

A Continuous, Quantitative Fluorescent Assay for Plant Caffeic Acid *O*-Methyltransferases

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Plant caffeic acid *O*-methyltransferases (COMTs) use *S*-adenosylmethionine (ado-met), as a methyl donor to transmethylate their preferred (phenolic) substrates in vivo, and will generally utilize a range of phenolic compounds in vitro. Collazo et al. (*Anal. Biochem.* 2005, 342, 86–92) have published a discrete, end-point fluorescence assay to detect histone methyltransferases using *S*-adenosyl homocysteine hydrolase and adenosine deaminase as coupling enzymes and a thiol-specific fluorophore, Thioglo1, as the detecting reagent. Using this previous assay as a guide, we have developed and validated a facile, sensitive and real-time fluorescence assay for characterizing plant COMTs and in the process simplified the original assay as well by obviating the need for adenosine deaminase in the assay, and simultaneously converting an end-point assay into a continuous one. Our assay has been used to kinetically characterize recombinant sorghum COMT (Bmr-12) a key enzyme involved in cell wall lignification, and analyze COMT activity in maturing tillers from switchgrass plants. Data indicated that the calculated K_m and V_{max} values for the recombinant sorghum COMT using different substrates in the fluorescent assay were similar to published values for COMT enzymes from other plant species. Native COMT activity was greatest in internodes at the top of a tiller and declined in the more basal internodes. This new assay should have broad applicability for characterizing COMTs and potentially other plant methyltransferases that utilize ado-met as a methyl donor.

KEYWORDS: Assay; caffeic acid *O*-methyltransferase; coupled; extracts; facile; fluorescence; real-time; recombinant sorghum Bmr-12; switchgrass

INTRODUCTION

Lignins are the second most abundant natural polymer after cellulose (1) and represent a major source of energy in biomass. At the same time they negatively impact biomass deconstruction into liquid fuels such as ethanol or butanol (2–5). As a consequence, there has been an emphasis to understand the basic aspects of lignin biosynthesis, cell wall architecture and breeding for low lignin germplasm in a range of dedicated bioenergy crops (5–10).

Lignin biosynthesis in plants is carried out by a suite of enzymes that convert cinnamic acid into hydroxylated and methylated monolignol derivatives. There are two transmethylation reactions during the formation of monolignols, first during the synthesis of feruloyl-*S*-CoA from caffeoyl-*S*-CoA which is catalyzed by caffeoyl-CoA-*O*-methyltransferase, and during the formation of sinapyl aldehyde by transmethylation of 5-hydroxyconiferyl aldehyde by the enzyme caffeic acid *O*-methyltransferase (COMT) (11, 12). COMTs are a class of enzymes that catalyze the transmethylation of a number of phenolic compounds including monolignol precursors using *S*-adenosylmethionine (ado-met) as a methyl donor. Multiple COMT genes encoding structurally related proteins are usually present within

a single plant genome as part of a larger *O*-methyltransferase gene family (11, 13, 14). However, only one or at most a few of the COMT proteins appear to be directly involved in lignification (10, 15–17). Downregulation of lignin-specific COMT leads to a loss of *S*-lignin in most plants and an increased incorporation of 5-hydroxyconiferyl alcohol into lignin (12, 16–19), indicating that 5-hydroxyconiferyl aldehyde is probably the major native substrate for lignifying COMTs. In vitro, COMTs involved in lignification will utilize a number of other mono- and dihydroxylated phenolic substrates, which have been used to assay enzyme activity of purified proteins or plant extracts (20).

Here we describe and validate a real-time, continuous fluorescent assay to measure COMT activity. Other previously published radiochemical, HPLC, GCMS, fluorescence and colorimetric assays (20–25) either required radiochemicals or used discrete end points whereby product amount was measured in aliquots removed from an assay mixture and subsequently used to calculate rates. Our assay was adapted from the coupled protocol first described by Collazo et al. (26) which was developed to assay histone methyltransferases using ado-met as a methyl donor and *S*-adenosylhomocysteine hydrolase (SAHH) and adenosine deaminase as coupling enzymes. Although suitable for measuring a range of ado-met requiring enzymes, this original assay did not provide a continuous, real-time measurement of enzymatic activity. We anticipated that a direct coupling of free homocysteine to

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Thioglo1 could convert the end-point assay of Collazo et al. (26) to a continuous assay for COMT and one that could be readily adapted for plate-reading spectrofluorimeters.

MATERIALS AND METHODS

Recombinant Proteins. *Sorghum COMT (Bmr12).* The coding region for sorghum COMT (Bmr12) was amplified by PCR using the following primers forward 5'-CCAGATCTGATGGGGTGCACGGC-GGAGGACGTGGCGG-3', reverse 5'-GAGTGC GGCCGCTTACTT-GATGAAC TCGATGGCCC-3'; sorghum EST clone (ANR1_8_C01_A002; GenBank numbers CX607344 and CX607415) as the template. The amplicon was digested with *Bgl*II and *Nor*I and ligated into pET30a vector according to manufacturer's protocol (Novagen, Madison, WI). Automated sequencing was performed by University of Nebraska—Lincoln sequencing facility to verify sequence fidelity of the construct. An error introduced into ANR1_8_C01_A002 through cloning, which changed the codon for amino acid 282 from tyrosine to cysteine, was corrected using site-directed mutagenesis. Recombinant protein was produced in *Escherichia coli* Rosetta 2 cells (Novagen, Madison, WI), and purified essentially as described earlier (27). Purity of the final enzyme preparation was checked by gel electrophoresis under denaturing conditions (28). Purified recombinant sorghum COMT was dialyzed against 50 mM Tris, pH 7.5, and stored in aliquots at -80°C .

Sulfolobus SAHH. The *Sulfolobus* SAHH clone was obtained through a Materials Transfer Agreement with Dr. Raymond C. Trievel, Department of Biological Chemistry, University of Michigan, Medical School and was recombinantly produced and purified essentially as described by Collazo et al. (26).

Protein concentrations were determined using a colorimetric microplate assay using Coomassie blue and bovine serum albumin as a standard, as described by the manufacturer (Thermo Scientific, Rockford, IL).

Plant Materials. Switchgrass plants were grown in the field as described earlier (29). Flowering tillers were hand separated into internodes, with internode 1 being closest to the peduncle. Internodes were flash frozen in liquid N_2 , and stored frozen at -80°C until used.

Internodes were first ground with chunks of dry ice in a coffee grinder. This powdered material was placed at -80°C until the dry ice had sublimated (approximately 48 h), then poured into 50 mL plastic tubes for storage. When needed ~ 0.7 g of internode material was weighed, transferred to a precooled mortar and pestle and extracted with 1.4 mL of 50 mM Tris buffer, pH 7.5, containing 0.35 M sucrose. Homogenates were filtered through 1 layer of MiraCloth, and the solution was placed in 2.0 mL microfuge tubes and centrifuged at 13,500 rpm for 15 min in a refrigerated centrifuge (MicroGPR, Thermo Scientific, Rockford, IL). Clarified supernatants were used as a source of native COMT. Protein concentrations in extracts were determined as described above. Switchgrass COMT was partially purified by binding 250 μL of clarified, internode homogenates to a strong anion exchange membrane (Vivapure Q, MiniH, Sartoriusstedim Biotech., Goettingen, Germany) equilibrated with 50 mM Tris buffer, pH 7.5 containing 0.35 M sucrose. The membrane filter was washed with $2 \times 250 \mu\text{L}$ of equilibrating buffer. Bound proteins were sequentially eluted with $1 \times 250 \mu\text{L}$ of equilibrating buffer containing 0.25 and 0.6 M NaCl. The wash fractions were pooled. All three fractions were assayed for COMT activity using the standard assay solution (see above).

Enzyme Assays. Routine COMT activity analyses were performed under the following conditions in a final well volume of 150 μL : 100 mM Tris-Cl, pH 7.5, 1 μM SAHH, 150 nM recombinant sorghum COMT, 85 μM ado-met and $\sim 15 \mu\text{M}$ Thioglo1 (Covalent Associates Inc., Corvallis, OR) final concentrations were prepared. Thioglo1 was obtained as its maleimide derivative. Neither the maleimido form nor the hydrolyzed unconjugated Thioglo1 exhibits intrinsic fluorescence (<http://www.covalentassociates.com/thioglo.htm>). Thioglo1 was prepared as a stock solution in dimethylformamide, divided into aliquots, and kept in foil covered tubes at -80°C . Phenolic substrates were prepared as 0.5 to 5 mM stock solutions in 10% dimethylformamide in water (v/v) and used at the indicated concentrations. For routine assays, the final concentration of a phenolic substrate in each well was 250 μM . All of the components, except for the phenolic substrate, were mixed together to yield a bulk reaction mixture that was subsequently used for assays.

As a routine protocol, 10 μL of a stock substrate solution was first added to the appropriate wells of a black 96 well microplate (Microfluor 2 black, flat well, ThermoFisher Scientific, Waltham, MA), and enzyme assays were initiated by the addition of 140 μL of the reaction mixture described above to each well. Well contents were thoroughly mixed by pipet action, and the plate was placed in a Bio Tek Synergy HT fluorescence plate reader (Bio Tek Instruments, Winooski, VT) set at 37°C with a gain set to 80. Fluorescence was determined using an excitation filter 360 ± 40 nm and an emission filter 528 ± 20 nm. Fluorescence signal was measured every minute for 45 min with an instrument predesignated "moderate shake" for 5 s between each read. Raw fluorescence data was imported into Microsoft Excel (MS Office 2007) and analyzed. Fluorescence data were converted into picomoles of $-\text{SH}$ groups released based on a standard curve using glutathione as a standard.

For kinetic analyses, substrate concentrations were varied, but the total volume added per well was maintained at 10 μL . Generally 30 μL of plant extracts were assayed. All enzymatic analyses were performed in triplicate, and these experiments have been repeated at least thrice. All chemicals were reagent grade or better. All solutions, especially buffers and substrates and plant extracts, were prepared fresh prior to an assay. Recombinant enzymes were stored as aliquots at -80°C and thawed prior to use.

During the development and validation of our assay for COMT, we also found that adenosine deaminase was not required because of the rapid reaction between Thioglo1 and homocysteine. The reaction rate could be manipulated by varying the levels of both COMT at fixed SAHH levels or increasing SAHH at fixed levels of COMT in the assay well. We have optimized conditions that result in reproducible increase in fluorescence for a range of substrates and one that permitted the analyses of COMT activities in total plant homogenates.

GCMS Analyses. GCMS analyses were performed on assay solutions containing caffeic acid to determine if ferulic acid (the product derived from methylation of caffeic acid) could be detected in the assays, and to serve as an independent estimator of the effect of substrate concentration on product formation under our standardized assay conditions (see above). Briefly, assays were performed as described above and at the end of the fluorescence assay (~ 45 min), all assay wells in the microplate were acidified by the addition of 50 μL of 6 M HCl and the entire well contents ($\sim 200 \mu\text{L}$) were transferred to a 1.7 mL microfuge tube. The acidified solutions were back extracted thrice with 0.25 mL aliquots of ethyl acetate, and the organic solutions were pooled. A few crystals of anhydrous sodium sulfate were added to the combined organic phase, followed by centrifugation at 13,500 rpm for 5 min. The supernatant was transferred to a fresh tube and dried by centrifugal evaporation. The dried residue was silylated and analyzed using an Agilent G2570A integrated GCMS system equipped with a G2913A autoinjector module, 6850 series II GC, and a 5973 network mass spectrometer (Agilent, Palo Alto, CA) as described previously (29).

Data Analyses and Parameter Estimates. Individual reaction rates were calculated by fitting a 2-parameter line to the linear portion of the fluorescence data using linear least-squares. To identify this portion, regressions were performed sequentially every 5 min on the observed linear portion of the fluorescence curve to obtain predicted maximal velocity of reaction. The slope of this line was taken as an estimate of product formation over time. Reaction rates were then plotted vs substrate concentration. The Michaelis–Menten kinetic model was fit to this data using nonlinear least-squares in SigmaPlot 11.0 to provide estimates for the parameters K_m and V_{max} .

RESULTS AND DISCUSSION

Fluorescent COMT Assay. The essential features of our assay are shown in **Figure 1**, and the assay is based on the original method developed by Collazo et al. (26) to assay histone methyltransferases. We have modified this original method in two important ways: (1) we have discontinued the use of adenosine deaminase, and (2) we have utilized the fast reaction kinetics between Thioglo1 with free sulfhydryls to convert a discrete end-point assay into a reproducible real-time, continuous method to assay plant COMTs involved in cell wall lignification.

Reaction of Thioglo1 with Reduced Glutathione. Thioglo1 displayed a large dynamic fluorescence range when directly

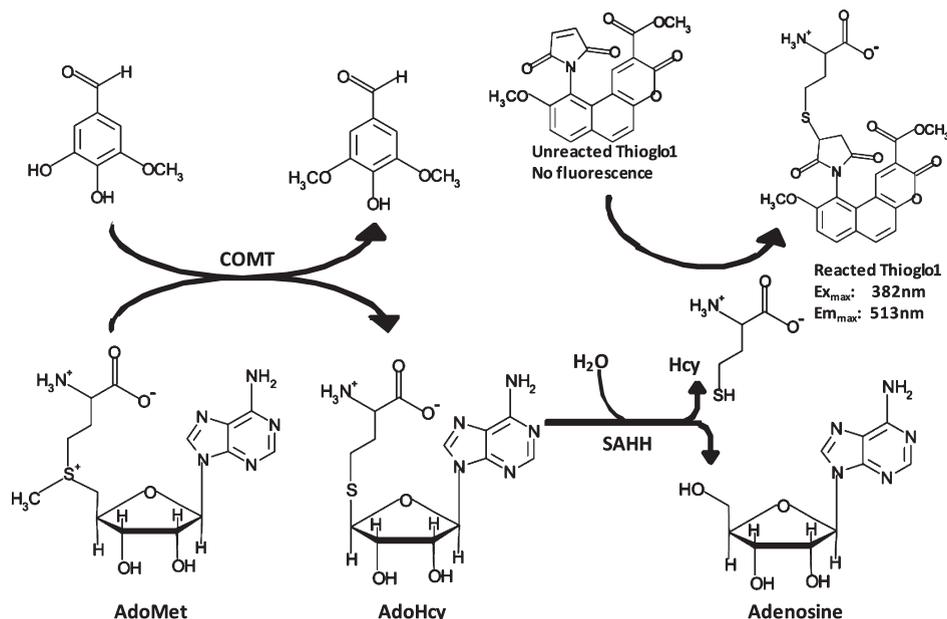


Figure 1. Schematic for coupled, fluorescent assay of plant caffeic acid *O*-methyltransferases. A source of plant caffeic acid *O*-methyltransferase (COMT) transmethyates a hydroxylated phenolic substrate to methoxylated form using ado-met as a methyl donor, converting ado-met to *S*-adenosylhomocysteine. *S*-Adenosylhomocysteine is hydrolyzed by the coupling enzyme *S*-adenosylhomocysteine hydrolase (SAHH) to homocysteine and adenosine. The free sulfhydryl group on a homocysteine molecule reacts with the maleimido form of the fluorophore, Thioglo1 forming a highly fluorescent conjugate with an excitation maximum (Ex_{max}) of 382 nm and an emission maximum (Em_{max}) of 513 nm.

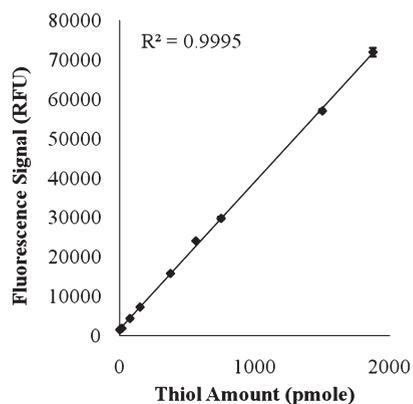


Figure 2. Standard curve for the reaction of free sulfhydryls with Thioglo1 using reduced glutathione as a standard.

derivatized with glutathione (**Figure 2**) showing linearity in fluorescence response over a 3 orders of magnitude (0–2000 pmol SH groups). Similar results have been reported earlier (30–32) for the reaction between Thioglo1 and free sulfhydryl groups.

Validation of Assay Using Recombinant Sorghum COMT.

We anticipated that background fluorescence arising from substrate (ado-met) and purified proteins would be minimal and the majority of the fluorescence signal could be attributed to the reaction between homocysteine and Thioglo1. Furthermore, the increase/decrease in fluorescence could be expected to be responsive to changes in COMT and phenolic substrate concentration source provided ado-met, Thioglo1, and SAHH were not limiting within the time frame of the assay.

In order to develop and validate this assay for COMT activities involved in wall lignification, we amplified the cDNA for a sorghum COMT (Bmr-12) that had been shown to control the deposition of S-lignin in sorghum plants (16, 17) and generated its recombinant protein. The expectation was that the behavior of this purified enzyme *in vitro* could be used to validate and define

the assay, as well as determine its broader applicability. A number of initial experiments were conducted to optimize the levels of enzymes and substrates required for reproducible detection of COMT activity (see Materials and Methods). We found that a final coupling enzyme concentration of 1 μ M SAHH and substrate concentrations of 85 μ M ado-met and 250 μ M phenolic substrate in the presence of 15 μ M Thioglo1 yielded excellent rates of quantifiable COMT activity (**Figure 3A**, solid line) even in the absence of the additional coupling enzyme adenosine deaminase (26), indicating that neither SAHH nor COMT activity was inhibited due to a buildup of products, and that there was minimal reaction between these proteins and Thioglo1. There appeared to be a small lag in the increase of fluorescence at the start of the assay. We assumed that this short lag was an inherent function of a coupled system. An increase in fluorescence in the reaction wells was dependent on the formation of homocysteine resulting from the hydrolysis of *S*-adenosylhomocysteine catalyzed by SAHH and the resulting reaction between homocysteine and Thioglo1. Initially, the levels of *S*-adenosylhomocysteine arising from demethylation of ado-met catalyzed by COMT would be low and the velocity of the forward reaction catalyzed by SAHH would be slow as well. However, as the levels of *S*-adenosylhomocysteine increased with time, the rate of fluorescence increase became linear. A similar small lag in fluorescence increase was observed even when *S*-adenosylhomocysteine was used a substrate for SAHH (not shown).

There was however, a noticeable, but small, initial increase in fluorescence in wells containing only ado-met and Thioglo1 (**Figure 3A**, dotted line) suggesting that the source of ado-met was contaminated to a limited degree with free sulfhydryl groups. However, this background increase was usually less than 50–80 pmol of sulfhydryl equivalents, and did not interfere with the assay. Control reactions lacking COMT also did not show any appreciable increase in fluorescence over time (**Figure 3A**, dashed line).

Assay Shows Linear Response to Increasing Levels of COMT.

Further validation of the assay was performed by varying the

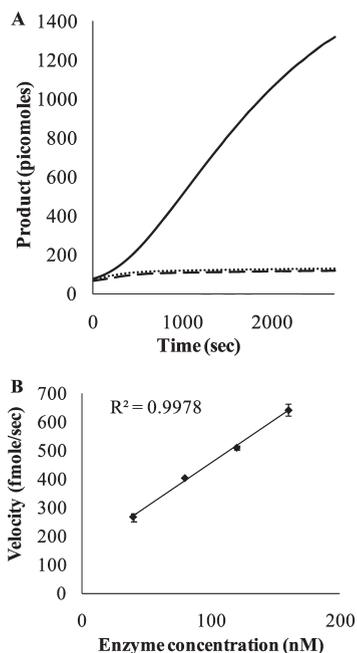


Figure 3. Validation of the coupled fluorescent assay using recombinant sorghum COMT. **(A)** Measurement of COMT activity using the fluorescent assay. The source of COMT was recombinant sorghum COMT (Bmr-12). The solid line is the formation of product (picomoles SH equivalents) in the full reaction mixture. The dotted line is the reaction arising from the substrate ado-met and Thioglo1 only. The dashed line is the control reaction in the absence of COMT. **(B)** Linear relationship between the formation of product in response to increasing amounts of recombinant COMT.

amount of recombinant sorghum COMT and determining the rate of change in fluorescence. There was a linear relationship between enzyme amount and fluorescence yields over a 4-fold change in enzyme loading (**Figure 3B**) indicating that the assay was robust and capable of measuring a relatively wide range of enzyme concentrations. Similar changes were observed at fixed COMT levels and increasing the amount of SAHH in the assay wells.

Increasing Caffeic Acid Concentration in the Assay Increases Fluorescence Yields. Having verified that the assay was responsive to enzyme levels, we studied the effects of differing substrate (caffeic acid) concentrations on fluorescence yields using the standard assay conditions described in Materials and Methods containing 150 nM COMT. Fluorescence yields increased with increasing substrate in the assay solution (**Figure 4A**), and the relative change in enzyme velocity appeared to be linear between 0 and 100 μM caffeic acid, after which time substrate concentration appeared to become saturating. Taken together, data shown in **Figures 3** and **4A** demonstrated that the fluorescence assay we have developed for plant COMTs was responsive to changes in both enzyme and substrate concentrations, was dependent on the availability of ado-met, SAHH and Thioglo1, and represented a ready and facile means to assay recombinant plant COMT known to be involved in cell wall lignification. However, it was necessary to demonstrate that ferulic acid, the product of the methylation of caffeic acid, was being generated in the assay wells (24).

Ferulic Acid Content in Assay Wells Increases in Parallel to Substrate Concentration. To verify that ferulic acid content increased with increasing levels of caffeic acid, well contents from an experiment similar to the data shown in **Figure 4A** were extracted for free phenolic acids and analyzed by GCMS. As shown in **Figure 4B**, ferulic acid content (ion signal) increased in

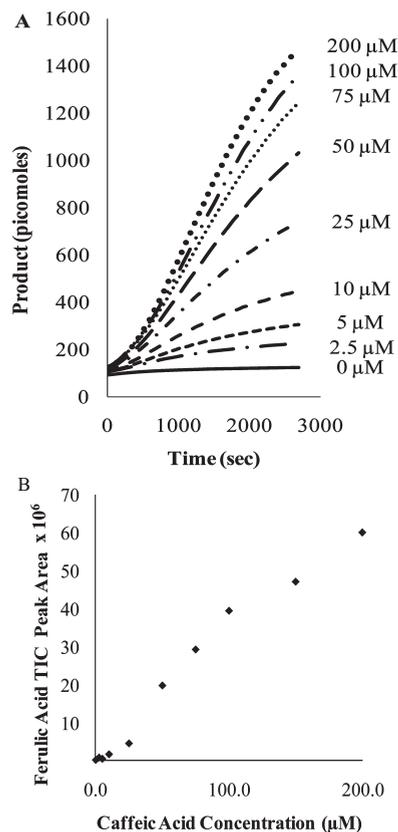


Figure 4. Influence of phenolic substrate concentration on reaction rate and formation of product. **(A)** The reaction rate was dependent on the initial concentration of caffeic acid in the assay. All wells contained the standard amounts of enzymes and other cofactors (see Materials and Methods). **(B)** GCMS analysis of the relative amounts of ferulic acid produced in assay wells as a function of initial caffeic acid content. Ferulic acid is the product formed by methylation of caffeic acid by COMT in the presence of ado-met. The relative amounts of ferulic acid (total ion-peak area) present were determined by GCMS as described in the Materials and Methods.

proportion to the concentration of caffeic acid in the assay solution in the presence of active COMT and the other required ingredients of the assay, indicating enhanced product formation in response to higher initial substrate concentrations.

Evaluation of Substrate Specificities of Recombinant Sorghum COMT. We next utilized differently hydroxylated phenolic substrates to characterize recombinant sorghum COMT. The target substrate for COMT *in vivo* is thought to be 5-hydroxyconiferyl aldehyde (14); unfortunately this substrate is not commercially available. Kota et al. (20) in an important study had shown that a number of alternate phenolics could serve as equivalent substrates for COMTs involved in lignification, and reliably used to evaluate enzyme kinetic constants. Using this earlier study as a guide, we evaluated the affinity of recombinant sorghum COMT toward four other phenolic substrates, caffeic acid, caffeoyl alcohol, 3,4-dihydroxybenzaldehyde and 3,4-dihydroxy-5-methoxybenzaldehyde (that mimics the endogenous substrate, 5-hydroxyconiferyl aldehyde). Recombinant sorghum COMT efficiently utilized all four substrates (**Figure 5**) and displayed greatest affinity ($K_m = 4.5 \mu\text{M}$) for 3,4-dihydroxy-5-methoxybenzaldehyde and least affinity for 3,4-dihydroxybenzaldehyde ($K_m = 38.0 \mu\text{M}$). Caffeoyl alcohol was a better substrate than caffeic acid. Interestingly, the V_{max} was greatest when either caffeoyl alcohol or 3,4-dihydroxy-5-methoxybenzaldehyde was used as substrate ($> 930 \text{ pkat mg}^{-1}$ enzyme; **Figure 5**). The kinetic values determined by the Thioglo1-dependent fluorescent assay for sorghum COMT (Bmr-12)

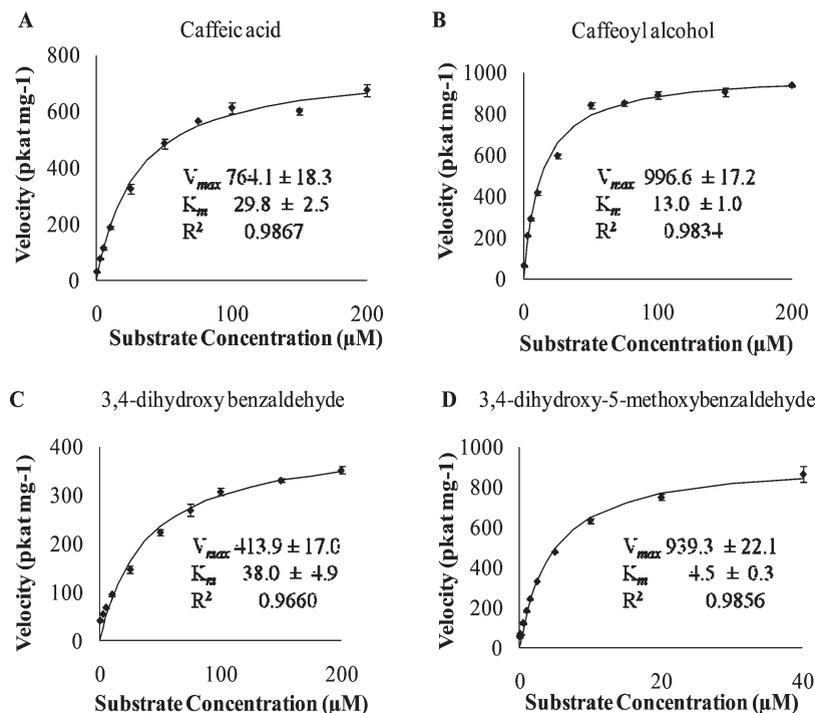


Figure 5. Determination of kinetic constants for recombinant sorghum COMT (Bmr-12) using the real-time fluorescent assay and four phenolic substrates. K_m and V_{max} values for sorghum Bmr-12 enzyme were determined using four different phenolic substrates. The enzyme showed greatest affinity for 3,4-dihydroxy-5-methoxybenzaldehyde and least affinity for 3,4-dihydroxybenzaldehyde. The calculated K_m and V_{max} (\pm SE) values were similar to literature values reported for comparable COMTs using other assays.

protein were in a similar range as those reported previously for other lignifying COMTs (14, 20, 22, 33).

Native COMT Activity in Switchgrass Internode Extracts. It was of interest to see if this new COMT assay would work with total and partially purified plant homogenates, which could be expected to contain measurable amounts of free and protein-bound thiols and potentially other interfering compounds, such as chlorophyll which might quench fluorescence. Free thiols would react with Thioglol increasing nonspecific background fluorescence, and could be slow, due to limited access to free thiols present in moderately accessible sites in proteins. Obviously, desalting of plant homogenates would remove most small, non-protein thiols, but could still contain other compounds that might interfere with the assay. Also, measuring COMT activity in crude plant homogenates would be dependent on the actual amount of COMT proteins present, and low activity may not be detected because increasing the amounts of plant homogenates could quench fluorescence signal in the assay, or cause a sufficiently high background. All of these different scenarios were tested with switchgrass extracts.

Crude, undesalted switchgrass internode extracts displayed COMT activity when caffeic acid or 3,4-dihydroxy-5-methoxybenzaldehyde was used as substrate (Figure 6A). Greater activity was observed when 3,4-dihydroxy-5-methoxybenzaldehyde was used as a substrate. The rate of fluorescence increase was positively correlated to the amount of protein present in the assay (Figure 6B). Although there was a longer lag phase in reactions containing crude homogenates as compared to assays containing pure COMT protein, enzyme activity present in plant extracts could be readily calculated. We assume that this lag was dependent on a slower rate of product formation, utilization of supplied phenolic substrates by other enzymes, such as polyphenoloxidases, and quenching of fluorescence by components present in plant extracts.

Using 3,4-dihydroxy-5-methoxybenzaldehyde as a substrate, the relative activity in developmentally distinct switchgrass

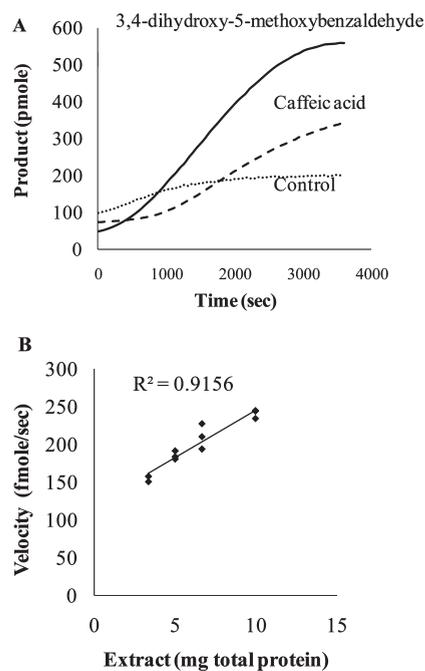


Figure 6. Evaluation of COMT activity in clarified switchgrass stem extracts. (A) Rate of product formation as a function of phenolic substrate. Activity was greater when 3,4-dihydroxy-5-methoxybenzaldehyde was used as substrate (solid line) as compared to caffeic acid (dashed line). Background fluorescence was greater when crude plant extracts were used (dotted line). (B) Rate of product (—SH equivalents) was dependent on the amount of plant protein present in the assay.

internodes obtained from flowering tillers was examined. The internode subtending the peduncle was labeled as internode 1. Highest COMT activity of $32.4 \text{ pmol s}^{-1} \text{ mg}^{-1}$ protein was

Table 1. COMT Activity in Switchgrass Internode Extracts^a

internode	activity (pmol s ⁻¹ mg ⁻¹)
1	32.4 ± 0.9
2	25.5 ± 0.4
3	27.7 ± 0.9
4	25.5 ± 0.4
5	25.7 ± 0.7
6	27.7 ± 0.7

^a Switchgrass extracts were prepared from individual internodes and assayed as described in Materials and Methods, using standard assay conditions. All assays were performed in triplicate, and the data are shown ±SE.

Table 2. Partial Enrichment of Switchgrass COMT by Ion-Exchange Chromatography Using Spin-Filters^a

fraction	activity (pmol s ⁻¹ mg ⁻¹)
crude extract	25.5 ± 0.4
0.25 M salt elution	61.4 ± 3.0
0.60 M salt elution	9.5 ± 0.4

^a Switchgrass stem extracts were prepared as described in Materials and Methods and passed over an anion exchange membrane. The flow through was collected. The membrane was sequentially washed with equilibrating buffer, followed by buffer containing 0.25 M NaCl and 0.60 M NaCl. Triplicate aliquots from each fraction was assayed for COMT activity using standard assay conditions (see Materials and Methods). Switchgrass COMT was partially purified and was mostly eluted in the 0.25 M NaCl fraction.

observed in the internode 1 extracts. Internodes 2–6 contained approximately similar but somewhat lower COMT activity (Table 1). Internode 1 is expected to be most active metabolically, although lignification appears to be a continuing process over the entire life cycle of a tiller (10, 29). COMT activity data appear to be consistent with these earlier studies.

We were able to quickly and partially purify switchgrass COMT using spin-filters containing anion exchange membranes (Table 2). This simple procedure yielded an approximately 2.5-fold enrichment of native COMT in the 0.25 M NaCl elution. COMT activity was not detected in the unbound (flow through + wash) fractions. There was minimal activity in the high-salt elution. These data indicate that if needed plant COMT activity can be enriched prior to assay.

However, as shown in Figure 6A, there was an increase in background fluorescence when plant homogenates were used. Fluorescence quenching appeared to be a potential problem, especially when phenolic substrates with lower affinities were used (for example: caffeic acid vs 3,4-dihydroxy-5-methoxybenzaldehyde) because the time required to detect a positive increase in fluorescence was delayed if caffeic acid was used as a substrate. We had noticed that some plant extracts (even desalted or enriched for COMTs by ion-exchange) did not yield any measurable activity. Addition of recombinant COMT assay to wells failed to overcome this lack of activity. It is unclear what components in these plant extracts were responsible for these observations, and if these components either quenched fluorescence of the derivatized Thioglo1 or inhibited COMT and/or SAHH. As an example, sorghum extracts prepared in an identical manner to switchgrass extracts did not yield an increase in fluorescence signal, whereas low activity was detected in extracts prepared from *Arabidopsis* stems. These data suggest that some experimentation may be necessary to optimize these protocols for a plant species. However, for many other plant samples the assay provides a robust estimate of COMT activity and should prove useful in evaluating plant germplasm in breeding programs for changes in COMT activity as it relates to cell wall lignification. This current method will obviously work well for enzymatically characterizing recombinant and/or partially purified proteins.

ABBREVIATIONS USED

Ado-met, *S*-adenosylmethionine; Bmr-12, brown-midrib-12; COMT, caffeic acid *O*-methyltransferase; GCMS, gas chromatography–mass spectrometry; SAHH, *S*-adenosylhomocysteine hydrolase.

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