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# *O*-Methylation of Flavonoids Using DnrK Based on Molecular Docking

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**Abstract** *O*-Methylation is a common substitution reaction found in microbes as well as in mammalians. Some of the *O*-methyltransferases (OMTs) have broad substrate specificity and could be used to methylate various compounds. *DnrK* from *Streptomyces peucetius* encodes an anthracycline 4-*O*-methyltransferase, which uses carminomycin as a substrate, and its crystal structure has been determined. Molecular docking experiments with *DnrK* using various flavonoids were successfully conducted, and some of the flavonoids such as apigenin and genistein were predicted to serve as substrates. Based on these results, *O*-methylations of various flavonoids with the DnrK were successfully carried out. The methylation position was determined to be at the hydroxyl group of C7. Important amino acid residues for the enzymatic reaction of DnrK with apigenin could be identified using site-directed mutagenesis. Molecular docking could be useful to predict the substrate specificity ranges of other OMTs.

**Keywords:** DnrK, flavonoid, *O*-methyltransferase

*O*-Methyltransferase (OMT) is one of the most ubiquitous enzymes, widely distributed in nature, and catalyzes the transfer of a methyl group onto an hydroxyl group of an acceptor. Acceptors are as variable as the types of OMTs. For example, secondary metabolites such as monolignols, flavonoids, and alkaloids serve as acceptors in plants [5]. Likewise, catechol OMT is a well-known example as an acceptor in humans [8]. Microorganisms also contain OMTs, but only a few of them, except those involved in antibiotic biosynthesis, have thus far been characterized [11, 12, 16]. Most plant OMTs are substrate-specific and regioselective, whereas OMTs from mammalians and microorganisms have a broad spectrum of substrates. However, OMT from ice plants was shown to methylate only various phenolic compounds including flavonoids and phenylpropanoid [6]. Flavonoids are phytochemicals, and modification of flavonoids has drawn attention owing to their pharmaceutical and nutritional importance. In addition, because of the low solubility of the flavonoids, several approaches have been tried to increase their solubility [4, 9].

The molecular basis of regioselctivity with various OMTs has received special attention. X-ray structures from plant OMTs to mammalian catechol OMTs could provide some clues towards understanding the substrate specificity and influencing factors in determining the regioselectivity of individual OMTs [3, 15, 19, 20]. However, there has been no report about the engineering of OMTs to change their substrate specificity and regioselectivity.

*DnrK* is involved in the biosynthesis of daunorubicin, an aromatic polyketide antibiotic produced by *Streptomyces peucetius* [13, 14]. In addition, daunorubicin was used as a chemotherapy agent for a variety of cancers. It has been known that *Dnrk* encodes carminomycin 4-*O*-methyltransferase, which shows substrate specificity toward anthracyclines, ε-rhodomycin and carminomycin. Since the backbone of flavonoids overlaps with that of rhodomycin and the structure of carminomycin is similar to that of flavonoids, we have examined whether *DnrK* could be used to methylate flavonoids as substrates. In addition, the crystal structure and the three-dimensional structure of DnrK have already been determined [7], thus we could conduct molecular docking experiments with several flavonoids. In the present study, we have examined the methylation of various flavonoids with DnrK, based on molecular docking experiment, and determined the important amino acids for the reaction.

## MATERIALS AND METHODS

### Cloning and Expression of *DnrK*

The full length of *DnrK* was amplified by polymerase chain reaction (PCR) with primers (5'-ATGAATTCATGA-CAGCCGAACCGACGGT-3' as a forward primer and 5'-CGTGTGTCAGGCGCCGGT-3' as a reverse primer; GenBank

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**Table 1.** Primers for the mutagenesis of DnrK.

| Mutation | Primers | Relative activity (%) |
|---|---|---|
| Tyr142Phe | GCAAGCCGTTCTACGAGGACCTGGC | 55 |
| Asp162Val | GCTCGCCTGCGACCAGGACGTCG | 171 |
| Asn256Ile | TCGTCCTCCTCAACTGGCCGGACCA | 32 |
| Arg302Leu | AGAGCTCGATCTGCGGATGCTGGTCTTCC | 2 |

*Mutagenized positions are shown in lower-case letters.

Accession No. L40425) using Proofstart pfu DNA polymerase (Stratagene, La Jolla, CA, U.S.A.). Genomic DNA of *S. peucetius* was used as a template. The restriction site EcoRI was attached to the forward primer (underlined). Subsequently, the PCR product was subcloned into pGEMT-easy, and sequenced. After digestion of the PCR product with EcoRI, it was subcloned into the EcoRI/SmaI site of pGEX 5X-1 (Amersham Biotech, Piscataway, NJ, U.S.A.). Induction and purification of the recombinant *DnrK* were conducted as described by Kim *et al.* [10]. The point mutations were carried out with a QuikChange II XL site-directed mutagenesis kit (Stratagene). The primers used for mutagenesis are listed in Table 1.

**Enzymatic Reaction and Analysis of Reaction Products**

*O*-Methyltransferase reaction was carried out in a reaction mixture containing 20 μg of the purified protein, 50 μM of flavonoids, and 100 μM of *S*-adenosyl methionine (SAM) at 37°C for 16 h. Then, the reaction mixture was extracted with ethylacetate. The organic phase was collected and evaporated completely. The resulting substance was dissolved in methanol. Subsequently, the final products were analyzed using high-performance liquid chromatography (HPLC) as described by Lee *et al.* [12].

**Molecular Docking**

All ligands were sketched using the sketch module in the SYBYL package (version 7.2), and conformational searches were performed by grid search, which calculates energies by systematically changing the dihedral angles of each ligand using a standard TRIPOS force field. Among them, the lowest energy structures were selected as the conformer for the FlexX studies. Finally, all ligands were fully optimized using the standard TRIPOS force field with Gästeiger-Hückel charges until the energy gradient converged to below 0.05 kcal/mol.

Crystal structure of the enzyme DnrK complexed with SAM and 4-methoxy-ε-rhodomycin T (M-ε-T) was obtained from the Protein Data Bank (PDB) with the accession number of 1TW2. For docking study, the FlexX module was used, and this gives the best poses at the binding site by an incremental algorithm with flexible conformations. The binding site for calculations was defined with default parameters as all atoms of 1TW2 within 15 A° of SAM and a substrate. The formal charge of each compound was assigned during the calculations by FlexX, and all FlexX parameters were set to standard conditions. Sampling was done with 100 poses per ligand and, after scoring by the original FlexX scoring functions, thirty poses were saved in mol2 files for further analysis. All stored poses were rescored using the CScore module of SYBYL 7.2 comprising five different scoring functions including Dock, Chem, FlexX, PMF, and Gold. In order to eliminate conformations with impossible torsion energy values, we introduced torsion energy constraints by setting up the maximal torsion energy term to a default value (20 kJ/mol).

## RESULT AND DISCUSSION

**Molecular Docking Study of *DnrK* with Flavonoids**

DnrK transfers a methyl group into a hydroxyl group of carminomycin or ε-rhodomycin T (ε-T). Carminomycin (or ε-T) is a glycosylated cyclic polyketide containing four rings. Since the structure of carminomycin (or ε-T) is similar to that of (iso)flavonoids, it was assumed that DnrK could also use an (iso)flavonoid as a substrate (Fig. 1). In order to test this assumption, molecular docking experiment was carried out using crystal structure of DnrK (PDB Accession No. 1TW2) as a receptor and 16 flavonoids as ligands (Table 2). The result of the docking study shows that, as expected, flavonoids and the ligand (4-methoxy-ε-rhodomycin T, M-ε-T) in 1TW2 share the same binding

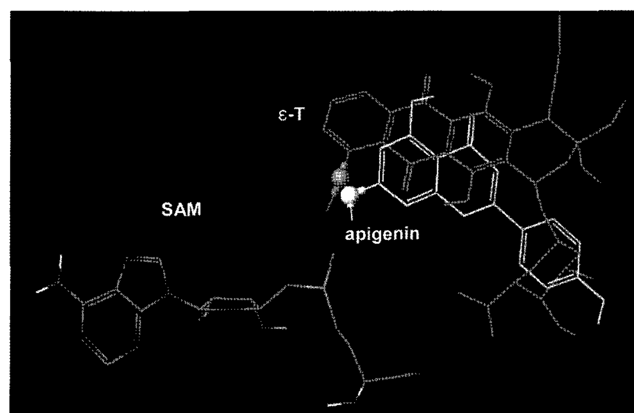


**Fig. 1.** Superimposed structures of ε-T (dark gray) and apigenin (light gray) docked into the active site of 1TW2.

**Table 2.** Docking of DnrK with various flavonoids.

| Entry | Flavonoids | Methylation position predicted by docking |
|---|---|---|
| 1 | Apigenin | 7-OH |
| 2 | 7-*O*-Glc-apigenin | Not docked |
| 3 | 7-*O*-Me-apigenin | Not docked |
| 4 | 7,4'-di-*O*-Me-apigenin | Not docked |
| 5 | 2(*S*)-Naringenin | 7-OH |
| 6 | 2(*R*)-Naringenin | 7-OH |
| 7 | 2(*S*)-7-*O*-Glc-naringenin | Not docked |
| 8 | 2(*R*)-7-*O*-Glc-naringenin | Not docked |
| 9 | Genistein | 7-OH or 4'-OH |
| 10 | 7-*O*-Glc-genistein | 4'-OH |
| 11 | Kaempferol | 7-OH |
| 12 | 3-*O*-Glc-Kaempferol | 7-OH |
| 13 | Luteolin | 7-OH or 3'-OH |
| 14 | 7-*O*-Glc-luteolin | Not docked |
| 15 | 4'-*O*-Glc-luteolin | 7-OH |
| 16 | Quercetin | 7-OH |

mode. Thus, every flavonoid aglycon tested was successfully docked at the active site of DnrK, whereas flavonoid-glucoside conjugates failed to do so. From the fact that, except for kaempferol, the most favorable methylation site was 7-OH of flavonoids, it is obvious that the flavonoid-glucose conjugates (Table 2, entries 2, 4, 7, 8, 14) or methoxy flavonoids (Table 2, entry 3) with substituents attached at the 7-OH of flavonoid fail to bind to the target enzyme. The isoflavone genistein (Table 2, entry 9) shows two possible binding modes in which 7-OH and 4'-OH are located at the methylation site of the enzyme. Moreover, as ring B of isoflavone projects into a different angle compared with other flavonoids, the docking mode of the isoflavone is completely different from other flavonoids. Taken together, genistein 7-*O*-glucoside (Table 2, entry 10) binds exceptionally to the target enzyme, with its 4'-OH group located at the methylation site of DnrK. The docking mode of kaempferol 3-*O*-glucoside (Table 2, entry 12) is worthwhile to note, because its 3-*O*-glucoside is located at the wide open space out of the binding pocket where the hydrophilic glucoside interacts with water molecules to enhance the binding affinity. The fact that flavonoid aglycones were docked into the binding pocket of DrnK is in contrast to the early assumption that DnrK acts solely on substrates containing amino sugars [2].

### Reaction of *DnrK* with Flavonoids

The molecular docking result clearly showed that flavonoid could be a substrate. In order to confirm the molecular docking results experimentally, the *DnrK* gene was amplified and sequenced. Then, the PCR product was subcloned into *E. coli* expression vector pGEX. The recombinant *DnrK* was successfully expressed and purified into near

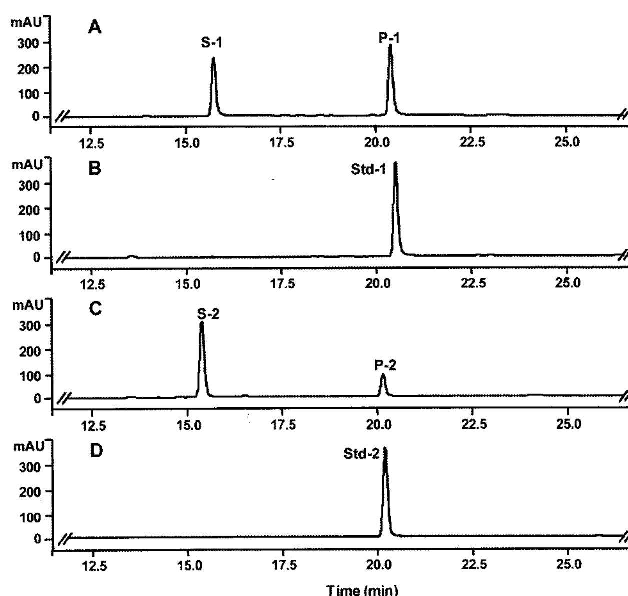


**Fig. 2.** HPLC profile of apigenin and genistein reactions products with DnrK.

**A.** HPLC profile of apigenin reaction product (S-1, apigenin; P-1 reaction product). **B.** Authentic 7-*O*-methoxyapigenin (Std-1). **C.** HPLC profile of genistein reaction product (S-2, genistein; P-2 reaction product). **D.** Authentic 7-*O*-methoxygenistein (Std-2).

homogeneity by SDS-PAGE analysis (data not shown). Several flavonoids were used to find out whether the recombinant DnrK could methylate any of them. As predicted by the molecular docking experiment, luteolin, luteolin 4'-*O*-glucoside, kaempferol, kaempferol-3-*O*-glucoside, and apigenin underwent reactions and produced corresponding products. However, substrates containing a methyl group or glucose at the 7-hydroxyl group, such as 7-methoxyapigenin and luteolin 7-*O*-glucoside, did not serve as a substrate, which agreed with molecular docking.

The methylation position was determined by comparing the retention time of the reaction product with that of the authentic compound. For example, HPLC analysis of the apigenin reaction product showed it had the same retention time as 7-*O*-methoxyapigenin (Fig. 2A). It indicated that the methylation position is at the hydroxyl group of C7. Genistein was predicted to be methylated either at the 7-hydroxyl group or at 4'-hydroxyl, based on molecular docking study. HPLC analysis of the genistein reaction product showed the same retention time as 7-*O*-methyl genestein, indicating that the methylation position is at the hydroxyl group of C7 (Fig. 2C).

### Sitedirected Mutagenesis of DnrK to Identify the Important Amino Acids for the Reaction with Flavonoids

The crystal structure of DnrK complexed with methyl donor (SAM) and ligand (methyl acceptor, Me-ε-T) (PDB accession code: 1TW2) shows that four amino acids

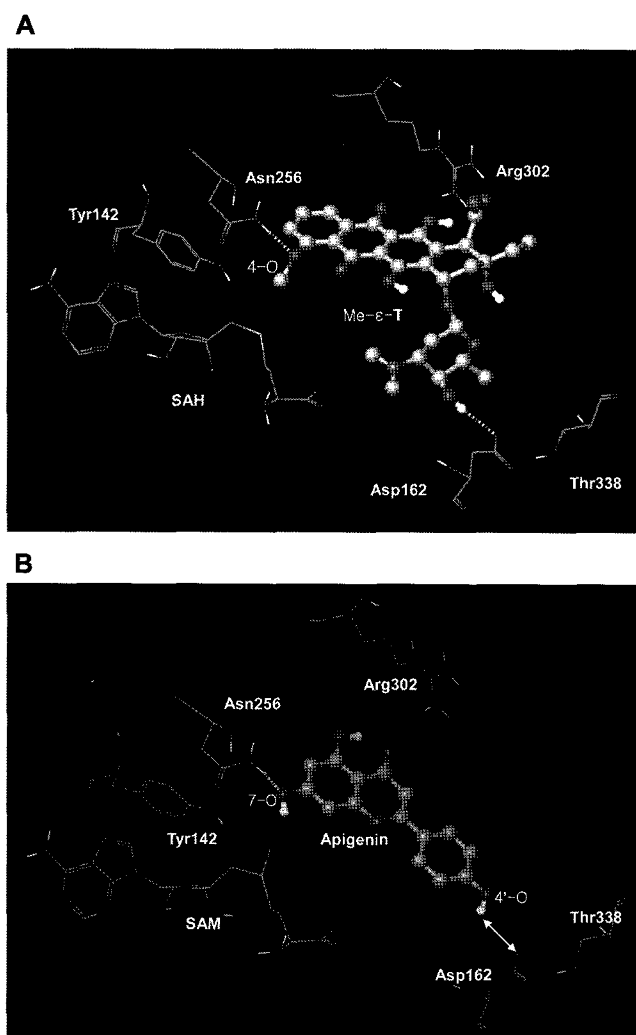


**Fig. 3. A**. Structure of apigenin docked at the active site of *DnrK*. Interactions of the apigenin with Tyr142, Asn256, and Arg302 are conserved, but Asp162 does not interact with flavonoids. **B**. Structure of apigenin docked at the active site of *DnrK*. Interactions of apigenin with Tyr142, Asn256, and Arg302 are conserved, but Asp162 does not interact with flavonoids.

(Tyr142, Asp162, Asn256, and Arg302) are in direct interaction with the ligand (Fig. 3A): Tyr142 and Asn256 are believed to stabilize the transition state of the methyl transfer reaction by interaction with the transient methyl cation through charge transfer and with the methyl acceptor (4-*O* of Me-ε-T) through hydrogen bonding, respectively. The transfer of one proton from the methyl group to an acceptor, known as acid/base catalysis, has been accepted as a reaction mechanism for methyltransferases including *O*-methyltransferases [19] and some of the *N*-methyltransferases, [18] and tyrosine at 142 was a plausible candidate to be methylated. However, mutation in Tyr142 into trytophane did not abolish the enzyme activity of DnrK for carminomycin [6]. Based on this result and orientation of substrate, cosubstrate, and DnrK, Dnrk was suggested to use proximity and orientation effects as an enzymatic mechanism instead of the acid/base mechanism found in other *O*-methyltransferases. For example, Arg302, located away from the reaction site, is hydrogen bonded to the hydroxyl group of carbon 10 of Me-ε-T and helps DnrK bind its substrate at the active site. On the other hand, Asp162 is likely to be involved in recognition of the substrate through hydrogen bonding with a sugar residue of Me-ε-T.

The similar structural feature of Me-ε-T and flavonoids (Fig. 3B) warrants similar binding modes of flavonoids to DnrK and similar roles of the three amino acid residues (Tyr142, Asn256, and Arg302) upon methylation of flavonoids. In other words, Tyr142 and Asn256 are predicted to be involved in stabilization of the reaction intermediate, and Arg302 forms a hydrogen bond with the oxygen of C4. However, the sugar-recognition site (Asp162) might affect the binding modes of flavonoid aglycons to DnrK in a different way because of the structural difference between the sugar moieties of Me-ε-T and ring B of the apigenin, which overlap in the same binding site (Fig. 1). The docking modes of apigenin show that the electronegative carboxylate anion of Asp162 and 4'-*O* of apigenin are located in close contact with each other and presumably result in unfavorable electrostatic interactions.

Thus, in order to evaluate the roles of these amino acids in binding of flavonoids and the methyl transfer reaction, site-directed mutagenesis was carried out (Tyr142Phe, Asp162Val, Asn256Ile, and Arg302Leu), and the mutant proteins were successfully expressed and purified. As expected, mutations of Arg302Leu resulted in the almost complete loss of activity of DnrK when apigenin was used as a substrate (Table 1), indicating that formation of a hydrogen bond between arginin302 and oxygen at carbon 4 of the substrate is critical for the proper arrangement of its substrate into the substrate-binding pocket. Mutation in Tyr142 into Phe resulted in about 45% loss of activity with apigenin. This result is consistent with the previous mutation study with carminomycine, and suggests that DnrK appears to use a similar mechanism for the *O*-methylation toward carminomycin and apigenin. Mutation of Asn256 into Ile lost about 70% of its activity, indicating that proper arrangement of cosubstrate is also critical for the enzymatic reaction. However, mutation in Asn256 increased the enzymatic activity more than two folds, presumably due to the relief of the unfavorable electrostatic interaction between 4'-*O* of apigenin and the carboxylate anion of Asp162 (Fig. 3B).

Protein engineering has been a valuable tool, but random mutation methods such as error-prone PCR [1] and gene shuffling [17] have been prevalent. It is due to the lack of sufficient structural information of proteins and methods for predicting three-dimensional structures. Since the number of newly determined protein structures has been growing rapidly and molecular modeling based on the homology

could be applied to predict the three-dimensional structures of proteins, our new approach would be a valuable tool to predict substrates without doing any wet experiments. In addition, site-directed mutagenesis followed by molecular docking experiment could be useful in attempting to find substrates of enzymes available from GenBank and improve the reactivity of enzymes.

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## REFERENCES

1. Arnold, F. H. and A. A. Volkov. 1999. Directed evolution of biocatalysts. *Curr. Opin. Chem. Biol.* **3:** 54–59.
2. Connors, N. C., P. L. Bartel, and W. R. Strohl. 1990. Biosynthesis of anthracyclines: Carminomycine 4-O-methyltransferase, the terminal enzymic step in the formation of daunomycine. *J. Gen. Microbiol.* **136:** 1895–1898.
3. Ferrer, J.-L., C. Zubieta, R. A. Dixon, and J. P. Noel. 2005. Crystal structure of alfalfa caffoyl coenzyme A 3-*O*-methyltransferase. *Plant Physiol.* **137:** 1009–1017.
4. Graefe, E. U., H. Derendorf, and M. Veit. 1999. Pharmacokinetics and bioavailability of the flavonol quercetin in humans. *Int. J. Clin. Pharmacol. Ther.* **37:** 219–233.
5. Ibrahim, R. K., A. Bruneau, and B. Bantignies. 1998. Plant *O*-methyltransferase: Molecular analysis, common signature and classification. *Plant Mol. Biol.* **36:** 1–10.
6. Ibdah, M., X.-H. Zhang, J. Schnot, and T. Vogt. 2003. A novel $Mg^{2+}$-dependent *O*-methyltransferase in the phenylpropanoid metabolism of *Mesembryanthemum crystallinum*. *J. Biol. Chem.* **278:** 43961–43972.
7. Jansson, A., H. Koskiniemi, P. Mäntsälä, J. Niemi, and G. Schneider. 2004. Crystal structure of a ternary complex of DnrK, a methyltransferase in daunorubicin biosynthesis, with bound products. *J. Biol. Chem.* **279:** 41149–41156.
8. Jeffery, D. R. and J. A. Roth. 1984. Characterization of membrane-bound and soluble catechol-*O*-methyltransferase from human frontal cortex. *J. Neurochem.* **42:** 826–832.
9. Kang, S., S. Lee, C. Kwon, and S. Jung. 2006. Solubility enhancement of flavonoids by cyclosophoraose isolated from *Rhizobium meliloti* 2011. *J. Microbiol. Biotechnol.* **16:** 791–794.
10. Kim, D. H., B.-G. Kim, Y. Lee, J. Y. Ryu, Y. Lim, H.-G. Hur, and J.-H. Ahn. 2005. Regiospecific methylation of naringenin to ponciretin by soybean *O*-methyltransferase expressed in *Escherichia coli*. *J. Biotechnol.* **138:** 155–162.
11. Lee, H. J., B. G. Kim, and J.-H. Ahn. 2006. Molecular cloning and characterization of *Bacillus cereus O*-methyltransferase. *J. Microbiol. Biotechnol.* **16:** 619–622.
12. Lee, Y. J., B.-G. Kim, Y. Park, Y. Lim, H.-G. Hur, and J.-H. Ahn. 2006. Biotransformation of flavonoids with *O*-methyltransferase from *Bacillus cereus*. *J. Microbiol. Biotechnol.* **16:** 1090–1096.
13. Otten, S. L., K. J. Stutzman-Engwall, and C. R. Hutchinson. 1990. Cloning and expression of daunorubicin biosynthesis genes from *Streptomyces peucetius* and *S. peucetius* subsp. *caesius*. *J. Bacteriol.* **172:** 3427–3434.
14. Stutzman-Engwall, K. J. and C. R. Hutchinson. 1989. Multigene families for anthracycline antibiotic production in *Streptomyces peucetius*. *Proc. Natl. Acad. Sci. USA* **86:** 3135–3139.
15. Vidgren, J. L., A. Syeriseon, and A. Liljas. 1994. Crystal structure of catechol *O*-methyltransferase. *Nature* **368:** 354–357.
16. Yoon, Y., Y. S. Yi, Y. Lee, S. Kim, B. G. Kim, J.-H. Ahn, and Y. Lim. 2005. Characterization of *O*-methyltransferase ScOMT1 cloned from *Streptomyces coelicolor* A3(2). *Biophys. Biochem. Acta* **1730:** 85–95.
17. Yuan, L., I. Kureck, J. English, and R. Keenan. 2005. Laboratory-directed protein evolution. *Microbiol. Molec. Biol. Rev.* **69:** 373–392.
18. Zhang, X., L. Zhou, and X. Cheng, 2000. Crystal structure of the conserved core of protein arginine methyltransferase PRMT3. *EMBO J.* **19:** 3509–3519.
19. Zubieta, C., X. Z. He, R. A. Dixon, and J. P. Noel. 2001. Structures of two natural product methyltransferases reveal the basis for substrate specificity in plant *O*-methyltransferases. *Nat. Struct. Biol.* **8:** 271–279.
20. Zubieta, C., P. Kota, J.-L. Ferrer, R. A. Dixon, and J. P. Noel. 2002. Structural basis for the modulation of lignin monomer methylation by caffeic acid/5-hydroxyferulic acid 3/5-*O*-methyltransferase. *Plant Cell* **14:** 1265–1277.