Enhanced expression of a calcium-dependent protein kinase from the moss *Funaria hygrometrica* under nutritional starvation

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Among the downstream targets of calcium in plants, calcium-dependent protein kinases (CDPKs) form an interesting class of kinases which are activated by calcium binding. They have been implicated in a diverse array of responses to hormonal and environmental stimuli. In order to dissect the role of CDPKs in the moss *Funaria hygrometrica*, a polymerase chain reaction (PCR)-based approach was adopted to clone the gene. Using degenerate PCR primers against conserved regions of CDPKs, a 900 bp amplicon was obtained from the genomic DNA of *Funaria*. Southern hybridization under low stringency conditions indicated the presence of several CDPK related sequences in the *Funaria* genome. This observation is consistent with reports of multigene families of CDPKs in other plants. The 900 bp fragment was subsequently used to isolate a 2·2 kb partial genomic clone of the CDPK gene from *Funaria*. The genomic clone encodes an open reading frame (ORF) of 518 amino acids. Interestingly, unlike other CDPK genes from plants, the entire 1·5 kb ORF is not interrupted by introns. The deduced amino acid sequence of the *Funaria* gene shows extensive homology with CDPKs from higher plants, 73% identity with the *Fragaria* CDPK and 71% identity with CDPK isoform 7 of *Arabidopsis*. Phylogenetic analysis revealed that the *Funaria* CDPK is closer to the CDPKs from higher plants like strawberry and *Arabidopsis* as compared to those from lower plants such as the liverwort *Marchantia*, the green alga *Chlamydomonas* or another moss *Tortula*. Northern analysis shows enhanced expression of the CDPK transcript within 24–48 h of starvation for nitrogen, phosphorus or sulphur. So far the only other kinase which is known to be induced by nutrient starvation in plants is the wpk 4 which is a snf-1 related kinase (SnRKs). To our knowledge this is the first report that implicates a CDPK in the starvation response.

1. Introduction

Calcium is an important second messenger in plants that mediates signaling pathways elicited by hormonal as well as environmental stimuli (reviewed by Bush 1995). Calcium also plays a critical role in various developmental processes like pollen tube growth (Picton and Steer 1983) and cytokinin induced bud formation in *Funaria* (Saunders and Hepler 1983). Among the downstream targets of calcium, the calcium dependent protein kinases (CDPKs) have emerged as the most predominant gene family in plants (Harmon *et al* 2000). CDPKs are a group of serine threonine kinases that have an N-terminal catalytic and a C-terminal calcium binding domain linked by an auto-

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Abbreviations used: CDPK, calcium dependent protein kinase; HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LCM, low calcium medium; LNM, low nitrate medium; LPM, low phosphate medium; LSM, low sulphate medium; MMG, minimal medium with glucose; NR, nitrate reductase; ORF, open reading frame; PCR, polymerase chain reaction; SnRK, sucrose non fermenting (snf) related kinase; SPS, sucrose phosphate synthase.

The nucleotide sequence reported in this paper has been submitted to the GenBank database (Accession No. AF276999).

inhibitory region. The kinase domain is preceded by a variable region, which differs both in sequence and size among different CDPK isoforms. The calcium binding region contains EF hand motifs and is also called the calmodulin-like domain. In the absence of calcium, the autoinhibitory region inhibits the kinase activity (Harmon *et al* 1994). The kinase is activated by the binding of calcium to the EF hands which brings about a conformational change that relieves the autoinhibition.

Interestingly, CDPKs have so far been reported only in plants and protists but are absent in the completely sequenced genomes of *Cenorhabditis elegans*, *Drosophlia melanogaster* and *Saccharomyces cerevisiae*. The CDPK genes are highly conserved across species and are encoded by multigene families. There are about 40 CDPK isoforms in *Arabidopsis*, nine in maize and three in rice and soybean. The recombinant CDPK isoforms *a*, *b* and *g* of soybean have been tested for their calcium binding affinity in the absence of substrates and found to have K_d values of 51, 1·4, and 1·6 μM, respectively (Lee *et al* 1998). Using isoform specific antibodies against the rice CDPKs, OsCDPK2 and OsCDPK11, it has been demonstrated that the two isoforms have different patterns of expression during seed development and in response to light (Frattini *et al* 1999). Such differences in substrate specificity, calcium binding affinity and tissue or subcellular localization of the various isoforms have been implicated to contribute to the multiplicity of function. CDPKs have been implicated in several aspects of plant growth and development (Harmon *et al* 2000). Studies involving the transient transfection of maize protoplasts with the *Arabidopsis* CDPK isoforms, CDPK1 and CDPK1a, have led to the establishment of their role in the stress response pathway (Sheen 1996). Recent work has implicated CDPKs in the Avr9 elicitor induced defense pathway (Romeis *et al* 2000). On the basis of induction of transcript levels, CDPKs have been implicated to play a role in mediating signalling elicited by the phytohormones such as, auxin (Botella *et al* 1996), ABA, cytokinin and gibberellin (Yoon *et al* 1999). Abiotic stresses like mechanical strain (Botella *et al* 1996), salt stress and dehydration (Urao *et al* 1994) have also been shown to induce transcription of CDPK genes in mung bean and *Arabidopsis*, respectively. Earlier work from our laboratory has shown the presence of multiple calcium dependent protein kinases in the protonema of *Funaria* (D'Souza and Johri 1999). In order to understand the role of CDPKs in the moss protonema we have isolated and characterized a partial genomic clone of a CDPK. The up-regulation of the transcript levels of this CDPK by nutrient starvation has been demonstrated by Northern analysis. To our knowledge, this is the first report that implicates a CDPK in the starvation response.

2. Materials and methods

2.1 *Plant material and growth conditions*

The experiments were carried out using the protonema of the moss *Funaria hygrometrica* Hedw. (Cell line J-2). The cultures were maintained in low-calcium medium (LCM) as described by Handa and Johri (1977). For the starvation experiments, cells were grown in media deficient in either of the nutrients such as nitrate, phosphate or sulphate. Before inoculating the LCM-grown cells into either of these media, they were washed with same medium in which they were inoculated. The low nitrate medium (LNM), low phosphate medium (LPM) and low sulphate medium (LSM) contain 0·5 mM nitrate, 24 μM phosphate and 8 μM sulphate, respectively. Cells grown in minimal medium with glucose (MMG) containing 8·4 mM nitrate, 1·8 mM phosphate and 1 mM sulphate served as control. The cells were inoculated at an initial density of 0·5 mg/ml in each of these media. During the first 48 h the cells grown in any of the media: LNM, LPM, LSM or MMG, grew at the same rate. This ensured that within 48 h, cultures grown in different media for the same duration of time, had identical cell densities. For the nutrient deprivation experiments, the cultures were harvested at identical cell densities of the treated and the control (MMG). After collection, the cells were frozen, ground in liquid nitrogen and stored at – 80°C till further use.

2.2 *Extraction of DNA and polymerase chain reaction*

Chloronema cells of *Funaria* were harvested at a density of 2 mg/ml and genomic DNA was prepared according to Dellaporta *et al* (1984) and subjected to CTAB purification. The polymerase chain reaction (PCR) conditions were as follows: 1·5 mM $MgCl_2$, 0·2 mM dNTP, 100 pmole of each primer, 25 ng of *Funaria* genomic DNA, 2·5 U of Taq polymerase (Promega, Madison, USA)/100 μl reaction. The cycling conditions consisted of denaturation for 2 min at 94°C, followed by 45 cycles of denaturation for 45 s at 94°C, annealing at 54°C for 1 min and extension at 72°C for 1·5 min and then 12 min of extension at 72°C. The reactions were carried out using a thermocycler (Stratagene, USA). The sequences of the degenerate primers D1 and D2 were as follows:

(D1) 5′ GGIGTIATGCA(T/C)(C/A)GIGA(T/C)
(T/C)TIAA(A/G)CCIGA(A/G)AA 3′

(D2) 5′ GTIAT (A/G)AAICCIGAICC(A/G)TC(T/C)
TT(A/G)TC 3′

These were obtained from Bangalore Genei. The PCR products were cloned in the pGEM-T vector from Promega, USA.

2.3 *Southern hybridization*

The genomic DNA of *Funaria* was digested with the enzymes *Xho*I, *Hin*dIII and *Eco*RI (Promega, USA), fractionated on a 0·7% agarose gel and transferred to a nylon membrane (Amersham, UK) (Sambrook *et al* 1989). The 900 bp PCR product was radiolabelled with α-P^{32} dATP (BRIT, India) by the random primer labelling kit (Amersham) and used as a probe. Southern hybridization was carried out at 65°C in a solution containing 6 × SSC, 5 × Denhardt's solution, 0·5% SDS and 0·1 mg/ml of denatured salmon sperm DNA. The blot was washed in 0·1 × SSC and 0·1% SDS at 65°C for 15 min for the high stringency condition and in 0·5 × SSC and 0·1% SDS for 20 min at 55°C for the low stringency condition.

2.4 *Northern hybridization*

Total RNA was prepared from protonema grown under different nutrient levels using the RNeasy Plant minikit (Qiagen). The RNA was fractionated on a formaldehyde/agarose (1·2% agarose) gel and blotted onto a nylon membrane (Sambrook *et al* 1989). Hybridization was carried out at 42°C in a solution containing 6 × SSC, 5 × Denhardt's solution, 0·5% SDS, 50% formamide and 0·1 mg/ml of denatured salmon sperm DNA. The blot was washed in 2 × SSC, 0·1% SDS at 65°C for 20 min followed by a wash in 0·1 × SSC, 0·1% SDS at 65°C for 20 min. The Northern blots were scanned and the relative mRNA levels were estimated on the basis of signal intensities that were normalized with the values of the intensities of the 28S rRNA of the ethidium bromide stained gels. The analysis was done using the image analysis software Adobe photoshop, version 5·5.

2.5 *Genomic library construction and screening*

Genomic DNA of *Funaria* was partially digested with *Sau*3AI and then size fractionated by sucrose gradient centrifugation in order to select fragments in the size range of 15 to 23 kb. Subsequently the DNA was partially filled-in with dGTP and dATP using Klenow polymerase (Amersham) and ligated to the λGEM-11 vector (Promega). Plaques were screened by hybridization with the 900 bp PCR product as the probe (Sambrook *et al* 1989). Subclones of the inserts present in the positively hybridizing plaques were made in the pGEM7Zf(+) vector (Promega, USA).

2.6 *DNA sequencing*

The genomic DNA clones were sequenced by an automated DNA sequencer (model 377, Perkin Elmer) according to the manufacturer's instructions by Bangalore Genei.

3. Results

3.1 *PCR amplification of a fragment of a CDPK gene*

The degenerate primers D1 and D2 were designed against the conserved subdomains VIb and the third EF hand, corresponding to the sequences GVMHRDLKPEN and DKDGSGYIT, respectively. Using the above primers, three amplicons of approximate sizes 900, 800 and 600 bp, respectively, were obtained from the genomic DNA of *Funaria* of (figure 1). The present communication reports the analysis of only the 900 bp amplicon, referred to as P1. The annealing temperature of the polymerase chain reaction was varied from 44°C to 54°C and the P1 fragment could be amplified under all the conditions. Amplification of P1 under high stringency conditions of PCR, namely at high temperatures such as 54°C, suggests that P1 represents authentic amplification of a CDPK gene. The P1 insert was cloned in the pGEM-T vector and sequenced. The sequence of P1 confirmed that it was a CDPK.

3.2 *Genomic Southern analysis of* Funaria *CDPK*

Southern hybridization of genomic DNA digested with *Xho*I, *Hin*dIII and *Eco*RI with the P1 probe was per-

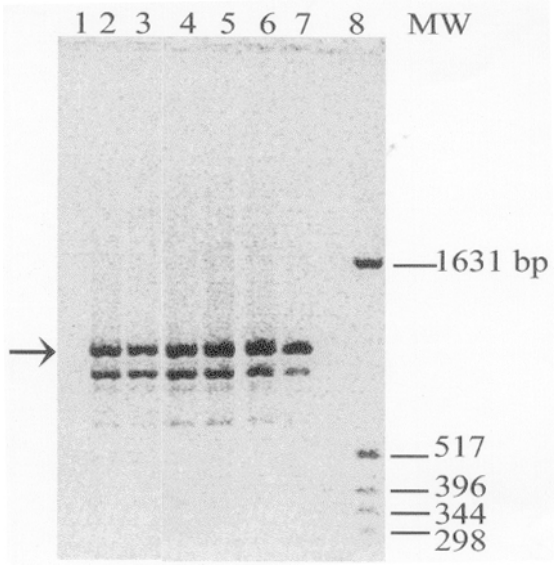

Figure 1. PCR amplification of genomic DNA of *Funaria* with primers D1 and D2. Lane 1: negative control, PCR done without genomic DNA, lanes 2, 3, 4, 5, 6, 7: PCR done at annealing temperatures of 44, 46, 48, 50, 52 and 54°C, respectively. Lane 8: molecular weight markers. The 900 bp fragment is marked by an arrow.

formed under low and high stringency conditions of hybridization (figure 2). As expected, under high stringency conditions single bands were observed in all three digests, which confirmed the presence of the CDPK gene in the *Funaria* genome. Multiple bands were observed in the digests under low stringency conditions indicating the presence of several CDPK-related sequences in the genome. This observation is consistent with reports of multigene families of CDPKs in other plants.

3.3 *Construction and screening of a genomic library of* Funaria

Genomic DNA of *Funaria* was partially digested with *Sau*3AI and subjected to size fractionation by sucrose density gradient centrifugation. DNA Fragments in the size range of 15 to 23 kb were selected and ligated to the *l*Gem-11 vector. Hence a genomic library of *Funaria* was constructed. In order to isolate the full-length gene, 250,000 plaques of this library were screened by hybridization with the radiolabelled P1 probe. Three positive clones were identified and purified to homogeneity. These clones were subjected to restriction analysis and hybridization to P1. The clone with the longest CDPK insert of 2·5 kb was subcloned using *Kpn*I into the vector pGEM7Zf(+) and sequenced. This led to the isolation of a 2·2 kb partial genomic clone of the *Funaria* CDPK.

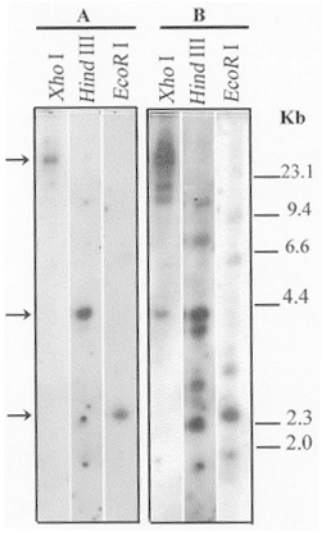

Figure 2. Southern blot analysis. 15 µg of genomic DNA of *Funaria* was digested with *Xho*I, *Hin*dIII and *Eco*RI, electrophoresed on a 0·7% agarose gel and transferred onto a nylon membrane. The membrane was probed with radiolabelled P1. The positions of the DNA molecular weight markers are indicated in kb. The blot was washed in 0·1 × SSC and 0·1% SDS at 65°C for 15 min for the high stringency condition (**A**) and in 0·5 × SSC and 0·1% SDS for 20 min at 55°C for the low stringency condition (**B**). The arrows indicate positions of single bands in the above three digests in A.

3.4 *Northern analysis of the* Funaria *CDPK*

The expression pattern of the *Funaria* CDPK transcript was examined by Northern analysis. Chloronema cells were grown in MMG and as expected, an increase in the duration of growth was accompanied by a rise in cell density of these cultures. Samples collected at different intervals of time were subjected to Northern analysis using the P1 probe. A 2·6 kb transcript was detected in all the lanes but interestingly the transcript levels showed a rise with an increase in the cell density (figure 5A). There was a 2·3-fold increase in the transcript level in the sample at the cell density of 22 mg/ml, compared to the level in the sample at the cell density of 2·6 mg/ml. Since increased cell density in these cultures leads to a reduction in the availability of nutrients, it is conceivable that the transcript level was responding to the nutritional status of the medium. In order to check this possibility, cultures were grown in media, that were deficient in either of the three important macro-nutrients nitrogen, phosphorus and sulphur, these media have been referred to as LNM, LPM and LSM, respectively. Consistent with our hypothesis, the transcript of the *Funaria* CDPK gene was found to be up-regulated when the cells were grown in LNM, LPM or LSM as compared to the MMG grown control cells (figure 5B, C). In LNM, the transcript was up-regulated after 24 h of treatment whereas in LSM and LPM the effect was observed after 48 h. After 48 h of growth in the LNM, the FhCDPK transcript was induced by 1·6-fold as compared to the level in MMG-grown cells. Growth for 48 h in the LSM and LPM caused a 1·9-fold and 2·2-fold rise in the transcript levels, respectively, in comparison with transcript levels in cells grown in MMG.

4. Discussion

Since CDPKs are highly conserved across species, a PCR based approach was adopted to clone the gene. The use of degenerate PCR primers designed against sequences in the kinase domain and in the calcium binding region ensured the amplification of only CDPKs. A 900 bp fragment of a CDPK gene was amplified from the genomic DNA of *Funaria*. This fragment (P1) was subsequently used to obtain the 2·2 kb partial clone from the genomic library of *Funaria*.

This genomic clone encodes an ORF of 518 amino acids. The entire ORF was found not to be interrupted by introns, unlike the CDPK genes of isoforms 6, 9 and 19 in *Arabidopsis* or the maize CDPK gene which contain several introns. This gene has a 3′ untranslated region of 624 bp

followed by a putative polyadenylation sequence, AATAAT. The ORF has all the features characteristic of CDPKs. It encodes 39 amino acids of the N-terminal variable region followed by the 11 kinase subdomains in the catalytic region and the four EF hands in the regulatory region. The autoinhibitory region consists of 30 amino acids in contrast to the usual number of 31 in other CDPKs. The significance of this observation is not understood.

The deduced amino acid sequence of the *Funaria* CDPK shows extensive homology with other CDPKs namely, 73% identity with the *Fragaria* CDPK and 71% identity with CDPK isoform 7 of *Arabidopsis* (figure 3). It shows greater homology to the higher plant CDPKs

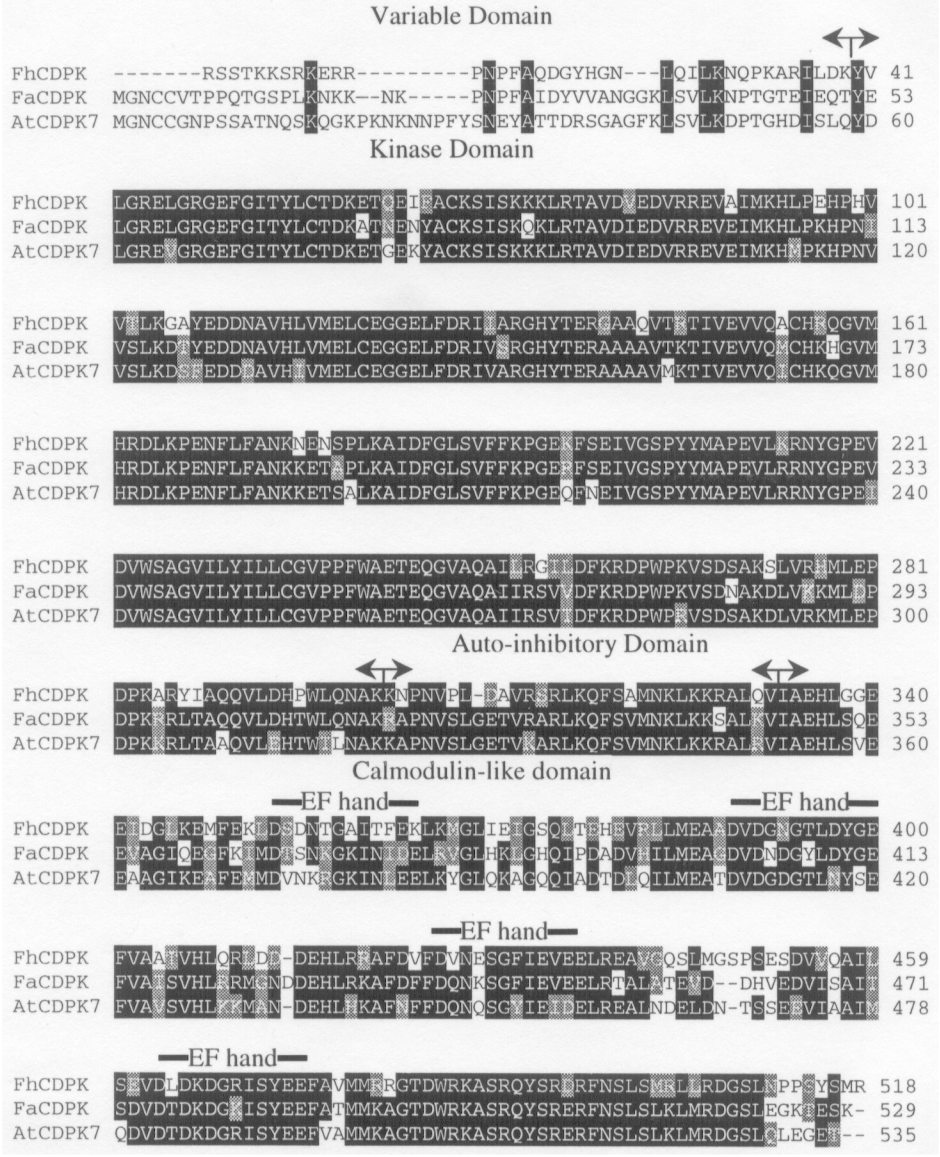

Figure 3. Comparison of the deduced amino acid sequences of CDPKs from *Funaria hygrometrica* (FhCDPK, GenBank Acc. No. AF276999); *Fragaria annanasa* (FaCDPK, GenBank Acc. No. AAB88537) and the isoform 7 of *Arabidopsis thaliana* (AtCDPK7, GenBank Acc. No. AAB03247). The alignment was done using Clustal W version 1·8 (Thompson *et al* 1994) and coloured with BoxShade. The boundaries of the variable, kinase and the autoinhibitory domains are demarcated by arrows. The EF hand motifs in the Calmodulin like domain are marked by lines. Dashes represent gaps introduced to maximize the alignment. Black and gray shaded backgrounds indicate amino acid residues that are identical or conservative changes, respectively, in the three sequences.

than to the green alga *Chlamydomonas* (50% identity, GenBank S57488), or the liverwort, *Marchantia* (64% identity; GenBank BAA81749) or the moss, *Tortula* (59% identity; GenBank AAB70706) enzymes. The kinase and the autoinhibitory regions in the protein encoded by the *Funaria* CDPK show 84% and 73% identity with the respective regions in the *Fragaria* enzyme, while the sequence identity in the calmodulin like domain is only 60% between the two CDPKs. A comparison of the deduced amino acid sequences of the kinase domains of the *Funaria* CDPK with various members of the CDPK superfamily and a few other kinases is presented in figure 4. It is clear from the dendrogram that the *Funaria* CDPK shows maximum homology to the CDPKs from the angiosperms such as strawberry and *Arabidopsis*. Studies by Knight *et al* (1995) have shown that the stress response pathway is well conserved between the moss *Physcomitrella* and the cereal wheat. These two lines of evidence strongly suggest that the signaling mechanisms are well conserved between the mosses *Funaria* and *Physcomitrella*

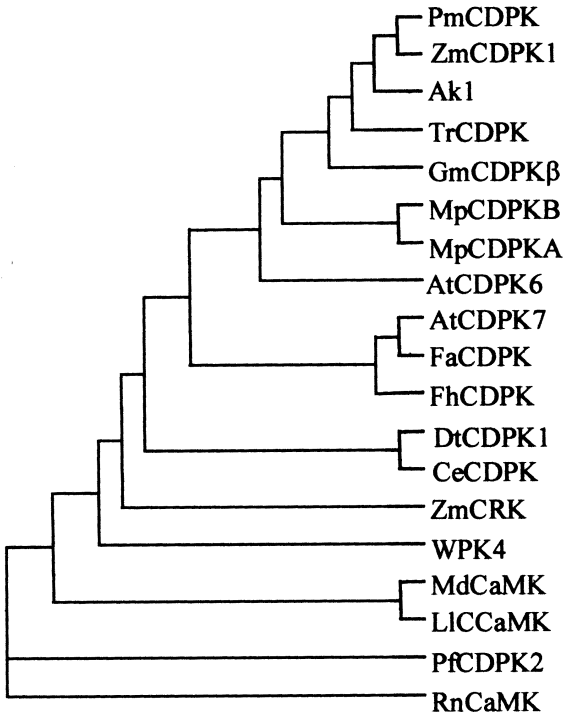

Figure 4. Dendrogram showing the phylogenetic relationships between the *Funaria* CDPK and other CDPKs, calmodulin dependent kinases (CaMK), calcium calmodulin dependent kinases (CCaMK), CDPK related kinases (CRK) and wpk4. The deduced amino acid sequence of the kinase domains of these molecules were compared and aligned using Clustal W and the dendrogram was generated on the basis of the alignment using the TreeView software (Page 1996). The kinases shown in the dendrogram are as follows: *Picea mariana* CDPK (PmCDPK), *Zea mays* CDPK1 (ZMCDPK1), *Arabidopsis thaliana* CDPK (AK1), *Tortula ruralis* CDPK (TrCDPK), *Glycine max* CDPKβ (GmCDPKβ), *Marchantia polymorpha* CDPK-B (MpCDPKB), *Marchantia polymorpha* CDPK-A (MpCDPKA), *Arabidopsis thaliana* CDPK6 (AtCDPK6), *Arabidopsis thaliana* CDPK 7 (AtCDPK7), *Fragaria ananassa* CDPK (FaCDPK), *Funaria hygrometrica* CDPK (FhCDPK), *Dunaliella tertiolecta* CDPK1 (DtCDPK1), *Chlamydomonas eugametos* CDPK (CeCDPK), *Zea mays* CRK (ZmCRK), *Malus domesticus* CaMK (MdCaMK), *Lilium longiflorum* CCaMK (LlCCaMK), *Plasmodium falciparum* CDPK2 (PfCDPK2), *Rattus norvegicus* CaMK (RnCaMK).

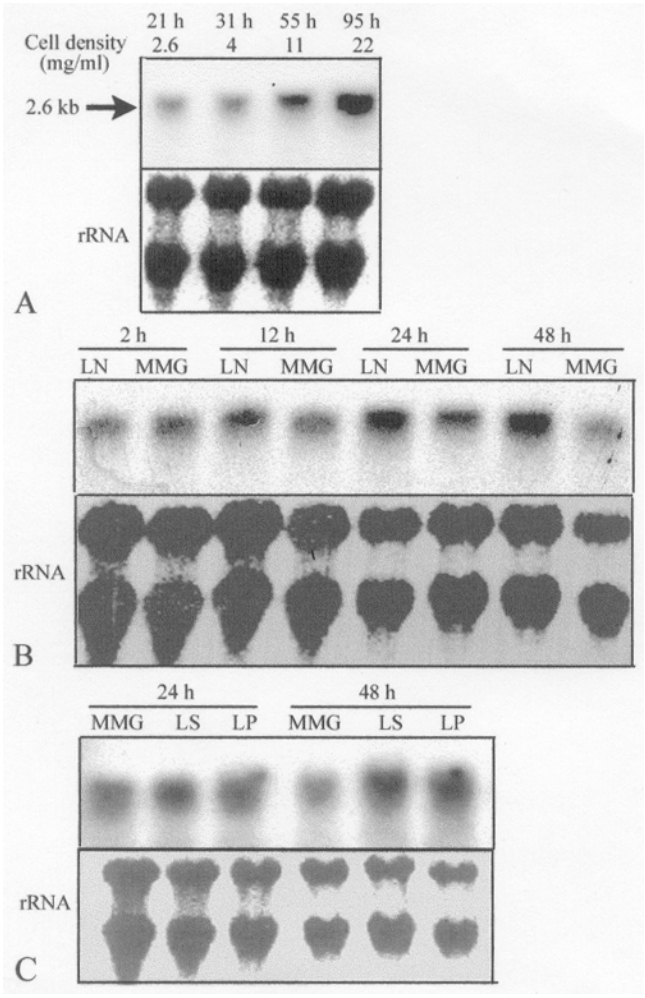

Figure 5. Expression of *Funaria* CDPK mRNA. About 45 µg of total RNA was electrophoresed on a 1·2% agarose gel containing formaldehyde and transferred onto a nylon membrane. (**A**) The cultures were collected after 21, 31, 55 and 95 h of growth in MMG when the cell densities were 2·6, 4, 11 and 22 mg/ml, respectively. (**B**) The cultures were grown in LN and MMG media for 2, 12, 24 and 48 h and the cell densities were 0·6, 1·2, 1·7 and 3·5 g/ml, respectively. (**C**) The cultures were grown in LP, LS and MMG media for 24 and 48 h and the cell densities were 1·7 and 3·6 mg/ml, respectively. The blots were probed with P1. Ethidium bromide staining of rRNA are presented to demonstrate equal loading in all lanes. The 2·6 kb transcript of the *Funaria* CDPK is marked by an arrow.

and higher plants like *Arabidopsis* and wheat. So it would be interesting to study if CDPK isoforms from *Arabidopsis*, particularly AtCDPK7, respond to starvation. Mosses provide the unique opportunity of a morphologically simple system, which seems to be endowed with molecular mechanisms matching the complexity of those in angiosperms. Our findings further highlight the importance of mosses as model systems to study signaling pathways.

Interestingly, the *Funaria* CDPK has a lysine (at overall position 364, figure 3) instead of the very well conserved glutamic acid at position 12 of the calcium binding loop in the first EF hand. In the *Plasmodium* CDPK, an E to K transition at an identical position in the first EF hand has been shown to drastically reduce the calcium affinity and kinase activity of the enzyme (Zhao *et al* 1994). As the consequences of such changes in the EF hands can not be extrapolated from one system to another (Harmon *et al* 2000), the consequence of this change on the calcium binding property of the *Funaria* CDPK remains to be understood.

Northern analysis indicates that the expression of the *Funaria* CDPK transcript is up-regulated by nutrient deprivation. The only other kinase, which is induced by nutrient starvation in plants, is the wpk 4 (wheat protein kinase 4) from wheat, which belongs to the SnF-1 related kinase 3 (SnRK3) family. The transcript of wpk4 is also induced by light and cytokinin (Sano and Youssefian 1994). The *Funaria* CDPK was found to respond differently and the transcript levels were unaffected by darkness and cytokinin (data not shown). Recent work of Ikeda *et al* (1999) has shown that wpk4 can phosphorylate and thereby inactivate 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR). HMGR catalyses a key step in the isoprenoid biosynthetic pathway. Nutritional starvation is known to lead to a down-regulation of anabolic processes.

The delay in the up-regulation (24 to 48 h) of the FhCDPK transcript levels suggest that it is more likely to be associated with the acclimation response rather than the starvation sensing mechanism. Similar to the role of wpk4 in inactivation of HMGR, it is plausible that the FhCDPK also plays a role in down-regulating anabolic pathways in the acclimation response to nutrient starvation. In this context, it may be pointed out that the spinach CDPK has been shown to inactivate NR (nitrate reductase) by phosphorylation under *in vitro* conditions (Douglas *et al* 1998). Calcium dependent phosphorylation and subsequent inactivation of the spinach sucrose phosphate synthase (SPS) has also been demonstrated (McMichael *et al* 1995). NR and SPS catalyse key steps in the nitrogen assimilation and sucrose synthesis pathways, respectively. Against the backdrop of these observations, we would like to propose that the *Funaria* CDPK could be involved in the down regulation of NR and SPS during starvation conditions.

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