



# Defining the role of a caffeic acid 3-O-methyltransferase from *Azadirachta indica* fruits in the biosynthesis of ferulic acid through heterologous over-expression in *Ocimum species* and *Withania somnifera*

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## Abstract

**Main conclusion** The recombinant caffeic acid 3-O-methyltransferase gene has been cloned and characterized from Neem. The gene is involved in ferulic acid biosynthesis, a key intermediate component of lignin biosynthesis.

**Abstract** *Azadirachta indica* (Neem) is a highly reputed traditional medicinal plant and is phytochemically well-known for its limonoids. Besides limonoids, phenolics are also distinctively present, which add more medicinal attributes to Neem. Caffeic acid is one of such phenolic compound and it can be converted enzymatically into another bioactive phytomolecule, ferulic acid. This conversion requires transfer of a methyl group from a donor to caffeic acid under the catalytic action of an appropriate methyltransferase. In this study, caffeic acid 3-O-methyltransferase gene from Neem (*NCOMT*) fruits has been isolated and heterologously expressed in *E. coli*. The recombinant *NCOMT* enzyme was purified, which exhibited efficient catalytic conversion of caffeic acid into ferulic acid, a highly potential pharmaceutical compound. The purified recombinant enzyme was physico-kinetically characterized for its catalysis. The analysis of tissue-wide expression of *NCOMT* gene revealed interesting pattern of transcript abundance reflecting its role in the development of fruit tissues. Further, *NCOMT* was heterologously overexpressed in *Withania somnifera* and *Ocimum species*, to analyze its role in ferulic acid biosynthesis *in planta*. Thus, the study provides insight for the endogenous role of *NCOMT* in ferulic acid biosynthesis *en route* to lignin, an important structural component. To the best of our knowledge, *NCOMT* pertains to be the first enzyme of the secondary metabolism that has been purified and kinetically characterized from Neem. This study may also have important prospects of applications as the observation on heterologous expression of *NCOMT* showed its involvement in the maintenance of the *in vivo* pool of ferulic acid in the plants. Thus, the study involving *NCOMT* opens up new dimensions of metabolic engineering approaches for the biosynthesis of potential therapeutically important phytomolecules in heterologous systems.

**Keywords** Ashwagandha · *Azadirachta indica* · Caffeic acid · Caffeic 3-O-methyltransferase · Ferulic acid · Neem · *Ocimum species* · *Withania somnifera*

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## Abbreviations

COMT Caffeic acid 3-O-methyltransferase  
SAM S-adenosyl-L-methionine  
IPTG Isopropyl β-D-1 thiogalactopyranoside

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MJ Methyl jasmonate  
SA Salicylic acid

## Introduction

Caffeic acid 3-O-methyltransferase (COMT) belongs to the family of S-adenosyl-L-methionine (SAM) dependent O-methyltransferase and catalyzes the conversion of caffeic acid into ferulic acid, an important step in the biosynthesis of lignin. Lignin is a heteropolymer, composed of three main units p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S). The composition and structure of lignin polymer differs considerably within and among plants due to variation in monomeric ratio especially in S and G units. Ferulic acid is a major metabolic intermediate in the route to lignin biosynthesis in plants (Ou and Kwok 2004; Kumar and Pruthi 2014; Li et al. 2015). COMTs are considered as multifunctional enzymes, which can perform methylation of several substrates such as caffeic acid, coniferyl alcohols, free acids and aldehydes as well as methoxylation of other derivatives of syringyl subunits (Li et al. 2015).

There are limited reports available in respect to isolation and characterization of *COMT* cDNA from plant sources such as *Ligusticum chuanxiong*, *Populus tremula*, *Iris hollandica*, *Arabidopsis thaliana*, *Medicago sativa* etc. (Kumar and Pruthi 2014; Li et al. 2015). *Arabidopsis thaliana* COMT (AtCOMT) has been reported to exhibit significant N-acetylserotonin methyltransferase activity also, catalyzing the conversion of N-acetyl serotonin into its methylated product (Byeon et al. 2014). In most of the reports, the involvement of COMT enzymatic activity was shown towards the lignin biosynthesis. COMT is also known as an enzyme of commercial significance for its applications for the production of ferulic acid, a potential therapeutic and nutraceutical compound (Ou and Kwok 2004; Kumar and Pruthi 2014).

Ferulic acid is one of the most abundant phenolic acid in plants and is used as an ingredient of many drugs, in functional foods and nutraceuticals (Kumar and Pruthi 2014). As a potential therapeutic agent, ferulic acid is in the experimental stage for its potential action in the treatment of serious diseases like diabetes, cancer, neuro-degenerative, cardiovascular etc. (Ou and Kwok 2004; Kumar and Pruthi 2014). In plants, ferulic acid is rarely present in free form, as usually it is encountered in the cross linked form like cross-linked with polysaccharides, and proteins etc. Therefore, its cross-linking property is used in the preparation of complex gels in food applications (Ou and Kwok 2004; Kumar and Pruthi 2014). Ferulic acid has been approved in several countries as a food additive to prevent lipid peroxidation due to its

effective scavenging activity towards free radicals (Srinivasan et al. 2007). Commercial significance of ferulic acid in pharmaceutical and food industry necessitates exploration of multiple opportunities of usage of alternative plant resources and approaches for its production.

The *Azadirachta indica* (Neem) is a medicinal tree well known for its characteristic triterpenoids called limonoids (tetranortriterpenoids). Besides, the plant is also reported to have notable quantities of various phenolics such as gallic acid, tannic acid, caffeic acid, ferulic acid, chlorogenic acid, quinones, quercetin, kaempferol, epicatechin and 4-caffeoyl quinic acid, hydroquinone, protocatechuic acid etc. Many of the phenolics have been shown to possess diverse biological activities such as anti-fungal, anti-feedant, anti-bacterial, anti-oxidant, anti-mutagenic and anti-carcinogenic (Singh et al. 2005; Krishnan et al. 2011; Narnoliya et al. 2014). Therefore, Neem could be a potential biore-source for the production of phenolics such as ferulic acid.

Despite diverse applications of Neem, till date there is no detailed report available on molecular attributes of any enzyme from the plant in the perspectives of biochemical/catalytic characteristics as a purified native or recombinant preparation. Recently, genome, transcriptome and ESTs data resources of Neem become available in the public domain, which could be used for the identification and elucidation of biosynthetic pathways related to secondary metabolites such as limonoids and phenolics (Krishnan et al. 2011, 2012; Narnoliya et al. 2014; Rajakani et al. 2013, 2014). Transcriptomics and genomics analysis provide information about genes and transcripts related to specific pathways present in that particular organism which could be further used for the improvement of quality and quantity of related metabolites. Such genomics and transcriptomics data from several medicinal plants including *Ocimum* species, *Withania somnifera* (known as Ashwagandha) and Neem have become available, which provides valuable information about their important biosynthetic pathways (Narnoliya et al. 2014, 2017; Sangwan et al. 2018; Chandra et al. 2020). Our earlier studies have shown the elevated terpenoids accumulation in synchronization with the enhanced expression of genes of their metabolic pathways, using *W. somnifera* and *Ocimum* as heterologous experimental system as a proof of concept (Bansal et al. 2018).

In case of Neem, however, none of the enzymes related to either primary or secondary metabolism appears to have been kinetically characterized for their catalytic reaction till date. Therefore, the present study was aimed to isolate and characterize NCOMT from Neem as a beginning for the enzymological study of the plant and examine the impact of metabolic engineering of heterologous plant systems with Neem NCOMT. Accordingly, an important *NCOMT* gene was identified and cloned from *A. indica* fruit tissue. The recombinant NCOMT was purified from *E. coli*, genetically

transformed to express NCOMT, was physico-catalytically characterized. Further, the investigations on the impact of transient over-expression of the gene in heterologous plants- *Withania somnifera* and *Ocimum species* (*O. sanctum*, *O. basilicum*, and *O. kilimandscharicum*) along with a stable transformation in *O. gratissimum* reflected its preeminent role in ferulic acid biosynthesis. Therefore, NCOMT may hold the potential to serve as a tool/target for the metabolic engineering for the improvement in the production of ferulic acid.

## Materials and methods

### Plant material and chemicals

*Azadirachta indica*, *Withania somnifera* and *Ocimum species* (*O. sanctum*, *O. basilicum*, *O. kilimandscharicum* and *O. gratissimum*) were grown and maintained at the experimental farm of CSIR-Central Institute of Medicinal and Aromatic Plant, Lucknow, India. Samples were immediately flash-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use. All the kits and chemicals were purchased from Thermo Scientific (Revert Aid cDNA synthesis kit), Applied Biosystems (SYBR green ROX master mix), Sigma (CTAB, IPTG, caffeic acid, ferulic acid, methyl jasmonate, salicylic acid), Merck (trifluoroacetic acid) and HiMedia (indole-3-acetic acid, abscisic acid, tris buffer,  $\text{MgCl}_2$ ).

### Total RNA isolation and cDNA synthesis

Total RNA was extracted from Neem fruit tissue by the modified CTAB method (Rajakani et al. 2013), and was quantitatively as well as qualitatively analyzed by Nano Drop-1000 spectrophotometer. Thereafter, DNase treated total RNA was used for cDNA synthesis by Revert Aid cDNA synthesis kit (Thermo Scientific) according to the manufacturer's instructions.

### Isolation of full-length NCOMT cDNA from Neem fruit

Degenerate primers were used for the amplification of core fragment which was made full length using 3' and 5' RACE approach (Bansal et al. 2018). The full-length cDNA of NCOMT was amplified by using full-length primers, designed from the assembled NCOMT sequence and confirmed by sequencing.

### Sequence analysis and 3D structure prediction

NCBI Blastx tool (<http://blast.ncbi.nlm.nih.gov/Blast/>) was used for searching similarities between NCOMT cDNA and

other plants OMT(s) sequences present in the NCBI database. ClustalW2 tool (<http://www.ebi.ac.uk/Tools/msa/clustalw2>) was used for sequence alignment. The ORF (open reading frame) finder graphical analysis tool of NCBI ([www.ncbi.nlm.nih.gov/projects/gorf](http://www.ncbi.nlm.nih.gov/projects/gorf)) was used to predict the coding region of the NCOMT gene. Molecular mass and isoelectric point (pI) was determined by Compute pI/Mw tool hosted at ExPasy web browser (<http://cn.expasy.org/tools/protparam.html/>). A 3D homology-based model was generated by Phyre2 (<http://www.sbg.bio.ic.ac.uk/phyre2>) using caffeic acid/5-hydroxyferulic2 acid 3/5-o-methyltransferase (PDB: c1kyzC) of *Medicago sativa* as a template. The generated NCOMT model was superimposed with its template using UCSF Chimera package. Enzyme binding site was predicted by 3D ligand site (<http://www.sbg.bio.ic.ac.uk/~3dligandsite/>). Evolutionary relationships were assessed between NCOMT and OMT(s) from other plants through constructing a phylogenetic tree by the maximum likelihood method using MEGA 5.01 software (<http://www.megasoftware.net>).

### Heterologous expression and purification of NCOMT in *E. coli*

The full-length NCOMT gene was used for genetic transformation of *E. coli* BL21 (DE3) cells and overexpression of the gene was induced by 0.8 mM IPTG at  $16^{\circ}\text{C}$  for an overnight duration, according to manufacturer's instructions. Crude protein was extracted from induced culture and the recombinant protein was purified through Ni-NTA affinity column chromatography (Qiagen, Hilden, Germany). All the eluted fractions were screened for the presence of protein ( $A_{280}$ ) as well as for its catalytic activity. Concentration of purified protein preparation was determined by Bradford's method (Bradford 1976). The purified recombinant NCOMT protein was verified by Western blotting. In brief, eluted proteins samples were subjected to SDS-PAGE and transferred the protein bands to PVDF membrane. The membrane was washed with PBS solution and transferred to the primary antibody (Anti-His antibodies,  $0.5\ \mu\text{g ml}^{-1}$ ) solution and later on, after washing with PBC-Tris buffer, into the solution of the secondary antibody (alkaline phosphatase enzyme-linked anti-rabbit IgG antibodies,  $0.2\ \mu\text{g ml}^{-1}$ ). Blotted NCOMT protein was screened for binding with anti-His antibodies which was further detected by BCIP/NBT chromogenic reaction on the membrane.

### Enzymatic assay and reaction product estimation

The NCOMT standard reaction mixture consisted of 2 mM caffeic acid, 0.5 mM SAM (S-adenosyl-L-methionine) and  $1\ \mu\text{g}$  purified recombinant NCOMT in 100 mM Tris buffer (pH 7.5) containing 2 mM  $\text{MgCl}_2$  and 5 mM DTT (Lee et al. 2014). The reaction mixture was incubated at

37 °C for 60 min and the reaction product was extracted by ethyl acetate and analyzed by reverse-phase HPLC with a RP-C18 column (Nova-Pak, 4 mm, 3.9 × 150 mm, Waters). The reaction product was resolved using the HPLC operational parameters of methanol (A) and water (B) in the presence of 0.3% trifluoroacetic acid (TFA) as mobile phase in gradient mode with a flow rate of 0.8 ml min<sup>-1</sup> and detection wavelength was 320 nm. The reaction products were also validated according to the method developed by Janicsak et al. (2013). Reaction mixtures lacking substrate or enzyme served as control. To study the catalytic kinetics of this enzyme, the standard assay reaction composition and conditions were altered as the specific requirement. The effect of pH on NCOMT activity was studied by using assay buffers of varied pH, and for thermostability, varied temperature range was used. The varying concentration of caffeic acid and SAM were used in the enzymatic assay to deduce  $K_m$  and  $V_{max}$  values for both the substrates.

### Expression analysis of NCOMT transcripts in Neem tissues and under elicitor treatment

Gene-specific primers were designed for NCOMT using Beacon Designer 8.0 for the quantitative real-time PCR (qRT-PCR) analysis. Total cDNA was synthesized using 5 µg of DNase treated RNA, extracted from different tissues. Neem tissues were treated with different concentrations of various elicitors such as methyl jasmonate (MJ), salicylic acid (SA), indole-3-acetic acid (IAA), abscisic acid (ABA). Wounding (30 min, 1, 2, 4, 8, 12 and 24 h) and UV (30 min, 1, 2, and 5 h) were also used for expression analysis. Reaction mixture for qRT-PCR was prepared in a 20 µl of total reaction volume, consisting of ~100 ng cDNA as template, 10 µl of SYBR green ROX master mix (ABI Biosystems) and 5 pM of each gene-specific primer. Reactions for qRT-PCR were set in triplicate using cycling conditions (95 °C for 10 s for one cycle, 95 °C for 15 s and 50 °C for 1 min for 40 cycles and for melting curve analysis 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s). The reaction was performed in a Step One™ real-time PCR system (Applied Biosystems). The expression of the gene was normalized against β-actin gene as an endogenous control. The relative gene expression levels were indicated by relative quantification (RQ) values, which were calculated following the  $2^{-\Delta\Delta CT}$  method (Pfaffl 2004).

### Construction of NCOMT over-expression construct in pBI121

The NCOMT was cloned in pJET1.2 cloning vector (Thermo Scientific) and then digested with BamHI and SacI restriction enzymes and ligated into pBI121 vector, predigested with BamHI and SacI restriction enzymes. The ligated

pBI121-NCOMT construct was transformed into *Agrobacterium tumefaciens* strain EHA101 by using the freeze-thaw method and positive colonies were confirmed by PCR and sequencing. Thereafter, positive clones were used for plant transformation study as earlier (Bansal et al. 2018).

### Genetic transformation of NCOMT in *W. somnifera* and *Ocimum sp.*

NCOMT was transiently over-expressed in *W. somnifera* and *Ocimum species* (*O. sanctum*, *O. basilicum*, *O. kilimandscharicum* and *O. gratissimum*) using young and healthy leaves. Stable transgenic lines were also generated in *O. gratissimum* using NCOMT. The genetic transformation of *W. somnifera* and *Ocimum sp.* was carried out as per the protocol of Mishra et al. (2012, 2015) and Bansal et al. (2018), respectively. Transformed tissues were used for molecular and biochemical analysis. The transgenic nature of the lines was confirmed by PCR analysis using genomic DNA as template and primer set of *nptII* gene. Expression of NCOMT was checked in transformed leaf tissues by real-time PCR as described above. Untransformed and empty vector transformed leaf tissues were used as controls and three independent biological experiments with triplicates were performed. For metabolite analysis, ferulic acid was extracted from transformed leaf tissues by the extraction process reported by Janicsak et al. (2013) and analyzed by HPLC as described earlier.

### Statistical analysis

All the experimental data obtained are the mean of at least three independent biological replicates (enzyme assay and real-time experiments) and the results are presented with standard deviation. *t*-test was used to analyze significance, at  $P < 0.05$  for (\*),  $P \leq 0.01$  for (\*\*), and  $P \leq 0.001$  for (\*\*\*) with the help of GraphPad Prism 7.03 software.

## Results

### Cloning of NCOMT and sequence analysis

A partial amplicon of ~600 bp was obtained by PCR using cDNA library of *A. indica* fruit tissue as a template and degenerate primers. Further, 3' RACE and 5' RACE primers resulted in amplification of ~500 bp amplicons for both sides which were assembled with partial amplicon (internal fragment) to obtain a full-length NCOMT. The full-length NCOMT contained 1284 bp with 35 bp 5' UTR, 148 bp 3' UTR region and 1098 bp coding region encoding a polypeptide of 366 amino acid residues. The full-length NCOMT gene sequence exhibited significant homology with



O-methyl transferases (OMTs) from other plants such as *Citrus aurantium* (85%), *Citrus sinensis* (85%), *Jatropha curcas* (85%), *Gossypium arboreum* (85%), *Ricinus communis* (84%) and *Prunus mume* (84%).

The theoretical pI and molecular weight of the NCOMT protein were computed to be 6.21 and 39.93 kDa, respectively. Generally, plant OMTs contain three signature motifs referred to as S-adenosyl-1-methionine (SAM) binding, catalytic and phenolic substrate binding motifs. Multiple sequence alignment of NCOMT amino acid sequence with other plant OMTs showed the presence of all the three signature motifs (Fig. S1). The SAM binding motif [VDVGGGXG] was observed to be located in approximately middle of the protein (amino acid residues from 206 to 213), phenolic substrate binding residues in the enzyme were W (267), IMLAHN (320 to 325) and the catalytic site residues were His (270), Glu (298) and Glu (330), respectively.

### Molecular modeling and evolutionary analysis

A 3D homology-based protein model was generated using caffeic acid/5-hydroxyferulic2 acid 3/5-O-methyltransferase (PDB: c1kyzC) as a template (Fig. 1a). A total of 355 (97%) residues of NCOMT could be modeled with 100% confidence level by the single highest scoring template. The superimposition of NCOMT model on the template (PDB: c1kyzC) showed an almost perfect match and their spatial differences indicated its sequence variability (Fig. 1b). Binding site prediction studies revealed the involvement of at least 21 amino acid residues in the binding of a substrate (Fig. 1c). The phylogenetic tree generated through the maximum likelihood method based on

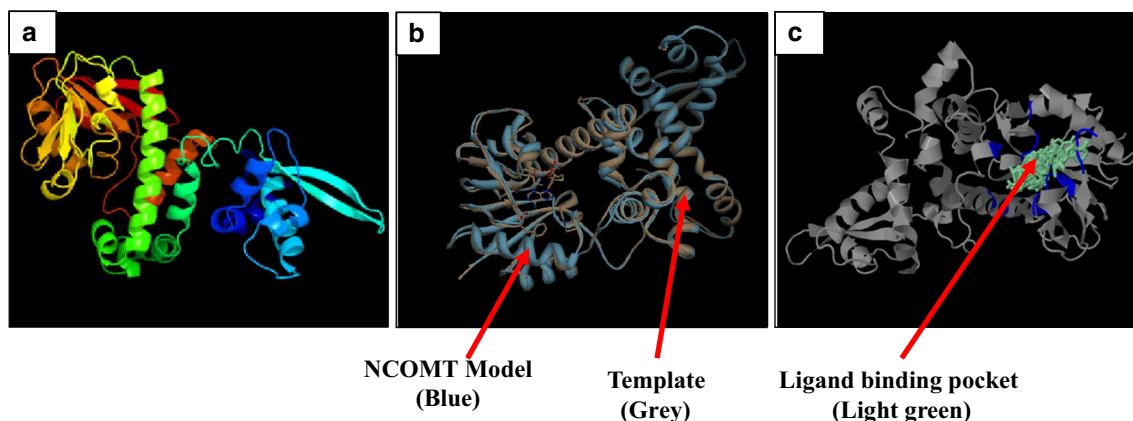
the Jones-Taylor-Thornton model indicated that NCOMT clustered together with *Citrus aurantium* OMT (Fig. 2). Both, the genus *Citrus* and *Azadirachta*, fall under the same order Sapindales.

### Recombinant NCOMT protein purification and catalytic activity

The recombinant pET28a-NCOMT construct was expressed heterologously in *E. coli* BL21 (DE3) cells as an N-terminal polyhistidine-tagged protein. The recombinant NCOMT protein was extracted from IPTG induced cultures and confirmed on 12% SDS-PAGE. Further, Ni-NTA eluted recombinant enzyme preparation was observed to be homogeneous by its state of purity as revealed by a single polypeptide (~40 kDa). The results from Western blotting using anti His-antibody also confirmed the enzyme as a monomeric protein (Fig. S2). The recombinant NCOMT protein was assayed for its catalytic attributes with respect to the acceptance of caffeic acid as an acceptor substrate for methyl group transfer from SAM. Ferulic acid as a product of the catalytic reaction was identified through HPLC (Fig. 3) as well as TLC (Fig. S3).

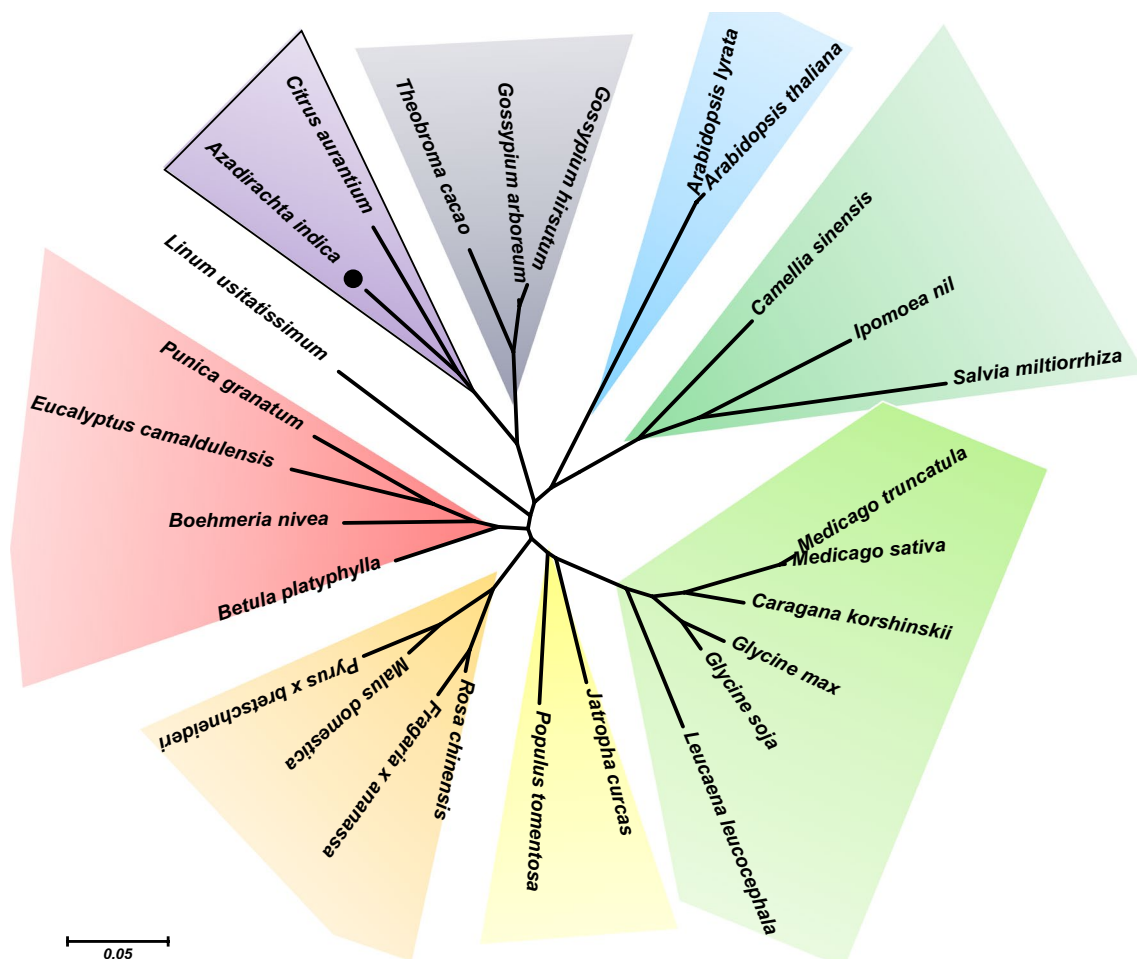
### Kinetic attributes of NCOMT

The catalytic activity of NCOMT was analyzed at various pH and temperatures using caffeic acid as substrates (Fig. 4a, b). The highest activity was observed at pH 7.5 with 100 mM Tris-Cl as assay buffer (Fig. 4a). NCOMT showed maximum activity at 37 °C and only 40% activity was remained at 50 °C. Although 30% catalytic activity was retained at 60 °C and at 70 °C, activity was completely lost. For determining, the thermo-stability, temperature-based



**Fig. 1** Homology based 3D modeling of NCOMT. **a** 3D structure of NCOMT predicted by Phyre2. Caffeic acid/5-hydroxyferulic2 acid 3/5-O-methyltransferase (PDB: c1kyzC) was used as a template. **b** Superimposition of NCOMT on the template. **c** Ligand binding

pocket (light green) predicted by 3DLigandSite, residues involved as active site are depicted in blue. Visualizations of modeled structures were performed by UCSF Chimera package



**Fig. 2** Phylogenetic analysis of *Azadirachta indica* caffeic acid 3-O-methyltransferase with other plants OMT proteins. Phylogeny of NCOMTs was inferred through the maximum likelihood method using MEGA 5 software. A total of 27 protein sequences used for evolutionary analysis from following plants viz *Azadirachta indica* (NCOMT), *Citrus aurantium* (ADK97702), *Jatropha curcas* (ACT87981), *Gossypium arboreum* (KHG13289), *Gossypium hirsutum* (ACT32029), *Boehmeria nivea* (ABG27066), *Eucalyptus camaldulensis* (ACY66932), *Betula platyphylla* (AGG91492), *Punica granatum* (AID68566), *Rosa chinensis* (BAC78827), *Malus*

*domestica* (ABI54119), *Theobroma cacao* (EOY23716), *Fragaria x ananassa* (AAF28353), *Pyrus x bretschneideri* (AGS44640), *Populus tomentosa* (AFZ78575), *Caragana korshinskii* (AEV93478), *Glycine max* (AEI54336), *Medicago truncatula* (AES72647), *Glycine soja* (KHN23296), *Medicago sativa* (ACY06328), *Linum usitatissimum* (AGO50639), *Leucaena leucocephala* (ABS57468), *Arabidopsis lyrata* (EFH40568), *Arabidopsis thaliana* (NP\_200227), *Camellia sinensis* (ADN27527), *Ipomoea nil* (BAE94403) and *Salvia miltiorrhiza* (AEO14870)

assays were performed by incubating the protein at various temperatures for 30 min. The catalytic activity was substantially retained till incubation at 60 °C whilst it was completely lost at 70 °C (Fig. 4b).

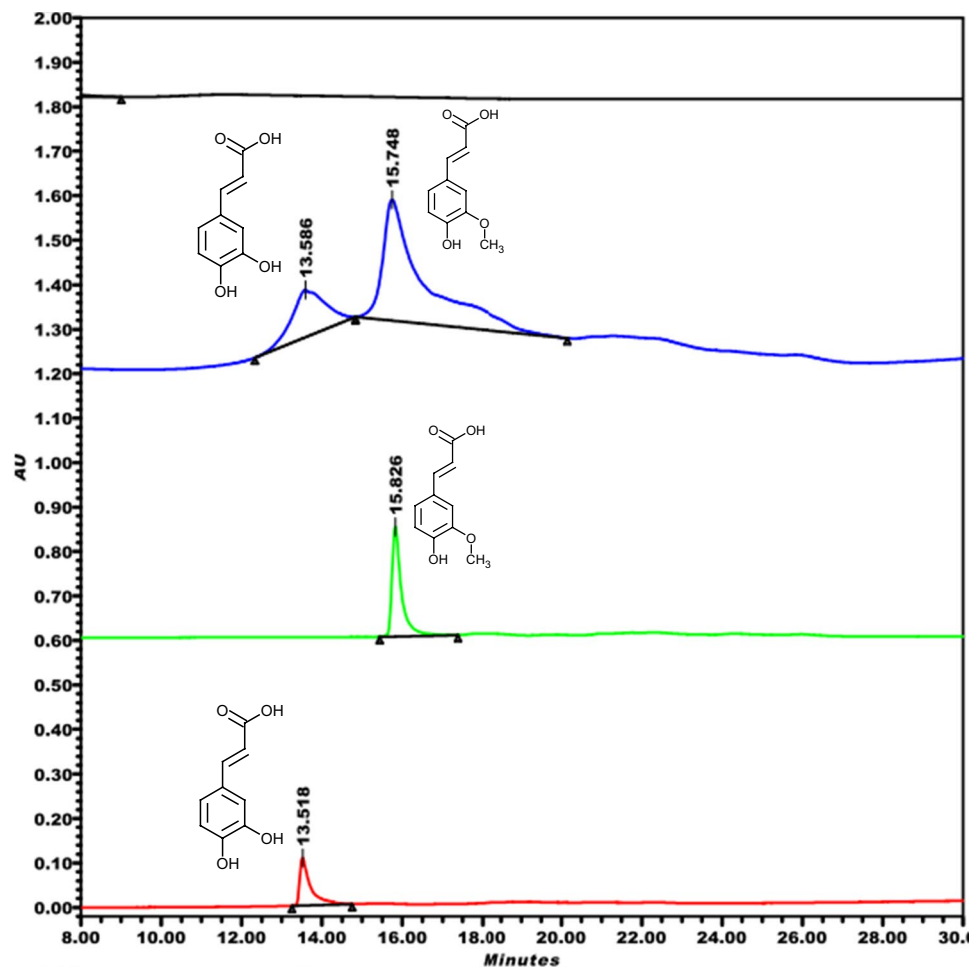
The substrate saturation curve of the enzyme was hyperbolic for caffeic acid but with a slight lag of activity response at an initial lower concentration. Yet the substrate saturation curve of the enzyme for SAM was normal hyperbolic. Thus, the enzyme followed almost normal Michaelis–Menten kinetics. The  $K_m$ ,  $V_{max}$  and  $k_{cat}$  values of NCOMT enzyme with caffeic acid and SAM were determined via double reciprocal plot (Fig. 4c, d). The  $K_m$  values of NCOMT for caffeic acid and SAM were observed to be 7.14 mM and

208  $\mu$ M, respectively, while the  $k_{cat}$  values for the two substrates were computed to be 256.32 and 44.32  $s^{-1}$ , respectively. The  $V_{max}$  values for caffeic acid and SAM were 7.69 and 1.42  $\mu$ M  $min^{-1} mg^{-1}$ , respectively. The kinetic parameters of the NCOMT catalyzed reaction gave an estimate of catalytic efficiency ( $k_{cat}/K_m$ ) of the recombinant enzyme as  $3.59 \times 10^4$  and  $2.13 \times 10^5 M^{-1} s^{-1}$  toward caffeic acid and SAM, respectively.

### NCOMT expression analysis

The pattern of *NCOMT* gene expression was analyzed by qRT-PCR in Neem tissues, different developmental stages

**Fig. 3** NCOMT enzymatic assay and analysis through HPLC. Red line indicates caffeic acid, green line indicates ferulic acid, blue line indicates enzyme assay and black line indicates control assay without substrate



of fruit and different fruit parts (Fig. 5a–c). Results revealed that *NCOMT* was constitutively expressed at high level in fruit tissues followed by stem and leaf tissues. The expression of *NCOMT* was much higher in fruit tissues than in buds. Stem, leaf and flower tissues have 28, 4.5 and 2-folds higher transcript abundance than bud tissue (Fig. 5a). Expression of *NCOMT* was continuously increased from immature green to mature ripen yellow stage of fruit development. *NCOMT* transcript abundance was almost 2, 3 and 10 fold higher in mature green, half ripen yellow green and fully ripen yellow fruit in comparison to immature green fruits (Fig. 5b). Elevated expression levels of *NCOMT* were observed in tegmen tissue, followed by endocarp and cotyledons. Interestingly, fruit mesocarp, epicarp and embryo showed a similar pattern having lower transcript abundance (Fig. 5c).

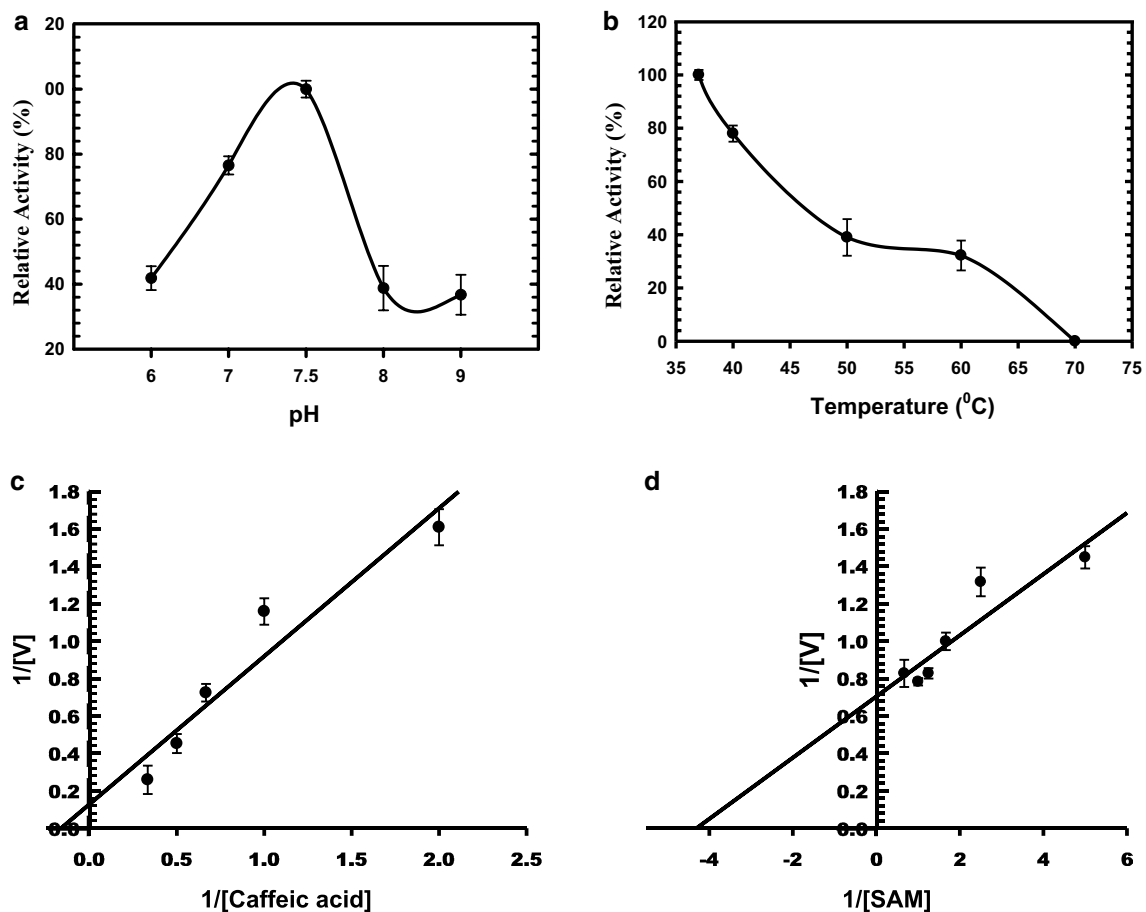
### Effect of elicitors on *NCOMT* transcript profiles

The exposure of fruit to different concentrations of MJ and SA led to up regulation of *NCOMT* transcripts (Fig. 6a, b). The exposure to wounding was unable to alter the transcript

levels of *NCOMT* (Fig. 6c). Under UV stress conditions, the levels of *NCOMT* transcript was increased up to threefold after 30 min and subsequently reached up to a maximum level of 5.5-folds after 1 h of exposure. Extended UV exposure for 2 h exhibited a slight decline in *NCOMT* transcript levels (4.5-fold) and after 5 h of exposure it remained about 4-fold (Fig. 6d). The abundance of *NCOMT* transcript was up regulated after IAA treatment. The expression was increased up to 20-fold at 100  $\mu$ M IAA and at 200  $\mu$ M IAA, the expression level was raised up 50-fold. The maximum transcript abundance (300-fold) was observed at 500  $\mu$ M concentration of IAA (Fig. 6e). However, the *NCOMT* transcript was unaffected after ABA treatment (Fig. 6f).

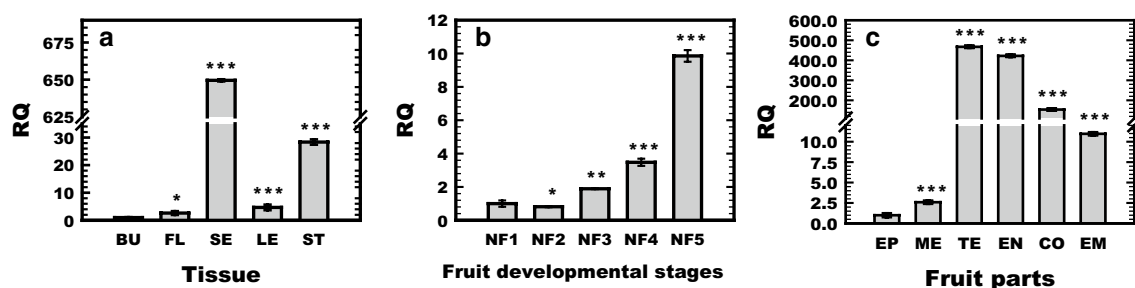
### Over-expression of *NCOMT*

*NCOMT* was cloned in *pBI121* plant expression vector and the gene construct was used for transient transformation by using leaf tissues of *W. somnifera* as well as different species of *Ocimum* (*O. sanctum*, *O. basilicum*, *O. kilimandscharicum* and *O. gratissimum*). Stable transgenic lines of *O. gratissimum*, containing *NCOMT* gene were generated



**Fig. 4** Kinetic parameters of recombinant NCOMT enzyme using caffeic acid as substrate and SAM as co-substrate. **a** Effect of pH on NCOMT activity. **b** Effect of temperature on NCOMT activity. **c** Double reciprocal plots of substrate saturation kinetics for substrate.

**d** Double reciprocal plots of substrate saturation kinetics for cofactor. [*t*-test was used to analyze significance, at  $P < 0.05$  for (\*),  $P \leq 0.01$  for (\*\*) and  $P \leq 0.001$  for (\*\*\*) with the help of GraphPad Prism 7.03 software.]



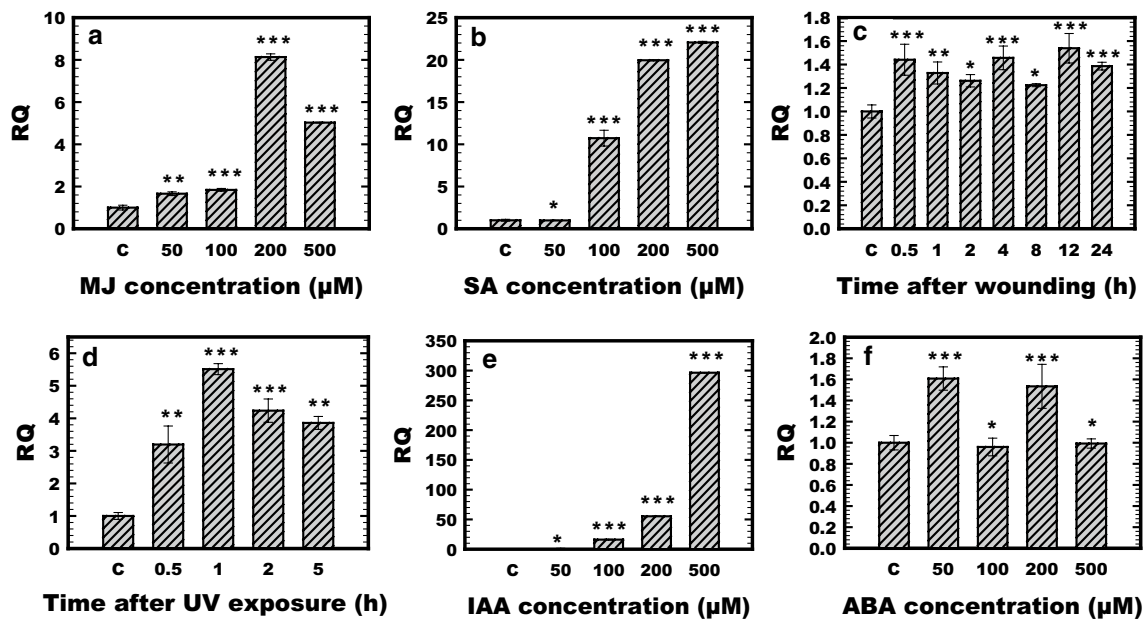
**Fig. 5** Real-time expression profile representing the transcript abundance of *NCOMT*. **a** Expression profile in different tissues. **b** Expression profile in different developmental stages of fruit. **c** Expression profile in different fruit parts. *BU* bud; *FL* flower; *SE* fruit; *LE* leaf; *ST* Stem; *NF1* immature green fruit; *NF2* mid mature green fruit;

*NF3* mature green fruit; *NF4* half ripen yellow green fruit; *NF5* fully ripen yellow fruit; *EP* epicarp; *ME* mesocarp; *TE* tegmen; *EN* endocarp; *CO* cotyledons and *EM* embryo. [*t*-test was used to analyze significance, at  $P < 0.05$  for (\*),  $P \leq 0.01$  for (\*\*) and  $P \leq 0.001$  for (\*\*\*) with the help of GraphPad Prism 7.03 software.]

and confirmed by the presence of the *nptII* gene through PCR analysis. In the present study, three independent lines of the transiently transformed leaf tissues and five lines of

stable transformed leaf tissues were examined. Overexpression of *NCOMT* gene in *W. somnifera* was confirmed by real-time PCR, suggesting 2- to 2.5-fold higher expression, and





**Fig. 6** Real-time expression profile representing the transcript abundance of *NCOMT* in elicitor treated fruits. **a** Methyl jasmonate (MJ). **b** Salicylic acid (SA). **c** Wounding. **d** UV treatment. **e** Auxin (IAA)

treatment. **f** Abscisic acid (ABA) treatment. All experiments were performed in triplicate with three biological repeats

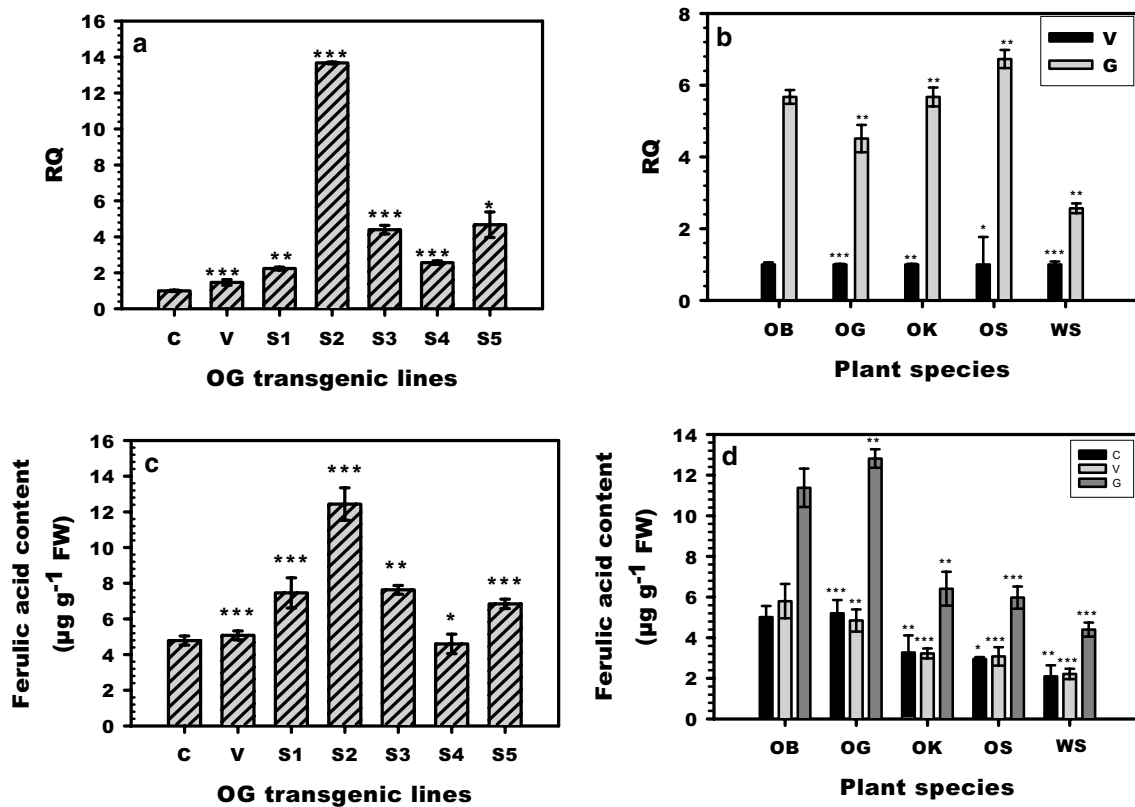
transformed *Ocimum sp.* also have ~4–14-folds enhanced transcript levels compared to controls (Fig. 7a, b). The metabolite analysis was performed through estimation of the ferulic acid content in transgenic *W. somnifera* leaves as well as in *Ocimum sp.* leaves (*O. sanctum*, *O. basilicum*, *O. kilimandscharicum* and *O. gratissimum*). About up to ~1.5–2.5 fold increase was noted for ferulic acid content in transformed leaf tissues of *W. somnifera*, *O. sanctum*, *O. basilicum*, *O. kilimandscharicum* and *O. gratissimum* as compared to un-transformed leaf tissues (Fig. 7c, d).

### Discussion

COMT enzyme converts caffeic acid into ferulic acid, a key component of lignin. Ferulic acid is also used in pharmaceuticals, nutraceuticals and food industries. It has been reported to exhibit a notable range of biomedical activities such as antioxidant, antidiabetic, anticarcinogenic, antiallergic, antimicrobial, antiviral, antiaging, vasodilator, hepatoprotective, anti-inflammatory, antithrombotic etc. (Srinivasan et al. 2007; Kumar and Pruthi 2014). It is also used as a preclusion agent for food discoloration, a growth enhancer as well as a precursor of vanillin, the high demand flavoring agent (Kumar and Pruthi 2014). Natural sources of ferulic acid are limited, therefore it is desired to explore and examine other sources/approaches of its production. In this study, an O-methyl transferase named NCOMT was isolated and characterized from Neem, which exhibited significant

potential for the biogenesis of ferulic acid from caffeic acid. The *NCOMT* gene, isolated from cDNA library of *A. indica* fruit, contains 1098 bp open reading frame encoding polypeptide of 366 amino acids. Other plants OMT such as *L. chuanxiong*, *Vanda mimi Palmer*, *Iris hollandica* have 362, 367, 365 amino-acids and 5.94, 5.74, 5.54 isoelectric points, respectively, which is quite close to the NCOMT isoelectric point (Yoshihara et al. 2008; Aiman et al. 2015; Li et al. 2015). A significant sequence similarity (> 80%) of *NCOMT* confirms it as caffeic acid O-methyltransferase gene (COMT). Generally, plant O-methyltransferases possess three signature motifs as SAM-binding, catalytic and phenolic substrate binding motifs (Kim et al. 2006; Byeon et al. 2015). Multiple sequence alignment of NCOMT with other plants OMT sequences indicates the presence of all three-signature motifs in appropriate manner. The SAM binding motif situated at the middle position and phenolic substrate binding and catalytic site binding motifs are present at the C terminal of NCOMT sequence. All these motifs are identical to other plants COMT enzymes, and strongly suggest this as caffeic acid O-methyltransferase enzyme. Previous reports indicate that COMT(s) lack any signal sequence for subcellular localization and are localized in the cytosol such as rice COMT, *Vanda Mimi Palmer* OMT, Arabidopsis OMT (Lee et al. 2014; Aiman et al. 2015; Byeon et al. 2015).

Homology modeling suggests that NCOMT model is perfectly matched with caffeic acid/5-hydroxyferulic2 acid 3/5-O-methyltransferase model of *Medicago sativa* (PDB: c1kyzC) whose crystal structure is already available in



**Fig. 7** Analysis of transient and stable transformed *NCOMT* in *Ocimum* species and *W. somnifera*. **a** Expression analysis of *gus* gene in stable lines of *O. gratissimum*. **b** Expression analysis of *gus* gene in transiently transformed tissues from different *Ocimum* species and *W. somnifera*. **c** Estimation of ferulic acid in stable transgenic lines of *O. gratissimum*. **d** Estimation of ferulic acid in transiently transformed tissues from different *Ocimum* species and *W. somnifera*. C untrans-

formed control; V empty vector transformed control; G *NCOMT* gene transformed tissue; S1–S5 Stable lines of *O. gratissimum*; OB *O. basilicum*; OS *O. sanctum*; OK *O. kilimandscharicum*; OG *O. gratissimum*; WS *Withania somnifera*. [*t*-test was used to analyze significance, at  $P < 0.05$  for (\*),  $P \leq 0.01$  for (\*\*), and  $P \leq 0.001$  for (\*\*\*) with the help of GraphPad Prism 7.03 software.]

PDB database. These results strongly indicated that the *NCOMT* protein may also be functional in accordance with the *Medicago sativa* COMT protein. Binding site prediction analysis proposes participation of almost 21 residues as a functional substrate binding site. It was previously reported in *Medicago sativa* that Lys-265, Asp-206, Asp-231 and Asp-251 are the key residues, which are involved in SAM binding (Zubieta et al. 2002). Here, in the present investigation, we also observed a similar structural composition in predicted substrate binding site as Lys-266, Asp-207, Asp-232 and Asp-252, which strongly supports *NCOMT* enzyme as kinetically active. Evolutionary relationship of *NCOMT* with other COMT protein sequences clustered it with *Citrus aurantium* OMT, which falls under the same order Sapindales.

Homogeneity of the purified recombinant *NCOMT* protein was confirmed by SDS-PAGE and Western-blot analysis. Purified *NCOMT* protein was used for enzyme assay to confirm the reaction product as ferulic acid. *NCOMT* enzyme follows the Michaelis–Menten kinetics and displays

a hyperbolic substrate saturation curve for its substrate as well as cofactor. The  $K_m$ ,  $V_{max}$  and  $k_{cat}$  values of the *NCOMT* enzyme with caffeic acid and SAM are quite comparable with other plants COMT enzymes. Previous studies on OMTs reported an elevated  $K_m$  with significant catalytic efficiency like OMT-II; 1.1 and OMT-II; 2.2 enzyme from *Thalictrum tuberosum*, catalyzing caffeic acid at  $K_m$  of 0.6 and 1.1 mM, respectively (Frick and Kutchan 1999). Several other studies have revealed that COMT(s) were also able to methylate N-acetylserotonin (Byeon et al. 2014). Thus, COMT may catalyze multiple reactions with significant catalytic efficiency as shown in *Arabidopsis*: it catalyzes methylation of caffeic acid and N-acetylserotonin with  $K_m$  values of 103 and 233  $\mu$ M, respectively (Byeon et al. 2014, 2015). In another report, it was shown that *A. thaliana* caffeic acid O-methyltransferase (*AtCOMT*) enzyme converted N-acetylserotonin into 5-methoxytryptamine with low catalytic activity having a  $K_m$  of 3.996 mM (Lee et al. 2014). Therefore, COMT is a multifunctional enzyme and able to catalyze methylation of an array of substrates including

phenolics, flavonoids, and aryl alkylamines (Byeon et al. 2014).

The COMT enzyme exhibited a wide range of catalytic efficiency ( $k_{cat}/K_m$ ) ranging from  $\text{mM}^{-1} \text{s}^{-1}$  to  $\text{nM}^{-1} \text{s}^{-1}$ . The COMT enzyme of sweet basil exhibited  $0.27 \text{ nM}^{-1} \text{ s}^{-1}$  catalytic efficiency (Gang et al. 2002), VanOMT3 isolated from *Vanilla planifolia*  $0.6 \text{ mM}^{-1} \text{ s}^{-1}$  (Li et al. 2006), COMT of *Clarkia breweri*  $9.93 \text{ nM}^{-1} \text{ s}^{-1}$  (Wang and Pichersky 1998), and a much higher efficiency was shown for *Rauvolfia serpentina* OMTs (Wiens and Luca 2016). The higher catalytic efficiency made NCOMT to be a catalytically efficient enzyme.

Optimum NCOMT activity is recorded at pH 7.5, which is equivalent to OMT(s) from other plants such as sweet basil and *Iris hollandica* OMT; both have maximal enzyme activity at pH 7.5–8.0 (Gang et al. 2002; Yoshihara et al. 2008). Recently, two OMT(s) characterized from *R. serpentina* roots showed significant variation in pH optima. RsOMT1 showed maximum enzymatic activity between pH 7.5–10 while RsOMT3 exhibited between 6.5–8.0 pH (Wiens and Luca 2016). Thermo-stability results of NCOMT exhibited  $37^\circ\text{C}$  as its optimum temperature. At higher temperature, its enzyme activity gradually decreases and at  $70^\circ\text{C}$ , activity is completely lost. Previous studies of COMT suggest that it remains active at  $60^\circ\text{C}$ , at higher temperatures integrity of enzyme is destroyed and unable to perform catalytic conversion. The OMT(s) characterized by *R. serpentina* lost their catalytic activity above  $60^\circ\text{C}$  (Wiens and Luca 2016). Reports of LcCOMT enzyme isolated from *Ligusticum chuanxiong* had its optimum pH at 7 and temperature at  $37^\circ\text{C}$  (Li et al. 2015).

Abundance of NCOMT transcripts was determined in tissues, fruit ontogeny, fruit parts and elicitor treated fruits. Fruit ontogeny specific expression pattern of NCOMT reveals that continuous increment in expression from immature to fully mature (ripen) fruits. When expression was analyzed within fruit parts, it showed interesting results. The tegmen part of fruit had a maximal level of expression followed by endocarp and cotyledons, but, interestingly, mesocarp, epicarp and embryo exhibited very low expression. This indicates that NCOMT has a tissue-specific role in Neem plants.

The signaling molecules, MJ and SA, are known for modulating the expression of genes involved in defense responses including secondary metabolites. Treatment of MJ and SA modulators resulted in the induction of NCOMT gene expression. These observations are in agreement with earlier reports of the application of MJ treatment for increasing phenolics production. Elevated level of COMT expression was observed in *Hibiscus cannabinus* after exposure of MJ and SA (Kim et al. 2013).

In general, wounding induced genes which were implicated in lignin biosynthesis but the caffeoyl-coenzyme A

3-O-methyltransferase gene from switchgrass displayed similar transcript abundance in leaf and stem tissue after exposing to wounding stress (Liu et al. 2016). Here, wounding exposure was unable to alter the expression level of NCOMT. UV stress slightly increased the level of NCOMT transcripts but IAA up-regulated notable transcript levels. ABA treatment had similar results as wounding and was not able to enhance remarkable abundance. *Ligusticum chuanxiong* OMT showed enhanced expression under different stress conditions (Li et al. 2015).

The most common function of COMT enzyme is biosynthesis of ferulic acid, an intermediate component of lignin biosynthesis. Although it exhibits maximal enzyme activity toward caffeic acid substrate, it can significantly convert N-acetylserotonin into melatonin. This is also confirmed by in vitro experiments in Arabidopsis and rice (Lee et al. 2014; Byeon et al. 2014, 2015). Several reports are available exhibiting the significance of the COMT enzyme in the regulation of lignin content. Down regulation of *Brachypodium distachyon* COMT reduced total lignin quantity, thus enhanced ethanol yield observed from plant biomass (Trabuocco et al. 2013). Knocking out of Arabidopsis COMT results in a lower production of melatonin compared to wild type, suggesting its involvement in melatonin pathway as well (Byeon et al. 2014).

Ferulic acid profiling was performed in different tissues of Neem. Several reports are available showing production of ferulic acid in varying range in plant tissues (Kumar and Pruthi 2014). Ferulic acid was not detected in flowers of Neem, yet a substantial level ( $0.38\text{--}1.16 \mu\text{g g}^{-1}$  fresh weight) was observed in fruit parts, raw fruit (green fruit) epicarp, mesocarp and seed (Singh et al. 2005). These results were quite comparable with the results on NCOMT transcripts abundance, which reveals minimal expression in epicarp followed by mesocarp (pulp) and maximal in seed parts (tegmen, endocarp, cotyledons and embryo) of green fruits. Singh et al. (2005) reported maximal yield of ferulic acid in ripen fruit ( $18.51 \mu\text{g g}^{-1}$  fresh weight), which is approximately 8.8-fold higher than in raw green fruit ( $2.09 \mu\text{g g}^{-1}$  fresh weight). The expression of NCOMT is about ten fold higher in ripen yellow fruits in comparison to unripe green fruits. Thus, NCOMT expression profile clearly indicates that the maturation of flowers is accompanied by a higher requirement of ferulic acid content as observed by Singh et al. (2005) in Neem tissues, probably for cellular multifunctionality, especially for lignin biosynthesis.

NCOMT was transiently overexpressed in *W. somnifera* leaf tissue and its expression pattern was observed to be almost similar with ferulic acid content. Over-expressed *W. somnifera* leaf tissue contained a higher levels of NCOMT expression as well as ferulic acid as compared to untransformed controls. To probe the functional role of NCOMT in ferulic acid biosynthesis, it was transiently transformed in *Withania somnifera*

and *Ocimum species* (*O. sanctum*, *O. basilicum*, *O. kilimandscharicum* and *O. gratissimum*) and stably transformed in *O. gratissimum*, to evaluate its over-expression impact on ferulic acid content. In this study, ferulic acid could be enhanced up to ~1.5–2.5 fold in transgenic lines of heterologous tissues. It is in agreement with previous reports exhibiting enhanced levels of ferulic acid or lignin in transgenic plants, transformed by *COMT* gene (Oraby and Ramadan 2015). Silencing of *COMT* genes of *Brassica napus* resulted in the reduction of the lignin content without affecting seed oil composition (Oraby and Ramadan 2015). Other COMTs were also involved in catalytic conversion of important molecules as shown earlier (Byeon et al. 2014, 2015). Here we report the first gene from the reputed medicinal plant Neem, *NCOMT*, which was cloned and physico-kinetically characterized. It was shown that *NCOMT* was involved in ferulic acid biosynthesis from caffeic acid, a key step in lignin biosynthesis. It could also serve as a new source for the biosynthesis of ferulic acid, a therapeutic agent (Srinivasan et al. 2007). In the present study, we demonstrated that metabolic engineering can be used to improve the production of ferulic acid. We discussed two approaches for ferulic acid production from caffeic acid using the *COMT* enzyme: first through isolation of the recombinant enzyme from engineered microbes (*E. coli*), followed by enzymatic assay in an *in-vitro* system, and second through the genetic transformation of the *NCOMT* gene in suitable plants (*Withania somnifera* and *Ocimum sp.*). This study provides insights into the ferulic acid production which plays a major role in lignin biosynthesis.

## Conclusions

In this study, a *NCOMT* gene of 1098 bp was identified, isolated and cloned from *A. indica* fruit tissues. *NCOMT* was heterologously expressed in *E. coli* and the purified recombinant protein catalyzed the conversion of caffeic acid into ferulic acid. *In-silico* structural analysis of *NCOMT* suggested that the 3D structure of *NCOMT* is closely similar to OMT from *Medicago sativa*. Further, the *NCOMT* enzyme was kinetically characterized. It exhibited a significant catalytic efficiency ( $k_{cat}/K_m$ ). The observed optimum pH was 7.5 and temperature was 37 °C for *NCOMT* with caffeic acid as substrate. The transcript abundance of *NCOMT* in different Neem tissues revealed maximal expression in fruit tissues, followed by fully mature fruit and tegmen tissues, respectively. The elevated level of *NCOMT* expression was observed in most of the elicitor treated fruit tissues. For *in-planta* functional characterization of *NCOMT*, it was transiently over-expressed in *W. somnifera* and *Ocimum species* (*O. sanctum*, *O. basilicum*, *O. kilimandscharicum* and *O. gratissimum*), and stable transformations of *O. gratissimum* were generated. The transformed tissues were able to accumulate an enhanced ferulic acid content

with enhanced levels of *NCOMT* expression as compared to untransformed tissues.

## Author contributions statement

RSS and NSS planned the experiments and prepared the manuscript. LKN, JSJ and SB were involved in performing the experiments and in the compilation of data. All the authors critically read the paper and approved.

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## Compliance with ethical standards

**Conflict of interest** All authors declare that there is no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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