Ca²⁺-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 Ca²⁺-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.

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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, 2). 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 Ca²⁺ 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 Ca²⁺ ionophore Ca²⁺-ionomycin or Ca²⁺-A23187 but not by Ca²⁺ 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 Ca²⁺-activated protein kinases (PKs) mediate stress signal transduction in plants, I coexpressed four constitutively active Ca²⁺-dependent
PKs (CDPKs) (14-16) and monitored the HVA1 promoter activity. Plant CDPKs share extensive sequence identity with the mammalian multifunctional Ca²⁺-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 Ca²⁺ 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).

![Diagram of PKs (CDPKs)](http://www.sciencemag.org/cgi/content/full/274/5294/1900/fig/fig3)

**Fig. 3.** Constitutive ATCDPK1 and ATCDPK1a activate stress signal transduction. (A) Structural comparison of plant CDPKs and mammalian CaMKII. Domains: H, hinge; N, NH₂-terminal domain for some CDPKs; J, junction; and EF, EF-hand Ca²⁺-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 [³⁵S]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.

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 Ca²⁺-dependent PK activity, and the truncated AK1/ATCDPK has Ca²⁺-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 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 [³⁵S]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 Lys⁴⁰ (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. 4A). Other serine-threonine PPs such as PPI, PP2A, and PP2B might be required to completely counteract the effect 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.

I used maize leaf protoplasts and the vital reporter GUS 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.

REFERENCES AND NOTES


5. J. Sheen, EMBO J. 12, 3497 (1993) [Medline].


8. The barley HVA1 promoter was obtained by PCR with the use of barley genomic DNA and two primers: 5'-TCCACCGAGATGCCGACGCA-3' and 5'-GGTGGAAGGCGATTTTCGCGAC-3'. The HVA1 promoter and the SGFP were fused at the ATG Nco I site. Four clones were selected and tested for stress responses and gave identical results.


11. J. Sheen, data not shown.


13. J. Sheen, unpublished data.


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'-GAAGATCTATGGTAATCTTGGTGA-3' and 5'-GTCGACGGCTGTCGACTGAGGAGCAGTGTCAATCTGACTACG-3' and 5'-GTCGACGGCTGTCGACTGAGGAGCAGTGTCAATCTGACTACG-3'; and ATCDPK1 and ATCDPK1α, 5'-GAAGATCTATGGTAATCTTGGTGA-3' and 5'-GTCGACGGCTGTCGACTGAGGAGCAGTGTCAATCTGACTACG-3' and 5'-GTCGACGGCTGTCGACTGAGGAGCAGTGTCAATCTGACTACG-3'.

19. I thank M. Minet for an outstanding site.


21. Each kinase tested was incubated with the DHA tag and nanomole synthase terminator sequence. Kinase fragments used were as follows: ATCDPK (AK1), amino acids 1 through 413; ATCDPK1, amino acids 1 through 274; ATCDPK1α, amino acids 1 through 274; ATP-Kb, 5'-GAAGATCTATGGTAATCTTGGTGA-3' and GTCAAGGGCTATCTTCTTTAGGAACCATGAATG-3'; ASK1, 5'-GAAGATCTATGGTAATCTTGGTGA-3' and GTCAAGGGCTATCTTCTTTAGGAACCATGAATG-3'; ASK2, 5'-GAAGATCTATGGTAATCTTGGTGA-3' and GTCAAGGGCTATCTTCTTTAGGAACCATGAATG-3'; and ASK2, 5'-GAAGATCTATGGTAATCTTGGTGA-3' and GTCAAGGGCTATCTTCTTTAGGAACCATGAATG-3'. The sequence of ATCDPK1α was 5'-GAAGATCTATGGTAATCTTGGTGA-3' and GTCAAGGGCTATCTTCTTTAGGAACCATGAATG-3'; ASK1, 5'-GAAGATCTATGGTAATCTTGGTGA-3' and GTCAAGGGCTATCTTCTTTAGGAACCATGAATG-3'; ASK2, 5'-GAAGATCTATGGTAATCTTGGTGA-3' and GTCAAGGGCTATCTTCTTTAGGAACCATGAATG-3'; and ASK2, 5'-GAAGATCTATGGTAATCTTGGTGA-3' and GTCAAGGGCTATCTTCTTTAGGAACCATGAATG-3'. All cDNAs were fused to the DHA sequence YPYDPDYAGYPDPYDVPYA (single-letter abbreviations for the amino acid residues are: A, Ala; D, Asp; G, Gly; P, Pro; V, Val; and Y, Tyr) at the StuI site.