Ca^{2+}-Dependent Protein Kinases and Stress Signal Transduction in Plants

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Stress responses in plants involve changes in the transcription of specific genes. The constitutively active mutants of two related Ca^{2+}-dependent protein kinases (CDPK1 and CDPK1a) activate a stress-inducible promoter, bypassing stress signals. Six other plant protein kinases, including two distinct CDPKs, fail to mimic this stress signaling. The activation is abolished by a CDPK1 mutation in the kinase domain and diminished by a constitutively active protein phosphatase 2C that is capable of blocking responses to the stress hormone abscisic acid. A variety of functions are mediated by different CDPKs. CDPK1 and CDPK1a may be positive regulators controlling stress signal transduction in plants.

In plants, the hormone abscisic acid (ABA) or environmental stresses such as drought, cold, and salinity can induce expression of a number of highly conserved genes in vegetative tissues (*1*). The accumulation of these gene products could protect plants from stress-induced damage (*1*, *2*). Many of these genes are also expressed during embryogenesis and seed development and may be important for seed desiccation and dormancy (*1*, *3*). Cis-acting elements and trans-acting factors regulate these stress-inducible genes (*1*, *3*, *4*); however, the molecular mechanisms controlling stress signal transduction remain elusive.

In isolated maize leaf protoplasts (*5*), responses to multiple stresses can be monitored with the use of green fluorescent protein (GFP) as a vital reporter (*6*). A chimeric gene was generated by fusion of the barley ABA-responsive (HVA1) promoter (*7*, *8*) to a synthetic GFP sequence (*6*) (forming HVA1-SGFP). The barley HVA1 gene is activated by multiple stress signals in vegetative tissues (*7*). After electroporation into maize leaf protoplasts of the plasmid DNA carrying HVA1-SGFP, the expression of GFP was enhanced by exposure to cold, high salt, dark, and ABA (Fig. 1). Expression from plasmids generated by fusing the maize ubiquitin promoter (*9*) and the β-glucuronidase gene (forming UBI-GUS) (*10*) or from UBI-SGFP was not affected (*11*).

Previous studies have suggested that Ca^{2+} might be a second messenger in multiple stress responses in plants (*12*). As shown in Fig. 2, the expression of HVA1-SGFP in maize protoplasts was substantially increased by the Ca^{2+} ionophore Ca^{2+}-ionomycin or Ca^{2+}-A23187 but not by Ca^{2+} alone in the incubation medium. The same treatment did not influence UBI-SGFP expression (Fig. 2) but inhibited the expression of GFP controlled by a stress-repressible photosynthetic gene promoter (*5*, *13*).

To determine whether Ca^{2+}-activated protein kinases (PKs) mediate stress signal transduction in plants, I coexpressed four constitutively active Ca^{2+}-dependent PKs (CDPKs) (*14–16*) and monitored the HVA1 promoter activity. Plant CDPKs share extensive sequence identity with the mammalian multifunctional Ca^{2+}-calmodulin–dependent PKII (CaMKII) (*17*). However, instead of bearing a calmodulin binding site, this family of PKs carries a calmodulin-like domain at the COOH-terminus (Fig. 3A) (*14–16*), which allows response to Ca^{2+} signals directly without calmodulin. Currently, CDPKs are the most prevalent serine-threonine PKs found in higher plants (*14–16*). Although their physiological roles remain unclear, they are induced by cold, drought, and ABA (*12*, *15*).

Eight *Arabidopsis* PKs with full-length coding sequences available (*15*, *16*, *18–20*) were chosen for the experiments. Two CDPKs (ATCDPK1 and ATCDPK1a) are very closely related (96% amino acid similarity), whereas the other two CDPKs (AK1/ATCDPK and ATCDPK2) have more divergent sequences (78 and 75% amino acid similarity, respectively, to ATCDPK1). It has been shown that AK1/ATCDPK and ATCDPK2 possess Ca^{2+}-dependent PK activity, and the truncated AK1/ATCDPK has Ca^{2+}-independent (constitutively active) PK activity in vitro (*15*, *16*). However, the PK activity of ATCDPK1 has not been demonstrated by in vitro assays because it does not phosphorylate common PK substrates (*15*). The effect of four other *Arabidopsis* PKs (ATPKa, ATPKb, ASK1, and ASK2) that share significant homology with the ABA-inducible PK (PKABA1) (*20*), which is speculated to mediate ABA signal transduction, were also tested. These PK cDNAs were obtained by polymerase chain reaction (PCR), and at least two clones of each cDNA were used for transient expression analysis. Truncated forms containing all 11 PK domains, analogous to the construction of a constitutively active mutant of CaMKII in mammals (*17*) (Fig. 3A), were inserted into the plant expression vector with a strong constitutive promoter, 35SC4PPDK (*5*, *6*, *21*). The putative regulatory domains of these

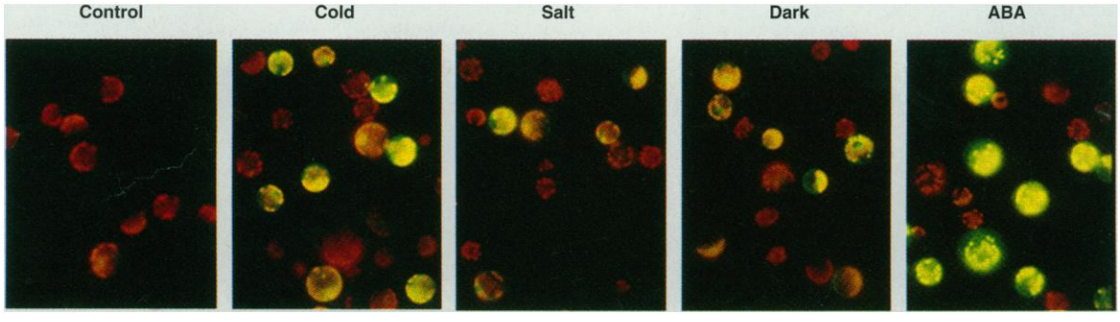

Fig. 1. Stress signaling in maize leaf protoplasts visualized by GFP expression. Maize leaf protoplasts were electroporated with the plasmid DNA carrying HVA1-SGFP and divided (10^5 cells/ml per sample) for various treatments as follows: constant light [15 microeinsteins (μE) m^{-2} s^{-1}] at 23°C for 16 hours (control); 0°C for 4 hours, followed by 12 hours at 23°C (cold); 0.2 M NaCl for 3 hours, washing, and incubation for 13 hours (salt); constant darkness for 16 hours (dark); and 100 μM of ABA for 16 hours (ABA). About 50% of the protoplasts, showing green or yellow fluorescence after the induction, were transiently transformed. The control and untransfected protoplasts showed only red autofluorescence from chlorophyll. GFP expression was visible with 1 μM of ABA (*11*). The protocol for transient expression analysis with maize leaf protoplasts has been described previously (*5*, *6*). About 10^5 protoplasts from each treatment were observed with a fluorescent microscope as described elsewhere (*6*). The experiment was repeated three times with similar results.

PKs were deleted (*21*). To allow monitoring of expression, these PKs were fused in-frame to a double hemagglutinin (DHA) epitope tag (*21*) at the COOH-terminus. The expression of the eight PKs in transfected maize leaf protoplasts was demonstrated by immunoprecipitation of [^{35}S]methionine-labeled proteins with the monoclonal antibody to HA (Fig. 3B).

To quantitate the effect of PKs on stress signaling, another chimeric gene with the HVA1 promoter and the luciferase coding sequence (HVA1-LUC) (*22*) was generated. Coexpression experiments were performed by electroporation of the reporter (HVA1-LUC) and the effector (35SC4PPDK-PK-DHA) plasmids together into maize leaf protoplasts. The results showed that ATCDPK1 and ATCDPK1a, but not the other six PKs, could activate LUC expression controlled by the HVA1 promoter (Fig. 3C). The identical set of PKs without the DHA tag gave the same results (*11*). The expression of UBI-GUS as an internal control was not affected (*11*).

To show that the PK activity is important for the activation of the stress-inducible HVA1 promoter, a null mutation was made by site-directed mutagenesis to eliminate the adenosine triphosphate (ATP) binding site Lys^{40} (K40) (*15*, *17*) in ATCDPK1. The kinase mutation (K40M) did not affect the expression of the protein (Fig. 4A), but it could no longer activate the expression of HVA1-SGFP (Fig. 4B). The expression of UBI-SGFP was not affected by ATCDPK1 or the ATCDPK1 mutant (*11*). This result indicates that the PK domain of ATCDPK1 is required and sufficient to recognize specific protein substrates mediating stress signal transduction. The deleted regulatory domain is probably involved in PK activity control in response to stress signals (*14*–*17*).

To further investigate the idea that ATCDPK1 and ATCDPK1a are positive regulators in plant stress signal transduction, I tested the effect of a specific and constitutively active *Arabidopsis* protein phosphatase 2C (PP2C) (*13*, *23*), which is capable of abolishing ABA responses. As shown in Fig. 4C, HVA1-LUC expression activated by ABA was repressed by the constitutively active PP2C (*13*). The constitutive PP2C, but not its null version (*13*), decreased but did not abolish HVA1-LUC expression enhanced by ATCDPK1 (Fig. 4C). Other serine-threonine PPs such as PP1, PP2A, and PP2B might be required to completely counteract the effect of ATCDPK1, which could be a convergent point of multiple stress signaling. The same results were obtained with ATCDPK1a, and the expression of the internal control UBI-GUS was not affected (*11*). As the genes involved in stress responses are highly conserved in plants, the role of ATCDPK1 and ATCDPK1a in stress signal transduction may extend to various cell types of diverse plant species. Thus, the manipulation of specific CDPK activities might have important agricultural applications in protecting crop plants from stress damage and yield loss.

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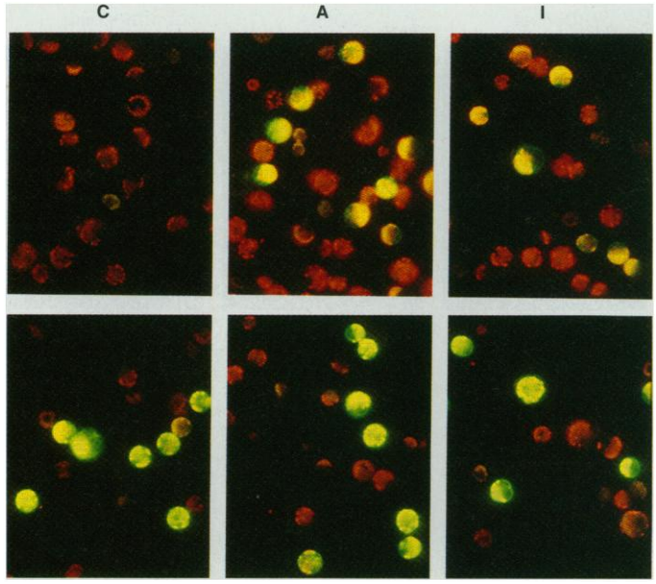

Fig. 2. Intracellular Ca^{2+} elevation activates stress signaling. Maize leaf protoplasts transfected with HVA1-SGFP (top row) or UBI-SGFP (bottom row) were treated with 1 mM Ca^{2+} (C), 1 mM Ca^{2+} plus 100 nM A23187 (A), or 1 mM Ca^{2+} plus 100 nM ionomycin (I). Protoplast transient expression was the same as described previously (*5*, *6*). About 10^5 protoplasts from each treatment were observed with a fluorescent microscope (*6*). The experiment was repeated twice with similar results.

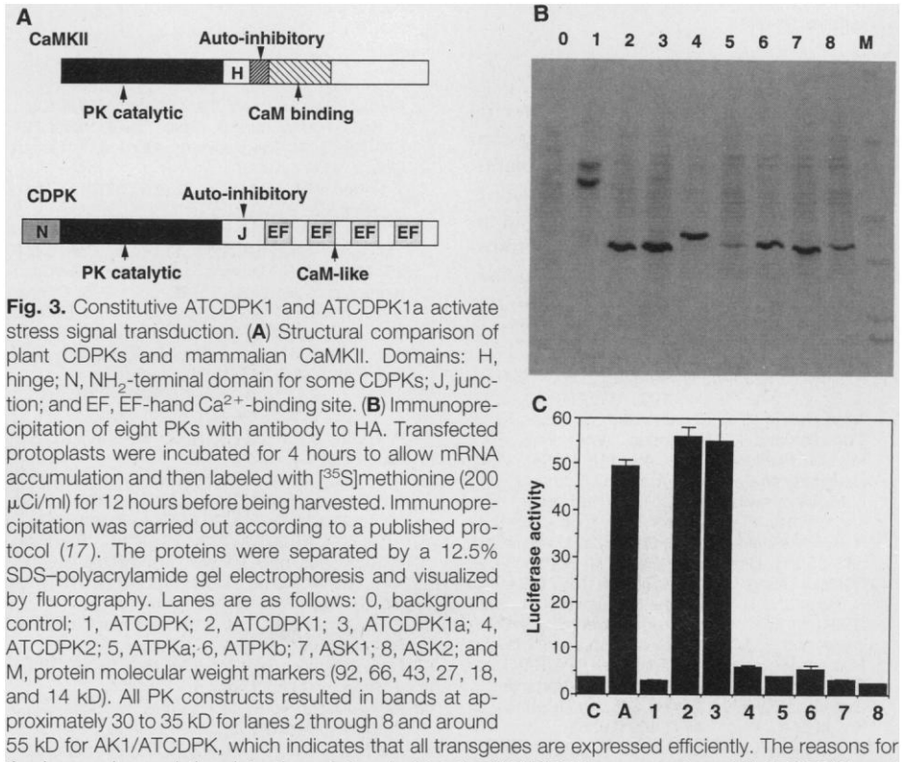

Fig. 3. Constitutive ATCDPK1 and ATCDPK1a activate stress signal transduction. (**A**) Structural comparison of plant CDPKs and mammalian CaMKII. Domains: H, hinge; N, NH_2-terminal domain for some CDPKs; J, junction; and EF, EF-hand Ca^{2+}-binding site. (**B**) Immunoprecipitation of eight PKs with antibody to HA. Transfected protoplasts were incubated for 4 hours to allow mRNA accumulation and then labeled with [^{35}S]methionine (200 μCi/ml) for 12 hours before being harvested. Immunoprecipitation was carried out according to a published protocol (*17*). The proteins were separated by a 12.5% SDS–polyacrylamide gel electrophoresis and visualized by fluorography. Lanes are as follows: 0, background control; 1, ATCDPK; 2, ATCDPK1; 3, ATCDPK1a; 4, ATCDPK2; 5, ATPKa; 6, ATPKb; 7, ASK1; 8, ASK2; and M, protein molecular weight markers (92, 66, 43, 27, 18, and 14 kD). All PK constructs resulted in bands at approximately 30 to 35 kD for lanes 2 through 8 and around 55 kD for AK1/ATCDPK, which indicates that all transgenes are expressed efficiently. The reasons for the larger size and the detection of the doublet for AK1/CDPK are not clear. (**C**) ATCDPK1 and ATCDPK1a activate stress-inducible transcription. Maize leaf protoplasts were transfected with HVA1-LUC alone and incubated without (C) or with (A) 100 μM of ABA. HVA1-LUC was also co-electroporated with the PK constructs (bars 1 through 8) and incubated without ABA. Relative LUC activities from duplicated samples are shown. Cell lysates from 2000 protoplasts were used for LUC (*22*) and GUS assays (*5*, *10*). The experiment was repeated three times with similar results.

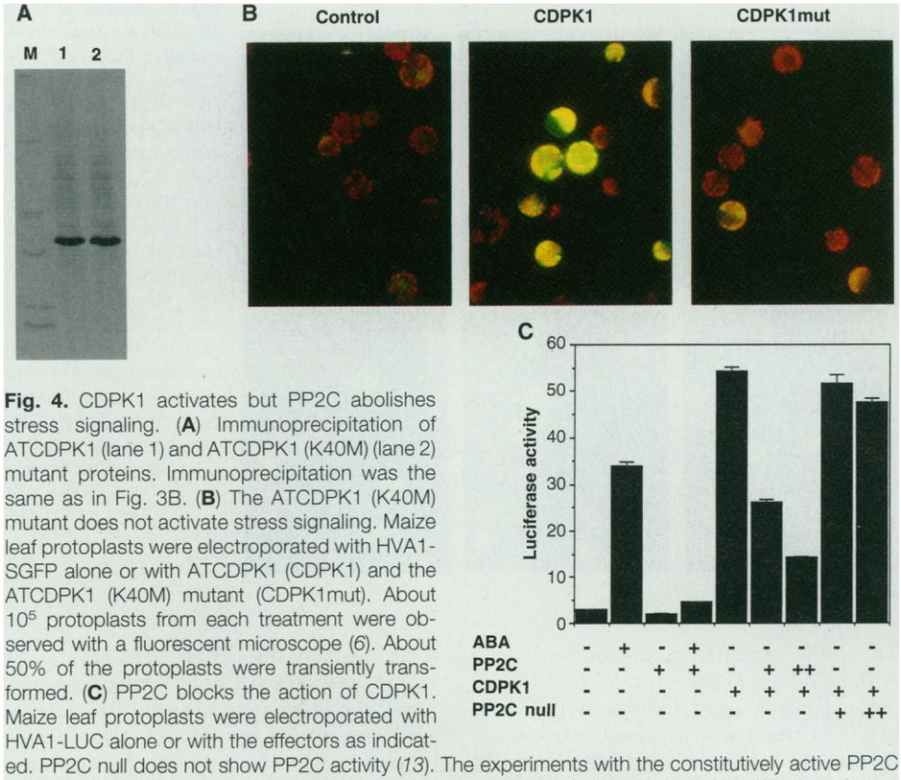

Fig. 4. CDPK1 activates but PP2C abolishes stress signaling. (**A**) Immunoprecipitation of ATCDPK1 (lane 1) and ATCDPK1 (K40M) (lane 2) mutant proteins. Immunoprecipitation was the same as in Fig. 3B. (**B**) The ATCDPK1 (K40M) mutant does not activate stress signaling. Maize leaf protoplasts were electroporated with HVA1-SGFP alone or with ATCDPK1 (CDPK1) and the ATCDPK1 (K40M) mutant (CDPK1mut). About 10^5 protoplasts from each treatment were observed with a fluorescent microscope (*6*). About 50% of the protoplasts were transiently transformed. (**C**) PP2C blocks the action of CDPK1. Maize leaf protoplasts were electroporated with HVA1-LUC alone or with the effectors as indicated. PP2C null does not show PP2C activity (*13*). The experiments with the constitutively active PP2C or the null PP2C were performed with two concentrations of plasmid DNA (+, 20 μg and ++, 60 μg). LUC and GUS assays were performed with cell lysates from 2000 protoplasts (*5, 10, 22*) Relative LUC activities from duplicated samples are shown. The experiment was repeated three times with similar results.

I used maize leaf protoplasts and the vital reporter GFP to dissect evolutionarily conserved signaling pathways in higher plants. The elucidation of intracellular signaling mechanisms at the cellular level should facilitate the understanding of signal transduction pathways in whole plants.

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18. Various PK cDNAs were obtained by PCR with the use of an *Arabidopsis* cDNA library. The primers were as follows: AK1 or ATCDPK, 5′-GAAGATCTATGGGTA-ATACTTGTGTTGGA-3′ and 5′-GTCAAGGCCTGTC-GACTTGAACCCATGG-3′; ATCDPK1 and ATCDP-K1a, 5′-GCGGATCCATGGCTAATCAAACTCAGATC-AGCG-3′ and 5′-GTCAAGGCCTCATCAGTGAGAAC-ATGTTC-3′; ATCDPK2, 5′-GCGGATCCATGGAGAC-GAAGCCAAACCCTA-3′ and 5′-GTCAAGGCCTTGC-TTGTTCATCGACAATCC-3′; ATPKa, 5′-CATGCCAT-GGCTCCGGCGACTAATTCACCG-3′ and 5′-GTC-AAGGCCTATTCTTCAAGAACCATTTATCG-3′; ATP-Kb, 5′-GCGGATCCATGGCTCGAGCTCCGGTGACC-A-3′ and 5′-GTCAAGGCCTATTCTTCAAGAACCAA-CTATG-3′; ASK1, 5′-GCGGATCCATGGCTAAGTCA-GAGCTGGTGAAAG-3′ and 5′-GTCAAGGCCTATTC-TTTAGGAACCATGAATG-3′; and ASK2, 5′-GCG-GATCCATGGCTAAGTATGACGTTGTCAAGG-3′ and GTCAAGGCCTATTCTTCAAGTACCACGG-3′. The sequence of ATCDPK1a was determined for both strands with an automatic sequencing facility.
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21. Each kinase tested was inserted after the 35SC4PPDK promoter and before the DHA tag and nopanine synthase terminator sequence. Kinase fragments used were as follows: AtCDPK (AK1), amino acids 1 through 413; ATCDPK1, amino acids 1 through 274; ATCDPK1a, amino acids 1 through 274; ATCDPK2, amino acids 1 through 289; ATPKa, amino acids 1 through 284; ATPKb, amino acids 1 through 283; ASK1, amino acids 1 through 265; and ASK2, amino acids 1 through 265. One strand of the sequence for the DHA tag was 5′-CCTTACCCATACGACGTTCCAGACTACGCTGGTT-ACCCATACGACGTTCCAGACTACGCT-3′. All cDNAs were fused to the DHA sequence YPYDVPDYAGYPY-DVPDYA (single-letter abbreviations for the amino acid residues are: A, Ala; D, Asp; G, Gly; P, Pro; V, Val; and Y, Tyr) at the Stu I site.
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