Molecular and biochemical characterization of LeCRK1, a ripening-associated tomato CDPK-related kinase

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Abstract

A cDNA clone (*LeCRK1*), encoding a novel isoform of calcium-dependent protein kinase (CDPK), was isolated by screening a tomato (*Lycopersicon esculentum*) cDNA library. The protein derived from the full-length sequence indicated that it belongs to the family of CDPK-related kinases (CRKs) and the predicted amino acid sequence shows a modular organization of the protein consisting of different characteristic domains. The kinase domain of LeCRK1 shares a high degree of similarity with the catalytic domain of CDPKs. In contrast to canonical members of the family, LeCRK1 has a degenerate sequence in the C-terminal calmodulin-like domain. LeCRK1 protein was shown to be a functional kinase, but, consistent with the lack of calcium-binding activity, its autophosphorylation activity did not require calcium. LeCRK1 harbours an amphiphilic amino acid region revealed to be a functional calmodulin-binding site by *in vitro* assay. A putative myristoylation/palmitoylation sequence has been identified at the N-terminus. Expressing an LeCRK1::GFP fusion protein in the protoplast resulted in its targeting to the plasma membrane. Site-directed mutagenesis of critical amino acids of the myristoylation/palmitoylation consensus sites led to the accumulation of the mutated protein in the cytoplasm, suggesting that the native protein is anchored to the plasma membrane by acylated residues. Expression studies revealed significant accumulation of *LeCRK1* transcripts during fruit ripening, although transcripts were also detected in stem, leaf, and flower. *LeCRK1* mRNA level in leaves was slightly induced by ethylene and salicylic acid, and upon mechanical wounding and cold treatment. It is noteworthy that *LeCRK1* mRNAs were undetectable in different tomato-ripening natural mutants such as *NR*, *Rin*, and *Nor*, suggesting a role in the ripening process.

Key words: CDPK, fruit ripening, *Lycopersicon esculentum*.

Introduction

Plant protein kinases belong mainly to the large family of serine/threonine kinases (Hardie, 1999) which is subdivided into several groups on the basis of structural, biochemical, and physiological properties. Calcium may control the activity of plant protein kinases through indirect or direct interaction with the enzyme. Indirect interactions involve calmodulin, a calcium-binding protein. Direct interaction implicates a multi-family protein referred to as calcium-dependent protein kinases (CDPKs) that fall into the serine/threonine class of protein kinases found mainly in plants and in some protozoans (Harmon *et al.*, 2001). More than 40 distinct CDPKs have been predicted in the complete *Arabidopsis* genome (Cheng *et al.*, 2002), and their multiplicity might be related to specialization of individual isoforms through differences in calcium sensitivity, substrate specificity, subcellular and tissue distribution (Harmon *et al.*, 2000). Such an hypothesis is strengthened by the modular organization of CDPKs that typically contain four distinct domains. The N-terminal variable domain is followed by a protein kinase domain, an auto-inhibitory domain, and a calcium-binding domain. In typical members

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of the CDPK family, the calcium-binding domain is similar to calmodulin in sequence and contains four calcium-binding motifs named EF-hands. The direct activation of CDPK enzymes by calcium occurs through the intramolecular binding of the calmodulin-like (CaM-like) domain to the auto-inhibitory domain, resulting in the release of inhibition. There is another type of protein kinase related to conventional CDPK, where the calcium-binding domain is more similar to visinin (only three EF-hands) than to calmodulin. In these kinases, the auto-inhibitory domain contains a calmodulin-binding site, and calmodulin stimulates the kinase activity (Harmon *et al.*, 2000). Finally, some members of the CDPK family, the so-called CDPK-related kinases (CRKs), exhibit low conservation of the EF-hand motifs in the CaM-like domain, and are unresponsive to calcium (Furumoto *et al.*, 1996).

Being involved in the study of the molecular basis of fruit development and ripening, the authors of this paper reasoned that protein phosphorylation is a key-step in the overall process, as already substantiated by different lines of evidence (Giovannoni, 2001). Fruit ripening, as well as other plant growth and development processes, are genetically regulated by a subtle interplay of intrinsic regulators and environmental cues (Brady, 1987; Lelièvre *et al.*, 1997; Giovannoni, 2001; Jones *et al.*, 2002). The plant hormone ethylene plays a major role in regulating the ripening process of climacteric fruit such as the tomato, and it is now well established that it exerts its effect through the activation of a cascade of protein kinases (Stepanova and Ecker, 2000). In the tomato, the ethylene receptor, ETR histidine kinases, and the down-stream components of the ethylene transduction pathway, LeCTR serine/threonine kinases, are encoded by small multigene families (Giovannoni, 2001). It is noteworthy that only *NR* (Lashbrook *et al.*, 1998) and *LeCTR1* (Leclercq *et al.*, 2002) were shown to be regulated during ripening. In addition to ethylene, it has also been suggested for a long time that calcium might play a role in fruit ripening (Ferguson, 1984; Burns and Pressey, 1987; Conway, 1987). In this connection, an ethylene-responsive and ripening-regulated gene (*ER66*), encoding a calmodulin-binding protein, has been isolated from tomato (Zegzouti *et al.*, 1999), the homologue of which has been subsequently characterized in *Arabidopsis* as a DNA-binding protein (Reddy *et al.*, 2000; Yang and Poovaiah, 2000). Moreover, protein phosphorylation and calcium requirements have also been reported to play a major role in ethylene-dependent responses (Raz and Fluhr, 1992, 1993). These data are highly suggestive of the crosstalk between ethylene and calcium signalling pathways that may take place not only in the ripening process but also in ethylene-mediated stress responses.

Screening a tomato fruit cDNA library using degenerated primers led to the isolation of a cDNA clone encoding a novel CRK named LeCRK1 (accession number AY079049). Unlike previously characterized CDPKs from tomato (Chico *et al.*, 2002; Rutschmann *et al.*, 2002), LeCRK1 has degenerate EF-hand motifs, displayed no calcium requirement for its auto-phosphorylation activity and was found to be targeted to the plasma membrane. *LeCRK1* transcript accumulation patterns suggest a potential role in signalling associated with fruit ripening and responses to abiotic stress.

Materials and methods

Plant material and treatments

Lycopersicon esculentum (cv. Ailsa Craig) plants were grown in soil under standard glasshouse conditions. Ethylene and methyl jasmonate (MetJA) treatments were performed for 5 h in 25 l sealed glass boxes. Tomato plants about 30 cm high were treated with 50 μl l^{-1} ethylene and control plants were exposed to air alone. For treatment with MetJA, 1 μl of 5 mg l^{-1} of MetJA (Duchefa) was diluted in 25 μl absolute ethanol and the total volume was applied to a Whatman paper inside the box. The control experiment was performed using absolute ethanol only. For salicylic acid (SA) treatment (5 h), plants were sprayed with 4 mM solution of SA in water or with water alone for control plants. For abiotic stress, leaves were wounded with a razor blade and left for either 15 min or 5 h. Cold treatment was performed by growing the tomato plants at 5 °C for either 1 or 2 d. After treatment, tissues were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

LeCRK1 isolation

Degenerated primers were chosen on two well-conserved regions among the protein kinase family, the conserved region of the serine/threonine kinase (HRDLKxxN: PK 5′: CCACMGVGACCTGAARCCKGAGAA with M:A/C; V:A/C/G; R:A/G; K:G/T) and another conserved region (PK 3′: ACWCCRVATGMCCAHACRTCHG with W=A/T; R:A/G; V:A/C/G; M:A/C; H:A/T/C). A polymerization chain reaction was performed on a mixture of fruit cDNAs. The amplification products were sequenced, and specific primers were chosen in order to screen a λ-Zap cDNA tomato fruit library (constructed by Clontech). The longest fragments corresponding to each cDNA were cloned and sequenced.

Northern blot and RT-PCR analysis

Total RNA was extracted from 0.5 g of tissue ground in liquid nitrogen and extracted with phenol as described by Verwoerd *et al.* (1989), except that the extraction buffer was 100 mM TRIS-HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, and 1% SDS. Total RNA (8 μg) was fractionated on a 1.2% (w/v) agarose gel containing formaldehyde in MOPS buffer (Sambrook *et al.*, 1989) and then transferred onto GeneScreen membranes (NEN Life Sciences) following the manufacturer's instructions. Probes were labelled with [^{32}P]dCTP using a random-primer kit (Ready-to-Go, Pharmacia). Blots were hybridized in a buffer containing 0.3 volume of 1 M sodium phosphate buffer pH 7.2; 0.7 volume of 10% SDS, and 0.02 volume of 0.5 M EDTA pH 8; with a fragment of the 5′ region of each of the three genes, *LeCRK1, E8* (Cordes *et al.*, 1989; Tournier *et al.*, 2003), and a *basic glucanase* (Jia and Martin, 1999; Ding *et al.*, 2002) labelled with [^{32}P]dCTP using a random-primer kit (Ready-to-Go, Pharmacia). To check for equal loading, a reverse picture of the ethidium bromide-stained gel was used. Washes were carried out under high stringency (Sambrook *et al.*, 1989). RT-PCR analysis was carried out as described in Zegzouti *et al.* (1999) and Jones *et al.* (2002).

Expression of LeCRK1 fusion protein in Escherichia coli *and purification*

Full-length *LeCRK1* was amplified by PCR; the amplified product was then introduced into pBAD-TOPO expression vector (Invitrogen) and used to transform *E. coli* TOP10F' strain. The fusion protein was purified from arabinose-induced cultures by immobilized metal-affinity chromatography (Ni-NTA, Qiagen) according to the manufacturer's instructions. The purified protein preparation was analysed by SDS–PAGE using Coomassie blue staining. Western blot analysis of the fusion protein was also performed using anti-V5 antibodies (Invitrogen), and immunoreactive polypeptides were detected by chemiluminescence assay (ECL kit, Amersham). Protein concentration was determined by a protein assay kit (Bio-rad) and the purified protein fraction was supplemented with 20% glycerol and stored frozen at −80 °C.

Calcium and calmodulin-binding assay

The calcium-binding capacity of both LeCRK1 and calmodulin was examined after SDS-PAGE and subsequent electro-transfer of the purified fusion protein on a nitrocellulose membrane. The membrane was washed three times in a buffer consisting of 5 mM imidazole, 60 mM KCl, and 5 mM $MgSO_4$, and then incubated in 2 ml of the same buffer supplemented with 50 µCi [^{45}Ca]Cl_2 (Amersham). After 60 min incubation at room temperature, the membrane was washed three times in 30% ethanol, and exposed to Hyperfilm-MP (Amersham) for 16 h.

Calmodulin was produced as a GST fusion protein in *E. coli* transformed with pGEX-2TK expression vector bearing the VU-1 calmodulin gene (Roberts *et al.*, 1985). In addition to GST tag, the fusion protein contains the recognition sequence for the catalytic subunit of cAMP-dependent protein kinase for a direct labelling in the presence of [γ-^{32}P]ATP. The GST-CaM was affinity purified from bacterial lysates by binding to glutathione Sepharose 4B, and the affinity support loaded with the fusion protein was incubated in the presence of [γ-^{32}P]ATP and bovine heart kinase (Sigma) following the manufacturer's instructions. [^{32}P]Cam was released from glutathione Sepharose by thrombin cleavage of the fusion protein, and used as a probe in the calmodulin-binding assay. For measuring CaM-binding activity to LeCRK1 under non-denaturing conditions, various amounts of soluble proteins from bacterial lysates were directly spotted on nitrocellulose membranes. After blocking with 5% skimmed milk in TTBS buffer (10 mM TRIS-HCl, pH 8, 150 mM NaCl, 0.5% Tween 20), the membrane was incubated overnight at 4 °C with 40 µCi [^{32}P]CaM in an overlay buffer consisting of TTBS, 1% milk, and 1 mM $CaCl_2$ or 5 mM EGTA. The membrane was then washed three times in the overlay buffer and exposed to Hyperfilm-MP (Amersham) for 16 h.

Kinase activity assay

Kinase activity was analysed by incubating 1 µg of fusion protein in TRIS-HCl 50 mM pH 7.5, 10 mM $MgCl_2$, 0.5 mM DDT, 0.1% Tween 20, 100 nM ATP, and 1 µCi of [γ-^{32}P] ATP (Amersham) in a final volume of 25 µl. Alternatively, the kinase activity was assayed towards exogenous protein substrates (10 µg casein, myelin basic protein or histone H1) in the presence or the absence of 1 mM $CaCl_2$, 0.5 µg bovine brain calmodulin (Sigma). After 30 min incubation at 30 °C, the reaction was quenched by adding Laemmli SDS-PAGE sample buffer, boiled at 95 °C for 5 min, and analysed by SDS-PAGE. The reaction products were detected by autoradiography after exposure of dried gel to Hyperfilm-MP (Amersham).

Phosphoamino acid analysis

^{32}P-labelled phosphorylated proteins were fractionated by SDS-PAGE, and LeCRK1, and casein bands were excised from stained and dried gels and allowed to swell in 500 µl 0.05 M ammonium bicarbonate for 5 min. The gel pieces were homogenized and supplemented with 500 µl 0.05 M ammonium bicarbonate, 50 µl β-mercaptoethanol, and 10 µl 10% SDS. Samples were boiled for 10 min and shaken overnight at 37 °C. The phosphorylated proteins were precipitated with 20% (w/v) ice-cold TCA in the presence of BSA (20 µg) as a carrier protein. After washing the pellet with 100% ethanol, the proteins were hydrolysed in 6 N HCl for 1.5 h at 110 °C in a screw-capped microcentifuge tube, dried in a Speed-vac concentrator and then dissolved in 10 µl of H_2O. Unlabelled phosphoserine, phosphothreonine, and phosphotyrosine (10 mM) were added to each sample and amino acids were separated by TLC (thin-layer chromatography) (Silika gel 60, Merck). Migrations were performed five times in the same migration buffer (140 ml absolute ethanol and 60 ml 28% ammonium), drying between each migration. Labelled phosphoamino acids were detected by autoradiography, and standard phosphoamino acids were visualized by spraying TLC plates with 0.25% ninhydrin in acetone.

Transient expression of LeCRK1::GFP fusion proteins

The ORFs (open reading frames) of wild-type LeCRK1, G2A, and C4A/C5A mutants were cloned between *Xba*I and *Bam*HI restriction sites of the pGreen vector (John Innes Institute). The corresponding ORFs (598 AA) were amplified by PCR (Vent polymerase, Clontech) from a cDNA of tomato using primers LeCRK1-5′ with an *Xba*I restriction site (5′-TTTTTCTAGATTTTTCTCGATGGGGCAG-TGTTGCAGTAAGGG-3′) and with an *Bam*HI restriction site (5′-TTTTGGATCCGATGATGTCTTGTGC-3′). The two N-terminal mutated versions of *LeCRK1* were obtained using LeCRK1-3′ as reverse primer and either LeCRK1(G2A) 5′-TTTTTCTAGATT-TTTCTCGATGG**C**GCAGTGTTG-CAGTAAGGG-3′ or LeCRK1 (C4A/C5A) 5′-TTTTTCTAGATTTTTCTCGATGGG-GCAG**CG**-T**GC**CAGTAAGGGTGTTTCTGGTGAAGATGG-3′ as forward primers. This vector allows the transient expression of protein in C-terminal fusion with enhanced GFP. For the protoplast transfection assay, suspension-cultured tobacco BY-2 cells were cultured in standard conditions, the cells were digested into 10 ml of an enzyme solution containing 1.0% (w/v) cellulase 345 (Cayla, France), 0.2% (w/v) pectolyase Y-23 (Seishin Pharmaceutical, Tokyo), 0.6 M mannitol, and 25 mM MES-KOH, pH 5.5, at 37 °C for 90 min. Tobacco protoplasts were isolated and transfected by a modified polyethylene glycol method as described previously (Negrutiu *et al.*, 1987; Abel and Theologis, 1994). Typically, 0.3 ml of protoplast suspension (0.5×10^6) was transfected with 50 µg sheared salmon sperm carrier DNA and 30 µg of DNA plasmid carrying 35S::LeCRK1::GFP or 35S::GFP (control) constructs. The transfected protoplasts were incubated at 25 °C for 16 h. All transient expression experiments were repeated at least three times with similar results. Confocal fluorescent images were obtained with a Leica (Leica TCS SP2, Leica DM IRBE; Leica Microsystems, Wetzlar, Germany) confocal scanning laser microscope. The samples were illuminated with an argon ion laser (488 nm wavelength) for GFP fluorescence. The emitted light was collected between 500 and 525 nm.

Results

Cloning and sequence analysis of LeCRK1 cDNA

A tomato fruit cDNA library was screened by PCR using degenerate oligonucleotide probes designed from conserved sequences of the catalytic domain of serine/threonine protein kinases. Using two primers corresponding to the kinase subdomains VI and IX resulted in the isolation of a cDNA clone with a 200 bp insert. Sequence analysis indicated that the isolated clone displayed strong homology

to protein kinases. The full-length cDNA was obtained by PCR using a tomato fruit cDNA library and primers based on the isolated cDNA and library vector sequences. Ultimately, the longest cDNA clone obtained, named LeCRK1, was 2 kb in length and had an ORF of 1794 bp encoding a predicted protein of 598 amino acids with a molecular weight of 66.9 kDa. The LeCRK1 predicted protein is related to CDPK and is highly similar to *Daucus carota* DcCRK (Lindzen and Choi, 1995) and *Zea mays* ZmMCK1 (Lu *et al.*, 1996) with 67% and 62% overall amino acid identity, respectively. A phylogenetic tree was built using amino acid sequences of both true CDPK and CRKs. Figure 1A indicated clearly that LeCRK1 belongs to CRKs which form a distinct group. Members of this group display a common degenerated CaM domain, and biochemical data obtained from DcCRK and ZmMCK1 confirmed that the kinase activity of these proteins did not require calcium. The sequence alignment shown in Fig. 1B indicates that the LeCRK1 protein shares all of the structural features of CDPKs, including a minimally conserved N-terminal domain, a central kinase domain, an autoinhibitory domain, and a C-terminal CaM-like domain. LeCRK1 also has a putative myristoylation signal at the N-terminus (Thompson and Okuyama, 2000). The kinase domain is a typical serine/threonine kinase containing the 11 canonical subdomains (Hanks and Quinn, 1991). By contrast, the C-terminus of LeCRK1 shows only low sequence similarity with the CaM-like domain of other tomato CDPKs (LeCPK1 and LeCDPK1 in Fig. 1B). The four calcium-binding motifs (EF-hand) found in the C-terminus of CDPKs are only partially conserved in the LeCRK1, indicating that LeCRK1 is structurally distinct from common CDPKs. In addition, the normally well-conserved auto-inhibitory domain connecting the kinase domain to the CaM-like domain in CDPKs is also degenerated in LeCRK1, except for a basic amino acid region that functions as a pseudo-substrate for the CaM-like domain in CDPKs (Yoo and Harmon, 1996; Binder *et al.*, 1994). It has also been reported that the basic peptide sequence in the junction domain may form a basic amphiphilic alpha-helix which can bind CaM (Lu *et al.*, 1996).

Calcium- and calmodulin-binding activity

The *LeCRK1* ORF was introduced into a bacterial expression vector to produce a fusion protein bearing a C-terminal V5 epitope and 6×His tag. After induction with arabinose, the fusion protein was isolated using the 6×His tail as an affinity tag. In order to examine whether the degenerate EF-hands were capable of binding calcium, electroblotted LeCRK1 was incubated with [$^{45}Ca^{2+}$]. Binding of the

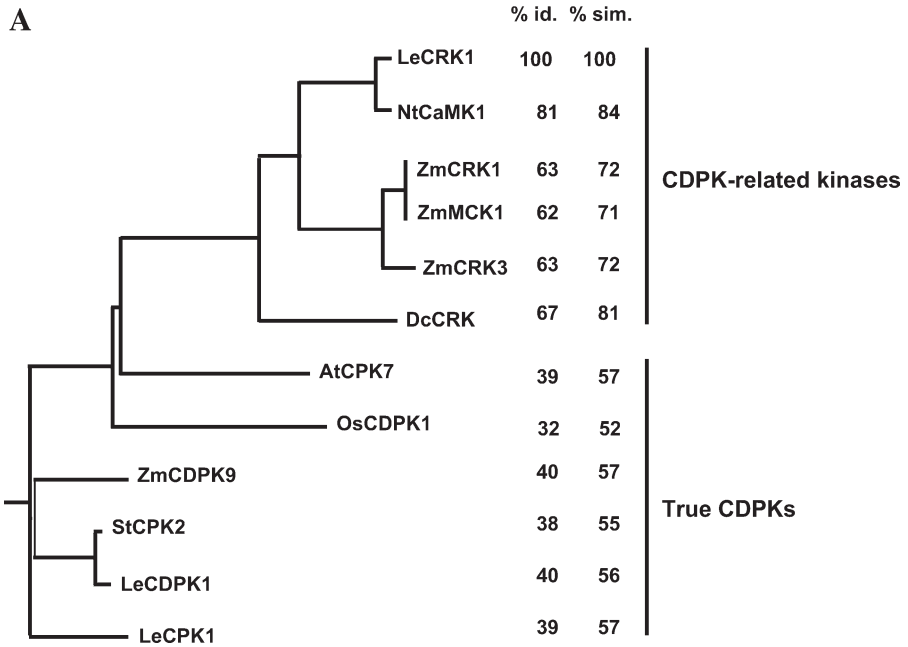

Fig. 1. Phylogenic analysis and structural features of LeCRK1. (A) Phylogenetic tree of CDPK and related proteins. Amino acid sequences from true CDPK and CRKs were aligned using the Clustal W program (http://clustalw.genome.ad.jp/) and the corresponding tree was built using the Phylodendron program (http://www.es.embnet.org/Doc/phylodendron/treeprint-form.html). CDPK and CDPK-related sequences from different species were used such as OSCDPK1 (P53682), StCPK2 (AAL09044), AtCPK7 (NP568281), LeCPK1 (AJ308296), LeCDPK1 (AF363784), ZmCDPK9 (AA12715), ZmCRK1 (BAA12691), NtCaMK1 (AAL30818), ZmCRK3 (BAA12692), DcCRK (X83869), LeCRK1 (AY079049). ZmMCK1 (S82324). The percentage of identical (% id.) and similar (% sim.) residues between LeCRK1 and other sequences was calculated using the program described in: http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html. (B) Amino acid sequence alignment of LeCRK1 and CDPK from carrot (DcCRK, accession no X83869; Lindzen and Choi, 1995), maize (ZmMCK1, accession number S82324; Lu *et al.*, 1996) and tomato (LeCPK1, accession number AJ308296; Rutschmann *et al.*, 2002; and LeCDPK1, accession number AF363784; Chico *et al.*, 2002). The consensus N-myristoylation signal is boxed. Roman numbers designate the position of kinase subdomains. The autoinhibitory domain and EF-hand motifs are also indicated. Identical amino acids and conservative replacements are shown with black and grey shading, respectively.

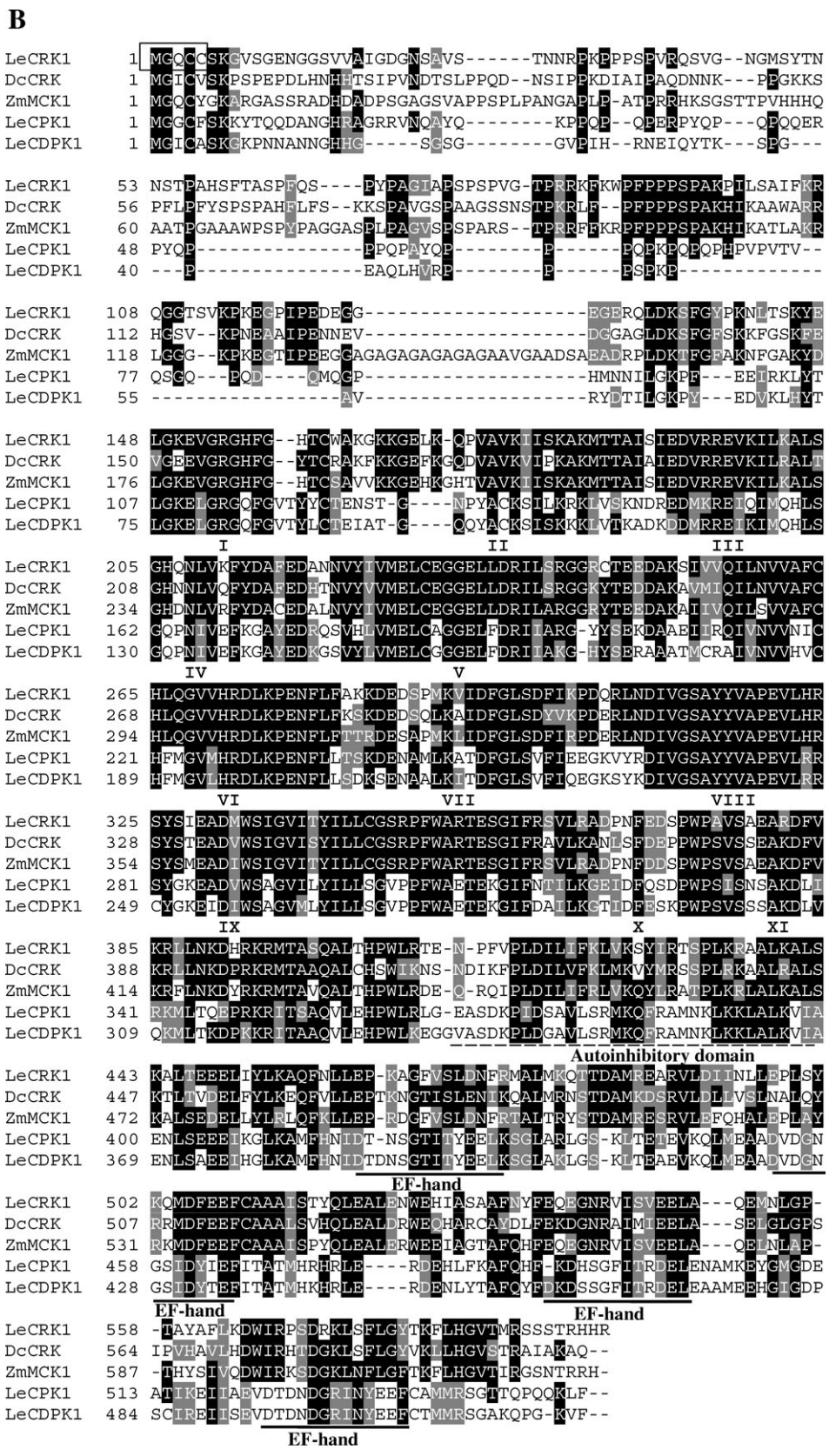

Fig. 1. *(continued)*

radiolabelled [$^{45}Ca^{2+}$] was observed for CaM, used as a positive control, but not for LeCRK1, indicating that LeCRK1 had impaired calcium-binding motifs (Fig. 2A).

Sequence analysis of the predicted LeCRK1 protein revealed the presence of a basic amphiphilic amino acid region, a structural feature found in many calmodulin-binding proteins (O'Neil and Degrado, 1990). This region is rich in basic and hydrophobic residues. A helical-wheel projection of this segment (LKRAALKALSKALT$_{446}$) showed a cluster of hydrophobic amino acids on one side of the alpha-helix and a concentration of positively charged amino acids on the opposite side, as observed in calmodulin-binding peptides. In order to assess whether the LeCRK1 protein was capable of binding calmodulin, soluble proteins

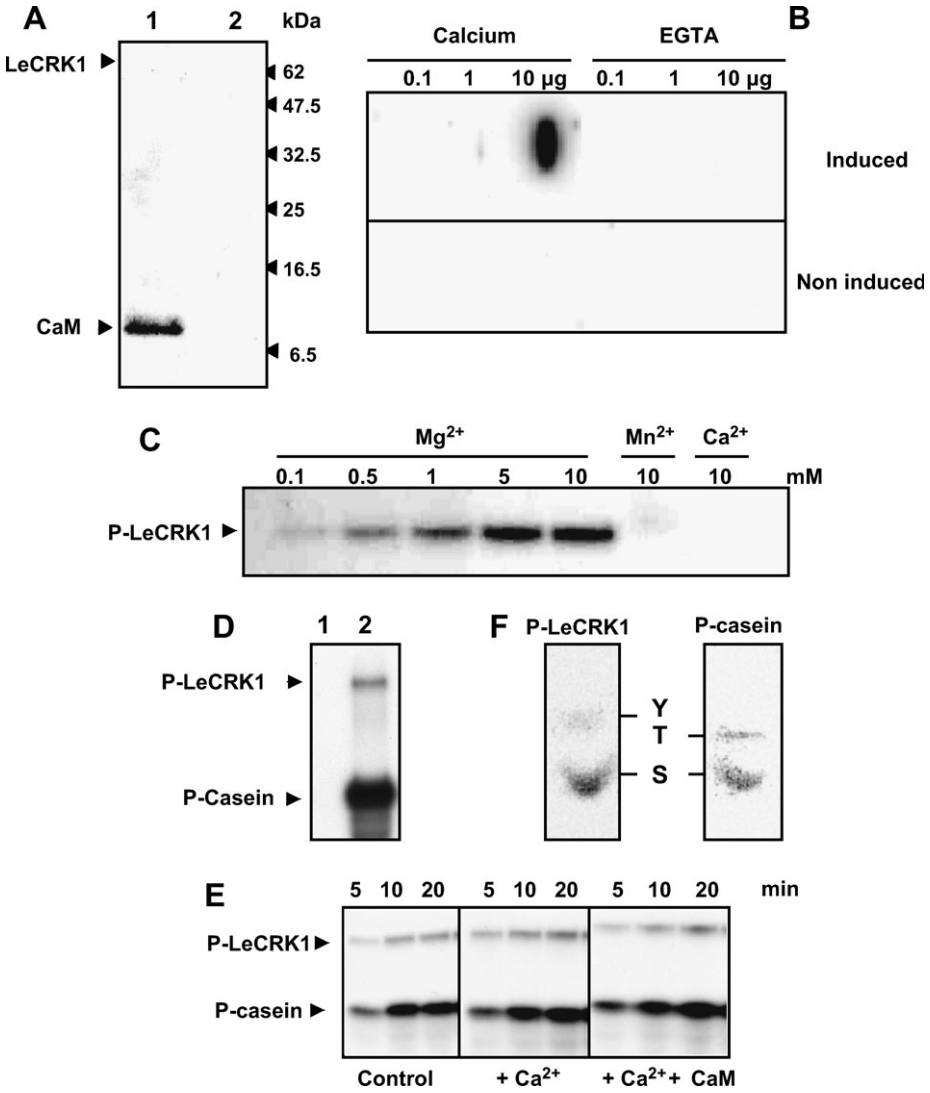

Fig. 2. Biochemical properties of LeCRK1. (A) Calcium-binding assay. Purified calmodulin (2 µg) produced as GST fusion protein in *E. coli* (lane 1) or purified LeCRK1 (5 µg) produced in *E. coli* as 6×His-tagged protein (lane 2) were separated on SDS-PAGE. Following transfer onto nitrocellulose membrane and incubation in the presence of $^{45}CaCl_2$, bound calcium was detected by autoradiography. Molecular weight markers are given on the right side and the position of full-length LeCRK1 and CaM proteins are indicated on the left. (B) Calmodulin-binding activity. Bacterial lysates were obtained from *E. coli* strains expressing the *LeCRK1* fusion construct and grown in inducing or in non-inducing medium. Aliquots of soluble fraction (0.1, 1, and 10 µg protein) from each cell lysate sample were spotted onto nitrocellulose membrane. The membrane was incubated with a ^{32}P-radiolabelled CaM probe in the presence of either $CaCl_2$ (1 mM) or EGTA (5 mM) and bound CaM was detected by autoradiography following membrane washing. (C) Autophosphorylation analysis. Purified LeCRK1 (1 µg) was incubated in buffer containing 1 µCi [γ-^{32}P]ATP and either $MgCl_2$, $CaCl_2$, or $MnCl_2$ (from 0.1 up to 10 mM). Because no autophosphorylation was observed with $CaCl_2$ or $MnCl_2$, the figure shows only the 10 mM concentration. After SDS-PAGE, autophosphorylation was detected by autoradiography. (D) LeCRK1 phosphorylation activity using casein as substrate. Protein fraction (1 µg) from non-induced (lane 1) or induced bacteria (lane 2) were incubated in buffer containing 1 µCi [γ-^{32}P]ATP, 10 mM $MgCl_2$, and casein (10 µg). After SDS-PAGE, autophosphorylation and kinase activity were detected by autoradiography. (E) Effect of calcium and calmodulin on LeCRK1 kinase activity. Purified LeCRK1 (1 µg) was incubated in buffer containing 1 µCi [γ-^{32}P]ATP, 10 mM $MgCl_2$ and casein (10 µg) in the absence (control) or presence of 1 mM $CaCl_2$ with or without 0.5 µg CaM. After 5, 10, and 20 min incubation at 30 °C, aliquots of kinase assay were withdrawn and analysed by SDS-PAGE and subsequent autoradiography. (F) Determination of phosphorylated residues. Phosphoamino acids from phosphorylated LeCRK1 protein and casein were separated by TLC and visualized by autoradiography (Y, threonine; S, serine; T, tyrosine).

from bacteria expressing *LeCRK1* were isolated and used in a calmodulin-binding assay. As shown in Fig. 2B, lysate from bacteria expressing *LeCRK1* exhibited a calcium-dependent calmodulin-binding activity that can be abolished in the presence of EGTA. As a control, lysate from non-induced bacteria failed to show calmodulin-binding activity. These data indicate that LeCRK1 behaved as a calmodulin-binding protein which cannot bind calcium in the absence of calmodulin.

Kinase activity

To ascertain whether LeCRK1 has protein kinase activity, partially purified bacterially expressed LeCRK1 protein was incubated in a buffer containing divalent cations and radio-labelled ATP. As shown in Fig. 2C, LeCRK1 was capable of autophosphorylation only in the presence of Mg^{2+} but not in the presence of Mn^{2+} or Ca^{2+} alone. Furthermore, the protein preparation efficiently phosphorylated conventional protein kinase substrates such as casein (Fig. 2D). Autophosphorylation activity as well as casein phosphorylation were not observed in the protein fraction recovered from non-induced bacteria (Fig. 2D). Neither autophosphorylation nor casein kinase activity was obviously affected by the addition of calcium or calmodulin (Fig. 2E).

Amino acids analysis

To determine the phosphorylated residues, autophosphorylated LeCRK1 protein and phosphorylated casein were hydrolysed with acid and the resulting products were separated by TLC. As shown in Fig. 2F, autophosphorylation occurred mostly on serine residue while casein phosphorylation occurred preferentially on serine but also on threonine residues. These data indicate that LeCRK1 is an active serine/threonine protein kinase.

Subcellular localization of LeCRK1

To investigate the *in situ* subcellular localization of LeCRK1, confocal laser scanning microscopy combined with transient heterologous expression of constructs fusing in-frame the coding sequences of LeCRK1 and green fluorescent protein (GFP) were transfected into BY-2 tobacco cells. When expressed in BY-2 tobacco protoplasts under the control of the double cauliflower mosaic virus 35S promoter, the LeCRK1::GFP fusion protein was targeted to the plasma membrane (Fig. 3B). This pattern was clearly distinct from that observed when GFP alone was expressed (Fig. 3D). To investigate the structural basis of membrane association, Gly2 and Cys4/Cys5, the potential N-terminal sites for myristoylation and palmitoylation of LeCRK1, respectively, were changed to Ala by site-directed mutagenesis. These mutations dramatically altered the normal targeting of the protein. As shown in Fig. 3C, changing the Gly2 residue by an Ala residue resulted in a cytoplasmic localization of the mutated protein. By contrast, when Cys4/Cys5 at the potential palmitoylation sites, were changed into Ala, the mutant protein was targeted mainly to the cell periphery, but fluorescence was also observed all over the cytoplasm (Fig. 3D). Taken together, these data strongly suggest that LeCRK1 is localized at the plasma membrane, and that dual acylation of the protein is required for proper membrane association.

Expression pattern of LeCRK1

Expression analysis by RT-PCR using the *Ubi3* gene as the internal control revealed that *LeCRK1* mRNA was detected in various plant tissues such as leaf, stem, root, flower, and red tomato fruit but not in mature green fruit (Fig. 4A). Moreover, the level of *LeCRK1* transcripts increased substantially during fruit ripening (Fig. 4B). *LeCRK1* transcripts accumulated to the highest levels in fruit collected at the red stage [69 d post-anthesis (dpa)] and thereafter declined. Interestingly, *LeCRK1* mRNA were undetectable in fruit from several tomato ripening mutants such as *Nr* (*Never-ripe*), *Nor* (*Non-ripening*), and *Rin* (*Ripening inhibitor*). The expression pattern of *LeCRK1* paralleled that of *E8*, a ripening-associated gene, although *E8* transcripts were dramatically more abundant (Fig. 4B).

Accumulation of *LeCRK1* transcripts in response to abiotic stimuli and hormonal treatments was also investigated in leaves. Figure 4C shows that *LeCRK1* transcripts increased with 5 h mechanical wounding and strongly accumulated following cold exposure (2 d at 5 °C). A 5 h treatment with ethylene (50 $\mu l\ l^{-1}$) or SA (4 mM) slightly induced *LeCRK1* transcript accumulation relative to control treatments, air and water, respectively. By contrast, MetJA (4 $\mu l\ l^{-1}$) treatment did not affect *LeCRK1* mRNA levels. A stress-inducible gene, *glucanase*, used as a positive control for the stress and hormone treatment (Jia and Martin, 1999; Ding *et al.*, 2002), showed increased accumulation of its transcripts in all treatments performed (Fig. 4C).

Discussion

CDPKs have been isolated from protozoa, algae, and a wide variety of higher plant species. The calmodulin-like domain in most CDPKs contains Ca^{2+}-binding EF-hands that allow the protein to function as a Ca^{2+} sensor (Cheng *et al.*, 2002; Ludwig *et al.*, 2004). CDPKs generally have four EF-hands, although one, two or three functional EF-hands are also common. Each functional EF-hand is thought to bind one calcium ion. Plant CRKs have been identified in *Oryza sativa* (Zhang *et al.*, 2002), *Zea mays* (Lu *et al.*, 1996), and *Daucus carota* (Lindzen and Choi, 1995). They differ from other CDPKs primarily because they display degenerated EF-hand motifs which affect their ability to bind Ca^{2+}. LeCRK1 is the first member of this group to be isolated from tomato. It differs significantly from previously characterized CDPKs from tomato, both in the C-terminal CaM-like domain and in the N-terminal variable domain (Chico *et al.*, 2002; Rutschmann *et al.*, 2002).

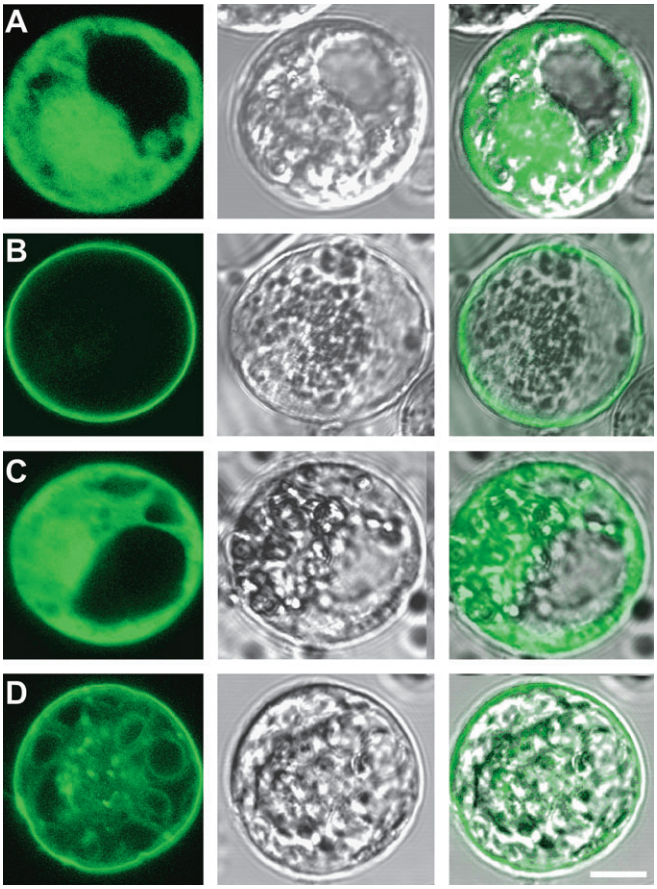

Fig. 3. Subcellular localization of LeCRK1-GFP fusion proteins. GFP-fusion proteins were transiently expressed in protoplasts from BY-2 tobacco cells and localization was analysed by confocal laser scanning microscopy (left panels). Light pictures (middle panels) and merged pictures of the green fluorescence channel with the corresponding light micrographs are shown on the right panels. (A) Control cells with GFP alone; (B) wild-type LeCRK1; (C) the Gly2Ala site-directed mutant of LeCRK1; (D) the Cys4Ala/Cys5Ala site-directed mutant of LeCRK1. The scale bar indicates 10 μm.

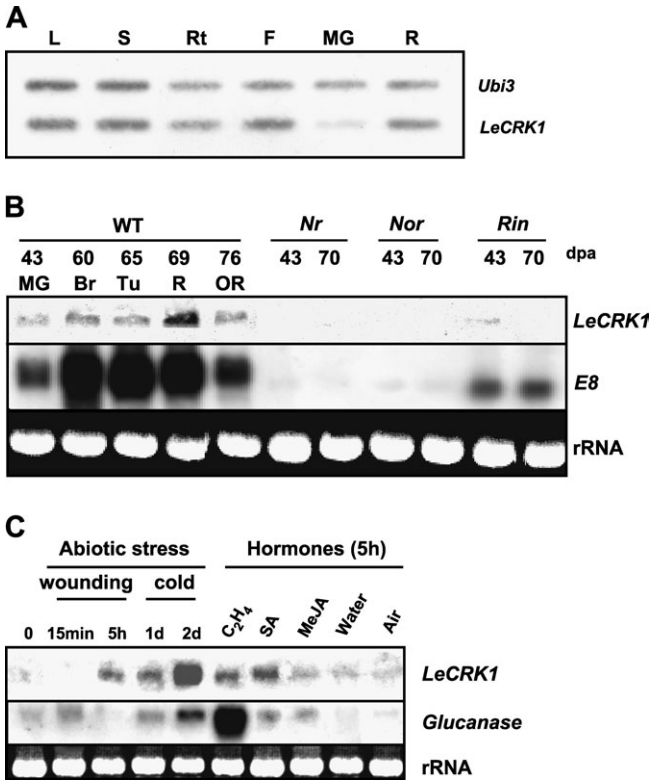

Fig. 4. Expression pattern analysis of *LeCRK1*. (A) RT-PCR analysis of *LeCRK1* mRNA accumulation in leaf (L), stem (S), root (Rt), flower (F), mature green (MG) and red (R) fruit from tomato plants. *Ubi3* was co-amplified as an internal control (Jones *et al.*, 2002). (B) Steady-state of *LeCRK1* mRNA during tomato fruit ripening (WT). Northern analyses were carried out using fruit at the following ripening stages: mature green (MG), breaker (Br), turning (Tu), red (R), and overripe (OR). *LeCRK1* transcript accumulation was also analysed in natural ripening mutants, *NR* (*Never-Ripe*), *Rin* (*Ripening Inhibitor*), and *Nor* (*Non-Ripening*), at two different ripening stages. The fruit stages are also indicated by days post-anthesis (dpa). Expression of *E8,* a ripening-related gene, was analysed in the same samples. (C) Northern blot analysis of *LeCRK1* transcript levels in leaves in response to a 5 h ethylene (C_2H_4), salicylic acid (SA), and methyl jasmonate (MetJA) treatments. The control treatments were carried out with air for gas hormones and with water for soluble hormone. For abiotic stress, wound treatment was applied for either 15 min or 5 h and cold treatment was applied for 1 d and 2 d. The expression of basic *glucanase*, a stress-induced and ethylene-responsive gene was also analysed. For each northern blot analysis, equal loading of the RNA samples on the gel was checked by ethidium bromide-staining of the ribosomal RNA (rRNA).

The kinase domain of LeCRK1 contains all highly conserved sequence motifs characteristic of serine/threonine kinases. It is shown here that LeCRK1 is capable of autophosphorylation *in vitro* and is also active on conventional substrates such as casein, as well as myelin basic protein and histones (data not shown) in the presence of Mg^{2+}. It is noteworthy that, despite the presence of both putative calcium- and calmodulin-binding motifs, kinase activity was neither stimulated by the addition of calcium nor by calmodulin and persisted in the presence of EGTA, indicating that, at least *in vitro*, LeCRK1 activity is not calcium-dependent. This contrasts with the calcium-dependent activity of most CDPKs (Harmon *et al.*, 2000), but is in line with observations of other CRK and is most likely the result of the degenerate EF-hands in the CaM-like domain.

Despite the sequence divergence from typical CDPK, LeCRK1 was found to bind calmodulin in a Ca^{2+}-dependent manner. Functional calmodulin-binding sites have been identified within the auto-inhibitory domains of CDPK from soybean (Yoo and Harmon, 1996) and *Arabidopsis* (Binder *et al.*, 1994) and this basic amphiphilic sequence motif is well conserved in LeCRK1. Given that the *in vitro* kinase activity of LeCRK1 was not affected by the addition of calmodulin, the significance of calmodulin binding remains unclear. It appears, however, that Ca^{2+}-dependent calmodulin binding is unable to mimic the Ca^{2+}-dependent intramolecular activation associated with functional EF-hands in other CDPK proteins. Zhang *et al.* (2002) have suggested that CaM binding to a rice CRK,

OsCBK, may act as an accessory form of regulation, in which CaM-binding promotes phosphorylation of the protein by other kinases or, alternatively, that CaM-binding induces a conformational change altering cellular location or substrate specificity.

Generally there is only minimal conservation of the N-terminal domain of CDPK proteins and little is known about its function. However, in addition to Ca^{2+} activation, CDPK regulation can also rely on protein targeting to a specific subcellular compartment, and amino acids within the N-terminal domain have been shown to be involved in subcellular targeting of a number of CDPKs (Martin and Busconi, 2000; Dammann *et al.*, 2003, Raices *et al.*, 2003). CDPKs have been found in various subcellular compartments including the cytosol, nucleus, cytoskeleton, and membrane fractions (Putnam-Evans *et al.*, 1989; Schaller *et al.*, 1992; Martin and Busconi, 2001). Like many other CDPKs, LeCRK1 has a myristoylation consensus sequence and a potential palmitoylation site at its N-terminus, indicating putative association to membranes. *In vitro* modification by myristoylation has been observed in CDPKs from various plant species, and lipid modification has been shown to be essential for CDPKs targeting to membranes such as the plasmalemma and the endoplasmic reticulum (Martin and Busconi, 2000; Lu and Hrabak, 2002). In addition, N-terminal myristoylation has also been shown to facilitate protein–protein interactions (Johnson *et al.*, 1994). Many CDPKs have potential amino terminal acylation sites, and lipid modification by myristoylation and palmitoylation has been reported to play a critical role in the association of CDPKs with membranes (Thompson and Okuyama, 2000; Dammann *et al.*, 2003, Raices *et al.*, 2003). The present data on the subcellular localization studies carried out using a GFP-tagged protein indicated that LeCRK1 is exclusively located in the plasma membrane, and that this targeting is directed by the N-terminal consensus myristoylation sequence. Using site-directed mutagenesis, it was demonstrated that alteration of the amino terminal myristoylation site of LeCRK1 prevents membrane association. LeCRK1 also contains potential N-terminal palmitoylation sites and mutation of the associated residues results in destabilizing the genuine targeting of the protein, indicating that palmitoylation is essential for stabilizing the anchoring of the protein to the plasma membrane. Therefore, proper targeting of LeCRK1 to the plasma membrane requires a dual acylation mechanism.

Plants have many CDPK isoforms which have been shown to respond to both environmental and endogenous signals and to elicit cellular responses to these factors (Harmon *et al.*, 2000; Ludwig *et al.*, 2004). Investigations into the expression pattern of *LeCRK1* revealed both developmental and environmental regulation. The increase in *LeCRK1* transcript accumulation during late stages of fruit ripening also suggests its involvement in the ripening process. That LeCRK1 is associated with ripening is corroborated by the absence of detectable *LeCRK1* transcripts in the ripening mutants *Nr* (Wilkinson *et al.*, 1995), *Nor* (Tigchelaar *et al.*, 1978), and *Rin* (Vrebalov *et al.*, 2002) known to have major defects in the ripening process. Interestingly, *E8*, a ripening-regulated gene (Deikman and Fischer, 1988) also shows increased expression during ripening and dramatic inhibition in the tomato-ripening mutants. While calcium is generally seen as protective in reducing the rate of plant senescence and fruit ripening (Ferguson, 1984), direct evidence for its involvement in the ripening process is still lacking. Interestingly, screening for ethylene-regulated genes in tomato fruit has recently allowed the isolation of a cDNA clone (ER66) that shows ripening and ethylene-dependent expression (Zegzouti *et al.*, 1999). An *Arabidopsis* clone highly homologous to *ER66* has been shown to encode a calmodulin-binding protein that may function as transcriptional regulator based on its nuclear localization and the presence of a DNA-binding motif (Yang and Poovaiah, 2000; Reddy *et al.*, 2000). These new findings raise a real possibility that calcium is involved in the signalling mechanism, driving the ripening process. The isolation of LeCRK1, a calmodulin-binding protein, reinforces this hypothesis since it is shown here that it is up-regulated during fruit ripening.

LeCRK1 transcript levels are low in unstressed leaves but increase in response to wounding and cold treatment. Cold treatment has previously been shown to enhance the activity of a rice CDPK (Martin and Busconi, 2001), and over-expression of the rice CDPK, OsCDPK7, confers cold and salt tolerance in the transgenic tissues (Saijo *et al.*, 2000). A cDNA clone encoding a CDPK from strawberry has also been shown to be expressed during fruit ripening and upon cold treatment (Llop-Tous *et al.*, 2002). *LeCRK1* transcript levels increased in leaves of tomato plants treated with the hormones ethylene and SA. *CDPK* mRNA accumulation has been shown previously to be induced by GA, ABA, cytokinin (Yoon *et al.*, 1999), indole-3-acetic acid (Davletova *et al.*, 2001), and brassinolide (Yang and Komatsu, 2000). Both ethylene and SA are implicated in eliciting responses to both biotic and abiotic stresses and may play a role in the stress induction of *LeCRK1*.

Transcripts for a previously identified tomato *CDPK*, *LeCDPK1*, increase transiently in plants subjected to mechanical wounding, both at the wound site and in non-wounded leaves (Chico *et al.*, 2002). The increase observed in *LeCDPK1* mRNA upon wounding correlates with an increase in the activity of a soluble CDPK detected in extracts of tomato leaves. *CDPKs* from many species have been shown to be involved in stress responses (Cheng *et al.*, 2002) and induction of *LeCRK1* gene expression by cold and wounding in tomato leaves may also indicate a role of the encoded protein in abiotic stress responses. Full elucidation of the physiological function of the *LeCRK1* gene will require the generation of transgenic lines altered in its expression; however, down-regulation of this gene in

the ripening mutants is indicative of its role in signalling pathways associated with the ripening process.

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