Purification and characterization of a Ca^{2+}-dependent/calmodulin-stimulated protein kinase from moss chloronema cells

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We have demonstrated the presence of a Ca^{2+}-dependent/calmodulin-stimulated protein kinase (PK) in chloronema cells of the moss *Funaria hygrometrica*. The kinase, with a molecular mass of 70,000 daltons (PK70), was purified to homogeneity using ammonium sulphate fractionation, DEAE-cellulose chromatography, and calmodulin (CaM)-agarose affinity chromatography. The kinase activity was stimulated at a concentration of 50 µM free Ca^{2+}, and was further enhanced 3–5-fold with exogenously added 3–1000 nm moss calmodulin (CaM). Autophosphorylation was also stimulated with Ca^{2+} and CaM. Under *in vitro* conditions, PK70 phosphorylated preferentially lysine-rich substrates such as HIIIS and HVS. This PK shares epitopes with the maize Ca^{2+}-dependent/calmodulin-stimulated PK (CCaMK) and also exhibits biochemical properties similar to the maize, lily, and tobacco CCaMK. We have characterized it as a moss CCaMK.

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1. Introduction

Controlled changes in cellular calcium concentrations have been identified as important components of signal transduction pathways. Calcium ions, widely recognized as a second messenger, are known to regulate a variety of cellular and physiological processes in plants (Ranjeva and Boudet 1987; Poovaiah and Reddy 1993; Bootman and Berridge 1995). The responses of Ca^{2+} are mediated by a group of Ca^{2+}-binding proteins which include Ca^{2+}-dependent protein kinases (CDPKs) and calmodulin (Roberts and Harmon 1992). CDPKs have been found to be unique to plants and belong to the family of serine/threonine kinases. They possess a conserved N-terminal catalytic domain, a junctional domain which is involved in autoinhibition, and a C-terminal calmodulin (CaM)-like sequence that binds Ca^{2+} (Harmon *et al* 2000). Calmodulin is a highly conserved and most widely distributed Ca^{2+}-binding protein (Wayne and Fromm 1998). It is an important sensor of intracellular Ca^{2+}, and, upon activation, functions as a regulatory element for its target proteins. A small group of CaM-binding proteins in plants is composed of Ca^{2+}/CaM-dependent protein kinases (CaMKs) (Lu and Feldman 1997; Lu *et al* 1996; Watillon *et al* 1993). The first CaMK in plants was cloned from apples and it shared a high degree of homology with the mammalian CaMKII (Watillon *et al* 1992). Two homologues of CaMKII are present in maize roots (MCK1, Lu *et al* 1996; MCK2, Wang *et al* 2001). The pattern of their mRNAs accumulation suggests role in growth and development.

A novel type of CaMKs in plants are the calcium-dependent/calmodulin-stimulated kinases (CCaMKs). The latter was for the first time cloned from tobacco and lily

Keywords. Calcium-dependent/calmodulin-stimulated protein kinase; chloronema; *Funaria hygrometrica*; moss CCaMK

Abbreviations used: CaM, calmodulin; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CCaMK, calcium-dependent/CaM-stimulated protein kinase; CDPK, calmodulin domain-like protein kinase; KM14, 14 amino acid synthetic peptide; LIEF-1*a*, Lily elongation factor-1 alpha; Phos, Phosvitin; PK, protein kinase.

anthers (Patil *et al* 1995). Its cDNA was found to code for a protein of 520 amino acids. The predicted structure contained a catalytic domain followed by two regulatory domains, a CaM-binding domain and a visinin-like domain with the three EF-hand motifs that are necessary for Ca^{2+}-dependent phosphorylation and its maximum activation (Ramachandiran *et al* 1997; Liu *et al* 1998). The CaM-binding domain had a high homology (79%) to CaMKII from animals. The expression of the tobacco CCaMK is known to be regulated temporally and spatially during development (Poovaiah *et al* 1999). The search for proteins that interact with the lily CCaMK using the yeast two-hybrid system (Wang and Poovaiah 1999) led to the isolation of a cDNA clone (LIEF-1*a*) which was highly homologous to the eucaryotic elongation factor-1 alpha. The lily CCaMK phosphorylates LIEF-1*a* in a Ca^{2+}/CaM-dependent manner. A 72 kDa CCaMK has also been purified to homogeneity from etiolated maize coleoptiles. It shares properties with the CCaMK from lily and tobacco (Pandey and Sopory 1998). The maize CCaMK is an important component of the light-signalling pathway and the level of the kinase decreases with red light irradiation (Pandey and Sopory 2001). Thus, there is overwhelming evidence for the role of several calcium-regulated PKs during plant development.

We have been studying the signal transduction mechanism associated with the auxin- and abscisic acid (ABA)-mediated developmental switches in the protonema of the moss *Funaria hygrometrica*. In mosses such as *Physcomitrella patens* and *Funaria hygrometrica*, calcium acts as an intracellular messenger in cytokinin-induced vegetative bud formation (Schumaker and Gizinski 1995). Calcium-free medium (buffered with EGTA), the extracellular Ca^{2+} antagonist lanthanum (La^{3+}) and the Ca^{2+} channel inhibitors, D600 and verapamil, all block bud formation in the protonema of *F. hygrometrica*. The calmodulin inhibitor trifluoperazine stops cytokinin-induced budding more effectively than the related compound chlorpromazine. It is concluded that besides a rise in intracellular calcium, activation of calmodulin also occurs in the cytokinin-induced bud formation (Saunders and Hepler 1983). Our earlier studies demonstrating the presence of multiple Ca^{2+}-dependent protein kinases in the moss protonema confirms the existence of a Ca^{2+}-messenger system in *Funaria* (D'Souza and Johri 1999). Further, a CDPK of 44 kDa has been implicated in the auxin-induced differentiation process (D'Souza and Johri 1999). A clone has also been isolated for another CDPK (FhCDPK) which is regulated by starvation (Mitra and Johri 2000). Here, we describe the characterization of another PK whose activity is dependent on Ca^{2+}, but is further enhanced 3–5-fold by moss calmodulin. This PK has a molecular mass of 70,000 daltons; it is unlikely to be a classical CaMK similar to the one found in animal systems but shares epitopes with the maize CCaMK. It is argued here that it can be classified as a moss CCaMK. The properties are consistent with its role in Ca^{2+}-signalling.

2. Materials and methods

Chemicals used for media were of analytical reagent grade and were obtained from Sarabhai M Chemicals Ltd, and Glaxo Laboratories (India) Ltd. Ammonium sulphate was of enzyme grade (extra-pure) and obtained from Sisco Research Laboratories Pvt. Ltd. All the other chemicals used were obtained from Sigma Chemical Company, USA, GIBCO Research, USA, E. Merck (India) Ltd. and Pharmacia, Sweden. [*g*-^{32}P]ATP (specific activity, $1{\cdot}3 \times 10^{14}$ Bq·mmol^{-1}) was obtained from Bhabha Atomic Research Centre, Mumbai. Calmodulin was purified from the chloronema cells of the moss *F. hygrometrica*.

2.1 *Growth of chloronemal cells and crude extract preparation*

Protonema cultures of the moss *F. hygrometrica* Hedw. were maintained in a low-calcium medium (LCM) and sub-cultured once in 3 or 4 days (Handa and Johri 1977). The chloronema cells were inoculated at a density of 0·1 mg·ml^{-1}, grown in minimal medium containing glucose (MMG) under continuous light at 25 ± 1°C and harvested after 3 or 4 days at a final density of 1·5 to 2·5 mg·ml^{-1}. The cells were pooled and collected by filtration under vacuum, dried by gently pressing the cells between 5–10 layers of tissue paper, weighed, frozen immediately in liquid nitrogen and stored at – 80°C until use.

The frozen cells were ground with a mortar and pestle in liquid nitrogen to a fine powder and the crude extract was prepared (D'Souza and Johri 1999). The suspension was left in ice for 15 min and mixed intermittently. The homogenate was then spun at 36,600 *g* for 15 min at 4°C. The supernatant was collected and kept in ice. The pellet was re-extracted with PK buffer (15% sucrose; 10 mM Tris-Cl, pH 7·5; 0·02% NaN_3, 2·5 mM $MgCl_2{\cdot}7H_2O$; 1 mM PNPP; 10 mM NaF; 1 mM PMSF; 1 mM DTT; and 5% *n*-propanol) and the supernatants were pooled. The combined supernatant fraction, referred to as cell-free propanol extract (CFPE), was used as a source of PK. Protein content was estimated using the dye-binding method (Bradford 1976).

2.2 *Enzyme purification*

The CFPE was fractionated using ammonium sulphate. The proteins precipitating at 60–70% saturation were

recovered by centrifugation at 36,600 *g* for 15 min at 4°C and the pellets were dissolved in 100–1000 µl of PK buffer. It was dialyzed against the dialysis buffer (10 mM Tris-acetate, pH 7·6, 2·5 mM Mg-acetate; 1 mM *b*-mercaptoethanol) with 2 changes overnight at 4°C. The dialyzed fraction was loaded on to a 2 × 60 cm DEAE-cellulose column equilibrated with the buffer containing 25 mM Tris-acetate, pH 7·6; 5 mM Mg-acetate, 2·5 mM *b*-mercaptoethanol. A total of 40–60 mg of protein was adsorbed on a 60 ml of DEAE-cellulose. After adsorption, the column was washed extensively and the elution was carried out with KCl using a gradient concentration. The gradient was generated using 200 ml each of buffer and 0·4 M KCl containing equilibration buffer. Eighty fractions of 5 ml each were collected and 10 µl of each were assayed for protein kinase activity in the presence and absence of calcium. The fractions showing the high PK activity were pooled and dialyzed at 4°C overnight against the dialysis buffer. The dialyzed fractions showing the maximum Ca^{2+}-dependent PK activity were used further. A 15 ml calmodulin-agarose column was equilibrated with equilibrating buffer (25 mM Tris-acetate, pH 7·6; 5 mM magnesium acetate, 2·5 mM *b*-mercaptoethanol; 0·1 mM $CaCl_2$). The fractions after the DEAE-cellulose chromatography step were loaded on to a calmodulin-agarose column in the presence of $CaCl_2$. The column was washed extensively and elution was carried out with the buffer containing 0·25 mM EGTA. Three ml fractions were collected and each was assayed for Ca^{2+}-dependent PK activity. The fractions showing the highest level of Ca^{2+}-dependent PK activity were analysed by SDS-PAGE, and the gel was stained by the silver-staining method.

2.3 In vitro *PK assay*

A typical phosphorylation reaction consisted of fraction or sample containing 50 µg protein, 60 µM ATP, 1×10^5 Bq·ml^{-1} [*g*-^{32}P]ATP (specific activity $1{\cdot}3 \times 10^{14}$ Bq·mmol^{-1}), 75 µM EGTA (for calcium-lacking treatments), 150 µM $CaCl_2$ (for treatments with calcium) and an exogenous substrate (casein, histones, phosvitin, BSA or KM14) when needed. The final volume of 50 µl was made up with PK buffer. The reaction was initiated by adding a cocktail consisting of labelled ATP, non-labelled ATP and PK buffer. Incubation was done at 25°C for 30 min and later 15 or 20 µl of the reaction mixture was spotted on a 25 mm diameter filter disc of Whatmann No. 3 filter paper pre-coated with 3 mM ATP. The filters were immediately transferred to cold 5% TCA and 20 mM sodium pyrophosphate solution (TCA/PP) and left for 1 to 1·5 h. The filters were transferred to fresh TCA/PP and boiled for 5 min. Ultimately, the discs were rinsed once more with fresh TCA/PP, dried and the radioactivity was determined. Suitable controls were included in each of the experiments to determine the non-specific binding of the labelled ATP to the filters. All experiments have been successfully repeated at least twice. The specific activity is expressed in terms of units·mg^{-1} protein such that 1 unit (U) corresponds to one pmol of ATP incorporated per min. The proteins phosphorylated *in vitro* were also analysed by SDS-PAGE followed by autoradiography.

2.4 *Western blotting and probing with antibodies*

The proteins separated by electrophoresis were transferred onto a nitrocellulose sheet using 50 mA current for 16–20 h. The blot was dried, probed immediately or stored at 4°C till further use. Blocking was carried out in TBST (50 mM Tris-Cl, pH 7·5; 50 mM NaCl; 2 mM $CaCl_2$; 0·1% Tween-20) containing 5% defatted milk for 2 h. Incubation with primary antibody (anti-PK70 or maize anti-CCaMK) was carried out in TBST containing 5% defatted milk at a dilution of 1 : 5000 for 3 h at 25°C and then overnight at 4°C. Next morning, the blot was washed with TBST, 3 times – 10 min each time. The blot was then incubated in TBST with 5% defatted milk for 2 h, using biotinylated anti-rabbit IgG at a dilution of 1 : 1000. Meanwhile, two drops (approximately 100 µl) from each avidin (A) and biotin (B) solutions were mixed in 10 ml of TBST and incubated at 25°C for 30 min. After thorough washes of the blot with TBST, it was incubated for 30 min with the A+B mixture. This was followed by several washes with TBST and TBS, and developed in TBS containing 5 mg di-amino benzidine and 4 µl H_2O_2 per 10 ml solution. The blot was then washed thoroughly in double-distilled water and dried between blotting papers.

3. Results

3.1 *Purification of CCaMK*

Our earlier studies had indicated that the supernatant of the cell-free aqueous extract of chloronema cells from the moss protonema contained 70–80% of PK activity while the remaining was associated with the pellet. Detergents and 5% *n*-propanol were individually employed to extract the activity from the pellet. Whereas detergents inhibited PK activity, use of 5% *n*-propanol at 25°C for 5 min led to the recovery of almost 90% of the total measurable PK activity (D'Souza 1993). Using the in-gel kinase assay, this cell-free propanol extract (CFPE) showed the presence of multiple Ca^{2+}-dependent PKs (D'Souza and Johri 1999) and was therefore used as a source of Ca^{2+}-dependent PKs for purification. When the CFPE was fractionated using ammonium sulphate, the 60–70%

Table 1. Ca^{2+}-dependent PK activity from chloronema cells. The cell-free propanol extract (CFPE) and various ammonium sulphate fractions (0–40%, 40–60%, 60–70%) were checked for Ca^{2+}-dependent PK activity (275 μM free Ca^{2+}) with or without casein as a substrate. Fold response refers to the stimulation in activity after the addition of Ca^{2+}.

		– Casein			+ Casein		
Fraction	Total protein (μg)	Units mg^{-1} protein	Total units	Fold response	Units mg^{-1} protein	Total units	Fold response
CFPE	180						
– Ca^{2+}		1·63	294		26·45	4773	
+ Ca^{2+}		4·47	807	2·7	33·36	6013	1·3
0–40%	28						
– Ca^{2+}		1·26	36		11·88	337	
+ Ca^{2+}		2·89	82	2·3	13·68	388	1·2
40–60%	132						
– Ca^{2+}		2·72	359		26·11	3448	
+ Ca^{2+}		7·23	955	2·7	31·70	4186	1·2
60–70%	29						
– Ca^{2+}		0·50	14		4·74	137	
+ Ca^{2+}		2·09	60	4·2	16·90	487	3·6

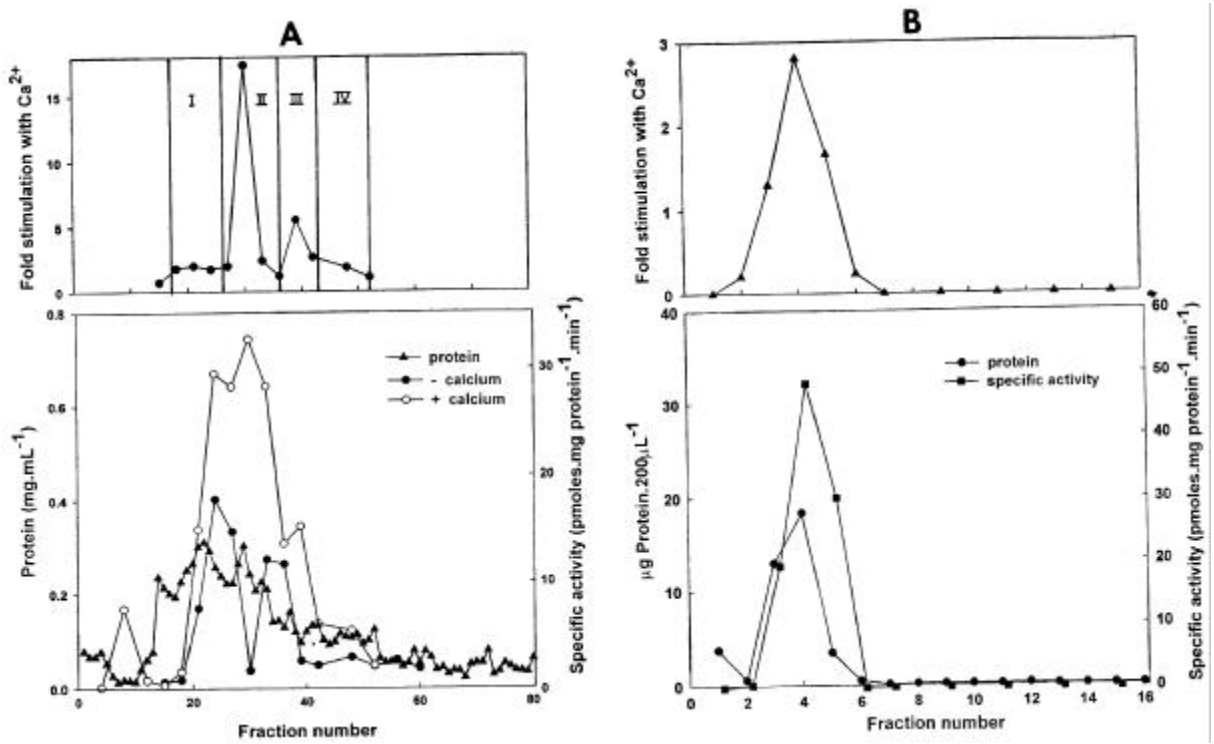

Figure 1. Purification of PK70 using DEAE-cellulose and CaM-agarose chromatography. Approximately 60 mg of proteins from chloronemal cell extract, precipitating at 60–70% ammonium sulphate, were chromatographed on a DEAE-cellulose column. The protein content and PK activity profiles of each fraction (30 μl) are shown. From pool II, 1·92 mg of total protein was loaded on the CaM-agarose column and washed with the buffer containing $CaCl_2$. Elution was carried out with buffer containing EGTA. One ml fractions were collected and checked for Ca^{2+}-dependent protein kinase activity. (**A**) The profile after the DEAE-cellulose chromatography. (**B**) The profile after CaM-agarose chromatography. PK activity was determined using casein as substrate.

fraction showed the highest stimulation with Ca^{2+} using casein and endogenously present substrates (table 1). This fraction was further purified using DEAE-cellulose chromatography. The Ca^{2+}-dependent PK activity was assayed in the eluates and four sets of combined fractions were prepared (marked I–IV in figure 1A). Eluates from each set were combined, and pool II alone showed CaM-dependent PK activity with HIIA as a substrate (data not shown). The PK was next purified by affinity chromatography using CaM-agarose (figure 1B). The fractions 3–5 showed the highest Ca^{2+}-dependent PK activity and there was a 3-fold stimulation in the presence of calcium ions. A silver-stained gel (figure 2) showed a polypeptide of 70 kDa as the major polypeptide. It is referred to as PK70. Only traces of other higher molecular weight polypeptides were present. The combined fractions 4 and 5 were used for further experiments. This fraction was 44-fold pure and showed a 10-fold enhancement in activity with Ca^{2+} (table 2).

The stability of the protein showed characteristic features. During storage at 4°C, the affinity purified PK70 was observed to undergo an activation process. The

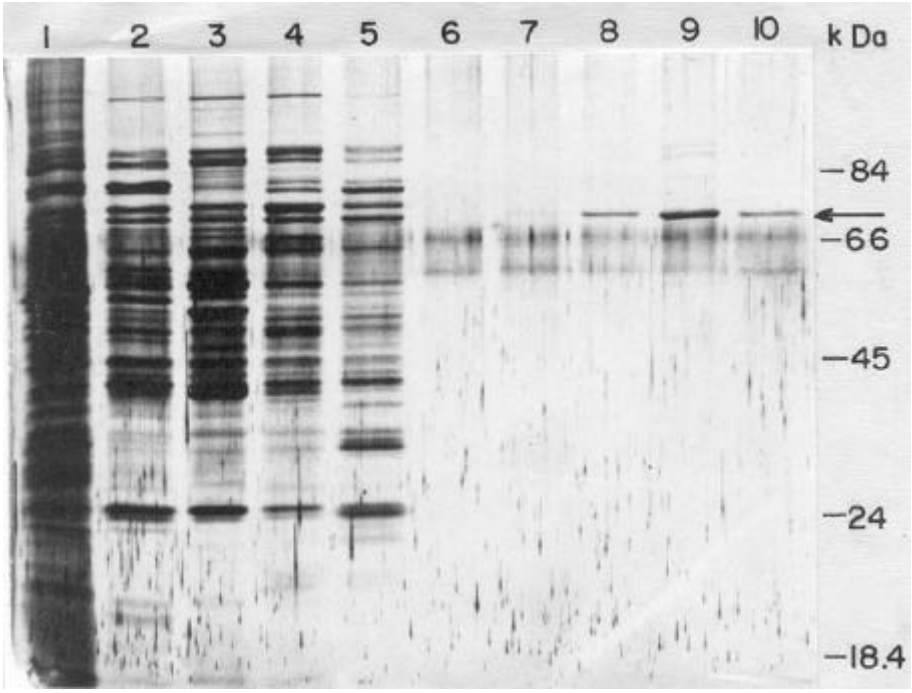

Figure 2. Silver-stained gel of the proteins at different stages of PK purification. Fractions at different stages of purification were electrophoresed by SDS-PAGE. Crude extract (lane 1), 60–70% ammonium sulphate fraction (lane 2), DEAE fractions-I, II and III (lanes 3–5 respectively) and CaM-agarose fractions 1–6 (lanes 6–10 respectively). Lane 9 represents the combined CaM-agarose fractions 4 and 5. The arrow indicates the position of the PK70. Protein concentration is 50 μg in lanes 1–5 and 0·25 μg in lanes 6–10.

Table 2. Purification profile of PK70.

			PK activity Units mg^{-1} protein				
Fraction	Volume (ml)	Protein (mg)	– Ca^{2+}	+ Ca^{2+}	Fold response	Fold purification	Yield (%)
Crude extract	273	846·3	1·1	2·3	2	1	100
Ammonium sulphate fraction	70	61·6	0·83	3·4	4	1·5	11
DEAE-cellulose eluate	83	32·2	0·22	4·4	20	2	8
CaM-agarose eluate	5·5	0·055	9·6	100	10	44	0·3

activity increased 2–4-fold during the 9 days of storage but was lost very rapidly later within a day (table 3). It was especially unstable to freezing and thawing, since the activity was completely lost upon a single freeze-thaw cycle (data not shown).

Table 3. Stability of PK70. PK70 activity was measured on different days after purification. The assays were carried using 2·5 μg of PK70 at 25°C for 20 min.

Days after purification	Specific activity (pmol mg protein^{-1} min^{-1})
2	11·2
3	18·9
4	27·2
5	33·6
6	35·4
7·5	37·8
9	38·3
10	5·2

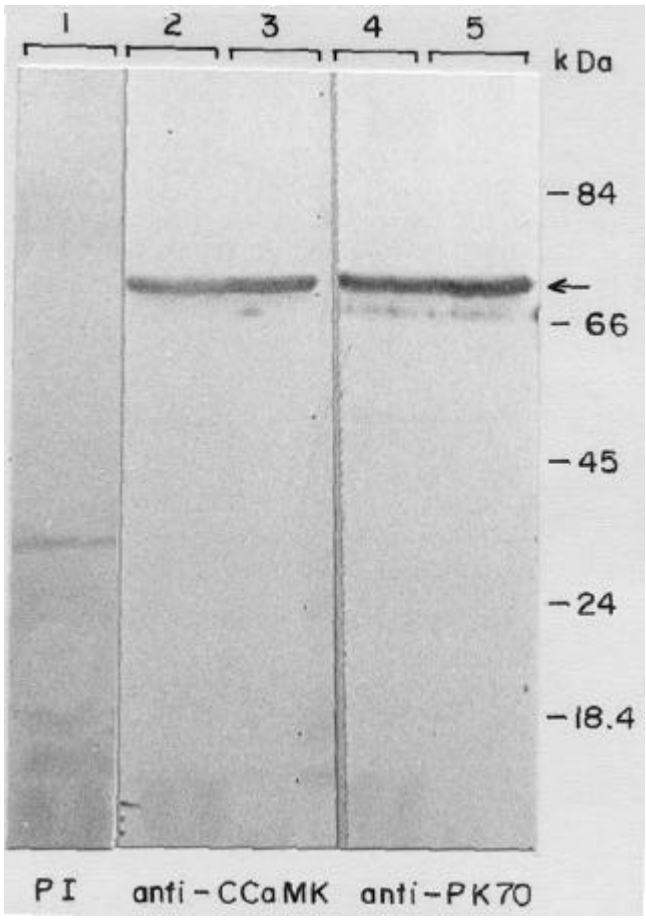

Figure 3. Cross-reactivity of PK70 with anti-CCaMK from maize. Two batches of purified PK70 (batch 1, lanes 2 and 4; batch 2, lanes 3 and 5) were electrophoresed on a 10% SDS-PAGE, blotted onto nitrocellulose and probed with anti-CCaMK from maize and anti-PK70 from moss. PI, pre-immune serum.

A polyclonal monospecific antibody against the maize CCaMK was used to check for cross-reactivity of PK70 using the enhanced biotin-avidin system. Duplicate strips of two batches of purified PK70 were probed, one with the anti-CCaMK antibody from maize and the other with the anti-PK70 antibody prepared in the laboratory. On both the strips, purified band at 70,000 daltons were detectable, suggesting that the moss PK70 shares epitopes with the maize CCaMK (figure 3).

3.2 *Effect of Ca^{2+} and CaM on the stimulation of PK activity*

The affinity purified PK70 was checked for Ca^{2+} utilization using the Ca^{2+}-EGTA buffer (final free Ca^{2+} concentration varied between 0 and 150 μM). When the Ca^{2+}-dependent PK activity was checked using HIIA or HIIIS as substrate, the PK70 activity increased rapidly beyond 40 μM free Ca^{2+} and was optimum at about 50–55 μM (figure 4A). The requirement of Ca^{2+} for the moss Ca^{2+}-dependent PK was observed to be far higher than that reported for most of the other Ca^{2+}-dependent PKs from plants, which responded optimally to a free Ca^{2+} concentration of 0·1–5 μM (Harmon *et al* 2000; Pandey and Sopory 1998). In order to resolve this discrepancy, we decided to investigate the effect of CaM in the presence of sub-optimal levels of Ca^{2+}. In the presence of 25 μM free Ca^{2+} (approximately one-half of optimal concentration), the activity was found to be enhanced 3–5-fold when 3–1000 nM purified moss CaM was added (figure 4B). It is worth mentioning that the sub-threshold level of Ca^{2+} used in this experiment (25 μM) has no effect on PK70 activity. Thus, we seem to be dealing with a Ca^{2+}-regulated CaM-dependent PK in the moss.

3.3 *Substrate specificity*

When assayed in the absence of exogenous substrate, PK70 underwent autophosphorylation. Among different substrates, lysine-rich histones (HIIIS and HVS, table 4) were preferentially utilized. The analysis of products by SDS-PAGE suggests that substrate phosphorylation can occur independently of autophosphorylation (figure 5). It may be pointed out that one does not know the proportion of the PK70 that may already be present in the phosphorylated state in the affinity-purified enzyme. Casein and several histones (HIIIS, HVS, HVIIS), but not BSA and phosvitin, showed Ca^{2+}-dependent substrate phosphorylation. In the presence of other substrates such as

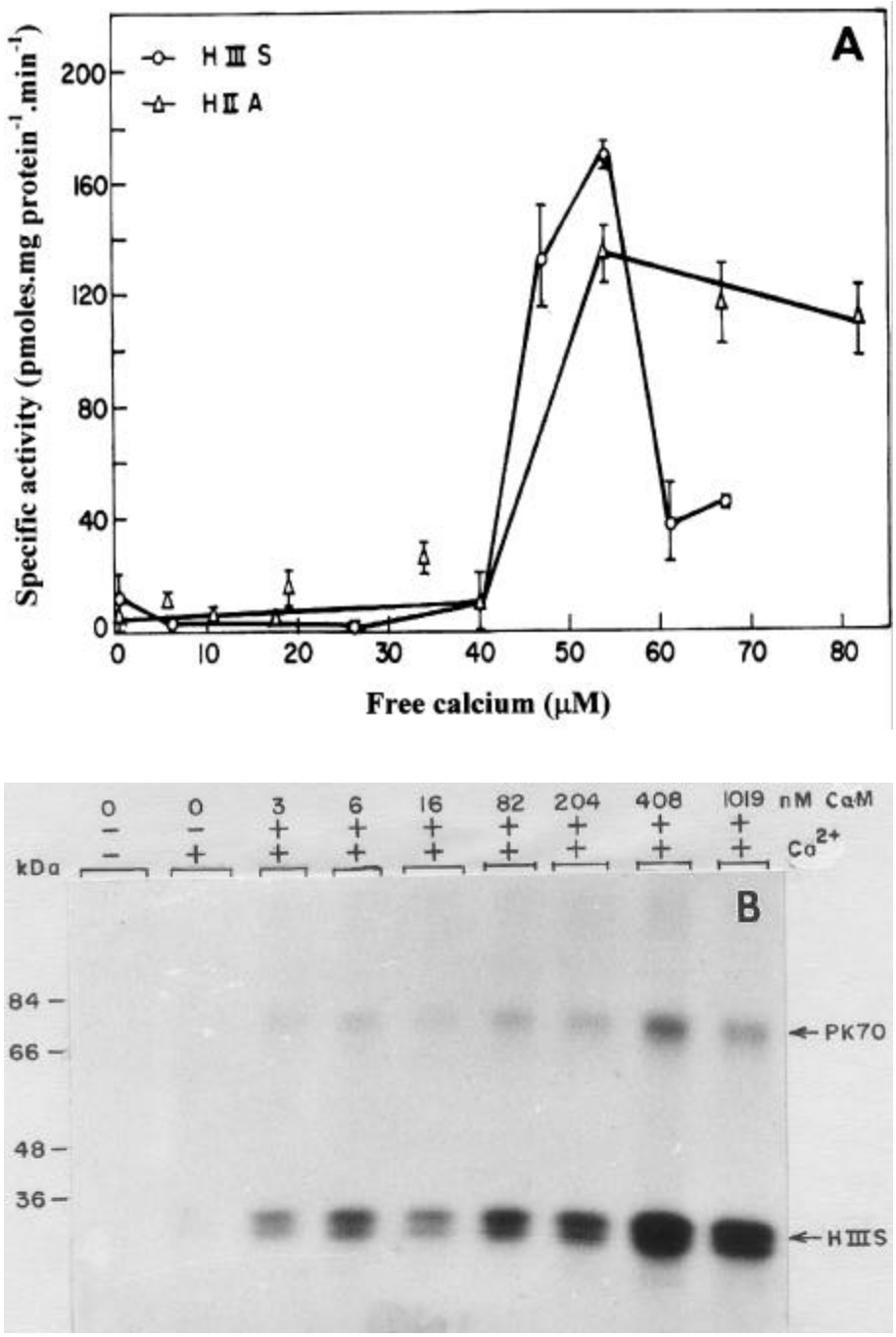

Figure 4. Effect of moss CaM on PK70 activity. (**A**) The combined fractions 4 and 5 (figure 2, lane 9) were assayed in the presence of different amounts of free Ca^{2+} using Ca-EGTA buffer. Two μg of PK70 protein was added to each tube containing HIIA or HIIIS as substrate. (**B**) The effect of moss CaM was investigated by incubating 2 μg of purified PK70 with 3 to 1019 nM of CaM in the presence of 23 μM free Ca^{2+} and 5 μg HIIIS for 20 min at 25°C. The *in vitro* phosphorylated products were analysed by SDS-PAGE and autoradiography.

Table 4. Substrate utilization by PK70. The PK activity was checked in the presence or absence of 50 μM free Ca^{2+}, using 2 μg of purified PK70 and 5 μg substrate per assay.

	PK activity (units mg^{-1} protein)	
Substrate	EGTA	EGTA + Ca^{2+}
None	0	22·5
Casein	54·0	233·0
BSA	0	42·0
Phosvitin	71·0	72·0
KM14	0	4·6
Histone IIA	9·6	100·0
Histone IIIS	92·0	688·0
Histone VS	121·0	367·0
Histone VI	15·0	113·0
Histone VIS	20·0	121·0
Histone VIIS	0	175·0
Histone VIIIS	0	63·0

casein, HIIIS and HVIS, autophosphorylation was inhibited, while with substrates such as HIIA, HVS, HVI and HVIIS it was enhanced. However, autophosphorylation was strictly Ca^{2+}-dependent with all the substrates used.

4. Discussion

We have described here the purification of PK70, which is identified to be a calcium-dependent/calmodulin-stimulated protein kinase from the chloronema cells of the moss *F. hygrometrica*. Of the various CaMKs known in plants, PK70 seems closest to the CCaMK purified from the maize coleoptiles (Pandey and Sopory 1998). The maize CCaMK and PK70 could be purified using more or less similar protocols. They share similar molecular size, exhibit optimum activity in the pH range of 7·5–8·0. A comparison of the properties of CCaMKs, given in table 5 shows that PK70 also shares properties with the CCaMKs isolated from the lily and tobacco anthers (Patil *et al* 1995). The magnesium ions are required for the activity of these PKs and casein and HIIIS were the preferred substrates.

PK70 from moss was found to be strictly a Ca^{2+}-dependent histone kinase, whose activity was enhanced several-folds in the presence of 3 to 1000 nM exogenously added moss CaM. On the other hand, the maize CCaMK responded to 3–30 nM of CaM in the presence of 0·01 to 0·1 μM free Ca^{2+}, and the lily CCaMK showed a K_a value of 150–200 nM for calmodulin (Pandey and Sopory 1998; Sathyanarayanan *et al* 2000). Ca^{2+} is required for the autophosphorylation of all these CCaMKs, including PK70. Unlike the maize PK, the autophosphorylation of PK70 increased with increasing CaM concentration. In case of lily CCaMK whereas Ca^{2+} binding at nanomolar range to the visinin-like domain led to the autophosphorylation and an increased affinity for CaM (Sathyanarayanan *et al* 2001), in case of moss CCaMK far higher levels of Ca^{2+} were present when the effect of CaM was tested. A close examination of the data in moss shows that there is hardly any discrepancy. CaM at 3–500 nM enhanced autophosphorylation and substrate phosphorylation; a calcium level of 12–2000 nM will be adequate for the optimal activity of the moss PK70. This calcium concentration is in the same range as that used for CCaMK in lily.

The autophosphorylation of the lily CCaMK led to an increase in its activity by 5-fold. Ca^{2+} had a dual effect on the stimulation of its activity. In the presence of Ca^{2+}, CaM is activated and stimulates CCaMK activity. In the absence of CaM, Ca^{2+} alone stimulates autophosphorylation of CCaMK, which further increases Ca^{2+}/calmodulin-dependent kinase activity (Takezawa *et al* 1996). Whereas Ca^{2+} was required for autophosphorylation, Ca^{2+} and CaM both were required for substrate phosphorylation (Ramachandiran *et al* 1997). The tobacco CCaMK protein expressed in *Escherchia coli* also showed Ca^{2+}-dependent autophosphorylation and Ca^{2+}/CaM-dependent substrate phosphorylation (Liu *et al* 1998). With PK70, both autophosphorylation and HIIIS phosphorylation increased steadily in the presence of 3–1000 nM moss CaM, the highest stimulation being at about 400 nM (figure 4B). At sub-threshold levels, Ca^{2+} alone was insufficient for the autophosphorylation or HIIIS phosphorylation. However, at optimal level (50 μM) it was required for autophosphorylation and substrate phosphorylation (figure 5). Interestingly, the Ca^{2+}-dependent autophosphorylation was observed to be either enhanced or inhibited depending on the type of substrate (figure 5). For example, HIIA, HVS, HVI and HVIIS enhanced autophosphorylation; while casein, HIIIS and HVIS inhibited it. The effect of Ca^{2+} on substrate phosphorylation was also variable. Whereas casein and histones such as HIIIS, HVS, HVIIS showed Ca^{2+}-dependent phosphorylation, others such as BSA and phosvitin were not affected. It appears that autophosphorylation and substrate phosphorylation are independent events. The observed response of PK70 to exogenously provided calcium or calmodulin makes it a very versatile calcium-sensing element capable of responding to a wide range of calcium levels. With sub-threshold levels such as 25 μM free Ca^{2+}, it responded to 3–1000 nM CaM, while at higher levels of free Ca^{2+} (more than 35–40 μM), the PK70 activity was independent of exogenous CaM. It is thus capable of responding to Ca^{2+} alone or to a Ca^{2+}/CaM complex. Depending on its substrate phosphorylation capacity, one can interpret that it could conceivably phosphorylate various physiological substrates differentially. This suggests that

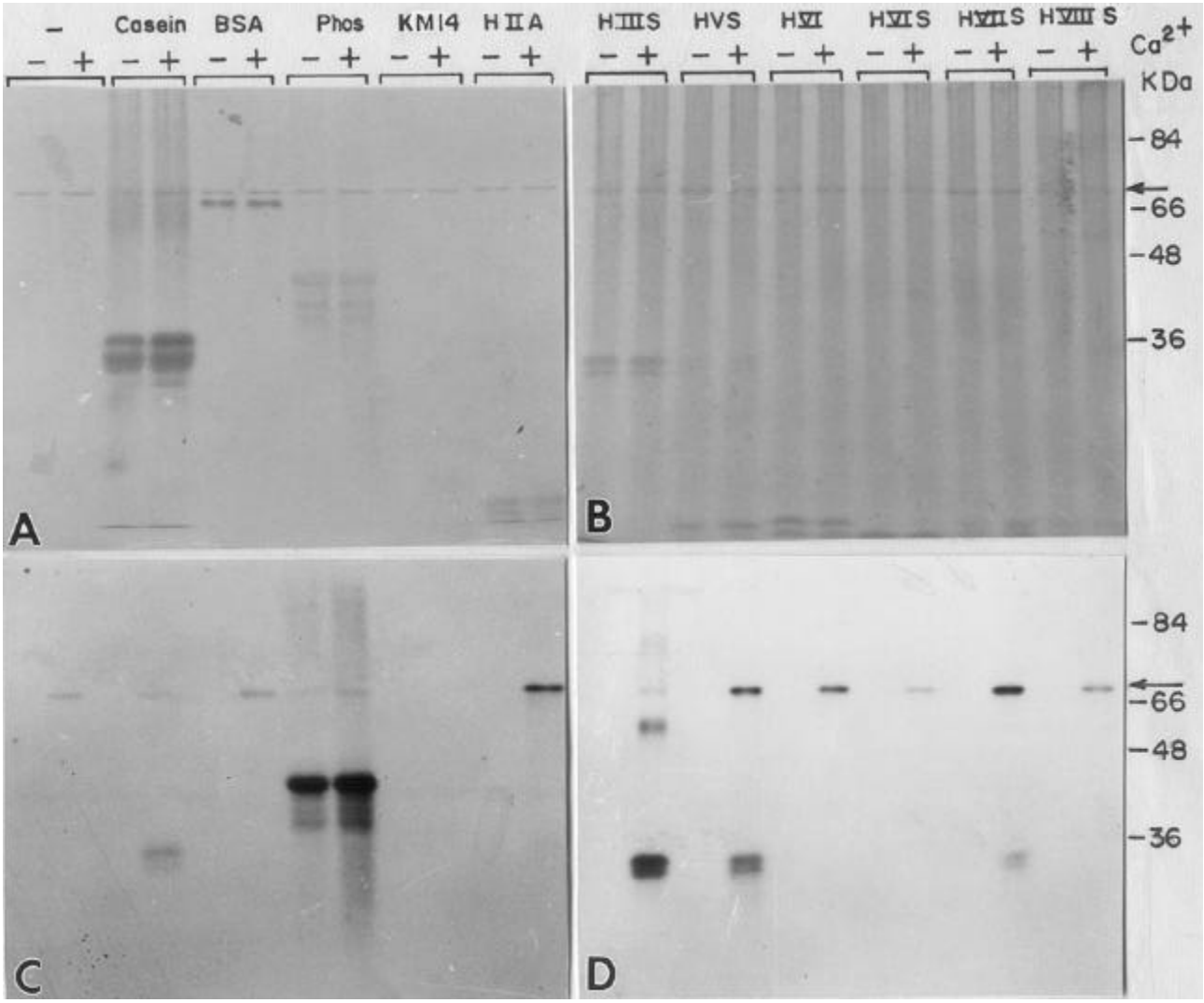

Figure 5. Qualitative analysis of the substrates utilized by PK70. The *in vitro* phosphorylated products using different substrates were electrophoresed by SDS-PAGE. Phosphorylation was carried out in the absence or presence of 50 μM free Ca^{2+} using 5 μg of various substrates and 2 μg of purified PK70. Incubation was carried out at 25°C for 20 min. (**A**) and (**B**) are the Coomassie-blue stained gels; while (**C**) and (**D**) are the autoradiographs.

PK70 may have several regulatory roles in the moss system. The experiments described here, have made it possible to assign specific roles to calcium and CaM. So far, the CCaMK from maize has been the best-characterized one biochemically (Pandey and Sopory 1998); and has recently been shown to be a component of the light-induced signalling pathway (Pandey and Sopory 2001). Though PK70 seems to be closest in properties to CCaMKs described in plants, its precise role in moss development and calcium/CaM signalling is yet to be understood.

Our earlier studies have suggested that two distinct CDPKs participate in different responses of the moss *F. hygrometrica*. PK44 plays a role in the caulonema differentiation (D'Souza and Johri 1999), while the expression of FhCDPK transcript was enhanced within 24–48 h of chloronemal starvation for nitrogen, phosphorus or sulphur (Mitra and Johri 2000). FhCDPK, seems to be involved in the starvation process. Both these studies reveal the presence of a Ca^{2+}-messenger system (D'Souza and Johri 1999). The presence of PK70 further supports the presence of this system, and the biochemical properties indicate that it is a signalling component. Further, we wish to suggest that the response of PK70 through interaction with CaM seems to be physiologically relevant

Table 5. Comparison of the properties of CCaMK from the lily, tobacco, maize and moss PK70.

Properties	Lily and tobacco	Maize	Moss
Cloned/purified	Cloned	Purified	Purified
Mr (kDa)	56	72	70
Mg^{2+} requirement	Required	Required	Required
pH optimum	Not reported	7·5	8·0
Substrates utilized	HIIIS, HIIA, casein	HIIIS, syntide-2, casein	HIIIS, HIIA, casein
Ca^{2+} requirement	Ca^{2+} for autophosphorylation and Ca^{2+}/CaM for substrate phosphorylation	Ca^{2+} for autophosphorylation and substrate phosphorylation	Ca^{2+} for autophosphorylation and substrate phosphorylation
CaM requirement	Responds to 150–200 nM CaM in the presence of 0·5 μM Ca^{2+}	Responds to 3–30 nM of CaM in the presence of 0·01–0·1 μM free Ca^{2+}	Responds to 3–1016 nM CaM in the presence of 25 μM free Ca^{2+}
Autophosphorylation status	Increases in the presence of substrate and CaM	Ca^{2+} is required for autophosphorylation, but is not significantly altered with CaM	Increases in the presence of specific substrates and CaM
Stability	Stable at 4°C for a few days	Not reported	Stable for 9 days at 4°C

because the plant cells in general maintain nanomolar levels of endogenous calmodulin (Hepler and Wayne 1985).

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References

Bootman M D and Berridge M J 1995 The elemental principles of calcium signaling; *Cell* **83** 675–678

Bradford M M 1976 A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of dye binding; *Anal. Biochem.* **72** 248–254

D'Souza J S 1993 *Studies on the Calcium/calmodulin-dependent protein kinase from the moss protonema*, M.Sc. Thesis, Bombay University, Mumbai, India

D'Souza J S and Johri M M 1999 Ca^{2+}dPKs from the protonema of the moss *Funaria hygrometrica*. Effect of indoleacetic acid and cultural parameters on the activity of a 44 kDa Ca^{2+}dPK; *Plant Sci.* **145** 23–32

Handa A K and Johri M M 1977 Cyclic adenosine 3′ : 5′-monophosphate in moss protonema; *Plant Physiol.* **59** 490–496

Harmon A C, Gribskov M and Harper J F 2000 CDPKs – a kinase for every Ca^{2+} signal?; *Trends Plant Sci.* **5** 154–159

Hepler P K and Wayne R O 1985 Calcium and plant development; *Ann. Rev. Plant Physiol.* **36** 397–439

Liu Z, Xia M and Poovaiah B W 1998 Chimeric calcium/calmodulin-dependent protein kinase in tobacco: differential regulation by calmodulin isoforms; *Plant Mol. Biol.* **38** 889–897

Lu Y-T and Feldman L J 1997 Light-regulated root gravitropism: a role for, and characterization of a calcium/calmodulin-dependent protein kinase homolog; *Planta* **203** S91–S97

Lu Y-T, Hidaka H and Feldman L J 1996 Characterization of a calcium/calmodulin-dependent protein kinase homolog from maize roots showing light-regulated gravitropism; *Planta* **199** 18–24

Mitra D and Johri M M 2000 Enhanced expression of a calcium-dependent protein kinase from the moss *Funaria hygrometrica* under nutritional starvation; *J. Biosci.* **25** 331–338

Pandey S and Sopory S K 1998 Biochemical evidence for a calmodulin-stimulated calcium-dependent protein kinase in maize; *Eur. J. Biochem.* **255** 718–726

Pandey S and Sopory S K 2001 *Zea mays* CCaMK: autophosphorylation-dependent substrate phosphorylation and down-regulation by red light; *J. Exp. Bot.* **52** 691–700

Patil S, Takezawa D and Poovaiah B W 1995 Chimeric plant calcium/calmodulin-dependent protein kinase gene with a neural visinin-like calcium-binding domain; *Proc. Natl. Acad. Sci. USA* **92** 4897–4901

Poovaiah B W and Reddy A S N 1993 Calcium and signal transduction in plants; *Crit. Rev. Plant Sci.* **12** 185–211

Poovaiah B W, Xia M, Liu Z, Wang W, Yang T, Sathyanarayanan P V and Franceschi V R 1999 Developmental regulation of the gene for chimeric calcium/calmodulin-dependent protein kinase in anthers; *Planta* **209** 161–171

Ramachandiran S, Takezawa D, Wang W and Poovaiah B W 1997 Functional domains of plant chimeric calcium/calmodulin-dependent protein kinase: regulation by autoinhibitory and visinin-like domains; *J. Biochem.* **121** 984–990

Ranjeva R and Boudet A M 1987 Phosphorylation of protein in plants: regulatory effects and potential involvement in stimuli/response coupling; *Ann. Rev. Plant Physiol.* **38** 73–93

Roberts D M and Harmon A C 1992 Calcium modulated proteins: targets of intracellular calcium signals in higher plants; *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **43** 375–407

Sathyanarayanan P V, Cremo C R and Poovaiah B W 2000 Plant chimeric Ca^{2+}/Calmodulin-dependent protein kinase. Role of the neural visinin-like domain in regulating autophosphorylation and calmodulin affinity; *J. Biol. Chem.* **275** 30417–30422

Sathyanarayanan P V, Siems W F, Jones J P and Poovaiah B W 2001 Calcium-stimulated autophosphorylation site of plant chimeric calcium/calmodulin-dependent protein kinase; *J. Biol. Chem.* **276** 32940–32947

Saunders M J and Hepler P K 1983 Calcium antagonists and calmodulin inhibitors block cytokinin-induced bud formation in Funaria; *Dev. Biol.* **99** 41–49

Schumaker K S and Gizinski M J 1995 1,4-Dihydropyridine binding sites in moss plasma membranes. Properties of receptors for a calcium channel antagonist; *J. Biol. Chem.* **270** 23461–23467

Takezawa D, Ramachandiran S, Paranjape V and Poovaiah B W 1996 Dual regulation of a chimeric plant serine/threonine kinase by calcium and calcium/calmodulin; *J. Biol. Chem.* **271** 8126–8132

Wang L, Liang S and Lu Y -T 2001 Characterization, physical location and expression of the genes encoding calcium/calmodulin-dependent protein kinases in maize (*Zea mays* L.); *Planta* **213** 556–564

Wang W and Poovaiah B W 1999 Interaction of plant chimeric calcium/calmodulin-dependent protein kinase with a homolog of eukaryotic elongation factor-1*a*; *J. Biol. Chem.* **274** 12001–12008

Watillon B, Kettmann R, Boxus P and Bunny A 1992 Cloning and characterization of an apple (*Malus domesticus* [L.] Borkh) cDNA encoding a calmodulin-binding protein domain similar to the calmodulin-binding region of type II mammalian Ca^{2+}/calmodulin-dependent protein kinase; *Plant Sci.* **81** 227–235

Watillon B, Kettmann R, Boxus P and Bunny A 1993 A calcium/calmodulin-binding serine/threonine protein kinase homologous to the mammalian type II calcium/calmodulin-dependent protein kinase is expressed in plant cells; *Plant Physiol.* **101** 1381–1384

Wayne A S and Fromm H 1998 Calmodulin, calmodulin-related proteins and plant responses to the environment; *Trends Plant Sci.* **3** 299–304

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