Plant calcium-dependent protein kinase-related kinases (CRKs) do not require calcium for their activities

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Received 14 August 1996; revised version received 20 September 1996

Abstract In plants, calcium-dependent protein kinases (CDPKs) make up a large family that is characterized by a C-terminal calmodulin(CaM)-like domain. Recently, a novel carrot cDNA clone encoding an atypical CDPK, which has a significantly degenerate sequence in the CaM-like domain, was found and named CDPK-related protein kinase (CRK) [Lindzen, E. and Choi, J.H. (1995) Plant Mol. Biol. 28, 785–797]. We obtained two different cDNA clones from maize which encode CRKs. For the first enzymatic characterization of CRK, a maize cDNA clone was expressed in *E. coli*. The recombinant protein efficiently phosphorylated casein, a conventional protein substrate. Notably, in this in vitro phosphorylation assay, the kinase activity did not require calcium as an activator. Thus, CRKs were suggested to be novel calcium-independent protein kinases having a degenerate CaM domain, the function of which remains to be elucidated.

Key words: Calcium-dependent protein kinase; CDPK-related protein kinase; Bacterial expression; cDNA cloning; Maize

1. Introduction

Calcium-dependent protein kinases (CDPKs) represent a third family of calcium (Ca^{2+})-regulated protein kinases [1,2]. They are biochemically distinct from protein kinase C and Ca^{2+}/calmodulin(CaM)-dependent protein kinases since they require neither lipid nor calmodulin for activation. Within a single polypeptide, CDPK contains a catalytic domain for serine/threonine phosphorylation, a junctional domain involved in autoinhibition, and a CaM-like regulatory domain with four Ca^{2+}-binding EF-hands. In plants CDPKs comprise the most abundant Ca^{2+}-regulated protein kinases, though their physiological functions are largely unknown.

During the course of our study on the maize CDPK which was suggested to be involved in the regulatory phosphorylation of phosphoenolpyruvate carboxylase for C4 photosynthesis [3], we incidentally isolated two different cDNA clones from maize. One was presumably of full length (2.2 kb) and the other was partial (2.1 kb), both being more abundantly expressed in roots than leaves as judged from Northern blot analysis. They were found to encode atypical CDPKs which had recently been found in carrot and named CDPK-related kinase (CRK) [4]. CRK was discriminated from CDPK through its degenerate sequence in the CaM-like domain including four Ca^{2+}-binding EF-hands. Although the activities of CRKs were speculated to be Ca^{2+}-dependent [4], their enzymatic properties have not been examined. Thus, we decided to confirm and to characterize CRK activity by means of functional expression in *Escherichia coli*. A full-length cDNA was inserted into an expression vector so that it was in-frame with the N-terminus of β-galactosidase. A cell-free extract of *E. coli* transformed with the recombinant plasmid efficiently phosphorylated several conventional substrate proteins such as casein, histone III S, myelin basic protein and myosin light chain. Unexpectedly, however, the kinase activity was neither enhanced by Ca^{2+} nor inhibited by excess EGTA.

2. Materials and methods

2.1. Cloning and sequencing of maize protein kinases

Four cDNA fragments encoding putative CDPKs were amplified by PCR using degenerate primers (accession numbers: D87043, D87044, D87045 and D87046) and a maize library as templates (Y. Saijo, S. Hata, J. Sheen and K. Izui, unpublished). A maize (*Zea mays* H84) leaf cDNA library in λgt10 [4] was screened with a mixture of ^{32}P-labeled cDNA fragments as probes, under low stringency conditions, as described [5]. A faintly positive clone, designated as ZmPK2, was found. Since ZmPK2 lacked a 5′ part, we next screened a maize root cDNA library in λgt10 (constructed from roots of 6-day-old seedlings by T. Masuda of this laboratory) using ^{32}P-labeled ZmPK2 as a probe. 18 positive spots were detected for 4.5×10^5 clones, and two recombinant phages with relatively long cDNA inserts, designated as ZmCRK1 and ZmCRK3, were further analyzed. After subcloning the cDNAs into pBluescriptII, exonuclease III-generated deletions were prepared with an Erase-a-base kit (Promega). The nucleotide sequence was determined for both strands using an ABI PRISM Dye Terminator Cycle Sequencing Kit and a DNA Sequencing System 373A (Perkin Elmer).

2.2. Expression of ZmCRK3 in E. coli

The protein-coding sequence of the original ZmCRK3 plasmid was not in-frame with the N-terminus of β-galactosidase on the vector. Therefore, the plasmid was cut with *Sac*I, and then successively treated with exonuclease III, S1 nuclease, Klenow polymerase and T4 DNA ligase. The resulting plasmid, designated as ZmCRK3E, lacked 28 base pairs compared to ZmCRK3 and was proved to have the correct open reading frame by determination of its nucleotide sequence. *E. coli* XL1- Blue cells transformed with ZmCRK3E were grown in LB medium containing 1 M sorbitol, 25 mM glycine betaine, 50 μg/ml ampicillin and 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. After precultivation at 37°C for 30 h in 5 ml of the medium, they were grown at 37°C for 3 h and then at 25°C for an additional 3 h in 50 ml of the medium. Enzyme extracts were prepared essentially as described [7,8] except for minor changes in the extraction buffer, viz. the addition of 0.5 mM phenylmethanesulfonyl fluoride, omission of

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Abbreviations: CDPK, calcium-dependent protein kinase; CRK, CDPK-related kinase

The nucleotide sequence data reported in this paper have been submitted to the DDBJ/GenBank/EMBL databases under the accession numbers, D38452 (ZmPK2), D84507 (ZmCRK1) and D84508 (ZmCRK3).

PII S0014-5793(96)01090-3

50 mM EDTA and lysozyme treatment. The protein concentration was determined with a Bio-Rad Protein Assay using bovine serum albumin as a standard. The standard reaction mixture (final volume, 10 μl) comprised 0.1 mM [γ-^{32}P]ATP (2 μCi), 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 μg substrate protein (α-casein, histone IIIS, myelin basic protein or myosin light chains (Sigma)), and the crude enzyme, 10 μg protein. It was incubated at 30°C for 30 min and subjected to 10% SDS-PAGE, the radioactivity then being determined by autoradiography or with a Bio-imaging analyzer (Fuji).

2.3. Other methods

For Northern blotting, 3.5 μg each of poly(A)$^+$ RNA was separated by electrophoresis in denaturing 1% agarose gels, and then transferred to a Hybond-N$^+$ membrane (Amersham). The membrane was probed with a ^{32}P-labeled ZmPK2 fragment (1 kb in length; within the coding region) and then washed under high stringency conditions as previously described [6]. Genetyx-Mac software (Software Development) was used for both analysis of the sequence data and construction of a phylogenetic tree of protein kinases.

3. Results

In the initial screening experiment, a cDNA clone, designated as ZmPK2, for a putative protein kinase was isolated. Although ZmPK2 was truncated at its 5′ end, the cDNA (1.9 kb in length) showed an open reading frame for a protein kinase domain followed by a novel C-terminal domain (see legend to Fig. 1). Interestingly, the nucleotide sequence of ZmPK2 did not correspond to that of any probe which encodes a putative CDPK, showing only weak similarity. Since our efforts to obtain the 5′ part of the ZmPK2 gene were unsuccessful, we examined the mRNA level of ZmPK2 in some parts of maize seedlings. Northern blotting with the coding region of ZmPK2 as a probe showed that a single band, corresponding to 2.2 kb, was much more strongly detected for roots than for leaves (data not shown). Therefore,

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ZmCRK3        1:MGQCYGKAGGASSRRADHDDAVAPPSPLPANGAPTPPQQPATPGRRKSGSATPV-HHQAAT---TAWPSPYPAGGASPLP 76
ZmCRK1        1:-----------------------.........---.-L....R.H....T...H......PGAA.............. 53
carrot CRK    1:..I.VS.PSPEPDLHNH.TSIPVNDTS..PQDNSI..KDI.I.AQ-DN-NKP.G-KKSPFLPFYSPS.AHFLFSKK..AV 77
ATHCALLIPR    1:-------------MGNTCVGPSRNGFLQSVSA.MWR.RDGDDSASMSN.-DIASE-AVSGE-LRSRLS-DEVQNKPPEQV 63
                                                          *

ZmCRK3       77:AGVSPSPARSTPRRFFKRPFPPPSPAKHIKATLAKRLGGGKPKEGTIPEE-----------G-GAG--V-AADSAEAERP 141
ZmCRK1       54:..................................................GGAGAGAGAGA.A...AA.G.......D.. 133
carrot CRK   78:GSPAAGSSN...K.L.--.............AW.R.H.SV..N.AA...N--------NEVD-.-.-A-G---------- 133
ATHCALLIPR   64:TMPK.GTDVE.KD.EI-.TESK.ETLEE.-SLES.PETKQET.SE.K..-SKPDP-PAKPKKPKHMKR.SS.-GLRT.SV 138
                                *  *        *     *                **

                                         I                      II                 III         IV
ZmCRK3      142:LDKTFGFANNFGAKYDLGKEVGRGHFGHTCSALVKKGEYKGHAVAVKIISKAKMTTAISIEDVRREVKILKALSGHNNLV 221
ZmCRK1      134:........K.......................V.....H...T..................................D... 213
carrot CRK  134:...S...SKK..S.FEV.E........Y..R.KF....F..QVD...V.P........A...........R..T...... 213
ATHCALLIPR  139:.QRK---TE..KEF.S..RKL.Q.Q..T.-FLC.E.--TT.KEF.C.S.A.R.LL.DEDV......IQ.MHH.A..P.VI 212
                  *        *      *   * * ** *      *    *    * * * * * *   ******  *   * ** *

                                         V                                   VI
ZmCRK3      222:KFYDACEDALNVYIVMELCEGGELLDRILARGGRYTEVDAKAIVVQILSVVSFCHLQGVVHRDLKPENFLFATRDESAPM 301
ZmCRK1      214:R..........................................E....I.......A...................T........ 293
carrot CRK  214:Q....F..HT...V...............S...K...D....VMI...N..A...................KSK..DSQL 293
ATHCALLIPR  213:SIKG.Y..VVA.HL...C.A....F...IQ.-.H...RK.AELTRT.VG..EA..SL..M...........VSKH.DSLL 291
                    * **    *  *** * **** ***  * * ***  *      *  **  **  ** ***********   *

                     VII                  VIII             IX                       X
ZmCRK3      302:KLIDFGLSDFIRPDERLNDIVGSVYYVAPEVLHRSYSMEADIWSIGVITYILLCGSRPFWARTESGIFRSVLRADPNFDD 381
ZmCRK1      294:.......................A.......................................................... 373
carrot CRK  294:.A.......YVK...........A.............T...V.....S...................A..K.NLS..E 373
ATHCALLIPR  292:.T......M.FK..DVFT.V...P........RKR.GP...V..A...V....S.VP....E..Q...EQ..HG.LD.SS 371
                  * ******    **    * *** ********   *  *** ** *** **** *  **** ** ***  **    *

                                    XI
ZmCRK3      382:SPWPSVSAEAKDFVKRFLNKDYRKRMTAVQALTHPW-LRD-EQRQIPLDILVFRLVKQYLRATPLKRLALKALSKALRED 459
ZmCRK1      374:....................................-...-..........I..........................S.. 451
carrot CRK  374:P......S.KA....L...P......A...C.S.-IKNSNDIKF.......K.M.V.M.SS..RKA..R....T.TV. 452
ATHCALLIPR  372:D....I.ES...L.RKM.VR.PK..L..H.V.C...VQV.GVAPDK...SA.LSRM..FSAMNKF.KM..RVIAES.S.E 451
                   **** *   * *   *  *  ** ** * * * *          ***       *           **    *

ZmCRK3      460:ELLYLRLQFKLLEP-RDGLVSLDNFRTALTRYVTDAMRESRVLEFLHALDPLAYRKMDLEEFCAAAISPYQLEALESWEE 538
ZmCRK1      452:..............-...F..............S............Q...E.......F.................R... 530
carrot CRK  453:..F..KE..V....TKN.TI..E.IKQ..M.NS....KD....DL.VS.NA.Q..R..F.......L.VH.....DR..Q 532
ATHCALLIPR  452:.IAG.KEM.NMIDADKS.QITFEELKAG.K.VGAN-LK..EI.DLMQ.A.VDNSGTI.YK..-I..-T-LH.NKI-ER.D 526
                  *   *   *         *         * *        * *              *  **  **     *     *

ZmCRK3      539:IAGTAFQHFEQEGNRVISVEEL--AQELNLAPT-H-YSIVQDWIRKSDGKLNFLGFTKFLH-G-VTIRGSNTRRH----- 607
ZmCRK1      531:......................--.........-.-...........................-.-..........----- 599
carrot CRK  533:H.RC.YDL..KD...A.MI...---.S..G.G.SIPVHAVLH....HT....S...YV.L..-.-.ST.AI-AKAQ----- 602
ATHCALLIPR  527:HLFA..TY.DKD.SGY.TPD..QQ.C.EFGVEDVRIEELMR.VDQDN..RIDYNE.VAMMQK.SI.GGPVKMGLEKSFSI 606
                      *   *   *   *   ** * *              *      **            *

ZmCRK3          :----
ZmCRK1          :----
carrot CRK      :----
ATHCALLIPR  607:ALKL                                                                            610
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Fig. 1. Amino acid sequence alignment of ZmCRK3, ZmCRK1, carrot CRK [4] and *Arabidopsis* CDPK(ATHCALLIPR) [10]. The 5′ end of ZmPK2 (see text) corresponds to amino acid 125 in the ZmCRK1 sequence. Dots indicate identical residues with those above. Dashes denote gaps introduced to facilitate the alignment. Roman numerals designate the positions of kinase subdomains [11]. A consensus N-myristoylation signal and four typical EF-hands are boxed.

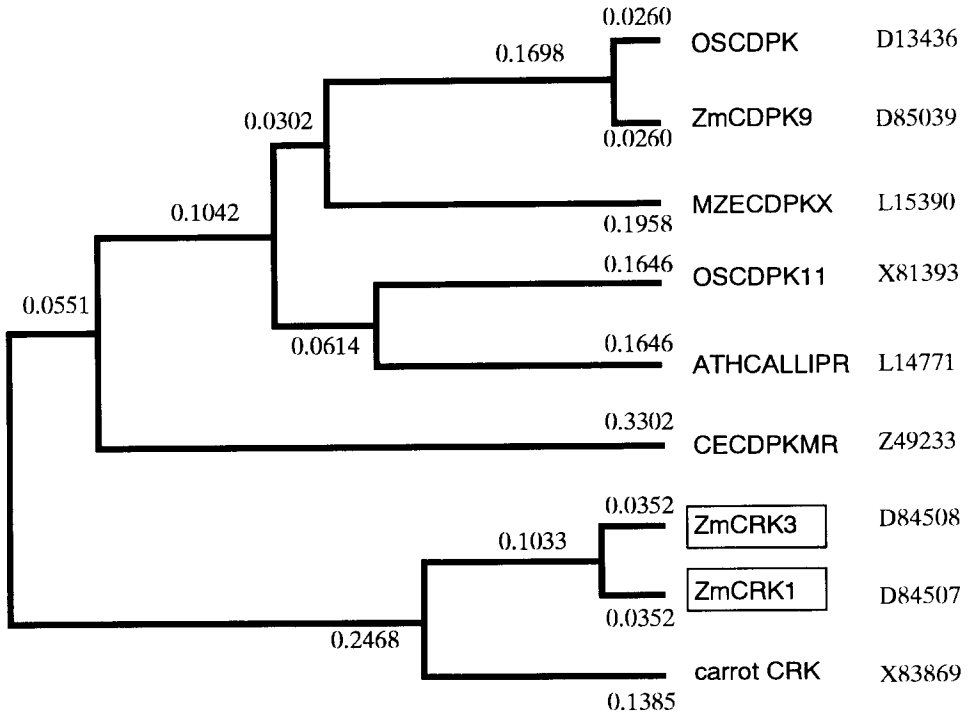

Fig. 2. Phylogenetic tree for the kinase domains of CRKs and other 'CaM group' kinases [12]. The UPGMA method [13] was applied using Genetyx-Mac software. The values near the branches give branch lengths as the numbers of amino acid substitutions per site. Accession numbers of the DDBJ/GenBank/EMBL data bases are indicated on the right.

we next screened a maize root cDNA library in λgt10 using the coding region of ZmPK2 as a probe and obtained two clones, designated as ZmCRK1 and ZmCRK3. Subcloning and sequencing showed that ZmCRK1 was a longer partial clone (2.1 kb in length) and encoded the same protein as ZmPK2. On the other hand, ZmCRK3 (2.2 kb in length) contained the entire coding sequence for a 607 amino acid polypeptide with a calculated molecular mass of 67.4 kDa. The predicted protein of ZmCRK3 was related to, but significantly different from, that of ZmCRK1 (see Fig. 1). Although no in-frame stop codon was found upstream of the first ATG codon of ZmCRK3, we assume that it represents the translation initiation codon because the size of the cDNA excellently corresponds to that of the transcript estimated by Northern blot analysis. Since ZmCRK3 and ZmCRK1 are very similar (95% identical at the nucleotide level in the coding region), the results of Northern blotting described above suggest that both molecular species are mainly expressed in roots.

Fig. 1 shows the amino acid sequence alignment of ZmCRK3, ZmCRK1 and similar kinase polypeptides. Evidently, our maize cDNAs encoded members of the protein kinase superfamily. After their amino acid sequences had been completely deduced, it turned out that our clones were highly homologous to the recently cloned carrot CRK [4]. The overall amino acid identity of ZmCRK3 with carrot CRK was 58%. The identities in the N-terminal region (1–148th residues), the kinase domain (149–417th residues), and the C-terminal region consisting of junctional and 'CaM-like domains' (418–607th residues) were 30, 77 and 55%, respectively. In contrast, the identity of CaM-like domain of ZmCRK3 (149 residues) with maize CaM was only 21%.

Closer inspection of the sequences revealed that both ZmCRK3 and carrot CRK have putative myristoylation signals at their N-termini, and that the N-terminal region is proline-rich (22–26 residues out of 148 residues) and the motif PFPPPSPAKHIKA is strictly conserved among the maize and carrot CRKs. The kinase domain is a typical serine/threonine kinase domain with 11 canonical subdomains. On the other hand, their C-terminal CaM-like domains are quite atypical, when compared with the CaM-like domain of CDPK of *Arabidopsis thaliana*. Although all invariant residues required for Ca^{2+}-binding [9] are conserved in all EF-hands of CDPK, they are only partially conserved in the corresponding region of CRKs. The junctional domain, which is known to be well-conserved among CDPKs [10], is also degenerate in CRKs. Except for the carrot CRK, a database search did not detect significantly homologous proteins to the proline-rich and C-terminal domains.

Fig. 2 shows a phylogenetic tree of the catalytic domains of CRKs, CDPKs and other protein kinases. The CRKs clearly belong to a distinct subfamily. Another phylogenetic tree based on the amino acid sequences of entire polypeptides showed a similar pattern (data not shown). The carrot CRK was once reported to be more similar to CDPKs than to *Plasmodium* putative CDPK [4]. However, the *Plasmodium* enzyme is highly degenerate so that it is not considered to be a typical member of the CDPK subfamily [11].

The expression plasmid, ZmCRK3E, encoded 18 amino acids of β-galactosidase, 46 amino acids of an additional peptide derived from the polycloning site and 5′ non-coding region, and 607 amino acids of the entire kinase protein. As described under Section 2, a cell-free extract of *E. coli* transformed with a recombinant ZmCRK3 plasmid was prepared. As shown in Fig. 3, the enzyme preparation efficiently phos-

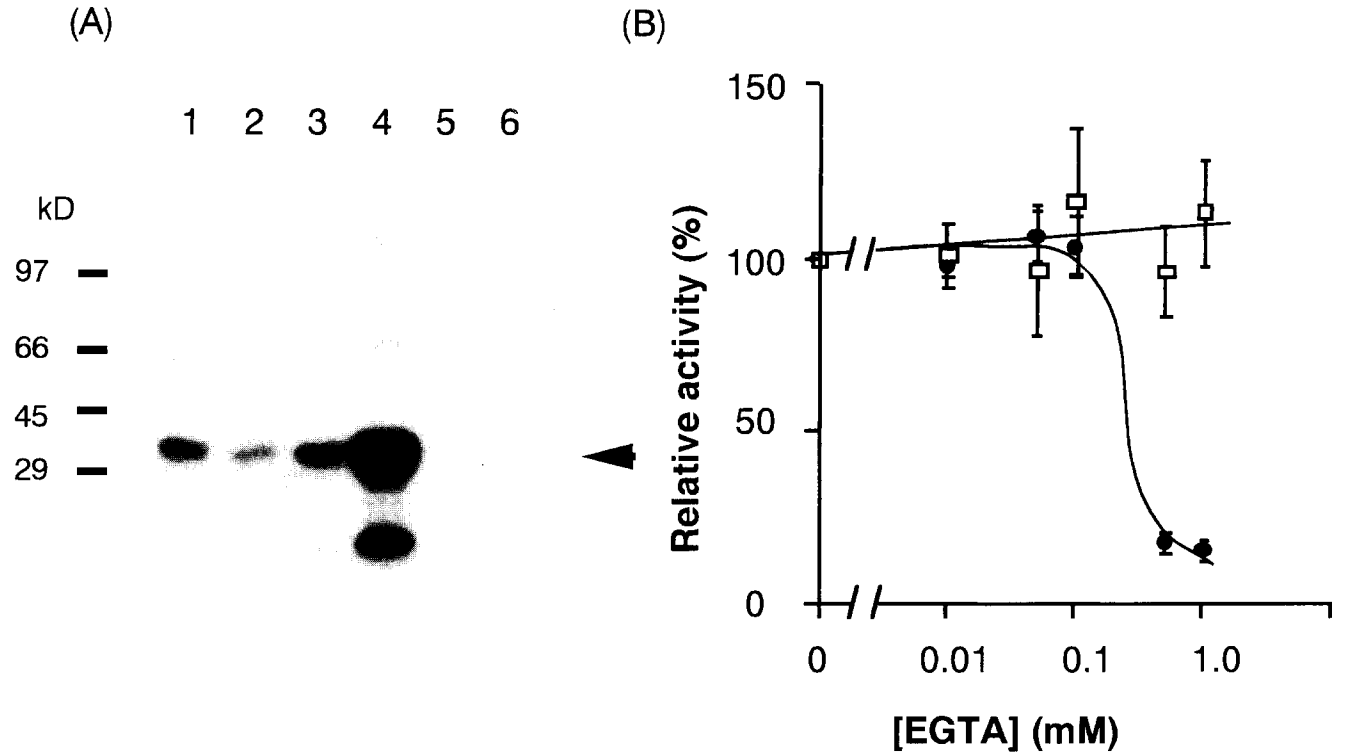

Fig. 3. Effects of Ca^{2+} and EGTA on protein kinase activities. (A) Autoradiogram of the reaction products after SDS-PAGE analysis. The casein kinase activities of extracts of *E. coli* transformed with ZmCRK3E (lanes 1–3), an expression plasmid of a maize CDPK (ZmCDPK9; lanes 4,5), and pBluescriptII without an insert (lane 6) were determined. The incubation was carried out as described under Section 2 with 0.1 mM Ca^{2+} (lanes 1,4,6), 0.1 mM Ca^{2+} plus 1 mM EGTA (lanes 3,5), or 1 mM Ca^{2+} (lane 2). The arrow indicates the position of casein. (B) Variation of kinase activities with increasing concentrations of EGTA. The standard reaction mixtures for ZmCRK3E (□) and ZmCDPK9 (●) contained 0.1 mM Ca^{2+}. The incorporation of ^{32}P from [γ-^{32}P]ATP into casein was determined. The error bar indicates S.E.M. for 3–6 assays.

phorylated casein, a conventional substrate protein, in the presence of 0.1 mM Ca^{2+} (lane 1). Under the conditions employed, the other exogenous substrates such as histone IIIS, myelin basic protein and myosin light chains were also phosphorylated (data not shown). As described elsewhere (Y. Saijo, S. Hata, J. Sheen and K. Izui, unpublished), the activity of a typical CDPK, ZmCDPK9, was almost completely inhibited by the addition of an excess amount (1 mM) of EGTA (lanes 4,5). In contrast, the ZmCRK3 activity was not affected by EGTA (lanes 1,3). The inclusion of 1 mM Ca^{2+} was not activating but rather inhibitory (lanes 1,2). Therefore, it was demonstrated that ZmCRK3 does not require calcium as a cofactor.

4. Discussion

In this study, for the first time, we isolated two maize cDNAs encoding CRKs from a monocotyledonous plant. These results substantiate the prediction of Lindzen and Choi [4] that CRKs are widely conserved among angiosperms, forming a small gene family. Furthermore, we demonstrated the protein kinase activity of a maize CRK by means of prokaryotic expression of the enzyme (Fig. 3).

CRKs are structurally distinct from common CDPKs (Fig. 1). Lindzen and Choi [4] also speculated that the 'calmodulin-like' domain of CRKs may be functional in a calcium-dependent manner, despite its degeneracy. In contrast to their prediction, ZmCRK3 was very active in the presence of an excess amount of EGTA (Fig. 3). However, it should be borne in mind that the in vitro phosphorylation activity does not always reflect the in vivo character of the enzyme. For example, Urao et al. [14] expressed a typical CDPK protein in *E. coli*, but they could not detect the enzyme activity even in the presence of Ca^{2+}. In addition, it is still possible that Ca^{2+} associates with the CRK protein. Thus, we cannot even rule out the possibility that CRKs might phosphorylate their natural substrates, which are unknown at present, in a calcium-dependent manner. Nevertheless, on the basis of the results of the in vitro phosphorylation assay (Fig. 3), together with the dendrogram (Fig. 2), we would like to suggest that CRKs make up a distinct subfamily of calcium-independent serine/threonine kinases in the protein kinase superfamily. It would be of interest to clarify the function of the degenerate calmodulin-like domain.

CRKs may associate with plasma membranes, since they have an N-terminal myristoylation motif (Fig. 1). Regarding the expression of CRK genes, we have only preliminary data indicating that they are mainly expressed in roots. Obviously, elucidation of their physiological roles is of great importance.

Acknowledgements: We wish to thank Dr. S. Yanagisawa, Y. Saijo and J. Sheen for help during the initial stages of this work. This work was supported in part by research grants from the Ministry of Education, Science, Sports and Culture of Japan.

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