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# A cost-effective colorimetric assay for phenolic *O*-methyltransferases and characterization of caffeate 3-*O*-methyltransferases from *Populus trichocarpa*

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ABSTRACT

*S*-Adenosyl-L-methionine (AdoMet)-dependent *O*-methyltransferases (OMTs) catalyze the transmethylation of a variety of phenolics in bacteria, plants, and humans. To rapidly characterize phenolic OMT activities, we adapted Gibbs' reagent, the dye originally used for detecting phenols, to develop a convenient assay method for measuring the catalytic properties of enzymatic transmethylation of phenolics. We demonstrated that Gibbs' reagent reacted with phenolics yielding distinct absorptive characters that we used to further develop the assay to monitor the reactivities of phenolic OMTs. To validate the method, we identified two caffeate/5-hydroxyferulate 3/5-*O*-methyltransferases (COMTs) from the black cottonwood, *Populus trichocarpa*. Together with a few other plant type I OMTs, we demonstrated that our Gibbs' reagent-mediated colorimetric assay could reliably determine the functions and kinetic parameters of phenolic OMTs. Because Gibbs' reagent reacting with different regioselectively modified phenolics displays different colorimetric properties, the assay method can be used to monitor both substrate specificity and the regioselectivity of phenolic OMTs.



*S*-Adenosyl-L-methionine (AdoMet)[1]-dependent methyltransferases are essential for modifying a variety of biological molecules, including myriad small molecule metabolites, carbohydrates, nucleic acids, and proteins [1–3]. A number of AdoMet-dependent methyltransferases in bacteria, animals, and plants are involved particularly in metabolizing various phenolics and polyphenolics [3–6]. Enzymatic methylation essentially determines the specific physiological functions of the resultant molecules and changes their lipophilicity [5]. A typical example of a phenolic *O*-methyltransferase (OMT) in plants is caffeate/5-hydroxyferulate 3/5-*O*-methyltransferase (COMT); this enzyme was demonstrated to methylate a range of monomeric phenolics with propanoid tails conferring different functionalities, that is, carboxylate, aldehyde, and alcohol moieties [7–9]. Whereas COMT methylates caffeic acid and 5-hydroxyferulic acid *in vitro*, it predominates *p*-cinnamaldehyde and cinnamyl alcohol methylations *in planta* for monolignol biosynthesis [10,11]. Moreover, in working on different lignin monomeric precursors, the enzyme exhibits exclusively regioselectivity for *meta* (3 or 5)-hydroxyl methylation [12]. Besides COMT, a few other OMTs were characterized with specific activity for the transmethylation of different allylphenolic compounds, including isoeugenol, eugenol, and chavicol (i.e., those chemicals imparting aroma or flavor to the plants) [13,14]. The characterized (iso)eugenol and chavicol 4-*O*-methyltransferases from the wildflower *Clarkia breweri* (fairy fans) [13] and from sweet basil (Lamiaceae family) [14] exhibit distinct substrate preferences and regiospecificity for the 4-hydroxyl methylation of phenylpropenes.

Plant phenolic OMTs recently were classified into the type I methyltransferase family based on protein structure–function studies [3]. The enzymes in this family commonly encompass symmetric homodimers composed of two larger subunit sizes (38–43 kDa); their enzymatic activity does not require divalent cations. The enzymes within this family act on a variety of substrates, primarily phenolics and polyphenolics; indeed, the range of the possible substrates for type I enzymes may be much larger because many of the family members have been identified only from sequence similarity.

The functional characterization of phenolic OMTs routinely relies on radioactive assays that measure the incorporation of $^{14}$C- or $^{3}$H-labeled methyl groups from AdoMet into the particular phenolic substrates or requires the use of analytic instruments such as high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS) [15,16]. Although the radiometric assay is highly sensitive, it is relatively laborious and, thus, not suitable for high-throughput applications; furthermore, the radioactive wastes generated from the assay are environmentally hazardous. On the other hand, HPLC-

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[1] *Abbreviations used:* AdoMet, *S*-adenosyl-l-methionine; OMT, *O*-methyltransferase; COMT, caffeate/5-hydroxyferulate 3/5-*O*-methyltransferase; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; SAH, *S*-adenosyl-l-homocysteine; IEMT, (iso)eugenol 4-*O*-methyltransferase; cDNA, complementary DNA; IPTG, isopropyl-β-d-1-thiogalactopyranoside; BSA, bovine serum albumin; PCR, polymerase chain reaction; TCA, tricarboxylic acid; DTT, dithiothreitol; UV, ultraviolet; ESI, electrospray ionization; RT–PCR, reverse transcription polymerase chain reaction; SbOMT, *Sorghum bicolor O*-methyltransferase.

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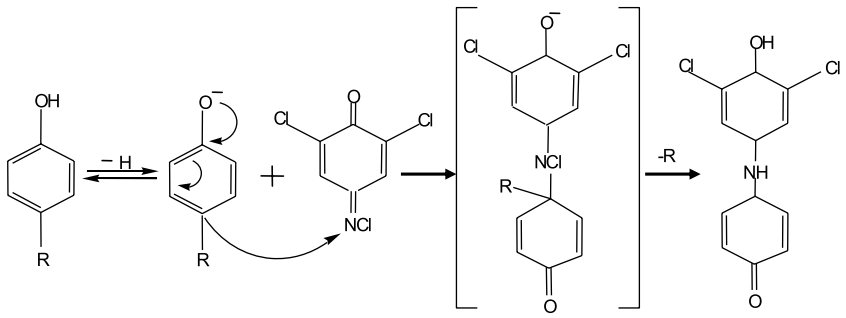


**Fig. 1.** Scheme of the proposed mechanism for the reaction of Gibbs' reagent with phenolics. R, substitutive residues.

or LC–MS-based chromatographic detections require expensive instrumentation that not all laboratories can easily access. To overcome these technical barriers, spectroscopic-based methyltransferase assays were developed and were essentially based on detecting the enzymatically decomposed chemical species of the by-product *S*-adenosyl-L-homocysteine (SAH) of transmethylation [17–19]. Because the assay procedures necessarily employ coupling enzymatic reactions, their sensitivity and convenience is somewhat compromised.

Genomics and functional genomics studies have led to the annotation of a large number of methyltransferase genes from different plant species in which the encoded enzymes are potentially responsible for modifying a variety of phenolics and polyphenolics. However, for many of them, the precise biochemical functions need validation. Moreover, structure–function studies on phenolic/polyphenolic OMTs [12,20,21] support the rational design of novel functional OMTs. Therefore, both the genome-wide functional characterization of the putative OMTs and the high-throughput functional screening of the laboratory-evolved OMT variants would benefit from a more convenient, cost-effective assay.

Gibbs' reagent (2,6-dichloro-*p*-benzoquinone-4-chloroimine) has long been used for detecting phenol and its derivatives [22]. The reaction yields 2,6-dichloroindophenol, the compound responsible for the development of a bright blue color [23,24]. The dye complex is formed by an oxidative coupling reaction, generating a *p*-quinoid species that requires a free *para*-hydroxyl from the phenyl ring for initiating a dehydrogenative reaction (Fig. 1). Theoretically, substitution of the hydroxyl groups at different positions of the phenolic ring may change the chemical properties of the phenolic compounds, thereby affecting dehydrogenation and the formation of the dye complex. In particular, modifying (e.g., by methylation) the *para*-hydroxyl moiety may abolish the radical initiation and, thus, the formation of the dye complex. By monitoring the changes in absorbance of the dye complex, we may be able to directly correlate and determine OMT activities.

Here we describe the development of a simple colorimetric assay method for determining phenolic OMT activity based on Gibbs' reagent. To validate the method, we identified two new COMT homologs from *Populus trichocarpa,* a tree grown as a potential bioenergy crop for producing biofuels. Using poplar COMTs together with (iso)eugenol 4-*O*-methyltransferase (IEMT) from *C. breweri,* we explored the applications of the Gibbs' reagent-based colorimetric method for the rapid determination of the enzyme's substrate specificity, regioselectivity, and catalytic kinetics.

## Materials and methods

### *Chemicals*

The following chemicals were purchased from Sigma (St. Louis, MO, USA): phenol, isoeugenol, eugenol, coniferyl alcohol, sinapyl alcohol, *p*-coumaric acid, caffeic acid, ferulic acid, 4-methoxycinnamic acid, 4-hydroxy-3-methoxyphenyl acetic acid, 3,4-dimethoxy phenyl acetic acid, 2,6-dichloroquinone-4-chloroimide (Gibbs' reagent), and AdoMet. BugBuster and Bradford were purchased from Novagen (Madison, WI, USA) and Bio-Rad (Hercules, CA, USA), respectively.

### *Preparation of reagent*

A solution of 0.4% (w/v) Gibbs' reagent was prepared in ethanol and stored at 4 °C. A stock solution of 10 mM AdoMet was made in acidic water (pH 3.2). All of the phenolics were dissolved in methanol and stored at −20 °C.

### *Isolation, expression, and purification of COMTs from P. trichocarpa*

We performed a BLAST search of COMT sequences from *P. trichocarpa* using the gene sequence of *Medicago sativa* COMT (GenBank Accession No. P28002). Two gene models were identified (estExt_fgenesh4_pm.C_LG_XII0129 and estExt_fgenesh4_pg.C_LG_XV0035) and denoted as *PtCOMT1* and *PtCOMT2.* Total RNA samples were extracted from the stems of *P. trichocarpa* and treated with DNase I (New England Biolabs, Ipswich, MA, USA), and 1 μg of RNA was used for complementary DNA (cDNA) synthesis by the reverse transcription reaction. Subsequently, full-length cDNAs corresponding to both gene models were amplified by the PCR reaction against 1 μl of reverse transcription product under the following conditions: 98 °C for 50 s, followed by 35 cycles of 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 60 s, and then a final extension at 72 °C for 10 min, with the gene-specific primers for *PtCOMT1* (forward primer 5′-CCGCGTGGATCCATGGGTTCGACAGGTGAA-3′; reverse primer 5′-CAAGAAAGCTGGGTTTAGTTCTTGCGGAATTCAATGAC-3′) and for *Pt COMT2* (forward primer 5′-CCGCGTGGATCCATGGGTTCAACAGGTGAAACTCAG-3′; reverse primer 5′-CAAGAAAGCTGGGTTTAGGCCTGCTTGCGGAAT-3′). The amplified cDNAs were subcloned into a modified pET28a(+) expression vector compatible with Gateway cloning in which the expression cassette was driven by the T7 promoter, followed by the ribosomal binding site and sequences encoding the 9-histidine tag and the in-frame fusion *ccdB* gene with attR1 and attR2 recombinant sequences at its N and C termini, respectively [25]. The constructs then were transferred into an *Escherichia coli* BL21(DE3) strain. The 100-ml *E. coli* cultures were induced by 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at $OD_{600} \sim 1.0$ and were incubated at 25 °C for 16 h. The cells were harvested and the cell pellets were frozen at −80 °C before use. The cells were resuspended in 50 mM Tris–HCl (pH 8.0) containing 500 mM NaCl, 10% glycerol, 1% Tween 20, 1% Triton X-100, 100 μg/ml lysozyme, 10 μg/ml DNase, 20 mM imidazole, and 1× protease cocktail inhibitor (Complete EDTA-free Protease Inhibitor Cocktail tablets, Roche Diagnostics, Basel, Switzerland). The cells were disrupted by sonication, and the supernatant was collected by centrifugation at 19,500 rpm for 45 min. The protein was purified in an $Ni^+$–agarose affinity column as described previously [26]. The protein content was determined