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Cloning of two chickpea cDNAs encoding calcium-dependent protein kinase isoforms

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In plants, calcium-dependent protein kinases (CPKs) constitute a unique family of enzymes consisting of a protein kinase catalytic domain fused to carboxy-terminal autoregulatory and calmodulin-like domains. We isolated two cDNAs encoding calcium-dependent protein kinases (CaCPK1 and CaCPK2) from chickpea. cDNAs encoding the two isoforms share 55.2% identity at the nucleotide level and 51.1% identity at the amino acid level. CaCPK1 and CaCPK2 predicted protein sequences are 556 and 540 amino acids in length with corresponding molecular weight of 62 and 61 kDa respectively. The CaCPK1 possesses four EF-hands whereas CaCPK2 has three EF-hands. The predicted amino acid sequence of CaCPK1 is highly homologous to *Dacus carota* CPK (84%) and CaCPK2 to *Arabidopsis thaliana* CPK7 (80%). Southern analysis showed that CaCPK1 and CaCPK2 proteins are both encoded by single copy of genes in the chickpea genome.

Keywords: Calcium-dependent protein kinase isoforms, cDNA cloning, chickpea (*Cicer arietinum*), EF-hands, Southern blot analysis.

CALCIUM acts as a second messenger in the signal transduction of a variety of environmental stimuli[1]. Molecular decoders of calcium signals are the calcium-binding proteins, which include protein kinases regulated by calcium. Four major classes of Ca^{2+}-regulated protein kinases have been characterized in plants are: calcium-dependent protein kinases (CPKs), CPK-related kinases (CRKs), calmodulin-dependent protein kinases (CaMKs), and calcium and calmodulin-dependent protein kinases (CCaMKs). Of these four classes, the predominant forms of Ca^{2+}-regulated protein kinases in plants are CPKs, which are identified as Ser/Thr protein kinase family. They have been implicated as key elements in signalling processes. CPKs have acquired CaM independence due to the presence of internal high affinity Ca^{2+}-binding sites[2]. They have a variable N-terminal domain, a catalytic domain, an autoinhibitory region and a calmodulin-like domain[3–7]. From an evolutionary standpoint, it has been suggested that genes encoding CPKs have evolved using the fusion of a gene encoding the catalytic/autoinhibitory domain of Ca^{2+}/CaM-dependent protein kinase and a gene encoding a CaM-like protein[8,9].

Many CPKs have been cloned and characterized from a wide variety of plant species including soybean[3], *Arabi-*

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dopsis[7], maize[10,11], rice[12,13], mungbean[14], potato[15], strawberry[16], cucumber[17], zucchini[18] and tobacco[19,20]. There has been considerable progress in plant CPK studies from identifying the new isoforms and cloning corresponding genes to clarify their specific roles in signal transduction cascades involving plant growth and development, and various stress responses. In *Arabidopsis*[6,7] and in rice[21], it has been found that there are 34 and 29 CPK isoforms respectively. Different CPK isoforms in a given plant species may be distinct in their expression patterns and physiological functions.

In this communication, we report the isolation and characterization of two cDNAs from chickpea that encode two CPKs (CaCPK1 and CaCPK2). These two isoforms are 556 and 540 amino acid residues in length respectively and 55% identical. The most notable differences in the primary structures of these two isoforms are the CaCPK1 has a 79 amino acid variable domain and CaCPK2 has a 63 amino acid variable domain. CaCPK1 contains four EF-hands whereas CaCPK2 has three EF-hands.

A cDNA library of chickpea (*Cicer arietinum* L. cv. *Kabuli*) stem constructed in a Uni-ZAP XR vector was a gift from Tom W. Okita (Washington State University, USA). For library screening, a rice CPK cDNA, OsCPK2[13] was randomly labelled with [α-^{32}P] dCTP using random primer labelling kit (Fermentas, Germany) and used as a probe to screen membrane lifts of 2×10^5 plaques grown in *E. coli* XL-1 Blue MRF′ cells. Hybridization and washing were performed as described previously[17]. Of the five plaques affording positive signals, two of them were purified through two additional cycles of hybridization. The purified λZAP II clones were *in vivo* excised as pBluescript SK (–) phagemids and transformed into *E. coli* SOLR cells (Stratagene). The plasmids were sequenced and the two cDNAs were compared at both nucleic acid and amino acid levels by using PILEUP and GAP programs[22] and found to be partial, lacking 5′ ends. To obtain 5′ terminal sequence of the two truncated clones, 5′ RACE was performed by using the FirstChoice™ RLM-RACE kit (Ambion, USA) according to the manufacturer's instructions with sequence-specific reverse primers. The 5′ RACE products were cloned into pTZ57R T/A cloning vector (Fermentas) and sequenced.

DNA sequencing was carried out by the dideoxy chain termination method[23] using fluorescent nucleotides in an automated DNA sequencer, PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencer. For comparison and analysis of the sequence data the following programs were used: BLAST[24], FASTA, GAP, MAP, SEQED and TRANSLATE of Genetics Computer Group (GCG), Wisconsin, version 7.0[22]. Multiple sequence alignment was performed using the ClustalW, European Bioinformatics Institute, at the ExPasy site (http://www.expasy.ch). Phylogenetic tree was created according to the neighbour-joining method clustering strategy[25].

Genomic DNA was isolated from chickpea seedling leaves[26]. Ten microgram portions was digested with restriction enzymes *Eco*RI, *Hin*dIII, *Kpn*I, *Bam*HI and *Xba*I, electrophoresed through 0.8% (w/v) agarose gels and blotted under denaturing conditions on to Hybond N^+ nylon membranes (Amersham). We used PCR amplified cDNA probes labelled with [α-^{32}P]-dCTP using a random primer labelling kit (Fermentas) as follows: CaCPK1 cDNA nucleotides 1–222 (5′-specific sequence corresponding to N-terminal variable domain) and CaCPK2 cDNA nucleotides 1–198 (5′-specific sequence corresponding to N-terminal variable domain). Hybridization was performed in a solution of 6 × SSC, 5 × Denhart's reagent, 0.5% (w/v) SDS and 100 µg/ml denatured salmon sperm DNA. After overnight incubation at 56°C, the filters were washed twice with 2 × SSC containing 0.5% (w/v) SDS for 5 and 15 min respectively at room temperature, once with 0.1 × SSC containing 0.5% (w/v) SDS at 37°C for 30 min, once with 0.1 × SSC containing 0.5% (w/v) SDS at 68°C for 30 min and followed by a final wash with 0.1 × SSC at room temperature for 5 min. The filters were then dried and wrapped in a thin plastic bag before exposure to autoradiographic film.

Sequence data have been deposited at the EMBL/GenBank under accession numbers AY312268 for CaCPK1 and AY312269 for CaCPK2.

A chickpea cDNA library constructed in Uni-ZAP XR vector[27] was screened by plaque hybridization with rice CPK cDNA (OsCPK2) as the probe. Two of the positive plaques were purified and *in vivo* excised as pBluescript phagemids and their inserts were completely sequenced. The cDNA insert of the first clone was found to be 1559 bp in length and consisted of a 1059 bp open reading frame without an initiating codon and terminating in an amber codon TGA. The cDNA insert of the second clone was found to be 1463 bp in length and consisted of a 1089 bp open reading frame without an initiating codon and terminating in a stop codon TAG. Since the reading frames of both clones did not begin with a methionine start codon and there was no 5′-untranslated region, these clones were considered to be partial ones. Additional sequences of 5′ ends of the cDNAs were obtained by 5′ RACE. Thus, the combined sequences of the partial clones and the 5′ RACE products were found to be 2228 and 2144 bp respectively for the two clones, and we designated the clones as CaCPK1 and CaCPK2. Full-length cDNA of CaCPK1 consists of 60 bp of 5′-untranslated region, 1668 bp of coding region, 481 bp of 3′-untranslated region and 19 bp of poly (A) tail, encoding a polypeptide of 556 amino acids with a calculated molecular weight of 62 kDa and an isoelectric point of 5.92. Full-length cDNA of CaCPK2 consists of 150 bp of 5′-untranslated region, 1620 bp of coding region, 353 bp of 3′-untranslated region and 21 bp of poly (A) tail, encoding a polypeptide of 540 amino acids with a calculated molecular weight of 61 kDa and an isoelectric point of 6.2. Two cDNA clones

are 55.2% identical at the nucleotide level. The 5′- and 3′-untranslated regions are highly diverse and show only 28.5 and 43.2% identities respectively.

The deduced chickpea CaCPK1 and CaCPK2 proteins contain all the characteristics of a calcium-dependent protein kinase (Figure 1). The N-terminal variable domain consists of 79 amino acids in CaCPK1 and 63 amino acids in CaCPK2. The kinase catalytic domain is 276 residues in CaCPK1 and 262 residues in CaCPK2. The C-terminus of the predicted polypeptide of CaCPK1 contains a calmodulin-like domain consisting of 170 amino acids with four highly conserved Ca^{2+}-binding EF-hands, while CaCPK2 contains 184 amino-acid spanning calmodulin-like domain with three highly conserved Ca^{2+}-binding EF-hands. An autoinhibitory junction domain of 31 amino acids in length joined the kinase domain and the calmodulin-like domain of both isoforms of CaCPKs. Comparison of the CaCPK1 and CaCPK2 sequences to other known CPK proteins in the databases showed that CaCPK1 is 84% homology to DcCPK from carrot (Figure 2 *a*)[28], 83% homology to NtCPK1 from tobacco[19], 80% homology to OsCPK2 from rice[13] and 53% homology to AtCPK7 from *Arabidopsis*[9], while CaCPK2 has 80% homology to AtCPK7 from *Arabidopsis*[8], 66% homology to NtCPK1 from tobacco[19], 55% homology to OsCPK2 from rice[13] and 50% homology to DcCPK from carrot[28].

In order to determine the functional relationship among CPK genes, a phylogenetic tree was created according to the neighbour-joining method clustering strategy[25], using ClustalW program with the deduced amino-acid sequences from cloned CPKs. This analysis included sequences from monocots and dicots (maize, rice, zucchini, tobacco, *Arabidopsis*, soybean, mungbean, strawberry, sweet potato and carrot), a moss (*Tortula ruralis*) and an alga (*Chlamydomonas eugametos*). Analysis of the phylogenetic tree revealed that this particular family of plant kinases could be divided into various subgroups. The various maize, rice and *Arabidopsis* CPK isoforms were found through the phylogram, with CaCPK1 being more closely related to DcCPK and NtCPK1 with CaCPK2 coming in a separate subgroup with more close relative-

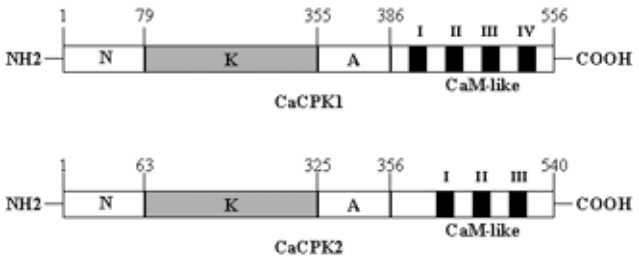

Figure 1. Schematic diagram showing the four predicted structural domains in CaCPK1 and CaCPK2. Numbers denote the positions of amino-acid residues. N, Amino-terminal variable domain; K, Kinase domain; A, Autoinhibitory domain; CaM, Calmodulin. The bars within the CaM-like domain represent the EF-hand Ca^{2+}-binding sites.

ness to AtCPK7 (Figure 2 *b*). The distribution of different members of the CPK family from a given species among the different groups suggests that the group might have different function(s), and CPK within a group might have similar function(s). Since CaCPK1 and CaCPK2 come in different groups, it is likely that these isoforms could perform different functions in chickpea.

In order to examine the copy number of *CaCPK1* and *CaCPK2* genes in the chickpea genome, Southern blot analysis was performed using isoform-specific probes. For Southern blot analysis of CaCPK1, chickpea genomic DNA was digested with *Eco*RI, *Hin*dIII, *Kpn*I and *Xba*I that do not cut the variable domain of CaCPK1 cDNA, and *Bam*HI that cut the variable domain of CaCPK1 cDNA once. One band was detected in the DNA digested with *Eco*RI, *Hin*dIII, *Kpn*I and *Xba*I and two bands were detected in the DNA digested with *BamH*I (Figure 3 *a*), suggesting that a single copy *CaCPK1* gene is present in the chickpea genome. For Southern blot analysis of CaCPK2, the genomic DNA was digested with *Eco*RI, *Hin*dIII, *Kpn*I and *Bam*HI that do not cut the variable domain of CaCPK1 cDNA. One band each was detected in the DNA digested with *Eco*RI, *Hin*dIII, *Kpn*I and two bands were detected in the DNA digested with *Bam*HI even though there was no restriction site in the variable domain of CaCPK2 cDNA probe used for hybridization (Figure 3 *b*). This is because of the presence of an intronic region having *Bam*HI restriction site in the variable domain of *CaCPK2* gene. These results suggested that only one copy of *CaCPK2* gene is present in the genome of chickpea.

Recently, it has been demonstrated that the CPK cascades play important roles in stress responses in plants. Expression of OsCPK7, a rice CPK was induced by cold and salinity in both shoots and roots of 10-day-old rice seedlings[29]. When the OsCPK7 was overexpressed in rice, plants showed increased tolerance to cold and salt[30]. The response of CPK gene expression to various stresses varied among individual members. For example, AtCPK10 and AtCPK11 are involved in mediating drought and salt stress signalling; while AtCPK30 (AtCPK1a) is involved in cold, salt and abscisic acid (ABA)-induced pathways[31]. *Nicotiana tabacum* CPKs – *NtCPK2* and *NtCPK3* – two CPK genes from tobacco, are involved in mediating defense and osmotic stress signalling pathways whereas *NtCPK1* mediates an array of signals including GA_3, ABA, cytokinin, wounding, fungal elicitors and salt stress[32,33]. We have recently reported that CaCPK1 was expressed in all tissues examined and is induced in response to salt, fungal spore and BA treatments; while CaCPK2 was almost undetectable in flowers and fruits and is induced by dehydration stress, BA and GA_3 treatments, suggesting a function for these CaCPK1 and CaCPK2 isoforms in the corresponding signalling pathways[34]. It is worthy of mention that autophosphorylation and phosphorylation of histone III-S by the purified recombinant CaCPK1 and CaCPK2 produced[35] in *E. coli* occurred only in the pres-

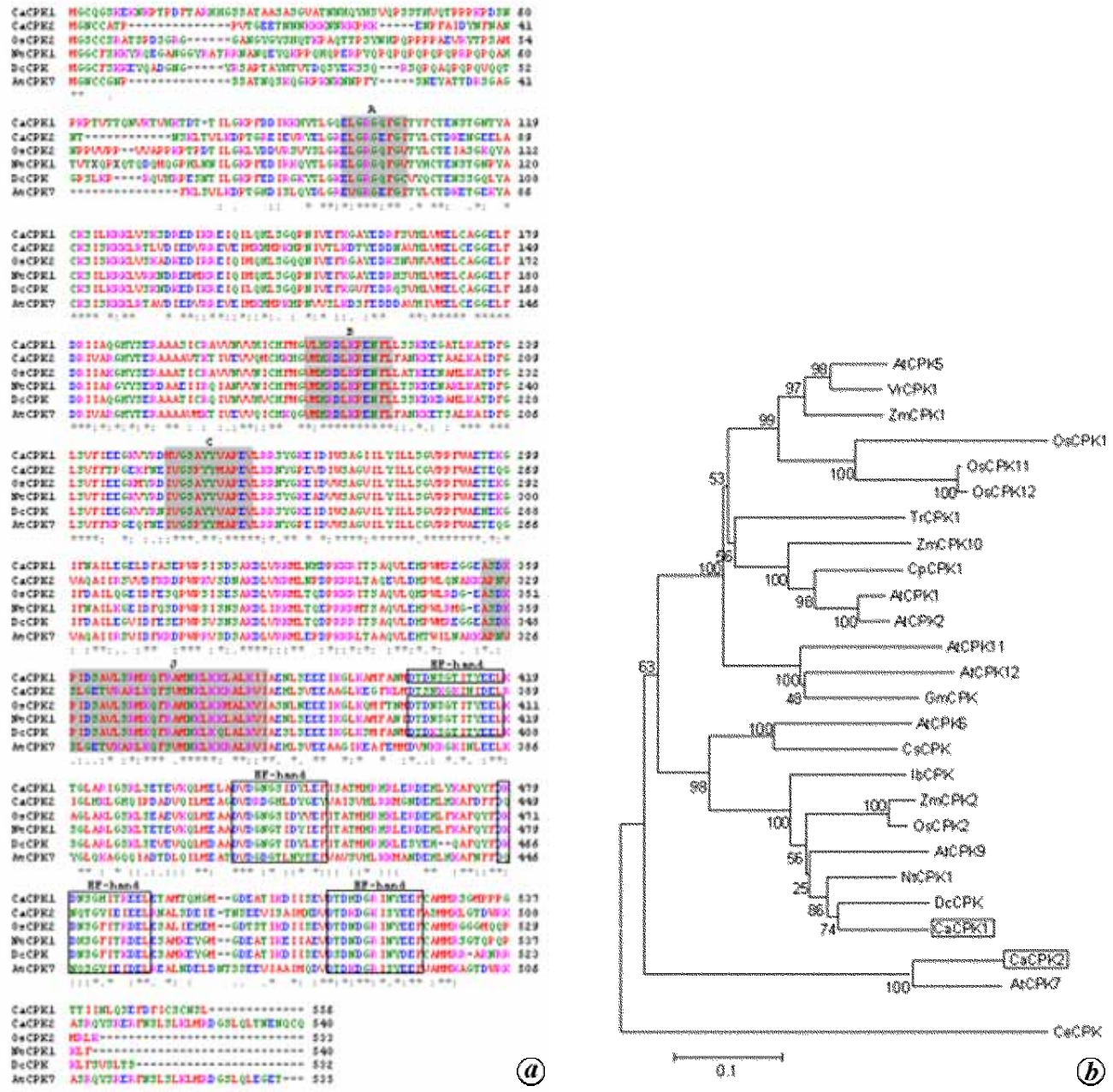

Figure 2. Sequence comparison of CaCPK1 and CaCPK2 with other plant CPKs. ***a***, Alignment of the deduced amino-acid sequences of CaCPK1 and CaCPK2 with other plant CPK proteins. Asterisks denote positions of identical amino acids. Single or double dots represent similar amino acids. Gaps, indicated by dashes (---) were introduced to maximize alignment. Diagnostic kinase sequences (A, B and C) and junction regions (J) are grayed and EF-hands are boxed. The EMBL database accession numbers of CaCPK1, CaCPK2 and other plant CPKs used for alignment are as follows: *Cicer arietinum* (CaCPK1, AY312268; CaCPK2, AY312269); *Oryza sativa* (OsCPK2, X81394); *Nicotiana tabacum* (NtCPK1, AF072908); *Dacus carota* (DcCPK, X56599) and *Arabidopsis thaliana* (AtCPK7, U31836). Sequences were aligned by ClustalW program located at ExPasy site (http://www.expasy.ch). ***b***, A rooted phylogenetic tree of CPK proteins illustrating the relationship between the cloned plant CPKs. The phylogenetic tree was constructed using neighbour-joining method[25]. Numbers indicate the per cent bootstrap replicates supporting the node. Root of the tree is arbitrarily placed. CaCPK1 and CaCPK2 are highlighted by box. Accession numbers of CPK sequences used to generate the phylogenetic tree are as follows: *A. thaliana* (AtCPK1, L14771; AtCPK2, U31833; AtCPK5, U31834; AtCPK6, U31835; AtCPK7, U31836; AtCPK9, U31751; AtCPK11, D21806; AtCPK12, U20388); *Zea mays* (ZmCPK1, D84408; ZmCPK2, U28376; ZmCPK10, ZMA7366); *O. sativa* (OsCPK1, AF319481; OsCPK2, X81394; OsCPK11, X81393; OsCPK12, AF048691); *Glycine max* (GmCPK, M64987); *Cucurbita pepo* (CpCPK1, U90262); *Cucumis sativus* (CsCPK, AY027885); *Vigna radiata* (VrCPK1, U08140); *D. carota* (DcCPK, X56599); *Ipomea batatus* (IbCPK, D87707); *N. tabacum* (NtCPK1, AF072908); *Tortula ruralis* (TrCPK1, U82087); *Chlamydomonas eugametos* (CeCPK1, Z49233), and this work, *C. arietinum* (CaCPK1, AY312268; CaCPK2, AY312269).

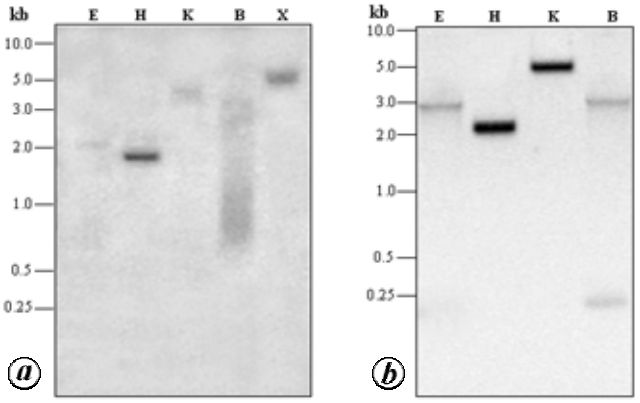

Figure 3. Genomic southern blot analyses of *CaCPK1* (***a***) and *CaCPK2* (***b***) genes. Aliquots (15 µg) of chickpea genomic DNA were digested with *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Bam*HI (B) and *Xba*I (X), electrophoresed on 0.8% (w/v) agarose gels, transferred onto nylon membranes and hybridized with the cDNA probes corresponding to the N-terminal variable domain of CaCPK1 (residues 1–222) or CaCPK2 (residues 1–198). The position of DNA standards is indicated on the left and their size is given in kb.

ence of Ca^{2+}. It has also been demonstrated that they differ in their kinetic and Ca^{2+} binding properties[35]. The major challenge of the future will be to overexpress the CaCPK1 and CaCPK2 in chickpea lines to confer salt and drought tolerance, and/or resistance to fungal pathogens in transgenic chickpea plants.

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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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