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# Calcium-Dependent Protein Kinase Genes in Corn Roots

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## Summary

Two cDNAs encoding $Ca^{2+}$-dependent protein kinases (CDPKs), CRPK1 and CRPK2 (corn root protein kinase 1 and 2), were isolated from the root tip library of corn (*Zea mays* L., cv. Merit) and their nucleotide sequences were determined. Deduced amino acid sequences of both the clones have features characteristic of plant CDPKs, including all 11 conserved serine/threonine kinase subdomains, a junction domain and a calmodulin-like domain with four $Ca^{2+}$-binding sites. Northern analysis revealed that *CRPK1* mRNA is preferentially expressed in roots, especially in the root tip; whereas, the expression of *CRPK2* mRNA was very low in all the tissues tested. *In situ* hybridization experiments revealed that *CRPK1* mRNA is highly expressed in the root apex, as compared to other parts of the root. Partially purified CDPK from the root tip phosphorylates syntide-2, a common peptide substrate for plant CDPKs, and the phosphorylation was stimulated 7-fold by the addition of $Ca^{2+}$. Our results show that two CDPK isoforms are expressed in corn roots and they may be involved in the $Ca^{2+}$-dependent signal transduction process.

*Key words: Calcium, corn, protein kinases.*

*Abbreviations:* CDPK = $Ca^{2+}$ dependent protein kinase; CRPK = corn root protein kinase.

## Introduction

Calcium, a universal second messenger has been shown to mediate a number of signal transduction processes in plants (Poovaiah and Reddy, 1987, 1993; Gilroy and Trewavas, 1994). Signal-induced changes in cytosolic $Ca^{2+}$ are sensed by a group of $Ca^{2+}$-binding proteins that are involved in the regulation of diverse cellular activities. $Ca^{2+}$-dependent protein kinases (CDPKs) are widely distributed in plants and are believed to be involved in various physiological processes (Roberts and Harmon, 1992). CDPK purified from different plants exhibits $Ca^{2+}$-dependent, but calmodulin-independent, kinase activity and efficiently phosphorylates H1 histone. CDPK also phosphorylates synthetic peptides which have a Basic-Xaa-Xaa-Ser/Thr motif such as peptides derived from glycogen synthase (Polya et al., 1989; Putnam-Evans et al., 1990; Harmon et al., 1994).

Several CDPK cDNAs have been isolated from higher plants (Harper et al., 1991; Suen and Choi, 1991; Poovaiah and Reddy, 1993). The predicted structure of the CDPK polypeptide is composed of three distinct domains: a serine/threonine kinase domain, a junction domain, and a $Ca^{2+}$-binding regulatory domain that resembles calmodulin (Roberts, 1993). It is suggested that the presence of the calmodulin-like domain makes CDPK exhibit similar biochemical characters to calmodulin. For instance, CDPK is known to bind to hydrophobic matrices such as phenyl-Sepharose in a $Ca^{2+}$-dependent manner. Its activity is inhibited by calmodulin antagonists such as W7 and trifluoperazine, possibly because of interactions of these drugs with the calmodulin-like domain. The junction domain between the kinase and calmodulin-like domains contains an autoinhibitory sequence which blocks binding of the substrates to the catalytic site of the kinase. Upon binding $Ca^{2+}$, the calmodulin- like domain directly interacts with the sequence near the autoinhibitory domain and releases the catalytic site from the inhibition.

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(Harmon et al., 1994; Harper et al., 1994). Structural and biochemical properties of CDPK suggest that this kinase plays key roles in $Ca^{2+}$-mediated signal transduction in plants (Roberts, 1993).

Some CDPKs are known to be expressed in an organ specific and a stage specific manner during plant development, while others are stage independent and induced by environmental stress (Kawasaki et al., 1993; Urao et al., 1994; Estruch et al., 1994; Monloy and Dhindsa, 1995). Physiological roles of CDPKs are not clearly understood, but their subcellular localization implies that they might control the function of the plasma membrane and cytoskeletal proteins (Roberts and Harmon, 1992).

$Ca^{2+}$ gradients have been shown to occur across the curvature zone of roots and it is suggested that $Ca^{2+}$-dependent phosphorylation may be involved in inducing gravitropic curvature (Scolum and Roux, 1983; Poovaiah and Reddy, 1987). Merit corn roots show light-dependent gravitropic curvature and have been used for studies on signal perception and transduction (Feldman and Gildow, 1984; McFadden and Poovaiah, 1988). We have cloned two cDNAs that code for CDPK isoforms from Merit corn roots, which are structurally similar to other known plant CDPKs. Furthermore, we present data showing the differential mRNA expression of one of the isoforms (*CRPK1*) in the root tip by northern analysis and *in situ* hybridization.

## Materials and Methods

### *Plant materials*

Corn (*Zea mays* L. cv. Merit) seeds were a gift from the Asgrow Seed Company, Kalamazoo, MI. Seeds were imbibed in water for 12 h and were germinated in the dark at room temperature for 3 or 4 days. Seedlings were grown on vermiculite in the light for 8 days. Different plant parts were excised and immediately frozen in liquid nitrogen.

### *Polymerase chain reaction (PCR)*

λgt11 and λZAP cDNA libraries were prepared from corn roots and used for PCR amplification. PCR was performed using degenerate oligonucleotide primers corresponding two regions of conserved amino acid sequences, GGELFD and DLKPEN from the catalytic domain of serine/threonine protein kinases. The amplification reaction contained 1× PCR buffer (Cetus Corporation), 200 µmol/L dNTPs, 50 pmoles of each primer, 1.5 mmol/L $MgCl_2$, 1 µL cDNA library ($7.1\times10^5$ pfu) and 1 unit of Taq DNA polymerase in a 50 µL total reaction volume. The cycling profile was 30 cycles of 94 °C for 30 sec, 48 °C for 1 min, and 72 °C for 1 min, followed by a final extension step carried out at 72 °C for 10 min. The PCR fragment was subcloned into pBluescriptII KS+ plasmid and nucleotide sequences were determined using the dideoxynucleotide chain terminator method (Sanger et al., 1977).

### *cDNA library screening*

Approximately $1\times10^6$ plaques were screened using $^{32}P$-random prime labeled DNA fragments as described by Sambrook et al. (1989). After three rounds of screening, hybridizing plaques were selected. The cDNA inserts were subcloned into pBluescriptII KS+ for nucleotide sequencing.

### *Southern analysis*

Corn genomic DNA (10 µg) was digested with different restriction enzymes and separated on a 0.8 % agarose gel. The digested DNA was transferred onto a nylon membrane and hybridized with $^{32}P$-labeled CDPK cDNA probes as described by Reddy et al. (1993).

### *Northern analysis*

Total RNA was isolated by phenol-SDS method (Verwoerd at al., 1989) and poly(A)$^+$ RNA was purified by oligo(dT) cellulose column chromatography (Sambrook et al., 1989). Northern analysis was performed using $^{32}P$-labeled antisense RNA probe as described by Takezawa et al. (1995).

### *In situ hybridization*

Four day-old corn roots were fixed and dehydrated as described by Cox and Goldberg (1988). The tissues were embedded in a paraffin block and tissues were sectioned at 5 µm thickness with a microtome. These sections were mounted onto gelatin coated slides, deparaffinized and rehydrated. The sections were treated with 1 µg/mL proteinase K for 30 min at 37 °C, followed by washing with 2×SSC. The cDNA probe was labeled with digoxygenin (Boehringer Mannheim) and Klenow enzyme for 12 h at 37 °C. Prehybridization and hybridization were performed as described by Cox and Goldberg (1988). After washing with 0.1×SSC, the sections were incubated with anti-DIG antibody (1:500) in TBST containing 1% BSA for 3 h in a moist chamber. The hybridization signals were detected by Protein G Gold (1:100) (Sigma Chemical) and silver enhancement reagents (Pharmacia LKB Biotech.).

### *Partial purification of corn root CDPK*

Corn root CDPK was partially purified by ammonium sulfate precipitation and phenyl-Sepharose column chromatography as described by Guo and Roux (1990). Root tissues were homogenized in a buffer containing 50 mmol/L Tris-Cl (pH 7.5), 2 mmol/L EDTA, 1 mmol/L DTT 0.1 % Triton X-100 and 0.5 mmol/L PMSF, and the homogenate was centrifuged at 10,000 $g_n$ for 10 min at 4 °C. The supernatant was further centrifuged at 40,000 $g_n$ for 1 h at 4 °C to obtain soluble proteins. Solid ammonium sulfate was added to the supernatant to 50 % saturation and incubated on ice for 2 h. The mixture was centrifuged at 10,000 $g_n$ for 30 min and the pellet was dissolved in the loading buffer containing 25 mmol/L Tris-Cl (pH 7.5), 1 mmol/L DTT and 1 mmol/L $CaCl_2$. After removal of insoluble materials by centrifugation at 10,000 $g_n$ for 10 min, the proteins were loaded onto a phenyl-Sepharose CL-4B column. The column was extensively washed with the buffer containing 25 mmol/L Tris-Cl (pH 7.5), 1 mmol/L DTT 0.5 M NaCl and 1 mmol/L $CaCl_2$, and the proteins were eluted by buffer containing 25 mmol/L Tris-Cl (pH 7.5), 1 mmol/L DTT and 2 mmol/L EGTA. Active fractions were collected, and concentrated using Centricon-30 (Amicon Inc.) to remove small molecular weight proteins. Glycerol was added to 20 % and the enzyme was stored at 70 °C until use.

### *Protein kinase assays*

CDPK assays were performed by measuring incorporation of $^{32}P$ into a synthetic peptide syntide-2 (Hashimoto and Soderling, 1987), which is efficiently phosphorylated by most plant CDPKs. The 20 µL reaction mixture contained 50 mmol/L Hepes (pH 7.5), 10 mmol/L magnesium acetate, 2 µL enzyme, 1 mmol/L [$\gamma^{32}P$] ATP (100–200 cpm/pmol), 100 µmol/L syntide-2, and either 0.5 mmol/L $CaCl_2$ or 2 mmol/L EGTA. The reaction was carried out for 10 min at 30 °C. The reaction mixture was spotted onto P-81 phosphocellu-

lose paper disc and $^{32}P$ incorporation was determined by measuring radioactivity in scintillation liquid.

## Results

### *Cloning and sequence analysis*

Degenerate oligonucleotide primers corresponding to the highly conserved regions, GGELFD and DLKPEN were used in PCR to amplify CDPK cDNA fragments. Two DNA fragments of 141 bp long, were cloned and sequenced. The sequence comparison revealed that the deduced amino acid sequences of both the clones share high homology with other plant CDPKs and animal $Ca^{2+}$/calmodulin-dependent protein kinases. Two cDNA clones (*CRPK1* and *CRPK2*) were obtained by screening corn root cDNA libraries using these 141 bp fragments as probes. The *CRPK1* (1527 bp) encodes a 480 amino acid polypeptide (Poovaiah and Reddy, 1993); whereas the *CRPK2* cDNA encodes a 513 amino acid polypeptide (Fig. 1). Primary amino acid sequences of both the clones contain all eleven subdomains conserved in eukaryotic serine/threonine protein kinases (Hanks et al., 1988). Sequence analysis revealed that this kinase domain has high homology to plant CDPKs. Both *CRPK1* and *CRPK2* also have a putative junction domain and the calmodulin-like domain with four $Ca^{2+}$-binding sites (Fig. 1). The *CRPK1* clone is incomplete because it does not contain the methionine codon at the $NH_2$-terminal to the subdomain I. The first 50 amino acid sequence of CRPK2 does not have significant homology to other plant CDPKs. CRPK1 amino acid sequence has 75 % similarity (60 % identity) to the CRPK2. CRPK1 also has 77 % and 82 % amino acid similarity with carrot and soybean CDPKs, respectively (Suen and Choi, 1991; Harper et al., 1991).

### *Southern analysis of CRPK1 and CRPK2*

To determine the approximate copy number of *CRPK1* and *CRPK2*, Southern blot analyses were carried out (Fig. 2). Corn genomic DNA digested with *Bam*HI, *Eco*RI or *Hind*III was hybridized with $^{32}P$-labeled *CRPK1* and *CRPK2* cDNAs as probes. Despite that *CRPK1* and *CRPK2* share 60 % identity at the nucleotide level, these clones showed distinct patterns of hybridizing bands in each digest, when the hybridization and washing were carried out at high stringent condition. The results indicated that these clones are coded by a few copies in the genome.

### *Highest expression of CRPK1 mRNA in the root tip*

Northern analyses were carried out using 5 µg total RNA to study the expression of *CRPK1* and *CRPK2* mRNAs in

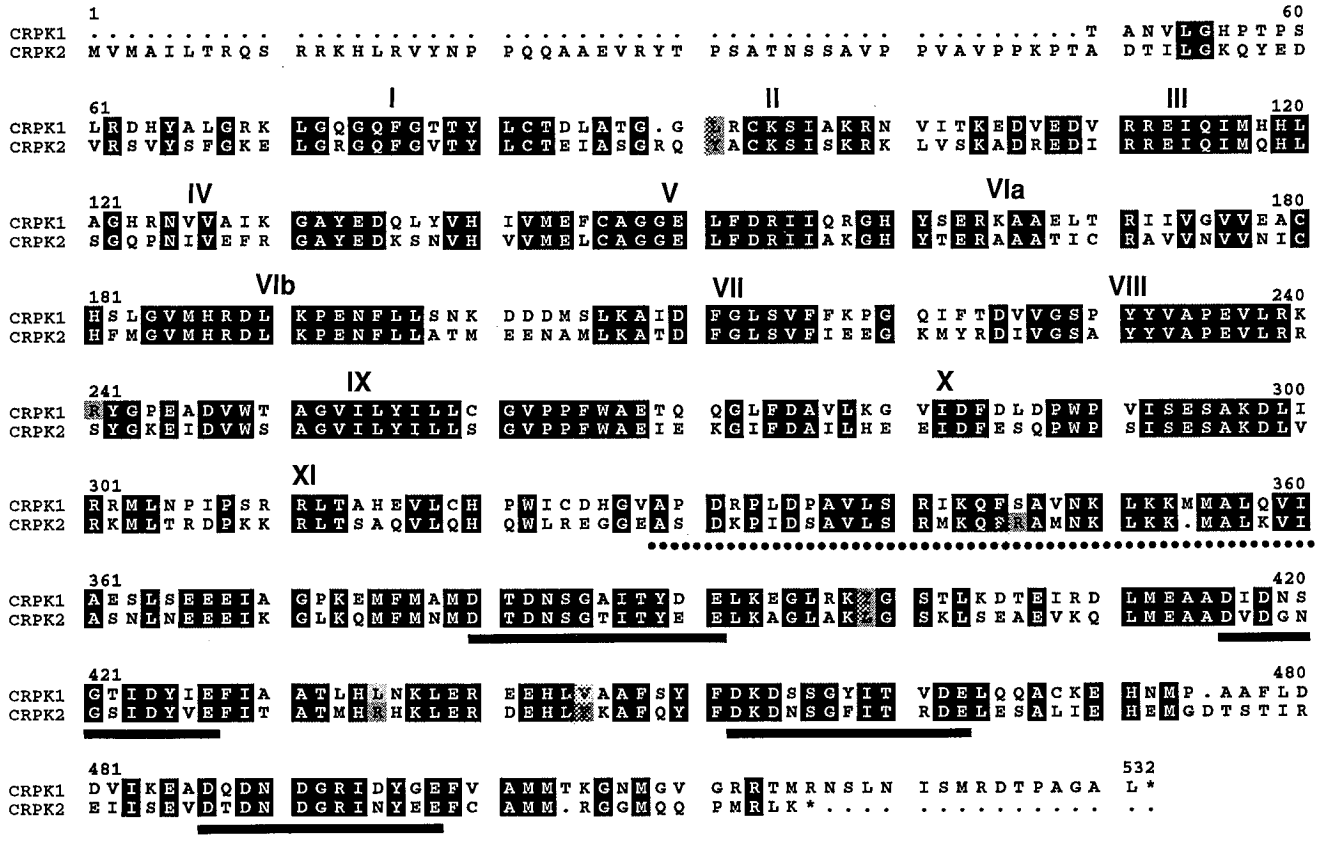


**Fig. 1:** Deduced amino acid sequence of *CRPK1* and *CRPK2*. Subdomains I to XI for serine/threonine protein kinase are marked. The junction domain is underlined with a large dotted line (· · · ·). Four EF-hand motifs are underlined (solid lines).

leaves and roots from 8 day-old seedlings. *CRPK1* mRNA (~2 kb) is expressed in roots but the expression was very low in leaves (Fig. 3 A). The size of the hybridizing signal appeared in a similar position when root poly $(A)^+$ RNA was used instead of total RNA. The amount of *CRPK1* mRNA in roots from 8 day-old seedlings and 3 day-old roots germinated in the dark, were similar. We also compared the *CRPK1* mRNA expression in the root tip and root base. Several independent experiments indicated that the root tip accumulates high amounts of *CRPK1* mRNA as compared to the root base. Results from a typical experiment are shown in Fig. 3 B. These results suggest that there is a gradient of the accumulation of *CRPK1* transcript in different parts of roots. We did not detect *CRPK2* mRNA by northern analysis, probably because the amount of mRNA expression is very low. When we performed RNase protection assay, a more sensitive RNA detection method, the signal indicating *CRPK2* mRNA expression in roots was detected, but the expression level was much lower than *CRPK1*. Therefore, we did not study *CRPK2* mRNA expression further.

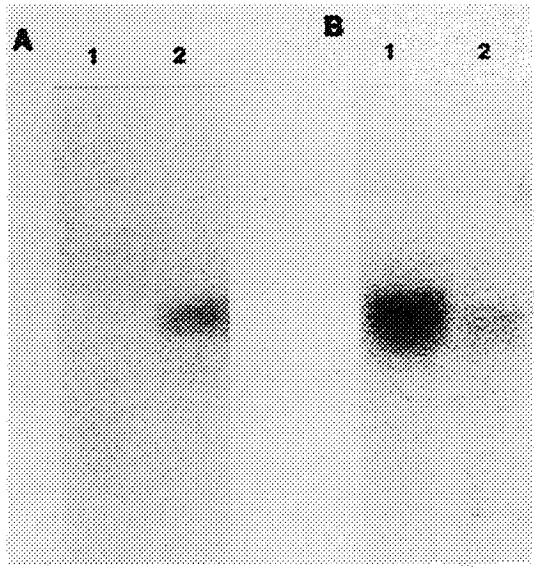


**Fig. 3:** Northern analysis of *CRPK1*. Total RNA (5 µg) denatured by glyoxal-DMSO was separated on 1% agarose gel and northern analysis was performed using $^{32}$P-labeled *CRPK1* antisense RNA probe. **A.** RNA samples from 8 day-old seedlings. Lane 1, leaf; Lane 2, root. **B.** RNA samples from 3 day-old roots were used. Lane 1, root tip; Lane 2, root base.

### *In situ localization of CRPK1 mRNA*

To study the abundance of the *CRPK1* transcript in different regions of root tissues, *in situ* hybridization experiments were carried out. The *CRPK1* mRNA was not uniformly expressed; the apical portion of the root accumulated high levels of *CRPK1* mRNA; whereas, the transcript level was reduced in the quiescent center and the prevascular and pericortical regions (Fig. 4).

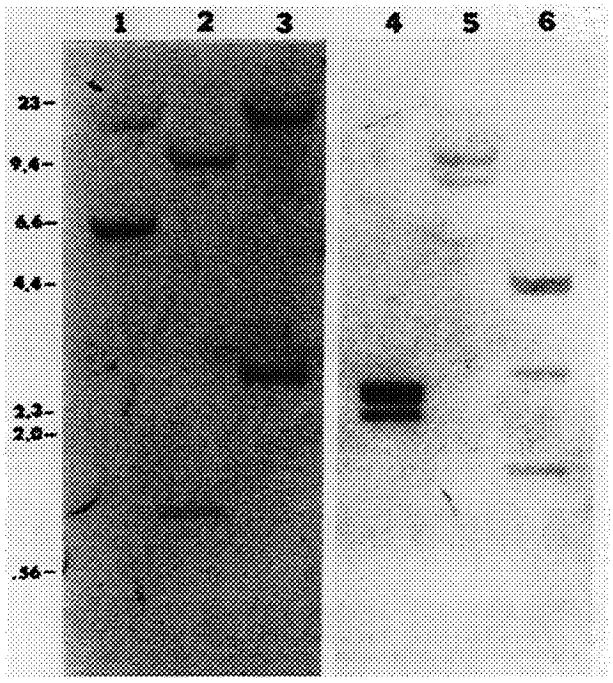


**Fig. 2:** Southern blot analysis of corn CDPKs. Corn genomic DNA (5 µg) digested with *Bam*HI (lanes 1 and 4), *Eco*RI (lanes 2 and 5), and *Hind* III (lanes 3 and 6) were hybridized with $^{32}$P-labeled cDNA of *CRPK1* (lanes 1–3) and *CRPK2* (lanes 4–6). The positions of molecular size markers are shown in kb on the left.

### *Activity of partially purified corn root CDPK*

Results of northern and *in situ* hybridizations indicated that the root tip contains higher levels of CDPK message than other parts of roots. CDPK was partially purified from corn root tip by ammonium sulfate precipitation and $Ca^{2+}$-dependent phenyl-Sepharose column chromatography. The partially purified root tips CDPK phosphorylated syntide-2 and the phosphorylation was stimulated about 7-fold by $Ca^{2+}$ (Fig. 5 A). The enzyme also phosphorylates H1 histone in a $Ca^{2+}$-dependent manner (data not shown). To study the $Ca^{2+}$-dependent phosphorylation, syntide-2 phosphorylation was compared between the root tip and the root base. The results shown in Fig. 5 indicate that the root tip has higher CDPK activity than the root base.

## Discussion

The results presented here show that corn root expresses two genes encoding different isoforms of CDPK. Both *CRPK1* and *CRPK2* encode a protein that is structurally similar to other plant CDPKs with conserved catalytic and regulatory domains (Fig. 1). The calmodulin-like domain of CRPK1 and CRPK2 contains four intact EF-hand motifs with conserved $Ca^{2+}$-ligating amino acids at x, y, z, −y, −x, and −z positions (Roberts, 1993). This suggests that the calmodulin-like domain of CRPK1 and CRPK2 undergoes conformational changes upon binding $Ca^{2+}$, similar to calmodulin.

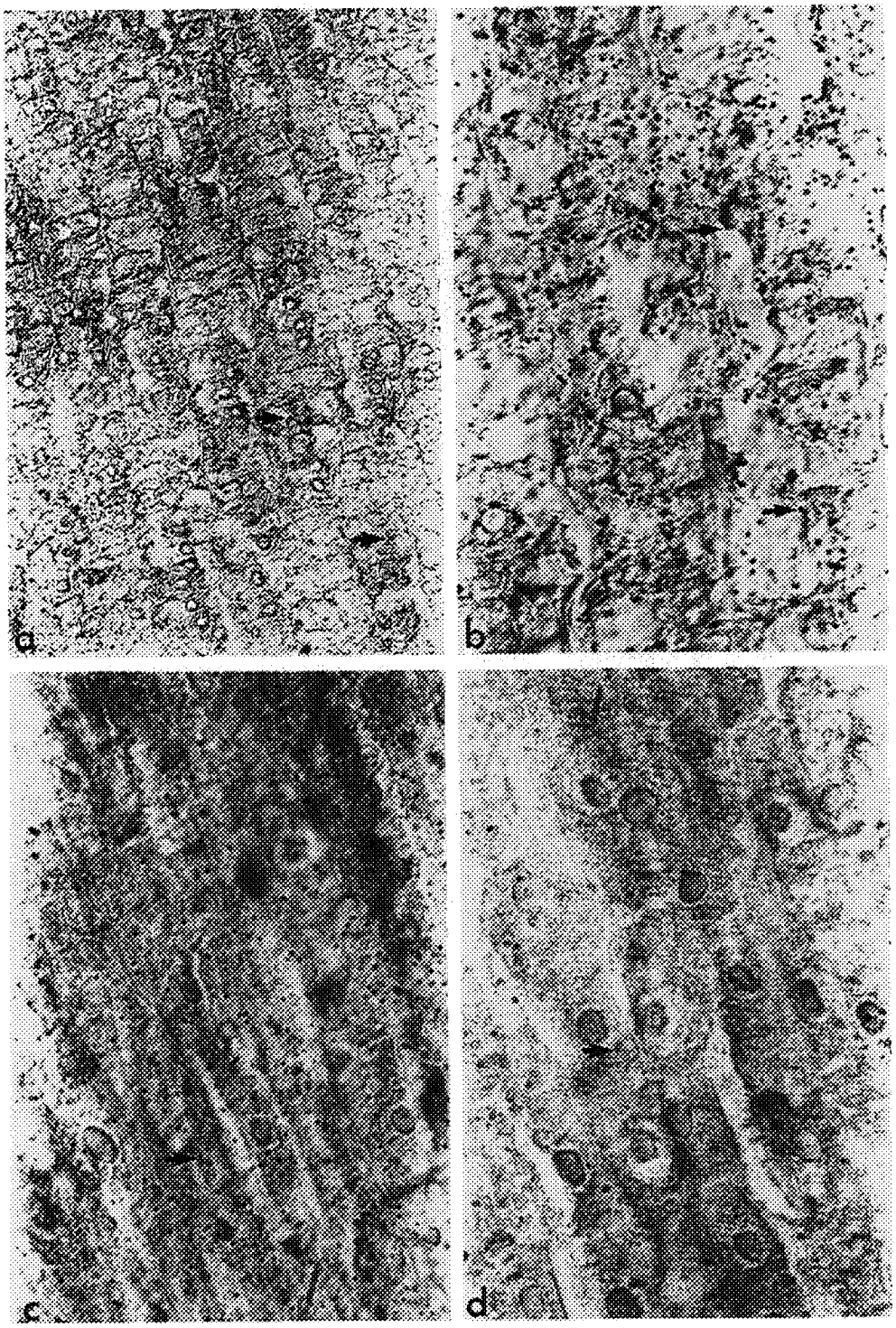


**Fig. 4:** *In situ* hybridization of *CRPK1*. The dots in the pictures represent *CRPK1* mRNA. Plates (a) and (b) show the mRNA levels in the corn root tip at low (125×) and high (312.5×) magnifications, respectively. Plates (c) and (d) (312.5×) show decreasing mRNA levels towards the base of the corn roots.

Southern analysis at high stringent condition revealed that these clones are coded by a small number of genes (Fig. 2). However, several bands were detected when we performed the Southern hybridization at low stringent condition. Considering that other plant CDPKs belong to a gene family (Urao et al., 1994), it is likely that corn also has other CDPK

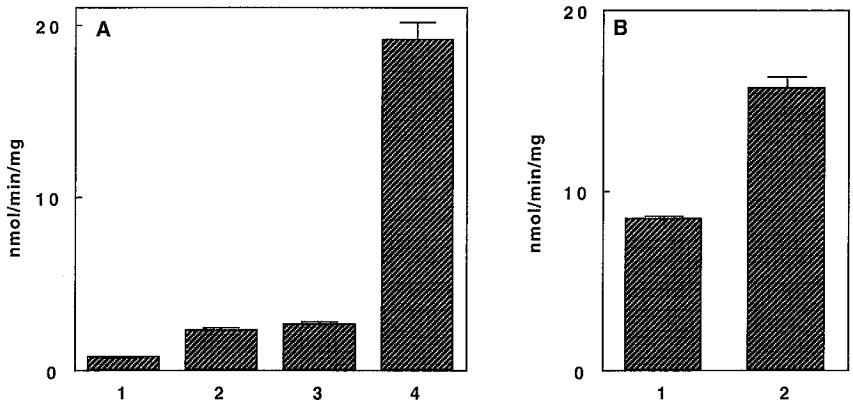


**Fig. 5: A.** $Ca^{2+}$-dependence of corn root tip CDPK. Partially purified corn root tip CDPK was assayed for phosphorylation either in the presence or absence of $Ca^{2+}$. Column 1, ($-Ca^{2+}$); Column 2; ($+Ca^{2+}$); Column 3, ($-Ca^{2+}$) (+syntide-2); Column 4, ($+Ca^{2+}$) (+syntide-2). **B.** Comparison of CDPK activity in the root tip (column 1) and root base (column 2). The values are represented as the difference of $^{32}P$ incorporation into syntide-2 between ($-Ca^{2+}$) and ($+Ca^{2+}$) reactions.

isoforms. Although northern analysis indicated that *CRPK1* mRNA is preferentially expressed in root tips (Fig. 3), it is likely that corn has other CDPK isoforms which are expressed in other organs (Estruch et al., 1994). The root tip is likely to be involved in the perception and transduction of external stimuli such as light and gravity (Scolum and Roux, 1983; Poovaiah and Reddy, 1987). $Ca^{2+}$- and $Ca^{2+}$/calmodulin-dependent phosphorylation is postulated to play a role in transducing gravity stimulus and inducing asymmetric growth of specific cells leading to a gravitropic response (Friedmann and Poovaiah, 1991). CRPK1, whose mRNA is highly expressed in the root tip (Fig. 3 B), may be one of the components involved in the mediation of the gravitropic response. We also attempted to study *CRPK1* mRNA expression during the light-dependent gravitropic curvature. Dark grown corn roots, after exposure to light, showed a detectable increase in *CRPK1* mRNA (data not shown). In contrast to *CRPK1, CRPK2* mRNA expression was much lower in the root tissue. *CRPK2* may be expressed in other cell types or tissues that were not tested during this investigation, or the expression may be induced in a signal-responsive manner.

Studies using the partially purified CDPK from corn roots, revealed that CDPK activity in the root tip was higher than the root base (Fig. 5 B). Even though *CRPK1* expression was very high in the root tips, the increase in kinase activity was not correlated to the *CRPK1* mRNA level. This could be due to the presence of other isoforms that have not been cloned yet. It should also be noted that a certain amount of CDPKs in the root tip may be associated with membrane fractions (Battey, 1990; Schaller et al., 1992). It is important to identify target cells where CDPK isoforms are localized and furthermore, it is also important to resolve the subcellular localization of these isoforms using isoform-specific antibodies. The protein extract used in this study may contain a substantial amount of $Ca^{2+}$/calmodulin-dependent protein kinase that can also phosphorylate syntide-2. This is unlikely since we used phenyl-Sepharose affinity column for partial purification of the CDPK. Furthermore, the CDPK activity in corn roots was not inhibited by 100 µmol/L KN-93, a specific inhibitor for $Ca^{2+}$/calmodulin-dependent protein kinase II (Sumi et al., 1991; Lu et al., 1993). CDPK isoforms are known to exist in plants, but it is not clear how these isoforms are involved in cellular regulation. Some CDPK isoforms recognize a broad range of substrates while others have a narrow substrate specificity (DasGupta, 1994). CDPK isoforms may have specific targets in different organs and may control cellular functions such as proton pumping (Sussman, 1994) or regulate the activity of metabolic enzymes (Huber et al., 1994).

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# Transport, Cytoplasmic Accumulation and Mechanism of Action of the Toxin Eutypine in *Vitis vinifera* Cells

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## Summary

The mechanism of the toxic action of eutypine, 4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzaldehyde, a toxin produced by the fungus *Eutypa lata,* the agent of dying arm disease of the grapevine, was investigated with cell suspension cultures of *Vitis vinifera* cv. Gamay and $^{14}C$-labelled eutypine. The study of the chemical characteristics of eutypine revealed that the toxin is a weak acid (pKa = 6.2), which also has a lipophilic character (water/octanol partition coefficient of 86). Eutypine was rapidly taken up by the cells and accumulated against a concentration gradient. Uptake showed no saturation at high eutypine concentration, and neither structural analogues of eutypine nor protein modifying reagents had an inhibitory effect on eutypine uptake. These data suggest a mechanism of passive diffusion for eutypine uptake. The eutypine accumulation observed in cells can be partly explained by an ion trapping mechanism related to the ionization state of the toxin. It has been demonstrated that some eutypine molecules insert into cellular lipids, i.e. cell membranes. Such partition of eutypine in the cells might play a major role in the expression of its toxicity. Eutypine seems to affect the functioning of mitochondria by an uncoupling effect or by inhibiting succinate dehydrogenase activity. These results suggest that eutypine has the same properties as the uncoupling agents that act as mobile proton carriers. The reduction of the energetic charge following eutypine action could explain the symptoms observed in diseased plants.

*Key words: Uptake, transport, uncoupling, grapevine, eutypine, toxin, Eutypa lata, Vitis vinifera.*

*Abbreviations:* CCCP = carbonylcyanide *m*-chlorophenylhydrazone; DIDS = 4,4′-diisothiocyano-2,2′-stilbenedisulfonate; DMO = 5,5-dimethyloxazolidine-2,4-dione; MES = 2-(N-morpholino)ethanesulfonic acid; pCMB = *p*-chloromercuribenzoic acid; SDH = succinate dehydrogenase; TLC = thin layer chromatography; TRIS = Tris-(hydroxymethyl)amino methane.

## Introduction

Eutypine, 4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzaldehyde, is a toxic compound secreted by *Eutypa lata* (Pers. : Fr.) Tul., the fungus responsible for Eutypa dieback of the grapevine (Renauld et al., 1989; Tey-Rulh et al., 1991). This disease is currently causing a large-scale economic problem throughout the world by reducing the yield and vegetative growth of grapevines (Munkvold et al., 1994). Through pruning wounds, ascospores of the fungus infect and colonize first the xylem tissue, then the cambium and phloem in the vine trunk and arms (Moller and Kasimatis, 1978). After an incubation period of 3 years or more a canker forms around the infected wound and the symptoms appear on the herba-