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Two genes that encode Ca^{2+}-dependent protein kinases are induced by drought and high-salt stresses in *Arabidopsis thaliana*

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Abstract Two cDNA clones, cATCDPK1 and cATCDPK2, encoding Ca^{2+}-dependent, calmodulin-independent protein kinases (CDPK) were cloned from *Arabidopsis thaliana* and their nucleotide sequences were determined. Northern blot analysis indicated that the mRNAs corresponding to the *ATCDPK1* and *ATCDPK2* genes are rapidly induced by drought and high-salt stress but not by low-temperature stress or heat stress. Treatment of *Arabidopsis* plants with exogenous abscisic acid (ABA) had no effect on the induction of *ATCDPK1* or *ATCDPK2*. These findings suggest that a change in the osmotic potential of the environment can serve as a trigger for the induction of *ATCDPK1* and *ATCDPK2*. Putative proteins encoded by *ATCDPK1* and *ATCDPK2* which contain open reading frames of 1479 and 1488 bp, respectively, are designated ATCDPK1 and ATCDPK2 and show 52% identity at the amino acid sequence level. ATCDPK1 and ATCDPK2 exhibit significant similarity to a soybean CDPK (51% and 73%, respectively). Both proteins contain a catalytic domain that is typical of serine/threonine protein kinases and a regulatory domain that is homologous to the Ca^{2+}-binding sites of calmodulin. Genomic Southern blot analysis suggests the existence of a few additional genes that are related to *ATCDPK1* and *ATCDPK2* in the *Arabidopsis* genome. The ATCDPK2 protein expressed in *Escherichia coli* was found to phosphorylate casein and myelin basic protein preferentially, relative to a histone substrate, and required Ca^{2+} for activation.

Key words *Arabidopsis thaliana* · Protein kinase
Water stress · Ca^{2+}-dependent activity

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Introduction

Plants respond to drought conditions by modulating a number of physiological and biochemical processes. Water deficit induces stomatal closure in leaves, which reduces further water loss. Stomatal closure is induced by changes in the volume of guard cells. To optimize CO_2 fixation in photosynthesis while minimizing water loss, guard cell volume responds within minutes to change in light intensity, CO_2 concentration, humidity, and plant hormones such as abscisic acid (ABA) and auxin. Cell volume is controlled osmotically, largely by the influx or efflux of K^+ (MacRobbie 1989; Blatt 1990). External stimuli, including the plant hormone ABA, which cause stomatal closure, also cause increases in cytoplasmic Ca^{2+} (McAinsh et al. 1990; Gilroy et al. 1991; Trewavas and Gilroy 1991). The increase in free Ca^{2+} in the cytoplasm may trigger a signal for stomatal closure.

Recently, several reports have described genes that respond to water deficit at the transcriptional level (Bray 1988; 1991; Mundy and Chua 1988; Guerrero and Mullet 1988; Close et al. 1989; Bartels et al. 1990; Guerrero et al. 1990; Skriver and Mundy 1990). In order to study molecular mechanisms of the signal transduction pathways that link dehydration stress and gene expression, we have cloned and characterized genes that respond to dehydration in *Arabidopsis thaliana*. We have identified nine independent cDNAs that are responsive to dehydration (RD clones; Yamaguchi-

Shinozaki et al. 1992; Shinozaki et al. 1993). Northern blot analysis has revealed highly variable timing of the induction of *RD* genes and has shown that six of these genes respond to ABA while three do not (Yamaguchi-Shinozaki et al. 1992; Shinozaki et al. 1993). It appears that Ca^{2+} may function as a second messenger in the signal transduction pathways that mediate drought-induced expression of *rd* genes. We attempted to isolate cDNAs encoding Ca^{2+}-related proteins, such as Ca^{2+}-dependent protein kinase, calmodulin-dependent protein kinase (CaM kinase), calmodulin (CaM), and Ca^{2+} channel protein, in order to understand the role of Ca^{2+} in the signal transduction pathways between the initial dehydration signal and gene expression.

Ca^{2+}-dependent, calmodulin-independent protein kinases (CDPK) or their genes have been found in various plants (Putnam-Evans et al. 1990; Harper et al. 1991; Suen and Choi 1991; Kawasaki et al. 1993; Harper et al. 1993). The CDPK proteins have two domains; one is a catalytic domain that contains conserved amino acid residues found in CaM kinases, and the other is a Ca^{2+}-binding domain that contains four typical E-F hand structures found in calmodulins (Roberts and Harmon 1992). In order to isolate cDNA clones encoding CDPKs that can function under water deficit conditions, we carried out polymerase chain reactions (PCR) with oligonucleotide primers corresponding to conserved regions of CDPKs, using as template cDNAs that had been prepared from dehydrated *Arabidopsis* plants. We isolated several PCR-amplified fragments of DNA that contain partial sequences of CDPKs. We screened a cDNA library prepared from dehydrated plants with the PCR fragments and isolated three cDNA clones that encode CDPKs.

In the present study, we report two CDPK genes that are rapidly induced by dehydration and high-salt conditions and the biochemical characteristics of these gene products. We also discuss the role of CDPKs in the signal transduction cascade during drought conditions.

Materials and methods

Dehydration and exposure to ABA, high salt, cold, and heat

A. thaliana (Columbia ecotype) was grown on GM agar plates (Valvekens et al. 1988) at 22° C for 3–4 weeks before stress treatments. For dehydration, plants were harvested from GM agar plates and then left on Whatman 3MM paper at 22° C and 60% humidity under dim light. For high salt treatment, plants were grown hydroponically in 250 mM NaCl or in water as a control under dim light. For ABA treatment, plants were grown hydroponically in 100 μM ABA under dim light. Plants to be subjected to cold and heat stresses were exposed to 4° C and 40° C, respectively, under continuous light. At various times after initiation of each of these treatments, plants were harvested and frozen in liquid nitrogen.

Preparation of a cDNA library and DNA templates for PCR

Total RNA was isolated from dehydrated, intact rosette plants, according to the method of Nagy et al. (1988), and was purified by chromatography on oligo(dT) cellulose, as described elsewhere (Maniatis et al. 1982). Double-stranded cDNA was synthesized from poly(A)$^+$ RNA using a cDNA synthesis System Plus (Amersham International, UK). cDNA libraries were constructed using a cDNA cloning system λgt11 (Amersham) and λZAPII (Stratagene, La Jolla, Calif., USA). DNA templates for amplification by PCR were prepared from the cDNA libraries by phenol extraction and CsCl gradient centrifugation, and were amplified by PCR using primers that corresponded to the two adaptors of the insert cDNAs (Mizoguchi et al. 1993).

PCR

Oligodeoxyribonucleotide primers, 5′-GGIGTIATGCA(T/C)(C/A)GIGA(T/C)(T/C)TIAA(A/G)CCIGA(A/G)AA-3′ and 5′-GTIAT(A/G)AAICCIGAICC(A/G)TC(T/C)TT(A/G)TC-3′, corresponding to the amino acid sequences GVMHRDLKPEN (subdomain VIb) and DKDGSGYIT (third E-F hand), respectively, which are conserved in CDPKs, were synthesized. The primers were phosphorylated with T4 polynucleotide kinase, as described elsewhere (Maniatis et al. 1982). PCR was carried out in a 100-μl reaction mixture containing 10 mM TRIS-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each of dNTPs, 100 pmol each primer, 5 ng template and 2.5 U AmpliTaq (Perkin Elmer Cetus, Norwalk, Conn., USA). As template, cDNA prepared from *Arabidopsis* rosette plants after dehydration for 10 h was employed. The reaction mixture was overlaid with 100 μl of liquid paraffin and subjected to 30 cycles of amplification at 94° C for 1 min (denaturation), at 43° C for 1 min (annealing), at 72° C for 2 min (polymerization), followed by a final incubation at 72° C for 7 min. The amplified DNA was purified in a 5% polyacrylamide gel and cloned into the *Sma*I site of pBluescript SKII (Stratagene).

Cloning and DNA sequencing

An *Arabidopsis* cDNA library (constructed with λZAPII) prepared from rosette plants, that had been dehydrated for 1 h was screened by plaque hybridization, as described by Maniatis et al. (1982). PCR-amplified fragments were used as probes after labeling with α-[^{32}P]dCTP with a random primer kit, according to the manufacturer's instructions (Boehringer, Mannheim, Germany). Positive plaques were purified and the inserts were subcloned into pBluescript phagemid by an in vivo excision process, according to the manufacturer's instructions (Stratagene). DNA sequences were determined using the dye-primer cycle sequencing method using a DNA sequencer (model 373A; Applied Biosystems, San Jose, Calif., USA). The GENETYX software system (Software Development, Tokyo, Japan) was used for the analysis of DNA and amino acid sequences.

Southern and Northern analysis

Genomic Southern analysis was done as described elsewhere (Maniatis et al. 1982). Thirty micrograms of total RNA was fractionated in a 1% agarose gel containing formaldehyde and was blotted onto a nitrocellulose filter (Maniatis et al. 1982). The filter was hybridized with [^{32}P]-labeled PCR-amplified fragments in 50% formamide, 5 × SSC, 25 mM sodium phosphate buffer, pH 6.5, 10 × Denhardt's solution, and 250 μg/ml of denatured salmon sperm DNA at 42° C. The filter was washed twice with 0.1 × SSC, 0.1% SDS at 60° C for 15 min and subjected to autoradiography.

Construction and expression of the recombinant CDPK proteins

A 1.7-kb *Bam*HI fragment containing the full-length cDNA of cATCDPK2 was cloned into the *Bam*HI site of pGEX-1 vector

(Smith and Johnson 1988). A 1.7-kb *Kpn*I fragment containing the full-length cDNA of cATCDPK1 as blunt-ended by treatment with Klenow fragment and was then cloned into the *Sma*I site of pGEX-2T vector (Smith and Johnson 1988). *Escherichia coli* JM109 cells (Maniatis et al. 1982) were used as hosts for expression of the recombinant proteins. Overnight cultures of *E. coli* cells carrying the recombinant plasmid were inoculated at 1:10 dilution in 100 ml of 2 × YT medium (16 g Bacto tryptone, 10 g Bacto yeast extract, 5 g NaCl/l) than contained 25 μg/ml ampicillin, and were grown for 1 h at 37° C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and incubation was continued for an additional 12 h at 15° C. The cells were harvested, washed, and resuspended in 1 ml of MTPBS (150 mM NaCl, 15 mM Na_2HPO_4, 4 mM NaH_2PO_4). The cell suspension was sonicated twice for 10 s each on ice and Triton-X100 was added to a final concentration of 1%, and centrifuged at 15000 rpm for 15 min at 4° C. The supernatant was mixed with 80 μl of glutathione-Sepharose (Pharmacia, Uppsala, Sweden) and rocked for 10 min at 4° C. The Sepharose beads were washed 3 times with MTPBS and the fusion protein was eluted from the beads by incubation for 10 min at 4° C with 150 μl of 50 mM TRIS-HCl, pH 8.0 containing 5 mM reduced glutathione. Glycerol was added to a final concentration of 40% and the purified protein was stored at −80° C.

In vitro phosphorylation assays

Casein (dephosphorylated, Sigma), myelin basic protein (Sigma), and histone III-S (Sigma) were used as substrates for in vitro phosphorylation assays. Phosphorylation reactions were carried out at 37° C for 10 min in 20 μl of a reaction mixture containing 25 mM TRIS-HCl, pH 8.0, 10 mM $MgCl_2$, 0.3 μCi γ-[^{32}P]ATP (5000 Ci/mmol, Amersham), 0.01 mM ATP, 10 μg substrate, and 0.1 μg purified recombinant GST-ATCDPK protein in the presence of 0.21 mM $CaCl_2$ (+Ca buffer) or 0.21 mM EGTA (−Ca buffer). The reaction was stopped by adding an equal volume of 2 × SDS sample buffer (Laemmli 1970) and the reaction products were separated by SDS-PAGE (15% polyacrylamide gel). The gel was washed with a solution containing 5% TCA and 1.65% sodium pyrophosphate, and was then washed with distilled water. The labeled products were visualized by autoradiography and their radioactivities were estimated with a Bio-Image analyzer (Fujix BAS-2000, Fuji Film, Tokyo, Japan).

Results

Isolation of cDNAs that encode putative Ca^{2+}-dependent protein kinase from dehydrated *Arabidopsis* plants

The remarkable conservation of *CDPK* genes make it possible to use PCR to isolate homologous sequences from *Arabidopsis*. For the PCR, we used two primer DNA sequences encoding the conserved catalytic domain VIb and the third E-F hand of the Ca^{2+}-binding domain of CDPKs with, as templates, cDNAs prepared from *Arabidopsis* rosette plants that had been dehydrated for 10 h. Two PCR-amplified fragments, PC33 and

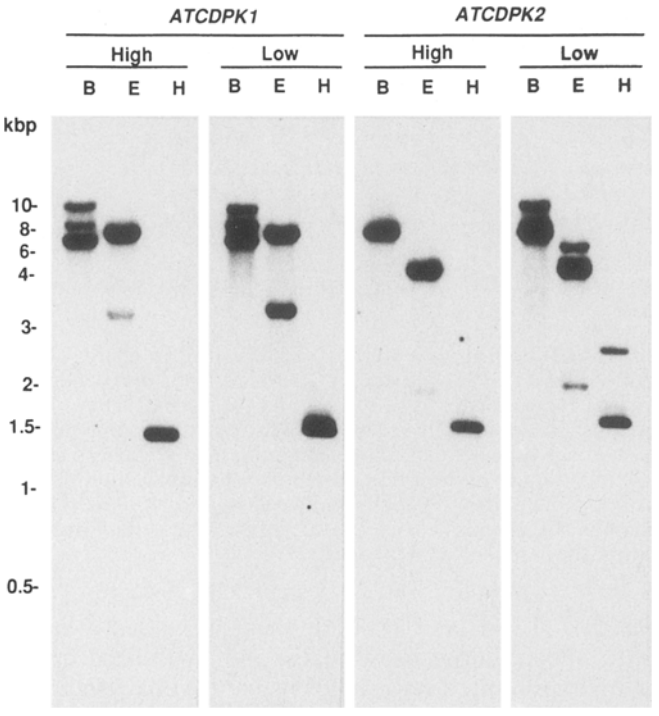

Fig. 1 Southern blot analysis of genomic sequences that correspond to *ATCDPK1* and *ATCDPK2*. Genomic DNA was digested with *Bam*HI (*B*), *Eco*RI (*E*), and *Hin*dIII (*H*), fractionated in 0.7% agarose gels and transferred to nitrocellulose membranes. Filters were hybridized with [^{32}P]-labeled polymerase chain reaction (PCR) fragments, PC33 and PC37, at 42° C and washed with either 0.5 × SSC, 0.5% SDS at 50° C (low stringency) or 0.1 × SSC, 0.1% SDS at 65° C (high stringency). *High* and *Low* represent high- and low-stringency hybridization conditions, respectively. The sizes of DNA markers are indicated in kbp

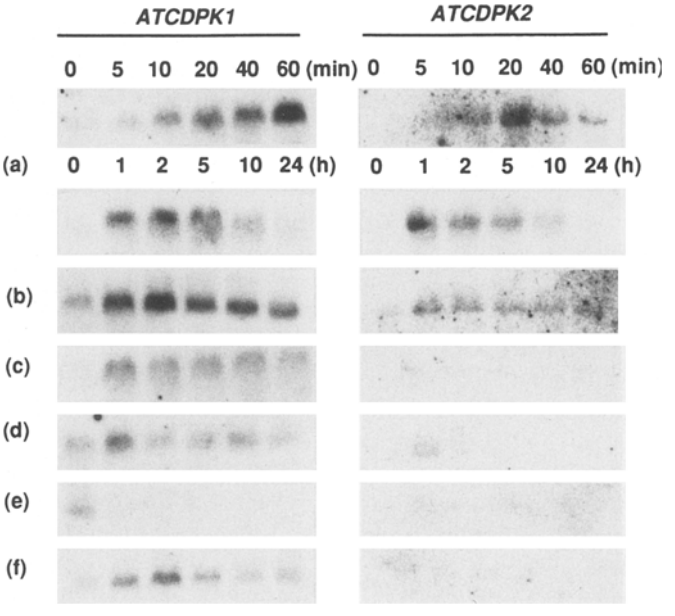

Fig. 2 Expression of *ATCDPK1* and *ATCDPK2* in response to dehydration, exogenous abscisic acid (ABA), salt, cold and heat stresses. Each lane was loaded with 30 μg of total RNA from 3- to 4-week-old unbolted *Arabidopsis* plants which had been dehydrated (a), transferred from agar plates to hydroponic growth in 250 mM NaCl (b) or in 100 μM ABA (c), transferred to and grown at 4° C (d) or at 40° C (e), or transferred from agar plates to hydroponic growth in water (f). The number above each lane indicates the duration of treatment prior to isolation of RNA. RNA was analyzed by Northern blotting with gene-specific probes, PC33 and PC37

A

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CCAGACTCAAAAGAATCAAAACCATCTTCAAAGCCGAAAAAACCCAATCGAGATCGGAAATTAAACCCATTCGCCGGAGATTTCACCAGATCCCCAGCTC     100
CAATACGTGTTCTCAAAGATGTAATCCCTATGAGCAATCAAACTCAGATCAGCGACAAATACATCTTAGGTCGTGAATTAGGTCGAGGCGAATTCGGAAT     200
                                  M  S  N  Q  T  Q  I  S  D  K  Y  I  L  G  R  E  L  G  R  G  E  F  G  I
CACTTACCTCTGTACTGATCGTGAAACCCACGAAGCTTTAGCTTGCAAATCGATTTCAAAGCGAAAGCTTCGAACAGCTGTCGATATCGAAGACGTTCGT     300
 T  Y  L  C  T  D  R  E  T  H  E  A  L  A  C  K  S  I  S  K  R  K  L  R  T  A  V  D  I  E  D  V  R
CGTGAGGTAGCGATTATGTCTACTTTACCTGAGCATCCAAACGTAGTTAAGCTTAAGGCTAGTTATGAGGATAACGAGAACGTGCATCTGGTTATGGAGC     400
R  E  V  A  I  M  S  T  L  P  E  H  P  N  V  V  K  L  K  A  S  Y  E  D  N  E  N  V  H  L  V  M  E  L
TTTGTGAAGGAGGTGAGCTTTTTGATCGGATTGTTGCTAGAGGACATTACACGGAGCGTGCTGCTGCAGCTGTTGCGAGAACGATTGCTGAGGTTGTGAT     500
  C  E  G  G  E  L  F  D  R  I  V  A  R  G  H  Y  T  E  R  A  A  A  A  V  A  R  T  I  A  E  V  V  M
GATGTGTCACTCTAATGGAGTTATGCATCGAGATTTGAAACCTGAGAATTTCTTGTTTGCTAATAAAAAGGAGAATTCTCCACTAAAGGCTATTGATTTT     600
 M  C  H  S  N  G  V  M  H  R  D  L  K  P  E  N  F  L  F  A  N  K  K  E  N  S  P  L  K  A  I  D  F
GGCTTGTCTGTGTTCTTCAAACCTGGAGATAAGTTTACAGAGATTGTAGGAAGTCCGTATTATATGGCTCCAGAAGTGTTGAAGAGAGATTATGGACCAG     700
G  L  S  V  F  F  K  P  G  D  K  F  T  E  I  V  G  S  P  Y  Y  M  A  P  E  V  L  K  R  D  Y  G  P  G
GGGTTGATGTGTGGAGTGCCGGAGTTATTATCTATATCTTGCTCTGTGGTGCTCCTCCGTTTTGGGCTGAGACTGAACAAGGTGTTGCTCTTGCGATCTT     800
  V  D  V  W  S  A  G  V  I  I  Y  I  L  L  C  G  A  P  P  F  W  A  E  T  E  Q  G  V  A  L  A  I  L
GCGGGGAGTTCTTGATTTTAAGAGAGACCCTTGGCCTCAGATATCAGAGAGTGCCAAGAGCCTTGTGAAGCAGATGTTGGATCCTGATCCGACTAAGCGG     900
 R  G  V  L  D  F  K  R  D  P  W  P  Q  I  S  E  S  A  K  S  L  V  K  Q  M  L  D  P  D  P  T  K  R
TTAACTGCTCAGCAAGTGTTAGCTCACCCATGGATACAGAATGCAAAGAAAGCTCCCAATGTTCCTTTAGGAGATATAGTCAGATCTAGGTTGAAGCAGT    1000
L  T  A  Q  Q  V  L  A  H  P  W  I  Q  N  A  K  K  A  P  N  V  P  L  G  D  I  V  R  S  R  L  K  Q  F
TCTCTATGATGAACAGATTCAAAAAGAAAGTTCTTCGTGTAATTGCGGAGCACTTGTCTATTCAAGAGGTTGAAGTGATAAAGAACATGTTCTCACTGAT    1100
   *
 S  M  M  N  R  F  K  K  K  V  L  R  V  I  A  E  H  L  S  I  Q  E  V  E  V  I  K  N  M  F  S  L  M
GGATGATGACAAGGATGGTAAAATAACTTACCCGGAACTCAAAGCTGGGCTTCAGAAGGTCGGTTCACAACTTGGTGAACCAGAGATCAAAATGTTGATG    1200
 D  D  D  K  D  G  K  I  T  Y  P  E  L  K  A  G  L  Q  K  V  G  S  Q  L  G  E  P  E  I  K  M  L  M
GAAGTGGCGGATGTCGATGGAAATGGGTTTCTGGATTATGGAGAGTTTGTAGCTGTGATAATTCACTTGCAGAAGATAGAGAATGATGAACTTTTCAAAC    1300
E  V  A  D  V  D  G  N  G  F  L  D  Y  G  E  F  V  A  V  I  I  H  L  Q  K  I  E  N  D  E  L  F  K  L
TAGCTTTTATGTTTTTCGACAAAGATGGAAGTACATACATTGAACTTGATGAGCTACGGGAAGCTTTAGCGGATGAGTTAGGCGAGCCAGACGCCAGTGT    1400
  A  F  M  F  F  D  K  D  G  S  T  Y  I  E  L  D  E  L  R  E  A  L  A  D  E  L  G  E  P  D  A  S  V
TCTAAGCGACATCATGCGTGAAGTTGACACTGACAAGGACGGACGTATAAACTATGATGAGTTTGTGACGATGATGAAAGCTGGAACTGACTGGAGAAAG    1500
 L  S  D  I  M  R  E  V  D  T  D  K  D  G  R  I  N  Y  D  E  F  V  T  M  M  K  A  G  T  D  W  R  K
GCATCGAGACAATATTCAAGAGAGAGGTTCAAAAGCTTAAGCATTAACTTGATGAAAGATGGGTCATTGCATCTCCATGACGCTCTCACTGGACAAACTG    1600
A  S  R  Q  Y  S  R  E  R  F  K  S  L  S  I  N  L  M  K  D  G  S  L  H  L  H  D  A  L  T  G  Q  T  V
TTCCTGTTTAAATTTATTCGTTATCACCAAAAACAGAGCAATGCTCCGTTTTTTCCCCATTTCATAAATTGGGAATTTTCCGGGCTTGTTCTTTGAGGGA    1700
  P  V
TGGGAATTTTACGGAAGCTATGGTTCTTTACATATATAAACATTTTACATTGTATTTTGTATGTAATGTTTTGTTCAAAGGTTGTATTTTTATTGTCTCA    1800
AAGCCCCTAAACCAAAGAGTCAAAGGAAAGATCTTTATTACAAAAAAAAA                                                        1850
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PC37, contained sequences homologous to regions of the catalytic domain and the Ca^{2+}-binding domains of CDPKs.

The corresponding full-length cDNAs were recovered from a cDNA library that had been prepared from *Arabidopsis* rosette plants dehydrated for 1 h. From a total of 5×10^5 plaques, we obtain 14 and 4 positive clones that hybridized to PC33 and PC37 probes, respectively (data not shown). The cDNA inserts were subcloned into the pSKII$^-$ vector. We sequenced the largest insert from each group and named them cATCDPK1 and cATCDPK2. Their corresponding genes were named *ATCDPK1* and *ATCDPK2*, respectively.

Analysis of *Arabidopsis CDPK* genes by Southern blotting

The number of *CDPK* genes in the *Arabidopsis* genome was estimated by Southern blot analyses (Fig. 1). Nuclear DNA from *Arabidopsis* plants was digested with *Bam*HI, *Eco*RI, and *Hin*dIII, electrophoresed, blotted onto nitrocellulose membranes, and hybridized under both high- and low-stringency conditions using the PCR fragments as probes. Under high-stringency conditions, PC33 hybridized with one *Hin*dIII, three *Bam*HI and two *Eco*RI fragments; PC37 hybridized with one *Bam*HI, one *Eco*RI, and one *Hin*dIII fragment. However, under low-stringency conditions, a few extra bands of DNA were detected, suggesting that there may be a few additional *CDPK* genes in the *Arabidopsis* genome.

Fig. 3A, B Nucleotide and deduced amino acid sequences of two cDNAs, cATCDPK1 **A** and cATCDPK2 **B**. The DNA sequences include the putative coding regions and the 5′- and 3′-noncoding regions. The amino acid sequences of the putative coding regions are shown beneath the DNA sequences. The sequences corresponding to the primers that were used for amplification of conserved sequences by PCR are indicated by *arrows*. Putative autoinhibitory domains are *underlined* and possible autophosphorylation sites are indicated by *asterisks*

B

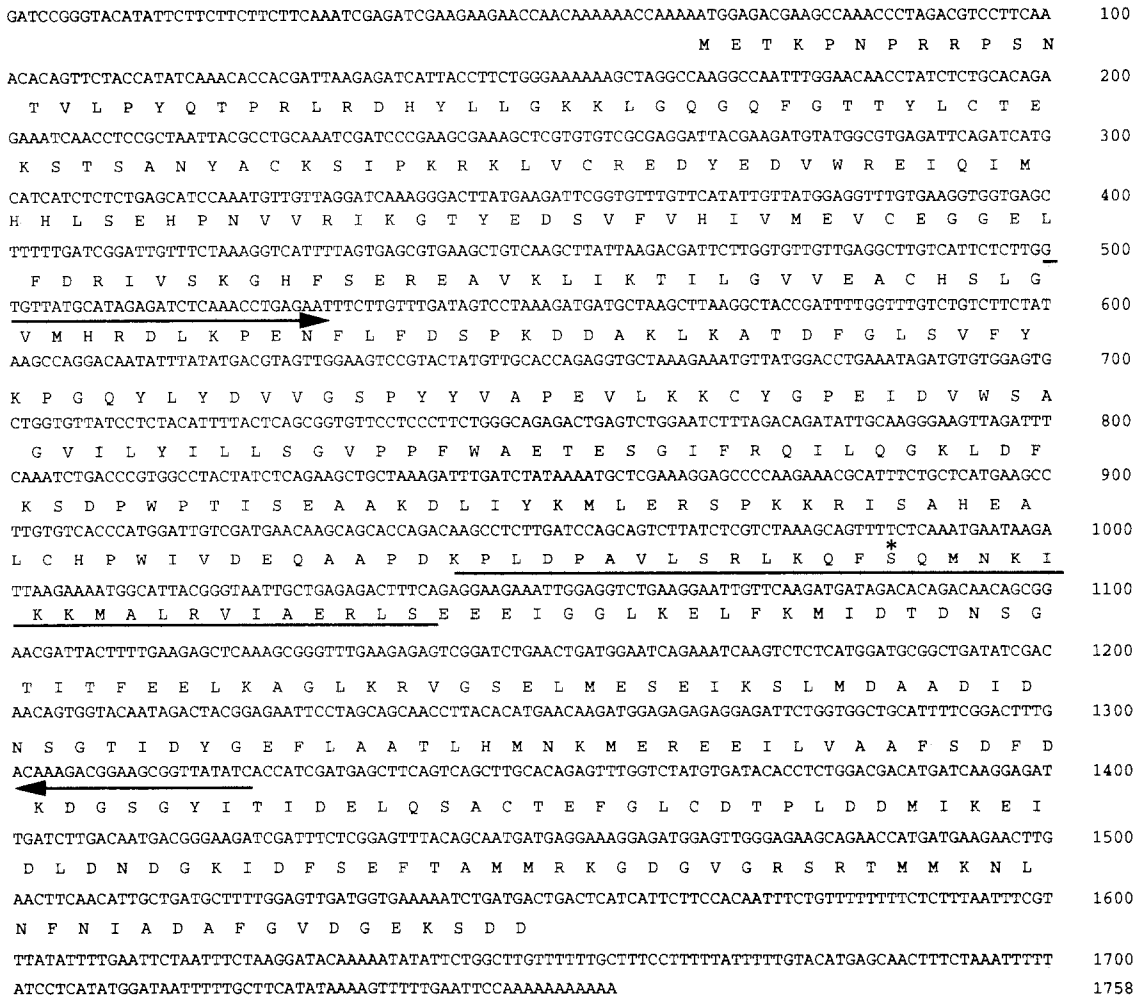

Fig. 3 (continued)

Analysis of the effects of dehydration, high salt, heat, cold, and exogeneous ABA on the expression of *ATCDPK1* and *ATCDPK2* genes

Since cATCDPK1 and cATCDPK2 clones were obtained by screening a cDNA library prepared from dehydrated plants, they may be induced by drought. This possibility was examined by Northern blot analysis. The *ATCDPK1* and *ATCDPK2* mRNAs appeared within 10 min of the start of dehydration and their levels increased for up to 2 h and 20 min, respectively (Fig. 2). These observations suggest that *ATCDPK1* and *ATCDPK2* genes are actually induced by water stress. Since many drought-inducible genes have been shown to be responsive to exogenous ABA (Skriver and Mundy 1990; Bray 1991), we similarly examined the effect of treatment with exogenous ABA on the expression of *ATCDPK1* and *ATCDPK2*. The results show that their expression was induced neither by exogenous ABA nor by a control treatment with water alone (Fig. 2). The effect of salt, low temperature, and heat stresses on the expression of *ATCDPK1* and *ATCDPK2* was also tested as described in Materials and methods. Figure 2 shows that *ATCDPK1* and *ATCDPK2* genes are induced under high-salt conditions. However, these two genes were not responsive to either low or high temperature. These results suggest that a change in the osmotic potential of the environment can serve as a trigger for the induction of *ATCDPK1* and *ATCDPK2*.

Sequence analysis of two cDNA clones that encode Ca^{2+}-dependent protein kinases

Figure 3 shows the nucleotide sequences of the two cDNAs and their corresponding deduced amino acid sequences. cATCDPK1 (Fig. 3A) contains a 1479-bp open reading frame, encoding a putative polypeptide having 493 amino acids and a predicted molecular weight of 55.7 kDa. cATCDPK2 (Fig. 3B) contains a 1488-bp open reading frame, encoding a putative polypeptide having 496 amino acids with a predicted molecular weight of 55.9 kDa. The coding regions of cATCDPK1 and cATCDPK2 are 59% identical at the nucleotide sequence level. No typical polyadenylation signals were found in their 3′ flanking regions.

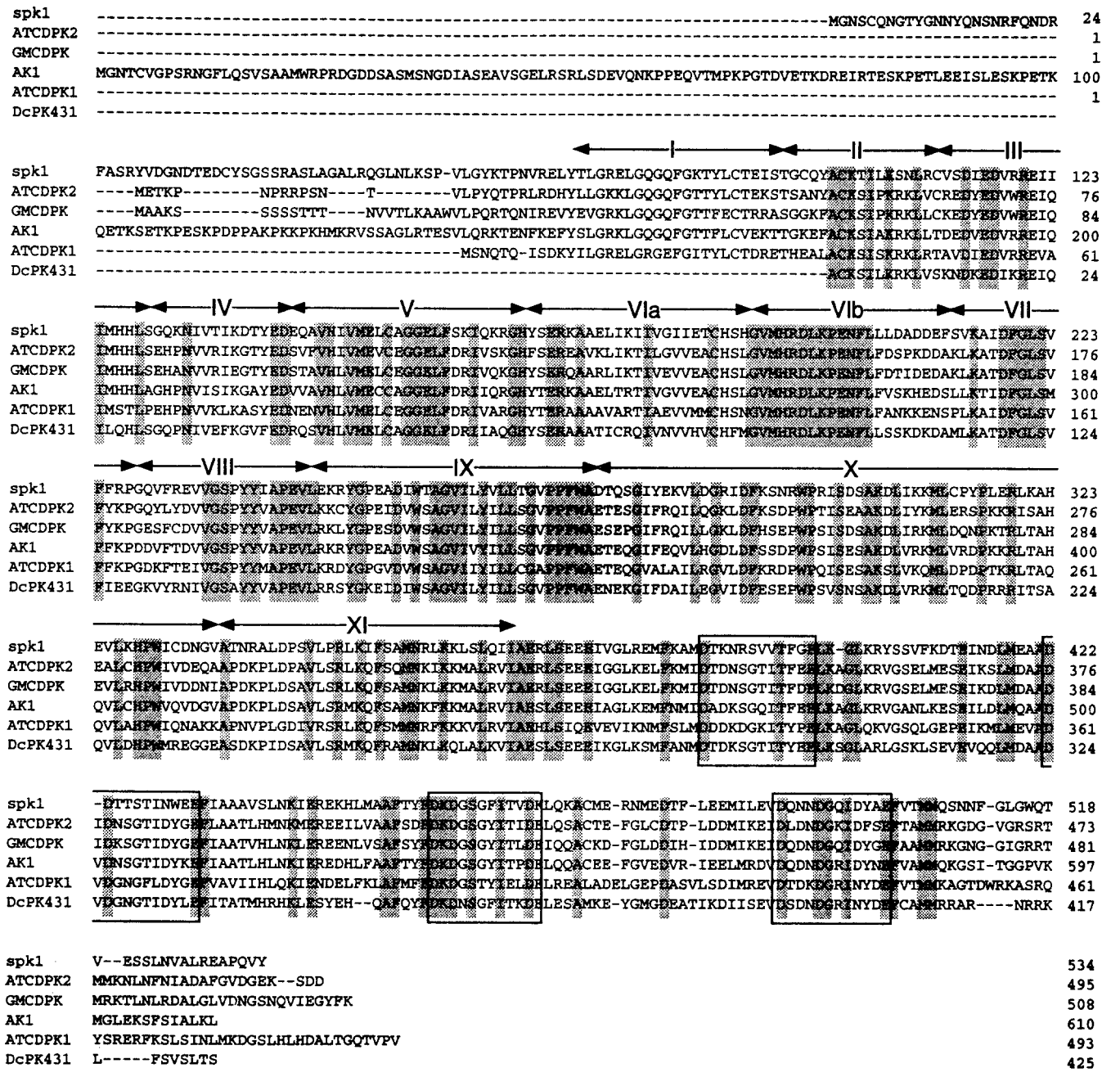

Fig. 4 Comparison of the deduced amino acid sequences of ATCDPK1, ATCDPK2, soybean Ca^{2+}-dependent, calmodulin-independent protein kinase (CDPK) (*GMCDPK*; Harper et al. 1991), carrot CDPK (*DcPK431*, Suen and Choi 1991), rice CDPK (*spk1*, Kawasaki et al. 1993) and *Arabidopsis* CDPK (*AK1*, Harper et al. 1993). *Shading* represents perfectly conserved amino acid residues and dashes indicate gaps introduced to maximize alignment. *Roman* numerals indicate the 11 major conserved subdomains of protein kinases identified by Hanks and Quinn (1991). Four Ca^{2+}-binding sites are *boxed*

Primary structure of putative ATCDPK1 and ATCDPK2 proteins

Figure 4 shows a comparison of the deduced amino acid sequences of the putative proteins encoded by *ATCDPK1* and *ATCDPK2* (named ATCDPK1 and ATCDPK2) with those of other CDPKs. ATCDPK1 and ATCDPK2 contain all the conserved amino acid residues in protein kinases and the 11 subdomains that are typical of the catalytic subunit of protein kinases (Hanks and Quinn 1991). Their catalytic domains most closely resemble the family of CaM kinases, which coincides with those of other CDPKs (Harper et al. 1991, 1993; Suen and Choi 1991; Kawasaki et al. 1993). A possible ATP-binding site was found in the N-terminal region of two CDPKs. CaM kinases contain a autoinhibitory domain composed of basic amino acids that inactivates kinase activity under Ca^{2+}-free conditions (Bennett and Kennedy 1987). The two *Arabidopsis* CDPKs also include the corresponding autoinhibitory domain (Fig. 3). A serine residue in the putative autoinhibitory domain may be an autophosphorylation site,

because phosphorylation of serine residue relieves autoinhibition by conformational changes (Soderling 1990).

The Ca^{2+}-binding proteins, including calmodulin and calpain, have four E-F hand Ca^{2+}-binding loops that have been shown to function as a Ca^{2+} sensor (Tufty and Kretsinger 1975; Babu et al. 1985). The C-terminal domains of ATCDPK1 and ATCDPK2, as well as the known CDPKs, resemble calmodulin, and have four E-F hand structures (Fig. 4). Each of the four Ca^{2+}-binding sites is flanked by polypeptides that form α-helices. The presence of Ca^{2+}-binding sites indicates that Ca^{2+} directly binds ATCDPK1 and ATCDPK2 proteins and regulates their kinase activities.

ATCDPK2 protein expressed in *E. coli* cells requires Ca^{2+} for activation

The proteins encoded by cATCDPK1 and cATCDPK2 were overexpressed by IPTG induction in *E. coli* cells as fusions with glutathione S-transferase (Smith and Johnson 1988), as described in Materials and methods. The ATCDPK1 and ATCDPK2 fusion proteins (named GST-ATCDPK1 and GST-ATCDPK2, respectively), were recovered mainly in the insoluble protein fraction. However, small amounts of the fusion proteins were recovered in the soluble fraction (Fig. 5), from which the fusion proteins were purified by use of glutathione-Sepharose beads. Purity was about 90% as judged from the gel pattern.

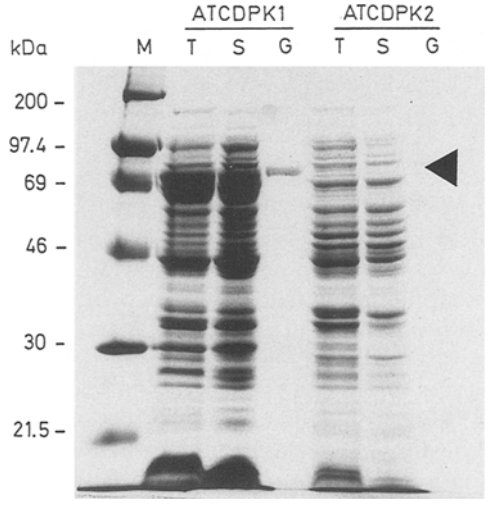

Fig. 5 Expression and purification of recombinant GST-ATCDPK1 and GST-ATCDPK2 proteins from *Escherichia coli* cells containing pGEX-CDPK1 and pGEX-CDPK2, respectively. The total cellular extracts (*T*) and the soluble fraction (*S*) of total cellular extracts prepared from IPTG-treated *E. coli* cells containing pGEX-CDPK1 (ATCDPK1) or pGEX-CDPK2 (ATCDPK2) were analyzed by SDS-PAGE. Fusion proteins (*G*) purified with glutathione-Sepharose beads from the soluble fraction were also analyzed by SDS-PAGE. The *closed triangles* indicate the electrophoretic positions of the full-length recombinant protein. The positions and sizes of molecular weight markers are also indicated in kDa

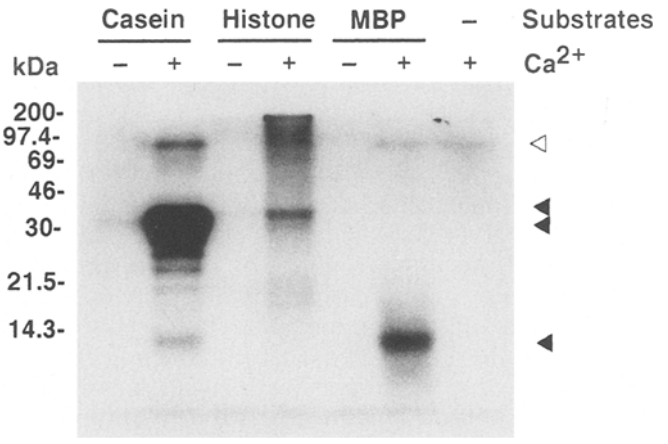

Fig. 6 Activation of the recombinant GST-ATCDPK2 protein by Ca^{2+}. Partially purified GST-ATCDPK2 was incubated for 10 min at 37° C with (+) or without (−) Ca^{2+} and γ-[^{32}P]ATP. The samples were then subjected to electrophoresis in a 15% polyacrylamide gel containing SDS and then the gel was autoradiographed. The *closed* and *open triangles* indicate the phosphorylated products and autophosphorylated CDPKs, respectively. The sizes of molecular weight markers are presented in kDa

To determine whether GST-ATCDPK1 or GST-ATCDPK2 had Ca^{2+}-dependent protein kinase activity, the fusion proteins were mixed with histone, casein, and myelin basic protein (MBP) in the presence of γ-[^{32}P]ATP. Each protein was phosphorylated only in the presence of Ca^{2+} (Fig. 6). Bands of 30 kDa casein and 10 kDa MBP were detected in the presence of Ca^{2+}, and the addition of Ca^{2+} stimulated the phosphorylation of casein with GST-ATCDPK2 by a factor of 46. A weak band, corresponding to GST-ATCDPK2, was also detected in the absence of substrates, indicating that autophosphorylation occurred. These observations indicate that GST-ATCDPK2 functions as a Ca^{2+}-dependent protein kinase in vitro. However, we could not detect significant stimulation of phosphorylation of any of the substrates with GST-ATCDPK1 (data not shown).

Discussion

We have screened a cDNA library prepared from dehydrated *Arabidopsis* rosette plants for sequences encoding CDPKs, and isolated two cDNAs, cATCDPK1 and cATCDPK2, whose genes are induced by dehydration stress and high-salt stress. Both the *ATCDPK1* and *ATCDPK2* genes respond to dehydration and high-salt conditions within 1 h (Fig. 2). In contrast, exogenous ABA treatment had no effect on the induction of *ATCDPK1* and *ATCDPK2*. Endogenous ABA induced by water deficiency in *Arabidopsis* is detectable by 2 h after dehydration and reaches a maximum level at 10 h (Kiyosue et al. 1994), providing further evidence that ABA is not involved in the induction of *ATCDPK1* and

ATCDPK2 by dehydration. These results suggest that a change in osmotic potential triggers the rapid induction of these two CDPK genes.

Both ABA-independent and ABA-responsive signal transduction pathways link dehydration and gene expression (Guerrero et al. 1990; Yamaguchi-Shinozaki et al. 1992; Yamaguchi-Shinozaki and Shinozaki 1993; Shinozaki et al. 1993). Low-temperature inducible genes have been shown to be regulated by three separate signal pathways, one of which is ABA-independent (Nordin et al. 1991; Gilmour and Thomashow 1991). Recently, we have identified a novel *cis*-acting element (DRE; Dehydration Responsive Element) involved in the rapid response of *rd29A* genes to dehydration and high salt. This element seems to function in induction by low temperature but not in its ABA-responsive expression (Yamaguchi-Shinozaki and Shinozaki 1994). and thus provides direct evidence for the existence of an ABA-independent pathway in dehydration-responsive expression. *ATCDPK1* and *ATCDPK2* are induced by dehydration through an ABA-independent signal pathway. However, they are not induced by low temperature (Fig. 2), which suggests that DRE is not likely to function in their rapid dehydration-responsive expression. We are in the process of analyzing the promoter region of *ATCDPK1* to identify *cis*-acting elements involved in the dehydration-responsive expression of *ATCDPK1*.

The GST-ATCDPK2 fusion protein expressed in *E. coli* was demonstrated to require Ca^{2+} for its protein kinase activity in vitro, which indicates that ATCDPK2 can function as Ca^{2+}-dependent protein kinase in vivo. GST-ATCDPK2 phosphorylated casein as a substrate more efficiently than histone or MBP (Fig. 6). Preference for casein over histone in substrate specificity has also been observed for *Paramecium* CDPK (Gundersen and Nelson 1987). However, purified soybean CDPK protein phosphorylates histone most efficiently (Putnam-Evans et al. 1990). These differences in substrate selectivity may be due to differences in enzyme properties. Another possibility is that the abnormal structure of the fusion protein causes the different substrate specificity. However, the GST-ATCDPK1 protein did not show any stimulation of phosphorylation in the presence of Ca^{2+}, which may be due to the abnormal structure of the recombinant protein or to a narrow range of substrate specificity.

It is likely that Ca^{2+} acts as a second messenger in the drought response of plant cells. The observed increase in free Ca^{2+} in the cytoplasm of guard cells suggests that it may trigger signals for stomatal closure (Trewavas and Gilroy 1991). Our results suggest that Ca^{2+}-dependent protein kinase is involved in the signal transduction pathways that mediate dehydration-induced biochemical changes in plants. Dehydration-inducible CDPKs can function in Ca^{2+}-mediated signal transduction pathways involved in drought tolerance. One possible function of ATCDPK1 and ATCDPK2 is the modulation of signal transduction involved in drought-inducible gene expression. Possible target proteins are *trans*-acting factors involved in gene expression or other protein kinases that function in signal transduction. Other possible target may be proteins involved in drought tolerance.

Four possible endogenous substrates of CDPKs have been identified, including nodulin-26 (Weaver et al. 1991; Weaver and Roberts 1992), tonoplast intrinsic protein (Johnson and Chrispeels 1992), plasma membrane H^+-ATPase (Schaller and Sussman 1988), and myosin (Putnam-Evans et al. 1990). The products of nodulin-26 (Sandal and Marcker 1988) and tonoplast intrinsic proteins (TIP) belong to the major intrinsic proteins (MIP) group, a superfamily of membrane proteins. MIPs are found in various organisms, including plants (Johnson et al. 1990; Yamamoto et al. 1990), animals (Gorin et al. 1984), *E. coli* (Muramatsu and Mizuno 1989), and yeast (Van Aelst et al. 1991). These proteins have been postulated to form channels that function in metabolite transport and water transport. The vacuolar membrane protein γ-TIP has been demonstrated to function as a water channel by expression of the protein in *Xenopus* oocytes (Maurel et al. 1993). One of the dehydration-responsive *Arabidopsis* genes (*RD28*) encodes a putative membrane protein that is a member of the MIP family (Yamaguchi-Shinozaki et al. 1992). RD28 protein may be one of the target proteins of the dehydration-induced ATCDPK1 and ATCDPK2 proteins. We are currently studying the phosphorylation of the RD28 protein by the recombinant ATCDPK proteins.

Recently, many genes that encode proteins involved in signal transduction pathways, such as calmodulin, protein kinase, and Ca^{2+}-ATPase, have been shown to be induced by environmental stimuli, such as touch, dehydration, and high salt, respectively. Three of the four touch-inducible genes (TCH) are reported to encode calmodulin homologues (Braam and Davis 1990); and two genes for the touch-inducible calmodulin homologues respond to Ca^{2+} and heat stress (Braam 1992). One protein kinase gene has been reported to respond negatively to light irradiation (Lin et al. 1991). Two genes encoding small GTP-binding proteins, *rab* homologs, in pea are also negatively regulated by red light (Yoshida et al. 1993). One transcript (PKABA1) in wheat, having homology to protein kinases, is known to be induced by both ABA and dehydration, and levels increase during seed maturation and the RNA accumulates in mature seeds (Anderberg and Walker-Simmons 1992). A gene encoding an endoplasmic reticulum-localized Ca^{2+}-ATPase of tomato has been shown to be induced by salt stress (Wimmers et al. 1992). Recently, we found that a gene encoding a *myb* transcription factor homologue, *Atmyb2*, is induced by ABA, dehydration stress, and salt stress at the transcriptional level (Urao et al. 1993); and in this report we also identified two *CDPK* genes that are induced by dehydration and high salt conditions. These results suggest that various genes, whose products are involved in signal transduction pathways, are induced at the transcriptional level in

plants by environmental signals or stresses; this is quite different from the situation in animal systems.

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