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Changes in Calcium-Dependent Protein Kinase Activity during in Vitro Tuberization in Potato[1]

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A soluble Ca^{2+}-dependent protein kinase (CDPK) was purified to homogeneity in potato (*Solanum tuberosum* L.) plants. Potato CDPK was strictly dependent on Ca^{2+} (one-half maximal activation 0.6 μM) and phosphorylated a wide diversity of substrates, in which Syntide 2 was the best phosphate acceptor (Michaelis constant = 30 μM). The kinase was inhibited by Ca^{2+}-chelating agents, phenotiazine derivatives, and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (one-half maximal inhibition = 0.25 mM). Polyclonal antibodies directed against the regulatory region of the soybean CDPK recognized a 53-kD polypeptide. In an autophosphorylation assay, this same band was strongly labeled with [γ-^{32}P]ATP in the presence of Ca^{2+}. CDPK activity was high in nontuberized plants, but increased 2.5-fold at the onset of tuber development and was reduced to one-half of its original activity when the tuber had completed formation. In the early stages of tuberization, Ca^{2+}-dependent phosphorylation of endogenous targets (specific bands of 68, 51, and 46 kD) was observed. These polypeptides were not labeled in nontuberizing plants or in completely formed tubers, indicating that this phosphorylation is a stage-specific event. In addition, dephosphorylation of specific polypeptides was detected in tuberizing plants, suggesting the involvement of a phosphatase. Preincubation of crude extracts with phosphatase inhibitors rendered a 100% increase in CDPK activity.

Tuberization is a complex process that results in the differentiation of a specialized shoot, the stolon, into a storage organ, the tuber. A wide variety of environmental and hormonal stimuli are known to be involved in the induction of tuberization (Menzel, 1985a, 1985b; Vreugdenhil and Struik, 1989). Experiments with single-node leaf cuttings from induced potato (*Solanum tuberosum* L.) plants suggested the possible role of Ca^{2+} as mediator of the tuberization stimulus (Balamani et al., 1986). In addition, Jena et al. (1989) demonstrated an increase of calmodulin mRNA in the stolon tip. However, the biochemical and molecular events involved in the signal transduction of this stimulus are not clearly understood.

Increasing evidence has established Ca^{2+} as a second messenger in plants (Roberts and Weaver, 1990; Muto, 1992; Poovaiah and Reddy, 1993). The concentration of free cytosolic Ca^{2+} in plant cells can be elevated by various external signals. Such increases in the concentration of free Ca^{2+} is one of the primary events in the transduction of many signals and can alter biochemical processes in plants by activating particular enzymes.

During eukaryotic evolution a multitude of specialized Ca^{2+}-modulated proteins have arisen that serve as receptors for Ca^{2+} signals. Ca^{2+} has been shown to affect protein phosphorylation in plants (Budde and Chollet, 1988), and a calmodulin-independent CDPK was first discovered in soybean (Harmon et al., 1987). Later studies demonstrated that this enzyme was prevalent in plants and contains a protein kinase catalytic domain and a Ca^{2+}-binding regulatory domain similar to calmodulin; both regions are separated by a junction domain. This unique molecular structure explains the direct activation of this enzyme by Ca^{2+} and clearly establishes CDPK as the prototype of a new class of protein kinases (Roberts and Harmon, 1992).

Regulatory phosphorylation can result in inactivation, activation, and/or changes in the allosteric properties of the target enzyme. Dephosphorylation of phosphorylated proteins by specific phosphatases offers a mechanism by which the biochemical pathways involving phosphorylation can be deactivated. The steady-state activity of the target enzyme is adjusted over a wide range by positive and/or negative effectors, influencing the rates of phosphorylation and dephosphorylation (Cohen, 1992).

In plants protein phosphorylation has been observed in vivo, and protein kinase activity has been demonstrated in vitro. However, in only a few cases has reversible phosphorylation been shown to be associated with the regulation of plant proteins of known functions. In this study a soluble CDPK was purified and characterized in potato plants that were grown under multiplication conditions; this activity was also present in plants that were induced to tuberize. Evidence supporting the presence of specific phosphatases is presented as well. Both enzymatic activities increase transiently in early stages of tuberizing plants,

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Abbreviations: CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CDPK, Ca^{2+}-dependent protein kinase; CDPKS, calmodulin-dependent protein kinase substrate; GS, peptide derived from glycogen synthase (residues 1–12); $K_{0.5}$, one-half maximal activation; MLCKS, myosin light chain kinase substrate, W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

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suggesting the direct involvement of Ca^{2+}-regulated phosphorylation/dephosphorylation at the onset of potato tuberization.

MATERIALS AND METHODS

Cultures and Extracts

Micropropagation of virus-free meristematic sprouts of potato (*Solanum tuberosum* L. var Spunta) was carried out in Murashige and Skoog (1962) medium containing 30 g L^{-1} Suc, and plants were grown in a growth chamber under a 16-h light photoperiod at 25°C (multiplication conditions).

Tuberization was induced with Murashige and Skoog medium containing 500 mg L^{-1} 2-chloroethyltrimethylammonium chloride and 80 g L^{-1} Suc (Tovar et al., 1985). Plants were grown in complete darkness under inducing conditions at 21 ± 2°C using stem cuttings and stolons. Four stages of the tuber-development process were defined by correlating the morphological aspect of the incipient tuber to the microscopic studies of longitudinal sections (Ulloa et al., 1996). Plants from each tuberization stage were harvested, rinsed with distilled water, ground in a mortar that was cooled with liquid nitrogen, and extracted with 10 mM Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$, 2 mM KCl, 2 mM β-mercaptoethanol, 3 mM EDTA, 1 mM EGTA, 5% (v/v) glycerol, 0.25 M Suc, 2% (w/v) polyvinylpolypyrrolidone, and protease inhibitors (0.1 mM PMSF, 1 mM benzamidine, 2 μg mL^{-1} soybean trypsin inhibitor, and 25 units mL^{-1} aprotinin) (buffer A). The suspensions (1 mL buffer g^{-1} of wet tissue) were centrifuged for 10 min at 2300*g* and the pellet was discarded. CDPK activity was then assayed in the supernatant fraction. In addition, the crude extract was phosphorylated with [γ-^{32}P]ATP and analyzed in SDS-PAGE.

Purification Procedures

Plants grown under multiplication conditions were harvested, rinsed with distilled water, ground in a mortar that was cooled with liquid nitrogen, and extracted with buffer A (1 mL buffer g^{-1} of wet tissue). The homogenate was centrifuged for 10 min at 1,000*g* and the pellet, containing cell debris, was discarded. The supernatant was centrifuged for 15 min at 12,000*g* and further centrifuged for 1 h at 105,000*g*. The resulting supernatant, designated S100 (73 mL, 0.43 mg mL^{-1}), was loaded onto a DEAE-celullose (DE-52) column (Whatman) (2 × 10 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, and protease inhibitors (buffer B). The column was washed with the same buffer and eluted step-wise (0.5-mL fractions) with buffer B containing increasing concentrations of NaCl (0.1–0.6 M). CDPK activity was assayed in all fractions, as described below.

The active fractions were pooled, dialyzed against 10 mM Tris-HCl, pH 7.4, and reloaded onto a Mono Q-Sepharose column (Pharmacia) equilibrated with the same buffer. The column was washed with the same buffer and was eluted with a linear gradient (0–0.7 M) of NaCl.

The peak with CDPK activity was dialyzed against 10 mM Tris-HCl, pH 7.4, containing 5 mM $CaCl_2$ and was loaded onto a phenyl-Sepharose column (Pharmacia) (bed volume = 5 mL) equilibrated with 10 mM Tris-HCl, pH 7.5, and 0.5 mM $CaCl_2$ (buffer C). The column was washed with 7 volumes of buffer C, 3 volumes of buffer C containing 0.15 M NaCl, 5 volumes of buffer C containing 5 mM EGTA, and was eluted with 5 volumes of 4 M urea. CDPK activity was assayed after dialysis against 10 mM Tris-HCl, pH 7.5. The active fraction was concentrated against PEG 8000 and was injected onto a Superdex 200 HR 10/30 filtration column (Pharmacia) equilibrated with 40 mM phosphate buffer, pH 7.2, containing 0.15 M NaCl (buffer D). The elution was performed with buffer D using a fast protein liquid chromatography system. Active fractions were concentrated against 2 M Suc. Samples from all of the purification steps were analyzed by 10% SDS-PAGE. Western blot analysis and autophosphorylation assays were performed with the phenyl-Sepharose preparation.

Alternatively, the DEAE fractions, which eluted at 0.3 M NaCl, were pooled and diluted (1:10) with 20 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 1 mM $CaCl_2$, and protease inhibitors (buffer E). This dilution (6.8 mg of protein) was loaded onto a Syntide-Sepharose affinity column (bed volume = 1 mL) equilibrated with buffer E. The column was washed and eluted with buffer E without Ca^{2+} followed by 20 mM Tris-HCl, pH 7.5, containing 1 mM β-mercaptoethanol, 0.3 M NaCl, and protease inhibitors. The enzyme was eluted with salt and the active fractions were concentrated and analyzed in 10% SDS-PAGE. Kinetic assays were performed with the Syntide-Sepharose preparation.

Chromatography on Superose 12

An aliquot (0.5 mL, 1 mg mL^{-1}) of the DEAE preparation, previously concentrated against 50% Suc, was dialyzed against 50 mM Tris-HCl, pH 7.5, containing 0.5 mM EGTA, 0.5 mM DTT, 0.1 M NaCl, and protease inhibitors and then loaded onto a Superose 12 gel filtration column (Pharmacia) equilibrated with the same buffer. The column was calibrated with the following internal standards: catalase (247 kD), lactic dehydrogenase (142 kD), malic dehydrogenase (70 kD), and Cyt *c* (12 kD).

Protein Kinase Activity Assays

Aliquots of the different fractions were 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 $pmol^{-1}$), 10 mM β-mercaptoethanol, and 25 μM Syntide-2 with the addition of 1 mM EGTA or 1 mM $CaCl_2$, in a final volume of 0.06 mL. Reactions were initiated by the addition of [γ-^{32}P]ATP and stopped as described previously (Ulloa et al., 1991). Assays were performed at 30°C for 10 min.

When acid substrates such as phosvitin or casein were used as acceptors, the reaction was stopped with 10% TCA according to Ulloa et al. (1987). The $K_{0.5}$ for Ca^{2+} was determined in the presence of EGTA-Ca^{2+} buffers according to Bartfai (1979).

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Western Blot Analysis

Aliquots from the Mono-Q or phenyl-Sepharose columns (30 or 4 μg, respectively) were electrophoresed in 10% SDS gels, and polypeptide transfer from polyacrylamide to nitrocellulose membranes was carried out as described previously (Ochatt et al., 1993). Blots were incubated with affinity-purified polyclonal antibodies directed against the calmodulin-like domain of soybean α CDPK (Bachmann et al., 1996), and with monoclonal antibodies directed against the catalytic domain of soybean CDPK (Putnam-Evans et al., 1990). The staining was done according to the method of Pratt et al. (1986). In addition, proteins were immunodetected with antibodies raised against rat CaMKII or rat αβγ protein kinase C and then developed with the alkaline phosphatase biotin-avidin complex from the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA).

Autophosphorylation Assay

Aliquots from the Mono-Q and the phenyl-Sepharose columns (30 or 4 μg of protein, respectively) were analyzed in 10% SDS-PAGE, transferred to PVDF membranes, and renatured according to Ferrell and Martin (1989). In situ phosphorylation was performed by incubating the membranes with a mixture containing 30 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM $CaCl_2$ or 5 mM EGTA, and [γ-^{32}P]ATP (50 μCi mL^{-1}, 6000 Ci $mmol^{-1}$) at room temperature for 30 min with agitation. The membranes were then washed and analyzed by autoradiography as described by Ferrell and Martin (1989).

Endogenous Phosphorylation Assays

Crude extracts of each tuberization stage (50 or 100 μg of protein) or from the plants grown under multiplication conditions (100 μg of protein) were incubated for 2 to 5 min at 30°C with 5 μM [γ-^{32}P]ATP (10,000 cpm $pmol^{-1}$) in 20 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, and 10 mM $MgCl_2$ with the addition of 1 mM EGTA or 1 mM $CaCl_2$ in a final volume of 50 μL. Reactions were stopped by the addition of cracking buffer and boiled for 2 min. Samples were resolved in 10% SDS-PAGE and the dried gels were autoradiographed.

Analytical Methods

Protein contents were determined by the methods of Lowry et al. (1951) or Bradford (1976) using BSA as a standard. Calmodulin was determined according to Téllez-Iñón et al. (1985). Pyruvate kinase activity was assayed with the 2,4-dinitrophenylhydrazine method (Leloir and Goldemberg 1960). SDS-PAGE was carried out according to Laemmli (1970). Gels were fixed and stained with Coomassie blue R-250 in 40% methanol containing 10% acetic acid (v/v). Except as otherwise indicated, prestained SDS-PAGE standards from GIBCO-BRL were used as molecular weight markers.

The Syntide-Sepharose column was prepared by coupling 5 mg of Syntide-2 to 1 g of cyanogen bromide-activated Sepharose 4B according to the manufacturer's protocol (Pharmacia). The amount of bound peptide was 94%.

Chemicals

Superose 12, Superdex 200 HR 10/30, Mono Q, phenyl-Sepharose CL-4B, and cyanogen bromide-activated Sepharose 4B were from Pharmacia. DEAE-cellulose (DE-52) was from Whatman. Kemptide, CDPKS, CDPKS analog, and MLCKS were from Peninsula Laborarories (Belmont, CA) Syntide-2 and GS were a kind gift from Dr. F. Baralle (International Center for Genetic Engineering and Biotechnology, Trieste). Histones, protamine, casein, phosvitin, PMSF, benzamidine, trypsin inhibitor, orthovanadate, β-glycerophosphate, ADP, chlorpromazine, trifluoperazine, 2,4-dinitrophenylhydrazine, and all chemicals for plant culture were from Sigma. Aprotinin (Trasylol, Bayer) was a gift from Gador Lab (Buenos Aires, Argentina). Compound 48/80 was from Calbiochem and W7 was from Seikagaku, Kogyo (Tokyo, Japan). All other reagents were of analytical grade. [γ-^{32}P]ATP was from New England Nuclear.

RESULTS

Soluble and particulate extracts of the potato plant (*S. tuberosum* L. var Spunta) grown under multiplication conditions were incubated with Histone IIIS and [γ-^{32}P]ATP in the presence of Ca^{2+}, EGTA, or cAMP and analyzed by SDS-PAGE. Histone phosphorylation was significantly increased in the presence of Ca^{2+} (Fig. 1, lanes 2 and 4), whereas cAMP had no effect (data not shown). The same results were observed when protein kinase activity was assayed in vitro on these crude fractions (data not shown).

To identify the protein kinase activity involved in these Ca^{2+}-dependent phosphorylations, the S100 fraction was chromatographed on a DEAE-cellulose column eluted stepwise with a saline gradient. Protein kinase activity was assayed with different substrates (Histone IIA S, Histone III S, Syntide-2, and Kemptide) in the presence of Ca^{2+} or EGTA. A Ca^{2+}-stimulated protein kinase activity eluted at 0.3 M NaCl, whereas calmodulin eluted at 0.45 M NaCl (Fig. 2A).

The peak from the DEAE column was assayed with EGTA or micromolar concentrations of free Ca^{2+}, using Syntide-2 as a substrate. Enzymatic activity was stimulated 8-fold in the presence of Ca^{2+}, and this increase could be

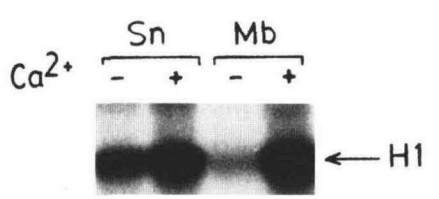

Figure 1. Histone phosphorylation by soluble (Sn) and particulate (Mb) extracts from potato plants grown under multiplication conditions (100 μg of protein in each lane). Incubations were carried out with 5 μM [γ-^{32}P]ATP and 1 mg mL^{-1} Histone III S and 1 mM Ca^{2+} (+) or 1 mM EGTA (−). Samples were analyzed by SDS-PAGE. Arrow indicates labeled histone.

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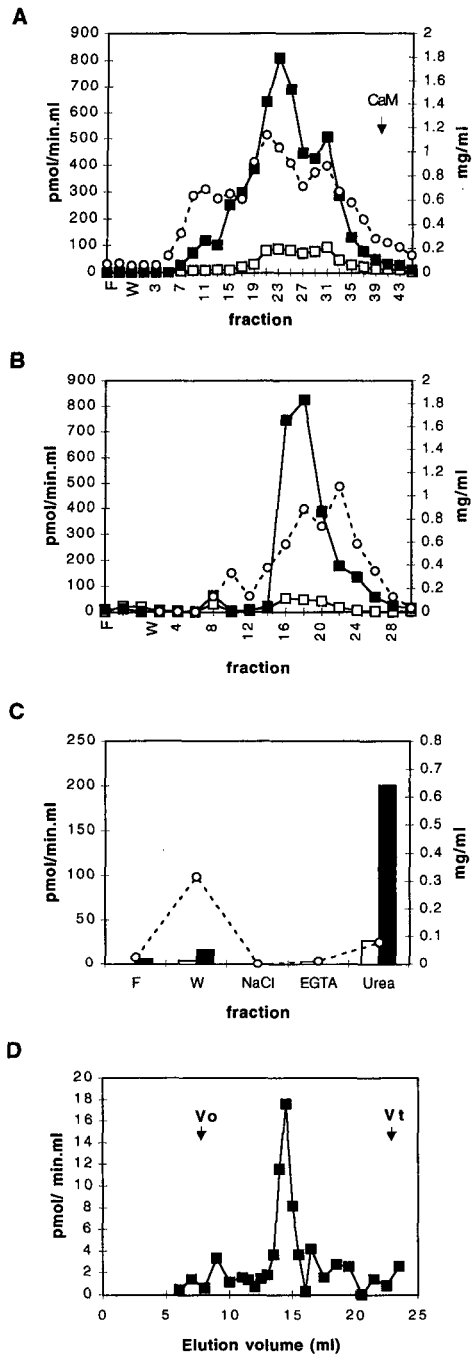

Figure 2. Sequential purification of the potato CDPK. A, Elution profile of the DEAE cellulose column. CaM, Calmodulin. B, Elution profile of the Mono-Q column. C, Elution profile of the phenyl-Sepharose affinity column. D, Elution profile of the Superdex 200 filtration column. CDPK activity was assayed with 1 mM EGTA (□) or 1 mM $CaCl_2$ (■). ○, Proteins.

completely reversed with EGTA. To study other possible modulators of the enzyme, exogenous calmodulin or phosphatidylserine/diacylglycerol was included in the assay in the presence of Ca^{2+} ions. Neither of these Ca^{2+} mixtures exceeded the activation obtained using free Ca^{2+} (data not shown). The possibility that endogenous calmodulin could be associated to the kinase and thus modulate its activity was analyzed. For this purpose, the DEAE preparation was loaded into a calmodulin-Sepharose affinity column and also analyzed with antibodies against CaMKII. The DEAE preparation was not recognized by the antibodies and did not bind to the affinity column (data not shown), indicating that this enzyme is not a calmodulin-dependent protein kinase. In addition, antibodies against $\alpha\beta\gamma$ protein kinase C did not recognize this enzyme.

A new type of Ca^{2+}-dependent, calmodulin-independent protein kinase, termed CDPK, has been characterized in plants (Roberts and Harmon, 1992). To test the possibility that the potato protein kinase corresponds to the CDPK family, the enzyme was purified to homogeneity (Fig. 3A). The DEAE preparation was dialyzed and chromatographed on a Mono-Q column. The elution profile shows a peak with CDPK activity eluting at 0.42 M NaCl (Fig. 2B).

A characteristic of the CDPKs is the binding to an affinity matrix typically used for calmodulin purification. The Mono-Q peak was dialyzed overnight against 10 mM Tris-HCl, pH 7.5, containing 0.5 mM Ca^{2+}, and the preparation was loaded onto a phenyl-Sepharose affinity column. The CDPK activity was eluted with 4 M urea (Fig. 2C). Previous attempts to elute the potato enzyme with EGTA, EDTA, or salt as used with other plant CDPKs (Polya et al., 1987; Battey, 1990; Putnam-Evans et al., 1990) were unsuccessful, showing that the potato protein kinase is a very hydrophobic enzyme.

The active fractions from the phenyl-Sepharose column were pooled, concentrated, and injected to a Superdex 200 filtration column. A peak with CDPK activity was eluted (Fig. 2D). To verify the purity of this peak, the sample was analyzed by SDS-PAGE and the gel was either stained with Coomassie blue (Fig. 3A) or silver-stained (data not shown). A unique band of 53 kD was stained in both cases.

The Mono-Q and the phenyl-Sepharose preparations were immunoblotted with polyclonal antibodies against the regulatory domain of the soybean αCDPK and with monoclonal antibodies against the catalytic domain. A 53-kD band was revealed with the polyclonal antibodies in both preparations (Fig. 3B, lanes a and b), whereas those directed against the catalytic domain could not recognize the potato enzyme (data not shown).

Some CDPKs are able to phosphorylate themselves. This was tested by autophosphorylation in a blot assay performed with the Mono-Q and the phenyl-Sepharose preparations. The transferred proteins were incubated in the presence of 1 mM $CaCl_2$ or 5 mM EGTA. A polypeptide of 53 kD was strongly labeled in the presence of Ca^{2+} (Fig. 3C, lanes c and d), and when EGTA was added to the assay the same band was almost undetectable (Fig. 3C, lanes a and b). The phosphorylated polypeptide had the same molecular mass as the band that was revealed in the western blot.

To analyze the polymeric conformation of the potato CDPK, the active fractions from the DEAE chromatography were pooled, dialyzed, and loaded onto a Superose 12 column. A single peak with CDPK activity eluted before the bulk of proteins was resolved (Fig. 4). An apparent

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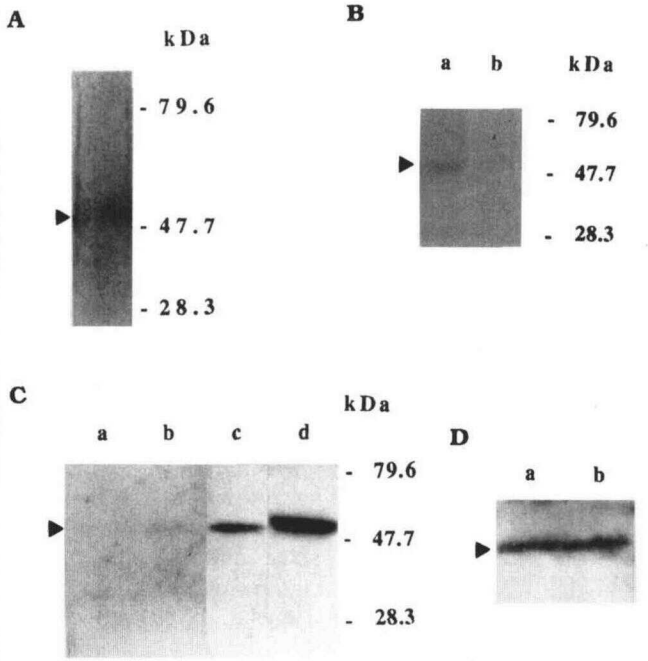

Figure 3. A, SDS-PAGE analysis of purified CDPK. Two micrograms of the Superdex 200 preparation was analyzed in a 10% gel stained with Coomassie brilliant blue R-250. B, Western blot analysis of the phenyl-Sepharose and Mono-Q preparations (4 and 30 μg of protein, lanes a and b, respectively) with polyclonal antibodies against the regulatory domain of the αCDPK from soybean. C, Autophosphorylation in blot assay. The Mono-Q preparation (30 μg of protein, lanes a and c) and the phenyl-Sepharose (4 μg of protein, lanes b and d) were electrophoresed in 10% SDS gels and transferred to Immobilon. The renatured membranes were incubated with [γ-^{32}P]ATP (50 μCi mL^{-1}, 6000 Ci mmol^{-1}) and 1 mM Ca^{2+} (lanes c and d) or 5 mM EGTA (lanes a and b). D, To study the Ca^{2+}-dependent mobility shift, the enzyme was previously incubated with 1 mM EGTA (lane a) or 1 mM Ca^{2+} (lane b), electrophoresed, and transferred. The renatured blot was then incubated with [γ-^{32}P]ATP (50 μCi mL^{-1}, 6000 Ci mmol^{-1}) and 1 m$M Ca^{2+}$. The membranes were exposed to x-ray film. Molecular mass markers were BSA (79.6), ovoalbumin (47.7), and carbonic anhydrase (28.3).

molecular mass of 60 kD could be estimated from the elution profile.

It is now possible to conclude that the potato protein kinase corresponds to the CDPK family; it is a monomeric enzyme with an apparent molecular mass of 53 kD according to SDS-PAGE. It has a regulatory domain similar to that of the soybean CDPK (Roberts and Harmon, 1992), it autophosphorylates in the presence of Ca^{2+}, it binds to hydrophobic matrices, and its activity is completely dependent on free Ca^{2+}.

CDPKs have a calmodulin-like domain, and it was reported that these enzymes exhibit a differential migration in SDS gels in the presence or absence of Ca^{2+} (Harmon et al., 1987). The authors reported the presence of two polypeptides with CDPK activity in soybean; one shifted when Ca^{2+} was present, and the other was unaffected. To study the Ca^{2+}-dependent mobility shift of the potato CDPK, the Mono-Q preparation was incubated with Ca^{2+} or EGTA, and after SDS-PAGE the proteins were transferred and incubated with Ca^{2+} and [γ-^{32}P]ATP. The same band was labeled in both lanes (Fig. 3D, lanes a and b), indicating that the potato enzyme shows no differential mobility shift and migrates at the same position, regardless of the presence of Ca^{2+}. This result is similar to that reported for the 50-kD CDPK from soybean (Harmon et al., 1987).

The CDPK activity was assayed with different exogenous acceptors of phosphorylation or without the addition of substrate in the presence or absence of Ca^{2+}. The Ca^{2+} ions increased the low levels of endogenous phosphorylation. Histone III S was the best exogenous protein substrate; however, synthetic peptides that mimic the phosphorylation site of the glycogen synthase enzyme, such as GS, Syntide-2, and CDPKS analog, were 5- to 8-fold better than Histone III S (Table I). The highest activation (14.3 times) was achieved using Syntide-2 as a substrate. It is surprising that peptide CDPKS, which differs in the ninth amino acid position (Ser instead of Ala or Val), was not a good substrate for the potato CDPK (Table I).

Compounds that affect Ca^{2+}-mediated processes were assayed on the potato CDPK in the presence of Ca^{2+} or EGTA. As shown in Table II, all of the drugs tested inhibited the activation of the enzyme in the presence of the divalent ion, as already reported for other CDPKs (Minichiello et al., 1989). W7, a calmodulin antagonist, completely inhibited the enzyme's activity at 2.5 mM, and one-half-maximal inhibition was at 0.25 mM. This concentration was 20 times higher than the one required to inhibit calmodulin (Hidaka and Tanaka, 1983).

Taking advantage of the high affinity of CDPK for Syntide-2, an alternative purification procedure was employed. The pooled DEAE fractions were loaded onto a Syntide-Sepharose affinity column. A highly purified CDPK eluted at 0.3 M NaCl, as depicted in Figure 5. The enzymatic activity was dependent on the presence of micromolar concentrations of Ca^{2+}. The following kinetic parameters were determined using this partially purified fraction: the K_m value for ATP was 30 $\pm$ 3 μM, the $K_{0.5}$ for Ca^{2+} was 0.6 μM, and the K_m for Syntide-2 was 30 μM.

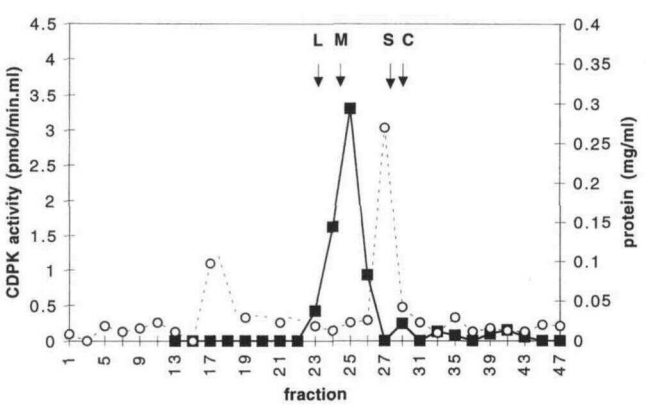

Figure 4. Fast protein liquid chromatography on a Superose 12 column. CDPK activity was assayed in the presence of 1 m$M Ca^{2+}$(■), and protein contents were determined (○). Arrows indicate protein markers used as internal standards. L, Lactic dehydrogenase; M, [illegible]; and C, Cyt *c*.

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Table I. *Substrate specificity of the soluble CDPK from potato*

All assays were carried out with the DEAE-cellulose preparation. CDPK was assayed under the standard assay conditions with 1 mM Ca^{2+} or 1 mM EGTA. Protein kinase activity is presented as percent of control rate in the presence of Lys-rich histone (H IIIS) and Ca^{2+} (1 pmol ^{32}P incorporated min^{-1}). The phosphorylation site (**S**) of the synthetic peptides is indicated: Syntide 2, PLARTL**S**VAGLPGKK; GS, PLSRTL**S**VAAKK; CDPKS analog, PLRRTL**S**VAA; MLCKS, KKRPQRAT**S**NVFS; CDPKS, PLSRTL**S**VSS.

Substrate	Final Concentration	Activity		Activation
		EGTA	Ca^{2+}	
		%		*-fold*
Without exogenous substrate		5	18	3.6
Proteins				
Histone III S	1 mg mL^{-1}	34	100	2.9
Histone IIA S	1 mg mL^{-1}	4	8	2
Histone VI S	1 mg mL^{-1}	4.5	13	2.9
Histone VII S	1 mg mL^{-1}	5	17	3.4
Histone VIII S	1 mg mL^{-1}	13	8	0.6
Protamin	1 mg mL^{-1}	3.5	10	2.8
Casein	1 mg mL^{-1}	20	41	2
Phosvitin	1 mg mL^{-1}	18	6	0.3
Synthetic peptides				
GS	25 μM	95	880	9.2
Syntide 2	25 μM	44	632	14.3
CDPKS analog	25 μM	39	520	13.3
MLCKS	25 μM	18	168	9.3
MBP	100 μM	31	57	1.8
CDPKS	25 μM	6	37	6.2
Kemptide	25 μM	7	28	4

Particulate and soluble extracts from leaves, stems, and roots were obtained to determine the localization of the enzyme in the plant. The soluble CDPK was localized mainly in the stem, and, in addition, CDPK activity was associated with the particulate fraction of leaves and stems (Fig. 6).

This CDPK was the only Ca^{2+}-modulated kinase activity detected in the soluble extracts of plants grown under multiplication conditions. In plants protein phosphorylation may regulate unique processes such as photosynthesis, photomorphogenesis, and gravitropism (Ma, 1993). To analyze whether this protein kinase participates in tuberization, CDPK activity was determined in the plants subjected to tuber-inducing conditions. Four stages of the tuberization process were defined (Ulloa et al., 1996).

The CDPK activity was assayed in crude extracts from different stages of tuber development and from plants grown under multiplication conditions used as a control (Table III). Significant variations in enzymatic activity were detected. At the onset of this process (stages 1 and 2) CDPK activity increased 2.5-fold compared with the control, whereas in completely developed tubers (stage 4) the activity was 50% lower than in the control. To verify that the variations detected in CDPK activity were not due to an increase in storage proteins, which reduces the relative CDPK activity on a per-milligram-protein basis, another potato cytosolic enzyme, pyruvate kinase (Cole et al., 1992), was assayed in the different stages and in the nontuberizing plants. There were no significant variations of this enzyme among the different stages (data not shown).

In addition, CDPK activity was assayed in the presence of 0.5 mM W7. An important inhibition (approximately 50%) was obtained with the naphthalene sulfonamide drug. This result, together with the assay conditions used, confirmed that CDPK was the predominant protein kinase involved in these phosphorylations (Fig. 7).

To study the endogenous targets of this protein kinase, crude extracts from each tuberization stage and from noninduced plants were incubated under conditions that favored CDPK activity. A negative control was carried out using EGTA in the assay mixture. In all extracts the phosphorylation of specific polypeptides increased in the presence of Ca^{2+} (Fig. 8, + lanes), but the pattern differed strikingly according to the culture conditions used. Three polypeptides (68, 51, and 40 kD) were phosphorylated in a Ca^{2+}-dependent manner in the early stages of tuberization (Fig. 8, lanes 1+ and 2+) and were absent in completely formed tubers (Fig. 8, lanes 3+ and 4+). These polypeptides were not labeled in noninduced plants (Fig. 8, lane

Table II. *Effect of calmodulin antagonists on CDPK activity*

The peak with CDPK activity from the DEAE-Cellulose column was preincubated during 30 min in an ice bath with the different inhibitors. The assay was carried out at 30°C during 10 min in the presence of 1 mM EGTA or 1 mM Ca^{2+} using 25 μM Syntide 2 as a substrate.

Inhibitor	CDPK Activity			
	EGTA		Ca^{2+}	
	pmol min^{-1} mg^{-1}	%	*pmol min^{-1} mg^{-1}*	%
–[a]	25.02	9.6	259.32	100
W7				
0.5 mM	6.04	2.3	87.31	33.7
2.5 mM	0.00	0.0	8.72	3.4
48/80				
100 μg mL^{-1}	6.20	2.4	144.90	55.9
1 mg mL^{-1}	4.06	1.6	26.52	10.2
TFP[b] 0.2 mM	0.00	0.0	33.68	13.0
CPZ[c] 0.5 mM	5.35	2.1	46.30	17.9

[a] –, Without inhibitor. [b] TFP, Trifluoperazine. [c] CPZ, Chlorpromazine.

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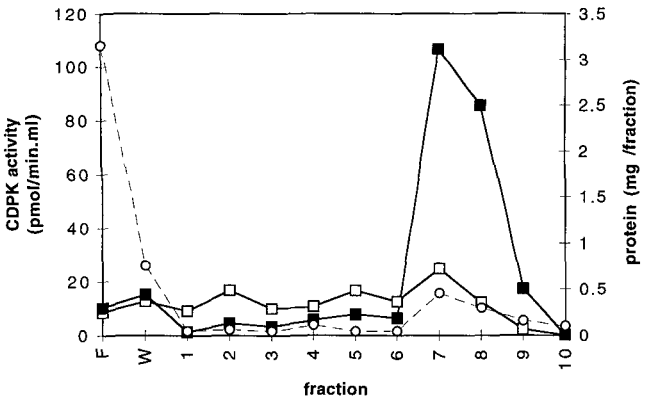

Figure 5. Elution profile of the Syntide-Sepharose affinity column. CDPK activity was assayed in the presence of 1 mM Ca^{2+} (■) or 1 mM EGTA (□). F, Flowthrough; W, wash; ○, protein.

M+). Both results suggest transient phosphorylation of specific polypeptides at the onset of the tuberization process. In addition, the overall labeling was stronger in the early stages of tuber development than in the later stages, even though the protein that was loaded in the gel was lower (50 μg instead of 100 μg), providing evidence for the high specific activity of CDPK at the start of tuber induction.

Another important event was the dephosphorylation of a 64-kD polypeptide that was present only in tuberizing plants. This polypeptide was labeled in the presence of EGTA (Fig. 8, lanes 1−, 2−, 3−, and 4−) and disappeared when Ca^{2+} was added to the reaction mixture.

Crude extracts of the four tuberization stages and of the plants that were cultured under multiplication conditions were preincubated for 30 min with two phosphatase inhibitors, β-glycerophosphate and orthovanadate, after which CDPK activity was assayed as before. The results shown in Table III demonstrate that the CDPK activity was doubled in the presence of these inhibitors in stages 1 and 2. The effect of the inhibitors was undetectable in stage 4 or in noninduced plants. These data strongly support the existence of a stage-specific phosphatase that is able to dephosphorylate Syntide-2. The simultaneous induction of both activities at the onset of tuberization suggests that protein kinase-phosphatase cascades act as a coordinate processing unit in plant cells.

DISCUSSION

In the present study, a soluble CDPK from potato plants grown under multiplication conditions is purified to homogeneity for the first time to our knowledge. This protein kinase was found to be similar to other members of the CDPK family in terms of substrate specificity, monomeric structure, apparent molecular mass, Ca^{2+}-dependent autophosphorylation, activation by Ca^{2+} ($K_{0.5}$ = 0.6 μM), and inhibition by phenotiazine derivatives or W7 (one-half maximal inhibition = 0.25 mM). In addition, the potato enzyme was recognized by polyclonal antibodies against the regulatory domain of αCDPK from soybean. This result confirms that the potato protein kinase belongs to the CDPK family.

A property that most CDPKs share with calmodulin is Ca^{2+}-dependent binding to hydrophobic resins such as phenyl-Sepharose (Roberts and Harmon, 1992). The potato protein kinase was tightly bound to a phenyl-Sepharose column and could be eluted only with 4 M urea. In this respect, the potato enzyme was more hydrophobic than the soybean, maize, or silver beet CDPKs, which could be eluted with EGTA (Polya et al., 1987; Battey, 1990; Putnam-Evans et al., 1990), and is similar to that of the halotolerant alga *Dunaliella tertiolecta*, which could be eluted only with 50% ethylene glycol (Yuasa and Muto, 1992).

Although antibodies raised against the regulatory domain of soybean αCDPK recognize the potato CDPK, a set of four monoclonal antibodies directed against the catalytic domain of soybean CDPK could not detect it. The different soybean CDPK isoforms vary in length and are 50 to 75% identical in the amino acid sequences of their catalytic and regulatory domains (Harmon et al., 1996). It is possible that the monoclonal antibodies were directed against epitopes that are not present in the catalytic domain of the potato enzyme. Further molecular studies should be performed to determine which isoform belongs to the potato CDPK.

Synthetic peptides were tested as substrates of the potato CDPK. The finding that peptide CDPKS was a very poor substrate for this enzyme while all of the other peptides were excellent acceptors prompted us to analyze the sequence of these peptides. The most relevant difference between these substrates and peptide CDPKS was a Ser in the ninth position in the latter, which created a charged environment near the phosphorylation site. This modification prevented the recognition of CDPKS as a phosphate acceptor.

The approach used to study the endogenous targets for CDPK in noninduced plants revealed that the phosphorylation of the majority of the polypeptides was increased in the presence of Ca^{2+}. Nevertheless, when the same experiment was carried out with the extracts of the different stages, the phosphorylation of specific polypeptides was induced in the presence of Ca^{2+} at defined stages (1 and 2) of the tuberization process. Concomi-

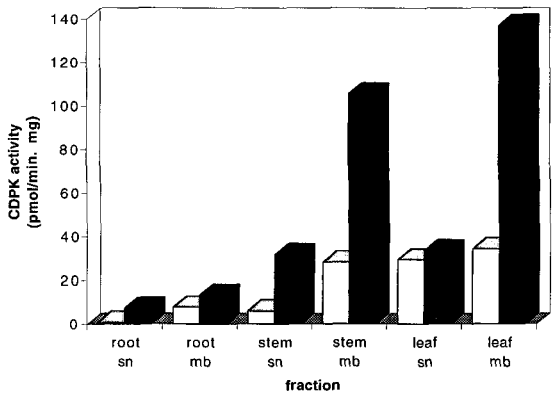

Figure 6. CDPK activity in soluble (sn) and particulate (mb) fractions of roots, stems, and leaves. Enzymatic activity was assayed in the presence of 1 mM Ca^{2+} (■) or 1 mM EGTA (□).

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Table III. *CDPK activity in the different stages of the tuberization process and potato plants grown under multiplication conditions assayed in the presence or absence of phosphatase inhibitors*

The crude extracts were preincubated during 30 min in an ice bath with or without phosphatase inhibitors (10 mM βGP and 2 mM orthovanadate). The enzymatic activity was determined under standard assay conditions (10 min, 30°C) in the presence of 1 mM Ca^{2+} or 1 mM EGTA using 25 μM Syntide-2 as a substrate. CDPK activity is presented as percent of the specific activity assayed with Ca^{2+} of the plant grown under multiplication conditions (100% = 40 pmol ^{32}P incorporated min^{-1} mg^{-1}).

Stage	CDPK Activity			
	Without PPase inhibitors		With PPase inhibitors	
	EGTA	Ca^{2+}	EGTA	Ca^{2+}
	%			
Multiplication conditions	40.3 ± 6.5	100.0	22.2 ± 6.3	77.5 ± 5.32
Stage 1	31.4 ± 2.4	95.2 ± 12.2	69.4 ± 9.6	203.5 ± 4.3
Stage 2	22.0 ± 3.8	89.6 ± 10.6	41.7 ± 4.2	187.7 ± 9.4
Stage 3	15.9 ± 3.1	33.0 ± 1.5	17.3 ± 3.2	56.1 ± 2.7
Stage 4	13.9 ± 7.2	52.2 ± 13.1	19.0 ± 5.9	53.4 ± 1.3

tantly, in these early stages the CDPK activity was 2.5- to 3-fold higher than in the nontuberized plants. A correlation can be established between the Ca^{2+}-dependent phosphorylation of these polypeptides, the CDPK activity, and the morphogenetic events that take place during tuberization. Changes in the pattern of gene expression during the tuberization process have been identified from the analysis of proteins extracted from stolon tips and tubers (Hannapel, 1991; Hendriks et al., 1991; Taylor et al., 1991). In addition, in vitro translation of RNA extracted from the early stages of tuberization (Hendriks et al., 1991; Taylor et al., 1991) and differential screening of cDNA clones that show enhanced expression in stolon tips in the very early stages of tuberization (Taylor et al., 1992) support the idea that changes in gene expression occur early in this process. It seems likely that during tuberization genes with a variety of functions are recruited. This may be necessary to bring about the complex processes of development associated with tuberization.

Other authors have previously demonstrated the inhibition of tuberization by calmodulin antagonists EGTA and Ca^{2+} ionophore A23187 and the reversibility of this effect. They suggested that Ca^{2+} and calmodulin were involved in tuberization and considered that the antagonists used in their experiments could also affect processes other than those mediated by calmodulin (Balamani et al., 1986). These results are in agreement with those reported in this paper and indirectly support the involvement of CDPK at the onset of tuberization.

Kawasaki et al. (1993) isolated a cDNA from a novel gene (*spk*), which was specifically expressed in developing rice seeds. The nucleotide sequence of *spk* was homologous to CDPK. This was the first report of a plant protein kinase gene specifically expressed according to tissue and developmental stage. Other authors (Estruch et al., 1994) cloned a CDPK from maize pollen and suggested that this tissue-

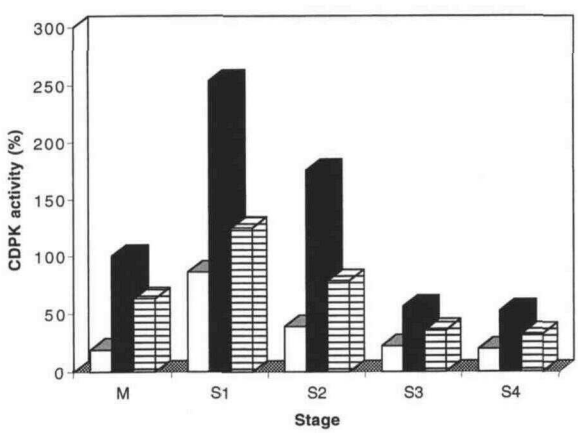

Figure 7. CDPK activity in the different stages of tuber development (S1, S2, S3, and S4) and in plants grown under multiplication conditions (M). Enzymatic activity was assayed with 1 mM EGTA (open bar), 1 mM Ca^{2+} (shaded bar), or 1 mM Ca^{2+} in the presence of 0.5 mM W7 (striped bar). Phosphatase inhibitors (10 mM β-glycerophosphate and 2 mM orthovanadate) were included in the reaction mixture. Protein kinase activity is presented as a percentage of the specific activity assayed in the presence of Ca^{2+} in plants grown under multiplication conditions (100% activity = 40 pmol ^{32}P incorporated min^{-1} mg^{-1}).

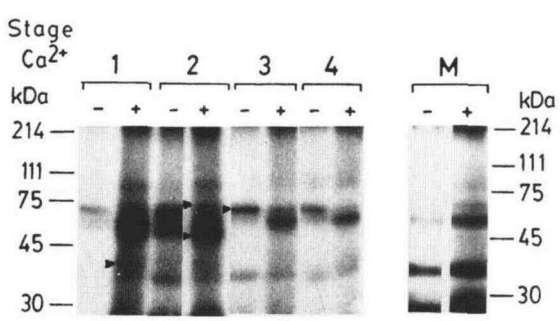

Figure 8. Endogenous phosphorylation in the different stages of tuber development (1, 2, 3, and 4) and in plants grown under multiplication conditions (M). Crude extracts of the different stages (1 and 2, 50 μg of protein; 3 and 4, 100 μg of protein) and of M (100 μg of protein) were incubated with 5 μM [γ-^{32}P]ATP (5000 cpm $pmol^{-1}$) and 1 mM Ca^{2+} (lanes +) or 1 mM EGTA (lanes −). Arrowheads indicate stage-specific phosphorylated or dephosphorylated peptides. Molecular mass markers are indicated. Gels were exposed for 96 h, except for the lanes corresponding to M (− and +) and to [illegible] h.

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specific CDPK protein is required for germination and pollen tube growth. Using a biochemical approach, we describe a protein kinase activity expressed in a temporal way in the developing potato tuber.

Another important result is the restricted localization of the soluble CDPK in the stems of plants grown under multiplication conditions. Potato tubers develop initially from an enlargement of pre-existing pith cells from the subapical region of a specialized shoot, the stolon. The fact that the soluble CDPK activity is highly expressed in this site at the time that the morphogenetic events occur supports a possible role for this protein kinase during the tuberization process.

The first visible sign of tuberization is a swelling in the subapical region of the stolon tip, which is caused by a switch in the plane of division. Recently, a tuber-inducing substance, termed tuberonic acid (a jasmonic acid derivative), has been suggested to function by disrupting cortical microtubules in stolon cells, allowing radial growth associated with tuberization (Koda et al., 1988; Matsuki et al., 1992). The mechanism by which microtubules undergo rapid shifts of alignment is crucial to understanding growth control in plants. The organization of cortical arrays must be coordinated with other cellular events; the evidence suggests that phosphorylation/dephosphorylation and Ca^{2+}/calmodulin appear to be involved (Cyr and Palevitz, 1995).

Taylor et al. (1991) reported that the pattern of expression of α-tubulin isotypes changes at a very early stage in the tuberization process, before there is any visible sign of swelling in the stolon tip. Mizuno (1994), studying the effects of GA_3 treatment on *Vigna angularis*, reported that one α-tubulin isotype could no longer be resolved on SDS-PAGE after the addition of GA_3. Because a kinase inhibitor prevented GA_3 from switching microtubules from longitudinal to transverse and also prevented the loss of the α-tubulin isotype, it was assumed that the missing isotype was phosphorylated. Phosphorylation of microtubule proteins may affect the cortical arrays by affecting nucleating activity and by influencing dynamicity. The target proteins for phosphorylation are unknown, but likely candidates are microtubule-associated proteins as well as tubulin itself (Cyr and Palevitz, 1995). The possibility that the potato CDPK is involved in these phosphorylations is an underlying hypothesis of work that will be developed in future studies.

A stage-specific dephosphorylation is also described in this paper. These experiments support the existence of phosphatase activities that might be responsible for the reversible phosphorylation of certain proteins. The phosphorylation of these stage-specific polypeptides could be the first step in a metabolic cascade that is triggered when external stimuli modify the cytosolic levels of Ca^{2+}.

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