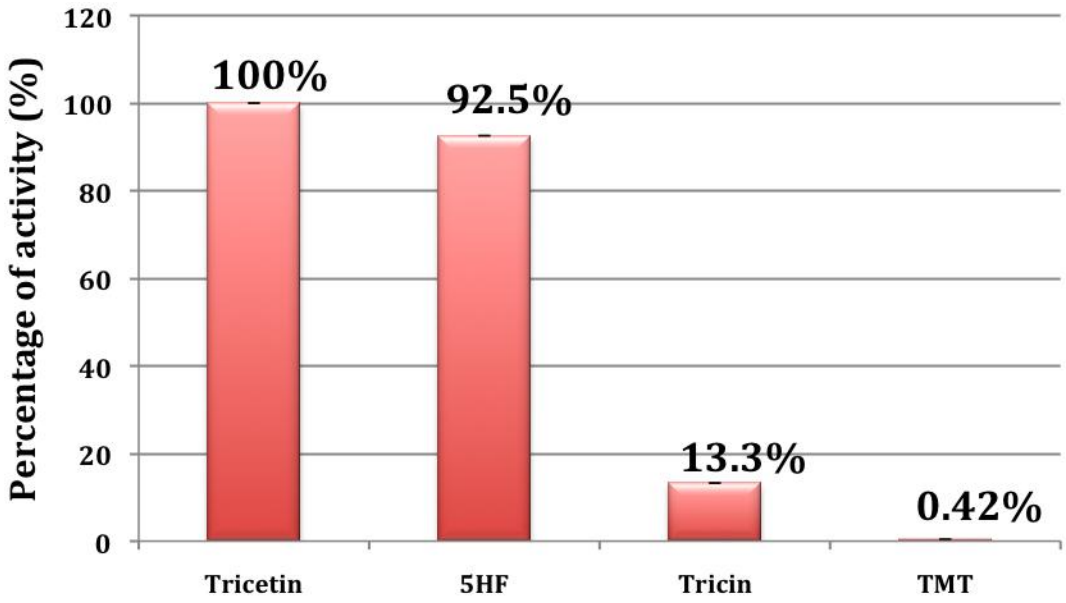
Michaelis-Menten plots of product inhibition results (plotted and calculated by the Prism software for enzyme kinetics studies) exhibited competitive inhibition pattern between tricetin and TMeT (Figure 12). An alternate preferred model suggested by the software is a mixed inhibition. The Ki is 53.21 µM ±13.3 µM for competitive inhibition.

**Figure 9 SDS-PAGE profiles of the recombinant protein and some mutants represented by equal amounts of the solubilized pellets***


1-Wild type, 2-E322I, 3- D263E, 4- H262F, 5- W259Y, 6- W259A, 7- G305S

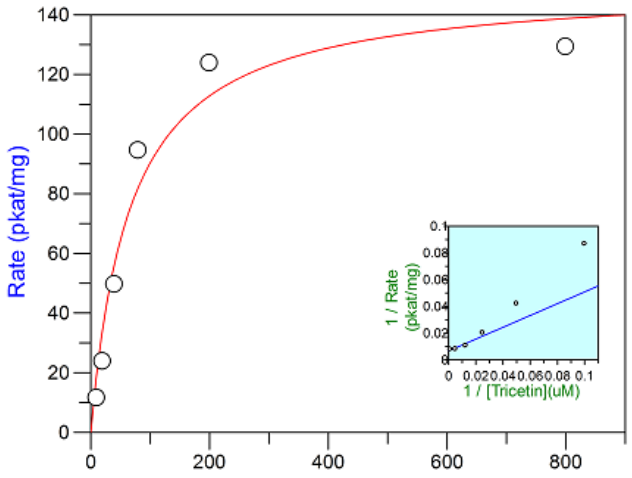
* These are the only bands seen on the SDS-PAGE gel.

**Figure 10 Relative activity of TaOMT2 with tricetin, 5HF, tricin and TMeT**

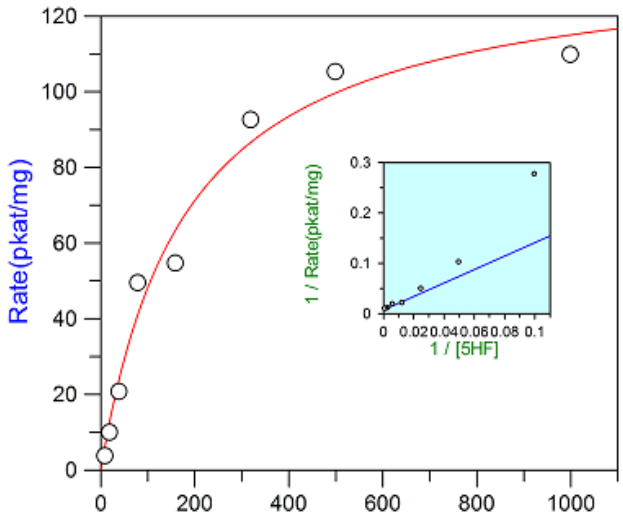



100% relative enzyme activity of wild type protein is equivalent to 140 pkat.mg$^{-1}$.

**Figure 11 Michaelis-Menten and Lineweaver-Burk plots of substrate interaction kinetics with wild type TaOMT2: (A) Tricetin, (B) 5HF**




**(A)**




**(B)**

**Table 3. Kinetic parameters of TaOMT2 for tricetin and 5HF as substrates**

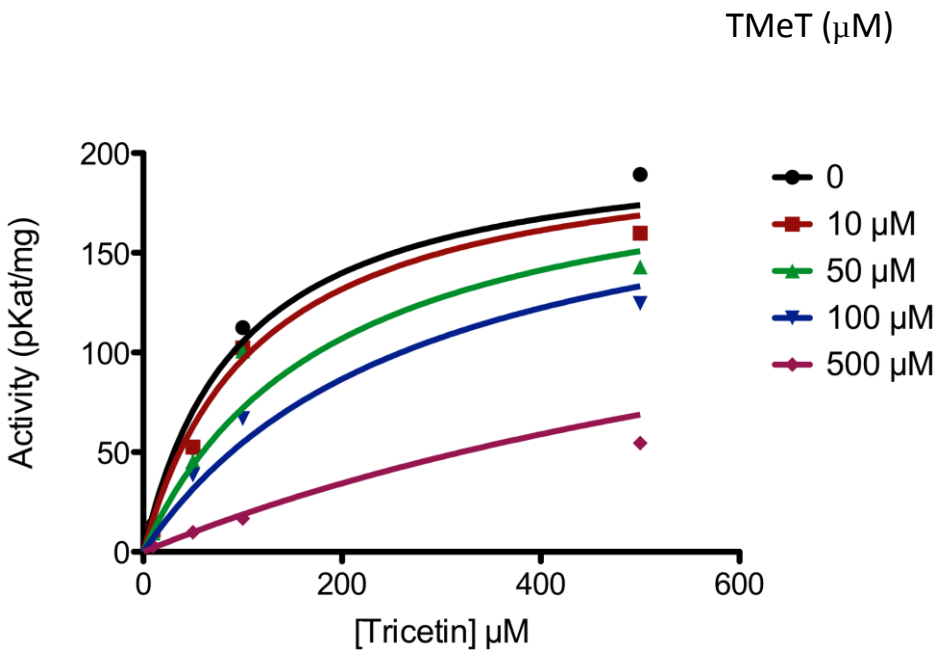
| | Km (μM) | Vmax (pkat/mg) | Vmax/Km |
|---|---|---|---|
| Tricetin | 54.79±11.6 | 137.082±5.85 | 2.50 |
| 5HF | 181.56±38.0 | 135.91±9.96 | 0.748 |
| Tricin | ---------- | ---------- | --------- |
| 3MeT | ---------- | ---------- | --------- |

------: kinetic parameters were not done for tricin and 3MeT

$K_m$ values (μM),

$V_{max}$ (pkat.mg$^{-1}$; pkat, the catalytic activity that raises the reaction rate by one pmol.s$^{-1}$)

**Figure 12 Product inhibition studies: Tricetin versus Trimethyltricetin**



### E.4.5 Relative activity of TaOMT2 mutants compared to the wild type with tricetin

After expression and purification, mutant proteins were assayed for OMT activity with tricetin as the substrate in order to evaluate the changes in activity between the variants and the wild-type (Figure 13). This assessment should determine the significance of the mutated residue in binding, catalysis or both. None of the mutants analyzed exhibited any increase of methylating activity compared to the wild type enzyme. Replacement of Asp263 with either glutamic acid or isoleucine resulted in a dramatic loss of activity, indicating its critical role in substrate binding. A conservative mutation of Asn124 to Gln showed significant loss of activity, indicating the redundancy of this residue in substrate binding since that mutation disrupts H-bonding with the 5-OH group of tricetin. The substitution of Try259 to Ala preserved ~80% of enzyme activity, indicating that Ala can still maintain the H-bonding network between Glu290 and His262. A loss of charge in E322 when substituted to Ile affected the H-bonding with neighbouring residues but did not abolish activity.

### E.4.6 Kinetic studies of TaOMT2 mutants

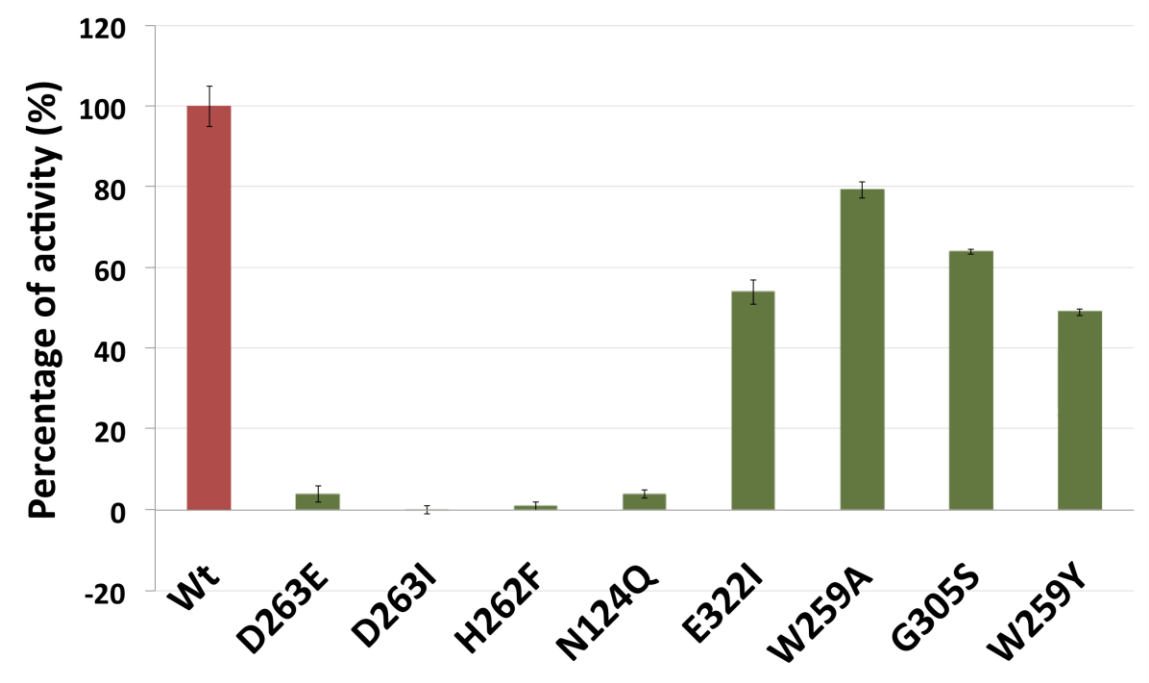
Kinetic analysis was performed in order to assess the importance and catalytic behavior of the mutated residues in relation to the binding of tricetin. Mutants exhibiting >40% relative activities of the wild type were selected for kinetic studies. Table 5 shows the apparent Km and Vmax values of the mutant proteins, obtained from the Michaelis Menten and Lineweaver Burk plots (Figure 14). All mutants exhibited a slightly lower

affinity towards tricetin and a lower Vmax than those of the wild type, which will be explained in details in the Discussion section.

### E.4.7 HPLC of mutants' reaction products with tricetin as substrate.

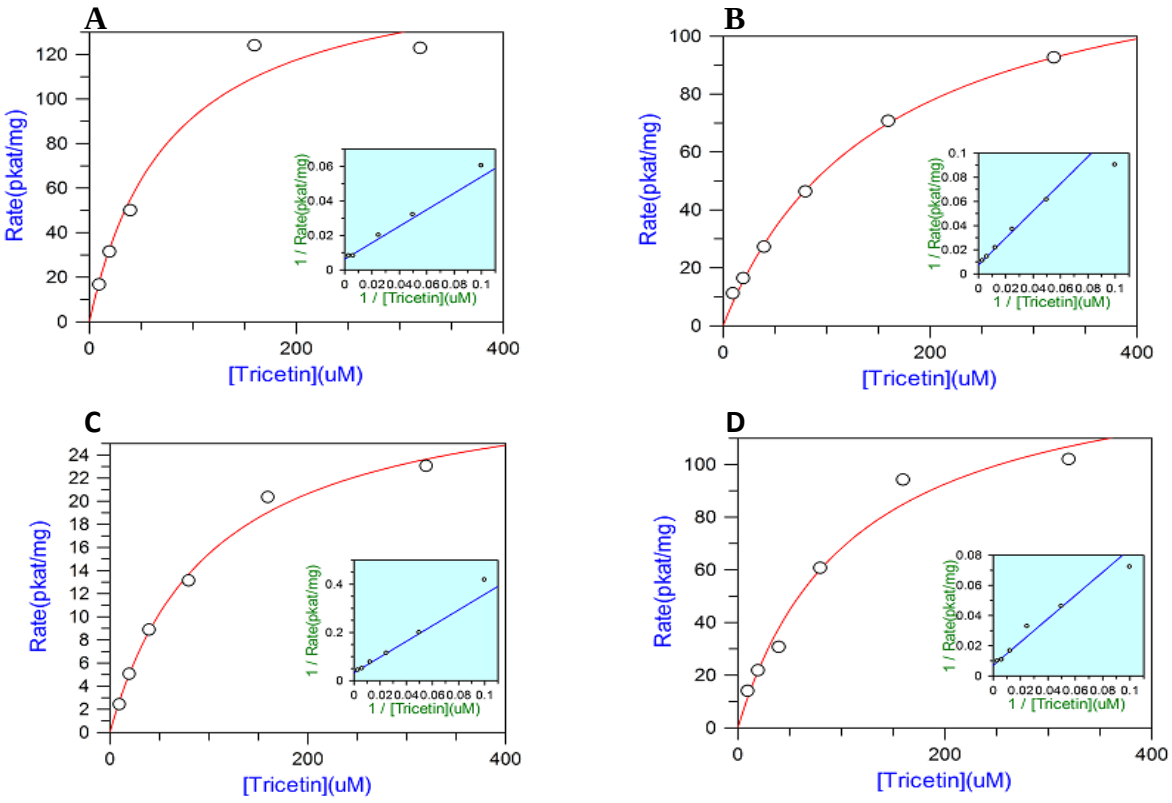
In order to investigate the effect of mutations on reaction products, mutants with a relative OMT activity of 40% or more were selected for HPLC analysis. HPLC of the non-labeled reaction products of a number of semi-preparative enzyme assays gave rise to two activity peaks, with tricin being the predominant product, as compared with a trace amount of the trimethyl ether derivative, TMeT (Figure 15 A). All variants of TaOMT2 showed a similar HPLC profile (Figure15-B), indicating that there was no change in product formation.

**Figure 13 Relative OMT activity of mutant proteins compared with the wild type TaOMT2**




100% relative enzyme activity of wild type protein is equivalent to 140 pkat.mg$^{-1}$.

**Figure 14 Michaelis-Menten and Lineweaver plots of tricetin interaction: (A) E322I, (B) G305S, (C) W259Y, (D) W259A**



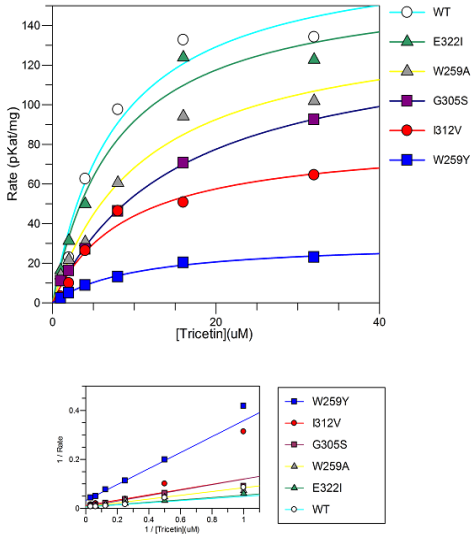
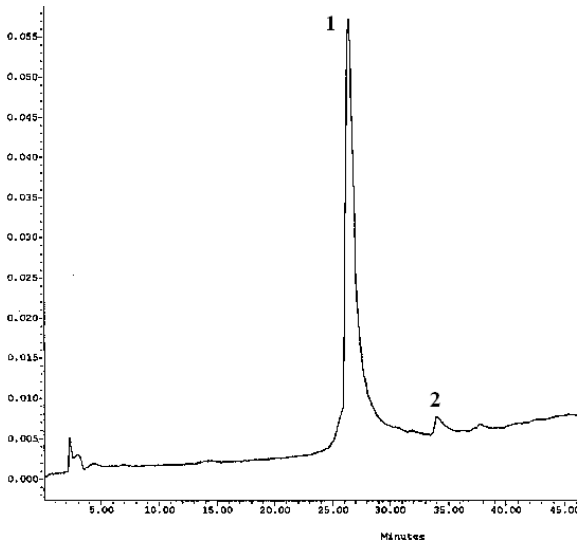



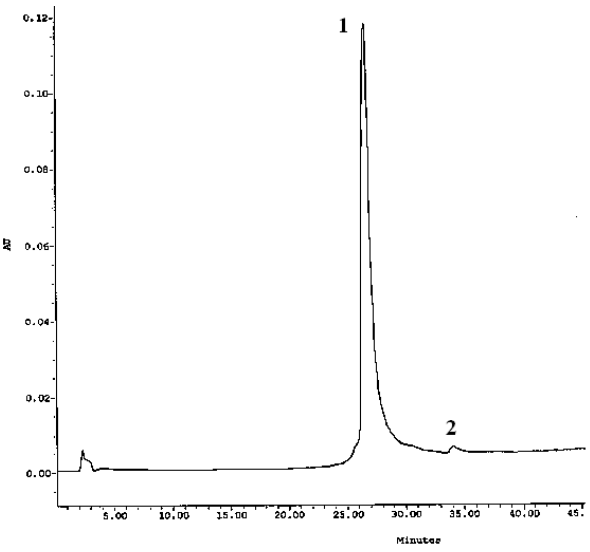
**Table 4. Kinetic parameters of mutant proteins with tricetin**

| | Km (µM) | Vmax (pkat/mg) | Vmax/km |
|---|---|---|---|
| **WT** | 77.9±25.1 | 179.5 ±21.9 | 2.304236 |
| **E322I** | 78.3±25.7 | 163.5±19.0 | 2.088123 |
| **W259A** | 111.2±28.3 | 143.9±15.7 | 1.294065 |
| **G305S** | 153.9±12.1 | 137.1±5.1 | 0.890838 |
| **W259Y** | 101.6±13.4 | 31.1±1.7 | 0.306102 |

**Figure 15 HPLC profiles of the enzyme reaction products of the wild type (A) and W259A (B) proteins assayed with tricetin as the substrate: 1, tricin; 2, trimethyltricetin**




(A)




**(B)**

1-Tricin, 2-Trimethyltricetin

### E.4.8 Differential TaOMT2 expression and activity in wheat leaves and influorescences

It was recently shown that tricin accumulates predominantly in influorescence tissues (A. Moheb, unpulished data). Since TaOMT2 synthesizes tricin as the major product of tricetin methylation, therefore, its enzyme activity and protein expression were evaluated in leaf and influorescence tissues in order to correlate the presence of tricin with the expression level of TaOMT2. Figure 16 shows a higher specific activity of TaOMT2 against tricetin in the influorescences, as compared to the leaves. In agreement with this finding, Figure 17 shows a concomitant accumulation of TaOMT2 protein in the influorescences.

### E.4.9 Activity of total methyltransferases accepting tricetin as substrate and western blot of TaOMT2 in cold acclimated wheat leaves.

In order to assess the function of TaOMT2 in cold acclimated wheat leaves, western blots and activity assays of TaOMT2 are used. Western blot analysis was performed on both winter and spring varieties, Claire and Bounty respectively. Non-acclimated plants grown for 0 and 8 days were chosen as controls for the 6, 12, 21 and 49 days cold-acclimated plants. Figure 18 shows that TaOMT2 exhibits a higher level of expression in cold-acclimated plants compared to the controls. In addition, TaOMT2

protein levels tend to increase in plants exposed to cold with the highest accumulation after 49 days of cold acclimation. The double bands seen at 0 day control might be the result of a truncation of TaOMT2. Although, it is difficult to assess the specific activity of TaOMT2 in wheat soluble extracts since it may contain many other methyltransferases accepting tricetin as substrate, activity of total methyltransferase using tricetin was investigated. Methyltransferase activity with tricetin as substrate and radiolabeled S-Adenosyl-L-Methionine as cosubstrate was determined in the soluble fractions of wheat leaves subjected to cold acclimation. It was predicted that the level of enzyme activity would be directly related to the amount of TaOMT2. In contrast, figure 20 shows that the activity of putative OMTs interacting with tricetin has a propensity to decrease in cold acclimated leaves.

**Figure 16 Activity of total methyltransferases accepting tricetin as substrate in wheat leaves and influorescences**

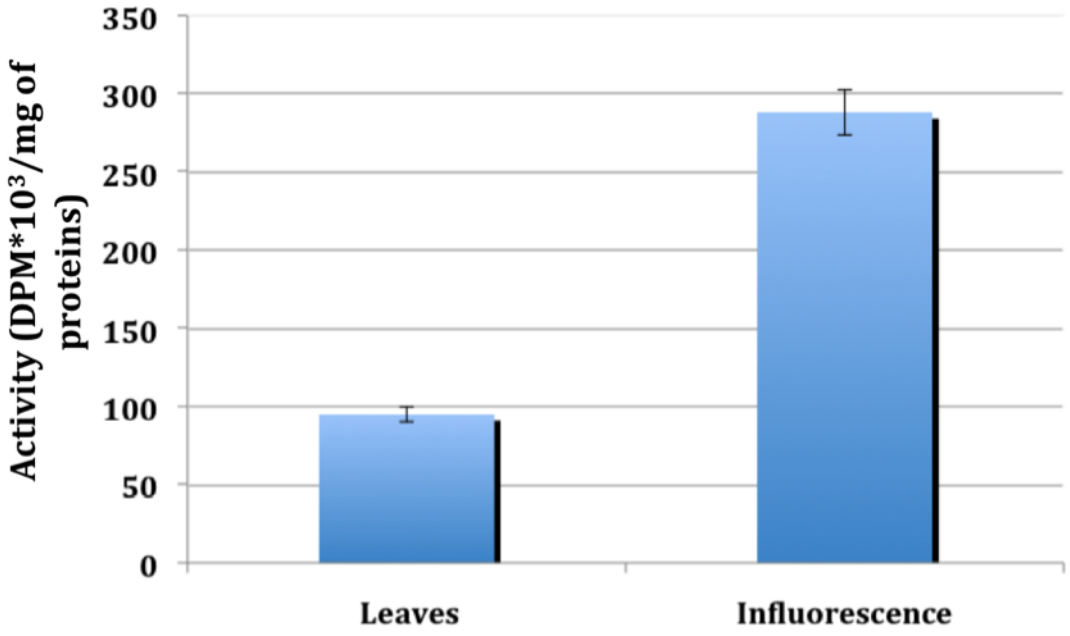
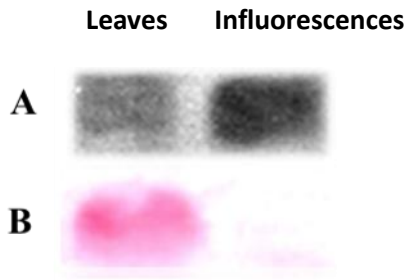
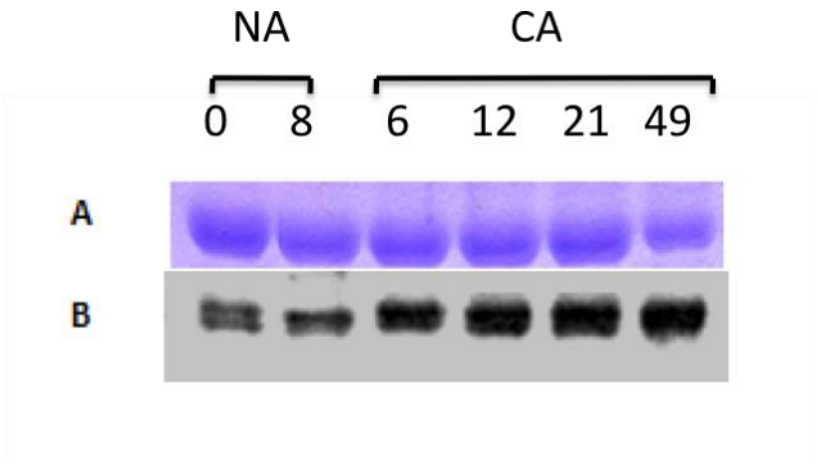


**Figure 17 Western blot of TaOMT2 in leaves and husks**




**A**, Immunoblot of TaOMT2. **B**, PVDF membrane stained with Ponceau Red showing Rubisco.

**Figure 18 Western blot of TaOMT2 in cold acclimated wheat leaves (Claire, winter wheat)**

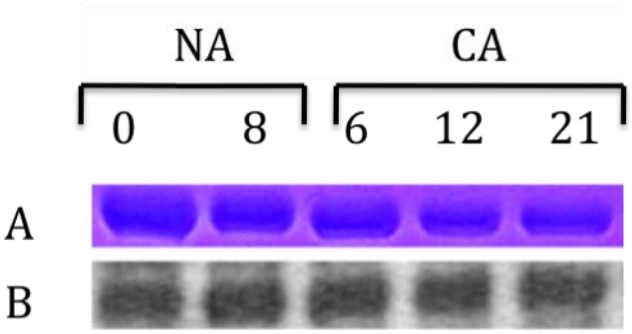



**NA**, non-acclimated; **CA**, cold-acclimated

**A**, SDS-PAGE gel after transfer stained with Coomassie blue showing Rubisco.

**B**, Immunoblot of TaOMT2 in winter wheat cv., Claire.

**Figure 19 Western blot of TaOMT2 in cold acclimated wheat leaves (Bounty, spring wheat)**

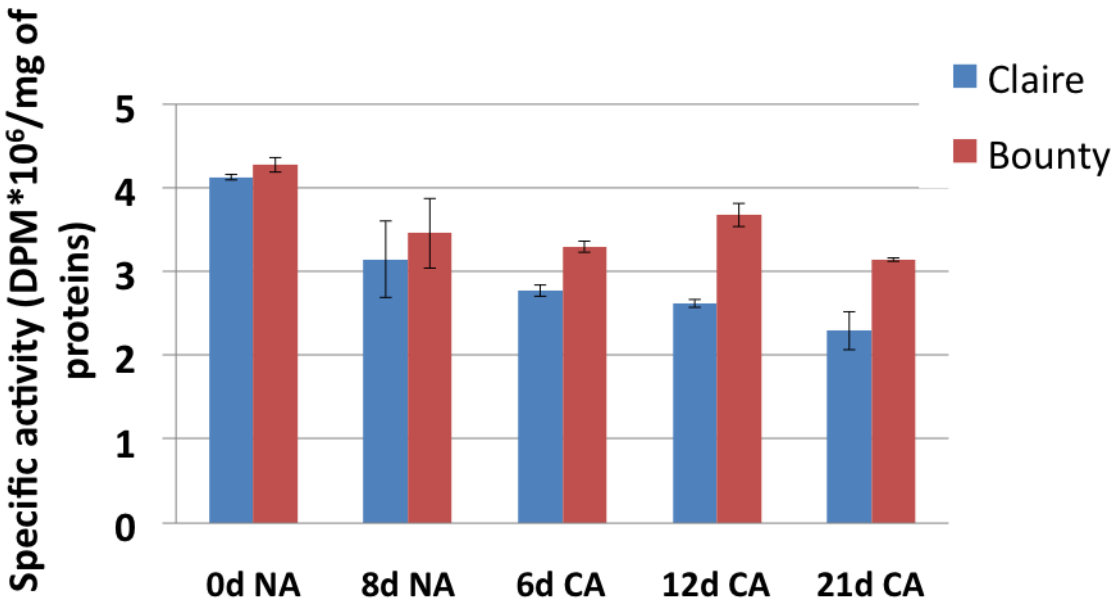



**NA**, non-acclimated; **CA**, cold-acclimated

**A**, SDS-PAGE gel after transfer, stained with Coomassie blue showing Rubisco.

**B**, Immunoblot of TaOMT2 in spring wheat cv. Bounty.

**Figure 20 Activity of methyltransferases accepting tricetin as substrate in cold acclimated wheat: Claire (winter variety); Bounty (spring variety)**



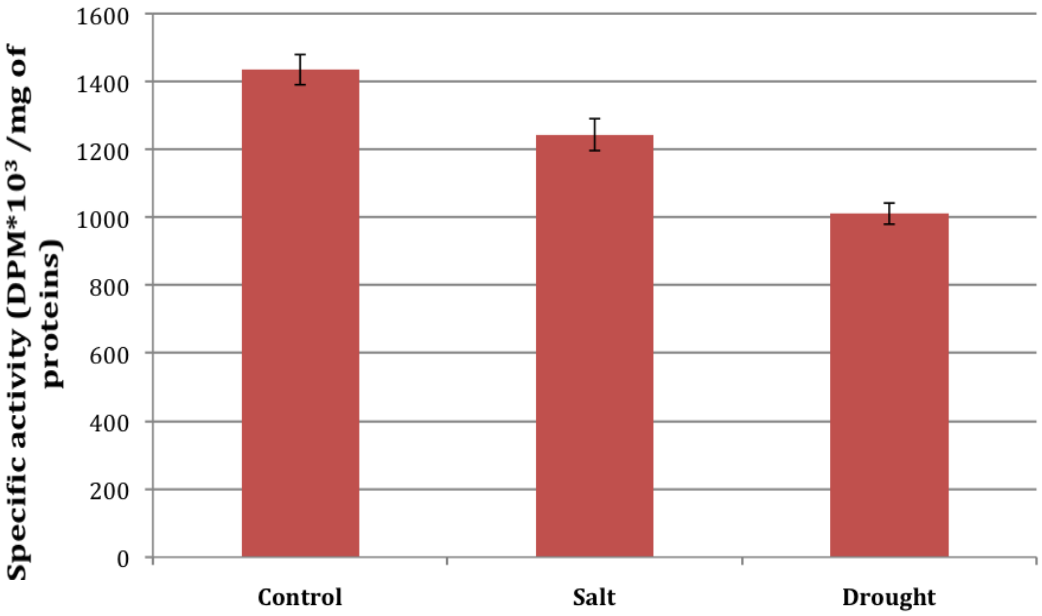

Total methyltransferase activity using tricetin as substrate with radiolabeled *S*-adenosyl-L-methionine as cosubstrate per mg of proteins in soluble fractions of wheat leaves during cold acclimation. Values represent the mean ± SE obtained from 2 independent experiments. 0 d NA, non acclimated plants grown for 7 days, 6 d CA, 6 day cold acclimated plants.

### E.4.10 Activity of total methyltransferases accepting tricetin as substrate and western blot of TaOMT2 in wheat leaves exposed to salinity and drought stresses.

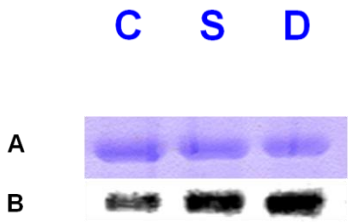
The enzyme activity of total methyltransferases accepting tricetin as substrate and radiolabeled SAM as co-substrate was assessed in wheat (Norstar cv) grown under conditions of high salinity and drought. Figure 21 shows a 10% and a 20% decrease in activity in leaf extracts for the salt stressed and for the drought stressed wheat respectively, compared to the control. The immunoblot of TaOMT2 in figure 21-B shows a similar expression pattern of TaOMT2 to the non-stressed wheat leaves.

**Figure 21 Biochemical characterization of methyltransferases interacting with tricetin: (A) Activity of methyltransferases, (B) Western blot of TaOMT2**

(A)




(B)




Legends: C-control, S-salt stress, D, drought stress **A.** SDS-PAGE gel after transfer stained with Coomassie Blue showing Rubisco. **B**-Immunoblot of TaOMT2.

## E.5 Discussion

One of the main objectives of the present work was to determine the structural and catalytic roles of the residues neighboring the active site and their relative importance in binding of tricetin and catalysis of sequential methylation. Purification of wild type TaOMT2 and its mutants gave reasonably high yields of enzyme proteins, except for His262. The behavior of mutants during purification was similar to that of the wild type, and the proteins obtained were almost pure in all cases. Mutant proteins remained tightly bound to the resin until eluted with imidazole containing buffer.

Kinetic parameters were obtained for the wild type TaOMT2 with tricetin and 5HF. 5HF had a dual function in this test: it was used as a substrate due to unavailability of sufficient amounts of selgin, since it possesses the same substitution pattern of the B-ring and the 3-C side chain of selgin and as a phenylpropanoid itself. TaOMT2, in presence with 5HF, exhibited a 93% enzyme activity relative to tricetin in agreement with the data (80-90%) reported by Zhou et al. (2006).

The relative methylating activities of TaOMT2 using tricin and trimethyl tricetin as substrates exhibited very low relative enzyme activities, ranging from 0.4 to 13% compared to tricetin. The major product of the reaction catalyzed by TaOMT2 was tricin. Methylation of 5HF to tricin is favored since the 3'- and 5'-hydroxyl groups are stereochemically equivalent. The stepwise methylation of tricetin starts at the 3'-OH because of its highest negative electron density, followed by 5'-OH methylation (Pople and Beveridge, 1970). In contrast, further methylation of tricin is the least favoured reaction, possibly due to steric hindrance resulting from introduction of a bulky methyl

group (Zhou et al., 2006). In addition, the enzyme reaction appears to be competitively inhibited by its product TMeT, which may explain the predominance of tricin as the major product of tricetin methylation (Figure 12).

Based on molecular modeling experiments and manual docking of each of the substrates, tricetin, selgin and tricin into the model, Asp263 was shown to play an important role in the binding of the three substrates, since its γ-carboxyl group forms H-bonds with the 3'-hydroxyl group of tricetin, 4'-and 5'-hydroxyls of selgin and the 4'-hydroxyl group of tricetin. Mutation of Asp263 to glutamic acid, did not serve the purpose of substrate binding, since this replacement completely abolished TaOMT2 activity. This may be due to the conflict between the catalytic His262-imidazole group and the extra methylene group of Glu, which would increase the distance between its carboxylic group and the imidazole ring of histidine. Replacing Asp263 to Ile also abolished activity, since the latter cannot form H-bonds with the hydroxyl groups of tricetin. The mutant H262F exhibited no enzyme activity due to the complete loss of protein expression as shown by SDS-PAGE. In addition, any substitution that alters the imidazole ring severely influenced the expression of TaOMT2 and consequently the catalytic activity of TaOMT2.

The molecular model shows that N124 interacts with the 4-OH and 5-OH groups of all substrates for orientation purpose, and its replacement with Gln resulted in loss of TaOMT2 activity. Such substitution may have resulted in disruption of the H-bonding with tricetin and consequently reduced substrate binding; although this mutation did not affect its heterologous expression in *E.coli*.

Mutants with a relative activity of >40% were selected for kinetic analysis. All mutants exhibited a reduced catalytic efficiency (Vmax/Km) compared to the wild type. Substitution of E322 with a non-polar residue such as isoleucine slightly affects H-binding with the neighboring residues; although affinity for tricetin and the maximal velocity were slightly lower than the wild type, thus suggesting that E322 is not a redundant residue for binding (refer to table 6).

In summary, it appears therefore that the network of amino acid residues selected for the mutagenesis study interacts with tricetin as follows: the 3'-OH and 5'-OH groups of tricetin are deprotonated by His262, thus facilitating transfer of the reactive methyl group of SAM to the phenolate anion. This general base is held in a catalytically productive position by H-bonding with Glu322. Asp263, the adjacent residue to His262, contributes to the orientation of the latter residue. Moreover, because of its low pKa value and its negative charge, Asp263 serves as a suitable active site residue. Other residues contributing to the negatively charged binding surface are Glu322, G305 and Glu290. The ß-$CNH^{3+}$ of Asn124 and the backbone of Gly305 seem to align the flavonoid A- and C-rings in a favorable position for substrate binding through a H-bonding network. Analysis of the mutant enzyme reaction products by HPLC revealed that none of the variants showed any shift in product formation. The major enzyme reaction product in all of the mutants was predominantly tricin, with a trace amount of TMeT, but no selgin (Figure 15). The fact that selgin does not accumulate indicates that selgin, the first methylated intermediate of tricetin methylation, does not leave the active site.

Changes in the H-bonding network, charge transfer and /or size of the target residue have significant effects on substrate binding and the catalytic activity of the mutant proteins but has no considerable differences in the product ratios between TaOMT2 and its variants with tricin being the predominant reaction product.

**Table 5. Significance of the putative residues of TaOMT2involved in binding and/or catalysis and changes in the properties of their mutant proteins**

| Wild type residues | Significance | Mutant proteins | Enzyme kinetics | | | | Properties of mutant proteins |
|---|---|---|---|---|---|---|---|
| | | | **(%)** | **Km** | **Vmax** | **Vmax/Km** | |
| Control | | | **100** | **77.9** | **179.5** | **2.30** | |
| $D^{263}$ | Important amino acid residue for substrate binding; forms H-bonds with all OH groups of tricetin | **$D^{263}I$** | 0.08 | | | | Ile263 cannot form a H-bond with 3'-OH group |
| | | **$D^{263}E$** | 4 | | | | Severe loss of activity is due to the conflict between the catalytic His262-imidazole group and Glu-$CH_2$ |
| $W^{259}$ | H-bonds with selgin 4'-OH and forms H-bond network with neighboring residues | **$W^{259}A$** | 55 | 111.2 | 143.9 | 1.29 | Ala can maintain the H-bonding network between Try259, Glu290and His262, whereas Tyr cannot |
| | | **$W^{259}Y$** | 49 | 101.6 | 31.1 | 0.30 | |
| $E^{322}$ | H-bonds with tricetin 5'-OH | **$E^{322}I$** | 54 | 78.3 | 163.5 | 2.0 | Loss of charge or change in the side chain affects the H-bonding with the neighboring residues, especially H262 |
| $G^{305}$ | H-bonds with tricetin 7-OH | **$G^{305}S$** | 64 | 153.9 | 137.1 | 0.89 | Change in polarity is less effective than chain length on catalytic activity |
| $H^{262}$ | Putative catalytic base involved in deprotonation of tricetin OH groups | **$H^{262}F$** | 1 | | | | Mutant protein lacks imidazole ring that is critical for proton transfer |

| | | | | |
|---|---|---|---|---|
| $N^{124}$ | H-bonds with O-4/O-5 of all substrates in order to reorient them to the most favorable position | **$N^{124}Q$** | 4 | Mutation disrupts the H-bonding with 5-OH group of tricetin resulting in decreased substrate binding |

In addition, we report here the biochemical characterization of TaOMT2 and its regulation during developmental stages and in response to abiotic stresses. The results obtained indicate that TaOMT2 was highly expressed in influorescence tissues and that its protein accumulation coincided with intracellular content changes of tricin (Amira Moheb, personal communication).

The relative concentration of tricin on upper canopy leaves of wheat plants were lower in early stages (leaf #5) of development as compared to later stages (flag leaves) of development (Estiarte et al.,1997). It has been demonstrated that tricin, accumulates in large amounts in the green influorescence (A. Moheb, personal communication). Concomitantly, the accumulation of TaOMT2 and its activity assays in presence of tricetin increased by almost three-fold in the influorescence tissues compared to the leaves. To our knowledge, this is the first report of the accumulation of TaOMT2 and tricin in wheat influorescences. These results suggest the importance of tricin and the enzyme involved in its biosynthesis, TaOMT2, during developmental stages of wheat.

Wheat influorescences are exposed to different external stresses such as uv radiation, herbivory, fungal, parasitic attack. In order to produce seed and continue with the succession of germination, wheat has developped different strategies to protect itself. The role of flavonoids in plant protection against light damage, and defense against herbivory was suggested (Harborne, 1991). Inhibition by tricin of the feeding activity of the aphid *Myzus persicae* (Dreyer and Jones, 1981), the brown plant hopper *Nilaparvata*

*lugens* (Bing et al., 2007) support this statement and suggest a possible protective function for tricetin methylation by TaOMT2 against possible fungal or parasitic attacks.

Protein kinases play essential roles in the control of cellular functions, including cell proliferation and differentiation, signal transduction, gene expression and metabolism. The *O*-methylation of tricetin by TaOMT2 is quite significant, considering the fact that this flavonoid has inhibitory effects on protein kinase C and phophoribulokinase (Khalil Kane, unpublished data). Therefore, the methylation of tricetin by TaOMT2 may be considered as a means of modulating the effect of tricetin on protein kinases, allowing them to function during the developmental stages of wheat.

The accumulation of TaOMT2 in winter wheat leaves during low temperature acclimation indicates that this enzyme might be involved in modulating tricin level during cold acclimation. Activity assays were performed using the most preferred substrate of TaOMT2, tricetin (Zhou et al., 2006). Results show that the activity of tricetin-specific methyltransferases was decreasing.

A literature search of tricin indicated its isolation from a rust-resistant wheat variety (Anderson et al., 1931), its accumulation during wheat pathogen attack (Harborne and Williams 1986); but no report had so far indicated its association with cold acclimation. During normal growth, tricin accumulates during growth and development and reaches its highest accumulation during the flowering stages. On the other hand, cold, as a stress factor has arrested this process, which may suggest the importance of tricin during the stages of flowering and seed formation.

The second most preferred substrate of TaOMT2 is 5HFA (Zhou et al., 2006), a lignin precursor (Lewis and Yamamoto, 1990). Lignin contributes to the strength of plant cell walls, facilitates water transport and impedes the degradation of wall polysaccharides and was shown to accumulate during cold acclimation (Hatfield and Vermerris, 2001; Campbell and Sederoff, 1996). N'Dong et al. (2002) showed that in addition to daphnetin methylation, OMT involved in 5HFA methylation exhibited the highest enzyme activity when rye plants were cold acclimated. Therefore, an accumulation of TaOMT2 might be involved in lignin biosynthesis indicating the participation of TaOMT2 during cold acclimation.

The expression level of TaOMT2 was investigated in spring and winter cultivars, Bounty and Claire respectively. Results showed an accumulation of TaOMT2 in cold acclimated winter wheat leaves, Claire compared to spring wheat variety, Bounty. This suggests that TaOMT2 is associated with the ability of winter cultivar to circumvent the effects of cold stress. In fact, it was reported that both ribosomal and soluble RNA increases were much more pronounced in winter wheat than in spring wheat during cold acclimation (Sarhan and Daoust, 1975). Moreover, spring cereals exhibit limited ability to acquire an increased resistance to low temperature-induced photoinhibition compared to the winter variety (Oquist et al., 1993). Given these reports, it is plausible to suggest a role for this enzyme in the protective mechanism required during cold acclimation of winter wheat compared to the spring variety.

The fact that immunoblot analyses and enzyme activity measurements revealed a constant expression and a decrease in activity in wheat leaves exposed to salinity and

drought stresses suggests that TaOMT2 does not participate in protecting the plant against salinity and/or drought stresses.

Tricin has been credited to many health-promoting properties (Zhou et al., 2010 and references therein). It was shown to interfere with intestinal carcinogenesis in $Apc^{min}$ mice by inhibiting cyclooxygenase enzymes, to inhibit growth of malignant breast tumour cells and colon cancer cells to mention a few (Cai et al., 2005, Hudson et al., 2000). The structural knowledge of TaOMT2 and its regulation during developmental stages and during abiotic stresses provide the basis for the metabolic engineering of tricin production as a nutraceutical in wheat.

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