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 stimuli–response signal transduction is discussed.

Results

Isolation of LeCBDGK *and* LeDGK1 *cDNAs*

Among the clones isolated by screening a cDNA library with ^{35}S-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 ^{35}S-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

(a)

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                          GAGGCTTAATTTCTGATTCTTCCGAAT    -61
ATCGCTCTGCATACGTAGCATAATTCATATACTTTTCCTTCTCAGCTGCAGCGGGAGTTA    - 1
ATGGCAGATTCTGAGTCCGAGCACAATAACATCCATAGAGACTTTTATATTCCTACTTAT     60
 M  A  D  S  E  S  E  H  N  N  I  H  R  D  F  Y  I  P  T  Y     20
ATACTTGCACCGAATGCTTCGAGCAACAGTTTACGTTTACCTGATGTACCAACATGCCCT    120
 I  L  A  P  N  A  S  S  N  S  L  R  L  P  D  V  P  T  C  P     40
GTGCTAGTGTTCGTCAACTCTAAAAGTGGAGGTCAACTTGGAGGAGAACTTCTTCGTACA    180
 V  L  V  F  V  N  S  K  S  G  G  Q  L  G  G  E  L  L  R  T     60
TTTCGCCATCTTCTTAACAAATATCAGGTTTTTGATTTGGGAGATGAAGCTCCTGATAGT    240
 F  R  H  L  L  N  K  Y  Q  V  F  D  L  G  D  E  A  P  D  S     80
GTGTTACGTAGACTATATTTGAATATAGAAAGGCTCAAGGGTAATGGTGATCACTTTGCC    300
 V  L  R  R  L  Y  L  N  I  E  R  L  K  G  N  G  D  H  F  A    100
GCCGAAATTGAAGAGCGAATGAAAATTATTGTTGCAGGTGGAGATGGAACGGCTGGCTGG    360
 A  E  I  E  E  R  M  K  I  I  V  A  G  G  D  G  T  A  G  W    120
CTTCTCGGAGTTGTATCTGATCTCAAACTATCTCAGCCACCACCAATTGCAACAGTACCT    420
 L  L  G  V  V  S  D  L  K  L  S  Q  P  P  P  I  A  T  V  P    140
TTGGGAACTGGAAATAACCTTCCATTTGCTTTTGGCTGGGGAAAGAAGAATCCAGGGACC    480
 L  G  T  G  N  N  L  P  F  A  F  G  W  G  K  K  N  P  G  T    160
GACCTTAACTCCGTCATCTCTTTTTTGAAGCAAGTAATGAACGCAAAGGAAATGAAGATG    540
 D  L  N  S  V  I  S  F  L  K  Q  V  M  N  A  K  E  M  K  M    180
GACAGTTGGCACATTCTTATGAGGATGAGGGCACCAAAAGTTGGTTCTTGTGATCCTGTT    600
 D  S  W  H  I  L  M  R  M  R  A  P  K  V  G  S  C  D  P  V    200
GCACCGCGTTGAATTGCCTCATTCTTTGCATGCCTTTCACCGGGTTTCTCCATCTGATGAA   660
 A  P  L  E  L  P  H  S  L  H  A  F  H  R  V  S  P  S  D  E    220
CTGAACGTGGAAGGTTTCCATACATTCCGTGGAGGTTTCTGGAATTACTTCAGCATGGGG    720
 L  N  V  E  G  F  H  T  F  R  G  G  F  W  N  Y  F  S  M  G    240
ATGGATGCACAAGTATCTTATGCATTTCACTCAGAGAGGAAAATGAACCCTGACAAATTC    780
 M  D  A  Q  V  S  Y  A  F  H  S  E  R  K  M  N  P  D  K  F    260
AAAAACCAACTTGTCAATCAGAGTTCATATTTAAAGCTAGGATGTACACAAGGATGGTTT    840
 K  N  Q  L  V  N  Q  S  S  Y  L  K  L  G  C  T  Q  G  W  F    280
TTTGCTCCTCTCATTCATCCTTCTTCAAAGAACATAGCTCAACTGACTAAGGTTAAGATA    900
 F  A  P  L  I  H  P  S  S  K  N  I  A  Q  L  T  K  V  K  I    300
ATGAAAAAGCAGGGTGGATGGCAAGACCTCCACATCCCACCCAGTGTCAGGTCAATTGTA    960
 M  K  K  Q  G  G  W  Q  D  L  H  I  P  P  S  V  R  S  I  V    320
TGCCTCAACTTGCCTAGCTTTTCCGGTGGACTAAATCCCTGGGGAACACCAAATAGCAAC   1020
 C  L  N  L  P  S  F  S  G  G  L  N  P  W  G  T  P  N  S  N    340
AAACGTCGATATAGGGACTTGACTCCTCCATTTGTAAACGATGGCCTTCTTGAGGTTGTT   1080
 K  R  R  Y  R  D  L  T  P  P  F  V  N  D  G  L  L  E  V  V    360
GGATTCAGAGATGCTTGGCATGGGATTGGTCCTCCTTGCTCCAAAAGGACATGGGACACGT  1140
 G  F  R  D  A  W  H  G  L  V  L  L  A  P  K  G  H  G  T  R    380
CTTGCCCAGGCGCATGGAATCCCGGTTTGAGTTTCAGAAAGGAGCAGCTGATCATACATTC  1200
 L  A  Q  A  H  G  I  R  F  E  F  Q  K  G  A  A  D  H  T  F    400
ATGAGGATTGATGGGGAACCCTGGAAGCAGCCTCTACCTGAAAATGATGACACCGTTGTG   1260
 M  R  I  D  G  E  P  W  K  Q  P  L  P  E  N  D  D  T  V  V    420
GTAGAAATTTCTCATCTAGGCCAAGTCAAGATGCTTGCTACCCACGATTGCAGAGCCAAA   1320
 V  E  I  S  H  L  G  Q  V  K  M  L  A  T  H  D  C  R  A  K    440
AGTATACATGATCCCTCAAGTCAATTCAGTCATGAAACTGATGAGGTCGATAGTGATGAA   1380
 S  I  H  D  P  S  S  Q  F  S  H  E  T  D  E  V  D  S  D  E    460
GAAAATTCGGTCAATGAAGAACAGAGGAAATTCGGGGCAGCAGATACTTTCAAAATTCCA   1440
 E  N  S  V  N  E  E  Q  R  K  F  G  A  A  D  T  F  K  I  P    480
GATGAGGTT|GATGTTTCTAGTCTCAGTTAA|TTCCATAATTCTTCTTTGTTCTGCTACCTC   1500
 D  E  V |D  V  S  S  L  S  * |                                  489
TATAGTACATTTGCTGTATAGTAGGCATAACAAGGCTCCATGTCGAATTTATAAGACATG   1560
GATATTTTAGAGGTTTTGCATAGAGAATTCGTGAACATTTGGTGTATAGGAGGAAGTTT    1620
GCTGACTTGGTGTATGATAAAGGAGATGCTAGAAGTCACCATTATAACCTTTAACAGGCT   1680
AGATTTTATTTAGCCAGAACATTATTTGGCTTTTAAAAAAAAAAAAAAAAAAAAAA      1736
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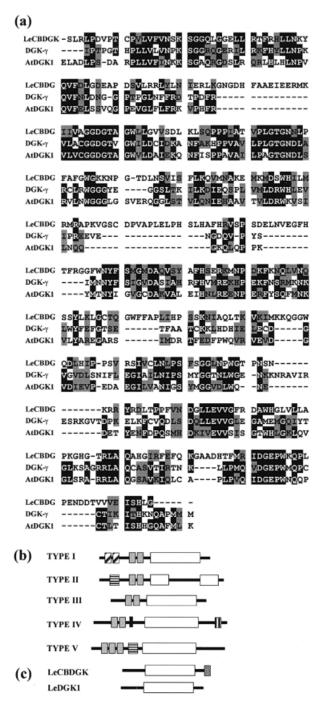
(b)

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AAGAGACAAAATAGGTCTCATGGGAGGAAGCCTAGACTATGGGCGCTATTAAGGAATTTG   1509
 K  R  Q  N  R  S  H  G  R  K  P  R  L  W  A  L  L  R  N  L    503
CTCGCCTTTCGTTTAGAGAGACATTGAATTTCATCCTAATGGAGTTGATATGTTGTATAT   1569
 L  A  F  R  L  E  R  H  *                                     511
GAGAAGAATGGTTTGAACTTTTTCTTCGTTCCCCCGTTTCCCTCTTTTGTGATGGATGTA   1629
ACTGTTTGTGAAGATCATCATTTAGCAATCACACATCAGTGCAATTAAGAAATGCTAGCA   1689
TTGGTCGCTGACTTAAAGTGACAGAGAGACATAGTAGAACTCCTGATATTTCTGGTACAA   1749
TTCCAGAGATATCATGGGCAGGTTAAATGCATTCAAGAAACTTGCACAGACAATAACAGA   1809
AAGAGTCCTTTGGCTATTAAAAAAAAAAAAAAAAAAAA                         1845
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Figure 1. Sequence of *LeDGK1* and *LeCBDGK* cDNAs isolated from tomato cells.
The cDNA sequences for both *LeDGK1* and *LeCBDGK* are identical from nucleotide positions 1–1449. (a) cDNA and deduced amino acid sequence for *LeDGK1*. The boxed region represents the nucleotide and deduced protein sequence unique to *LeDGK1*. (b) Nucleotide and deduced amino acid sequence for the unique C-terminal region of *LeCBDGK*, beginning at nucleotide position 1450. The CaM-binding domain of *LeCBDGK* is present within this region. Predicted stop codons are marked by asterisks and the predicted ATP-binding domain is underlined.

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).

(a)

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LeCBDGK  -SLRLPDVPT CPVLVFVNSK SGGQLGGELL RTFRHLLNKY
DGK-γ    ----IPTPGT HPLLVDVNPK SGGROGERIL RKFHYLLNPK
AtDGK1   ELADLPS-DA RPLLVFINKK SGAORGDSLR QRLHLHLNPV

LeCBDG   QVFDLGDEAP DSVLRRLYLN IERLKGNGDH FAAEIEERMK
DGK-γ    QVFNLDNG-G PTPGLNFFRD TPDFR----- ----------
AtDGK1   QVFELSSVQG PEVGLFLFRK VPHFR----- ----------

LeCBDG   ITVAGGDGTA GWLLGVVSDL KLSQPPPIAT VPLGTGNNLP
DGK-γ    VLACGGDGTV GWILDCIDKA NFAKHPPVAV LPLGTGNDLA
AtDGK1   VLVCGGDGTA GWVLDAIEKQ NFISPPAVAI LPAGTGNDLA

LeCBDG   FAFGWGKKNP G-TDLNSVIS FLKQVMNAKE MKMDSWHILM
DGK-γ    RCLRWGGGYE ----GGSLTK ILKDIEQSPL VMLDRWHLEV
AtDGK1   RVLNWGGGLG SVERQGGLST VLQNIEHAAV TVLDRWKVSI

LeCBDG   RMRAPKVGSC DPVAPLELPH SLHAFHRVSP SDELNVEGFH
DGK-γ    IPREEVE--- ---------- ---NGDQV-P YS--------
AtDGK1   LNQQ------ ---------- ----GKQLQP PK--------

LeCBDG   TFRGGFWNYF SVGVDAQVSY AFHSERKMNP DKFKNQLVNQ
DGK-γ    ----IVNNYF SIGVDASIAH RFHVVREKHP EKFNSRMKNK
AtDGK1   ----YMTNYI GVGCDARVAL EIHNLREENP ERFYSQFMNK

LeCBDG   SSYLKLGCTQ GWFFAPLIHP SSKNIAQLTK VKIMKKQGGW
DGK-γ    LWYFEFGTSE ------TFAA TCKKLHDHIE LECD-----G
AtDGK1   VLYARECARS ------IMDR TFEDFPWQVR VEVD-----G

LeCBDG   QDLHIE-PSV RSIVCLNLPS FSGGLNPWGT PNSN------
DGK-γ    VGVDLSNIFL EGIAILNIPS MYGGTNLWGE -NKKNRAVIR
AtDGK1   VDIEVE-EDA EGILVANIGS YMGGVDLWQ- -NE-------

LeCBDG   -------KRR YRDLTPPFVN DGLLEVVGFR DAWHGLVLLA
DGK-γ    ESRKGVTDPK ELKFCVQDLS DLLEVVGLE GAMEVGQIYT
AtDGK1   -------DET YENFDPQSMH DKIVEVVSIS GTWHLGKLQV

LeCBDG   PKGHG-TRLA QAHGIRFEFQ KGAADHTFMR IDGEPWKQPL
DGK-γ    GLKSAGRRLA QCASVTIRTN K----LLPMQ VDGEPWMQPC
AtDGK1   GLSRA-RRLA QGSAVKIQLC A----PLPVQ IDGEPWNQQP

LeCBDG   PENDDTVVVE ISHLG----- -
DGK-γ    ------CTIK ITHKNQAPMM M
AtDGK1   ------CTLT ISHHGQAFML K
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(b)
TYPE I
TYPE II
TYPE III
TYPE IV
TYPE V

(c)
LeCBDGK
LeDGK1

Recombinant LeCBDGK and LeDGK1 are catalytically active

In the presence of DAG and ^{32}P-γ-ATP, both LeCBDGK and LeDGK1 produced a ^{32}P-radiolabelled, 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 D63787, residues 351–684) and human DGK-γ (accession number N48667, 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), myristolylated, 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).
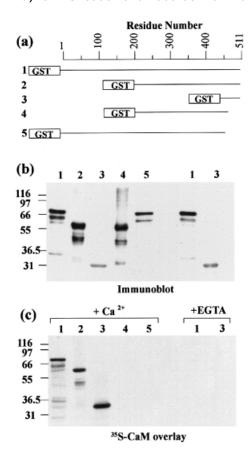
Figure 3. Delineation of the CaM-binding domain of LeCBDGK.
(a) Different regions of either *LeCBDGK* or *LeDGK1* were subcloned into an expression vector in-frame with glutathione-*S*-transferase (GST). The numbers on the left correspond to the different fusion protein constructs: (1) full-length LeCBDGK, residues 1–511; (2) LeCBDGK residues 200–511; (3) LeCBDGK residues 450–511; (4) LeCBDGK residues 200–496; (5) LeDGK1 residues 1–489. (b) Immunodetection of recombinant GST–DGK fusion proteins in *E. coli* total extracts (5 μg per lane) using anti-DGK antiserum. Lane numbers correspond to the constructs described in (a). (c) ^{35}S-CaM overlay assay. A duplicate protein blot to that described in (b) was incubated with recombinant ^{35}S-CaM in the presence (lanes 1–5 on the left) or absence (lanes 1 and 3 on the right) of 1 mM Ca^{2+}, dried, and exposed to X-ray film. Similar results were obtained in three separate experiments.

Figure 4. Enzyme activity of LeCBDGK and LeDGK1.
Purified recombinant LeCBDGK and LeDGK1 catalyse the *in vitro* synthesis of phosphatidic acid (PA) using DAG and ^{32}P-γ-ATP as substrates. Chloroform-soluble reaction products were separated by thin-layer chromatography and detected by a phospholipid-sensitive spray (left panel) or by autoradiography (right panel). Lane 1 shows the migration pattern of a PA standard (arrow). Lanes 2 and 3 correspond to samples from LeCBDGK and LeDGK1 assays, respectively. These reactions were spiked with unlabelled PA to allow for comparison with the migration of ^{32}P-labelled products detected by autoradiography (lanes 4–6) from the same samples presented in lanes 1–3. The results of a typical experiment are presented.

et al., 1996; Sakane *et al.*, 1991; Topham and Prescott, 1999), prompted us to examine whether Ca^{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^{2+}, or Ca^{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^{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-napthalenesulphonamide (W7) (data not shown). As Ca^{2+} alone, but not in conjuction with CaM antagonists, increased the fraction of LeCBDGK associated with membranes, we examined the possibility that Ca^{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).
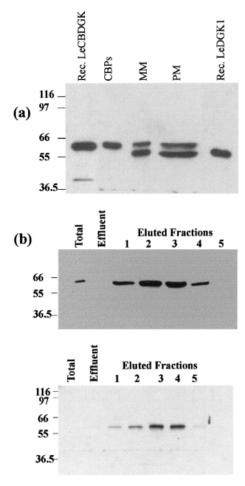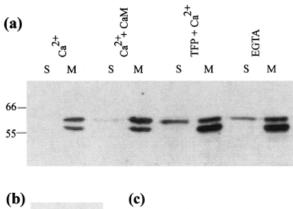
Figure 5. Immunodetection of LeCBDGK and LeDGK1 from plant cell extracts.

(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 electorphoretic 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 tomato 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 Ca^{2+} and
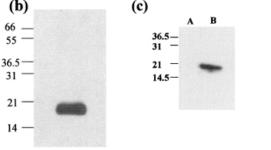

Figure 6. Recruitment of soluble LeCBDGK to membranes *in vitro*.
(a) Tomato cell homogenates (40 μg protein) were prepared as described in Experimental procedures. Homogenate samples were incubated in the presence of either 10 μM Ca^{2+} alone, 10 μM Ca^{2+} and 1 μM CaM, 50 μM TFP and 10 μM Ca^{2+}, or 5 mM EGTA, and then centrifuged at 100 000 *g* to yield soluble (S) and membrane (M) fractions and analysed by electrophoresis and immunodetection using anti-DGK antiserum. (b) Cell homogenate preparations (40 μg protein), from the same preparation as described in (a) were separated by electrophoresis and immunodetection was performed using anti-CaM monoclonal antiserum. (c) CaM and LeCBDGK co-immunoprecipitate from tomato cell soluble extracts. Pre-immune serum (lane A) or anti-DGK antiserum (lane B) was added to an aliquot of total soluble protein from tomato cell extracts in the presence of Ca^{2+}, and protein–antibody complexes were pelleted from solution using protein A–Sepharose beads and immunodetected using anti-CaM monoclonal antibodies. Results are from a single experiment and were reproduced four times.

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. LeBDGK and LeDGK1 were catalytically active as bacterially expressed recombinant proteins, establishing them unequivocally as members of the DGK
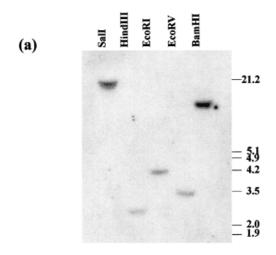
Figure 7. LeCBDGK and LeDGK1 are encoded by the same gene.
(a) Southern blot of tomato genomic DNA digested with various restriction enzymes and probed with a cDNA corresponding to the conserved region of *LeCBDGK* and *LeDGK1*. DNA markers (kb) are shown to the right. (b) Organization of the *LeCBDGK/LeDGK1* 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 TGA 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.*, 1995). 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

observed, and a number of reports have described the Ca^{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^{2+}) or EGTA were able to disrupt LeCBDGK movement to membranes *in vitro*, whereas Ca^{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^{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^{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^{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^{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^{2+}-sensitive and -insensitive DGK isoforms, thereby allowing flexibility of response during Ca^{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-trisphosphate (IP_3) receptor results in a form with a second CaM-

binding domain (Lin *et al.*, 2000). An additional example of alternative splicing which reflects the coordination of Ca^{2+} 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 IP_3, activate PKC and modulate the release of Ca^{2+} 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 phosphatidy-linositol-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 IP_3-induced intra-cellular Ca^{2+} 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, IP_3-induced intracellular Ca^{2+} 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 masto-paran, a heterotrimeric G-protein activator, stimulated a rapid increase in PA in *Chlamydomonas* cells due primar-ily 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 Ca^{2+} and CaM, possibly by facilitating the recruitment of DGKs to sub-strate DAG in membranes. The mechanism of LeCBDGK subcellular translocation remains unknown and is cur-rently being examined. PA might modulate upstream targets involved in modulating Ca^{2+} 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 Ca^{2+} and phos-pholipid 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 polyvinyl-polypyrrolidone 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 ^{35}S-labelled calmodulin and calmodulin-binding assays

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

CDNA library screening using ^{35}S-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 1×10^6 plaque-forming unit (pfu)) was screened for cDNAs encoding CaM-binding proteins using ^{35}S-CaM as a probe as described by Fromm and Chua (1992). The initial partial cDNA encoding *LeCBDGK* (nucleo-tides 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 ^{35}S-CaM overlay experiments. For enzyme assays, LeCBDGK was expressed from a pET5a vector (Pharmacia) and purified from soluble bacterial extracts by CaM-affinity chromato-graphy. LeDGK1 was subcloned into a pGEX-2TK vector, ex-pressed 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 $CaCl_2$ 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 $CaCl_2$). 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 $CaCl_2$ 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 $CaCl_2$ 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 $CaCl_2$ and 50 μM trifluoperazine (TFP), 10 μM $CaCl_2$ alone, or 10 μM $CaCl_2$ 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 ^{32}P-phosphatidic acid (PA) from ^{32}P-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 ^{32}P-γ-ATP (1.6 mCi mmol^{-1}, Amersham), 500 μM 1,2-dioleoyl-*sn*-glycerol (DAG) (Avanti Polar Lipids), 100 mM NaCl, 5 mM $MgCl_2$, 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). ^{32}P-phosphatidic acid was analysed by TLC using a PA standard (Avanti Polar Lipids) as described by Munnik *et al.* (1998b). Following separation, TLC plates were sprayed with phospholipid detection reagent (Vaskovsky and Kostetsky, 1968) to visualize the PA standard and then exposed overnight to r-Ray film. As ^{32}P-PA was the only radiolabeled 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 ^{32}P randomly 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′-ATGGCAGATTCTGAGTCC-3′ and 5′-TTGCACTGATGTGTGATTGC-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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