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Cloning and characterization of a maize pollen-specific calcium-dependent calmodulin-independent protein kinase

(pollen germination/protein phosphorylation)

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ABSTRACT **A calcium-dependent calmodulin-independent protein kinase (CDPK) has been cloned from maize (*Zea mays*). The sequence predicts a 550-amino acid (predicted molecular mass is 60 kDa) protein with two major functional domains: an N-terminal catalytic domain highly homologous to protein kinases and a C-terminal domain resembling calmodulins. Northern analysis shows that the expression of the maize CDPK gene is pollen specific and that its transcription is restricted to late stages of pollen development. Western blots reveal a major abundance of CDPK protein at the stage of pollen germination. *In vitro* germination and pollen tube growth are impaired upon addition of a calmodulin antagonist (calmidazolium), CDPK inhibitors (W-7), and antisense oligonucleotides directed against CDPK mRNA. These observations indicate that the function of the pollen-specific maize CDPK protein is required for germination and pollen tube growth.**

Pollen transmits the male genetic material in sexual reproduction of all higher plants, and pollen germination and tube growth are critical steps for a successful fertilization. Pollen germination and sustained tube growth occur only under specific physiological conditions that include high humidity, the presence of specific ions, and a continuous source of energy (1). Pollen tube growth is associated with cytoplasmic streaming, and cytological evidence supports the hypothesis that the motive force for this growth is the dynamic reorganization of the cytoskeleton (2). Pollen tube development requires Ca^{2+} (3, 4). In 1982, Picton and Steer (5) proposed that pollen tube growth depends on the state of contraction of the microfilaments at the tube tip, which is determined by the Ca^{2+} concentration. However, the mechanism of regulation of motility by Ca^{2+} remains poorly understood. In animals, Ca^{2+} is a well-known secondary messenger that transduces signals through activation of protein kinases dependent on Ca^{2+} and calmodulins (6). Each of these components has been found in plants, so Ca^{2+} may act in a similar fashion in plants and animals (7). In both animals and plants, several protein kinases have been identified which are Ca^{2+} dependent but calmodulin independent (CDPKs) (7). These CDPKs do not require calmodulin because they contain high-affinity Ca^{2+}-binding sites themselves.

The possible physiological functions of the plant CDPKs have been proposed according to their subcellular distribution. CDPKs have been found associated with chromatin (7), plasma membranes (8), and the plant cytoskeleton (9). The multiple subcellular localizations suggest that this protein family is involved in multiple signal transduction pathways. A soybean CDPK (10) is one of the best-characterized examples. Immunocytochemical staining with monoclonal antibodies against the soybean CDPK has shown its association with the F-actin network (9). This finding supports the hypothesis that CDPKs regulate the activity and structure of the plant cytoskeleton.

In this report, we describe the identification and cloning of a maize (*Zea mays*) cDNA and its genomic clone, which encodes a protein related to previously described plant CDPKs.† Its pollen-specific expression identifies the maize CDPK as one of the few cases of protein kinases that are tissue specific. We also show that the maize CDPK is crucial for germination and growth of the pollen tube. On the basis of these results, we propose that the maize CDPK may serve as a link between Ca^{2+} and pollen germination.

MATERIALS AND METHODS

cDNA and Genomic Cloning of the CDPK Gene. Poly(A)$^+$ RNA was isolated (11) from mature pollen (maize inbred line 211D). cDNA complementary to the pollen mRNA was obtained and cloned in λ gt10. From this pollen-specific cDNA library (10^5 plaques), a 1.4-kb cDNA was isolated, cloned in pBS (Stratagene), and sequenced by using the *Taq* cycle sequencing method (Applied Biosystems). When compared with sequences in the data bank, the serendipitously obtained clone (1.4-kb insert) exhibited homology with CDPKs. The genomic clone for CDPK (gCDPK, Fig. 1) was obtained by screening a maize genomic library in λ GEM-11 (Promega), using a random-primed ^{32}P-labeled DNA probe made from a 0.5-kb *Eco*RI fragment of the CDPK cDNA. Screening was performed in 6× SSC (1× SSC = 0.15 M NaCl/0.015M sodium citrate, pH 7.4) at 68°C for 12 h followed by washes at 50°C in 2× SSC/0.1% SDS for 30 min, and in 0.1× SSC/0.1% SDS for 30 min.

Sequence Analysis. DNA and peptide sequence analysis employed various programs of the GCG (Genetics Computer Group) sequence analysis software package, including FASTA and TFASTA for data base homology searches. The alignment of the protein sequences was done using the MEGALIGN DNASTAR program.

Nucleic Acid Preparations. Genomic DNA from young maize shoots of line 211D was isolated according to Shure *et al.* (12), digested to completion with restriction enzymes, electrophoresed through 0.7% agarose, and blotted onto nitrocellulose membranes (Micron Separations, Westboro, MA). Total RNA was isolated from maize roots, leaves, pollen, anthers, and silks as described (11). Total RNA was also isolated from microspores at different stages of development such as vacuolated microspore (late uninucleate microspore to early binucleate immature pollen), late binucleate, early and late trinucleate immature pollen, and mature and germinating pollen. *In vivo* stages were determined by using 4′,6-diamidine-2-phenylindole dihydrochloride as stain

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Abbreviation: CDPK, calcium-dependent calmodulin-independent protein kinase.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L27484).

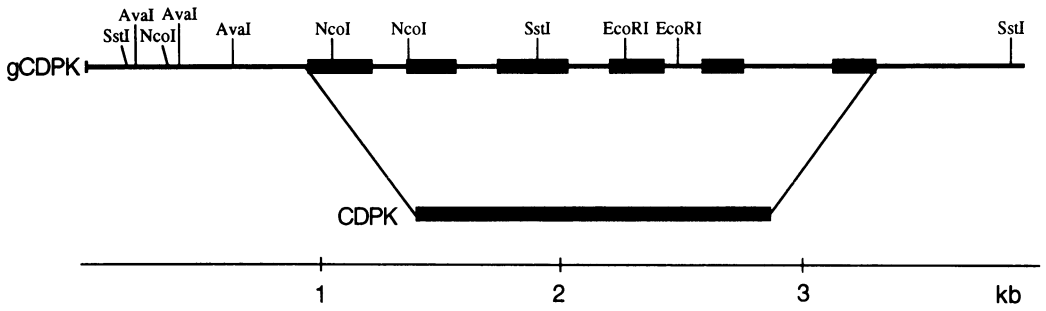

FIG. 1. Structure of the maize CDPK gene. The sequenced portion of the genomic clone gCDPK is shown with the position of selected restriction enzyme sites. Exons are represented as solid boxes. The cDNA clone is shown below the map of the gCDPK.

(13). RNAs were denatured with glyoxal, subjected to electrophoresis in a 1% agarose gel, and transferred to nitrocellulose membranes (11).

Hybridization Analysis. Southern and Northern blots were probed with random-primed ^{32}P-labeled DNA from 1.0- and 0.5-kb *Eco*RI fragments containing the 5′ and the 3′ ends of the CDPK gene, respectively. The hybridizations were performed at 68°C in 6× SSC (Southern) or at 42°C in 50% (vol/vol) formamide (Northern). After 12 h of incubation, the blots were washed in 2× SSC/0.1% SDS at 45°C for 30 min (low stringency) and in 0.1× SSC/0.1% SDS at 62°C for 30 min (high stringency).

Western Blot Analysis. Microspore staging was done as described above. Pollen was collected in Eppendorf tubes and proteins were extracted in 0.1 M Tris·HCl, pH 6.8/1% SDS buffer. After the samples had been heated at 100°C for 5 min, they were centrifuged (8,000 × *g*, 10 min) and the supernatants were collected. Proteins were separated by SDS/PAGE and transferred to Immobilon-P membranes (Millipore). Blots were blocked in 20 mM Tris·HCl, pH 7.5/0.15 M NaCl/0.02% NaN_3/5% nonfat dry milk. The maize CDPK protein was detected by using monoclonal antibodies (10 μg/ml) raised against soybean CDPK. The antibodies recognize an epitope located between amino acids 75 and 90 of the soybean CDPK (A. Harmon, personal communication). Immunodecorated protein bands were visualized by using a goat anti-mouse IgG secondary antibody conjugated to alkaline phosphatase (Sigma).

Germination and Pollen Tube Growth Test. Freshly collected mature pollen was transferred to 1 ml of liquid germination medium (35 mM sucrose/2.7 mM calcium chloride/1.6 mM boric acid) and incubated at a relative humidity of 90–100% at 26°C. To study the effects of the Ca^{2+} concentration on pollen germination or pollen tube growth, Ca^{2+} concentrations were varied from 5 μM to 10 mM either from the beginning of the germination process or from 30 or 45 min after pollen germination under control conditions. In additional experiments, the following compounds were added to the germination medium: 500 μM *N*-(6-aminohexyl)-5-chloro-1-napththalenesulfonamide (W-7), which is a calmodulin-binding compound and a CDPK inhibitor (10); 100 μM [bis-(4-chlorophenyl)methyl]-3-[2,4-dichlorobenzyloxy]imidazolium chloride (calmidazolium), an inhibitor of calmodulin; and 10 μM cytochalasin D, an inhibitor of actin polymerization.

For transient antisense experiments, the following oligonucleotides were used: 5′-CAGGAAGTTCTCGGGCTTGATGTC-3′ (sense ID#3); 5′-GACATCAAGCCCGAGAACTTCCTG-3′ (antisense ID#3); 5′-GAGGGTAATGGTCCCCTGTT-3′ (sense ID#9); 5′-AACAGGGGACCATTACCCTC-3′ (antisense ID#9); 5′-AGGCGCGTCTCCGTCTTCTTTGAT-3′ (sense JE#28); 5′-ATCAAAGAAGACGGAGACGCGCCT-3′ (antisense JE#28); 5′-TTCGCTGTCTGACAGCTTGGGCCC-3′ (sense JE#30); 5′-GGGCCCAAGCTGTCAGACAGCGAA-3′ (antisense JE#30); 5′-GTAAAACGACGGCCAGT-3′ (universal primer); and 5′-AACAGCTATGACCATG-3′ (reverse primer). The latter two will be designated as nonspecific oligonucleotides. ID#3, ID#9, JE#28, and JE#30 oligonucleotides correspond to amino acid positions 219–226, 423–429, 351–358, and 427–434, respectively, of the CDPK protein. These oligonucleotides were delivered into the growing pollen tubes 30–45 min after germination either by addition of 50 μg of each oligonucleotide to the medium or by the calcium phosphate precipitation method (14) using 5 μg of each oligonucleotide. Oligonucleotide penetration into the germinating pollen was assessed by radioactive labeling and electrophoresis. Incorporation of 0.1–0.3% of the administered radioactivity was obtained in germinating pollen after exposure for 4 h to oligonucleotides in a soluble form. The incorporation was 4–6% when the oligonucleotides were calcium-precipitated for 30 min and further incubated for 4 h in germination medium with no oligonucleotides.

RESULTS

Isolation and Analysis of cDNA and Genomic Clones. A 1.4-kb cDNA clone was isolated from a pollen-specific library. Sequence analysis of the insert revealed a 1349-bp sequence that corresponded to a partial cDNA with an open reading frame encoding a polypeptide exhibiting extensive homology to animal and plant calcium-dependent protein kinases (Fig. 2). By using the cDNA as probe, a 4165-bp genomic clone, designated gCDPK, was isolated. The sequence revealed the presence of five introns ranging from 83 to 214 bp (Fig. 1). The gCDPK allowed determination of the entire CDPK open reading frame (Fig. 2), which encoded a 550-aa polypeptide with a predicted molecular mass of 60 kDa.

Sequence Analysis. The maize CDPK contains a kinase domain from residues Gly-98 to Asp-356 that has 29–41% amino acid identity with catalytic domains of other serine/threonine protein kinases (Fig. 2). Adjacent to the kinase domain, there is a sequence spanning from Gly-389 to Leu-550 with 42–48% amino acid identity to calmodulins (Fig. 2). Although in Fig. 2 only one kinase and one calmodulin are presented, the comparisons have been extended to reported sequences of 11 kinases and 33 calmodulins. Maize CDPK shared high degrees of amino acid identity throughout the entire protein with four previously characterized CDPKs: 66% with a carrot CDPK (17), 64% with an *Arabidopsis* CDPK (16), 61% with a soybean CDPK (15), and 50% with a rice CDPK (18). In the case of the *Arabidopsis* CDPK, the first 125 amino acids were not considered in the comparison because they showed no homology with any of the domains of the CDPKs analyzed. The maize CDPK calmodulin-like domain has some distinctive features relative to other plant calmodulins. It has a threonine instead of a cysteine residue at position 413 and a tyrosine rather than a phenylalanine residue at position 485 (first and third calcium-binding box respectively; Fig. 2). These substitutions are also present in

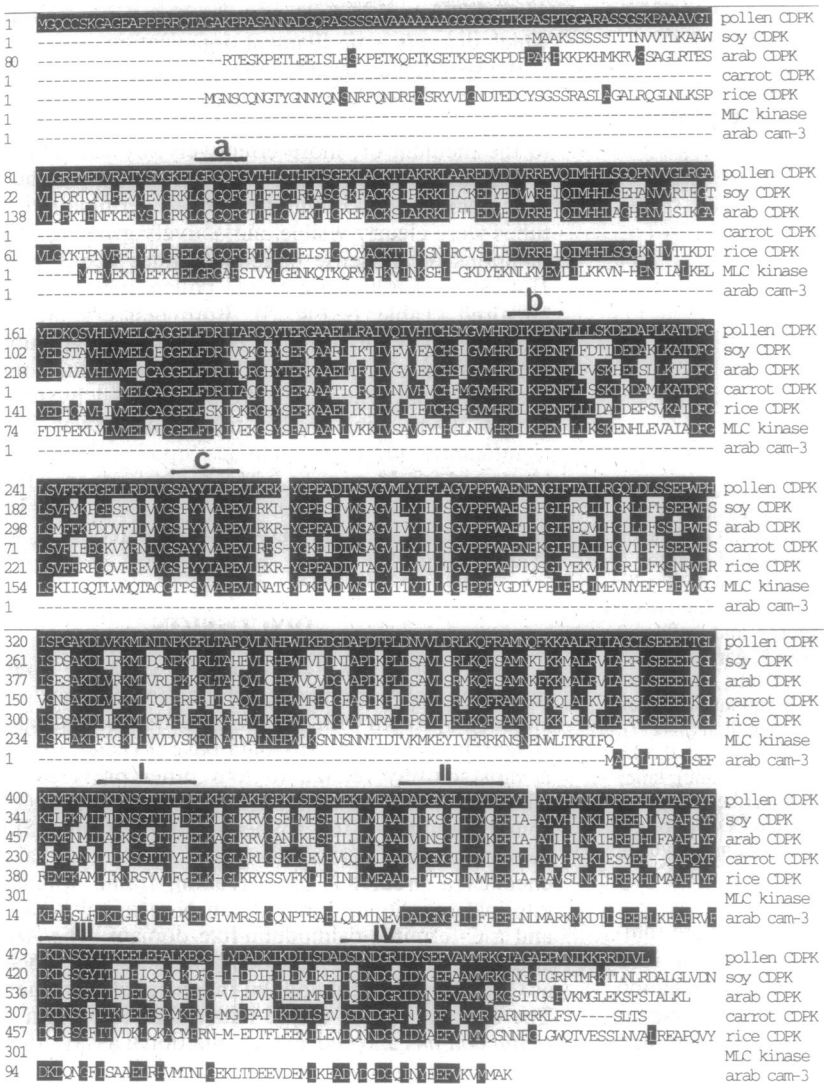

FIG. 2. Deduced amino acid sequence of the maize CDPK, aligned with the soybean (15), *Arabidopsis* (16), carrot (17), and rice (18) CDPKs. The alignment also includes the myosin light chain (MLC) kinase (19) and the *Arabidopsis* calmodulin 3 (20). The N-terminal first 125 aa of the *Arabidopsis* CDPK and the last 10 aa of the C terminus of the soybean CDPK are not included in the alignment. The alignment was performed by using the Clustal method with PAM 250 residue weight table. Residues that match maize pollen CDPK are shaded with solid black. Motifs a, b, and c are diagnostic kinase sequences, and motifs I, II, III, and IV are putative calcium-binding sites.

the soybean CDPK (15). Interestingly, these substitutions are characteristic of animal calmodulins (21).

Genomic DNA Hybridization Analysis. Blots of genomic DNA isolated from maize (211D) plants were probed with a 1.0-kb fragment that spans the kinase domain of the CDPK. When the blots were washed under nonstringent conditions (i.e., 2× SSC/0.1% SDS at 45°C for 30 min), they exhibited many bands (data not shown). These results indicated that there is a family of related genes with high homology within the kinase domain (7). As shown in Fig. 3, the number of bands was reduced to three when the blots were washed at higher stringency (0.1× SSC/0.1% SDS at 62°C for 30 min), indicative of very few copies (possibly three) of the CDPK gene in the maize genome. In the case of *Bam*HI-digested DNA, the fourth band is due to the presence of an internal *Bam*HI site in the gCDPK. gCDPK has no internal sites for *Sph* I, *Bgl* II, *Cla* I, and *Hin*dIII.

Developmental Regulation and Pollen-Specific Expression of CDPK. Spatial and temporal expression of the CDPK gene was analyzed by Northern hybridization (Fig. 4). A 1.8-kb transcript was detected only in RNA isolated from pollen, with no other tissue expressing the CDPK gene (Fig. 4*A*). Furthermore, a temporal analysis of the expression showed that CDPK transcripts become detectable at the early trinucleate stage of pollen development, and the steady-state level of CDPK mRNA was increased in the late trinucleate stage and in mature and germinating pollen (Fig. 4*B*). Western blot analysis revealed that the CDPK protein accumulates during the later stages of pollen development and its highest level occurs during pollen germination (Fig. 4*C*).

Germination and Pollen Tube Growth Analysis. Maize pollen germinated (40–75%) and pollen tubes grew adequately when the external Ca^{2+} concentrations were varied from 0.5 mM to 5 mM (Fig. 5). Higher or lower Ca^{2+} concentrations severely reduced germination rates (2–10%) and pollen tube growth (Fig. 5). The addition of calmodulin antagonists such as calmidazolium and W-7 [which is also a CDPK inhibitor (10)]

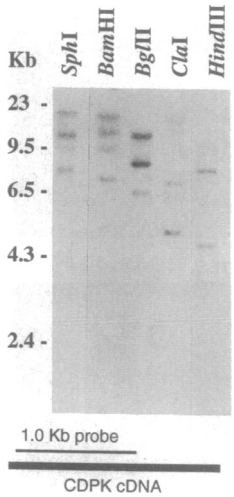

FIG. 3. Southern blot hybridization of genomic DNA. Each lane was loaded with 5 μg of maize DNA digested with the indicated enzyme. Blots were probed with a ^{32}P-labeled 1.0-kb fragment from the CDPK cDNA and washed at high stringency.

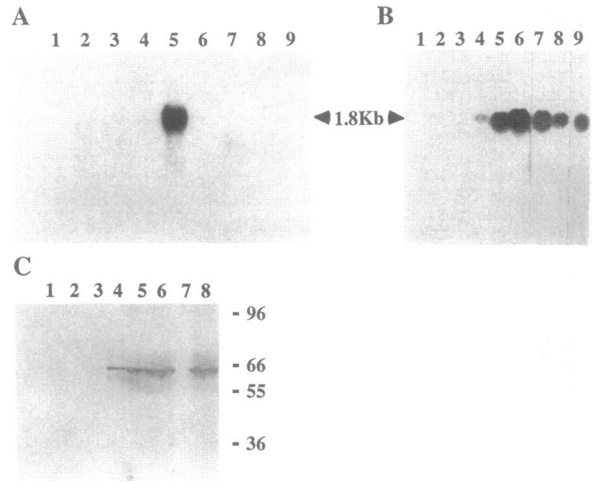

FIG. 4. Developmental and tissue-specific expression of the CDPK gene. (*A*) Northern blot analysis of total RNA (20 μg in each lane) isolated from different maize tissues: 1, inner leaf sheath; 2, inner leaf whorl; 3, green leaf; 4, anther; 5, immature pollen; 6, silk; 7, kernel; 8, pith; and 9, root. (*B*) Northern blot analysis of total RNA (10 μg per lane) isolated from maize microspores and pollen at different stages: 1, uninucleate microspore; 2 and 3, early and late binucleate immature pollen; 4 and 5, early and late trinucleate immature pollen; 6, mature pollen (211D); 7, germinating pollen (211D); 8, mature pollen (2N217); and 9, germinating pollen (2N217). CDPK transcripts were detected with both the 1.0- and 0.5-kb probes. (*C*) Western blot analysis of proteins (10 μg per lane); lanes 1, 2, 3, 4, 5, 6, and 8 correspond to the material described for lanes 1, 2, 3, 4, 5, 6, and 7 of *B*. Lane 7 of *C* is empty. Maize CDPK protein was detected with monoclonal antibodies raised against soybean CDPK (9). Masses are in kDa.

to the germination medium resulted either in complete inhibition (i.e., not detectable) of pollen germination or in a disruption of the pollen tube if added once pollen germination has started (Fig. 5). A similar effect of total inhibition and/or disruption of the pollen tube was obtained when the germination medium was supplemented with cytochalasin D (Fig. 5), which disrupts actin filament organization.

We specifically targeted the CDPK activity (mRNA levels and level of translation) by using antisense oligonucleotides delivered into the germinating pollen tube either by addition to the medium or, more effectively, by calcium phosphate precipitation. Oligonucleotides were added to the germination medium 30–45 min after pollen germination, so pollen tubes were clearly visible and actively growing. When combinations of antisense oligonucleotides ID#3 + ID#9 and JE#28 + JE#30 were used, pollen tube elongation was impaired (Table 1; Fig. 5). Both delivery methods were effective, but the negative effects on pollen tube growth appeared later (more than 2 h) when oligonucleotides were taken up than when they were delivered by precipitation (about 30–40 min). Pollen tube growth was impaired at significantly lower frequencies upon calcium phosphate treatment for 30 min, or when control nonspecific oligonucleotides (see *Materials and Methods*), sense ID#3 and ID#9 and sense JE#28 and ID#30 oligonucleotides, were used (Table 1; Fig. 5).

DISCUSSION

Maize CDPK Has Regulatory and Catalytic Domains. We report the isolation and characterization of a maize clone that encodes a protein with homology to kinases and calmodulins. A search of the GenBank data base revealed that this clone is most closely related to four other previously reported CDPK genes, isolated from *Arabidopsis* plants (17), carrot somatic embryos (16), soybean cultured cells (15), and rice developing seeds (18). Like these genes, the maize CDPK encodes a predicted open reading frame which contains multiple functional domains: an N-terminal kinase domain and a C-terminal calmodulin-like domain. The *Arabidopsis* and soybean CDPKs have been characterized biochemically as Ca^{2+}-regulated calmodulin-independent enzymes (10, 16). The overexpression of the maize CDPK protein in *Escherichia coli* has allowed similar functional assays that confirm

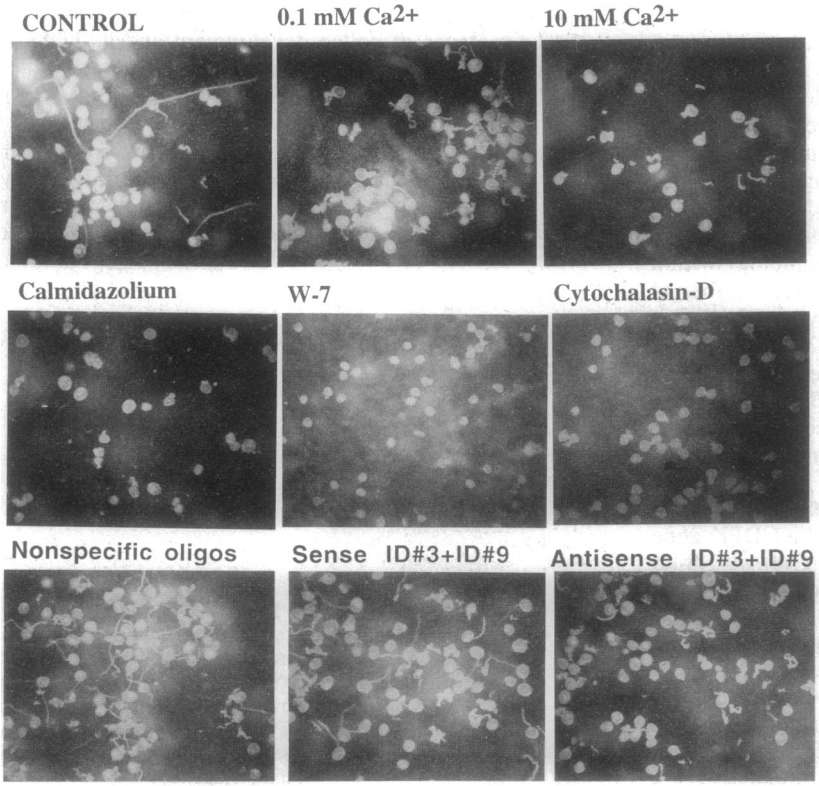

FIG. 5. *In vitro* maize pollen tube growth test. Effects of treatments affecting the calcium status of the pollen and pollen tube and/or the activity of the CDPK protein on the pollen tube growth are shown. (× 25.) Optimal germination and pollen tube growth occur at 2 mM external Ca^{2+} concentration (Control). Lower (0.1 mM) or higher (10 mM) Ca^{2+} concentrations severely affected pollen tube growth. The addition of compounds such as calmidazolium, W-7, or cytochalasin D also impaired pollen tube growth. In the experiment shown, the mentioned substances were added to the basal germination medium 30–45 min after pollen germination was initiated and the observation was made 3–4 h later. Pollen tubes grew well in the presence of either nonspecific or sense ID#3 + ID#9 oligonucleotides. Treatment of the pollen tubes with antisense oligonucleotides (ID#3 + ID#9) directed to CDPK resulted in a high frequency of disruption of the pollen tubes (see Table 1).

Table 1. Effect of oligonucleotides on the integrity of maize pollen tubes

Oligonucleotides	% disrupted pollen tubes		
	Exp. 1	Exp. 2	Exp. 3
None	5	2	4
Nonspecific primers	7	6	8
Sense ID#3	7	9	10
Sense ID#9	9	9	12
Sense JE#28	10	11	12
Sense JE#30	9	10	8
Antisense ID#3	15	24	28
Antisense ID#9	28	34	38
Antisense JE#28	32	36	39
Antisense JE#30	34	38	42
Antisense ID#3 + ID#9	69	58	61
Antisense JE#28 + JE#30	52	64	67

Pollen germination media were supplemented with 50 μg of each oligonucleotide 30 min after germination. Values represent the percentage of pollen tubes disrupted after 4 h of incubation with the oligonucleotides.

its Ca^{2+}-regulated protein kinase enzymatic activity (J.J.E., unpublished data). Calmodulins and kinases are usually encoded by gene families; their expression is differentially controlled and they may have distinct tissue distributions (7). Therefore, the presence of both domains on the same polypeptide has two potential advantages: at the expression level, it constitutes the tightest mechanism of coordination because both are expressed simultaneously; and at the functional level, the catalytic activity would be tightly controlled by the regulatory domain. Thus, these CDPKs may be capable of a faster and more versatile response to calcium variations, and they seem to fulfill all requirements to be ideal stimulus-coupling proteins.

Maize CDPK Is a Pollen-Specific Protein. Northern and Western analyses have shown that the expression of the maize CDPK gene is pollen specific and that it is temporally restricted to late stages of pollen development (Fig. 4*B*). Expression of a rice CDPK gene has been shown to be spatially and temporally regulated during seed development. These two cases of tissue-specific and developmentally regulated expression of CDPKs clearly point to the involvement of these regulatory/catalytic proteins in tissue-specific processes (18). In contrast, the soybean CDPK gene is expressed throughout the plant, from leaves to roots (9). Immunocytological localization of the CDPK protein with a soybean CDPK-monoclonal antibody indicated an association with the F-actin network in three different plant species, namely *Glycine*, *Allium*, and *Tradescantia* (9). The case of *Tradescantia* is particularly interesting because a CDPK-like protein has been characterized and colocalized with the actin microfilament system by using the same soybean monoclonal antibody. The maize CDPK is also recognized by monoclonal antibodies raised against soybean CDPK (Fig. 4*C*). This close relatedness of the maize CDPK to the soybean CDPK and its pollen-specific expression suggest that maize CDPK protein could be associated with the pollen microfilament system as well.

Maize CDPK Protein Is Involved in Pollen Germination. Germination and growth of the pollen tube require intense cytoplasmic streaming (2). These processes involve cytoskeleton reorganization and they are thought to be Ca^{2+} dependent (3). *In vitro* maturation of pollen is strongly affected by Ca^{2+} concentrations (13, 22), and germination and growth of pollen tubes are restricted to a specific range of Ca^{2+} levels (Fig. 5). These observations indicate that during pollen germination the sensing of Ca^{2+} is very important, and Ca^{2+}-binding regulatory proteins may be involved in this process. Indeed, the pollen-specific maize CDPK protein may serve a very specific function during germination and growth of the pollen tube by acting as a stimulus–response protein. The crucial role of the maize CDPK in pollen germination is further substantiated by experimental data showing that nonspecific inhibition of calmodulins by calmodulin-antagonists or a more specific inhibition of the CDPK by antisense oligonucleotides impaired germination and growth of the pollen tube (Table 1; Fig. 5). We do not know yet how this regulation occurs, but a possible working model could be as follows: maize CDPK senses Ca^{2+} variations modulating its kinase activity, leading to phosphorylation of appropriate substrate(s) involved in the cytoskeleton dynamics required for pollen tube growth.

Biochemical and immunocytological studies have indicated that CDPKs may be involved in the regulation of actin/myosin interactions in plants (7). However, the mechanism(s) underlying this regulation are poorly understood because the *in vivo* substrate(s) of the CDPKs have not yet been characterized. Because the expression of the maize CDPK is confined to pollen, this system could provide a valuable experimental model for the study of CDPK regulation of cytoskeleton function and other cellular processes. The biochemical characterization of the maize CDPK enzyme may afford new insight on how it regulates pollen germination as well as clues to identify its *in vivo* target(s).

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1. Stanley, R. G. & Linskens, H. F. (1974) in *Pollen: Biology, Biochemistry, Management*, ed. Linskens, H. F. (Springer, New York), pp. 3–12.
2. Heslop-Harrison, J. (1988) in *Sexual Reproduction in Higher Plants*, eds. Cresti, M., Gori, P. & Pacini, E. (Springer, Berlin), pp. 195–203.
3. Hepler, P. K. & Wayne, R. O. (1985) *Annu. Rev. Plant Physiol.* **36**, 397–439.
4. Picton, J. M. & Steer, M. W. (1983) *Protoplasma* **115**, 11–17.
5. Picton, J. M. & Steer, M. W. (1982) *J. Theor. Biol.* **98**, 15–20.
6. Stull, J. T., Nunnally, M. H. & Michnoff, C. H. (1986) in *The Enzymes: Control by Phosphorylation*, eds. Boyer, P. D. & Krebs, E. G. (Academic, New York), Vol. 17, pp. 113–166.
7. Roberts, D. M. & Harmon, A. C. (1992) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 375–414.
8. Schaller, G. E., Harmon, A. C. & Sussman, M. R. (1992) *Biochemistry* **31**, 1721–1727.
9. Putnam-Evans, C., Harmon, A. C., Palevitz, B. A., Fechheimer, M. & Cormier, M. J. (1989) *Cell Motil. Cytoskel.* **12**, 12–22.
10. Harmon, A. C., Putnam-Evans, C. & Cormier, M. J. (1987) *Plant Physiol.* **83**, 830–837.
11. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
12. Shure, M., Wessler, S. & Fedoroff, N. (1983) *Cell* **35**, 225–233.
13. Dupuis, I. & Pace, G. M. (1993) *Plant Cell Rep.* **12**, 564–568.
14. Tolou, H. (1993) *Anal. Biochem.* **215**, 156–158.
15. Harper, J. F., Sussman, M. R., Schaller, G. E., Putnam-Evans, C., Charbonneau, H. & Harmon, A. C. (1991) *Science* **252**, 951–954.
16. Harper, J. F., Binder, B. M. & Sussman, M. R. (1993) *Biochemistry* **32**, 3282–3290.
17. Suen, K.-L. & Choi, J. H. (1991) *Plant Mol. Biol.* **17**, 581–590.
18. Kawasaki, T., Hayashida, N., Baba, T., Shinozaki, K. & Shimada, H. (1993) *Gene* **129**, 183–189.
19. Tan, J. L. & Spudich, J. A. (1990) *J. Biol. Chem.* **265**, 13818–13824.
20. Perera, I. Y. & Zielinski, R. E. (1992) *Plant Mol. Biol.* **19**, 649–664.
21. Roberts, D. M., Lukas, T. J. & Watterson, D. M. (1986) *Crit. Rev. Plant Sci.* **4**, 311–339.
22. Pareddy, D. R. & Petolino, J. F. (1992) *Plant Cell Rep.* **11**, 535–539.