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SHORT COMMUNICATION

EXPRESSION OF CAFFEIC ACID *O*-METHYLTRANSFERASE GENE INVOLVED IN LIGNIN BIOSYNTHESIS OF SUGARCANE

K. Lakshmi* and A. Kalaivani

Abstract

Caffeic acid *O*-methyltransferase (COMT) plays a pivotal role in the lignification process of plants. In the present study, sugarcane *COMT* gene has been cloned, characterized and its protein has been expressed in a prokaryotic expression system (BL21). Phylogenetic analysis showed a very close evolutionary relationship between *COMT* sequences of *Miscanthus* and *Sorghum*. Using bioinformatic tools like ExPasy and Prosite, the sugarcane COMT was found to belong to the Class I S adenosyl- L- methionine (SAM)-dependent methyltransferases family with a predicted molecular weight of 39.98 kDa. The SDS PAGE displayed the COMT protein at the molecular weight of ~ 40 kDa as a proof of confirmation of the study.

Key words: Caffeic acid *O*-methyltransferase, lignin biosynthetic pathway, sugarcane, protein expression

Lignin is an impediment to the production of biofuels and forage digestibility (Fu et al. 2011). Due its cross linking, it blocks the access of cell wall degrading enzymes to cellulose and hemicellulose (Vanholme et al. 2010).This makes it necessary to employ harsh pretreatment to disrupt cell wall structure prior to enzyme hydrolysis. Lignin is an energy dense compound due to phenolic ring and thus high lignin content is desirable if the biomass is used for direct combustion. Aspects of lignification in sugarcane about which we still know relatively little include: the regulatory cascades that trigger lignification, the cellular biology, metabolic connections between monolignol biosynthesis, their transport to the apoplast and polymerization, as well as its interaction with other cell wall polymers such as non-cellulosic polysaccharides. Lignins are complex heteropolymers derived from three monolignols, namely *p*-coumaryl, coniferyl and sinapyl alcohol which produce *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively. The S content and the S to G ratio are critical parameters that measure for characterizing lignin composition in the cell wall of angiosperm plants (Chen and Dixon 2007). Although lignin is a key polymer providing the strength necessary for the plant's ability to grow upward, a reduction in lignin content down to 64% of the wild-type level in *Arabidopsis* was tolerated without any obvious growth penalty. In contrast to common perception, it was found that a reduction in lignin was not compensated for by an increase in cellulose, but rather by an increase in matrix polysaccharides (Rebecca et al. 2013).

Caffeic acid *O*-methyltransferase (COMT EC 2.1.1.68) is a pivotal enzyme which catalyzes the multi-step methylation of the C5 hydroxyl moiety of suitably hydroxylated phenolic rings of monolignols, leading to the preferential formation of S subunits. Consequently, COMT is one of the crucial enzymes

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for targeted down regulation/modification of the S/G ratio of the lignin biosynthetic pathway for biofuel production from lignocellulosic biomass (Chen et al. 2004). In terms of the catalytic mechanism, COMT is an S adenosyl- L- metheonine (SAM) dependent O-methyltransferase which utilizes a histidine (His) with catalytic activity (Zubieta et al. 2002). It uses SAM as the methyl group donor for its activity like most methyltransferases, after which SAM becomes S-adenosyl-L-homocysteine (SAH), one of the ligand components in the co complex. Decrease in COMT activity led to improved forage digestibility, suggesting that the down regulation of COMT might alter the overall cell wall organization in a way that walls become more accessible to bacterial enzymes (Piquemal et al. 2002). In a crop like sugarcane, it is essential to identify, clone and characterize the key genes to understand the regulatory reactions of lignin biosynthesis pathway which can ultimately be used for delignification of sugarcane biomass and its improved utilization for ethanol production or an increased expression for cogeneration.

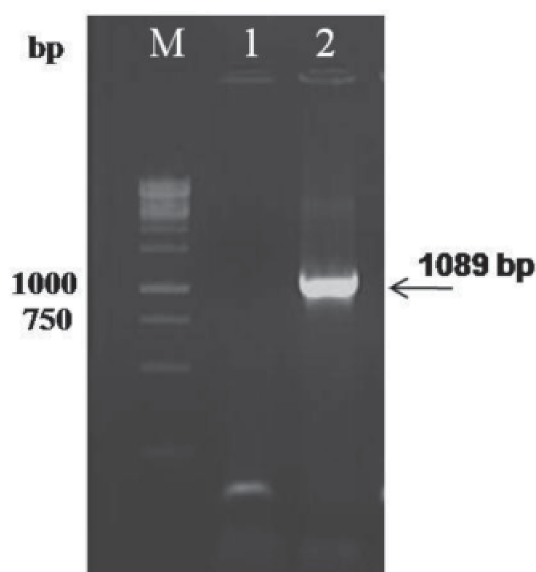

Fig. 1. PCR amplification of *COMT* gene from *Saccharum officinarum*. Lane M - 1kb DNA ladder, lane 1 - negative control (without template), lane 2 - amplification of *COMT* gene.

Degenerate primers and RACE (rapid amplification of cDNA ends) were used to clone the full-length of the *COMT* gene of 1089 bp from *Saccharum officinarum* (PIO 00-809) (Fig. 1); the sequence was submitted in NCBI genebank and the ID: KM370990 obtained (Lakshmi and Kalaivani 2015). Using this sequence, we have made an attempt in this present study to express the gene in bacteria (*E. coli* strain BL21), since the bacterial system is a convenient choice for the preliminary expression of foreign genes. To achieve this objective, the coding region of *COMT* gene fragment, which is bound by a start codon (ATG) in the 5' end and a stop codon (TAA) in the 3', was amplified using PCR with gene specific primers (forward primer-5' ttGAATTCATGGGCTCGACCGCC 3' and reverse primer - 5' tgAAGCTTTTACTTGATGAACTC GATGGCCCAG 3') with *Eco R*I and *Hind* III restriction sites, respectively. The amplified *COMT* gene was directionally sub-cloned into pET-28a using these restriction enzymes and ligated using T4 DNA ligase. The cloned construct was confirmed for the presence of insert with PCR and restriction digestion (Fig. 2), and the cloned construct (pET28a:*COMT*) was transformed into BL21 (DE3A). Among the transformed colonies, four were selected and grown individually in 5 ml of Luria bertani (LB) broth containing 100 µg/ml of kanamycin overnight at 37°C. An active culture was raised by inoculating 1% of the overnight grown colonies and maintaining at 200 rpm and 37°C until an OD of 0.5 was attained. Prior to isopropylthio-â-galactoside (IPTG) induction, 1 ml of un-induced culture was taken and treated as control. One milli molar IPTG was added to the remaining culture and kept at 200 rpm and 28ºC for 4 h for the expression of *COMT*. One ml of un-induced as well as induced cultures of different colonies were centrifuged at 6000 rpm for 15 min

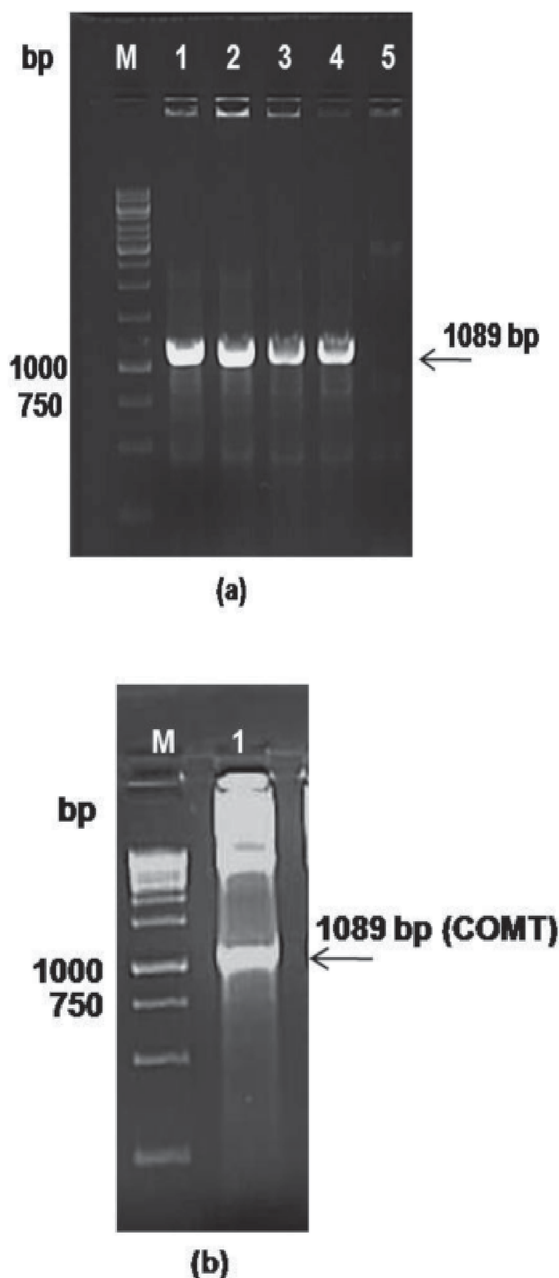

Fig. 2. Confirmation of cloning of *COMT* in expression vector pET 28a (a) PCR with gene specific primers: lane M - 1 kb DNA ladder, lane 1 to 4 – PCR product of *COMT* using transformed colonies as template, lane 5 – negative control (without template) (b) Confirmation of *COMT* clone by restriction digestion: lane M - 1kb DNA ladder, lane 1 - plasmid of transformed colony digested with *EcoR* I and *Hind* III

and the pellet was resuspended in 100 µl of SDS buffer and heated at 65°C for 5 min and loaded on 15% SDS-PAGE. Electrophoretic run was carried out for 2 h at 100 mA followed by coomassie brilliant blue staining and destaining. The gel image was scanned for documentation.

Sequence characterization of the *COMT* cDNA showed a 1089-bp fragment which codes for 362-amino acids. The clustal W results revealed the COMT was conserved between species and the blast analysis showed 98% similarity with SbCOMT (AF387790) from *Sorghum bicolor*; Comparison of this protein with maize COMT gave an identity of 91.16% over the entire amino acid sequence. ExPASy analysis indicated that the expected molecular weight of the deduced protein was 39.98 kDa with an isoelectric point (pI) of 5.42. Methyltransferases can be divided into five classes based on the structure of their catalytic domain (Schubert et al. 2003). PROSITE analysis performed to identify the class indicated that this COMT protein belongs to the Class I SAM-dependent methyltransferases family. Protein homology by domain architecture (Geer et al. 2002) of COMT showed specific hits on SAM or AdoMet-MTase, class I. AdoMet-MTases are enzymes that use SAM or AdoMet as a substrate for methyl transfer, creating the product S-adenosyl-L-homocysteine (AdoHcy). SignalP 4.0 study suggested the absence of signal peptide for subcellular localization; therefore, the COMT ortholog is thought to be a cytoplasmic protein. COMT protein sequences of nine plant species were used to generate a phylogenetic tree using Mega 5. The COMT ortholog showed the closest relationship to *Miscanthus sinensis* and belonged to a subcluster of three proteins that also included *Sorghum sudanense* and *Zea nicaraguensis* (Fig. 3). These findings strongly suggest that sugarcane COMT

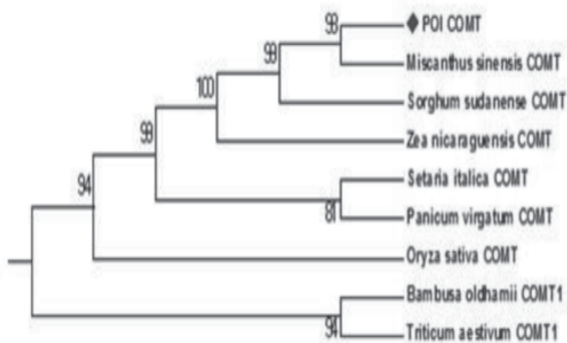

Fig. 3. Phylogenetic analysis of the amino acid sequences of POI COMT with the sequences from other plants. The tree was constructed by the neighbor-joining method of Clustal W and Mega 5. The numbers at the nodes indicate bootstrap value

ortholog is a COMT enzyme; therefore, it was designated as POICOMT.

The SDS PAGE displayed the protein expression pattern of transformed colonies 1-4 (Fig. 4). Compared to the un-induced samples, induced samples showed a thicker band at the molecular weight of ~ 40 kDa range which was exactly the same as that of the predicted molecular weight obtained from the bioinformatics studies. The expression of recombinant COMT in the pET expression vector ensures that the isolated gene from our laboratory could be used for metabolamic engineering. Manipulation of the enzymatic pathway of lignin biosynthesis for achieving reduced lignin content would possibly lead to an improved and cost effective biomass conversion protocol. The effects of *COMT* gene modification on reduction of S monomer level, which is linked directly to the lignin reduction, have been shown in other modified plant species (Guillaumie et al. 2008; Lu et al. 2010; Ma and Xu 2008 and Yoshihara et al. 2008). Therefore, the gene cloned earlier and characterized in this study can be utilized for developing strategies to produce transgenic plants with lower and/or modified lignin content.

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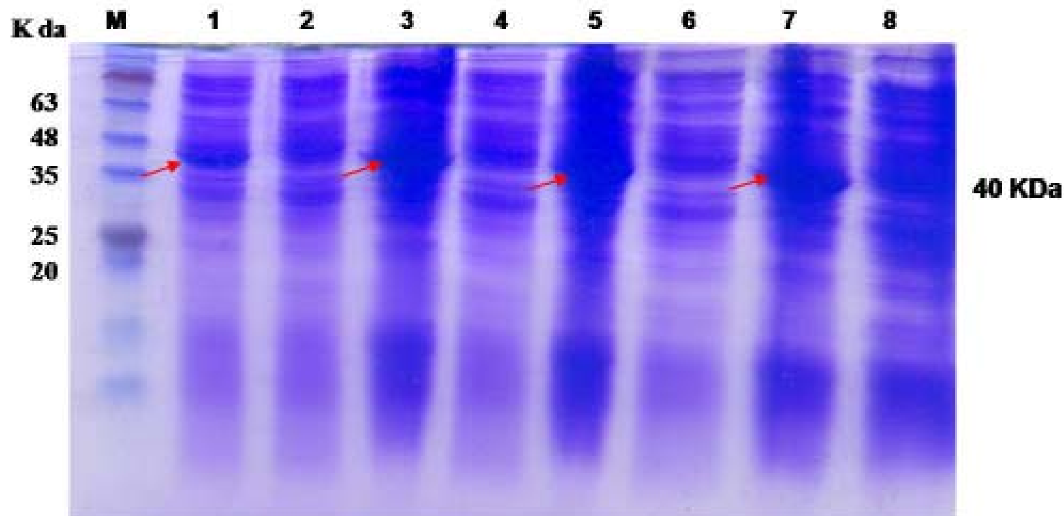

Fig. 4. SDS PAGE analysis of COMT proteins isolated from *E. coli* BL21. Lane M - protein marker, lane 1,3,5,7 - crude proteins of transformed colony 1,2,3,4 of BL 21 cells containing the pET28a vector + *COMT* gene induced with 1mM IPTG, lane 2,4,6,8 - crude proteins of transformed colony 1,2,3,4 of BL 21 cells containing the pET28a vector + *COMT* gene uninduced

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