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Biochemical evidence for a calcium-dependent protein kinase from *Pharbitis nil* and its involvement in photoperiodic flower induction

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Abstract

A soluble Ca^{2+}-dependent protein kinase (CDPK) was isolated from seedlings of the short-day plant *Pharbitis nil* and purified to homogeneity. Activity of *Pharbitis nil* CDPK (PnCDPK) was strictly dependent on the presence of Ca^{2+} ($K_{0,5}=4{,}9$ μM). The enzyme was autophosphorylated on serine and threonine residues and phosphorylated a wide diversity of substrates only on serine residues. Histone III-S and syntide-2 were the best phosphate acceptors (K_m for histone III-S = 0,178 mg ml^{-1}). Polyclonal antibodies directed to a regulatory region of the soybean CDPK recognized 54 and 62 kDa polypeptides from *Pharbitis nil*. However, only 54 kDa protein was able to catalyse autophosphorylation and phosphorylation of substrates in a Ca^{2+}-dependent manner. CDPK autophosphorylation was high in 5-day-old *Pharbitis nil* seedlings grown under non-inductive continuous white light and was reduced to one-half of its original when plants were grown in the long inductive night. Also, the pattern of proteins phosphorylation has changed. After 16-h-long inductive night phosphorylation of endogenous target (specific band of 82 kDa) increased in the presence of calcium ions. It may suggest that Ca^{2+}-dependent protein kinase is involved in this process and it is dependent on light/dark conditions.

Keywords: *Pharbitis nil*; Convolvulaceae; Japanese morning glory; Protein purification; Ca^{2+}-dependent protein kinase; Protein phosphorylation; Photoperiodic process

1. Introduction

The role of Ca^{2+} as a second messenger has been well established but the mechanisms by which Ca^{2+} controls different processes are only beginning to be understood. The changes in calcium ions level is sensed by array of Ca^{2+}-sensors, which decode calcium signal (Pandey and Sopory, 1998). The receptor and transducer of the calcium signal could be the Ca^{2+}-binding protein e.g. calmodulin or the protein kinases itself, which either by themselves or by modifying other proteins or factors transduce the signal downstream to elicit the final response (Sanders et al., 1999). Protein kinases are one of the main targets, which by their reversible phosphorylation/dephosphorylation activate other constituents of signal transduction pathways (Bootman and Berridge, 1994; Roberts and Harmon, 1992).

Soluble CDPKs have been purified to near homogeneity from various plant species (Harmon et al., 2000). Although, the biochemical activity of CDPKs has been fairly well characterized, the biochemical function(s) of individual Ca^{2+}-dependent protein kinases remains largely unknown. So far several lines of evidence suggest that these enzymes mediate, e.g. growth and development (Estruch et al., 1994; MacIntosh et al., 1996), abiotic stress signalling pathways (Chico et al., 2002; Martin and Busconi, 2001) and pathogen response (Romeis et al., 2000, 2001).

Flowering is a complex process that results in the differentiation of a vegetative shoot meristem into a generative one. A wide variety of environmental and hormonal stimuli are known to be involved in the induction of flowering. The physiological experiments suggested the role of Ca^{2+} and calcium-binding proteins as a mediator of flowering stimulus (Friedman et

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al., 1989; Tretyn et al., 1994). However, the biochemical and molecular events involved in the signal transduction of this stimulus are not understood. Beside calcium ions and calmodulin the involvement of calcium-dependent protein kinase (CDPK) in flower induction of *Pharbitis nil* was proposed (Tretyn et al., 1997). Therefore, the characterization of CDPK-mediated signalling pathways will require isolation and biochemical characterization of this enzyme from *P. nil* seedlings.

P. nil, a model short-day plant in most of the photoperiodic studies, does not only initiate flowers in response to a single inductive long night, but it is also responsive to photoperiodic induction at a very early stage of seedlings growth (5–6-day-old).

In the present work we describe the purification of a soluble 54 kDa calcium-dependent protein kinase (CDPK) from *P. nil* seedlings and the physical and kinetic properties, substrate specification and inhibitor influence. We also made a first step towards the identification of endogenous substrates, whose phosphorylation is controlled by light/dark conditions in a calcium-dependent manner.

2. Results and discussion

2.1. Purification of CDPK

CDPK has been purified to homogeneity from 5-day-old *P. nil* seedlings. The results of purification are summarized in Table 1. The standard purification procedure allowed recovery of 1% of the total available kinase activity, and yielded approximately 86 μg of purified CDPK from 1 kg of plant material. The protein kinase was purified 825-fold. In the presence of Ca^{2+} and histone III-S as substrate, specific activity values were greater than 6 nmol min^{-1} mg^{-1}.

Four column chromatography steps were used to purify CDPK. After ion-exchange chromatography the fractions with kinase activity were pooled and the protein kinase, arbitrarily named PnCDPK, was subsequently purified on a phenyl-Sepharose, Sephadex G-100 and Cibacron blue-sepharose columns. Many Ca^{2+}-binding proteins undergo a conformational change in the presence of Ca^{2+} such that a hydrophobic site is exposed (Moore and Dedman, 1982; Putnam-Evans et al., 1990) allowing the protein to bind to hydrophobic resins such as phenyl-Sepharose. CDPK bound to phenyl-Sepharose in the presence of Ca^{2+} and was eluted in buffers containing EDTA. High purification (110-fold) was received after chromatography on phenyl-Sepharose. Gel filtration on Sephadex G-100 resulted in a further 2-fold purification. The final purification was achieved by protein chromatography on Cibacron blue-sepharose, a resin to which CDPK binds very tightly. Calmodulin did not bind to that matrix (Putnam-Evans et al., 1990) and was absent in the purified CDPK pool after the Cibacron blue-sepharose.

As was shown, CDPK from *P. nil* tissue can be extensively purified by a procedure that critically involves Ca^{2+}-dependent chromatography on a hydrophobic matrix (phenyl-Sepharose CL-4B). This chromatography has previously been used for purification of a variety of soluble CDPKs (Frylinck and Dubery, 1998; Polya and Chandra, 1990; Putnam-Evans et al., 1990; Roberts and Harmon, 1992). This behaviour is consistent with the presence of Ca^{2+}-binding calmodulin-like domains on such CDPKs (Chang et al., 1995).

2.2. Determination of the subunit structure and molecular mass

SDS-PAGE was used to determine the homogeneity and molecular mass of the purified enzyme. Purified CDPK, which was eluted as a single peak of activity during column chromatography, resolved into two protein bands of molecular mass 54 and 62 kDa on 12% SDS gel (Fig. 1B, lane 10). Quantitative densitometry revealed that these two bands represented > 95% of the total protein presented in the preparation. Similar results were obtained with the CDPK from soybean and mango, where the purified enzyme resolved into two immunologically related bands of the 52 and 55 kDa (Putnam-Evans et al., 1990), or 49 and 45 kDa (Frylinck and Dubery, 1998) respectively. The following observation may be in support of this possibility that several CDPK preparations have contained two bands

Table 1
Summary of purification of calcium-dependent protein kinase from *Pharbitis nil*

Purification steps	Volume (ml)	Protein (mg)	Specific activity (pmol min^{-1} mg^{-1})	Total activity (pmol min^{-1})	Purification (*x*- fold)	Yield (%)
1. Crude homogenate	5470	3982	7.56	30464.8	1	100
2. Crude supernatant	5270	3529	9.4	29641.0	1.1	97
3. DEAE-cellulose	365	604	35.94	21711.3	4.7	71
4. Phenyl-Sepharose	84	5.32	833.3	4437.3	110	15
5. Sephadex G-100	26	2.15	1538.3	3296.9	203	10
6. Blue-Sepharose	12.5	0.086	6240.1	536.6	825	1

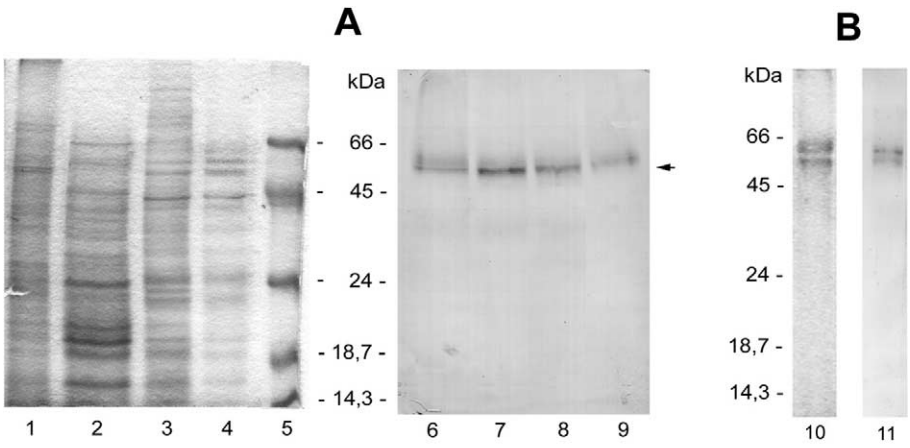

Fig. 1. SDS-PAGE and Western-blotting analysis of proteins from each stage of purification of PnCDPK (see Table 1). Proteins from various stages of purification were resolved by electrophoresis in a 12% poliacrylamide gel in the presence of SDS and stained with Coomassie blue (Panel A, lanes 1–4 and panel B, lane 10) or transferred to nitrocellulose and stained with polyclonal antibodies against calmodulin-like domain (CLD) CDPK from soybean (Panel A, lanes 6–9 and panel B, lane 11). Lanes 1 and 6, homogenate; lanes 2 and 7, DEAE-cellulose pool; lanes 3 and 8, phenyl-Sepharose pool; lanes 4 and 9, Sephadex G-100 pool; lanes 10 and 11, Cibacron blue-sepharose pool. Lane 5 contained molecular mass markers.

which molecular masses are about 55 kDa and the monoclonal antibodies to CDPK recognized both bands (Putnam-Evans et al., 1990). So preliminary characterization can indicate that two proteins appear to be related and may have resulted from proteolysis, or may represent different Ca^{2+} bound forms.

2.3. Characterization of CDPK by immunoblotting and in-gel kinase assays

To confirm that the kinase is a CDPK, the fractions from column chromatography were subjected to immunoblot analysis using polyclonal anti-CDPK antibody made against the calmodulin-like domain of soybean CDPK. Fig. 1A (lanes 6–9) shows that the antibody recognized one 54 kDa polypeptide received after the first three purification steps but two polypeptides with molecular masses of 54 and 62 kDa in enzyme fractions after the last column chromatography (Fig. 1B, lane 11). This immunoblotting experiment did not allow us to unequivocally identify the band corresponding to the CDPK because the antibody recognized two polypeptides. To solve this problem we did an in-gel assay. The results show that only the polypeptide with a molecular mass of 54 kDa had kinase activity (Fig. 2A). The absence of kinase activity for the 62 kDa peptide recognized by the anti-CDPK antibody suggests that the band corresponded either to a non-CDPK calmodulin-like domain containing protein or to an unsuccessfully renatured CDPK. Similar results were received for rice CDPK where from 56 and 64 kDa polypeptides only the 56 kDa had kinase activity (Martin and Busconi, 2001). We should mention here, what later experiments revealed, that protein from the gel was phosphorylated only on serine residue (Fig. 2C, lane 3). It suggested that only substrate phosphorylation was observed, because autophosphorylation occurred on both serine and threonine residues.

2.4. Autophosphorylation and substrate specificity

Most protein kinases can phosphorylate themselves in the presence of ATP (Putnam-Evans et al., 1990; Smith et al., 1993). Such reaction was observed when PnCDPK was tested. The enzyme was autophosphorylated on a gel with no exogenous substrate added. Separated proteins on the gel were incubated in the presence of 1 mM $CaCl_2$ or 5 mM EDTA. A polypeptide of 54 kDa was strongly labelled in the presence of Ca^{2+}, but when EDTA was added to the assay the same band was almost undetectable (Fig. 2B). The phosphorylated polypeptide had the same molecular mass as the band revealed in the western blotting. Autophosphorylation of this kinase, like other CDPKs (Anil et al., 2000; Binder et al., 1994; Frylinck and Dubery, 1998; Harmon et al., 1987; Yoon et al., 1999), was Ca^{2+}-dependent. A probable interpretation of this result is that the Ca^{2+}-dependent protein kinase is capable of autophosphorylation, and it may be an important way to regulate the enzyme activity (Soderling, 1990; Wang et al., 1998; Yuasa et al., 1995). However, details on the occurrence and function of autophosphorylation of plant protein kinases in vivo are not known (Harmon et al., 2000). ^{32}P-Amino acid analysis showed that *P. nil* CDPK was autophosphorylated on both serine and threonine residues but analyzed substrates were phosphorylated only on serine (Fig. 2C). Our results are similar to all known CDPKs (Putnam-Evans et al., 1990; Yoon et al., 1999; Yuasa et al., 1995), indicating that there are at least two phosphorylation sites on the enzyme.

During the next step we examined the catalytic activity of the 54 kDa protein using different exogenous

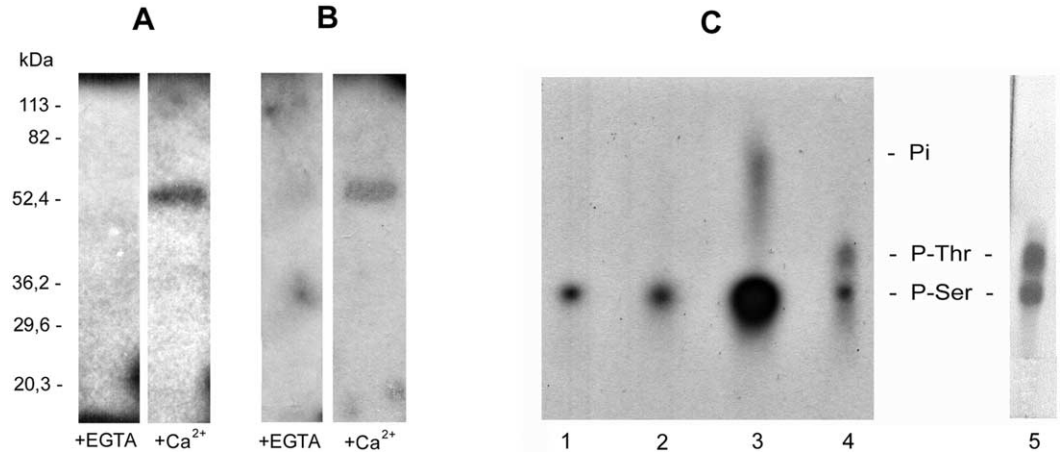

Fig. 2. Characterization of the PnCDPK. In-gel kinase assay a purified CDPK (1.6 μg) was electrophoresed in 12% SDS-poliacrylamide gel containing histone III-S (panel A) and for autophosphorylation assay in a gel without histone (panel B). Following SDS-PAGE enzyme was renatured and incubated with [γ-^{32}P] ATP in a presence of $CaCl_2$ or EDTA as described in "Experimental". Band on the autoradiogram indicates the position of kinase activity against histone on the gel (panel A) and autophosphorylated kinase (panel B). The M_rs of the labelled bands are indicated (B). (C) Identification of amino acids phosphorylated by PnCDPK. Phosphorylated substrates (histone III-S, MBP, dephosphorylated casein) and autophosphorylated CDPK were hydrolyzed in 6 M HCl and the amino acids were separated by thin-layer chromatography. The chromatograms were then exposed to X-ray film to detect the ^{32}P-labeled amino acids. Lane 1, hydrolysate of phosphorylated MBP; lane 2, hydrolysate of phosphorylated dephosphorylated casein; lane 3, hydrolysate of phosphorylated histone III-S; lane 4, hydrolysate of autophosphorylated CDPK; lane 5, standards (phosphoserine and phosphothreonine) visualized by ninhidrin staining.

acceptors of phosphorus in the presence of Ca^{2+}. We have found that histone III-S is the best exogenous protein substrate. However, synthetic peptides that mimic the phosphorylation site of the glycogen synthase enzyme such as syntide-2 were 3-fold better phosphorylated than histone III-S (Table 2). Other substrates like MBP, dephosphorylated casein and mixture of histones can be phosphorylated as well. All of these polypeptides are also substrates for other CDPKs (Anil et al., 2000; Martin and Busconi, 2001; Polya and Chandra, 1990; Putnam-Evans et al., 1990).

2.5. Effector and inhibitors studies

To investigate the effect of calmodulin and Ca^{2+}-dependent inhibitors on kinase activity, several experiments were performed.

The exogenous addition of CaM had no effect on enzyme activity (data not shown). On the other hand, this protein was inhibited by the calmodulin antagonists: chlorpromazine, W-7, trifluoperazine and calmidazolium (Fig. 3). Calmidazolium was the most effective inhibitor, resulting in 70% inhibition at a concentration of 0.1 mM. W-7, calmidazolium and trifluoperazine variously inhibited other plant CDPKs (Harmon et al., 1987; Polya and Chandra, 1990; Roberts and Harmon, 1992) but the concentration required for inhibition of CDPK were higher than those required for inhibition of calmodulin-dependent enzymes (Roberts and Harmon, 1992). It seems that catalytic domain of PnCDPK is similar to that of the calcium/calmodulin-dependent protein kinases, so calmodulin antagonists can inhibit activity of CDPK, while no external calmodulin is necessary for the activation of the enzyme (Frylinck and Dubery, 1998).

Table 2
Substrate specificity of CDPK from *Pharbitis nil*[a]

Substrate	Concentration	Activity (%)
Histone III	0.5 mg ml^{-1}	100
Casein	0.5 mg ml^{-1}	24.5
MBP	0.5 mg ml^{-1}	38.0
Mixture of histones	0.5 mg ml^{-1}	127.5
Syntide-2	50 μM	297.5

[a] CDPK assays were performed as described in "Experimental". Values are the means of duplicate determinations. 100% activity was 1.6 nmol min^{-1} mg^{-1} in a presence of histone III-S.

2.6. Kinetic properties

As was shown above the best substrate for PnCDPK is the histone III-S. The kinase activity vs histone III-S concentration curve was fitted to a Lineweaver-Burk curve with a K_m value of 0.178 mg ml^{-1} and V_{max} was 2016 pmol min^{-1} mg^{-1} (Fig. 4). It should be noted that kinetic data were obtained by assaying PnCDPK in standard assay conditions at a fixed ATP concentration (25 μM) and by varying the concentration of protein substrates.

The effect of Ca^{2+} concentration on the protein kinase activity was measured and the results are shown in Fig. 5. The activity was stimulated by the increasing concentration of Ca^{2+} and one-half maximal activation was 4.9 μM. These results confirm that *P. nil* CDPK is

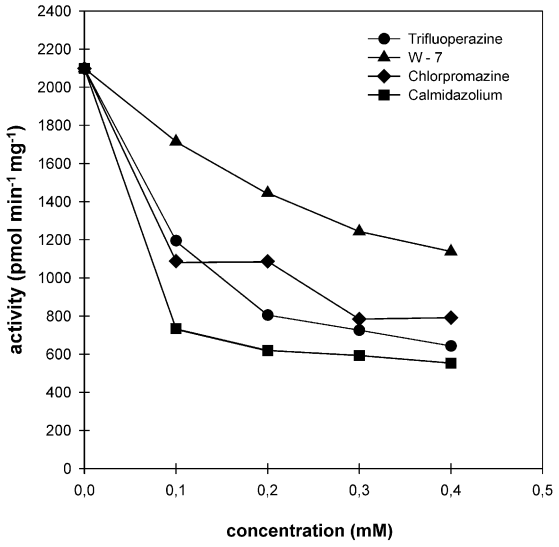

Fig. 3. Effects of various calmodulin antagonists on CDPK activity. The enzyme activity was tested in the presence of 1 mM $CaCl_2$ using 0.5 mg ml^{-1} histone III-S as substrate (100% activity = 2096 pmol min^{-1} mg^{-1}).

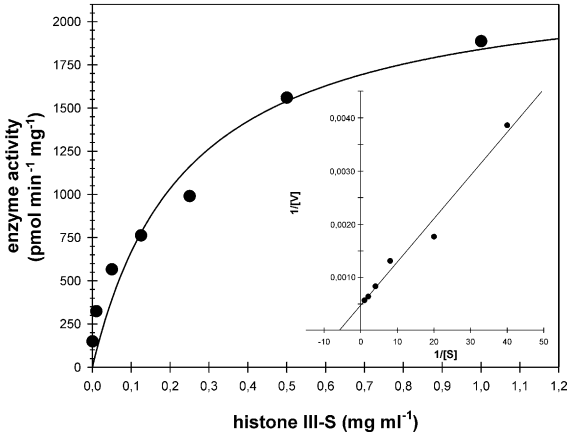

Fig. 4. Determination of K_m and V_{max} values for PnCDPK using different concentration of substrate. The protein kinase activity was assayed in the presence of various concentration of histone III-S as substrate. The inset shows a Lineweaver–Burk plot for the same values.

similar to some plant CDPKs in relation to kinetic properties (Bootman and Berridge, 1995; Harmon et al., 1987; Putnam-Evans et al., 1990).

2.7. Endogenous substrates

It has been shown that CDPK was not only detected in the soluble extracts but also in membrane fraction from *P. nil* (Sakamoto and Shibata, 1992). However, the involvement of CDPK in flower induction of this plant was not examined. In plants, protein phosphorylation may regulate unique processes such as photosynthesis, photomorphogenesis and gravitropism (Kopcewicz and Tretyn, 1998). To analyze whether this

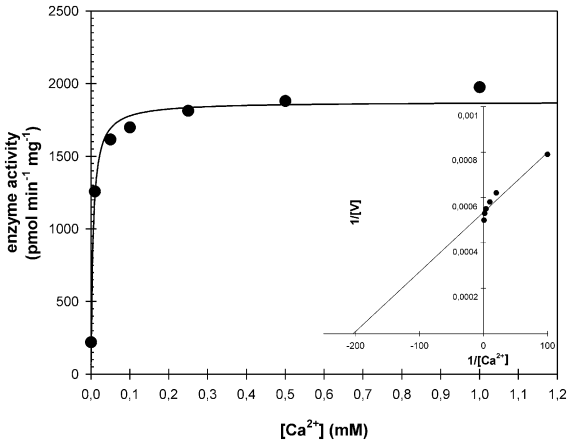

Fig. 5. Dependence on Ca^{2+} concentration for protein kinase activity of PnCDPK. The enzyme activity was assayed with a histone III-S as substrate in the presence of various concentrations of calcium ions. The inset shows a Lineweaver–Burk plot for the same values.

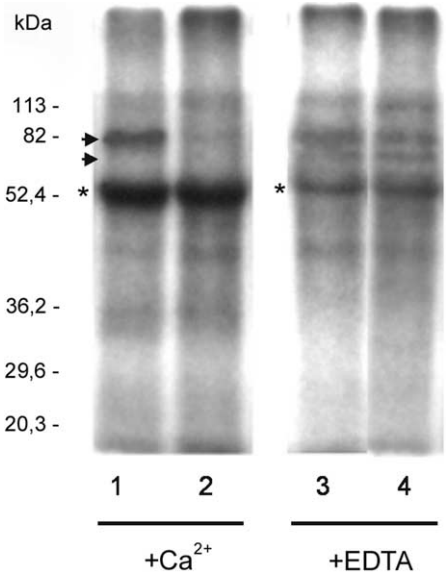

Fig. 6. Endogenous phosphorylation in seedlings of *P. nil* grown under inductive and non-inductive conditions. Crude extracts (40 μg) from induced (lanes 1 and 3) and non-induced (lanes 2 and 4) plants, incubated with [γ-^{32}P] ATP in a presence of $CaCl_2$ or EDTA as described in "Experimental" and electrophoresed in 12% SDS-PAGE. Asterisk (*) indicates autophosphorylated CDPK; arrows (→) indicate phosphorylated or dephosphorylated proteins.

protein kinase participates in induction of flowering, the CDPK activity and the endogenous targets of this kinase were assayed in crude extracts from plants grown under inductive or non-inductive conditions. The protein crude extracts were incubated under conditions that favoured CDPK activity or carried out using EDTA in the assay mixture (negative control). Significant changes in enzymatic activity were detected (Fig. 6). From these two light stages the phosphorylation of specific polypeptides increased in the presence of Ca^{2+}, but the pattern was different according to the experimental conditions. Two peptides (54, 82 kDa) were phosphorylated in a

Ca^{2+}-dependent manner. In non-induced plants the phosphorylation of 82 kDa peptide was very weak, but after 16-h-long inductive night phosphorylation increased. When EDTA was present the phosphorylation level of this band decreased. It may suggest that Ca^{2+}-dependent protein kinase is involved in this process and its activity is regulated by light/dark conditions. However, the low phosphorylation of 82 kDa protein still observed in non-induced plants and in the presence of EDTA, may indicate that there was another peptide (on the same molecular mass), whose phosphorylation was Ca^{2+} and light condition independent. The 54 kDa band was identified as a authophosphorylated CDPK (Fig. 6).

Another event was the dephosphorylation of the 76 kDa polypeptide. It was labeled in the presence of EDTA and disappeared when Ca^{2+} was added to the reaction mixture.

These results suggest transient phosphorylation of specific polypeptides at the onset of the flower induction processes.

Other authors have previously demonstrated the inhibition of flowering by calmodulin antagonists and calcium-dependent protein kinases inhibitors (Friedman et al., 1989; Tretyn et al., 1994). They suggested that Ca^{2+} and calmodulin were involved in flowering and considered that the antagonists used in their experiments could also affect processes other than those mediated by calmodulin, maybe by CDPK.

These results are in agreement with those reported in this paper and indirectly support the involvement of CDPK at the onset of flower induction. The phosphorylation of the specific polypeptides could be the first step in cascade that is triggered when external stimuli modify the cytosolic level of Ca^{2+}.

3. Conclusions

It has been well documented from physiological studies of *Pharbitis nil* flowering that Ca^{2+} plays a very important role in flower induction of this plant (Friedman et al., 1989; Kopcewicz and Tretyn, 1998; Tretyn et al., 1997). However, signalling intermediaries between Ca^{2+} and flowering inductive factor have not been identified.

So far several different laboratories purified and characterized Ca^{2+}-dependent kinase in plants. However, only Sakamoto and Shibata (1992) tried to characterize a specific Ca^{2+}-dependent protein phosphorylation in *P. nil*. They found that the membrane fraction isolated from hypocotyls of that plant showed protein kinase activity and that process was stimulated by Ca^{2+}.

Using a biochemical approach, we describe a protein kinase activity express in *Pharbitis nil* seedlings. The target proteins for PnCDPK are still unknown but our results suggest transient phosphorylation of specific polypeptides at the onset of the flower induction processes. The possibility that the *P. nil* CDPK is involved in the photoinduction processes is an underlying hypothesis of work that will be developed in future studies. The phosphorylation and dephosphorylation of above described proteins could be the first step in a metabolic cascade that is triggered when external stimuli modify the cytosolic levels of Ca^{2+}.

At the moment it is possible to conclude that the *P. nil* CDPK protein kinase corresponds to the CDPK family: (1) it is a monomeric enzyme with an apparent molecular mass of the 54 kDa according to SDS-PAGE, (2) it has a regulatory domain similar to that of soybean CDPK, (3) it is autophosphorylated in the presence of Ca^{2+}, (4) it binds to hydrophobic matrices, (5) its activity is dependent on free calcium. The present report also provides evidence for the regulation of phosphorylation of soluble 82 kDa protein by calcium-regulated protein kinases in a light/dark dependent manner. Further work is required now to establish the nature and function of polypeptide that is phosphorylated in the darkness in the presence of calcium.

4. Experimental

4.1. Plant material and reagents

Seeds of Morning glory (*P. nil* Chois. cv. Violet) (Marutane Co., Kyoto, Japan) were soaked in concentrated sulphuric acid for 50 min and then washed with running tap water for 3 h. They were left in water at 25 °C overnight. The swollen seeds were planted on a mixture of vermiculite and sand (2:1) in plastic pots, covered with Saran Wrap to maintain high humidity. For CDPK isolation seedlings were grown for 5 days in the darkness, then harvested and frozen in liquid nitrogen.

For in vitro phosphorylation of endogenous proteins *P. nil* seedlings were grown for 5 days on continuous white light (130 μmol m^{-2} s^{-1}; cool white fluorescent tubes, Polam, Poland). Some of these plants were exposed to 16-h-long inductive night. Thereafter plants were harvested and frozen in liquid nitrogen.

^{32}P-Labelled adenosine triphosphate ([γ-^{32}P] ATP) (4500 Ci $mmol^{-1}$) was obtained from ICN. DEAE-cellulose, Cibacron blue-sepharose CL-6B and P-81 filter paper were from Whatman. Sephadex G-100 was from Serve. TLC-cellulose (20×20×0.1 cm) was from Merck. Calibration proteins for sodium dodecyl sulfate-poliacrylamide gel electrophoresis (SDS-PAGE) were from Sigma and BioRad (SDS6 range 14 300–66 000 and Low Range 20 300–113 000). Other reagents were purchased from Sigma.

4.2. Enzyme purification

Calcium-dependent protein kinase was purified by methods modified for our purpose (Harmon et al., 1994; Putnam-Evans et al., 1990). All steps were carried out at 4 °C. One kilogram of *P. nil* seedlings were put into 3 l homogenization buffer (20 mM Tris, pH 7.2, 2.5 mM EDTA, 1 mM PMSF, 10 mM β-ME, 10 μg ml^{-1} leupeptin and 10 μg ml^{-1} aprotinin), homogenized for 3 min and then filtered through a double layer of muslin. To the resulting crude homogenate 2% PVP, 10% PEI were added and then centrifuged at 8000 × *g* for 45 min. The crude supernatant fraction was applied to a column of DEAE-cellulose equilibrated in 20 mM Tris, pH 7.2, and 2.5 mM EDTA and 10 mM β-ME. The column was washed with 2.5 l of equilibration buffer containing 0.05 M NaCl, and all proteins absorbed were eluted with 1.5 l of equilibration buffer containing 0.4 M NaCl. The fractions containing kinase activity were pooled and solid ammonium sulfate was added to 60% saturation. The mixture was stirred for 1 h and precipitated proteins were pelleted by centrifugation at 8000 × *g* for 40 min. The pellet was resuspended in 200 ml of 10 mM Tris (pH 7.2) and stirred for 30 min. The concentration of calcium was brought to 2 mM by addition of adequate amount of 1 M $CaCl_2$. The pH was monitored during the addition and was kept at 7.2 by 1 M Tris (pH 8.0). The solution was stirred for 1 h, and then clarified by centrifugation at 15 000 × *g* for 15 min. The supernatant was loaded on phenyl-Sepharose CL-4B column equilibrated in 10 mM Tris (pH 7.2), 2 mM $CaCl_2$ and 10 mM β-ME. Column was washed and kinase was eluted as previously described Putnam-Evans and coworkers (1990). Fractions containing CDPK activity were pooled and concentrated to 3 ml in Amicon ultrafiltration device using PM 10 membrane (Diaflo). After addition of NaCl and glycerol to final concentration of 0.2 M and 3%, respectively, the concentrated enzyme was passed through Sephadex G-100 column (1.5 × 80 cm) equilibrated in 20 mM Tris (pH 8.0), 2.5 mM EDTA, 200 mM NaCl and 10 mM β-ME. Leupeptin (10 μg ml^{-1}) and $MgCl_2$ (final concentration = 5.5 mM) were added to the G-100 pool of fraction containing CDPK activity and loaded onto a matrix Cibacron blue-sepharose CL-6B and chromatographed as previously described (Putnam-Evans et al., 1990).

For storage, 10% glycerol was added to the purified CDPK fraction, which was divided into aliquots and frozen at −80 °C.

4.3. Protein determination

Protein concentrations were measured by dye binding protein (Bradford, 1976), with bovine serum as a standard at 595 nm.

4.4. Enzyme assay

Activity was determined in vitro by measuring the incorporation of ^{32}P from [γ-^{32}P] ATP into different substrates. Calcium-dependent protein kinase assays were performed in a total volume of 50 μl containing 50 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 0.5 mg ml^{-1} histone III-S, 1 mM $CaCl_2$ or 5 mM EDTA. During purification the assays were performed in a standard buffer and 10 μl of each column fraction was added. Reactions were initiated by addition of 25 μM [γ-^{32}P] ATP (260–420 cpm $pmol^{-1}$) and assay mixtures were incubated for 15 min at 30 °C. Spotting 35 μl on P-81 filter terminated the reaction. The filters were washed with 5% phosphoric acid and 95% ethanol, dried and added to scintillation vials containing 4 ml scintillation cocktail and counted in a liquid scintillation counter (Wallac 1407).

For the other assays, reactions were carried out as described above but reaction mixtures contained additional effectors: exogenous substrates [histone III-S (0.5 mg ml^{-1}), dephosphorylated casein (0.5 mg ml^{-1}), MBP (0.5 mg ml^{-1}), histone mixture (0.5 mg ml^{-1}), syntide-2 (50 μM)] and inhibitors [W-7, calmidazolium, trifluoperazine and chlorpromazine (0.1- 0.4 mM for each)].

For some experiments the reaction was terminated by addition of sample buffer. The samples were boiled for 5 min and subjected to 12 or 15% acrylamide gels. The dried gels were autoradiographed with FOTON X-ray film, which was exposed at −80 °C for 1–2 weeks.

4.5. Kinetic studies

The assay of the kinetic of CDPK were conducted as described above taking a 0.6 μg of the purified enzyme and 25 μM [γ-^{32}P] ATP (270 cpm $pmol^{-1}$). Enzyme assays was performed in the presence of different concentration of calcium ions (0–1 mM) and histone III-S (0-1 mg ml^{-1}). The $K_{0,5}$ with histone III-S, K_m for histone III-S and V_{max} were determined from slopes and intercepts of the Lineweaver–Burk plots of 1/v vs. 1/[Histone III-S] or $1/[Ca^{2+}]$. Kinetic parameters were determined by averaging two independent assay results.

4.6. In-gel protein kinase assay

Purified CDPK (1.6 μg) was subjected to SDS-PAGE in 12% gel. Histone III-S (0.35 mg ml^{-1}) was added to the separation gel just prior to polymerization. For autophosphorylation assay the gel was prepared in the absence of substrate. After electrophoresis, SDS was removed by washing the gel for 30 min at room temperature with 20% isopropanol in 50 mM Tris (pH 8.0) and then 2 × 30 min in 50 mM Tris (pH 8.0), 5 mM β-ME (buffer A). Proteins were denatured by treating the

gel with 6 M guanidine-HCl in buffer A for 1 h at room temperature and then renaturated with 0.04% Tween 40 in buffer A at 4 °C overnight. Next, the gel was pre-incubated with 25 ml 50 mM HEPES (pH 7.4) containing 5 mM $MgCl_2$, 2 mM $MnCl_2$ and 2 mM DTT for 1.5 h at 30 °C and then in 4 ml of the same buffer 50 μCi [γ-^{32}P] ATP (4500 Ci mmol^{-1}) and 3 mM $CaCl_2$ or 5 mM EDTA for 1.5 h at 30 °C. Reaction was stopped by washing the gel with 1% sodium pyrophosphate in 5% trichloroacetic acid. The washed gels were dried and subjected to autoradiography.

4.7. In vitro phosphorylation of endogenous proteins

Aliquots of soluble fraction from induced and non-induced cotyledons (40 μg) were incubated in a final volume of 50 μl containing 50 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 25 μM ATP, 0.15 μCi [γ-^{32}P] ATP and 2 mM $CaCl_2$ or 5 mM EDTA. Reaction mixture was incubated 1 h at 30 °C and stopped by 50 μl 3 × Laemmli sample buffer addition and heating for 10 min in boiling water. The sample buffer contained DTT (the final concentration in the sample was 5 mM) instead of β-ME. Proteins were resolved in 12% (w/v) SDS-PAGE and analyzed by autoradiography. Phosphorylation of endogenous substrates was determined twice, as independent assays.

4.8. SDS gel electrophoresis and immunoblotting

Proteins were electrophoresed in 12 or 15% acrylamide gels (Laemmli, 1970) to determinate the following: the homogeneity of protein kinase after each fraction step, the existence of the subunits and their M_r, autophosphorylation of PnCDPK and phosphorylation of exogenous substrates, and M_rs of endogenous substrates.

Proteins were transferred to nitrocellulose by semi-dry system (BioRad; 15 min at 15 mA) using 25 mM Tris, 192 mM glycine and 20% (v/v) methanol (pH 8.3). Nitrocellulose was blocked in TBS (20 mM Tris, 100 mM NaCl) containing 5% non-fat dry milk and rinsed for 1 h with primary polyclonal antibodies against CLD domain of CDPK from soybean 1:200 (in TBS containing 1% non-fat dry milk), respectively (Bachmann et al., 1996). After three times washing in TBS, membrane was incubated for 1–2 h with alkaline phosphatase-conjugated secondary goat antibodies (1:15 000) and visualized using NBT/BCIP as a substrate in 10 ml buffer containing 100 Tris–HCl (pH 9.5) and 5 mM $MgCl_2$. Rinsing the blots in water terminated the reaction.

4.9. ^{32}P-amino acid analysis

Phosphorylation of CDPK itself and some exogenous substrate for CDPK was performed as described above. Gel fragments containing phosphoproteins were cut out of the gel and rehydrated in 10% methanol. The cellophane was removed and the fragments were redried. The proteins on the gel were subjected to hydrolysis in 6 M HCl for 2 h at 110 °C in sealed glass tubes. After hydrolysis, samples were lyophilized and the residue was washed two times with 100 μl distilled water and relyophilized. Residues were then dissolved in 10 μl distilled water. Thin-layer chromatography was performed on cellulose plates (20 × 20 cm; 0.1 mm thick on glass) in the following solvent system: 1 M propionic acid/ NH_4ON/ isopropanol (45:17.5:17.5) according to (Neufeld et al., 1989). Standards of phosphoserine (Ser-P) and phosphothreonine (Thr-P) (5 μg) were run in parallel and detected by reaction with 0.1% ninhydrin, whereas the ^{32}P-labelled amino acids were detected by autoradiography.

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