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Regulation of CDPK isoforms during tuber development

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Abstract

CDPK activities present during tuber development were analysed. A high CDPK activity was detected in the soluble fraction of early stolons and a lower one was detected in soluble and particulate fractions of induced stolons. The early and late CDPK activities displayed diverse specificity for *in vitro* substrates and different subcellular distribution. Western blot analysis revealed two CDPKs of 55 and 60 kDa that follow a precise spatial and temporal profile of expression. The 55 kDa protein was only detected in early-elongating stolons and the 60 kDa one was induced upon stolon swelling, correlating with early and late CDPK activities. A new member of the potato CDPK family, *StCDPK3*, was identified from a stolon cDNA library. Gene specific RT-PCR demonstrated that this gene is only expressed in early stolons, while the previously identified *StCDPK1* is expressed upon stolon swelling. This expression profile suggests that StCDPK3 could correspond to the 55 kDa isoform while StCDPK1 could encode the 60 kDa isoform present in swelling stolons. StCDPK1 has myristoylation and palmitoylation consensus possibly involved in its dual intracellular localization. Transient expression studies with wild-type and mutated forms of StCDPK1 fused to GFP were used to show that subcellular localization of this isoform is controlled by myristoylation and palmitoylation. Altogether, our data suggest that sequential activation of *StCDPK3* and *StCDPK1* and the subcellular localisation of StCDPK1 might be critical regulatory steps of calcium signalling during potato tuber development.

Introduction

Potato tuberization is an ideal model system to study the regulation of gene expression during organ development. Potato plants produce tubers as a result of the changing balance of endogenous growth regulators, which is brought about by the plant's ability to perceive changing environmental conditions (Cutter, 1978).

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AF115406 (*StCDPK1)*, AF418563 (*StCDPK2)* and AF518003 (*StCDPK3).*

During the early stages of tuber formation the stolons alter their growth habit, displaying a cessation of elongation and the initiation of subapical radial growth. Increased cell division and expansion are followed rapidly by a massive deposition of starch and storage proteins as a result of the coordinated expression of genes involved in starch and protein biosynthesis (Prat *et al*., 1990; Visser *et al*., 1994). The transformation of stolons into tubers impacts greatly on the whole plant physiology because developing tubers subsequently become the largest sinks present.

There have been many reports of the effects of growth regulators on tuberization in potato, however no unequivocal 'tuberising' factor has been identi-

fied (Jackson, 1999). This is perhaps due to the fact that tuberization is a complex sequence of independently regulated events (Ewing and Struik, 1992; Jackson, 1999). For example, the arrest of stolon growth (Vreugdenhil and Struik, 1989), the initiation of radial growth (Catchpole and Hillman, 1969; Mingo-Castel *et al*., 1976) and starch deposition (Müller-Röber *et al*., 1992; Park, 1990) are all events that have been shown to be capable of occurring independently of each other.

Calcium plays a main role in plant physiology (Poovaiah and Reddy, 1993; Bush, 1995; Sanders *et al*., 2002). In particular, it has been shown that intracellular calcium is necessary for tuber development and that calmodulin antagonists can inhibit this process (Balamani *et al*., 1986). Transient increases in cytosolic calcium, transduced via calcium-binding proteins, likely affect protein kinases to transduce various external signals (Sopory and Munshi, 1998).

In plants, calcium-dependent calmodulin-independent protein kinases (CDPKs) are key intermediates in calcium-mediated signalling that couple changes in Ca^{2+} levels to a specific response. These enzymes have a unique structure consisting of an amino-terminal catalytic domain fused to a carboxy-terminal calmodulin-like domain (CLD) with four EF-hand Ca^{2+}-binding sites, and require only micromolar concentrations of free Ca^{2+} for their activity (Harmon *et al*., 1986; Harper *et al*., 1991; Roberts and Harmon, 1992). CDPKs are found in various subcellular localizations which suggests that this family of serine/threonine kinases may be involved in multiple signalling pathways (Lu and Hrabak, 2002). Enhanced CDPK activity/expression has been linked to different stress responses (Sheen, 1996) and numerous environmental stimuli (Botella *et al*., 1996; Pestenacz and Erdei, 1996; Yoon *et al*., 1999). However, the signal transduction pathways involving these kinases still remain unclear and very little is known about which particular CDPK acts as the calcium sensor in each case (Cheng *et al*., 2002).

In a previous paper (MacIntosh *et al.*, 1996), we reported a temporal correlation between an increase in CDPK activity and the morphological changes associated with the onset of tuber development using *in vitro* cultured potato stolons. We have identified StCDPK1, an active CDPK isoform transiently induced in swelling stolons (Raíces *et al*., 2001). However, there was no temporal correlation between CDPK activity and *StCDPK1* transcriptional activation.

In this study we analysed the activity and expression of potato CDPK isoforms that follow different expression profiles during tuberization. Two CDPK activities, which display diverse specificity for *in vitro* substrates and different subcellular localization, were detected in early and induced stolons. *StCDPK3*, a new member of the StCDPK family, is transiently expressed in early stolons, correlating with the early enzymatic activity. *StCDPK3* expression declines upon stolon swelling, before the induction of *StCDPK1*, a later isoform which displays a dual subcellular localization possibly mediated by post-translational acylation.

Materials and methods

Plant material

Potato plants, *Solanum tuberosum* L. cv. Spunta, were cultivated in a greenhouse under a regime of 16 h light (25 °C) and 8 h dark (20 °C). Leaves, thin stolons, induced stolons with evident swelling tips (0.8–6 mm diameter, weighing less than 0.1 g) and mature tubers from potato greenhouse plants were analysed. Micropropagation of virus-free meristematic sprouts was carried out in MS medium containing 30 g/l sucrose as described (MacIntosh *et al*., 1996). Four *in vitro* stages of tuber development were obtained by growing plants in MS medium containing 80 g/l sucrose with the addition of 5 mg/ml chlorocholine chloride (Sigma) in total darkness. Thin stolons were grouped in stage 1 while stages 2–4 are equivalent with induced stolons from greenhouse plants (0.8–6 mm diameter, weighing less than 0.1 g). In general, we refer to thin stolons as early stolons and to those with an evident swelling tip as induced stolons.

Protein extraction and CDPK activity

Early stolons, induced stolons, mature tubers and leaves from greenhouse plants or *in vitro* tuberization stages were harvested, rinsed with distilled water, ground in a mortar cooled with liquid nitrogen and extracted as described (Chico *et al*., 2002). The suspensions (1 ml buffer per gram of wet tissue) were centrifuged for 10 min at 2500 × *g* and the pellet was discarded. The supernatant (crude extract) was centrifuged for 1 h at 20 000 × *g* and soluble and particulate fractions were obtained. Pellets were washed with 0.5 ml of extraction buffer and were then re-

suspended in a volume equal to 10% of the volume of the soluble fraction.

CDPK activity was assayed in a reaction mixture containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 μM [γ-^{32}P] ATP (specific activity 100 cpm/pmol), 10 mM 2-mercaptoethanol, 25 μM Syntide-2, with the addition of 1 mM EGTA or 1 mM $CaCl_2$, as described (MacIntosh *et al*., 1996). When indicated, other phosphate acceptors were used. Alternatively, fractions were incubated 5 min at 30 °C with 0.1 mg/ml histone H1, or 0.1 mg/ml tubulin, and 10 μM [γ-^{32}P] ATP (specific activity 500 cpm/pmol) in the presence of 1 mM EGTA or 1 mM $CaCl_2$. Reactions were stopped as described (Chico *et al*., 2002) and subjected to 12% SDS-PAGE. Signals were scanned with a phosphorimager Storm 820 (Amersham Pharmacia Biotech) and quantified with ImageQuant software.

Western blotting

Western blot analysis of the different samples (100 μg) was performed as described (Chico *et al*., 2002) with affinity-purified polyclonal antibodies (1:2000) directed against the calmodulin-like-domain (CLD) of soybean αCDPK (Bachmann *et al*., 1996). Blots were developed with ECL reagent from Amersham according to the manufacturer's procedure.

Recombinant MBP-StCDPK1 fusion proteins

PCR products corresponding to *StCDPK1* (ca. 1800 bp) were amplified with *Pfu* DNA polymerase (Promega) with primers containing *Bam*H1 and *Hin*dIII restriction sites and subcloned into pMAL-c2 expression vector (Biolabs), in frame with maltose-binding protein (MBP). The resulting fusion protein, MBP-StCDPK1, was obtained after induction of transformed BL21 *Escherichia coli* cell cultures with 1 mM IPTG for 2 h. A negative control was performed without IPTG. Induced and non-induced cultures (OD_{600nm} ca. 1) were pelleted and re-suspended in equal volumes of 1× cracking buffer. A 10 μl aliquot of each culture was subjected to 10% SDS-PAGE, transferred onto nitrocellulose membranes and incubated with an anti-soybean CDPK antibody (1:2000) as described.

Isolation and sequencing of StCDPK3

A λZAP II cDNA library constructed from mRNA from tuberizing stolon tips (Taylor *et al*., 1998) was screened with a potato fragment spanning the conserved CDPK catalytic sequence from sub-domain VIb onwards to the hinge region as described (Raíces *et al*., 2001). After three rounds of screening, a novel StCDPK clone designated *StCDPK3*, was purified and excised from hybridizing phage into *Escherichia coli* JM109 strain with helper phage R408 (Short *et al*., 1988). Double-stranded phagemid DNA for sequencing was prepared with the Qiagen Plasmid Midi Kit. Automated sequencing was performed with pBluescript SK+/− Reverse and T7 primers. Assembly and analysis of DNA sequence data was done with software available at NCBI's Advanced BLAST sequence similarity search.

RNA isolation, gene-specific RT-PCR for StCDPKs and Southern blot analysis

Total RNA was isolated from 0.1 g of early stolons, induced stolons and mature tubers from greenhouse plants with the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. After treating each RNA (10 μg) with DNAse-free RNAse (Promega), the corresponding cDNAs were synthesized with M-MLV-Reverse Trancriptase (Promega) with 2 μg of each DNAse-treated RNA as template. Control reactions to check equal amounts of cDNA were performed with specific primers (5′-ATGCAGATCTTTGTGAAGAC-3′ and 5′-ACCACCACGGAGACGGAG-3′) to amplify a 250 bp Ubiquitin fragment (20 cycles, annealing temperature 55 °C).

Semi-quantitative RT-PCR was carried out according to Bauer *et al*. (1994) with specific primers to exclusively amplify each StCDPK mRNA (*StCDPK1*, *StCDPK2* and *StCDPK3*). A common 5′ primer, 5′ *St* (5′-GGAAGCTGCTGATGTGGATGG-3′) present in the three StCDPK sequences was combined with three different 3′ primers derived from the 3′-UTR region of each kinase (UTRSt1, UTRSt2 and UTRSt3, indicated in Figure 4B). Control reactions with plasmids (5 ng) yield amplified fragments corresponding to *StCDPK1*, *StCDPK2* and *StCDPK3* of 419, 385 and 498 bp respectively. Conserved primers R5 (5′-GATTTTGGGCTGTCCATGTTCATT-3′) and R4 (5′-AAGTTCTTGAGCATCCTTGG-3′), indicated in Figure 4A, were used to amplify a 340 bp fragment used as positive control. PCRs were performed for 30 cycles with Platinum *Taq* polymerase (Invitrogen). The annealing temperature was 64 °C for *StCDPK1* and *StCDPK2* and 61 °C for *StCDPK3*.

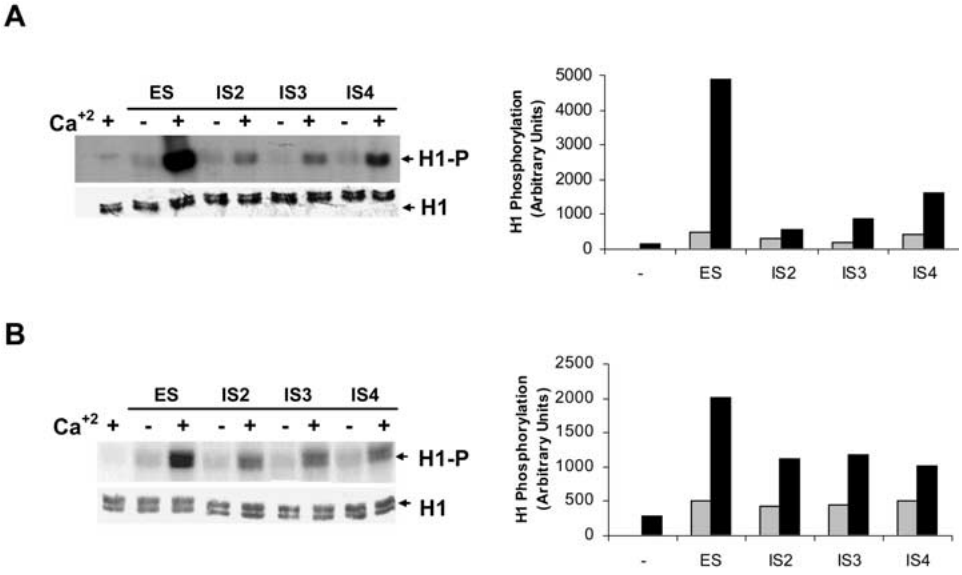

Figure 1. Histone H1 phosphorylation assays. Protein extracts (4 μg) from early (ES) and induced stolons (IS2-IS4) from *in vitro* tuberization stages (A) or from greenhouse plants (B) were incubated with 0.1 mg/ml histone H1 and 10 μM [γ-^{32}P] ATP in the presence of 1 mM EGTA (−) or 1 mM $CaCl_2$ (+). Phosphorylated samples (H1-P) were analysed on 12% SDS-PAGE. Histone loading (H1) stained with Coomassie blue is indicated. A negative control without protein extract was performed in each case (first lane). Relative H1 phosphorylation levels are plotted on the right. Signal was scanned with a phosphorimager Storm 830.

RT-PCR products were separated on 1% agarose gels, photographed and blotted under denaturing conditions onto Hybond N+ positively charged nylon membranes (Amersham) according to the manufacturer's procedures. Probes against *StCDPK1* and *StCDPK3* generated by PCR with primers R12 and R13 (indicated in Figure 4A) or R5 and UTRSt3, respectively, were labelled with [α-^{32}P]-dCTP (10^9 cpm/pmol) with the RadPrime DNA labelling system Kit (Invitrogen). Membranes were prehybridized in Church's buffer and hybridized for 2 h with the [^{32}P]-labelled probes at 65 °C in the same buffer. Stringent washes (2× SSC, 1× SSC and 0.1× SSC with 0.1% SDS) were performed for 20 min at 65 °C. Signals were scanned with a Storm 830 phosphorimager.

Construction of StCDPK1-GFP fusions and transient expression in onion cells

Translational C-terminal fusions with GFP to StCDPK1 were done by cloning PCR products corresponding to *StCDPK1* downstream of a duplicated 35S promoter at the *Xho*I and *Nco*I restriction sites of the pPK100 vector (Despres *et al.*, 2001). The 5′ primer used to amplify the wild-type StCDPK1 contained the *Xho*I restriction site and the first six amino acid residues of the kinase (5′-CCG**CTCGAG**GAGATAAATGGGTGTTTGTTTG-AGC-3′). Mutated forms of StCDPK1 with an altered myristoylation site, a palmitoylation site or both were generated by PCR amplifications with modified 5′ oligonucleotides. A common 3′ primer (5′-CATG**CCATGG**TTTCGAGTTCATCTCTTGTGA-3′) containing the *Nco*I restriction site was used for all the PCRs. Mutated versions of StCDPK1 were cloned in the same vector at the same position (Myr-StCDPK1-GFP, Pal-StCDPK1-GFP and Myr/Pal- StCDPK1-GFP). The four constructs were delivered into onion (*Allium cepa*) epidermal cells (Scott *et al.*, 1999) with a biolistic PDS-1000/HeTM particle gun (BioRad). Three bombardments were performed for each construction (ca. 15 transformed cells/experiment). Cells were observed 24 h later under a laser scanning confocal microscope (Leica DM RXA2). GFP fluorescence was monitored with a band pass filter (488 nm excitation line of a Krypton-Argon laser). Typically, 20–30 focal sections (1 μm each) were obtained for each cell and projected along the Z axis.

Analytical methods

Protein contents were determined with BioRad protein assay reagent. SDS-PAGE was carried out as described by MacIntosh *et al.* (1996). Pre-stained SDS-PAGE standards from Invitrogen were used as molecular weight markers.

Results

CDPK activities associated with early and induced stolons

CDPK activity increases in early stages of *in vitro* tuber development (MacIntosh *et al*., 1996). Recently, we observed the induction of StCDPK1 mRNA in later stages of tuber formation from greenhouse plants (Raíces *et al*., 2001). This temporal difference could be due to variations between *in vitro* and greenhouse culture conditions or could suggest the presence of several CDPK isoforms during the tuberization process.

An H1 phosphorylation assay, indicative of CDPK activity, was performed with soluble protein extracts obtained from early and induced stolons of greenhouse plants and crude extracts from *in vitro* tuberization stages. Both culture systems showed a similar pattern of CDPK activity during tuber development. Calcium-dependent H1 phosphorylation was observed in all stages and was strongly enhanced in early stolons from greenhouse (Figure 1A) and *in vitro* plants (Figure 1B). A lower CDPK activity was detected in induced stolons. Comparable levels of calcium-independent tubulin phosphorylation was observed in all tuberization stages indicating that all extracts were active (data not shown).

In addition, CDPK activity was assayed in soluble and particulate fractions of leaves, early stolons, induced stolons and mature tubers from greenhouse potato plants with Syntide-2 as substrate. High CDPK activity was detected in soluble fractions from early stolons and lower activities were detected in leaves, induced stolons or mature tubers (Figure 2A, Table 1). Particulate CDPK activity was also higher in early stolons, however a substantial activity (3.85 nmol ^{32}P incorporated per minute per mg protein) was also present in the membrane fraction of induced stolons (Figure 2B, Table 1). Subcellular fractionation revealed a significant increase in the proportion of CDPK activity present in the particulate fraction of induced stolons (Table 1). Specific CDPK activity was always higher when associated to the particulate fraction, but only 10–12% of total CDPK activity was associated to the particulate fraction of leaves and early stolons (Table 1).

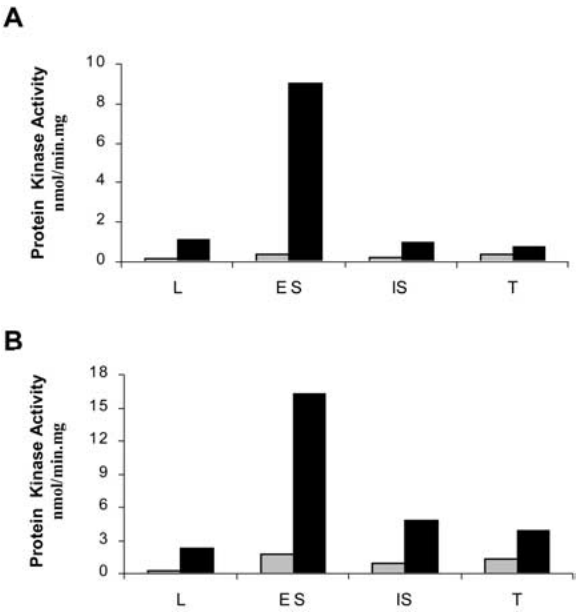

Figure 2. Protein kinase activity in soluble (A) and particulate (B) fractions of leaves (L), early stolons (ES), induced stolons (IS) and tubers (T) from greenhouse plants. A standard kinase assay was performed in the presence of 1 mM EGTA (grey bars) or 1 mM $CaCl_2$ (black bars) with Syntide-2 as substrate. Specific CDPK activity, expressed as nmol of ^{32}P incorporated per minute per mg protein, is the difference between the activities detected in the presence of calcium and EGTA. Values correspond to one experiment representative of three independent ones.

All these data suggest that there are different levels of CDPK activities in early and induced stolons and that the subcellular distribution of these activities varies among the different tissues.

Substrate specificity of different StCDPK activities

Substrate specificity of CDPK activities present in early and induced stolons were carried out using different peptides containing the motif **R**-X-X-**S/T**- (Roberts and Harmon, 1992) identified as a minimal sequence element recognized by many CDPKs (Table 2). Considering activity levels and activation fold, Syntide-2 was the best substrate for early StCDPK activity, followed by GS. The other peptides tested were poor phosphate acceptors. In contrast, late CDPK activity phosphorylated preferentially GS. The early CDPK displayed a higher activity with all substrates tested (Table 2). When Syntide-2 was used as

Table 1. Subcellular distribution of CDPK activities.

	Soluble fraction		Particulate fraction		% membrane-associated CDPK[b]
	Specific activity[a], nmol ^{32}P min^{-1} mg^{-1}	% total activity	Specific activity[a] nmol ^{32}P min^{-1} mg^{-1}	% total activity	
Leaves	0.98	90	1.98	10	ND
Early stolons	**8.60**	**88**	14.54	12	ND
Induced stolons	0.75	55–75	**3.85**	**25–45**	**10.5**
Tubers	0.40	92	2.51	8	ND

[a]CDPK activity is the difference between the activity measured in the presence of 1 mM Ca^{2+} and the activity measured in the presence of 1 mM EGTA with Syntide-2 as phosphate acceptor. The results shown in this table are representative of three independent experiments.
[b]% membrane-associated CDPK was calculated from western blot assays performed with the soybean anti-CDPK antibody with the Image Quant software.
ND, not detected.

phosphate acceptor, an almost 1:10 ratio was observed between early and late CDPK activities from greenhouse extracts (Figure 2 and Table 2). This ratio was reduced to 1:6 or 1:3 with GS or H1 substrates respectively. A similar difference in substrate preference was observed in CDPK activities from *in vitro* extracts (Table 2).

StCDPKs substrate specificity was also explored by analysing phosphorylation of endogenous proteins in crude extracts of *in vitro* tuberization stages. Four bands of 55, 26, 16 and 11 kDa were selectively phosphorylated in early stolons while two polypeptides of 60 and 14 kDa were only phosphorylated in induced stolons (data not shown). The different pattern of phosphorylated proteins observed in each stage could reflect the different calcium dependent phosphorylation/dephosphorylation events that take place during tuberization or could be due to developmental regulation of CDPK targets.

These results indicate that not only CDPK activities and their subcellular distribution differ during tuberization, but also that the specificities of the kinases are different, suggesting the presence of different isoforms in early and induced stolons.

Western blot analysis reveals different CDPK moieties in early and induced stolons

Western blot analyses were performed with a polyclonal antibody against the CLD domain of soybean αCDPK. This antibody recognizes many CDPKs from different plant species (Bachmann *et al.*, 1996) and cross-reacts with MBP-StCDPK1 recombinant protein (Figure 3A). *StCDPK1* encodes an active CDPK of 60 kDa which is only expressed in swelling stolons (Raíces *et al.*, 2001). Its CLD domain shares 59% identity and 79% similarity with the αCDPK one.

When protein fractions of greenhouse plants were analysed, two bands were revealed, one with an apparent molecular mass of 54/55 kDa in soluble extracts of leaves and early stolons, and one of about 60 kDa in soluble and particulate extracts of induced stolons (Figure 3B). No polypeptide was detected in mature tubers even using a rapid extraction of proteins in the presence of protease inhibitors.

In early stolons, both CDPK activity and the 55 kDa band were present in the soluble fraction (Table I). In induced stolons the 60 kDa protein was mainly present in the soluble fraction but CDPK activity was higher in the particulate fraction (Table 1). This could be explained by the presence of inhibitory factors in the soluble fraction, or of positive regulators in the membrane fraction. Indeed, phosphatase inhibitors increased CDPK activity in the soluble fraction (data not shown).

The two bands detected by Western blot could correspond to CDPK isoforms differentially expressed during tuberization or to phosphorylated versions of the same kinase. Autophosphorylation assays and treatments with phosphatase inhibitors ruled out this second possibility (data not shown), supporting the presence of different CDPK isoforms in the different stages of tuber development.

Several CDPK genes are expressed during stolon to tuber transition

Previously, we isolated two clones encoding different StCDPK isoforms from a stolon tip cDNA library, *StCDPK1* (Raíces *et al.*, 2001) and *StCDPK2*, which is highly expressed in leaves (Ulloa *et al.*, 2002). None

Table 2. Substrate specificity of StCDPK isoforms active during tuber development.

Substrate (25 μM)	Early stolons			Induced stolons		
	CDPK activity, nmol min^{-1} mg^{-1}	-fold activation	% P Syntide-2	CDPK activity, nmol min^{-1} mg^{-1}	-fold activation	% P Syntide-2
In vitro plants						
Syntide-2 PLARTLSVAGLPGKK	2.81	8–10	100	0.58	3–7	100
GS PLSRTLSVAAKK	2.66	8–10	94.9	0.99	6–12	169.5
MLCKs KKRPQRATSNVFS	0.77	7	27.4	0.36	4–5	62
CDPKs PLSRTLSVSS	0.03	3	0.9	0.01	1–2	2
Greenhouse plants						
Syntide-2 PLARTLSVAGLPGKK	6.49	7–12	100	0.67	2–6	100
GS PLSRTLSVAAKK	4.42	6–8	68.2	0.75	4–6	113
MLCKs KKRPQRATSNVFS	0.31	2–3	4.75	0.37	2	55.8
CDPKs PLRTLVSS	0.07	1	1.1	–	–	–

CDPK activity is the difference between the activity measured in the presence of 1 mM $Ca^{2+}$and the activity measured in the presence of 1 mM EGTA.
Fold activation is the ratio of activity measured in the presence of calcium to activity measured in the presence of EGTA.
The results shown in this table are representative of three independent experiments. The RXXS motif present in the synthetic peptides is shown.
Syntide-2, GS and CDPKs are peptides derived from glycogen synthase; Syntide-2 and CDPKs are calmodulin-dependent protein kinase II substrates; GS is a PKC substrate, MLCKs is a myosin light-chain kinase substrate. The PKA substrate kemptide was also used as phosphate acceptor but no significant calcium-dependent phosphorylation was detected.

of these transcripts were detected in early stolons suggesting that the early CDPK activity was not related to these isoforms.

New members of the StCDPK family were screened in a stolon cDNA library with a probe spanning a conserved CDPK region. A third clone, *StCDPK3*, containing characteristic features of the CDPK family was identified. *StCDPK2* and *StCDPK3* are partial clones that contain the catalytic domain (from sub-domains VI or VII onwards) fused to the junction region and the four EF-hand calcium-binding motifs of CLD (Figure 4A). Sequence analysis indicated that StCDPK1 and StCDPK2 are highly related (89% identities) while StCDPK3 is more distant (80% identities). The partial cDNA obtained for StCDPK3 allowed to compare equivalent regions from other CDPKs. Both, the CLD domain and the complete sequence of StCDPK3 share the highest identity with LeCPK1 (95–97%) and NtCDPK1 (93–94%) from tomato and tobacco respectively (Rutschmann *et al.*, 2002; Yoon *et al.*, 1999), and are highly homologous to subgroup II AtCPKs (AtCPK21 and AtCPK15; Cheng *et al.*, 2002). As StCDPK1, StCDPK3 shares 60% identity and 79% similarity with the CLD domain from αCDPK from soybean suggesting that the antibody should also recognize this new isoform.

The expression of StCDPK genes was studied by gene-specific RT-PCR (primers are indicated in Figure 4). Each primer pair amplified specifically each StCDPK isoform (data not shown). Total RNA from early stolons (ES), progressively induced stolons (IS1–IS3) and mature tubers of greenhouse plants were normalized with Ubiquitin mRNA primers. On ethidium bromide gels, both *StCDPK1* and *StCDPK2* were detected predominantly in IS2 and to a lesser extent in IS3, but *StCDPK1* was much more abundant than *StCDPK2*. *StCDPK3* was the only gene detected in early stolons (Figure 5A). In addition, RT-PCRs

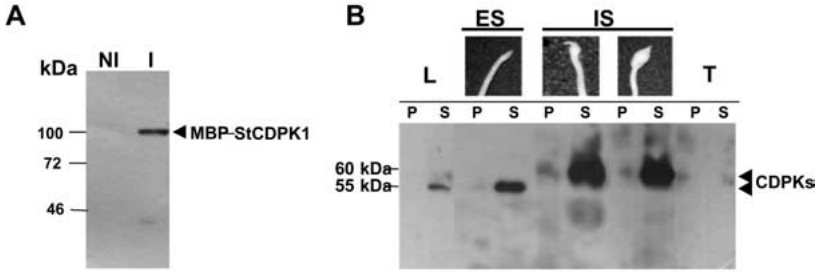

Figure 3. Western blot analysis. A. 10 μl aliquots of induced (I) and non-induced (NI) *E. coli* cell cultures transformed with pMAL-StCDPK1. B. Soluble (S) and particulate (P) protein extracts (100 μg) from leaves (L), early stolons (ES), induced stolons (IS) and tubers (T) from greenhouse plants. Protein extracts were subjected to electrophoresis and blotted. Membranes were incubated with a polyclonal antibody (1:2000) against the CLD domain of soybean CDPK.

performed against a conserved CDPK region in each stage (primers R4 and R5, Figure 4A) gave the expected result based on the expression of each isoform (Figure 5A).

Southern blot hybridization with *StCDPK1* showed a very faint signal in early stolons and in IS1 indicating that low levels of *StCDPK1* transcripts are also present in those stages. No cross-signal was observed for *StCDPK3* bands (Figure 5B, upper panel) but a strong signal was observed in IS2 and IS3 samples (Figure 5B). The *StCDPK3* probe revealed a strong signal in early stolons and a much fainter one in induced stolons (Figure 5B, lower panel). Again, no cross-hybridization of *StCDPK3* and the other genes was observed (Figure 5B, lower panel). As expected, both probes recognized the conserved CDPK fragments (Figure 5B). Thus, this new *StCDPK3* gene is mainly expressed in early stolons and the predominant expression of *StCDPK1* in induced stolons was confirmed.

These results indicate that different StCDPK isoforms are expressed during tuberization and that *StCDPK3* and *StCDPK1* follow a precise expression profile during stolon to tuber transition.

Membrane localization of StCDPK1 depends on myristoylation and palmitoylation

Our results show that the 60 kDa isoform and the late CDPK activity are partially associated with the particulate fraction. It was postulated that certain CDPKs have low activity when located in the cytosol but are activated upon translocation to the membrane (Harmon *et al*., 2000). This could be the case of the late CDPK isoform that is mostly present in the soluble fraction of induced stolons but is active when associated to the membrane fraction (Table 1). The correspondence between StCDPK1 expression profile and the presence of the 60 kDa protein in induced stolons suggests that StCDPK1 could be responsible for the late CDPK activity present in this tissue. StCDPK1 has a functional N-terminal myristoylation consensus (Raíces *et al*., 2001) and a putative palmitoylation site (Figure 4). Therefore it can be suggested that fatty acid acylation could be involved in StCDPK1 association to membranes.

To study the role of myristoylation and palmitoylation on StCDPK1 localization, epidermal onion cells were bombarded with wild-type StCDPK1 or its mutated versions (with altered myristoylation and/or palmitoylation sites) fused to green fluorescent protein, GFP (Figure 6A). Confocal analysis showed that the wild-type StCDPK1 is efficiently targeted to the cell periphery (Figure 6B and C). Plasma membrane localisation was further confirmed by treating the epidermal cells with sorbitol to promote cell plasmolysis. As can be observed in Figures 6F and 6G, the fluorescence corresponding to StCDPK1-GFP is restricted to the plasma membrane and its connections to the cell wall. In contrast, the mutated StCDPK1-GFP fusion proteins were not targeted to the plasma membrane. Soluble localization of StCDPK1 lacking the myristoylation consensus (Figure 6D) resembled that of GFP alone (data not shown) as observed for tomato LeCPK1 (Rutschmann *et al*., 2002). The palmitoylation-deficient mutant (Figure 6E) and the one lacking both consensus sequences (data not shown) also showed a soluble pattern.

These data demonstrate that both N-terminal myristoylation and palmitoylation are essential for plasma membrane targeting of StCDPK1 in onion cells. N-myristoylation was not enough for StCDPK1 membrane localization but might be important to further promote subsequent palmitoylation, a dynamic pro-

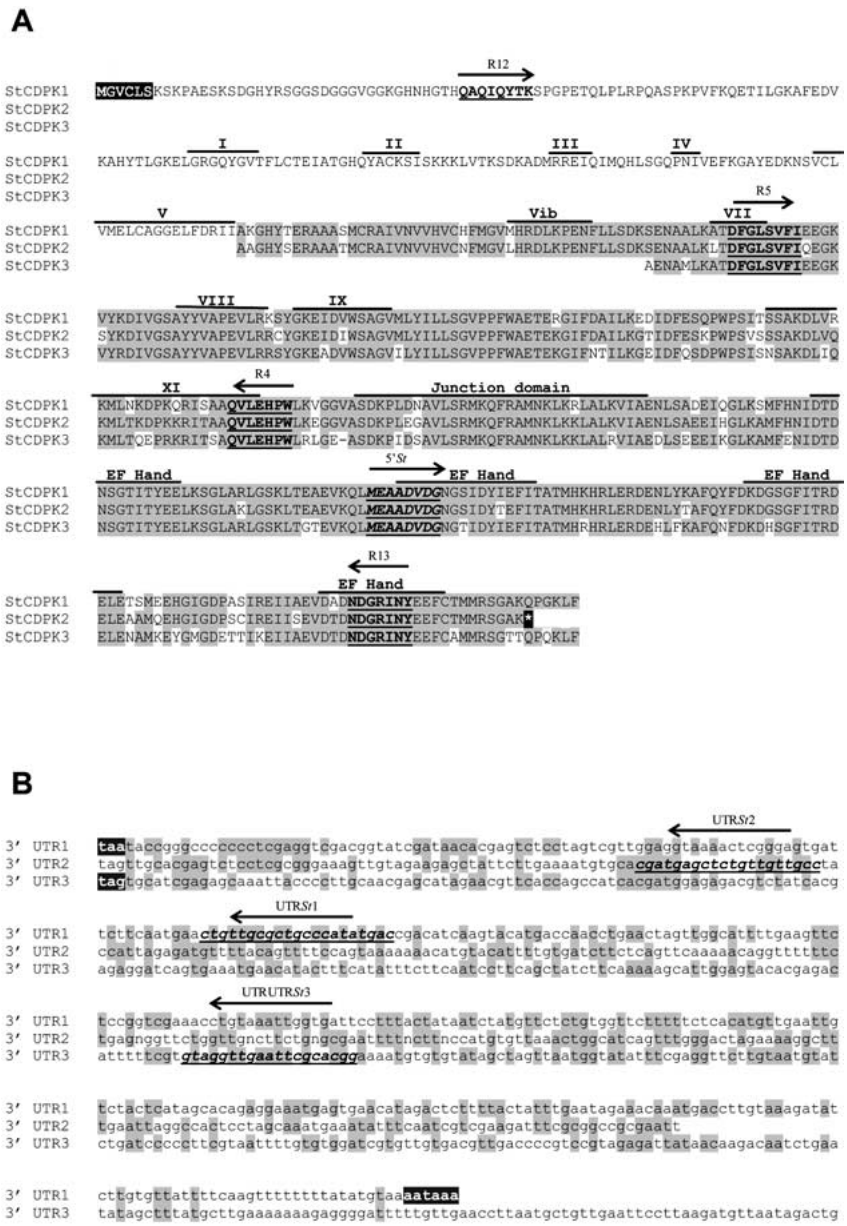

Figure 4. Sequence alignment of the potato CDPK isoforms. A. StCDPK1, StCDPK2 and StCDPK3 amino acid sequences were aligned. Catalytic sub-domains, the junction domain and the EF-hands are indicated. The Myr/Pal consensus of StCDPK1 is indicated with a black box. Identical residues are highlighted in grey. An asterisk in a black box indicates a stop codon in StCDPK2. Arrows indicate the position and direction of the primers used in RT-PCRs, amino acid sequences are underlined and in bold. Conserved primers R4 and R5 were used to amplify the three cloned StCDPKs, primers R12 and R13 were used to amplify the *StCDPK1* fragment used as probe, and 5′ St was the common 5′ primer used to amplify the three isoforms in the gene-specific RT-PCR. B. Alignment of 3′-UTR nucleotide sequences of each *StCDPK* cDNA. Arrows indicate the position of the 3′ primers used for gene-specific RT-PCR, primer sequences are underlined and in bold.

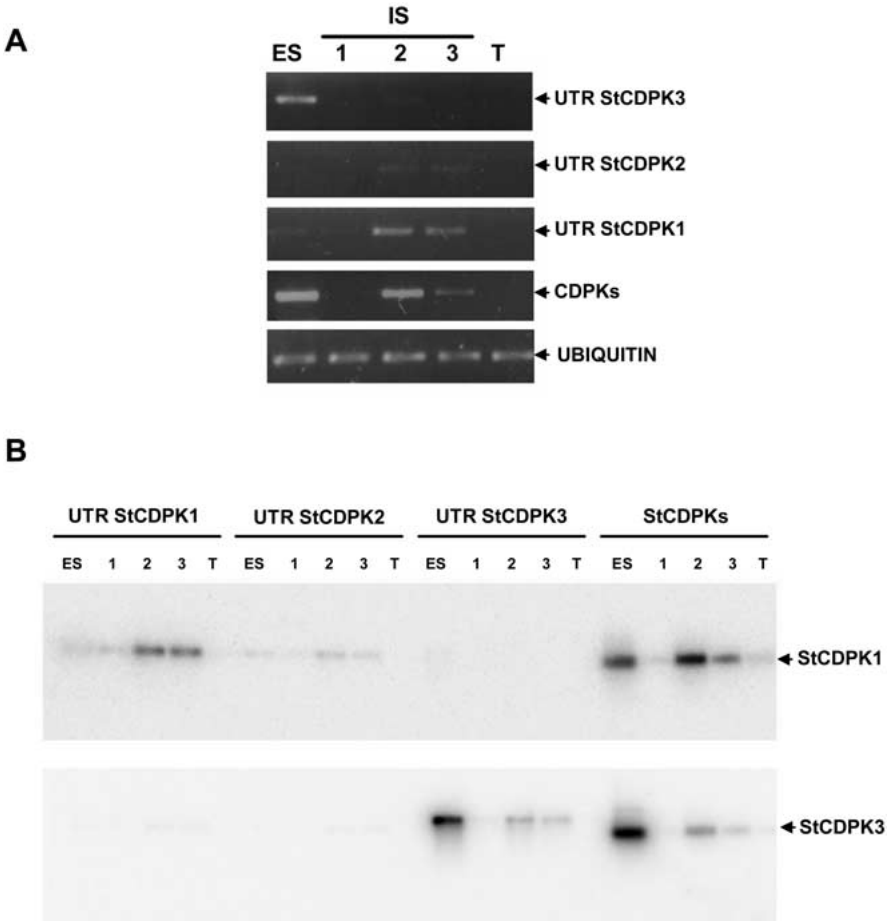

Figure 5. Expression of StCDPK isoforms during tuber development. A. Semi-quantitative RT-PCR analysis of total RNA extracted from early stolons (ES), three stages of induced stolons (IS) and tubers (T) from greenhouse plants. Equal amounts of total RNA were used and normalized using primers that amplify a constitutively expressed Ubiquitin gene. PCR reactions to exclusively amplify each *StCDPK* isoform from cDNAs were performed using the specific primers UTRSt1, UTRSt2 and UTRSt3 (indicated in Figure 4B) or with the conserved primers R4 and R5 (indicated in Figure 4A). The conserved primers were used to amplify all StCDPK isoforms. B. Southern blot 33 of PCR products. Membranes were hybridized with the ^{32}P-labelled *StCDPK1* or *StCDPK3* probes at 65 °C. Signal was scanned with a phosphorimager Storm 830.

cess that could regulate the reversible subcellular distribution of this kinase.

Discussion

CDPKs are present in many cellular processes such as mobilisation of starch during seed germination (Ritchie and Gilroy, 1998), regulation of actin tension (Grabski *et al*., 1998), plant defence (Romeis *et al*., 2000) and stress responses (Chico *et al*., 2002; Sheen, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997). CDPKs are also involved in a wide variety of growth and developmental processes regulated by calcium (Evans *et al*., 2001; Hepler *et al*., 2001). These include pollen tube growth (Estruch *et al*., 1994), pollen tube reorientation (Moutinho *et al*., 1998), embryogenesis, seed development and germination (Anil *et al*., 2000), and sexual organ development (Nishiyama *et al*., 1999). However, how specific CDPKs translate the information encoded in 'calcium signatures' to specifically affect metabolism and gene expression is mostly unknown (Cheng *et al*., 2002).

In this work, we analysed CDPKs present during potato tuberization. Two CDPK activities with different substrate specificity and subcellular distribution were found associated with early or induced stolons. The early CDPK activity, predominantly soluble, was more active than the later one and displayed equal preference for Syntide-2 and GS. In contrast, the late CDPK present in induced stolons preferentially phos-

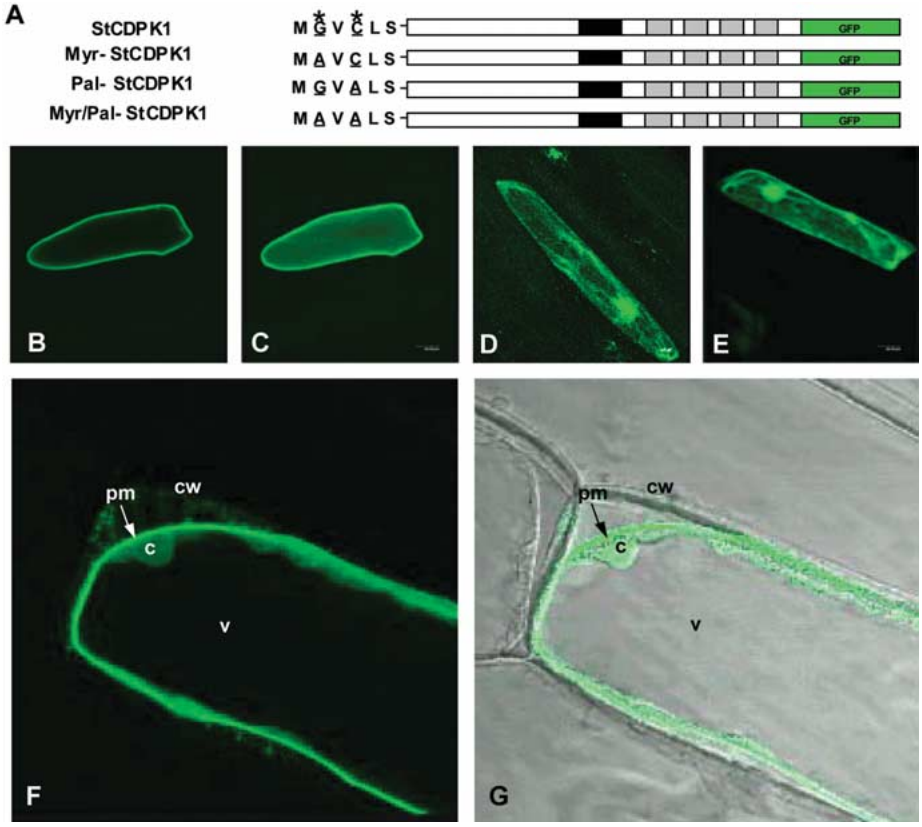

Figure 6. Subcellular localization of StCDPK1-GFP fusion proteins. Wild-type StCDPK1 and N-terminal myristoylation and palmitoylation mutants were transiently expressed as C-terminal GFP-fusion proteins in onion epidermal cells. A. Schematic representation of wild-type and mutant-GFP fusions used in this study. B–G. Localization of GFP-fusion proteins analysed by confocal laser scanning microscopy. Panel B is an optical section of 1 μm whereas panels C, D and E are the overlay of 29 1-μm sections. B, C, F and G. Wild-type StCDPK1. D and E. Myristoylation (D) and palmitoylation mutants (E) showed a similar pattern as soluble GFP. F and G. Cell plasmolysis induced by sorbitol confirmed the membrane localization of StCDPK1; panel G is a bright-field image to see the cell wall and the membrane. Three bombardment experiments were performed for each construction.

phorylated GS and displayed the highest activity in the particulate fraction. Western blot analysis revealed two CDPKs of 55 and 60 kDa in early and induced stolons that correlate with the CDPK activities detected in these tissues. In addition, the differential expression of *StCDPK3* (only transcribed in early elongating stolons) and *StCDPK1* (expressed upon swelling of the stolon tip) confirmed the existence of specific CDPK isoforms with restricted patterns of expression and activity during the events leading to tuber formation.

The two StCDPK isoforms of 55 and 60 kDa identified by Western blot analysis follow the same spatio-temporal expression profiles as *StCDPK3* and *StCDPK1*. It is possible to speculate that the *StCDPK3* gene product could be responsible for the high CDPK activity detected in early stolons and StCDPK1 for the late activity present in induced stolons. The fact that the molecular weight of *in vitro* translated StCDPK1 (Raíces *et al*., 2001) corresponds with the main band detected in Western blot analysis supports this hypothesis. Although *StCDPK2* was also detected in induced stolons, only basal transcript levels are present in this tissue.

There is general agreement about the importance of compartmentalization in signal transduction processes (Mochly-Rosen, 1995). The late CDPK activity present both in particulate and soluble fractions of swelling stolons shows more specific activity when localised in the membrane fraction, suggesting the presence of post-translational control mechanisms of enzymatic activity. Protein acylation is important for the subcellular localization of a variety of proteins (McCabe and Berthiaume, 1999; Thompson and Okuyama, 2000) and has recently been implicated in

CDPK targeting. Membrane localization of rice OsCPK2 was shown to be mediated by myristoylation and palmitoylation when expressed in different heterologous systems (Martin and Busconi, 2000). It was also shown that N-myristoylation of tomato LeCPK1 is required for efficient targeting to the plasma membrane *in vitro* (Rutschmann *et al*., 2002) and that myristoylation is likely to be involved in the membrane association of AtCPK2 with the endoplasmic reticulum (Lu and Hrabak, 2002). Upon transient expression in epidermal onion cells, a fusion of StCDPK1-GFP was efficiently targeted to the plasma membrane. Both myristoylation and palmitoylation of StCDPK1 were crucial for plasma membrane targeting. Co-translational myristoylation was not sufficient to anchor StCDPK1 to the lipid bilayer and additional palmitoylation was necessary to further promote membrane association.

Bombardment experiments argue that membrane localisation requires protein acylation but cannot be used to conclude about the subcellular distribution of StCDPK1 in potato stolons. Immunoblot data indicates that only 10% of the 60 kDa protein was present in the particulate fraction of induced stolons, whereas activity data show that 25–45% of total CDPK activity was associated to membranes in this tissue (Table 1). These data suggest that, in addition to fatty acid modifications, other factors could regulate the association of StCDPK1 with membranes. Such is the case for recoverin, a myristoyl switch protein whose association to membranes is regulated by calcium binding (Zozulya and Stryer, 1992). Calcium-dependent myristoyl/palmitoyl switches are ancient devices for shuttling proteins between the cytoplasm and the membrane in response to stimuli (Ames *et al.*, 1997; Godsel and Engman, 1999). This type of switch characterizes a subfamily of EF-hand calcium-binding proteins that in the calcium-bound state associate with the plasma membrane to interact with their targets (Calvert *et al*., 1995). When intracellular calcium level drops, the protein conformation changes and membrane association is lost (Tanaka *et al*., 1995). Thus, the binding of recoverin to its partner protein is modulated by the membrane accessibility of its fatty acid, which is regulated by the calcium-binding state of the protein. StCDPK1 is an EF-hand calcium-binding protein that suffers myristoylation and binds to hydrophobic matrixes in a calcium-dependent manner (Raíces *et al*., 2001). In addition, membrane localization of the CDPK isoform present in induced stolons affects kinase activity (Table 1). Therefore, it is tempting to suggest that like many myristoyl switch proteins, StCDPK1 subcellular localization could be mediated by a calcium dependent mechanism.

How do the different StCDPK isoforms recognize their substrates to trigger specific signalling events? The required specificity may be achieved by substrate affinity, accomplished by temporally and spatially restricted patterns of expression during tuber development or by targeting of CDPK isoforms to specific subcellular compartments. Altogether, our data suggest that sequential activation of specific CDPKs with distinct biochemical properties and subcellular localization could be essential for the co-ordination of multiple calcium signals triggered upon tuberization and opens new perspectives for the study of CDPKs regulation during this developmental process.

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