Evidence for localization of *N*-methyltransferase (MMT) of caffeine biosynthetic pathway in vacuolar surface of *Coffea canephora* endosperm elucidated through localization of GUS reporter gene driven by NMT promoter

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Three *N*-methyltransferases (NMTs) are involved in the formation of caffeine from xanthosine. We have earlier cloned the promoter for one of the NMT genes. This study is focused on the localization of NMT in coffee endosperm cells. Constructs bearing the NMT promoter with and without the first exon were made in pCAMBIA 1381. These constructs and pCAMBIA 1301 bearing the intron *uid*A gene driven by CaMV 35S promoter were electroporated into coffee endosperm and the activity of β-glucuronidase (GUS) localized. In tissues transformed with the construct containing promoter and first exon, enzymatic activity was localized on the outer surface of the vacuole. Interestingly, antibodies to the coffee NMT also specifically localized to the same region. In tissues bearing either the NMT–GUS construct without the first exon, or pCAMBIA 1301 with intron GUS, GUS activity was spread throughout the cytoplasm. The results suggest that NMT is targetted to external surface of the vacuole. It would be interesting to discover the localization of other enzymes involved in caffeine and chlorogenic acid biosynthesis for understanding their relationship, if any.

Keywords: *Coffea canephora*, genetic transformation, localization, *N*-methyltransferase, tissue electroporation.

COFFEA species contain caffeine (a purine alkaloid) in the beans, leaves and embryos. The sequential conversion of xanthosine to caffeine through 7-methylxanthosine, 7-methylxanthine and theobromine occurs through the action of *N*-methyltransferases (NMTs) that use *S*-adenosyl-L-methionine (SAM) as the methyl donor[1,2]. The cDNAs encoding 7-methylxanthosine synthase[1], theobromine synthases and caffeine synthase[3,4] have been cloned. Moreover, we have cloned the promoter for one of the NMT genes[5] and demonstrated the reporter gene expression driven by NMT promoter in *Nicotiana tabacum*.

However, information on cellular localization of coffee NMT is lacking. It has also been reported that chlorogenic acid (CGA) and caffeine are bound together in the cell[6]. Using NMR spectroscopy it was concluded that in aqueous medium the caffeine–potassium chlorogenate was best described as 'hydrophobically bound π-molecular complex'[7]. The free CGA and also the complex with caffeine are stored intracellularly in the central vacuole[8]. Caffeine accumulation to a certain extent correlates with the CGA concentration in the cells[9]. Therefore, there is a possibility of co-regulation of caffeine and CGA synthesis in coffee tissues. Endosperm tissues contain a large pool of caffeine.

The objective of this study has been to analyse the expression of the *uid*A reporter gene driven by NMT promoter in the presence and absence of the first exon of the NMT gene in relation to the localization of coffee NMT enzymes in the cells of coffee endosperm.

Certified seeds of *Coffea canephora* cv. S-274 were obtained from the Central Coffee Research Institute, Chikmagalur District, Karnataka, India and planted in the greenhouse. Green, unripe fruits were collected and the endosperm tissues were used in electroporation experiments. Electroporation was carried out according to an earlier report[10]. The endosperm tissues were taken with 100 µg/ml plasmid DNA in 350 µl electroporation buffer (pH 5.6) consisting of 70 mM aspartic acid, 5 mM calcium gluconate, 5 mM MES and 0.5 mM mannitol. The tissues were incubated for 1 h at 4°C prior to electroporation using a BioRad Gene Pulser. Electroporation was carried out[10] with single electric pulse of 500 V/cm, discharged from a 900-µF capacitor. Electroporation of endosperm without plasmid DNA served as a negative control. The tissues were incubated for an additional hour at 4°C after discharge.

The plasmid vectors pCAMBIA 1301 and pCAMBIA 1381 were procured from the Center for the Application of Molecular Biology to International Agriculture (CAMBIA), Canberra, Australia. The NMT promoter was isolated by PCR-based genome-walking[5]. A construct was made with the 938 bp PCR product comprising 29 bases of the adapter and 909 bases of the coffee genomic fragment (clone pETSSPI, accession no. DQ010010). The 938 bp PCR product was reamplified[5] from the clone pETSSPI using the primers ASP2 and GSPin. The PCR product was cloned into pTZ57R/T vector and sequenced for checking the orientation of the insert. The resulting construct was designated as pTZSSPI. A 979 bp *Eco*RI/*Bam*HI fragment spanning the –896 to +83 bp region (numbering related to ATG of the theobromine synthase gene, accession no. AB048794) was released from pTZ938. The +83 bp region comprised 75 bases of the first exon and 8 bases of the first intron. The *Eco*RI/*Bam*HI promoter fragment released from pTZSSPI was fused in frame with a cassette containing the *uid*A gene and the NOS terminator in pCAMBIA 1381 digested with *Eco*RI/*Bam*HI. The resulting translational fusion construct was designated as pPCTS938 (Figure 1 *a*).

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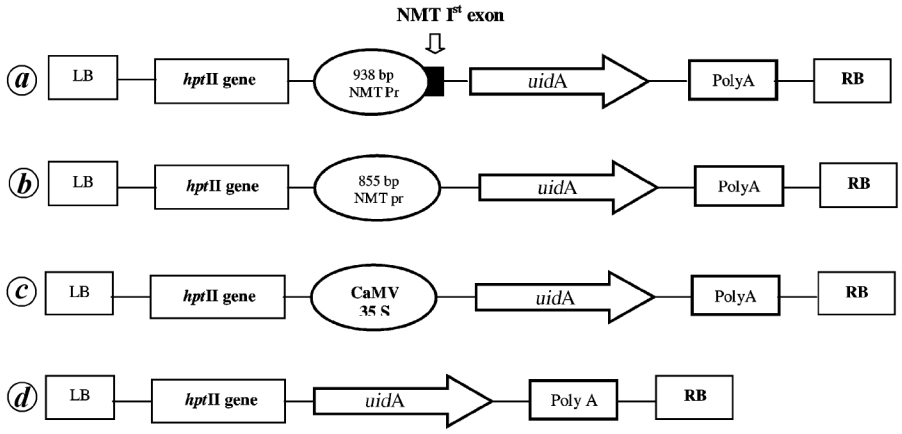

Figure 1. T-DNA region of constructs of (*a*) pPCTS938, (*b*) pPCTS855, (*c*) pCAMBIA 1301 and (*d*) pCAMBIA 1381.

Similarly, another construct was made in such a way that the first exon region was avoided in the *N*-terminal fusion to the *uid*A gene. The 855 bp PCR product was reamplified from the clone pETSSPI using the primers ASP2 and VUR (5′-TTCGATTGTCCTGCATATGA-3′). The primer VUR binds to the sequence at the end of the 5′ UTR, without extending into the first exon. The 855 bp PCR product was cloned into pTZ57R/T vector and sequenced for checking the orientation of the insert. An 896 bp *Eco*RI/*Bam*HI promoter fragment was released from pTZ855 and fused in frame to the *uid*A gene in pCAMBIA 1381 digested with *Eco*RI/*Bam*HI. The resulting translational fusion construct was designated as pPCTS855 (Figure 1 *b*).

The vector pCAMBIA 1301 contains the selectable marker gene hygromycin phosphotransferase (*hpt*II) under the control of the CaMV 35S promoter and CaMV 35S terminator; β-glucuronidase (GUS; *uid*A) gene with a catalase intron under the control of CaMV 35S promoter and NOS terminator (Figure 1 *c*). pCAMBIA 1381 contains a promoter less *uid*A coding region construct and was used as a negative control (Figure 1 *d*). All constructs were maintained in *Escherichia coli* DH5α and plasmid DNA isolation was carried out according to the reported methods[11].

GUS assay was performed by immersing the electroporated endosperm for 12 h at 37°C in a buffer containing 100 mM sodium phosphate (pH 7), 20 mM EDTA, 0.1% triton X-100, 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 20% methanol and 1 mM X-Gluc (5-bromo 4-chloro indolyl-D-glucuronide cyclo-hexamonium salt, Sigma, USA)[12]. Methanol was added to the reaction mixture to suppress endogenous GUS-like activity[13] and documented using a Leica (Wild M3Z) stereo-microscope. The samples were fixed in formaldehyde : acetic acid : 70% (v/v) ethanol (5 : 5 : 90) prior to free-hand sectioning and observed using an Olympus light microscope (BX40).

The method of Reeve[14] was followed for staining CGA. Free-hand sections were first treated with aqueous solution containing 10% sodium nitrite, 2% urea and 1% glacialacetic acid. After the appearance of a yellow colour, 2% NaOH was added to the sections. A deep cherry-red colour appeared in 2–3 min. The sections were observed using the Olympus light microscope. Immunocytolocalization of the NMT enzymes was performed using rabbit polyclonal anti-coffee theobromine synthase-2 (CTS2) antibodies (kind gift from Dr K. Mizuno). Immunolocalization was performed according to Anil *et al.*[15]. The sections were observed under Olympus microscope equipped with flourescens filters (Olympus, BX40; emission wavelength 450–480 nm, Excitation wavelength 510–550 nm).

GUS expression was not detected in endosperm electroporated without plasmid DNA and in endosperm electroporated with pCAMBIA 1381 vector harbouring a promoterless *uid*A gene (Figure 2 *a*). GUS expression was observed in endosperm transformed with *uid*A gene driven by coffee NMT promoter containing the first exon (Figure 2 *b*) and also in those tissues transformed with NMT promoter constructs devoid of the first exon (Figure 2 *c*). The results clearly demonstrate that the NMT promoter could drive the expression of reporter genes in endosperm tissues of *C. canephora*. GUS activity was observed in positive control endosperm electroporated with pCAMBIA 1301, wherein the *uid*A gene is driven by the CaMV 35S promoter (Figure 2 *d*).

In tissues electroporated with constructs containing the first exon sequence, GUS activity was targetted to the external surface of the vacuole (Figure 2 *e*). Deletion of the first exon resulted in a diffused expression of the *uid*A gene in the cytoplasm, not localized to the areas around the vacuole. Staining for CGA according to the method of Reeve[14], revealed localization of CGA in the vacuole (Figure 2 *g*). The NMTs of coffee were traced using antibodies raised against them and were found to be localized

surrounding the vacuole (Figure 3 *a* and *b*), as seen with GUS expression driven by NMT promoter containing the first exon (Figure 2 *e*).

In silico analysis of the first exon sequence of the coffee NMT revealed homology to that of methyltransferase from different plant species. Two motifs 'LHMN' and 'GETSYAKNS' were particularly conserved (Figure 4). The *N*-terminal of the tea caffeine synthase is FMNR-GEESSYAQNSQFTQV[16]. Leucine to phenylalanine can be the result of an SNP while retaining hydrophobicity in tea. It is intriguing that there are some reports of chloroplast location of caffeine synthase from tea. Our work indicates localization of the enzyme in the vacuolar surface.

The mechanisms for uptake and sequestration of caffeine in vacuoles are yet to be investigated. Binding of caffeine to CGA may allow accumulation of these compounds in vacuoles against a concentration gradient[17]. Baumann and co-workers[6,9] suggested that vacuolar compartmentation of purine alkaloids depends exclusively on the formation of complexes with CGA. Our results are in accordance with the above observations.

Mizuno *et al.*[4] reported isolation of caffeine synthase (CCS1) clone from coffee endosperm. However, the predicted amino acid sequences of CCS1 are only about 40% similar to those of tea caffeine synthase (TCS1). It was found that the tea caffeine synthase, which is a key enzyme in catalysing the final two steps of caffeine biosynthesis in tea, is located in the chloroplasts[17]. It is also known that the NMTs are SAM-dependent and SAM synthetase was also detected exclusively in the cytosol, and SAM is produced in the cytosol of tea leaves[17]. Subcellular localization of *C. arabica* methyl xanthine methyltransferase (CaMXMT) using the fusion protein of CaMXMT and GFP demonstrated its existence predominantly in the cytoplasm of onion epidermal cells. It was also predicted using the PSORT program that CaMXMT is localized in the cytoplasm[18]. Kolosova *et al.*[19]

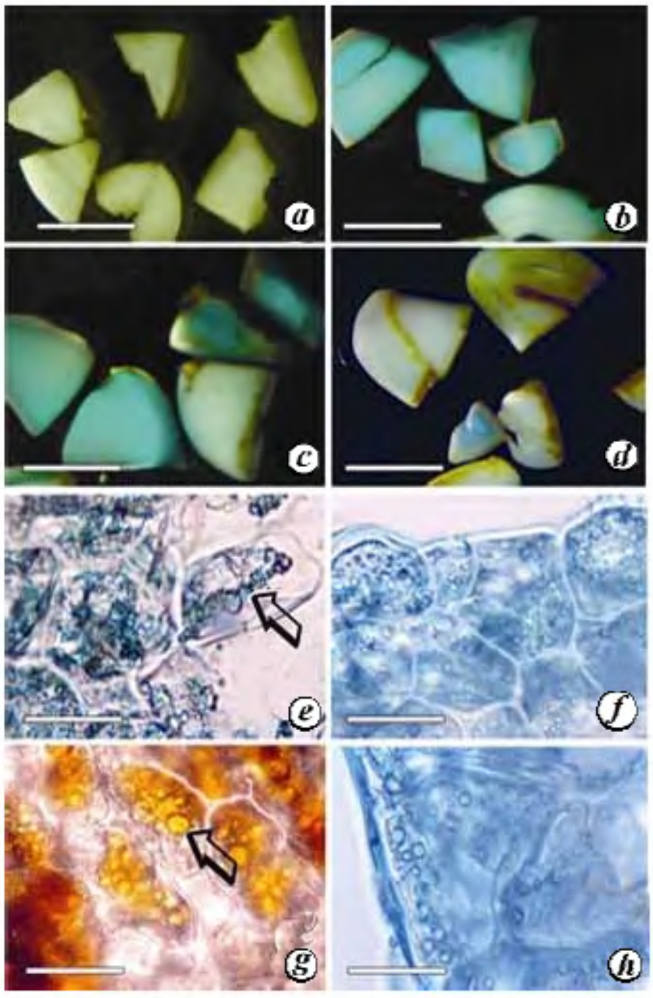

Figure 2. ***a***, *Coffea* transformed with promoterless *uid*A construct pCAMBIA 1381 (bar = 2.5 mm). ***b–d***, *Coffea* electroporated with GUS gene driven by (***b***) NMT promoter with the first exon; (***c***) NMT promoter without the first exon and (***d***) CaMV promoter (bar = 2.5 mm). ***e***, ***f***, Sub-cellular localization of GUS expression in endosperm tissues electroporated with GUS gene driven by NMT promoter (***e***) with the first exon and (***f***) lacking the first exon (bar = 30 µm). ***g***, Sub-cellular localization of chlorogenic acid (bar = 30 µm). ***h***, *Coffea* electroporated with GUS gene driven by CaMV promoter (bar = 30 µm).

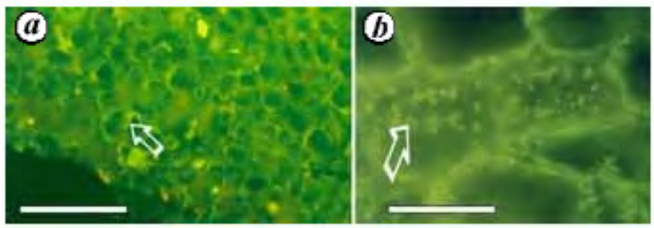

Figure 3. ***a***, Immunocytolocalization of NMT enzymes in endosperm tissues of *Coffea canephora* (bar = 180 µm). ***b***, Localization of NMT enzymes in a single endosperm cell (bar = 10 µm).

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 1. MEVMR-ILHMN-KGNGET-SYAKNS---
 2. MEVMR-ILHMN-KGNGET-SYAKNS---
 3. MEVMR-VLHMN-KGNGET-SYAKNST--
 4. MEVMR-VLHMN-KGNGET-SYAKNS---
 5. MELQE-VLHMN-EGEGDT-SYAKNASDN
 6. MELQE-VLHMN-GGEGEA-SYAKNSSFN
 7. MEVVE-VLHMN-GGTGDA-SYASNS---
 8. MNVEA-VLHMK-EGVGET-SYAKNST--
 9. MKVMKKLLCMNIAGDGET-SYANNSGL-
10. -------F-MNR---GEESSYAQNSQFT
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Figure 4. Alignment of putative signal peptides from nine different methyltransferases. 1. NMT *Coffea canephora*; **gil66774632**. 2. NMT *Coffea arabica*; **gil13365694**. 3. Jasmonic acid carboxyl methyltransferase *Capsicum annuum*; **gil77745528**. 4. *S*-adenosyl-L-methionine : jasmonic acid carboxyl methyltransferase *Arabidopsis thaliana*; **gil13676829**. 5. Methyl transferase *Brassica juncea*; **gil55442027**. 6. Jasmonate-*O*-methyltransferase *S*-adenosyl-L-methionine : jasmonic acid carboxyl methyltransferase floral nectary-specific protein; **gil56748931**. 7. *S*-adenosyl-L-methionine : salicylic acid carboxyl methyltransferase *Stephanotis floribunda*; **gil13235641**. 8. Putative *S*-adenosyl-L-methionine *Oryza sativa* japonica cultivar-group; **gil54291645**. 9. Benzoate carboxyl methyltransferase *Antirrhinum majus*; **gil54291645**. 10. Tea NMT *N*-terminal amino acid sequence, Kato *et al.*[16].

localized the *S*-adenosyl-L-Met : benzoic acid carboxyl methyltransferase to the cytoplasm of the epidermal cells of the snapdragon flower. The enzyme was located, as we have noticed for the caffeine NMTs in close association with the vacuole surface. Pimenta *et al.*[20] showed that though most of the barely *O*-methyltransferase was cytoplasmic, a portion was adsorbed on the vacuole.

In this study, the sub-cellular targetting of NMTs in coffee was demonstrated using GUS reporter constructs driven by NMT promoter with deletion of the first exon and by immunocytolocalization. We would expect that the different enzymes involved in caffeine production and also that of CGA would co-exist at the same site. Recently, we have reported genetic transformation of coffee using *Agrobacterium rhizogenes* harbouring a binary vector[21]. The findings of this study would be helpful in understanding the regulation of caffeine biosynthesis and also to utilize the NMT promoters to alter the expression of individual NMT genes in transgenic coffee plants in a tissue-specific manner.

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ACKNOWLEDGEMENTS. We thank the Department of Biotechnology, New Delhi, for financial assistance. V.K. and K.V.S. are grateful to CSIR, New Delhi for the award of Research Fellowships. We also thank Dr V. Prakash, Director, CFTRI, Mysore for institutional support.

Received 10 July 2006; revised accepted 2 April 2007

Population structure and genetic relationships among *Delphinium* species – A fast dwindling genus from northwestern Himalaya

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Delphinium* is a medicinally important genus, several species of which have been reported from the Himalayan region. We estimated population size and possible erosion of *Delphinium* species from Himachal Pradesh, northwestern Himalaya. The study elucidated genetic diversity in *D. denudatum* and studied phylogenetic relationships among *Delphinium* species inhabiting the area. The region was reported to inhabit seven *Delphi-

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