

Genomic and functional characterization of StCDPK1

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Abstract StCDPK1 is a calcium dependent protein kinase expressed in tuberizing potato stolons and in sprouting tubers. *StCDPK1* genomic sequence contains eight exons and seven introns, the gene structure is similar to *Arabidopsis*, rice and wheat CDPKs belonging to sub-group IIa. There is one copy of the gene per genome and it is located in the distal portion of chromosome 12. Western blot and immunolocalization assays (using confocal and transmission electron microscopy) performed with a specific antibody against StCDPK1 indicate that this kinase is mainly located in the plasma membrane of swelling stolons and sprouting tubers. Sucrose (4–8%) increased StCDPK1 protein content in non-induced stolons, however the

amount detected in swelling stolons was higher. Transgenic lines with reduced expression of StCDPK1 ($\beta 7$) did not differ from controls when cultured under multiplication conditions, but when grown under tuber inducing conditions some significant differences were observed: the $\beta 7$ line tuberized earlier than controls without the addition of CCC (GA inhibitor), developed more tubers than wild type plants in the presence of hormones that promote tuberization in potato (ABA and BAP) and was more insensitive to GA action (stolons were significantly shorter than those of control plants). *StCDPK1* expression was induced by GA, ABA and BAP. Our results suggest that StCDPK1 plays a role in GA-signalling and that this kinase could be a converging point for the inhibitory and promoting signals that influence the onset of potato tuberization.

The *StCDPK1* coding and genomic sequences have the GenBank Database accession numbers AF115406, and DQ507862.1 respectively.

Keywords Calcium-dependent protein kinases · *Solanum tuberosum* · Tuberization · Gibberellins

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Introduction

Plant cells are equipped with highly efficient mechanisms to perceive, transduce and respond to a wide variety of internal and external signals during their growth and development. Perception of signals via receptors results in generation of second messengers that control diverse cellular processes. Calcium (Ca^{2+}) is a crucial regulator in plants and nearly all environmental, hormonal and developmental stimuli use it as a signalling molecule.

There can be magnitudinal, spatial and temporal aspects of individual Ca^{2+} signals and these characteristics might be informative in ways that enable decoding of this ‘ Ca^{2+} signature’ to elicit specific and appropriate responses (Evans et al. 2001). Although such Ca^{2+} signatures may

partially explain the specificity of cellular responses triggered by a particular stimulus, the molecules that “sense” and “interpret” the Ca^{2+} signals provide additional specificity to the coupling of Ca^{2+} parameters to cellular responses. Plant protein kinases are a large and differentiated group of calcium sensors, among them, the Ca^{2+} -dependent protein kinase (CDPK) family plays distinct roles in mediating Ca^{2+} signalling (Klimecka and Muszyńska 2007).

CDPKs are monomeric proteins, with an N-terminal variable domain, a kinase domain, an autoinhibitory domain, a regulatory domain (CaM-LD, calmodulin-like domain) and a C-terminal domain of variable length (Harmon 2003). CDPKs are unique owing to the presence of CaM-LDs that are able to couple the calcium sensor directly to its responder (kinase). CDPKs are encoded by multigene families, for example in *Arabidopsis thaliana* there are 34 genes for CDPKs (Cheng et al. 2002; Hrabak et al. 2003) and 31 in *Oryza sativa* (Ray et al. 2007). A genome-wide analysis of *Arabidopsis* CDPKs provides an over-view of the diversity of this large multigene family and shows that gene duplication and subsequent divergence generated CDPKs with distinct functions. CDPKs are found in various subcellular localizations which suggest that this family may be involved in multiple signalling pathways (Lu and Hrabak 2002). Enhanced CDPK activity/expression has been linked to different stress responses and numerous environmental stimuli (Klimecka and Muszyńska 2007).

Potato tuberization is an ideal model system to study gene expression during organ development. Tuberization is a highly coordinated morphophysiological process occurring on the underground stolons under the influence of both extrinsic and intrinsic factors (Sarkar 2008). Tubers are underground modified stems that serve as a storage organ and as a vegetative propagation system. During the early stages of tuber formation 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.

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; Rodríguez-Falcón et al. 2006). Sugars and phytohormones play an important role in tuber formation (reviewed in Jackson 1999 and in Sarkar 2008; Fernie and Willmitzer 2001). Several lines of evidence implicate gibberellins (GAs) in the inhibition of

tuberization. All the environmental signals that regulate tuber development affect GAs concentration (Woolley and Wareing 1972; Krauss 1985; Menzel 1983, 1985). Gibberellins have been shown to inhibit tuberization using both in vivo and in vitro experiments (reviewed in Vreugdenhil and Sergeeva 1999). Antisense expression of a *GA 20-oxidase* gene (Carrera et al. 2000) or overexpression of a knotted-like homeobox gene (Rosin et al. 2003) that reduce the levels of bioactive gibberellins increased the rate of tuberization. Similarly, exogenous application of gibberellins decreased tuberization (Simko 1994; Xu et al. 1998) whereas application of the gibberellin biosynthesis inhibitor, paclobutrazol, increased tuberization (Simko 1994).

On the other hand, several studies using transgenic plants have provided evidence that increasing sugar concentrations can promote tuber formation (Müller-Röber et al. 1992; Sonnewald et al. 1997). In vitro experiments have shown that application of exogenous sucrose promotes tuberization (Garner and Blake 1989; Simko 1994; Xu et al. 1998; Raices et al. 2003c) and that the effects of sucrose on tuber formation are not due to alterations in the osmotic potential of the media (Perl et al. 1991). Sucrose has been proposed to affect tuberization, at least in part, via effects on the levels of bioactive gibberellins (Simko 1994; Xu et al. 1998).

Probably tuber development is the result of the relative balance of inducers (e.g. sucrose) and inhibitors (e.g. GAs) of tuberization (Jackson 1999). Sugar- and phytohormone-response pathways ‘interact’ in the regulation of many processes, however, little is known about the mechanisms by which they interact. Intracellular calcium is necessary for tuber development and calmodulin antagonists can inhibit this process (Balamani et al. 1986). An increase in cytosolic calcium concentration was reported in tobacco cells in response to sucrose (Furuichi and Muto 2005) and in GA-treated barley aleurone protoplasts (Gilroy and Jones 1992); thus it can be speculated that sucrose and GAs can induce a Ca^{2+} influx into the cytoplasm of potato cells.

In previous works 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 (MacIntosh et al. 1996). We analyzed the expression and activity of potato CDPK isoforms that follow different expression profiles during tuberization (Raices et al. 2003a). Among them, StCDPK1 was transiently expressed in swelling stolons (Raices et al. 2001) and its expression was induced by high sucrose (Raices et al. 2003b). In this paper we analyze the genomic structure of *StCDPK1* gene, its chromosome localization and we study how its expression is affected by the addition of different phytohormones. A genetically modified line, $\beta 7$, with low StCDPK1 expression produced more tubers when cultured under inducing

conditions and responded anomaly to GAs suggesting that this kinase could be part of the signalling cascade triggered by GAs.

Experimental procedures

Plant material

Virus-free meristematic sprouts from *Solanum tuberosum*, L. var. Spunta wild type and StCDPK1-transgenic potato plants were micropropagated on a modified MS (Murashige and Skoog 1962) medium with 3% (w/v) sucrose and 0.7% (w/v) agar under a 16 h light photoperiod at 23°C (multiplication conditions). 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). Induced stolons with progressively swelling tips (2–8 mm diameter) were classified as stages 1 to 4 (S1–S4). Tubers were harvested and stored in the cold room.

In vitro tuberization system

Single node cuttings of 30 days-old wild type or StCDPK1-transgenic potato plants were grown in tuberization medium: Murashige and Skoog salts supplemented with low nitrate concentration (82.5 mg l⁻¹ NO₃NH₄ and 95 mg l⁻¹ NO₃ K), 8% (w/v) sucrose, 0.7% (w/v) agar, pH 5.7) under complete darkness at 20°C during the indicated periods of time. Alternatively, 0.05 or 0.5 mg ml⁻¹ chlorocholine chloride (CCC); 0.05 or 0.5 μM GA3 gibberellic acid (GA); 7.6 μM abscisic acid (ABA) or 5 mg ml⁻¹ benzilaminopurine (BAP) were added to the culture media. In addition, the single node cuttings were grown in the presence of different sucrose concentrations (2, 4 or 6% w/v). To analyze the effect of a short GA-treatment, single-node cuttings grown during 13 days in tuberization conditions were transferred during 2 or 6 h to the same media with the addition of 0.5 μM GA.

StCDPK1 transgenic plants

The N-terminal variable domain of StCDPK1 (GenBank Database, accession number AF115406) spanning from the ATG to subdomain I of the kinase catalytic domain (~350 bp) was amplified using primers Pa18 (5'GGATCC TCTGGGGTTGTTGTTTTTC3') and Pa19 (5'TGGGTAGA GGTCAATATGGGGGGATCC3') and subcloned in pGEM T-easy (Promega). After digestion with *Bam*H1, the restriction fragment was subcloned in sense orientation into the pGUT binary vector (pUC18-derived plasmid containing the numbers 3' region of pBI 101.1) under the control of the specific tuber promoter of the Granule-Bound Starch

Synthase (GBSS). *Agrobacterium tumefaciens* (GV2260-6) mediated transformation of microtuber discs (50) was performed on MS medium supplemented with 5 μM zeatin riboside and 5 μM indoleacetic acid to induce the organogenesis. Discs were transferred to selection media (250 mg l⁻¹ cefotaxime and 50 μg ml⁻¹ kanamycin) 48 h post-infection, and then to fresh media every 20 days. The 54 transformant plantlets were transferred to individual flasks and grown in MS media with kanamycin. Presence of the transgene was confirmed in Southern blot assays or by PCR using genomic DNA as template and the following pair of primers that amplify a 600 bp fragment: P_{GBSS} (5'CTCACTCACACAGCTCAAC3'), specific for the GBSS promoter, and Pa19. Plants regenerated from untransformed in vitro shoots were used as wild-type controls.

Cloning of StCDPK1 gene

A *Solanum tuberosum* var. Desireé genomic library (Clontech) was screened using a full length *StCDPK1* coding sequence as a probe. Three rounds were performed to get a positive clone designated 4.1.b. The phage was purified following the instructions of the Lambda Midi Kit (QIAGEN). Automated sequencing was performed at the DNA Sequencing facility (University of Chicago, USA). Assembly and analysis of DNA sequence data was done using software available at the NCBI's Advanced BLAST sequence similarity search, designed to support analysis of nucleotide and protein databases. Clone 4.1.b (2,300 bp) lacked the 3' end of the gene and the kinase catalytic domain, therefore a PCR strategy was designed to obtain the full-length genomic sequence. Specific primers directed against the coding sequence of StCDPK1 (Mar1, Pa8, Pa9, Pa10, Pa11, Pa3, R10-2), the intronic sequences of clone 4.1.b. (Pa6, Pa12, Pa13) or the 3'UTR region of the gene (St1UTR) were used to amplify PCR fragments using genomic DNA as template (primers are indicated in Fig. 1A). The amplified fragments were subcloned in pGEM-Teasy (Promega), sequenced (Macrogen Inc., Korea) and compiled. Intronic sequences were identified using software to determine splicing sites. The complete genomic sequence (5952 nt) was submitted to GenBank (Accession number DQ507862.1).

DNA isolation and southern blot hybridization

Genomic DNA was extracted from greenhouse potato plants according to Murray and Thompson (1980). DNA (10 μg) was digested with restriction enzymes (*Eco*RI, *Bam*H1 and *Hind*III) (New England Biolabs, Beverly, MA), separated on 0.7% agarose gels and blotted under denaturing conditions onto Hybond N+ positively charged

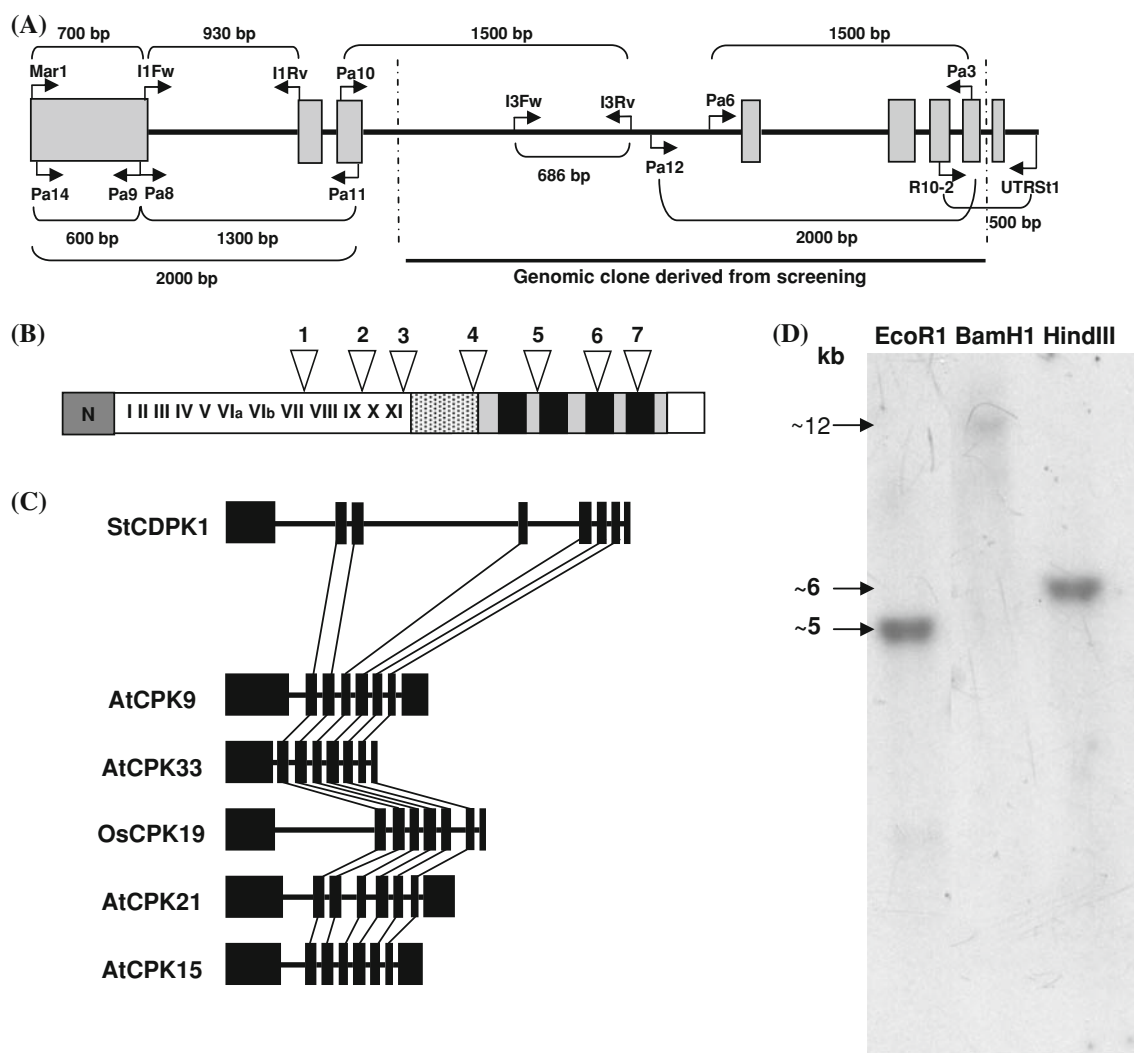


Fig. 1 Analysis of *StCDPK1* gene. **A** Schematic representation of *StCDPK1* gene cloning strategy. The genomic clone obtained by screening a genomic Clontech potato library is indicated. Primers used to amplify the different sequences using genomic DNA as template and the length of each amplicon is indicated. Exons are represented as grey boxes and introns as lines. **B** Genomic structure of *StCDPK1* gene. Intron locations (1–7) relative to the protein sequences are shown by triangles. The exact sizes of exons and introns can be found in Table 1. *Dark grey box*: N-terminal variable domain; *white*

box: protein kinase domains; I–XI, subdomains of the protein kinase domain are indicated; *dense dotted box*: junction domains; *light grey box*: calmodulin-like domain containing 4 EF hands (black). **C** Comparison of *StCDPK1* gene structure with *Arabidopsis* and rice CDPKs belonging to subgroup IIa. The intronic sequences are drawn to scale. **D** Southern blot hybridization. Genomic DNA of potato plants (12 μ g) was digested with *EcoRI*, *BamHI* and *HindIII* and separated on 0.7% agarose gels. After blotting, the DNA was probed with Amplicon II (930 bp). Size (in kb) of the bands is indicated

nylon membranes (Amersham) according to the manufacturer's procedures. Membranes were hybridized overnight with a [32 P]-labelled intronic sequence (INT 1) of the gene at 65°C in Church's buffer. Sequential stringent washes were performed (2 \times SSC, 1 \times SSC and 0.1 \times SSC with 0.1% SDS) during 30 min at 65°C and blots were exposed to X-OMAT Kodak films.

Chromosome mapping

The genetic location of *StCDPK1* was determined using the single strand conformation polymorphism (SSCP)

technique. Different pairs of primers, directed against exon-1 (Mar1, Pa9), intron-3 (I3Fw, I3Rv), or the 3' end of the gene (R10-2, 3'UTR), were used to amplify PCR fragments from three parental genotypes (M200-30, B11B and H11-9) of two different mapping populations (BCB and BCT, Bonierbale et al. 1994). Amplified fragments were denatured in formamide and the ssDNAs were analyzed by 5% MDE-PAGE (Cambrex, USA) and revealed with silver staining. The differences observed in the migration pattern reflect the nucleotide differences between the parental genotypes. Amplicon I3 showed two segregating bands in BCT population that were used for the

mapping assays. Localization was performed by co-segregation of amplicon I3 with RFLP and SSR markers previously assigned to the different chromosomes (Bonierbale et al. 1994; Feingold et al. 2005) using the Mapmanager QTX software (www.mapmanager.org).

Protein extraction

Tuberization stages from *Solanum tuberosum* transgenic and control in vitro plants and swelling stolons from greenhouse plants (S1–S4) were harvested, ground in a mortar cooled with liquid nitrogen and extracted with 50 mM Tris–HCl pH 7.5; 2 mM β -mercaptoethanol; 1 mM EDTA; 20% glycerol and protease inhibitors. The suspensions (1 ml buffer/g of wet tissue) were centrifuged 10 min at 1,000g and the pellet was discarded. The supernatant (crude extract) was centrifuged 1 h at 20,000g and soluble and particulate fractions (Sn-20 k and P-20 k) were obtained. Pellets were washed with 0.5 ml of extraction buffer and were then suspended in a volume equal to 10% of the volume of the soluble fraction. Alternatively, after the first centrifugation the supernatant was centrifuged at 10,000g and subsequently at 100,000g. Both pellets (P-10 k and P-100 k) were suspended in extraction buffer.

Isolation of plasma membrane fractions

Plasma membranes (PM) from sprouting tubers were prepared by partition in an aqueous polymer two phase system according to Larsson et al. (1994). Potato tubers (59 g) were homogenized with a blender and a Polytron homogenizer (PT 3000; Kinematica AG, Lucerne, Switzerland) in 150 ml of buffer A (50 mM Tris–HCl pH 8, 500 mM sucrose, 10% glycerol, 20 mM EDTA, 20 mM EGTA, 0.6% w/v polyvinylpyrrolidone, 5 mM DTT, 5 mM β -glycerophosphate) with protease inhibitors (complete mini EDTA free Protease Inhibitor Cocktail Tablets, Roche Applied Science). The homogenates were filtered through three layers of cheesecloth and centrifuged at 10,000g for 10 min. The supernatant was further centrifuged at 50,000g for 36 min and the resulting pellet (microsomal fraction) was suspended in 9 ml of buffer B (330 mM sucrose, 2 mM DTT in 5 mM potassium phosphate pH 7.8). The microsomal fraction was separated through an aqueous two-phase partitioning system containing 6.6% dextran T500 (Amersham Pharmacia Biotech), 6.6% polyethylene glycol P-3650 (Sigma), 5 mM KCl, 0.33 M sucrose, and 5 mM potassium phosphate buffer (pH 7.8). The resulting upper phases were designated the PM-rich fractions. These fractions were diluted with six volumes of buffer C (10 mM boric acid, 9 mM KCl, 300 mM sucrose, 2 mM DTT in 10 mM Tris–HCl pH 8.3 with protease inhibitors) and centrifuged at 100,000g for 36 min. The pellets were

suspended in buffer C and stored at -80°C . All procedures were carried out at $0-4^{\circ}\text{C}$.

Analytical methods

Protein contents were determined with BioRad protein assay reagent with BSA as the standard.

The hydrolytic activity of vanadate-sensitive ATPase (PM- H^{+} -ATPase) was used as a marker of PM. ATPase activity was determined in the PM-rich fractions and in microsomal (Mi) fractions of sprouting tubers at 37°C for 25 min as described (Larsson et al. 1994). The reaction mixture contained 5 μg of protein (PM or Mi), 50 mM MES (pH 6.5), 3 mM ATP, 2.5 mM MgSO_4 , 50 mM KCl, 1 mM NaN_3 , 0.1 mM Na_2MoO_4 , 160 mM sucrose and 0.02% Triton X-100. PM H^{+} -ATPase activity was assayed in the presence or not of 0.6 mM Na_3VO_4 and was expressed as the difference between the activity measured in the absence and in the presence of Na_3VO_4 . The Pi released during the reaction was assayed by a colorimetric method based on that of Fiske and Subbarow (1925).

CDPK activity was determined in soluble, microsomal and enriched plasma membrane fractions as described (MacIntosh et al. 1996). The reaction mixture contained 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 5 μM CaCl_2 . 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.

Anti-StCDPK1 polyclonal antibody

The StCDPK1 N-terminal variable domain (634 bp) was amplified using primers PaR1-*Bam*H1 (5'CGCGGATCCGAGCAAAAGTAAACCAGC3') and PaR2-*Xho*I (5'CCGC TCGAGCTCAGGCTTAAGATCACG3'), digested with *Bam*H1 and *Xho*I restriction enzymes (New England Biolabs) and subcloned into the pET22b+ vector (Novagen). The StCDPK1-6 \times His recombinant protein was expressed in *E. coli* BL21pLysS strain and purified by affinity chromatography using a nickel column (Ni-NTA agarose, Qiagen). The purified recombinant protein (0.8 mg/dose) was used as antigen to produce antibodies in rabbit. The polyclonal serum was purified using a Sepharose CL4B-Protein A column. The antibodies were used in Western blot (1:400) and immunolocalization (1:100) assays.

Western blot assays

SDS-PAGE was carried out as described by MacIntosh et al. (1996). Pre-stained SDS-PAGE standards from

Invitrogen or Fermentas Life Sciences were used as molecular weight markers. Western blot analysis of protein samples (50–100 µg) from tuberization stages (S1–S4) or from sprouting tubers was performed using affinity purified polyclonal anti-StCDPK1 antibodies (1:400).

Blots were developed with ECL reagent from Amersham (according to the manufacturer's procedure). The signal was scanned and quantified with ImageQuant software.

Immunolocalization assays

Swelling stolons (S4) were fixed overnight at 4°C with 4% paraformaldehyde in 0.05 M phosphate buffer, pH 7, washed three times with phosphate buffer (30 min each), dehydrated in a graded ethanol series with changes every 30 min and embedded in LR White resin (Polyscience, Inc. 17411) according to Harris (1994). Semithin longitudinal sections (7 µm) or ultra-thin sections were obtained using an ultramicrotome (Reichert-Jung, Vienna, Austria) with a glass knife.

Semithin sections were hydrated with 0.05% v/v Tween 20 in 1× TBS (TBS-Tw) during 10 min, blocked with 5% normal donkey serum in TBS-Tw during 2 h and rinsed three times with TBS-Tw (5 min each). After incubation with affinity purified polyclonal anti-StCDPK1 antibody (1:100 in TBS-Tw) during 2 h, sections were incubated in the dark for 1 h with a secondary anti-rabbit antibody conjugated to Cy3 (1:100 in TBS-Tw) (Jackson Lab.), rinsed three times with TBS-Tw, once with water and were left to dry. Mowiol 4-88 and cover slips were added and sections were kept overnight in the dark at room temperature. Control sections were treated as above but rabbit preimmune serum was used as primary antibody. Fluorescence was observed with a Leica DM LB epifluorescent microscope equipped with a mercury lamp (50 W, Wetzlar, Germany) and a filter system suitable for Cy3. Images were obtained with a CCD camera.

Ultrathin sections were mounted on Formvar-coated nickel grids (Polyscience, Inc. 7550N) for immunolabeling at the TEM level. Sections were first hydrated on a drop of Milli-Q-grade water for 3 min, washed with rinsing buffer twice for 3 min, blocked for 90 min with 1% gelatin in PBS at room temperature, washed with rinsing buffer twice (3 min each) and incubated overnight at 4°C with the purified rabbit anti-StCDPK1 primary antibody at a 1:100 dilution in the rinsing buffer. Next, sections were washed with rinsing buffer (five changes, 5 min each), incubated for 1 h on a drop of colloidal gold (10 nm)-conjugated goat antiserum to rabbit immunoglobulins diluted 1:100 in rinsing buffer at room temperature and washed again with rinsing buffer (five changes, 5 min each). After a 10-min

fixation with 1% glutaraldehyde in 0.1 M PBS, pH 7.2, sections were thoroughly washed with Milli-Q-grade water. Finally, grids carrying sections were counter-stained with uranyl acetate, and examined under a ZEISS EM 109 turbo (Wiesbaden, Germany) transmission electron microscope operating at an accelerating voltage of 90 kV. Control sections were treated as above, excluding the purified rabbit StCDPK1 immune serum.

RNA extraction

Total RNA was extracted from different tuberization stages or from dormant and sprouting tubes with the Total RNA Isolation, TRIzol, Reagent (Gibco-BRL) following the manufacturers' instructions.

Semiquantitative RT-PCR analysis

Total RNAs (10 µg) were pre-treated with DNase (RQ1 RNase-free DNase, Promega) and reverse transcribed with M-MLV-Reverse Transcriptase (Promega) using an oligo-dT primer. Control reactions to check equal amounts of cDNA template were performed using primers Ubi1 (5'ATGCAGATCTTTGTGAAGAC3') and Ubi2 (5'ACCA CCACGGAGACGGAG3') to amplify a 250 bp ubiquitin fragment (20 cycles, annealing temperature 55°C).

Semiquantitative RT-PCR was carried out according to Raices et al. (2003a) using specific primers to exclusively amplify *StCDPK1* and *StCDPK3*. A common 5' primer, 5'*St* (5'GGAAGCTGCTGATGTGGATGG3') present in both StCDPK sequences was combined with different 3' primers derived from the 3'UTR region of each kinase. PCRs (30 cycles) were performed with Platinum *Taq* polymerase (Invitrogen). Annealing temperature was 64°C for *StCDPK1* and 61°C for *StCDPK3*. Control reactions with plasmids (5 ng) yield amplified fragments corresponding to *StCDPK1* and *StCDPK3* of 419 and 498 bp respectively. In addition, the expression of *GA 2-oxidase (GA-2ox)* and *GA 20-oxidase (GA-20ox)* was determined using specific oligonucleotides. The clones containing *GA-2ox* and *GA-20ox* coding sequences were a kind gift of Dr. Salomé Prat.

RT-PCR products were separated on 1% agarose gels, blotted onto Hybond N+ positively charged nylon membranes (Amersham) and hybridised to specific radio-labelled DNA probes. Probes against *StCDPK1*, *StCDPK3*, *GA-2ox* and *GA-20ox* were generated by random priming using the RadPrime DNA labelling system kit (Invitrogen) from PCR products amplified from cDNA clones. Hybridization conditions were the same as in Raices et al. (2003a). Signal was scanned with a phosphorimager Storm 830 (Amersham Pharmacia Biotech) and quantified with ImageQuant software.

Results

Cloning and characterization of StCDPK1 gene

A partial genomic clone of *StCDPK1* (~2,300 bp) was obtained by screening a potato genomic library (Clontech). This clone contained four intronic regions and four exons that encoded the hinge region and the carboxyterminal regulatory domain of the kinase except for the last 75 bp (Fig. 1A). In order to obtain the complete sequence of the gene, different PCRs were performed using genomic DNA as template with primers directed against the 5' end of the kinase, the catalytic domain, the 3'UTR or the intronic regions (primers are indicated in Fig. 1A). Using this strategy we obtained the complete sequence of the gene (5,952 bp long from the ATG to the stop codon; GenBank Accession Number DQ507862, plus the 3'UTR of the gene that spans a 357 bp sequence to the polyadenylation signal, GenBank Accession Number AF115406). In order to reveal the gene structure of *StCDPK1*, mRNA splicing sites were mapped by comparing the coding and the genomic sequences.

StCDPK1 gene is organized in eight exons and seven intronic regions (Table 1, Fig. 1B). The intron–exon

junctions obeyed the GT/AG rule (Shapiro and Senapathy 1987). The predicted splicing sites of *StCDPK1* correspond to those reported for CDPKs from subgroup II-a, that includes 10 *Arabidopsis* isoforms (AtCPK9, 15, 19, 21, 22, 23, 27, 29, 31 and 33; Cheng et al. 2002), two rice isoforms (OsCPK12 and 19; Asano et al. 2005) and two wheat isoforms (TaCPK5 and 18; Li et al. 2008). The length of exons 2–7 is conserved in all these isoforms (Table 2, and schematic representation in Fig. 1C). Exon 1 contains the N-terminal variable domain and subdomains I–VII of the catalytic domain, exon 2 contains subdomains VIII and IX while exon 3 completes the kinase catalytic domain. Exon 4 encodes the hinge region, and the regulatory domain is encoded by exons 5–8 (Fig. 1B, EF hands are represented in black). *StCDPK1* can be considered a GC-poor gene; its overall GC content is 37%, being higher in exons (39–44%) than in introns (32–37%) (Table 1). The intronic regions present in *StCDPK1* are much longer than those present in the *Arabidopsis* CDPK genes (schematic representation in Fig. 1C). While these range from 21 to 50% of the gene sequence and represent in average 34% (<http://mips.gsf.de/cgi-bin/>) *StCDPK1* introns represent 74% of the total gene sequence.

PCR were performed with primers IIFw–IIRv, using genomic DNA from *S. tuberosum* potato plants (4n) or from two diploid parental lines, HB-171 and DB226, as template. The purified fragments (amplicon II, derived from intron 1; 930 bp) were digested with *Bam*H1, *Hind*III or *Eco*R1 to confirm that amplicon II does not present these restriction sites (data not shown). Genomic DNA digested with *Bam*H1, *Hind*III or *Eco*R1 was probed with amplicon II. Southern blot hybridization patterns were compatible with the presence of one copy of the *StCDPK1* gene per genome (Fig. 1D). In experiments performed with the full-length coding sequence as probe, several bands were revealed (data not shown; Raices et al. 2001) in accordance with the presence of several CDPK isoforms in the potato genome.

Table 1 Characteristics of *StCDPK1* gene

Exons	Length (bp)	GC content (%)	Introns	Length (bp)	GC content (%)
1	715	39	1	918	36
2	144	44	2	97	33
3	153	41	3	2301	35
4	116	41	4	779	35
5	168	43	5	97	37
6	128	39	6	82	35
7	100	41	7	79	32
8	75	39			

Table 2 Comparison of *StCDPK1* with *Arabidopsis* and rice CDPK genes belonging to subgroup IIa

Genes	Length (bp)					
	<i>StCDPK1</i>	<i>AtCPK9</i>	<i>AtCPK33</i>	<i>OsCPK19</i>	<i>AtCPK21</i>	<i>AtCPK15</i>
ATG-Stop	5950	2907	2158	3750	3288	2821
Exon 1	715	923	682	718	831	809
Exon 2	144	143	143	144	143	143
Exon 3	153	152	152	153	152	152
Exon 4	116	115	115	113	112	112
Exon 5	168	167	167	168	167	167
Exon 6	128	127	127	127	127	127
Exon 7	100	99	99	100	99	99
Exon 8	73	377	74	78	445	343

StCDPK1 mapping experiments were performed with amplicon I3 using the single strand conformation polymorphism (SSCP) technique. The data obtained from two linkage maps suggest that *StCDPK1* gene is located in the distal portion of chromosome 12. *StCDPK1* was localized at 13.2 cM from *StI079* marker (LOD score: 4.2) in the M200-30 map and at 16.7 cM of this same marker in H11-9 map (LOD score: 3.4). *StI079* is an unpublished microsatellite that is linked to STM2028 in both maps (at 10.8 cM in M200-30 and totally linked in H11-9). The fact that the same position was obtained using both maps (two independent events) supports this result; however to determine the exact location, mapping should be performed in a higher density map.

Immunologic analysis of StCDPK1

Our previous results indicate that *StCDPK1* is expressed in induced stolons and that the CDPK activity detected in tuberizing stolons was mainly associated to the particulate fraction. However Western blot assays performed with an antibody directed against the CLD of soybean α CDPK (Bachmann et al. 1996) suggested that CDPK protein was more abundant in the soluble fraction of tuberizing stolons (Raices et al. 2003a). A specific anti-*StCDPK1* antibody directed against the N-terminal variable domain and part of

the catalytic domain (including subdomain V) of *StCDPK1* was produced. *StCDPK1* mRNA (1,596 nt) encodes a predicted protein of 59 kDa (Raices et al. 2001) with an apparent molecular weight of 60 kDa when revealed with the anti-*StCDPK1* antibody (Fig. 2).

Proteins extracts from different tuberization stages (S1–S4) were centrifuged at 20,000g and Western blot assays were performed using the supernatants (100 μ g) or the particulate fractions (50 μ g) (Fig. 2A). The anti-*StCDPK1* antibody (1:400) detected a faint band in soluble extracts of tuberizing stolons (S1–S4) and a stronger and slightly faster migrating band in S3 and S4. This second band migrated as one of the two polypeptides (\approx 60 and 57 kDa) detected in the particulate fractions. Furthermore, the amount of the \approx 60 kDa band increased in particulate extracts of progressively tuberizing stolons (Fig. 2A, right panel) in accordance with the expression data (Raices et al. 2001). The lower band detected could correspond to another CDPK isoform. Though less protein was loaded, a stronger signal was detected in the particulate extracts suggesting that the higher CDPK specific activity determined in these fractions (Raices et al. 2003a, b, c) is due to the presence of more *StCDPK1* protein.

Protein extracts from S4 tuberizing stolons were subjected to differential centrifugation. The particulate (10 k and 100 k) and soluble (100 k) extracts were analyzed by

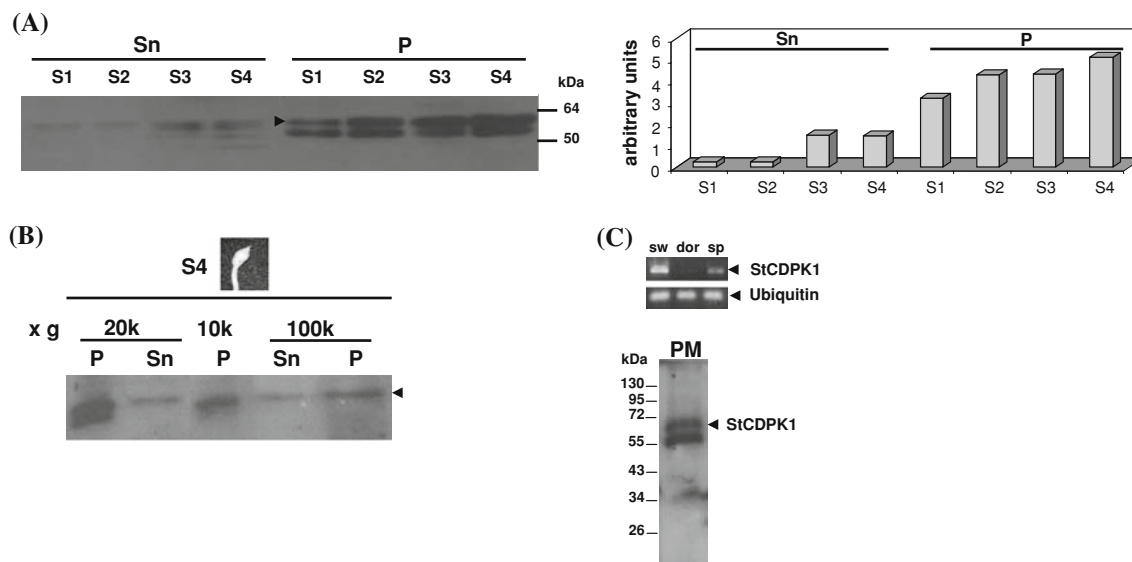


Fig. 2 Western blot analysis of *StCDPK1* in swelling stolons and sprouting tubers. Protein extracts were subjected to electrophoresis and blotted. Membranes were incubated with a polyclonal antibody (1:400) against the N-terminal domain of *StCDPK1* (A–C). The arrows indicate the 60 kDa *StCDPK1* band. **A** Soluble (S; 100 μ g) or particulate fractions (P; 50 μ g) from different tuberization stages (S1–S4) centrifuged at 20,000g were analyzed by Western blot (left panel). Molecular weight markers are indicated. Signal was scanned and quantified (right panel). **B** Protein extracts (50 μ g) from S4 tuberizing stolons were either centrifuged at 20,000g (20 k); or first at

10,000g (10 k) and then at 100,000g (100 k). Soluble (S) and particulate (P) fractions were analyzed. **C** RT-PCR analysis of *StCDPK1* was performed in swelling stolons (sw) and in dormant (dor) or sprouting (sp) tubers from greenhouse plants. Equal amounts of total RNA were used and normalized using primers that amplify a constitutively expressed Ubiquitin gene. PCR to exclusively amplify *StCDPK1* were performed using primers 5'*St* and 3'*St* (upper panel). The plasma membrane-rich (PM) fraction (30 μ g) from sprouting tubers was analyzed. Molecular weight markers are indicated (lower panel)

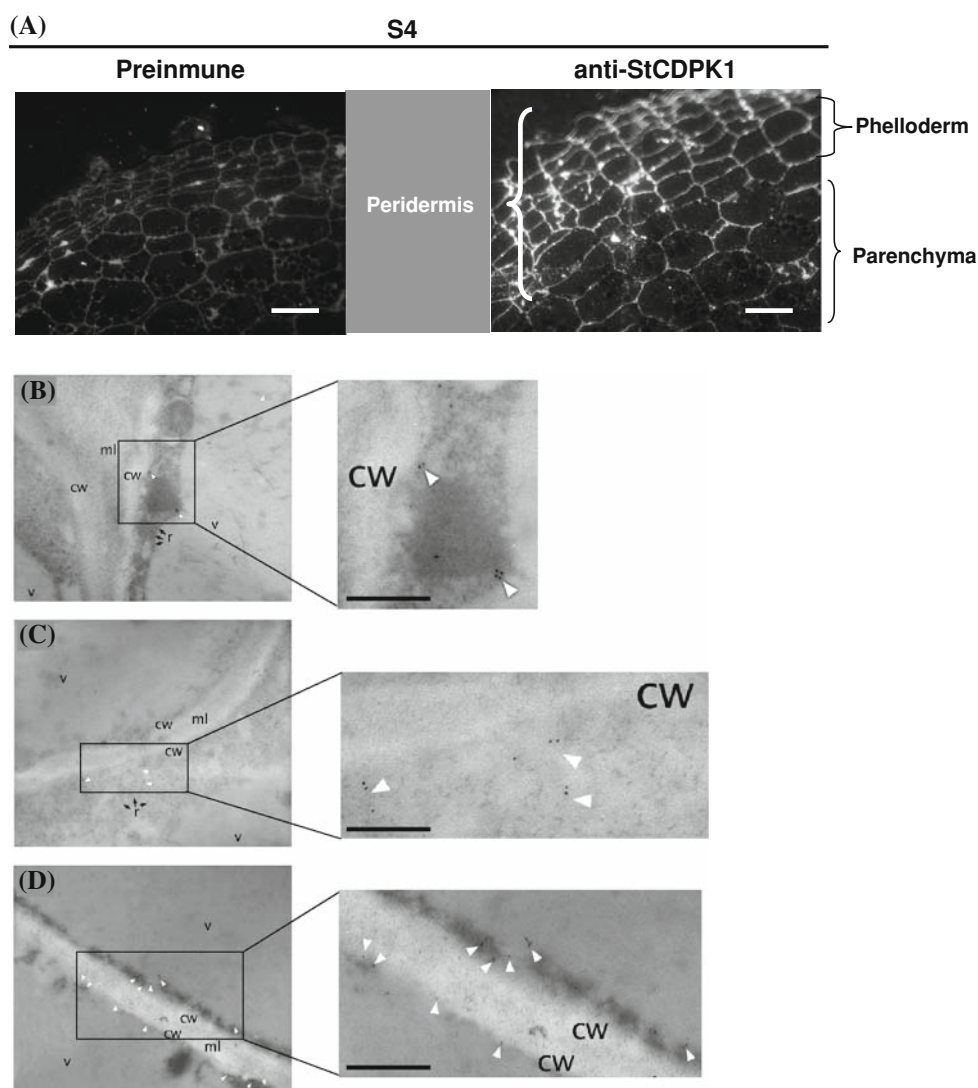
Western blot (Fig. 2B). Two bands are observed in the 10 k pellet while only the higher one is present in the microsomal fraction (P100 k); the same band is present in the cytosolic fraction (Sn100 k) but as before, the signal is fainter.

StCDPK1 is also expressed in sprouting tubers but not in dormant ones (Fig. 2C, upper panel). Plasma membrane fractions (PM) from sprouting tubers were purified according to the two-phase partitioning method. Vanadate-sensitive ATPase activity was used as marker of the plasma membrane ($33 \mu\text{moles Pi h}^{-1} \text{mg}^{-1}$). CDPK activity was higher in the PM-rich fraction ($12.1 \text{ nmoles } ^{32}\text{P incorporated.min}^{-1} \text{mg}^{-1}$) than in the soluble fraction ($2.8 \text{ nmoles } ^{32}\text{P incorporated.min}^{-1} \text{mg}^{-1}$) of sprouting tubers. The Western blot assay (Fig. 2C, lower panel) revealed two bands (60 and 57 kDa) in the PM-rich fraction from sprouting tuber while as before a very faint band was detected in the soluble fraction (data not shown). These

data indicate that *StCDPK1* protein and CDPK activity are associated to the plasma membrane.

In addition, longitudinal sections of S4 were incubated with anti-*StCDPK1* or preimmune serum. As shown in Fig. 3A, the signal is observed surrounding nuclei and in the periphery of the cells of both periderm and neighbouring parenchyma tissues. Ultra-thin sections of swelling stolons (S2 and S4) were incubated with anti-*StCDPK1* and incubated on a drop of colloidal gold (10 nm)-conjugated goat antiserum to rabbit immunoglobulins, counter-stained with uranyl acetate, and examined under a transmission electron microscope (Fig. 3B–D). *StCDPK1* was mainly located on the parietal cytoplasm and in the site corresponding to the plasma membrane. The protein was more abundant in S4 (Fig. 3D). Western blot data (Fig. 2B, C) are consistent with the association of *StCDPK1* to cell membranes so we could suggest that in vivo *StCDPK1* protein is mainly associated to plasma membrane.

Fig. 3 Immunolocalization analysis of *StCDPK1*. **A** Longitudinal sections ($7 \mu\text{m}$) of S4 tuberizing stolons were incubated with anti-*StCDPK1* antibody (1:100). An anti-rabbit antibody conjugated to Cy3 (1:100) was used as secondary antibody. Sections were observed by LM. Protein was mainly located in living cells of the peridermis. The bar indicates $50 \mu\text{m}$. **B** Ultrathin sections of S2 (**B, C**) and S4 (**D**) tuberizing stolons were incubated with the *StCDPK1* primary antibody at a 1:100 dilution and then on a drop of colloidal gold-conjugated goat antiserum to rabbit immunoglobulins diluted 1:150. Sections were observed by TEM. Protein was mainly located in living cells of the peridermis, on the plasma membrane and in peripheral cytosol. Bars: 300 nm (**B, C**), 500 nm (**D**). *cw* Cell wall; *ml* middle lamellae; *r*, ribosomes; *v*, vacuole. Arrows indicate gold particle labeling



StCDPK1 expression was up-regulated in potato plants transferred during 16 h to liquid MS medium with 8% sucrose under light conditions (Raices et al. 2003b). Single node cuttings were grown during 8 days in MS with increasing amount of sucrose (2–8%) under complete darkness. At the moment of harvest, non-induced stolons (NIS, where no swelling was observed) were collected from all conditions; however, in the 8% sucrose treatment some stolons were already induced to tuberize (IS). Total protein extracts were obtained from each condition, Western blot assays were performed and the bands were quantified (Fig. 4, upper and lower panels). An increase in StCDPK1 protein (1.5- to 2.5-fold) was observed in non-induced stolons treated with 4, 6 or 8% sucrose suggesting that sucrose regulates StCDPK1 expression even though there are no morphologic effects. However, when swelling of the stolon is evident (IS 8% Sucrose), the amount of StCDPK1 protein detected was 5-fold higher than in controls (2% sucrose) indicating that the kinase is developmentally regulated.

StCDPK1 transgenic plants

Our results suggest that StCDPK1 could be a component of the signalling cascades triggered during stolon to tuber transition. Thus we tried to obtain transgenic plants in

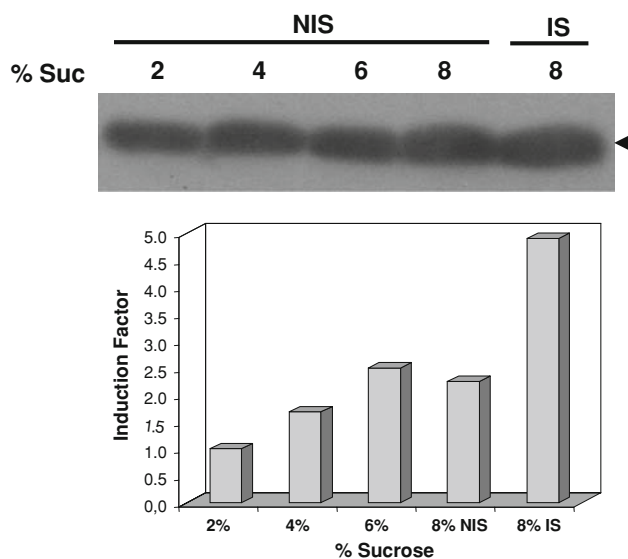


Fig. 4 Sucrose increases StCDPK1 protein content. Single node cuttings were grown during 8 days in MS with increasing amount of sucrose (4–8%) under complete darkness. Control plants were grown in 2% sucrose. At the moment of harvest, non-induced stolons (NIS) or induced stolons (IS) were collected and total protein extracts were obtained, subjected to electrophoresis and blotted. Western blot assays were performed with 50 μ g of protein extracts (upper panel). Membranes were incubated with a polyclonal antibody (1:400) against the N-terminal domain of StCDPK1. The arrows indicate the 60 kDa StCDPK1 band. Bands were quantified (lower panel)

which StCDPK1 expression was modified and analyze them in conditions that promote or inhibit tuber formation. To this end, the variable 5'end of the gene (350 bp) was subcloned in sense orientation into the pGUT binary vector (Fig. 5A, upper panel). *Agrobacterium* mediated transformation of tuber discs was performed and 50 transformed shoots were selected on Kanamycin MS media. The presence of the transgene was confirmed by PCR using primers directed against the GBSS promoter and the Pa19 specific primer that amplify a 600 bp fragment (Fig. 5A, lower panel) and by Southern blot (data not shown). Nodal stem cuttings from the different transgenic lines were cultured in vitro under tuber inducing conditions during 20 days; tuberizing stolons were harvested and total RNA was extracted. *StCDPK1*, *StCDPK3* and *ubiquitin* expression was analyzed by semiquantitative RT-PCR (Fig. 5B, left panel). *StCDPK1* expression was normalized relative to ubiquitin expression. *StCDPK1* transcript levels varied in the different β lines analysed but the lowest expression was observed in $\beta 7$ line (approximately one tenth of the expression observed in wild type plants (Fig. 5B, right panel) and this decrease in *StCDPK1* transcript levels was correlated with a decrease in StCDPK1 protein content (Fig. 5C).

Control and $\beta 7$ plants were grown in multiplication and tuber inducing conditions. While no difference was observed in the phenotypic characteristics (root and stem length, number of internodes and chlorophyll content) of the in vitro plants grown in multiplication conditions (Fig. 6), the $\beta 7$ line displayed a different behaviour when grown under tuber inducing conditions (8% sucrose, complete darkness, 20°C). Control and $\beta 7$ single node cuttings were grown at the times indicated in the presence of 8% sucrose, 8% sucrose with CCC, inhibitor of gibberellin synthesis (0.05 or 0.5 mg ml^{-1}), or 8% sucrose with GA3 (0.05 or 0.5 μM). At the moment of harvest, stolons were separated into non-induced or induced to tuberize and tuber weight was assayed. CCC promoted tuberization both in wild type and $\beta 7$ plants at the times analysed (Table 3, Fig. 7A), however, the average weight of the $\beta 7$ microtubers was moderately higher than that of wild type ones (13 or 29% according to the CCC concentration used; Fig. 7A). On the contrary, no visible signs of tuber development were observed when control or $\beta 7$ plants were grown in the presence of GA3 and longitudinal stolon growth was increased (Table 3, Fig. 7B). This is consistent with the inhibitory effect of GAs on tuber formation and with its promoting effect on stolon elongation. However, the length of the non-induced stolons was significantly shorter in the $\beta 7$ line with both GA3 concentrations (Fig. 7B right panel), and this effect was even more evident when stem cuttings were exposed to 0.5 μM GA3 for longer periods (Fig. 7B left panel).

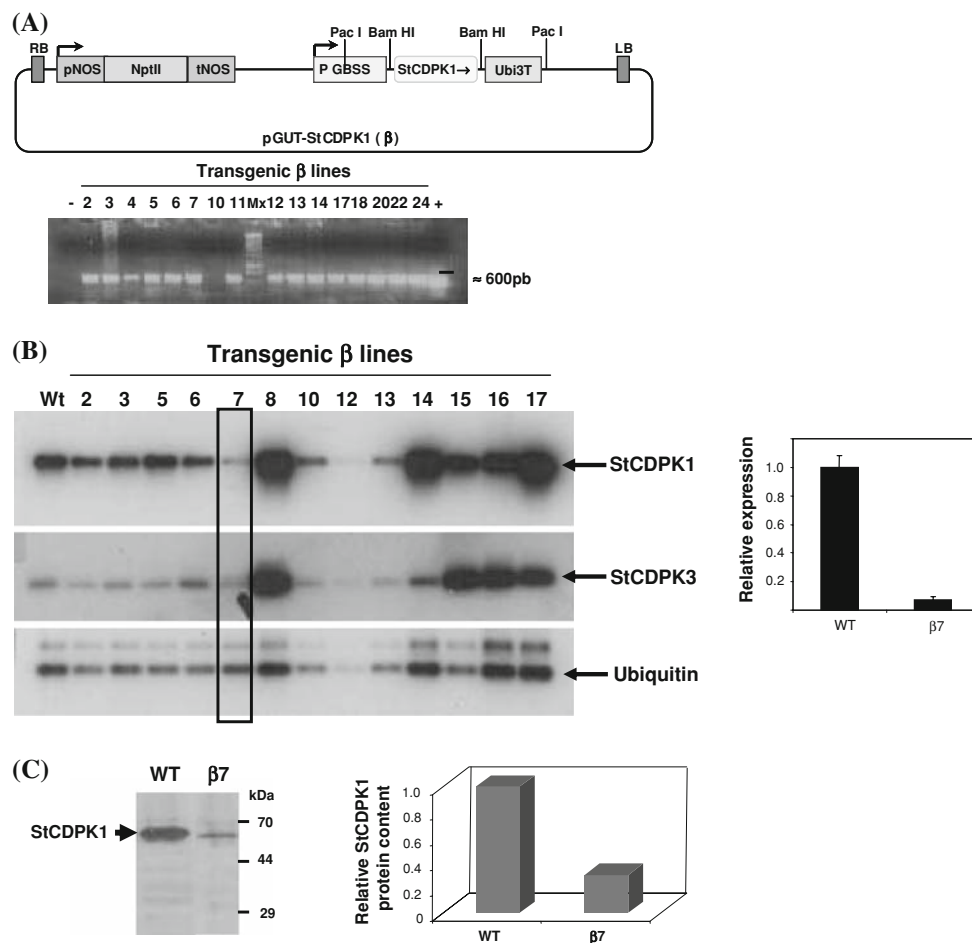


Fig. 5 Production of transgenic β plants. **A** Schematic representation of the pGUT vector containing the N-terminal region (350 bp) of StCDPK1 (upper panel). The presence of the transgene in the transformed lines was confirmed by PCR using primers directed against the GBSS promoter and the Pa19 primer that amplify a 600 bp fragment (lower panel). **B** *StCDPK1* relative expression in wild type (WT) and transgenic β plants. RT-PCR analysis of *StCDPK1*, *StCDPK3* and Ubiquitin was carried out with specific primers. RT-

PCR products were separated on 1% agarose gels, blotted and hybridised to specific radio-labelled DNA probes (left panel). The value of $\beta 7$ was normalized to the expression of WT plant (=1) (right panel). **C** Western blot analysis of total protein extracts (75 μ g) from wild type (WT) or $\beta 7$ induced stolons with a specific antibody against StCDPK1 (1:400). Molecular weight markers are indicated (left panel). Bands were quantified, StCDPK1 protein content for $\beta 7$ is normalized to that of the WT plant (=1) (right panel)

When grown in the presence of 8% sucrose, the $\beta 7$ line displayed a more pronounced tuberization response (84% of the $\beta 7$ plants tuberized compared to 54% of the wild type plants, Table 3). A similar experiment was performed in the presence of 6% sucrose during 10 days. In these conditions, wild type plants yielded no tubers, however 20% of the $\beta 7$ stolons were induced to tuberize. High sucrose concentrations (6 or 8%) affects the length of potato stolons, these were significantly ($P < 0.01$) shorter than those grown in 2% sucrose (Table 4). This effect was more noticeable in non-induced $\beta 7$ stolons.

The effect of ABA and BAP was also studied; single node cuttings from wild type and $\beta 7$ plants were cultured during 10 days under tuberization conditions in the presence of 7.6 μ M ABA or 5 mg ml⁻¹ BAP (Fig. 8). Both

hormones positively regulated tuber development being ABA more effective than BAP. In both conditions, $\beta 7$ plants developed more tubers than wild type plants.

Expression analysis of StCDPK1 in response to phytohormones

RNA was obtained from the different treatments (8% sucrose and 8% sucrose with the addition of ABA, BAP or GA) and StCDPK1 expression was assayed. Data are shown relative to expression of ubiquitin and in all cases controls (8% sucrose) are set as 1 (Fig. 9A). The expression of StCDPK1 was induced with the addition of the phytohormones (Fig. 9A, left panel). ABA treatment produced

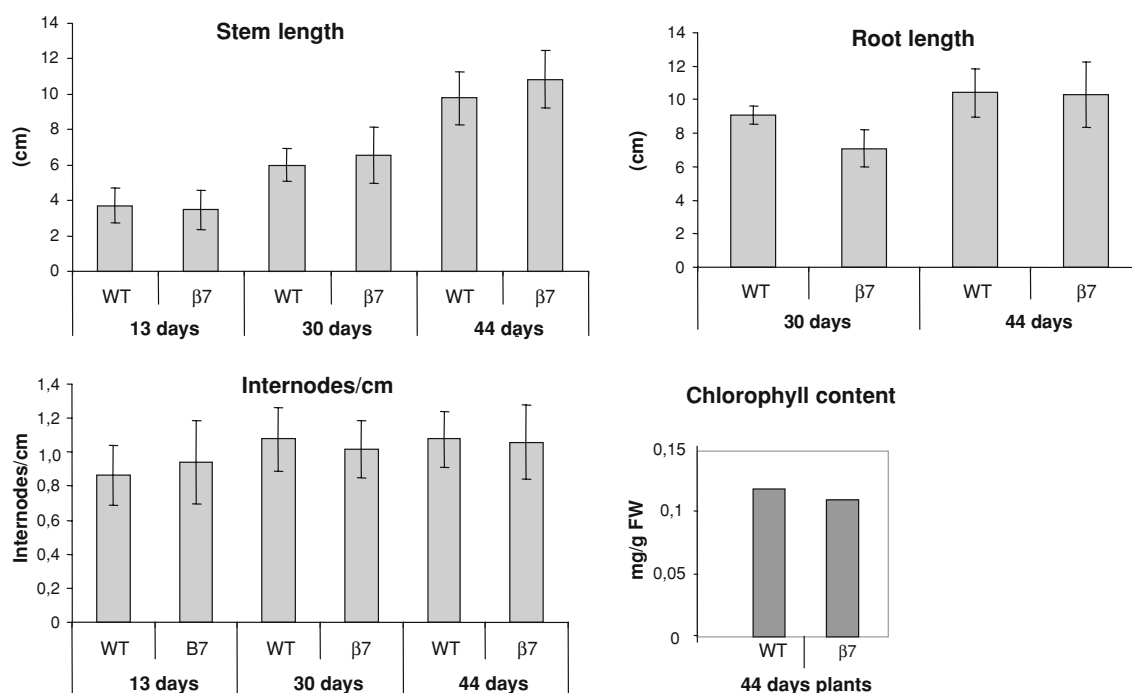


Fig. 6 Morphological characteristics of wild type and $\beta 7$ plants. Plants were grown under multiplication conditions for 13, 30 or 44 days. Stem length (cm), root length (cm), number of internodes (per cm) and chlorophyll content (mg g^{-1} FW, fresh weight) was determined

Table 3 Effect of sucrose (8%), CCC (0.5 mg ml^{-1}) or GA3 ($0.5 \mu\text{M}$) on tuber induction

WT					$\beta 7$				
Days in 8% SUC	<i>n</i>	NIS	IS	% of tuber induction	Days in 8% SUC	<i>n</i>	NIS	IS	% of tuber induction
8	31	13	18	58	8	32	12	20	62.5
10	36	18	18	50	10	36	1	35	97.2
24	30	14	16	53	24	39	4	35	90
Total	97	45	52	53.6	Total	107	17	90	84
Days in 8% SUC+CCC					Days in 8% SUC+CCC				
10	17	0	17	100	10	18	0	18	100
24	15	0	15	100	24	20	0	20	100
Total	32	0	32	100	Total	38	0	38	100
Days in 8% SUC+GA3					Days in 8% SUC+GA3				
10	16	16	0	0	10	14	14	0	0
15	21	21	0	0	15	30	22	0	0
Total	37	37	0	0	Total	44	36	0	0

the highest increase (almost 4-fold); which could be related to a synergistic effect of sucrose and ABA. However no induction in response to phytohormones was observed in the $\beta 7$ line (Fig. 9A, right panel).

Previous reports have proposed that CDPKs could participate in the GA transduction pathway (Abo-el-Saad and

Wu 1995; Khan et al. 2005; Zhang et al. 2005). Therefore, StCDPK1 expression was studied in wild type cuttings incubated during 10 days in tuber induction conditions (8% sucrose, complete darkness) but with the presence of GA3; and in cuttings grown during 13 days in tuber inducing conditions that were then transferred for 2 or 6 h to liquid

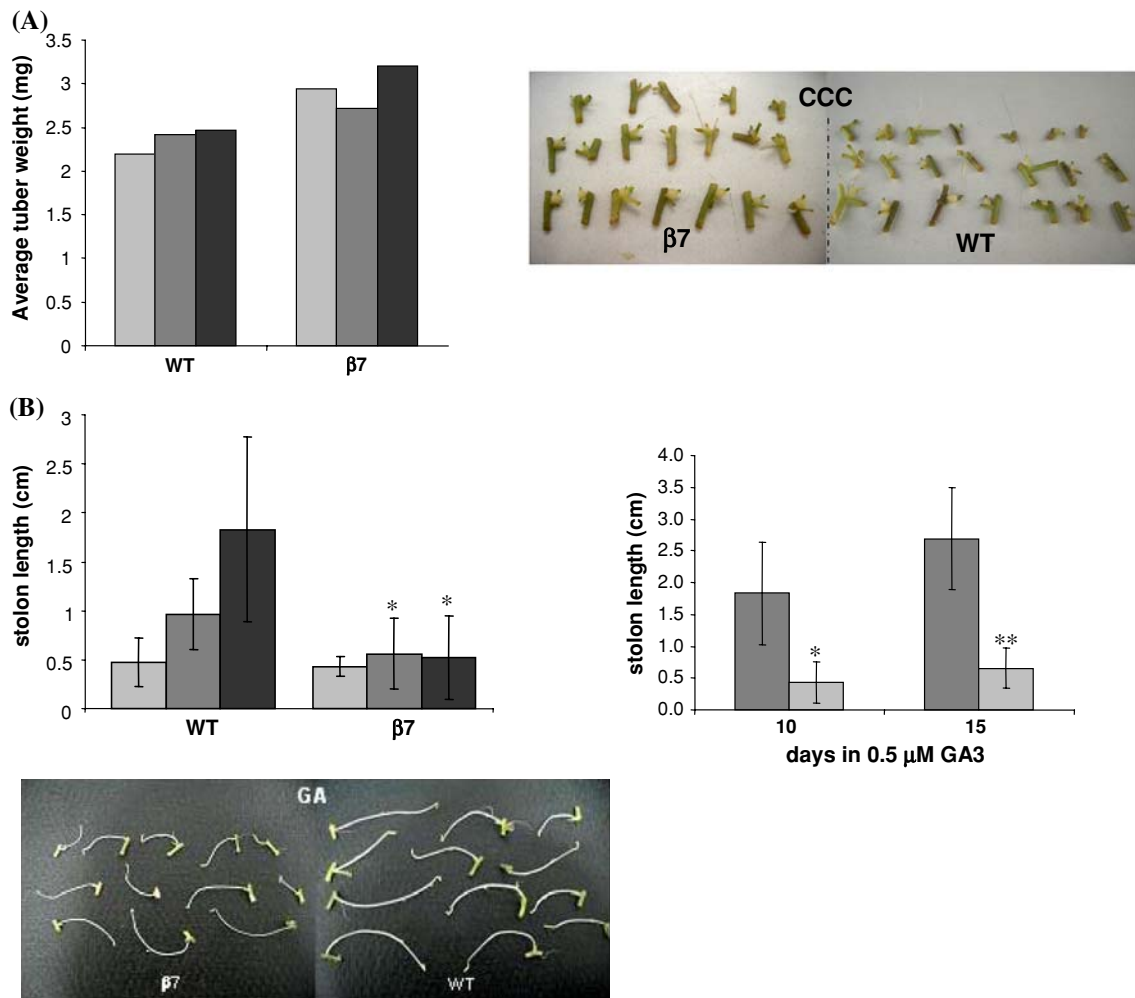


Fig. 7 Differential responses of wild type and $\beta 7$ stem cuttings grown under tuberization conditions to: **(A)** the addition of CCC (inhibitor of GA synthesis); **(B)** the addition of 0.05–0.5 μM GA3. **A** Average weight (mg) of wild type and $\beta 7$ microtubers from stem cuttings treated or not (Control = light grey bars) with 0.05 (dark grey bars) or 0.5 mg ml^{-1} (black bars) CCC (left panel). Photographs of CCC-treated wild type (WT) and $\beta 7$ microtubers (right panel). **B** Wild type ($n = 20$) and $\beta 7$ ($n = 20$) stem cuttings were cultured during 10 days with different GA3 concentrations and stolon length

(cm) was determined (left panel). Controls without GA treatment (light grey bars; $n = 8$); dark grey bars indicate 0.05 μM GA and black bars indicate 0.5 μM GA treatments. Asterisk indicates that the result is statistically significant ($P < 0.01$). Wild type (dark grey) and $\beta 7$ (light grey) stem cuttings were cultured during 10 or 15 days with 0.5 μM GA3 and stolon length (cm) was determined (right panel). Asterisk indicates that the result is statistically significant ($P = 0.0192$; ** $P = 0.008$). Photographs of GA3-treated wild type (WT) and $\beta 7$ stolons (lower panel)

Table 4 Effect of increasing sucrose concentrations on tuber yield and stolon length

Sucrose in the culture media	Tuber yield		Length of stolons (cm)			
	$\beta 7$	WT	$\beta 7$	n	WT	n
2%	0/23	0/22	2.7 ± 0.2	23	2.5 ± 0.2	22
4%	0/25	0/26	2.4 ± 0.15	25	2.7 ± 0.2	26
6%	6/31	0/29	2 ± 0.1	31	1.5 ± 0.3	29
8%	18/18	9/16	0.75 ± 0.25	18	1.5 ± 0.1	16

MS media with 8% sucrose with or without (control) 0.5 μM GA3. For the long treatment, wild type cuttings treated during 10 days with 8% sucrose were used as

controls. No induced stolons were present in the long treatment with GA. A 50% increase in *StCDPK1* expression was observed in response to the long treatment (data not shown), confirming the enhancement previously observed.

The expression of *StCDPK1*, *StCDPK3* and of two enzymes involved in the GA-biosynthetic pathway, *GA2-oxidase* and *GA20-oxidase*, was determined in the short treatment. Data are shown relative to expression of ubiquitin and in all cases controls (8% sucrose) are set as 1 (Fig. 9B). The *GA20-oxidase* in general, leads to the formation of a C19-GA which after 3-oxidation produces active GA (GA_4 and GA_1). These bioactive products are subsequently inactivated by 2-oxidation catalysed by GA

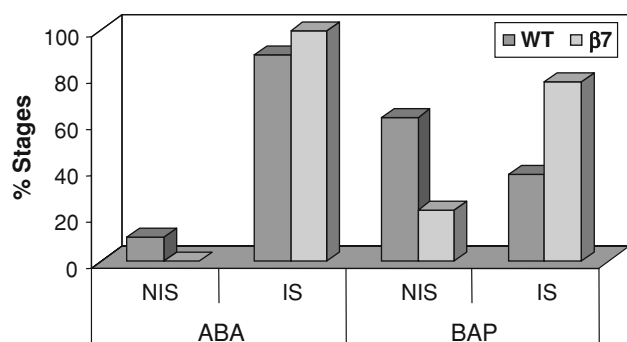


Fig. 8 Responses of wild type and $\beta 7$ stem cuttings grown under tuberization conditions to the addition of ABA and BAP. Percentage (%) of induced (IS) or non-induced stolons (NIS) was determined in wild type (WT, dark grey) and $\beta 7$ (light grey) internodes treated with $7.6 \mu\text{M}$ ABA or 5 mg ml^{-1} BAP

2-oxidase. A 50% increase in *GA2-oxidase* expression levels was observed after 2 h of treatment with GA3 in accordance to a positive feed-back, but after 6 h of treatment, *GA2-oxidase* expression returns to control levels. Regarding the expression of *GA20-oxidase*, a gradual increase was observed after the treatment. An almost 3-fold increase in *StCDPK1* expression was observed in the induced stolons exposed to GA for 2 h. After 6 h exposure, *StCDPK1* expression returned to basal levels. In contrast to

StCDPK1 expression, *StCDPK3* was not induced by GA3, moreover it was downregulated after 6 h of treatment. Altogether, these results suggest that *StCDPK1* could be involved in GA signalling upon tuber development.

Discussion

In this manuscript we describe for the first time the genomic organization of a CDPK gene in *Solanum tuberosum*, and the response of wild type plants and of a transgenic line with low *StCDPK1* expression to different tuberization conditions. *StCDPK1* is transiently expressed in swelling stolons (Raices et al. 2001) and the increase (5-fold) in *StCDPK1* protein observed in induced stolons confirms that it is developmentally regulated.

Based upon sequence homology CDPKs can be classified into four major subgroups (Cheng et al. 2002), though the significance of these subgroups with regard to biochemical or physiological function is not known (Hrabak et al. 2003). *StCDPK1* coding sequence is similar to *Arabidopsis* and rice CDPKs (AtCPK9, 80%, AtCPK33, 78%, OsCPK19, 79%) belonging to subgroup IIa. Its genomic structure was found to be identical to these genes. According to Li et al. (2008), CDPK genes belonging to the

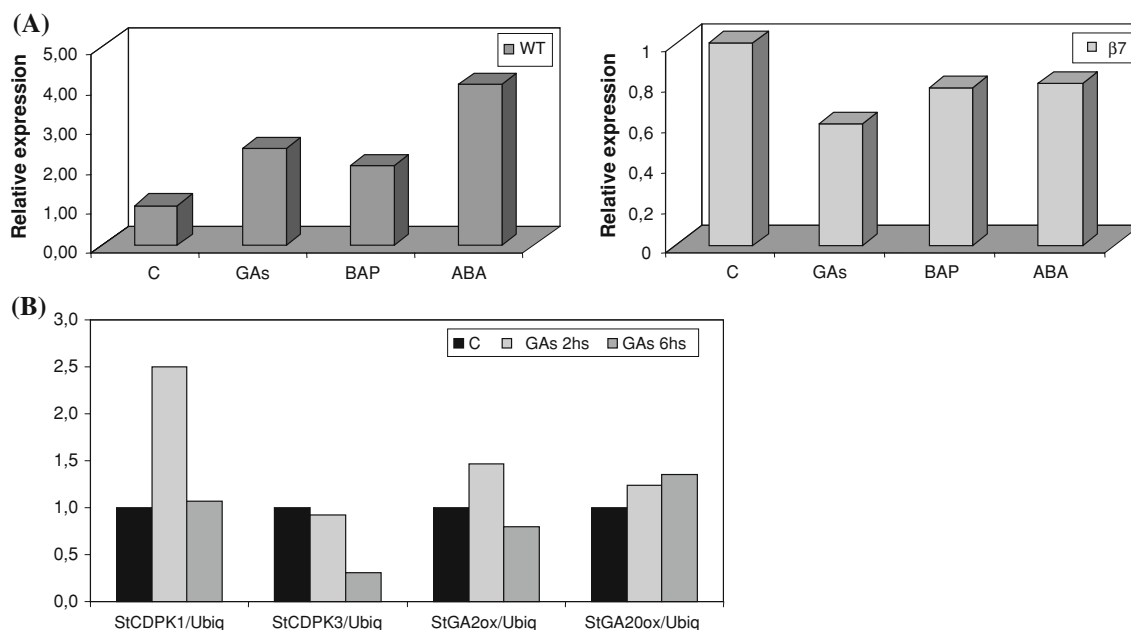


Fig. 9 Relative expression of *StCDPK1* in: **A** Wild type (right panel) and $\beta 7$ (left panel) plants grown during 10 days (long treatment) under tuberization conditions (8% sucrose, complete darkness) with the addition of phytohormones (GA, BAP and ABA). *StCDPK1* expression was normalized to Ubiquitin expression. In both cases, the expression in control conditions (8% sucrose) was set as 1. **B** Wild type plants grown during 13 days under tuberization conditions (8%

sucrose, complete darkness) and transferred to liquid MS media with 8% sucrose (control, black) or 8% sucrose+GA3 during 2 (light grey) or 6 (dark grey) hours (short treatments). In addition, the expression of *GA2-ox* and *GA20-ox* was determined in the short treatments. The expression of *StCDPK1*, *GA2-ox* and *GA20-ox* was normalized to Ubiquitin expression. The expression in control conditions (8% sucrose) was set as 1

same group had the same number of introns among wheat, rice and *Arabidopsis*. This is also true for *StCDPK1*, therefore not only the coding sequence but also the gene structure can be considered a characteristic shared by the group. Introns are inserted in nearly the same position in all these genes, however, there is a considerable variation in the size of the corresponding introns, some being very large, as is the case of *StCDPK1*. Though genome size may not necessarily be correlated with intron size, this could be one of the factors that influence the size of the potato genome (about 840 Mb).

Our mapping experiments indicate that *StCDPK1* is localized in the distal arm of chromosome 12. The fact that intronic sequences were used in these experiments guarantees that we have mapped this gene and not another member of the CDPK family. In *Arabidopsis*, wheat and rice genomes CDPKs are distributed in all chromosomes. Interestingly, in *Arabidopsis*, a gene cluster on the short arm of chromosome IV contains five genes (*AtCPK* 21, 22, 23, 27, and 31), all members of subgroup IIa. It would be interesting to determine if a similar organization is observed in the potato genome.

Modification with lipophilic moieties has been described as an important mechanism involved in membrane anchoring of cytoplasmic proteins, which tightly regulates their proper intracellular targeting (Gonzalo and Linder 1998; Smotrýs and Linder 2004). In the majority of the cases studied to date, dually acylated proteins are regulated through a palmitoylation–depalmitoylation cycle. Our previous results indicated that *StCDPK1* had a functional myristoylation and palmitoylation consensus and *StCDPK1::GFP* fusion protein was found to be associated with plasma membranes when expressed in onion epidermal cells (Raices et al. 2003a). Experiments performed with an artificial protein showed that association to the plasma membrane was only observed in dually acylated constructs (Navarro-Lérida et al. 2002). Western blot experiments show that *StCDPK1* is mainly associated to the microsomal fraction of swelling stolons or to a PM-rich fraction purified from sprouting tubers that displays higher CDPK activity than soluble fractions (4-fold). The anti-*StCDPK1* antibody detected a positive signal in the periphery of the cells from the periderm and neighbouring parenchyma tissues of swelling stolons. Moreover, TEM analysis of swelling stolons indicates that *StCDPK1* is located in the site corresponding to the plasma membrane of parenchyma cells. These results are consistent with *in situ* hybridization experiments showing that the *StCDPK1* mRNA was abundant in external cells of swelling stolons (Raices et al. 2003b). Altogether our results indicate that *in vivo* this kinase is targeted to the plasma membrane.

While *CDPK* genes and the proteins they encode have been identified in many species, the studies to clarify their

roles in plant growth and development are still under way. Different approaches are being used to investigate the functions of these proteins but these experiments can be complicated by the existence of multiple, closely related genes that may have similar functions. Knockouts in these kinase genes usually have not had an obvious phenotype, indicating either that the correct conditions to identify a phenotype were not tested or that the protein's function can be fulfilled by other related family members (Hrabak et al. 2003). In an effort to elucidate the role of *StCDPK1* in tuber formation, transgenic lines with reduced expression of *StCDPK1* ($\beta 7$) were produced. These lines did not differ from controls when cultured under multiplication conditions, however when grown under tuber inducing conditions some significant differences were observed. Most important is that the $\beta 7$ line tuberized earlier than controls without the addition of CCC (GA inhibitor), developed more tubers than wild type plants in the presence of hormones that promote tuberization in potato (ABA and BAP) and was more insensitive to GA action (stolons were significantly shorter than those of control plants).

Potato plants tuberize in response to endogenous growth regulators that reflect changing environmental conditions. According to the classical hypothesis, a substance is synthesized in the leaves under short-day conditions and transported to the stolons, where it triggers tuber formation (Struik et al. 1987). The exact nature of this transmissible signal is still unknown but it is most likely based on a mixture of both inducing and inhibiting types of signals (Ewing and Struik 1992; Jackson 1999; Martínez-García et al. 2002). Two pathways regulate tuberization; one is photoperiod-dependent and the other is GAs-dependent. Both pathways are coordinated and are necessary to direct this developmental process (Martínez-García et al. 2002). During the early stages of the process, GAs regulate the production and transport of the tuberizing signal, and in the later stages they modulate the late responses. Therefore, high GAs concentrations in the aerial portion of the plant determine the morphological changes while low GAs concentrations in the stolon tips allow tuber formation (Xu et al. 1998). Whether biologically active GAs are part of the transmissible signal or play a role in the production, sensitivity or transport of the tuberization-promoting or -inhibiting signals within the plant remains to be resolved.

Considerable progress has been made in understanding the pathways involved in GA biosynthesis and metabolism. Catabolism of GAs is an important factor that regulates the endogenous levels of bioactive GAs. Bioactive GAs are 2 β -hydroxylated to produce biologically inactive GAs, a step catalyzed by GA2-oxidase (Ross et al. 1995). *StGA2ox1* is upregulated during the early stages of potato tuber development prior to visible swelling and is

predominantly expressed in the subapical region of the stolon and growing tuber, thus a role for StGA2ox1 in early tuber initiation has been suggested. On the contrary, the transcript levels of StGA3ox2, an enzyme that catalyses the final step in the biosynthesis of bioactive GAs, is strongly downregulated at tuber onset (Kloosterman et al. 2007). The expression of *GA2-oxidase* genes from *Arabidopsis* was stimulated by the application of GA3 indicating a feedback regulation of these genes in order to maintain endogenous levels of bioactive GAs (Thomas et al. 1999). The increase in *StGA2-ox* expression observed after GA3 application (Fig. 9B) suggests that the potato gene is tightly regulated.

The GA signalling pathway essentially consists of three interacting players. GA receptors of rice (GID1) and *Arabidopsis* (Ueguchi-Tanaka et al. 2005; Nakajima et al. 2006) are able to interact, in a GA dependent way, with the DELLA proteins; these interactions solely depend on the presence of the DELLA-domain (Willige et al. 2007). DELLA proteins are inhibitors of GA responses, which are broken down in the presence of GAs, and this degradation is mediated by an E3 ligase, that is specified by an F-box protein such as SLY1 in *Arabidopsis* or GID2 in rice (McGinnis et al. 2003; Sasaki et al. 2003; Dill et al. 2004). In *Arabidopsis thaliana*, once DELLA proteins are destabilized, the nuclear transcription factor PIF4 accumulates in the nucleus and controls genes mediating cell elongation (de Lucas et al. 2008).

Protein kinases and protein phosphorylation have been shown to play an important role in the GA response. Typically; specific phosphorylation of target sequences is required for efficient F-box-mediated recruitment of target proteins to SCF E3 ubiquitin ligase complexes (Patton et al. 1998; Jackson and Eldridge 2002; Busino et al. 2003). Fu et al. (2004) suggest that the inherent (phosphorylation-independent) level of interaction between the DELLA and SLY1 proteins is further strengthened by phosphorylation. DELLA proteins are now known to be fundamental to the regulation of growth via several plant developmental regulatory pathways and are thought to serve as integrators of multiple growth regulatory signalling inputs (Achard et al. 2003; Fu and Harberd 2003).

It has been well documented that CDPK participate in early signal transduction and function as integration points for multiple stimuli (Cheng et al. 2002; Ludwig et al. 2004). The GA3-induced increase in Ca^{2+} influx observed in barley aleurone protoplasts (Gilroy and Jones 1992), provides a possible mechanism to link the GA3 response with activation of CDPKs. HvCDPK1 mediates the GA response of the aleurone through regulation of vacuolar function that lies downstream of transcriptional regulation (McCubbin et al. 2004). In rice, evidences that CDPKs are involved in the GA response pathway accumulate: the

activity of a plasma membrane CDPK in GA-treated rice seeds was higher than in untreated seeds (Abo-El-Saad and Wu 1995); CDPK activity also increased in rice leaves after GA treatment and the phosphorylation of seven proteins was enhanced by this treatment (Khan et al. 2005). CDPK and phosphorylation activities of some proteins were increased in the Rubisco activase sense transgenic rice plants by GA3-treatment (Sharma and Komatsu 2002). Antisense OsCDPK13 transgenic rice lines were shorter than the vector control lines, and the expression of Os-CDPK13 was lower in dwarf mutants of rice than in wild type. This rice isoform became phosphorylated in response to cold and GA and its expression and protein accumulation were up-regulated by GA3 treatment (Abbasi et al. 2004). In addition, the expression of two tobacco CDPK isoforms, NtCDPK1 (Yoon et al. 1999) and NtCPK4 (Zhang et al. 2005) and of *liCDPK2*, a CDPK gene from *Isatis indigotica*, was enhanced in response to GAs (Lu et al. 2006). In this paper we show that GA-treatments enhanced *StCDPK1* expression. The fact that, in vitro, $\beta 7$ plants tuberized earlier and, when exposed to GAs $\beta 7$ stolons were shorter than wild types, together with the increase observed in *StCDPK1* expression in response to GAs suggests that this CDPK isoform could modulate GA signalling.

However, *StCDPK1* expression was also positively modulated by tuberization-promoting phytohormones (ABA and BAP) and by sucrose (Raices et al. 2003b). At the protein level, a CDPK activity increased in sucrose-treated potato plants (Raices et al. 2003c) while StCDPK1 protein content increased (1.5- to 2.5-fold) in potato stolons in response to sucrose. ABA and sucrose promote accumulation of storage reserves in developing plant embryos (reviewed in Gibson 2004), and in potato stolons both stimulate tuberization. ABA functions as an antagonist of GAs, and sucrose regulates tuber formation by changing the GA level of the developing stolons and tubers (Xu et al. 1998a). Calcium is involved in ABA signal transduction (reviewed in Finkelstein et al. 2002; Himmelbach et al. 2003; Fan et al. 2004) and several *Arabidopsis* CDPK isoforms are involved in this response (Mori et al. 2006; Zhu et al. 2007). According to the expression data observed in wild type plants, it can be suggested that StCDPK1 could be a target of ABA action and that both ABA and sugar have a synergistic effect on the kinase expression.

Under in vitro conditions the time point of tuber formation appears to rely primarily on sucrose/GA interactions within the stolon tip (Xu et al. 1998) whilst under in vivo conditions additional regulatory factors are required to induce tuber formation. Sugar- and phytohormone-response pathways exhibit 'crosstalk' in the regulation of many processes. Our results suggest that StCDPK1 may act as a stimulus-response coupler in

calcium-regulated processes during potato tuberization. Under non-inducing conditions, GA inhibition prevails; stolons elongate and no swelling is observed. In our culture conditions GA inhibitory effect is stronger than the sucrose promoting effect because in the presence of 0.5 μM GA3 no swelling is observed. According to the results obtained with the $\beta 7$ line, StCDPK1 could be a positive regulator of the elongation response and a negative regulator of the tuberization process. In another developmental process, the formation of root nodules as result of the *Medicago-Rhizobia* interaction, a CDPK isoform, MtCPK3, acted as a negative regulator that controlled nodule number (Gargantini et al. 2006). Under inducing conditions, the relative balance of inducers and inhibitors of tuberization promotes stolon to tuber transition, elongation ceases and stolon swelling begins. StCDPK1 could also be involved in this process in view of the rise in transcript (Raices et al. 2003a, b, c) and protein levels (Fig. 4) observed in response to increasing sucrose concentrations (4–8%) and to the high expression of StCDPK1 detected in swelling stolons.

In both conditions (inhibiting or promoting), once cytosolic calcium has raised StCDPK1 could phosphorylate a response element and thus stimulate or inhibit an event or process. In the future, identifying the kinase substrate/s will help to elucidate this signalling pathway. A potato CDPK isoform, StCDPK5, induces the phosphorylation of StRBOHB and regulates the oxidative burst (Kobayashi et al. 2007). Recently it was shown that superoxide anion acts as a signal transducer via GA biosynthetic pathways for the regulation of plant growth and tuber development of potato (Kim et al. 2007). Thus, the possibility of a crosstalk with an oxidative burst-mediated redox signalling pathway during tuberization should be considered.

Other CDPK isoforms were shown to be modulated by different phytohormones. NtCDPK1 from tobacco is induced by GA, ABA and cytokinin (Yoon et al. 1999), it phosphorylates a regulatory subunit of the 26S proteasome of tobacco *in vitro*, and interacts with it *in vivo* (Lee et al. 2003). More recent studies showed that NtCDPK1 plays a role in lateral root development and root elongation (Lee et al. 2006). In *Arabidopsis*, AtCPK30 encodes a CDPK that is highly expressed in roots and is induced by ABA, IAA, 2,4-D, GA(3) and 6-BA treatments (Yuan et al. 2007). The fact that a CDPK gene can be induced by different hormones could suggest that the gene expression profile is not the central part of the regulation of CDPKs. Changes in the level of cytoplasmic Ca^{2+} or in the phosphorylation/dephosphorylation status of CDPK substrates could be important in the regulation of CDPK activity. Recently, the expression patterns of wheat CDPKs were analyzed under various conditions. Each CDPK isoform exhibited responses to multiple stress treatments, suggesting that they were major converging points for crosstalk

between different signal transduction pathways. On the other hand, multiple CDPKs seemed to be necessary to coordinate with one stress stimulus (Li et al. 2008). In particular, *StCDPK1* expression was induced by different hormones that are related to the tuberization process. Our results suggest that StCDPK1 could be a converging point for the inhibitory and promoting signals that influence the onset of potato tuberization.

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