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Delivery of *N*-methyltransferase and 11S globulin promoters of *Coffea canephora* Pex Fr. by tissue electroporation and analysis of transformational events

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A tissue electroporation system was optimized to deliver transgenes, and the expression of reporter gene driven by coffee *N*-methyltransferase (NMT) and 11S globulin promoters in somatic embryos of *Coffea canephora* was achieved. Plant transformation vector pCAMBIA 1301 was adopted for electroporation. Transient as well as stable expression of *uid*A gene was detected after electroporation using field strengths of 500 V/cm, 900 μF capacitance and 100 μg/ml of plasmid DNA. The efficiency of tissue electroporation was dependent on the type and developmental stage of the plant material. Spermidine treatment during electroporation increased transformation frequency twofold. Histochemical staining of GUS activity confirmed the expression of *uid*A gene in somatic embryos and endosperm tissues of *C. canephora*. Electroporation with pPCGB 959 (11S globulin promoter) resulted in 32% of explants showing GUS expression in endosperm tissues. The study demonstrated the ability of these promoters to drive the expression of the reporter gene. The results may be helpful for using these promoters to alter the expression of the NMT gene family through transcriptional gene silencing by RNA-directed DNA methylation, and also for using 11S globulin promoter for silencing NMT genes in a tissue-specific manner in transgenic coffee plants.

Keywords: *Coffea canephora*, 11S globulin promoter, *N*-methyltransferase, tissue electroporation.

COFFEE is a woody perennial crop and requires 4–5 years to yield fruit. *Coffea* species contain caffeine, a purine alkaloid. Caffeine is known to accumulate in beans as well as in the leaves and embryos. The caffeine biosynthesis pathway involves the following steps, viz. xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine as the major route to caffeine. The methylation steps are catalysed by *N*-methyltransferases (NMTs) that use *S*-adenosyl-l-methionine as the methyl donor[1]. The cDNAs encoding 7-methylxanthosine synthase, theobromine synthases and caffeine synthase[2–4] have been cloned and are found to possess close similarity. We have recently cloned the promoter for one of the *NMT* genes[5] and demonstrated reporter gene expression in *Nicotiana tobacum*. Similarly, the promoter for the seed-specific 11S globulin gene has been cloned[6]. This has opened up new avenues for developing transgenic plants with down regulation of caffeine synthesis in a seed-specific manner. The 11S globulins are major seed storage proteins in coffee beans. These storage proteins are also found in low levels in somatic and zygotic embryos of coffee[7]. Tissue-specific promoters such as 11S globulin could be good candidates to silence caffeine biosynthesis in a tissue-specific manner in the endosperm and embryos. As a prelude to this, it is essential to study the function of the isolated promoters in coffee tissues, especially in somatic embryos where caffeine is synthesized and down regulation of this pathway can be analysed in the early stage of transgenic plant development.

Electroporation is a DNA delivery technique that utilizes a high-intensity electric pulse to create transient pores in the cell membrane and hence facilitates uptake of DNA. The simplicity and efficiency of DNA delivery into plant protoplasts by this technique[8,9] has encouraged its application for targetting intact single cells as well as whole plant tissues[10,11]. Tissue electroporation has been success-

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fully used in transformation of zygotic and somatic embryos[12–14]. However, there are no reports on the analysis of 11S globulin and NMT promoters in coffee.

The objective of this study was to obtain rapid transformation of somatic embryos and endosperm tissues by tissue electroporation and to obtain gene expression driven by 11S globulin and NMT promoter in *Coffea canephora*. The study also demonstrated the expression of *uid*A gene under the control of 11S globulin or NMT promoter in embryos as well as in endosperm.

Authentic seeds of *C. canephora*, viz. S-274 were obtained from Central Coffee Research Institute, Chikmagalur District, Karnataka, India. The seeds were germinated *in vitro*. The callus and somatic embryos were produced according to reported methods[15]. Somatic embryos were used as explants for transformation experiments. Green, unripe fruits were collected and the endosperm segments were also used in electroporation experiments.

Electroporation was carried out according to the reported protocol of Fernandez and Menendez[14], with some modification in enzymatic degradation of cell wall. Electroporation was carried out on different tissues of *C. canephora* cultured *in vitro*. Pretreatment of somatic embryos was carried out according to Fernandez and Menendez[14]. Somatic embryo samples were incubated for 6 h in enzymatic solution containing 2% cellulase (Onozuka R-10 Yakult Pharmaceutical Industry Co, Ltd, Japan) and 1% pectinase (Sigma, USA) in 5 mM MES buffer, 0.5 M mannitol and 25 mM $CaCl_2$ at pH 5.8. Subsequently the samples were washed thoroughly with electroporation buffer containing 70 mM aspartic acid, 5 mM calcium gluconate, 5 mM MES and 0.5 mM mannitol (pH 5.6). The endosperm tissues were not subjected to cell-wall degradation treatment. Electroporation was performed with 100 µg/ml plasmid DNA in 350 µl electroporation buffer. The tissues were incubated 1 h at 4°C prior to electroporation using an electroporator (BioRad Gene Pulser Xcell system). Electroporation was carried out[14] with single electric pulse of 250, 500 or 750 V/cm, discharged from a 900 µF capacitor. Electroporation of endosperm without plasmid DNA served as a negative control. The embryos were incubated for an additional hour at 4°C after discharge. All the vectors were maintained in *Escherichia coli* DH5α and plasmid DNA isolation was carried out according to an earlier reported method[16]. The vector pCAMBIA 1301 was linearized by digestion with *Hin*dIII (MBI Fermentas, Lithuania). Influence of spermidine (Sigma, USA) was assessed by adding it in the electroporation buffer at a concentration of 0.2 mM.

The plasmid vectors pCAMBIA 1301 and pCAMBIA 1381 were procured from the Center for the Application of Molecular Biology to the International Agriculture of Canberra, Australia (CAMBIA). 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 (*uid*A) gene with a catalase intron under the control of CaMV 35S promoter and NOS terminator. pCAMBIA 1381 contains a promoterless *uid*A coding region construct and used as a negative control (Figure 1). NMT promoter (gi:59710567) 745 bp was used[5] in making promoter::GUS construct pPCTS745 in binary vector pCAMBIA 1381. Similarly, 959 bp 11S globulin promoter from coffee was isolated (gi:76365132) and used in making a promoter::GUS construct pPCGB959 in binary vector pCAMBIA 1381 and introduced into *C. canephora* somatic embryos and endosperm by tissue electroporation. Electroporation of tissues without plasmid DNA and with pCAMBIA 1301 was used as control. A promoterless *uid*A construct, pCAMBIA 1381 was also used as control.

GUS assay was performed after 24 h of electroporation. The tissue was immersed for 12 h at 37°C in a GUS assay 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

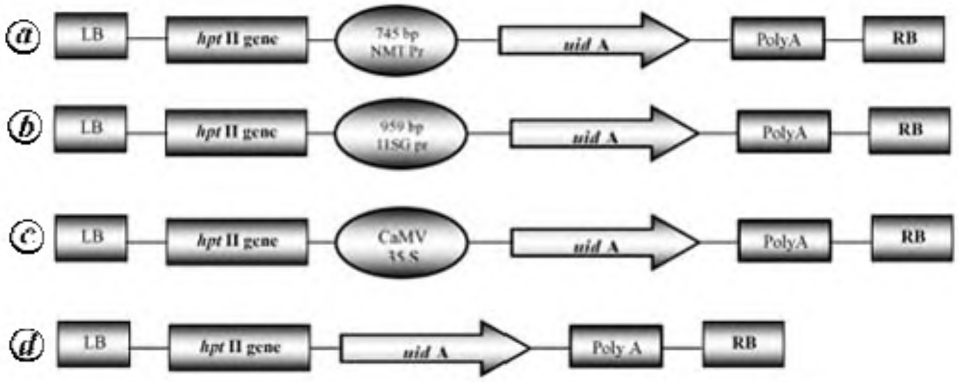

Figure 1. T-DNA region of constructs. (*a*) pPCTS745, (*b*) pPCGB959, (*c*) pCAMBIA 1301, and (*d*) pCAMBIA 1381.

Table 1. Influence of field strength and type of plasmid vector in electroporation-mediated gene delivery to *Coffea canephora* somatic embryos

Plasmid	Field strength[a] (V/cm)	Per cent explants with GUS expression	
		Without spermidine	With spermidine
pCAMBIA 1301 circular	250	0	0
	500	2.6	4.8
	750	1.2	3.2
pCAMBIA 1301 linear	250	0	0
	500	2.5	4.5
	750	0.4	1.3

[a]Electroporation was carried out at constant capacitance of 900 μF.
GUS expression was not observed in promoterless *uid*A constructs pCAMBIA 1381 as well as in coffee tissues electroporated without plasmid DNA.

Table 2. Transient expression of *uid*A reporter gene driven by NMT and 11S globulin promoter in different electroporated tissues of *C. canephora*

Construct	Nature of tissue used for electroporation	Per cent explants with GUS expression
pCAMBIA 1301	Globular embryos	3.1
	Torpedo embryos	3.5
	Endosperm	0.5
pPCTS 745	Globular embryos	2.8
	Torpedo embryos	3.5
	Endosperm	12
pPCGB 959	Globular embryos	1.5
	Torpedo embryos	5.6
	Endosperm	32

No GUS expression was observed in untransformed tissues and promoterless *uid*A containing vector pCAMBIA 1381.

cyclo-hexamonium salt, Sigma, USA)[17]. Methanol was added to the reaction mixture to suppress endogenous GUS-like activity[18].

A total of 15 explants in ten replicates were subjected to electroporation for each construct. The efficiency of electroporation-mediated transformation was determined by the percentage of somatic embryos/endosperm tissues expressing GUS.

The efficiency of DNA uptake of linear or circular plasmids upon electroporation at 250–750 V/cm field strengths was estimated after 48 h of electroporation, by analysing transient expression of the *uid*A gene in the cells. There was no visible GUS activity after a pulse of 250 V/cm. GUS activity was observed in somatic embryos electroporated at 500 V/cm (Table 1). Positive GUS staining was not detected in somatic embryos electroporated in the absence of any plasmid DNA (data not shown), and also in somatic embryos electroporated with pCAMBIA 1381 vector which harbours promoterless *uid*A gene (Figure 1 *a*). GUS assays demonstrated that cell-wall degradation using enzyme treatment was absolutely required for delivery of plasmid vectors and expression of transgenes in somatic embryos. There was no significant difference in transformation frequency in somatic embryos electroporated with linear or circular plasmid vectors (Table 1). The transformation frequency varied between 0.4 and 4.5% (Table 1) in somatic embryos. Spermidine (0.2 mm) enhanced transformation frequency twofold (Table 1). Transformation efficiency was high in torpedo-stage embryos (Table 2). Among the tested tissue types, electroporation-mediated gene delivery was effective in endosperm tissues with 12–32% transformation frequency, and an intense GUS staining.

No GUS expression was noticed in control embryos transformed with promoterless *uid*A construct (Figure 2 *a*). GUS expression was observed in somatic embryos transformed with *uid*A gene driven by coffee 11S globulin (Figure 2 *b*) and CaMV 35S promoter (Figure 2 *c*). GUS activity was observed in immature endosperm tissues electroporated with *uid*A gene driven by CaMV 35S promoter (Figure 3 *a*). Spermidine influenced transformation efficiency as is evident from the more intense GUS activity (Figure 3 *b*) observed in tissues when compared to those electroporated in the absence of spermidine (Figure 3 *a*). Our results clearly demonstrated the ability of 11S globulin (Figure 3 *c*) and NMT (Figure 3 *d*) promoters to drive transgenes into the endosperm tissues of *C. canephora*.

We attempted to regenerate the tissues electroporated with NMT promoter. The somatic embryos were cultured on secondary embryogenic medium[15] containing 5–20 mg/l^{-1} hygromycin. Transgenic secondary embryos were produced after a period of 4 months of culturing on medium containing 20 mg/l^{-1} hygromycin. The stable expression of *uid*A reporter gene was observed in transgenic secondary embryos (Figure 2 *d*). The overall efficiency of regeneration of transgenic secondary embryos was found to be 0.3%. The results clearly demonstrated that these promoters can be used for stable transformation of coffee with desired gene constructs. Whole-tissue electroporation as a means of gene delivery has an advantage over biolistics method. First, this offers an efficient means of delivering DNA to plant organs that may be sensitive to damage caused by biolistics, including heavy-metal toxicity due to

gold or tungsten. Also, this system is less expensive. Electroporation has been used successfully in various plant systems, including sweet potato[19], maize[11], cassava[20], rice[21,22] and barley[23]. D'Halluin *et al.*[11] and Arencibia *et al.*[22] reported the use of spermidine in the electroporation buffer to enhance the transformation efficiency. Our results also indicate that spermidine helps in increasing the transformation efficiency as is evident from intense GUS staining. Songstad *et al.*[24] showed transient expression of the *GUS* gene after electroporation in a buffer containing spermidine. It has been shown that spermidine can induce condensation and clustering of DNA molecules[25]. This may facilitate uptake of more DNA by the cells, and as a result, more intensive transgene expression. It is well known that caffeine and 11S globulin storage proteins are produced abundantly in endosperm tissues. However, the ability of 11S globulin and NMT promoters to drive transgene expression in coffee has not been demonstrated. In this communication we report the expression of the isolated promoters to drive the reporter gene (*uid*A) in somatic embryos. A high level of transgene expression in endosperm tissues electroporated with NMT construct (pCTS 745) and 11S globulin (pPCB 959) promoter constructs may be attributed to the greater ability of NMT and 11S globulin promoters to drive transgenes in the endosperm tissues.

Cloning of the promoter for the gene involved in the caffeine biosynthetic pathway has opened the possibility of studying the molecular mechanisms that regulate the production of caffeine in different tissues of *Coffea* sp. The NMT promoter sequence could be used for specific down-regulation of individual members of the NMT gene family through transcriptional gene silencing by RNA-directed DNA methylation[26], to develop caffeine-free coffee plants.

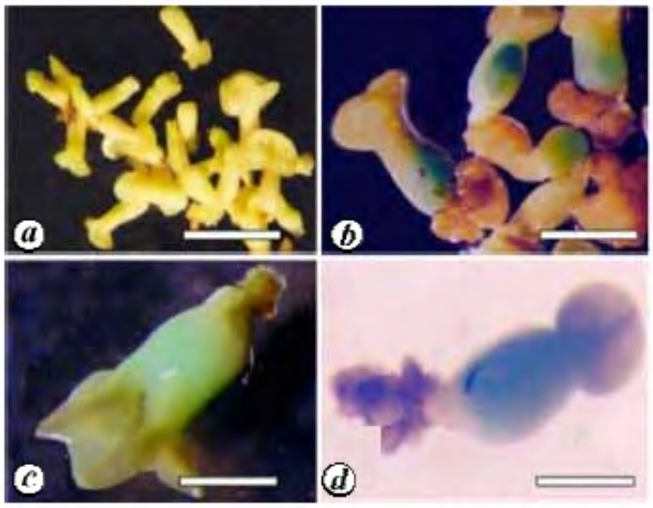

Figure 2. ***a***, *Coffea* transformed with promoterless construct pCAMBIA 1381 (bar = 3 mm). ***b***, ***c***, *Coffea* electroporated with *GUS* gene driven by (***b***) 11S globulin promoter (bar = 1.5 mm) and (***c***) CaMV promoter (bar = 0.8 mm). ***d***, Stable expression of *GUS* gene in regenerated transgenic secondary embryos of *Coffea* electroporated with *GUS* gene driven by NMT promoter (bar = 0.8 mm).

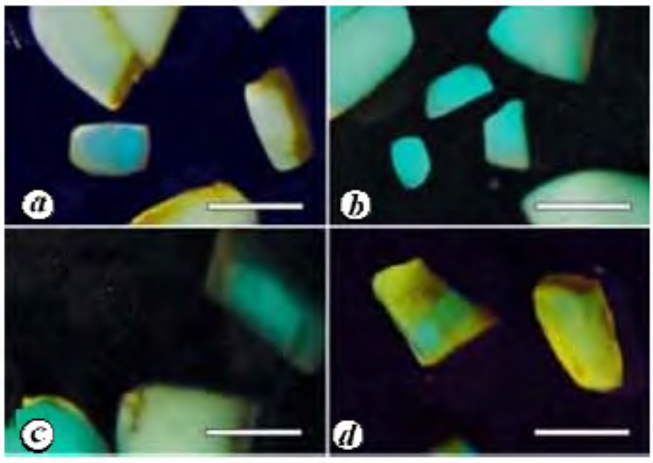

Figure 3. GUS staining in electroporated endosperm with control and different promoter constructs. *Coffea* endosperm tissues electroporated with *GUS* gene driven by (***a***) CaMV promoter without spermidine treatment (bar = 2 mm), (***b***) CaMV promoter with spermidine treatment (bar = 2 mm), (***c***) NMT promoter (bar = 2 mm) and (***d***) 11S globulin promoter (bar = 2 mm).

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Distribution and threat status of the cytotypes of *Pteris vittata* L. (Pteridaceae) species complex in India

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In India, *Pteris vittata* L. is very common and widely distributed. This fern is reportedly a 'species complex' and includes five cytotypes, viz. diploid, triploid, tetraploid, pentaploid and hexaploid with the basic number being 29 chromosomes. A survey of the literature indicated that some of the cytotypes were reported and collected only once or twice and never thereafter. In order to determine the current availability of these cytotypes, several collection excursions were undertaken, and a number of plants were collected from all over India and maintained in the fernery at National Botanical Research Institute (NBRI), Lucknow, India. In the absence of clear morphological characters specific to any cytotype, only the chromosome analysis of these plants confirmed their ploidy status. It was observed that only the tetraploid form was collected from different places. The hexaploid first reported in South India was not traceable during subsequent collection trips to this region. Similarly, in none of our collection trips did we come across any diploid form. In case of the triploid and pentaploid cytotypes, the only specimen available are those maintained in the NBRI fernery ever since their first report. The cytological abnormalities and reproductive failures along with other factors are probably responsible for the extinction of the cytotypes in their natural habitats. Thus in India, only the tetraploid cytotype is abundant while diploid and hexaploid are probably extinct. The triploid and pentaploid cytotypes, represented only by the limited specimens maintained at NBRI fernery, must also be considered as extinct in natural habitats.

Keywords: Brake fern, cytotypes, polyploidy, *Pteris vittata*, species complex.

THE common fern *Pteris* L., an almost exclusively tropical genus with 330 species and 3 hybrids, grows in both xeric and moist shady habitats as terrestrial perennials[1]. In India, about 45 species of *Pteris* have been reported, however, no comprehensive survey and analysis of diversity and biosystematics of the genus have been reported[2]. The Brake fern or *Pteris vittata* L. is a very common and widely distributed species occurring at higher altitudes up to 1600 m. The fern is also highly valued as an ornamental. It has recently been reported that *P. vittata* can accumu-

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