Journal of Experimental Botany, Vol. 52, No. 355, pp. 215–221, February 2001

Journal of Experimental Botany

Auxin and heat shock activation of a novel member of the calmodulin like domain protein kinase gene family in cultured alfalfa cells

Sholpan Davletova[1], Tamás Mészáros[1], Pál Miskolczi[1], Attila Oberschall[1,2], Katalin Török[1], Zoltán Magyar[1], Dénes Dudits[1,3] and Mária Deák[1,2]

[1] *Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences H-6701 Szeged, POB 521, Hungary*

[2] *BAY ZOLTAN Foundation for Applied Research, Institute for Biotechnology H-6701 Szeged, POB 2337, Hungary*

Received 12 June 2000; Accepted 5 September 2000

Abstract

A calmodulin like domain protein kinase (CPK) homologue was identified in alfalfa and termed *MsCPK3*. The full-length sequence of cDNA encoded a 535 amino acid polypeptide with a molecular weight of 60.2 kDa. The deduced amino acid sequence showed all the conserved motifs that define other members of this kinase family, such as serine-threonine kinase domain, a junction region and four potential Ca^{2+}-binding EF sites. The recombinant *MsCPK3* protein purified from *E. coli* was activated by Ca^{2+} and inhibited by calmodulin antagonist (W-7) in *in vitro* phosphorylation assays. The expression of *MsCPK3* gene increased in the early phase of the 2,4-D induced alfalfa somatic embryogenesis. Heat shock also activated this gene while kinetin, ABA and NaCl treatment did not result in *MsCPK3* mRNA accumulation. The data presented suggest that the new alfalfa CPK differs in stress responses from the previously described homologues and in its potential involvement in hormone and stress-activated reprogramming of developmental pathways during somatic embryogenesis.

Key words: *Medicago sativa*, CPK, stress, 2,4-D, phosphorylation, somatic embryogenesis.

Introduction

Formation of embryos from somatic plant cells is a unique, plant-specific developmental pathway that also provides an efficient way for plant regeneration in *in vitro* cultures. The molecular bases of this metabolic and developmental switch are poorly understood despite extensive research with different tissue culture systems (Sharma and Thorpe, 1995). The large body of previous tissue culture experiments emphasized the pivotal role of auxin-activated cell division in induction of an embryogenic state in cultured somatic cells (reviewed by Dudits *et al*., 1991). A growing number of experimental observations demonstrated the involvement of stress responses during somatic embryogenesis (Dudits *et al*., 1995), like activation of the heat shock system in alfalfa (Györgyey *et al*., 1991). Interestingly, several abscisic acid activated genes such as annexin, ferritin and aldose reductase were identified in a cDNA library from alfalfa cells exposed to embryogenic induction (Deák *et al*., 1995).

Both auxin and abscisic acid signalling pathways are dependent on Ca^{2+}-mediated events (Saunders, 1990; reviewed by Poovaiah and Reddy, 1993). Therefore, it is postulated that the Ca^{2+}-regulated kinase genes may also respond to hormonal and stress signals during embryogenic induction. In particular, the class of CAM-like domain protein kinases (CPKs) may be considered as potential components in these activation events. This unique class of plant specific kinases are encoded by a multigene family (reviewed by Roberts and Harmon,

[3] To whom correspondence should be addressed. Fax: +36 62 433 434. E-mail: Dudits@nucleus.szbk.u-szeged.hu
EMBL/GenBank Data Library accession number: X96723.

1992; Roberts, 1993; Harmon *et al*., 2000). So far 29 members have been found in *Arabidopsis* but the estimated number is 40 in this single species (Hong *et al*., 1996; Hrabak *et al*., 1996; Harmon *et al*., 2000). In addition to plants, CPK genes have also been cloned from protozoa (Zhao *et al*., 1993). Notably, the CPK genes are absent from the yeast and nematode genomes.

A number of putative endogenous CPKs substrates have been identified by biochemical approaches that suggest regulatory roles in gene expression, metabolism, cytoskeleton dynamics, and extracellular plant signalling (Harmon *et al*., 2000). The plant CPKs have been associated with plasma membrane (Schaller *et al*., 1992), cytoskeleton (Putman-Evans *et al*., 1989) and chromatin (Roberts and Harmon, 1992). The multiple subcellular localization also suggests that CPKs have diverse functions. Despite the cloning and characterization of many CPK genes, the function of these proteins is still largely a mystery. The crucial role of the CPK in signal transduction pathways controlling cell growth and germination has been shown in maize pollen by inhibition of the CPK with calmodulin antagonists and antisense oligonucleotides (Estruch *et al*., 1994). Involvement of CPKs in stress signal transduction has been concluded from studies on a constitutively active mutant of ATCDPK1 gene. This mutation activated the stress-inducible HVA1 promoter, while mutation in the kinase domain abolished this effect. These findings indicated the importance of the ATCDPK1 in transmission of stress signals (Sheen, 1996). Different environmental stresses have been reported to affect the activity of CPK genes. The mRNA level of two *Arabidopsis* CPK genes increased in plants subjected to salt or drought stresses (Urao *et al*., 1994). In mung bean, mechanical strain, salt, cycloheximide, and auxin treatment could induce the CPK gene expression (Botella *et al*., 1996). NtCDPK1 mRNA accumulation was stimulated by Ca^{2+}, wounding, auxin, cytokinin, gibberellin, and ABA (Yoon *et al*., 1999).

In this paper the cloning of an alfalfa cDNA that encodes a novel member of the CAM-like protein kinase family is reported. The Ca^{2+}-dependent activity and calmodulin inhibitor sensitivity of the kinase is demonstrated by using the recombinant enzyme expressed in bacteria. As shown by Northern hybridization data the corresponding gene is activated by a high concentration of auxin (2,4-D) or heat treatment. The data presented outline new aspects of molecular mechanisms of somatic embryogenesis that are equally based on auxin-and stress-related Ca^{2+}-signalling pathways.

Materials and methods

Cloning of alfalfa CPK cDNA homologues

RNA prepared from *Medicago sativa* RA3 microcallus suspension (MCS), treated with 10 mg l^{-1} 2,4-D for embryo induction, was used to construct a cDNA library in λ-ZAP II vector (STRATAGENE). Another cDNA library prepared from *M. sativa* RA3 somatic embryos was kindly provided by Dr H Hirt (Vienna). Both libraries were screened with a partial carrot CPK cDNA (kindly provided by Dr JH Choi; Suen and Choi, 1991) and several clones were isolated. The longest cDNA clone containing a full length CPK gene with an open reading frame of 1841 nucleotide, was analysed. The nucleotide sequence of the cDNA was determined by the dideoxynucleotide-chain-termination method (Sanger *et al*., 1977) as double stranded DNA using the Sequenase kit (US Biochemicals). DNA sequence analysis was carried out using PCGENE. DataBank searching was performed by using the BLAST system (Altschul *et al*., 1990).

Other cDNA clones used in the present studies

To obtain a probe for an alfalfa calmodulin, PCR primers were synthesized based on the published sequence (Barnett and Long, 1990) and the EcoRI-NsiI region of the cDNA between nucleotides 250–747 was amplified. The resulting PCR fragment was cloned into the EcoRI-NsiI site of pGEM-7 vector (Promega) and its identity was confirmed by sequencing. The cDNA clone of an alfalfa small heat shock *Mshsp18-1* gene was kindly provided by Dr J Györgyey (Györgyey *et al*., 1991). The soybean β-tubulin gene was kindly provided by Dr E Lam (Rutgers University, NJ) and histone H3 cDNA (Kapros *et al*., 1991).

Plant material, 2,4-D and stress treatments

Medicago sativa RA3 MCS was maintained on SH medium (Savouré *et al*., 1995) in the presence of 1 mg l^{-1} α-naphthalene acetic acid and 0.2 mg l^{-1} kinetin. Embryogenic 2,4-D treatment was applied as described earlier (Dudits *et al*., 1991; Györgyey *et al*., 1991). After 2 d of subculture 300 ml of liquid cell suspensions were exposed to 10 mg ml^{-1} 2,4-D treatment and after 1 h the cells were washed twice and transferred into hormone-free medium. Samples were taken from the same culture for 3 d and subsequently the microcalli were plated on hormone-free agar medium where somatic embryos developed within 3–4 weeks.

Heat shock treatment was applied by distributing the same *Medicago sativa* RA3 liquid culture at the beginning of the experiment into 100 ml flasks. 15 ml cell suspension was diluted with 15 ml 45 °C media and cultured further at 37 °C for 30 min. Control material was diluted with the same medium at room temperature. At least two independent hormone and heat induction experiments were carried out for gene expression analysis.

RNA preparation and Northern analysis

The total RNA was extracted according to the freezing phenol method (Maes and Messens, 1992) with slight modification to scale it down to the volume of Eppendorf tubes. Frozen tissue of 0.1–0.3 g was homogenized in liquid nitrogen in a small mortar with 0.5 ml of phenol (equilibrated to pH 4.9 with 3 M K-acetate). After diluting with 0.5 ml 1% SDS the samples were incubated for 15 min at 65 °C, then centrifuged for 10 min in an Eppendorf centrifuge at 4 °C. The supernatant was extracted with phenol/chloroform (1:1, v:v) twice, and precipitated with ¼ volume of 8 M LiCl overnight at 4 °C. The precipitates were dissolved in 100 µl ribonuclease free water, and a second LiCl precipitation was applied for safe elimination of DNA

contamination. 20 μg total RNA was run on 1% formaldehyde gel, containing 0.01% EtBr. Transfer of RNAs to Amersham Hybond N filters was carried out with the classic upward technique, and the ribosomal RNA pattern of the filters were examined under UV to verify the efficiency of transfer, and the quality of loaded RNA.

Radioactive labelling and hybridization was carried out according to standard protocols supplied by the company (Amersham).

Cloning of MsCPK3 *into bacterial expression vectors and purification of the recombinant protein*

The cDNA coding for the full length *MsCPK3* protein was cloned into pFLAG-ATS vector (IBI) in order to tag the N-terminal region of the protein with the eight amino acid FLAG antigen determinant. First, the 5′ non-coding region of the cDNA containing three stop codons was eliminated using a specific PCR oligonucleotide. The oligonucleotide synthesized corresponded to 17 nucleotides of the *MsCPK3* sequence beginning from the first methionine, carrying an additional six nucleotides, creating the XhoI site (5′-CCCTCGAGATGGG-GTGTTTGTTAAGTAA-3′). The second oligonucleotide used for the PCR reaction corresponded to the *MsCPK3* sequence from nucleotide 486–506 (5′-TTGATAAGCTCCTTTAAA-TTCAACAATATT-3′), covering the internal DraI site. The 0.46 kb PCR product was digested with the XhoI-DraI and ligated with the second 1.37 kb DraI-KpnI fragment of the cDNA in one step into the XhoI-KpnI site of pFLAG-ATS vector. The DNA sequence of the created pFLAG-*MsCPK3* construct was verified and contained the full-length cDNA without 5′- non-coding sequence. The purification of the FLAG tagged protein was performed from a crude extract of *E. coli* and was not efficient as most of the fusion product was recovered in inclusion bodies.

To obtain the purified protein in the soluble fraction, the GEX expression system (Pharmacia) was used. The *MsCPK3* cDNA with the attached FLAG tagging was amplified by PCR from the pFLAG-*MsCPK3* plasmid. One of the oligonucleotides corresponded to the FLAG sequence, carrying an extra BamHI site: 5′-CGGATCCATGGACTACAAGGACGAGGA-3′, while the other oligonucleotide corresponded to the C-24 region in the pFLAG-ATS vector. The resulting PCR product was treated with the Klenow fragment, then digested with the BamHI and was cloned into the BamHI-SmaI sites of pGEX-4T1 vector. The identity of the construct was confirmed by sequencing. The purification of the recombinant protein from bacteria by GST column was carried out according to the Pharmacia manual. The FLAG-*MsCPK3* protein was detected by Western analysis using anti-FLAG M1 and M2 monoclonal antibodies according to the manufacturer's protocol (IBI).

Kinase assay

Protein kinase activity was determined by *in vitro* detecting the incorporation ^{32}P from ($\gamma^{32}P$)ATP. The standard assay mixture (30 μl) for the *in vitro* phosphorylation assay contained 25 mM TRIS (pH 7.5), 10 mM $MgCl_2$, 50 mM NaF, 0.5 mM $CaCl_2$, 1 mg ml^{-1} histone type III-S (Sigma) or dephosphorylated β-casein (Sigma) as substrates, 10 μM ATP, 5 μCi ($\gamma^{32}P$) ATP, and 0.4 μg purified GST-*MsCPK3* fusion protein. The Ca^{2+}-free mixture contained 5 mM EGTA instead of $CaCl_2$. The effect of calmodulin inhibitor was studied by addition of 10 μM, 100 μM and 500 μM of W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide) to the reaction mixtures. The kinase reactions were started by the addition of CPK and stopped after 30 min at room temperature with 7.5 μl 5×SDS sample buffer. The samples were analysed by 10% SDS-PAGE and autoradiography.

Results

Cloning and sequence analysis of an alfalfa CPK cDNA

The alfalfa cDNA library prepared from the RNA of 2,4-D treated *Medicago sativa* RA3 microcallus suspension was screened with the carrot CPK probe (kindly provided by Dr Choi). The longest cDNA clone, named *MsCPK3*, is composed of 1841 nucleotides with an open reading frame that corresponded to a 535 amino acid long polypeptide (Fig. 1). Sequence analysis showed that the deduced polypeptide carried a serine-threonine kinase domain, a regulatory junction domain and four Ca^{2+}-binding EF-hands. Comparison with other sequences available in GenBank revealed that the most related genes are soybean *GmCDPKγ* (Lee *et al*., 1997), tobacco *NtCDPK1* (Yoon *et al*., 1999), *OsCPK2* from rice (Breviario *et al*., 1995), *Arabidopsis AtCDPK9*, and maize *ZmCDPK9*. Like several other members of CPK family, at the *N*-terminus *MsCPK3* contains the motif MGxxxS that defines a consensus sequence for a potential *N*-myristoylation site. The first 80 amino acids of the *N*-terminal region of *MsCPK3* share no homology with other sequences.

Purification of the recombinant protein and the in vitro *kinase assays*

To study the characteristics of *MsCPK3*, the FLAG-tagged *MsCPK3* protein was expressed in bacterial cells. Altough the presence of the proper FLAG-*MsCPK3* protein of approximately 60.2 kDa could be detected by immunoblot analysis (data not shown), the great majority of the recombinant protein formed inclusion bodies.

To obtain sufficient purified protein for biochemical characterization, the GST expression system was also applied. The p*GST-MsCPK3* plasmid construct contained the glutathione *S*-transferase sequence joined to the 5′ coding region of FLAG tagged *MsCPK3*. The gluthatione agarose affinity purified recombinant protein was analysed by SDS-PAGE and the 86 kDa size of the purified protein corresponded to molecular weight of GST (26 kDa) and FLAG-*MsCPK3* (60.2 kDa). Immunodetection with anti-FLAG antibodies confirmed that the protein obtained is the recombinant *GST-MsCPK3* (Fig. 2).

Substrate specificity of the CPK was tested by using histone and casein as substrates. Histone showed a higher phosphorylation level and was used for further experiments. Kinase activity of the recombinant protein was induced *in vitro* by Ca^{2+} (Fig. 3A). A CAM antagonist,

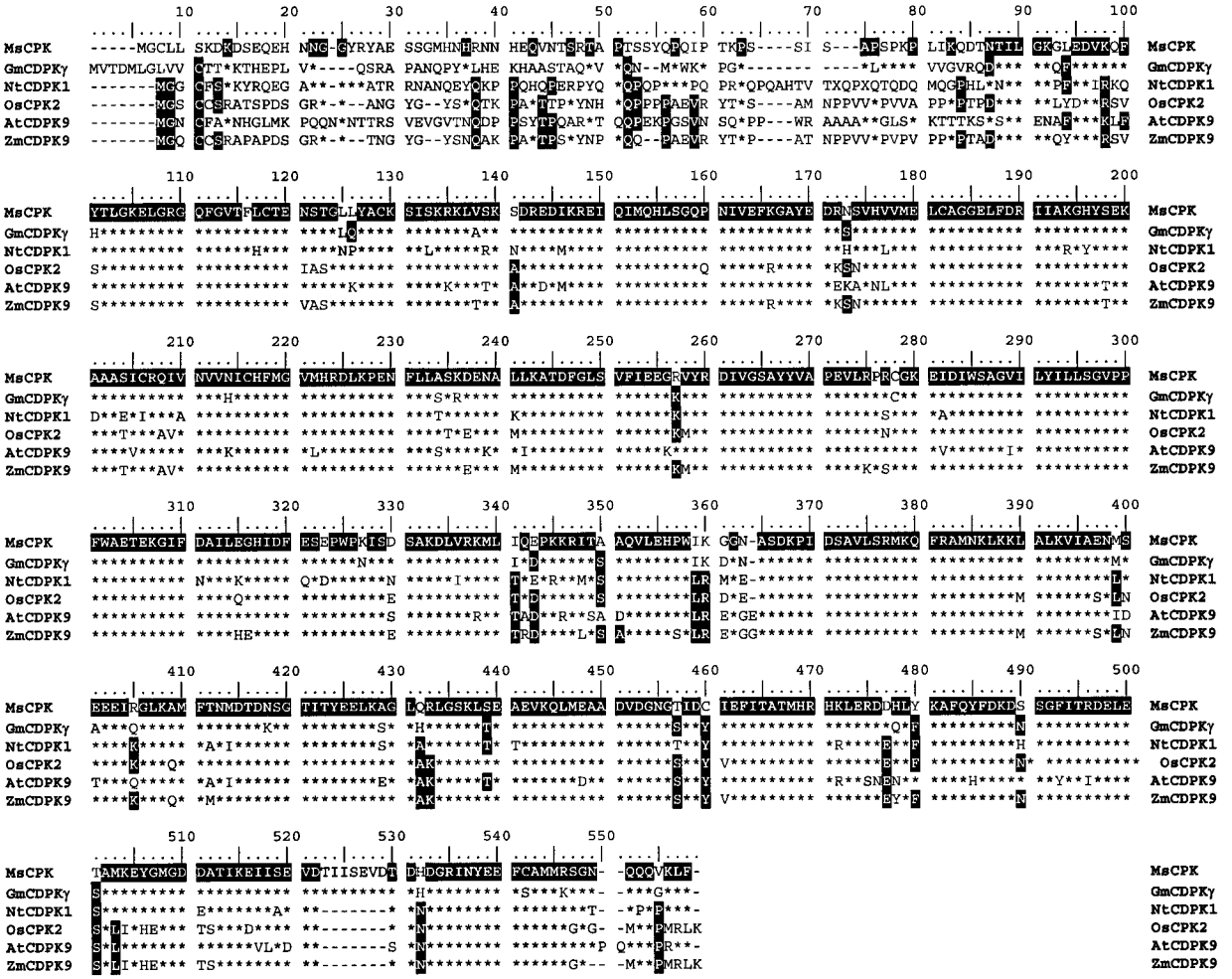

Fig. 1. Alignment of the predicted amino acid sequences of *MsCPK3* with the *GmCDPKγ* (Lee *et al*., 1997), tobacco *NtCDPK1* (Yoon *et al*., 1999), *OsCPK2* from rice (Breviario *et al*., 1995), *Arabidopsis AtCDPK9*, and maize *ZmCDPK9*. To obtain maximal similarity, gaps were introduced, represented by dashes. The same amino acids are marked by asterisks and black boxes indicate positions at which at least half of the sequences are identical.

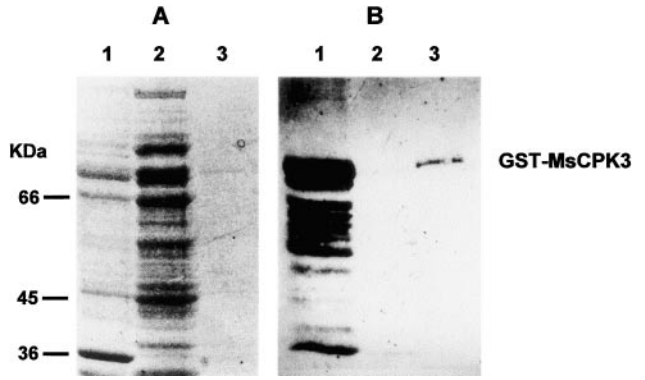

Fig. 2. Purification of *GST-FLAG-MsCPK3* protein from bacteria after overexpression of *MsCPK3* cDNA with pGEX system. The *MsCPK3* cDNA was expressed in *E. coli* as a fusion protein and purified by affinity chromatography as described. Proteins were separated by SDS-PAGE. (A) Staining of proteins with Coomassie blue after PAGE. (B) Immunodetection of the recombinant protein with anti-FLAG antibodies. Lanes: 1, protein in inclusion bodies; 2, proteins in the soluble fraction; 3, GST-bound proteins.

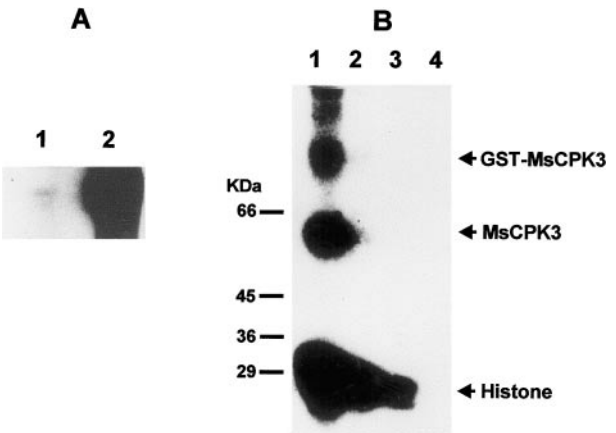

Fig. 3. Effect of Ca^{2+} and calmodulin inhibitors on the histone type III-S kinase and autophosphorylation activity of the recombinant *MsCPK3* expressed in *E. coli*. The kinase assays were performed as described in Materials and methods. (A) Lanes: 1, 5 mM EGTA; 2, 0.5 mM $CaCl_2$. (B) Lanes: 1, Control; 2, 10 μM W-7; 3, 100 μM W-7; 4, 500 μM W-7.

W-7, was applied at concentrations from 10 μM to 500 μM in the *in vitro* phosphorylation assays. Both the phosphorylation of the histone and autophosphorylation were inhibited in the presence of this compound (Fig. 3B). These results showed that the analysed CPK homologue exhibited a Ca^{2+}-dependent kinase activity and it could be inhibited by calmodulin antagonist W-7.

Treatment of alfalfa callus cells with high concentration of 2,4-D or heat shock caused a transient MsCPK3 *transcript accumulation*

For characterization of the expression pattern of *MsCPK3* gene during somatic embryogenesis, RA3 microcallus suspensions of *Medicago sativa were* treated with 10 mg l^{-1} 2,4-D for 1 h. RNA samples were collected at 30 min, 1, 2, 4, 8, 24, 48, and 72 h after embryogenic induction (Fig. 4A). The steady-state mRNA level of *MsCPK3* was analysed using the 5′ region of the *MsCPK3*cDNA as a gene-specific probe. Considerable *MsCPK3*mRNA accumulated when the cells were exposed to auxin treatment. This prompt response declined after 4 h of auxin shock in hormone-free medium. The expression of a small heat shock protein *Mshsp18-1* gene, was also induced, though the peak in the mRNA level appeared later, in samples from the 4 h and 8 h cultures. The level of calmodulin mRNA was almost constitutive during the analysed period.

To investigate the effect of other environmental stresses on *MsCPK3* mRNA accumulation, heat shock was applied for 30 min at 37 °C. As shown in (Fig. 2B), this treatment could also transiently activate the expression of the *MsCPK3* gene, with an expression peak at 30 min after induction. As expected, the small heat shock protein gene responded immediately to the treatment. The calmodulin mRNA was slightly decreased by the heat shock during the first 2 h, but then it was restored.

Other treatments of alfalfa MCS such as application of a lower amount of 2,4-D (1 mg ml^{-1}), kinetin (1 mg ml^{-1}), abscisic acid (150 μM) or salt (100 mM NaCl) did not significantly affect the expression of the *MsCPK3* gene (data not shown).

Discussion

In this study cDNA libraries constructed from somatic embryos and 2,4-D treated microcallus suspension were screened and a full-length clone of putative alfalfa CPK, designated *MsCPK3* was isolated. Although partial sequences of two alfalfa CPKs have been reported (Monroy and Dhindsa, 1995) the *MsCPK3* is the first full length CPK representative from this species. The deduced amino acid sequence of *MsCPK3* depicts a protein with a typical structure of CPKs. The comparison with other sequences available in GenBank (Fig. 1) showed very strong homology to several plant CPK proteins. These data are in agreement with previous findings that certain CPK variants in monocots and dicots are more closely related than the different members of the gene family within the same species. The amino acid sequences of these CPKs are almost the same in the kinase, regulatory and EF-hands domains while the N terminal region shares no homology.

Previous reports showed that many CPKs are associated with membranes (Schaller *et al*., 1992). A possible mechanism for targeting the proteins to the membranes is the *N*-myristoylation. Recently, the *in vitro* myristoylation of zucchini CPK was demonstrated (Ellard-Ivey *et al*., 1999). The presence of the *N*-terminal MGxxxS *MsCPK3* motif defines a possible *N*-myristoylation site, suggesting that *MsCPK3* may be targeted to a membrane compartment.

In attempts to prove the Ca^{2+} dependency of the isolated alfalfa kinase a recombinant protein was purified. The *GST-MsCPK3* expressed in *E. coli* required Ca^{2+} for its activity *in vitro*. The data presented suggest that *MsCPK3* can function as Ca^{2+}-dependent kinase *in vivo* as well. The calmodulin antagonist W-7 is widely used to study the role of calmodulin in different signalling pathways (Hidaka *et al*., 1981; Lam *et al*., 1989). Sensitivity of *MsCPK3* to this inhibitor indicates that calmodulin homologue Ca^{2+}-binding motifs are required for the kinase activity. These results further support that *MsCPK3* can be an active component of Ca^{2+}-dependent signal transduction in alfalfa. Similarly to previously

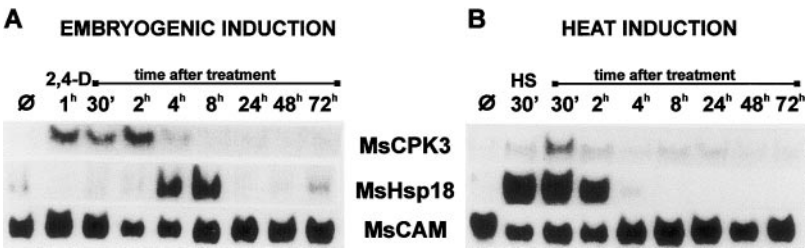

Fig. 4. Effect of high concentrations of 2,4-D and heat shock treatments on the expression of *MsCPK3* gene in *Medicago sativa* RA3 cell suspension. (A) 10 mg l^{-1} 2,4-D was applied for 1 h to the liquid microcallus suspension, then after three washes the cells were transferred into hormone-free medium. (B) Cell suspensions were treated at 37 °C for 30 min, then transferred to room temperature. Samples were taken 30 min, 2, 4, 8, 24, 48, and 72 h after the heat treatment. Control material was diluted with room temperature medium. The same filter was hybridized sequentially with labelled cDNAs encoding *MsCPK3*, Mshsp18-1 and MsCAM.

studied CPK from alfalfa, this recombinant kinase exhibited autophosphorylation activity (Bögre *et al*., 1988). This function might contribute to the amplification of the signal.

Changes in gene expression frequently reflect the biological function of the genes in different processes. In this work the expression of *MsCPK3* was induced by high concentration of 2,4-D treatment that was previously shown to induce somatic embryogenesis (Dudits *et al*., 1991). The kinetics of *MsCPK3* transcript accumulation showed a rapid and transient response that was frequently observed with stress-related genes. Indeed the high concentration of this synthetic auxin can also activate the expression of the classic small heat shock protein *Mshsp18-1*. The overall alarm of stress responses was further shown by the synthesis of mRNA from the heat shock transcription factor gene (HSF) in the same cells. The HSF transcript level followed a similar pattern to *MsCPK3* mRNA accumulation (D Dudits *et al*., unpublished results). Application of a lower amount of 2,4-D (1 mg l^{-1}), which still efficiently promoted cell division in callus tissues, but does not induce organized embryogenic structures in RA3 culture, did not cause a significant increase in the *MsCPK3* expression (data not shown). This suggests that the mRNA peak of *MsCPK3* observed during embryogenic 2,4-D induction may primarily be related to the stress effects of this compound. Induction of *Mshsp18-1* and an alfalfa heat shock transcription factor support the observation that various stresses might have significance during the early phase of embryogenic induction in *Medicago sativa* cells. However, 2,4-D induced somatic embryogenesis accompanied by increasing division activity of the cells, so the possibility can not be ruled out that the reactivation of cell cycle by 2,4-D can also be linked to the mRNA increase of *MsCPK3*. The relation between calcium signalling and cell division as well the potential role of CPKs have been discussed previously (Dudits *et al*., 1998).

Many CPK genes have been reported to be activated in response to environmental stresses. Thus, the alfalfa MSCK1 mRNA level increased during cold acclimation (Monroy and Dhindsa, 1995). Rice *OsCPK2* was shown to be regulated by light and anoxic treatment (Breviario *et al*., 1995; Frattini *et al*., 1999), while *VrCDPK1* was induced by mechanical strain, IAA, salt and cycloheximide treatment (Botella *et al*., 1996). Maize *ZmCDPK1* expression was induced by low-temperature stress and cycloheximide (Berberich and Kusano, 1997). Activation by cycloheximide has been shown earlier for several CPKs involved in signal transduction pathways (Mahadevan and Edwards, 1991). Two *Arabidopsis* CPKs, *ATCDPK1* and *ATCDPK2*, were induced in response to low temperature, salt and drought stress (Urao *et al*., 1994). In the work presented here the effect of several stress treatments (heat shock, high salt, low amount of 2,4-D, ABA, and kinetin) were studied. When heat shock was applied, *MsCPK3* and *Mshsp18-1* genes showed similar activation, though *Mshsp18-1* responded earlier by accumulating the mRNA within minutes (Fig. 4B). Interestingly, the expression of CPKs described in other plant species was not induced by heat shock. Other stress treatments resulting in activation of the CPKs from different species (Urao *et al*., 1994; Botella *et al*., 1996; Yoon *et al*., 1999) had no effect on *MsCPK3* expression under the conditions analysed in the present work. These differences in response to various external factors suggest that the alfalfa CPK might represent a novel type of these kinases, but it cannot be ruled out that large divergence in responses of various CPK genes originate from the use of different experimental systems.

Somatic embryogenesis involves initiation of an ontogenic programme in cultured plant cells and is strongly dependent on the reprogramming of gene expression required for hormone-induced cell division and differentiation. The present studies are concentrated on the early phases of embryogenic induction in *Medicago* cell suspensions, when the somatic cells become committed towards the completion of an embryo differentiation pathway. It was observed that the high 2,4-D application, altering the cell division pattern, also initiates the expression of *MsCPK3* and several stress-related genes. Taken together these data suggest that *MsCPK3* may be involved in signal transduction of auxin or/and stress-activated processes of developmental reprogramming in plant cells during somatic embryogenesis and cell division. Further studies are required to understand whether the occurrence of stress is a coincidence of the treatment, or whether it could be beneficial for the reprogramming of cells for differentiation.

Acknowledgements

We thank Dr Jung H Choi (School of Biology, Georgia Institute of Technology, Atlanta) for providing the carrot cDNA of CPK and Dr Heribert Hirt (University of Vienna) for providing an alfalfa cDNA library.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215,** 403–410.

Barnett MJ, Long SR. 1990. Nucleotide sequence of an alfalfa calmodulin. *Nucleic Acids Research* **18,** 3395.

Berberich T, Kusano T. 1997. Cycloheximide induces a subset of low temperature-inducible genes in maize. *Molecular and General Genetics* **254,** 275–283.

Botella JR, Arteca JM, Somodevilla M, Arteca RN. 1996. Calcium-dependent protein kinase gene expression in response to physical and chemical stimuli in mung bean (*Vigna radiata*). *Plant Molecular Biology* **30,** 1129–1137.

Bögre L, Oláh Z, Dudits D. 1988. Ca^{2+}-dependent protein kinase from alfalfa (*Medicago varia*): partial purification and autophosphorylation. *Plant Science* **58,** 135–144.

Breviario D, Morello L, Giani S. 1995. Molecular cloning of two rice cDNA sequences encoding putative calcium-dependent protein kinases. *Plant Molecular Biology* **27,** 953–967.

Deák M, Sutus M, Györgyey J, Dudits D. 1995. Involvement of stress-related and cell cycle genes in transition from somatic to embryogenic cells of alfalfa. *Acta Phytopathologica et Entomologica Hungarica* **30,** 119–122.

Dudits D, Bögre L, Györgyey J. 1991. Molecular and cellular approaches to the analysis of plant embryo development from somatic cells *in vitro*. *Journal of Cell Science* **99,** 475–484.

Dudits D, Györgyey J, Bögre L, Bakó L. 1995. Molecular biology of somatic embryogenesis. *In vitro* embryogenesis in plants. *Current plant science and biotechnology in agriculture*, Vol. 20. Kluwer Academic Publishers, 267–309.

Dudits D, Magyar Z, Deák M, Mészáros T, Miskolczi P, Fehér A, Brown S, Kondorosi É, Athanasiadis A, Pongor S, Bakó L, Koncz C, Györgyey J. 1998. Cyclin-dependent and calcium-dependent kinase families: response of cell division cycle to hormone and stress signals. In: Francis D, Dudits D, Inzé D, eds. *Plant cell division: molecular and developmental control*. London: Portland Press, 21–46.

Ellard-Ivey M, Hopkins RB, White TJ, Lomax TL. 1999. Cloning, expression and N-terminal myristoylation of CpCPK1, a calcium-dependent protein kinase from zucchini (*Cucurbita pepo* L.). *Plant Molecular Biology* **39,** 199–208.

Estruch JJ, Kadwell S, Merlin E, Crossland L. 1994. Cloning and characterization of a maize pollen-specific calcium dependent calmodulin independent kinase. *Proceedings of the National Academy of Sciences*, *USA* **91,** 8837–8841.

Frattini M, Morello L, Breviario D. 1999. Rice calcium-dependent kinase isoforms OsCDPK2 OsCDPK11 show different responses to light and different expression patterns during seed development. *Plant Molecular Biology* **41,** 753–764.

Györgyey J, Gartner A, Nemeth K, Hirt H, Heberle Bors H, Dudits D. 1991. Alfalfa heat shock genes differentially expressed during somatic embryogenesis. *Plant Molecular Biology* **16,** 999–1007.

Harmon AC, Gribskov M, Harper JF. 2000. CDPKs—a kinase for every Ca^{2+} signal? *Trends in Plant Science* **4,** 154–159.

Hidaka H, Sasaki Y, Tanaka T, Endo T, Ohno S, Fuji Y, Nagata T. 1981. *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide, a calmodulin antagonist, inhibits cell proliferation. *Proceedings of the National Academy of Sciences*, *USA* **78,** 4354–4357.

Hong Y, Takano M, Liu CM, Gasch A, Chye M-L, Chua N-H. 1996. Expression of three members of the calcium dependent protein kinase gene family in *Arabidopsis thaliana*. *Plant Molecular Biology* **30,** 1259–1275.

Hrabak EM, Dickmann LJ, Satterle JS, Sussmann MR. 1996. Characterization of eight new members of the calmodulin-like domain kinase gene family from *Arabidopsis thaliana*. *Plant Molecular Biology* **31,** 405–412.

Kapros T, Bögre L, Németh K, Bakó L, Györgyey J, Wu SC, Dudits D. 1991. Differential expression of histone H3 gene variants during cell cycle and somatic embryogenesis in alfalfa. *Plant Physiology* **98,** 621–625.

Lam E, Benedyk M, Chua N-H. 1989. Characterization of phytochrome-regulated gene expression in a photoautotrophic cell suspension: possible role for calmodulin. *Molecular and Cell Biology* **9,** 4819–4823.

Lee JY, Roberts DM, Harmon AC. 1997. Isolation of two new CDPK isoforms from soybean (*Glycine max* L.). *Plant Physiology* **115,** 314.

Maes M, Messens E. 1992. Phenol as grinding material in RNA preparations. *Nucleic Acids Research* **20,** 4374–4375.

Mahadevan LC, Edwards DR. 1991. Signalling and superinduction. *Nature* **349,** 747–748.

Monroy AF, Dhindsa RS. 1995. Low-temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25 °C. *The Plant Cell* **7,** 321–331.

Poovaiah BW, Reddy ASN. 1993. Calcium and signal transduction in plants. *Critical Reviews in Plant Sciences* **12,** 185–211.

Putman-Evans C, Harmon AC, Palevitz BA, Fechheimer M, Cormier MJ. 1989. Calcium-dependent protein kinase is localized with F-actin in plant cells. *Cell Motility and the Cytoskeleton* **12,** 12–22.

Roberts DM. 1993. Protein kinases with calmodulin like domains: novel targets of calcium signals in plants. *Current Opinions in Cellular Biology* **5,** 242–246.

Roberts DM, Harmon AC. 1992. Calcium modulated proteins: targets of intercellular calcium signals in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **43,** 375–414.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, *USA* **74,** 5463–5467.

Savouré A, Fehér A, Kaló P, Petrovics Gy, Csanádi Gy, Szécsi J, Kiss G, Brown S, Kondorosi A, Kondorosi É. 1995. Isolation of a full-length mitotic cyclin cDNA clone CycIIMs from *Medicago sativa*: chromosomal mapping and expression. *Plant Molecular Biology* **27,** 1059–1070.

Saunders MJ. 1990. Calcium and hormone action. *Symposium of the Society of Experimental Biology* **44,** 271–283.

Schaller GE, Harmon AC, Sussman MR. 1992. Characterization of a calcium- and lipid-dependent protien kinase associated with the plasma membrane of oat. *Biochemistry* **31,** 1721–1727.

Sharma KK, Thorpe TA. 1995. Asexual embryogenesis in vascular plants in nature. *Current plant science and biotechnology in agriculture*, Vol. 20. Kluwer Academic Publishers, 17–72.

Sheen J. 1996. Ca^{2+}-dependent protein kinase and stress signal transduction in plants. *Science* **274,** 1900–1902.

Suen KL, Choi JH. 1991. Isolation and sequence analysis of a cDNA for a carrot calcium-dependent protein kinase: homology to calcium/calmodulin dependent protein kinases and to calmodulin. *Plant Molecular Biology* **17,** 581–590.

Urao T, Katagiri T, Mizoguchi T, Yamaguchi-Shinozaki K, Hayashida N, Shinozaki K. 1994. Two genes that encode Ca^{2+}-dependent protein kinases are induced by drought and high-salt stresses in *Arabidopsis thaliana*. *Molecular and General Genetics* **244,** 331–340.

Yoon GM, Cho HS, Ha HJ, Liu JR, Lee HP. 1999. Characterization of NtCDPK1, a calcium-dependent protein kinase gene in *Nicotiana tabacum*, and the activity its encoded protein. *Plant Molecular Biology* **39,** 991–1001.

Zhao Y, Kapper B, Franklin RM. 1993. Gene structure and expression of an unusual protein kinase from *Plasmodium falciparum* homologous at its carboxyl terminus with the EF-hand calcium-binding proteins. *Journal of Biological Chemistry* **268,** 4347–4354.