Alternative splicing of a novel diacylglycerol kinase in tomato leads to a calmodulin-binding isoform

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Summary

Calmodulin is a regulatory protein activated during Ca\(^{2+}\) signalling. We have isolated a cDNA, designated LeCBDGK (*Lycopersicon esculentum* calmodulin-binding diacylglycerol kinase) encoding a novel calmodulin-binding protein with sequence similarity to diacylglycerol kinases from animals. Diacylglycerol kinases convert diacylglycerol to phosphatidic acid. We delineated the calmodulin-binding domain to approximately 25 residues near the C-terminus of LeCBDGK. We have also isolated a second diacylglycerol kinase cDNA, designated LeDGK1, identical to LeCBDGK, except that it lacks the calmodulin-binding domain. Both recombinant LeCBDGK and LeDGK1 were catalytically active *in vitro*. Anti-DGK antiserum detected two immunoreactive proteins associated with microsomal and plasma membrane fractions from cell suspensions. The higher molecular weight immunoreactive protein was also present in soluble extracts and bound to calmodulin–agarose in the presence of calcium, demonstrating that native LeCBDGK is a calmodulin-binding protein. In the presence of calcium, LeCBDGK associated with membrane cell fractions *in vitro*, but calmodulin antagonists disrupted this association, suggesting a possible role of calcium in the recruitment of LeCBDGK from soluble to membrane cell fractions. Native LeCBDGK and calmodulin co-immunoprecipitated from tomato soluble cell extracts, suggesting their interaction *in vivo*. The same gene encodes both LeCBDGK and LeDGK1 and the calmodulin-binding domain of LeCBDGK is encoded by a separate exon. Thus, alternative transcript splicing leads to calmodulin-binding and non-binding forms of diacylglycerol kinases in tomato. Possible roles of LeCBDGK and LeDGK1 in calcium and lipid signalling are discussed.

**Keywords:** calmodulin, signal transduction, diacylglycerol, tomato, splicing.

Introduction

Ca\(^{2+}\) functions as an intracellular secondary messenger whereby, in response to various stimuli, Ca\(^{2+}\) fluxes trigger a cascade of events that culminates in a physiological response (Berridge *et al.*, 1999; Trewavas and Malho, 1998). One mechanism for the translation of Ca\(^{2+}\) fluxes into cellular responses is by the activation of Ca\(^{2+}\)-binding regulatory proteins such as calmodulin (CaM). In animal cells, proteins under CaM control include kinases, a phosphatase, ion channels, transcription factors and others (Rhoads and Friedberg, 1997). In plants, comparatively few CaM-binding proteins have been characterized. However, it is noteworthy that plants possess unique CaM-regulated targets such as kinesin (*Reddy et al.*, 1996), glutamate decarboxylase (*Snedden et al.*, 1996) and a chimeric kinase (*Patil et al.*, 1995). CaM has been suggested to play a signalling role in plants in response to pathogen attack (*Bergey and Ryan*, 1999; Blumwald *et al.*, 1998; *Harding et al.*, 1997; *Heo et al.*, 1999), light (*Bowler et al.*, 1994; *Neuhaus et al.*, 1993), cold shock (*Polisensky and Braam*, 1996; *van der Luit et al.*, 1999) and a number of other stimuli (*Braam and Davis*, 1990; *Lu et al.*, 1995). Given the mounting evidence for the involvement of CaM in cellular signalling in plants, the list of downstream effectors, although expanding, is probably incomplete. In addition, plants possess a unique repertoire of CaM isoforms and CaM-like proteins (*Snedden and Fromm*, 1998; *Zielinski*, 1998), suggesting an important role for CaMs during signal transduction in plants. Thus, in order to elucidate the pathways mediated by Ca\(^{2+}\) and CaM in plants, it is necessary to identify and characterize the specific protein targets of CaM.
This report describes the isolation of a novel CaM-binding protein from tomato cells and its identification as a diacylglycerol (DAG) kinase (DGK). We demonstrate that two splice variants of this enzyme are present in tomato, and that one form possesses a CaM-binding domain. DGKs catalyse the phosphorylation of DAG to yield phosphatidic acid (PA). PA has been shown to accumulate rapidly in plant cells in response to stimuli (Munnik et al., 1998b), and recent reports suggest that PA may function as a signalling molecule in both plant and animal cells (Munnik et al., 1998a; Topham and Prescott, 1999). The possibility that the DGKs described in the present work may be involved in stimulus–response signal transduction is discussed.

Results

Isolation of LeCBDGK and LeDGK1 cDNAs

Among the clones isolated by screening a cDNA library with 35S-CaM was one predicted to encode a protein with sequence similarity to mammalian DGKs. We designated this clone LeCBDGK (Lycopersicon esculentum CaM-binding diacylglycerol kinase). We used the partial LeCBDGK clone to re-screen for a full-length cDNA, and, in doing so, also isolated a second putative DGK, designated LeDGK1, identical in sequence to LeCBDGK except for a region at the C-terminus. Figure 1 presents the cDNA and predicted amino acid sequences for both DGKs. The 5’ region upstream of the predicted start codon (for both DGK cDNAs) contains stop codons in all three reading frames, suggesting that the sequences represent complete open reading frames. LeCBDGK and LeDGK1 encode proteins of 511 and 489 residues, respectively (predicted molecular masses approximately 57.4 and 54.5 kDa), with the difference being due to a C-terminal extension in LeCBDGK. LeCBDGK and LeDGK1 showed sequence similarity to the catalytic domains of mammalian DGKs and AtDGK1, the only other plant DGK described to date (Katagiri et al., 1996) (Figure 2a). Eukaryotic DGKs comprise a diverse family that share a conserved catalytic region and are divided into five subtypes (Figure 2b) based upon their structural motifs (Topham and Prescott, 1999). Other than an ATP-binding site (Figure 1) and a catalytic domain (Figure 2b), LeCBDGK and LeDGK1 lack the other structural motifs previously identified in DGKs (Figure 2c).

The CaM-binding domain of LeCBDGK is near the C-terminus

CaM-binding domains are difficult to predict based upon sequence information and need to be determined empirically (Rhoads and Friedberg, 1997). GST fusion proteins, comprising different regions of LeCBDGK (or LeDGK1) (Figure 3a), were expressed in E. coli (Figure 3b), and total bacterial extracts were assessed for their ability to bind recombinant 35S-CaM. Although proteolysis products of the fusion proteins were observed in the total bacterial extracts, a clear pattern of CaM binding was observed. Full-length LeCBDGK or GST fusion proteins that included

![Figure 1. Sequence of LeDGK1 and LeCBDGK cDNAs isolated from tomato cells.](image-url)
at least the 25 C-terminal residues of LeCBDGK were able to bind $^{35}$S-CaM (Figure 3c). In addition, the binding of LeCBDGK to $^{35}$S-CaM was Ca$^{2+}$-dependent, providing further evidence that LeCBDGK is a bona fide CaM-binding protein. In contrast, total bacterial extracts strongly enriched in LeDGK1 GST fusion proteins did not bind $^{35}$S-CaM (Figure 3c).

**Recombinant LeCBDGK and LeDGK1 are catalytically active**

In the presence of DAG and $^{32}$P-$\gamma$-ATP, both LeCBDGK and LeDGK1 produced a $^{32}$P-radioactive, chloroform-soluble product that migrated on TLC as PA (Figure 4). Under various *in vitro* assay conditions, no effect of CaM was observed on recombinant LeCBDGK activity (not shown). The specific activities of recombinant LeCBDGK and LeDGK1 were comparable to previous reports (Bunting *et al.*, 1996; Ding *et al.*, 1997; Sakane *et al.*, 1991; Wissing and Wagner, 1992): between 5 and 20 nmol PA min$^{-1}$ mg$^{-1}$ protein, depending on the preparation.

**LeCBDGK and LeDGK1 differ in subcellular distribution**

Antiserum against the conserved region of the two DGK isoforms detected two immunoreactive proteins (approximately 58 and 55 kDa, respectively) in microsomal- and plasma membrane-enriched fractions (Figure 5a). The protein of approximately 58 kDa was also detected in soluble cell extracts and bound to CaM-agarose in a Ca$^{2+}$-dependent manner (Figure 5b, upper panel), suggesting that this protein is probably LeCBDGK, while the immunoreactive protein of approximately 55 kDa is LeDGK1. We examined the possibility that other plant species may also possess a CaM-binding DGK isoform, and used anti-DGK antiserum to detect an immunoreactive CaM-binding protein (molecular mass 58 kDa) from tobacco (Figure 5b, lower panel).

**CaM antagonists disrupt the interaction of native LeCBDGK with membranes**

LeCBDGK was observed in both soluble and membrane subcellular fractions and yet differs in sequence from LeDGK1 in possessing a CaM-binding domain near the C-terminus. This observation, coupled with the fact that certain mammalian DGKs translocate from soluble to membrane fractions in a Ca$^{2+}$-dependent manner (Flores

**Figure 2.** Sequence comparison of LeCBDGK to other DGKs.

(a) Comparison of the predicted catalytic domains of LeCBDGK (accession number AF198258, residues 30–427), Arabidopsis AtDGK1 (accession number D83787, residues 351–684) and human DGK-$\gamma$ (accession number N48867, residues 427–772). Identical amino acid residues are shaded in black, conserved changes in grey. Dashes represent gaps introduced to maximize alignment. (b) Schematic representation of the structural organization of mammalian DGKs. For simplicity, only a single member is presented for each family type. The conserved catalytic domain is shown as an open box. Different structural motifs are shown as filled boxes: EF hands (diagonal bars), cysteine-rich domain (grey), pleckstrin-homology domain (horizontal bars), myristoylated, alanine-rich C-kinase substrate homology domain (black), and ankyrin repeats (vertical bars). (c) The two tomato DGKs described in the present study, LeCBDGK possesses a C-terminal CaM-binding domain (stippled box).
et al., 1996; Sakane et al., 1991; Topham and Prescott, 1999), prompted us to examine whether Ca\textsuperscript{2+}/CaM binding might play a role in the subcellular localization of tomato DGKs. Using tomato cell homogenates, we observed that LeDGK1 remained in association with membranes under all treatments. In the presence of Ca\textsuperscript{2+}, or Ca\textsuperscript{2+} and CaM, LeCBDGK was almost exclusively associated with the membrane fraction (Figure 6a). In contrast, when homogenates were treated with EGTA, detectable levels of LeCBDGK remained in the soluble fraction. In addition, the presence of the CaM antagonist trifluoperazine (TFP) (in the presence of added Ca\textsuperscript{2+}), resulted in a substantial increase in the amount of LeCBDGK remaining in the soluble fraction (Figure 6a). Similar results were observed using another CaM antagonist, N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W7) (data not shown). As Ca\textsuperscript{2+} alone, but not in conjunction with CaM antagonists, increased the fraction of LeCBDGK associated with membranes, we examined the possibility that Ca\textsuperscript{2+} might be utilizing endogenous CaM present in our cell preparations to influence the association of LeCBDGK with membranes. Immunoblotting using anti-CaM antiserum revealed a significant amount of endogenous CaM in our preparations (Figure 6b), and thus led us to investigate whether soluble LeCBDGK interacted with CaM in vivo. In co-immunoprecipitation experiments, anti-DGK antiserum was able to precipitate CaM from the soluble fraction of tomato cell extracts, whereas pre-immune serum did not precipitate CaM (Figure 6c).

LeCBDGK and LeDGK1 are splice variants derived from the same gene

We investigated whether the two gene products may have been derived from the same gene due to alternative splicing as has been reported for human DGK (Ding et al., 1997). Southern blotting, using a cDNA probe covering an identical region in both LeCBDGK and LeDGK1 (nucleotides 1–1449), suggested that a single gene probably encoded both products (Figure 7a). A 9.3 kb region of tomato genomic DNA was amplified by PCR, and sequencing of this fragment revealed exons for both gene products (Figure 7b), suggesting that LeCBDGK and LeDGK1 are derived from the same gene by alternative splicing. RT–PCR confirmed the presence of both transcripts in tomato cells (not shown). Interestingly, the unique exon of LeCBDGK is the terminal exon and encodes the CaM-binding domain. All predicted introns conform to the 5’GU and 3’AG splice site consensus sequences for plants (Brown and Simpson, 1998).

Figure 5. Immunodetection of LeCBDGK and LeDGK1 from plant cell extracts.
(a) Anti-DGK serum detected immunoreactive proteins in the subcellular fractions of tomato cell extracts: microsomal membranes (MM), plasma membrane (PM), soluble calmodulin (CaM)-binding proteins (CBPs). MM, PM and CBPs were isolated as described in Experimental procedures. Proteins (30 μg of MM and PM, respectively, 10 μg of CBPs) were separated by SDS-PAGE and transferred to nitrocellulose for immunodetection. Aliquots (approximately 10 ng) of purified recombinant LeCBDGK (Rec. LeCBDGK) or LeDGK1 (Rec. LeDGK1), expressed in E. coli, were included for comparison of electrophoretic migration with native DGKs. Molecular mass markers (kDa) are shown on the left. (b) Anti-DGK antiserum detects an immunoreactive CaM-binding protein in both tomato and tobacco soluble cell extracts. CaM-binding proteins were isolated from concentrated soluble extracts of tobacco cells (upper panel) or unconcentrated soluble extracts of tobacco cells (lower panel) by CaM-affinity chromatography as described in Experimental procedures. Included in the immunoblot analysis were samples of soluble extract (Total) applied to the CaM column for tomato (approximately 40 μg) and tobacco (approximately 5 μg) and aliquots of proteins which did not bind to the CaM column (effluent). CaM-binding proteins eluted from the column by EGTA were collected in fractions (eluted fractions 1–5) and immunodetected using anti-DGK antiserum. Data are from a typical experiment and were reproduced three times.

Discussion

The present study demonstrates that tomato cells possess unique DGKs, including an alternatively spliced CaM-binding form, LeCBDGK, and raises the possibility that these DGKs may play a role in the coupling of Ca2+ and phospholipid signalling. DGKs in animals comprise a diverse family, and although a variety of structural motifs have been described, a CaM-binding domain has not previously been observed. LeCBDGK and LeDGK1 are also unique among eukaryotic DGKs in that they lack a cysteine-rich region. These domains are the site of DAG binding in protein kinase C (PKC) (Liu and Heckman, 1998), but it is not clear what function they perform in DGKs. Our work supports a recent finding (Sakane et al., 1996) suggesting that cysteine-rich regions are not necessary for DAG binding or phosphorylation by DGKs.

We delineated the CaM-binding domain of LeCBDGK to a region within 29 C-terminal residues and showed that a CaM-binding native DGK is present in both tomato and tobacco, suggesting that CBDGKs may be widespread among plant species. LeBGDK and LeDGK1 were catalytically active as bacterially expressed recombinant proteins, establishing them unequivocally as members of the DGK
observed, and a number of reports have described the Ca\textsuperscript{2+}-mediated recruitment of DGKs from cytosolic to membrane compartments (Flores et al., 1996; Ishitoya et al., 1987; Sakane et al., 1991; Topham and Prescott, 1999; Topham et al., 1998). Our study is the first to describe subcellular immunolocalization of a DGK in plants, although membrane-associated DGK activity in plants has been demonstrated (Kamada and Muto, 1991; Wissing and Wagner, 1992). We observed that CaM antagonists (in the presence of Ca\textsuperscript{2+}) or EGTA were able to disrupt LeCBDGK movement to membranes in vitro, whereas Ca\textsuperscript{2+} alone facilitated LeCBDGK translocation, suggesting the involvement of the endogenous CaM present in our preparations (Figure 6). In support of this hypothesis, using co-immunoprecipitation, we demonstrated that endogenous CaM and LeCBDGK are complexed together in soluble cell extracts, and thus may also interact in vivo. Collectively, our data indicate a possible role for the involvement of Ca\textsuperscript{2+}/CaM in recruiting LeCBDGK to membranes from the soluble fraction, and/or maintaining the interaction of LeCBDGK with membranes. The specific membrane(s) to which LeCBDGK is targeted is under investigation. In contrast, the association of LeDGK1 with membranes appears to involve a Ca\textsuperscript{2+}/CaM-independent mechanism, the nature of which is at present unknown. Although we cannot exclude the participation of other CaM-binding proteins in our samples, our findings are reminiscent of reports on the Ca\textsuperscript{2+}-dependent translocation of certain animal DGK and PKC isoforms from soluble to membrane compartments (Flores et al., 1996; Liu and Heckman, 1998; Sakane et al., 1991; Topham and Prescott, 1999). Thus, the Ca\textsuperscript{2+}-induced subcellular movements of PKCs and DGKs appear to be spatially and temporally coordinated during stimulus response. It is noteworthy that, in plants, both soluble and membrane-associated CaMs are present (Collinge and Trewavas, 1989), and the subcellular translocation of a CaM isoform has been reported recently (Rodriguez-Concepcion et al., 1999). Thus, LeCBDGK may associate with different subcellular pools of CaM in vivo. Furthermore, CaM binding has been shown to affect the membrane interaction of several animal proteins, including striatin (Bartoli et al., 1998), GAP-43 (Hayashi et al., 1997) and endothelial nitric oxide synthase (eNOS) (Venema et al., 1995). Interestingly, the CaM-binding domain of LeCBDGK is present as a short, separate exon on the LeCBDGK gene (Figure 7). Thus, alternative splicing may provide a mechanism to generate Ca\textsuperscript{2+}-sensitive and -insensitive DGK isoforms, thereby allowing flexibility of response during Ca\textsuperscript{2+} and phospholipid signalling. Similarly, human DGK was also recently shown to undergo alternative splicing (Ding et al., 1997). Moreover, alternative splicing of a CaM-binding inositol-1,4,5-triphosphate (IP\textsubscript{3}) receptor results in a form with a second CaM-

![Figure 7. LeCBDGK and LeDGK1 are encoded by the same gene.](image-url)

(a) Southern blot of tomato genomic DNA digested with various restriction enzymes and probed with a cDNA corresponding to the conserved region of LeCBDGK and LeDGK1. DNA markers (kb) are shown to the right. (b) Organization of the LeCBDGK/LeDGK1 gene. A genomic fragment (9.3 kb) was isolated by PCR and shown to contain the coding regions for both LeCBDGK and LeDGK1. The start codon is indicated (ATG), as are the alternative stop codons (TAA and TAG for LeDGK1 and LeCBDGK, respectively). Exon positions (shaded boxes) are indicated by roman numerals, and their respective lengths (bp) are presented beneath their relative position in the gene. Intron positions and sizes (solid line) are shown relative to exon positions. The terminal exon of LeCBDGK (open box) encodes the CaM-binding domain of LeCBDGK.

family. Although recombinant LeCBDGK (purified from E. coli) was not responsive to CaM under various in vitro assay conditions (not shown), we cannot exclude the possibility that CaM may regulate LeCBDGK activity in vivo. For example, CaM regulation of LeCBDGK may require post-translational modification and/or additional factors not present in our system. Alternatively, LeCBDGK activity may be regulated in vivo by a specific member of the growing family of CaM isoforms or CaM-like proteins present in plants (Snedden and Fromm, 1998; Zielinski, 1998). Indeed, differential activation or inhibition of CaM-dependent enzymes by plant CaM isoforms is well-documented (Cho et al., 1998; Lee et al., 1999). Another possibility raised by the present study is that CaM binding may serve to modulate some other aspect of DGK function, such as subcellular localization. It is noteworthy that DAG is found in membranes but not soluble cellular fractions, and thus the translocation of LeCBDGK to its substrate may be one means of activation. In animal cells, both soluble and membrane-associated DGKs have been

binding domain (Lin et al., 2000). An additional example of alternative splicing which reflects the coordination of Ca²⁺ and lipid signalling is that of another DAG-binding protein, dUNC13, where one splice variant possesses a CaM-binding domain (Xu et al., 1998) similar to the DGKs described in the present study.

Possible roles for DGKs in signalling are beginning to emerge. The products of phospholipase C (PLC), DAG and IP₃, activate PKC and modulate the release of Ca²⁺ from internal stores, respectively (Liu and Heckman, 1998). In animal cells, the conversion of DAG to PA by DGKs probably serves to down-regulate PKC, and evidence is mounting that PA itself may be a signalling molecule in both animals and plants (Munnik et al., 1998a; Topham and Prescott, 1999). In animals, some PLCs and phosphatidylinositol-5-kinase (PI-5-K) are activated by PA (Ishihara et al., 1998; Jones and Carpenter, 1993), and a PA-activated protein kinase involved in stimulating NADPH oxidase has recently been described (Erickson et al., 1999). Similarly, CaM is also involved in PI signalling where it activates PI-3-K (Joyal et al., 1997) and modulates IP₃-induced intracellular Ca²⁺ release (Patel et al., 1997). Although PI signalling is not as well-characterized in plant cells, there is evidence for the presence of protein kinases with PKC-like properties, IP₃-induced intracellular Ca²⁺ release has been documented, and phospholipase C and D isoforms have been implicated in stress signalling (Munnik et al., 1998a). Increased PA production in plants occurs in response to stimuli such as wounding, or exposure to phytohormones or fungal elicitors, but the roles of PA remain unclear (Munnik et al., 1998a). Importantly, PA is also produced by the action of phospholipase D (PLD), and evidence suggests that both PLD- and DGK-derived PA participate in intracellular signalling in plants. A recent report (Ritchie and Gilroy, 1998) showed that ABA-induced synthesis of PA in barley aleurones was mainly generated by PLD, whereas another study demonstrated that mastoparan, a heterotrimeric G-protein activator, stimulated a rapid increase in PA in Chlamydomonas cells due primarily to DGK activation (Munnik et al., 1998b). In light of our present findings, the rapid production of PA in plant cells in response to certain stimuli may involve Ca²⁺ and CaM, possibly by facilitating the recruitment of DGKs to substrate DAG in membranes. The mechanism of LeCBDGK subcellular translocation remains unknown and is currently being examined. PA might modulate upstream targets involved in modulating Ca²⁺ signals such as PLC or act upon downstream effectors. Nevertheless, the relative contributions of PLDs and DGKs to PA production in plants, and identification of PA targets, requires further investigation before roles can be assigned to DGKs and PLDs. Such studies are essential in order to further our understanding of the cross-talk between Ca²⁺ and phospholipid signalling in plants.

**Experimental procedures**

**Plant cell cultures and subcellular fractionation**

Tomato (*Lycopersicon esculentum* cv Moneymaker) and tobacco (*Nicotiana tabacum* cv Petit Havana SR1) cell-suspension cultures were grown as described previously (Parsons et al., 1999; Vera-Estrella et al., 1992). Soluble, microsomal and plasma membrane cell fractions were prepared as described by Lam et al. (1998), with the exception that 5 mM EGTA and 5% w/v insoluble polyvinylpolypyrrolidone were included in the homogenization buffer. The soluble cell fraction (the supernatant fraction following a 45 min centrifugation at 80 000 g) of tomato cells was concentrated to a protein content of approximately 2 mg ml⁻¹ using a concentration cell (Amicon) with a molecular mass cut-off of 3 kDa, and used for immunoprecipitation or the isolation of CaM-binding proteins by CaM-affinity chromatography. Tobacco cell extracts were not concentrated prior to CaM-affinity chromatography. All samples were either used fresh or were divided into aliquots, frozen in liquid nitrogen, and stored at −70°C.

**Preparation of recombinant ³⁵S-labelled calmodulin and calmodulin-binding assays**

Petunia ³⁵S-labeled CaM81 was prepared and purified from *E. coli* as described from Fromm and Chua (1992), and SDS-PAGE electrophoresis and ³⁵S-CaM-binding assays were performed as described by Baum et al. (1993).

**CDNA library screening using ³⁵S-calmodulin**

Total RNA was extracted from 3-day-old tomato cell cultures, and mRNA was isolated using a poly Atract kit (Promega Corp.) and used to generate a cDNA expression library (ZAP Express kit, Stratagene). This library (approximately 1 × 10⁸ plaque-forming unit (pfu)) was screened for cDNAs encoding CaM-binding proteins using ³⁵S-CaM as a probe as described from Fromm and Chua (1992). The initial partial cDNA encoding *LeCBDGK* (nucleotides 495–1845) was used to re-screen the library under high-stringency conditions to obtain a full-length cDNA that was then sequenced on both strands. Another full-length cDNA, nearly identical to *LeCBDGK*, designated *LeDGK1*, was also isolated during this re-screening.

**Expression of LeCBDGK and LeDGK in Escherichia coli**

Recombinant proteins were expressed in *E. coli* BL21 (DE3)-pLysS cells transformed with different pGEX-2TK (Pharmacia) vector constructs containing regions of either *LeCBDGK* or *LeDGK* (see figure legends) to generate GST fusion proteins. Bacteria were grown at 30°C in LB medium for 2–5 h following induction of protein expression with 0.1 mM isopropyl-β-D-thiogalactoside. As not all of the various GST fusion proteins were expressed in the soluble fraction of *E. coli*, total bacterial lysates (from 2 ml cultures) were mixed with SDS sample buffer, boiled for 5 min, and used in SDS-PAGE and transfer of proteins to nitrocellulose membranes for ³⁵S-CaM overlay experiments. For enzyme assays, *LeCBDGK* was expressed from a pET5α vector (Pharmacia) and purified from soluble bacterial extracts by CaM-affinity chromatography. *LeDGK1* was subcloned into a pGEX-2TK vector, expressed in *E. coli* as a soluble GST fusion protein, purified and proteolytically released from GST by thrombin lysis (as per
manufacturer's directions), and used in enzyme assays. The purity of enzyme preparations was assessed by SDS-PAGE and Coomassie blue staining.

**Calmodulin-affinity chromatography**

The soluble fraction from tomato or tobacco cell extracts, or from *E. coli* expressing recombinant LeCBDGK, was brought to a final CaCl₂ concentration of 1 mM and passed over a 0.5 ml bed-volume CaM–agarose (Sigma) column pre-equilibrated with buffer A (50 mM Tris–Cl, pH 7.5 containing 1 mM CaCl₂). The column was washed with 20 bed-volumes of buffer A supplemented with 0.2 M NaCl. CaM-binding proteins were eluted with 50 mM Tris–Cl buffer, pH 7.5, containing 2 mM EGTA.

**Production of polyclonal antibodies**

Antibodies were raised in rabbits against purified recombinant LeCBDGK and used for immunoblotting. Immunoreactive proteins were detected by chemiluminescence using peroxidase-conjugated anti-rabbit secondary antibodies (Sigma).

**Immunoprecipitation**

Co-immunoprecipitation of LeCBDGK and CaM from soluble cell extracts was performed as described by Baum et al. (1996), with the following modifications. A 250 µl aliquot (approximately 500 µg protein) of soluble tomato cell extract was diluted with 1 volume of 50 mM Tris pH 7.5 containing 200 mM NaCl, 1 mM PMSF, and CaCl₂ was added to a final free concentration of 1 mM. Antiserum against LeCBDGK, or pre-immune serum, was added (1:750 dilution), and the mixture shaken gently at 4°C for 1 h. Swollen protein A beads (50 µl) (Pharmacia) were added and samples incubated at 4°C with gentle shaking for 1 h. Samples were centrifuged at 12 000 g for 5 min, and the pellet was washed three times with 50 mM Tris–Cl pH 7.5 containing 1 mM CaCl₂ and 200 mM NaCl, and resuspended in SDS-PAGE loading buffer. Electrophoresis and immunoblotting (using anti-petunia CaM monoclonal IgG) were performed as described by Baum et al. (1996).

**Recruitment of soluble LeCBDGK to microsomal membranes**

Tomato cell extracts were prepared as described previously (Lam et al., 1998), centrifuged at 10 000 g for 20 min, and the supernatant fraction (termed cell homogenate) used for recruitment experiments. Aliquots (20 µl, containing approximately 40 µg total protein) were incubated for 20 min at room temperature in the presence of either 5 mM EGTA, 10 µM CaCl₂ and 50 µM trifluoperazine (TFP), 10 µM CaCl₂ alone, or 10 µM CaCl₂ and 1 µM recombinant petunia CaM81 with gentle shaking. Following incubation, samples were centrifuged at 100 000 g for 30 min. Pelleted and supernatant fractions were designated as microsomal membrane and soluble fractions, respectively, combined with SDS sample buffer, and used for electrophoresis and immunoblotting as described above.

**DGK activity assays**

Enzyme activity was assayed by measuring the production of 32P-phosphatidic acid (PA) from 32P-γ-ATP and DAG. Recombinant DGKs were assayed by a deoxycholate method similar to that described by Bunting et al. (1996). Assay mixtures (100 µl) contained 50 mM MOPS buffer (pH 7.5), 1 mM DTT, 2 mM 32P-γ-ATP (1.6 mCi mmol⁻¹, Amersham), 500 µM 1,2-dioleoyl-sn-glycerol (DAG) (Avanti Polar Lipids), 100 mM NaCl, 5 mM MgCl₂, 1 mM deoxycholate and approximately 1 µg of purified recombinant LeCBDGK or LeDGK1. In all assays, samples were incubated for 30 min at 30°C, and reactions stopped by the addition of 0.2 ml of 1 M HCl. Carrier PA (2 µg) was added, and chloroform-soluble products extracted (Sakane et al., 1991). 32P-phosphatidic acid was analysed by TLC using a PA standard (Avanti Polar Lipids) as described by Munnik et al. (1996b). Following separation, TLC plates were sprayed with phospholipid detection reagent (Vaskovsky and Kostetský, 1968) to visualize the PA standard and then exposed overnight to r-Ray film. As 32P-PA was the only radiolabelled product detectable by autoradiography, reactions were routinely analysed by scintillation counting of the chloroform-soluble phase.

**Southern hybridization**

Genomic DNA was isolated from tomato cells, 10 µg was digested with different restriction enzymes (see figure legends), and Southern blotting was performed using a 32P-labelled DNA probe corresponding to a region identical in LeCBDGK and LeDGK1 (nucleotides 1–1449) and standard procedures.

**Isolation of the LeCBDGK gene by PCR**

Template genomic DNA was prepared as described above. A genomic DNA fragment, covering the region from the start codon to a 3’ untranslated region in LeCBDGK, was amplified by PCR using the following primers: 5’-ATGGCGAGATTTCTGAGTCC-3’ and 5’-TTGACCTGATGTGATTGCG-3’ using a High Fidelity Expand Long Template PCR System (Boehringer Mannheim) according to the manufacturer’s instructions. The specific PCR product was subcloned into a TA-cloning vector (Invitrogen) and sequenced on both strands.

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**References**


GenBank database accession numbers AF198258 (LeCBDGK, cDNA), AF198259 (LeDGKI, cDNA) and AF198260 (LeCBDGK/LeDGKI gene).