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Structure and Function of NtCDPK1, a Calcium-dependent Protein Kinase in Tobacco

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Abstracts

We have isolated a cDNA encoding a calcium-dependent protein kinase (CDPK) in *Nicotiana tabacum*, which was designated *NtCDPK1*. Accumulation of the *NtCDPK1* mRNA was stimulated by various stimuli, including phytohormones, $CaCl_2$, wounding, fungal elicitors, chitin and methyl jasmonate. The *NtCDPK1* gene encodes a functional Ser/Thr protein kinase of which phosphorylation activity is strongly induced by calcium. By analyzing expression of the NtCDPK1-GFP fusion protein and by immunoblotting with antibody which reacts with NtCDPK1, we found that NtCDPK1 is localized in membrane and nucleus in plant cells. Silencing expression of the *NtCDPK1* transgene resulted in marked decrease of lateral root development in the transgenic tobacco plants. Yeast two hybrid screening using NtCDPK1 as a bait identified a tobacco homologue of proteasome regulatory subunit 21D7, designated Nt21D7. The *21D7* mRNA has been shown to be predominantly expressed in proliferating tissues in the cell cycle-dependent manner in carrot. The recombinant NtCDPK1 protein associated with Nt21D7 *in vitro*, and could phosphorylate the Nt21D7 protein *in vitro* in the presence of calcium, suggesting that Nt 21D7 protein is a natural substrate of NtCDPK1 in tobacco. These results suggest that NtCDPK1 may regulate cell proliferation processes, such as lateral root formation, by regulating specificity and/or activity of proteasome-mediated protein degradation pathway.

Introduction

Changes in cytosolic Ca^{++} concentration have been shown to be involved in plant responses to various stimuli, which include light, environmental stress, pathogen attack, and hormones such as gibberellic acid, abscisic acid (ABA), auxin, and cytokinin (for review see Bush, 1995). However, the downstream signal transduction pathways that connect changes in intracellular Ca^{++} concentration to changes in gene expression and cellular metabolism are largely unknown. Recently a large family of calcium-dependent protein kinase (CDPK) genes has been identified in various species of plants, implicating that CDPKs play important roles in signaling process of plants (Breviaro et al., 1995; Estruch et al., 1994; Harper et al., 1991; Hong et al., 1996).

CDPKs have a unique structure in which the N-terminal kinase domain is fused to the carboxy-terminal calmodulin (CaM)-like domain containing four EF-hand Ca^{++}-binding sites. Each EF hand consists of a loop of 12 amino acid residues, which is flanked by two alpha-helices. When the protein binds Ca^{++} via EF-hand motifs, it becomes activated. Thus unlike calcium/calmodulin-dependent protein kinases in animal and fungal cells, CDPKs do not depend on exogenous calmodulin for activity. A junction domain between the kinase and CaM-like domains functions as a pseudosubstrate autoinhibitor, which inhibits phosphorylation in the absence of calcium. By inhibiting phosphorylation, CDPKs are normally kept in a state of low activity. The extreme N-terminus of CDPKs may also contain a variable domain which differs greatly in length and sequence among

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CDPKs (Hong et al., 1996).

CDPK genes have been cloned from various plant species, such as *Arabidopsis* (Johnson and Chrispeels, 1992; Hong et al., 1996; Urao et al., 1994), carrot (Suen and Choi, 1991), rice (Breviaro et al., 1995), soybean (Harper et al., 1991), mungbean (Botella et al., 1996), and corn (Estruch et al., 1994). In many species, CDPK genes constitute a multigene family; *Arabidopsis* contains more than 15 members of CDPK genes isolated so far; soybean and rice have three and two members reported, respectively. The large number of CDPK genes suggest that the protein is involved in multiple signaling pathways with differential specificity (Roberts and Harmon, 1992). Despite the cloning and characterization of many CDPK genes, the functions of these proteins are still largely unknown. Plant CDPKs have been found associated with chromatin, plasma membrane, and the cytoskeleton. This multiple subcellular localization of CDPKs also suggests that they have multiple functions. In maize, pollen-specific CDPK has been implicated in pollen germination and pollen tube growth (Estruch et al., 1994). Using a transient expression system in maize protoplasts, two closely related *Arabidopsis* CDPKs, CDPK1 and CDPK1a, have been shown to be involved in stress signal transduction (Sheen, 1996).

To investigate a function of CDPKs in plant cells, we have isolated a CDPK cDNA from tobacco, designated *NtCDPK1*, and examined gene expression and the activity of its encoded protein. Transgenic tobacco plants expressing the *NtCDPK1* transgene were characterized. Yeast two hybrid screening was carried out in order to identify NtCDPK1-interacting protein in plant cells. Based on these results, a possible cellular function of NtCDPK1 was proposed.

Materials and Methods

RT-PCR, Northern blot analysis, Autophosphorylation and phosphoamino acid analysis, Immunoblotting, and In vitro phosphorylation and immunoprecipitation

These experiments were carried as described by Yoon et al. (1999).

Yeast two hybrid screening

The MATCHMAKER LexA two-hybrid system (Clontech, USA) was used to screen a tobacco flower cDNA library (complexity of 5 $\times$ 10^5 total recombinants) constructed in pB42AD plasmid (Clontech) with NtCDPK1 as a bait. The bait plasmid was constructed in pLexA (Clontech) using the *NtCDPK1* cDNA corresponding to the kinase domain of NtCDPK1. After amplification, total 2 $\times$ 10^7 transformants were screened, that yielded 58 independent clones (blue, Leu^+). PCR-amplification and sequencing of 40 independent clones revealed that they were divided into two different classes, and one class (consisting of 26 clones) encoded a polypeptide which was highly homologous to proteasome regulatory subunit 21D7 of carrot. The clone was designated Nt21D7. To verify the interaction, the pB42AD plasmid containing the Nt21D7 was transformed back into EGY48[p8op-lacZ] strain and the EGY48[p8op-lacZ] strain containing the pLexA/NtCDPK1 plasmid, and the transformants were tested for beta-galactosidase activity.

Generation of the NtCDPK1 transgenic plants

For OF lines, the full *NtCDPK* cDNA was PCR amplified using Pwo DNA polymerase. The PCR product contained *Bam*HI and *Kpn*I sites at the ends. After digestion with *Bam*HI and *Kpn*I, the PCR fragment was cloned into *Bam*HI and *Kpn*I sites of Ti-plasmid vector pMBP1 to generate fusion between CaMV35S promoter and the full *NtCDPK1* cDNA in the sense orientation. The fusion point was verified by sequencing. For KD lines, the partial *NtCDPK1* cDNA corresponding to the kinase domain of NtCDPK1 was PCR amplified and cloned to pMBP1 vector as described above. The two recombinant Ti-plasmids were separately introduced into *Agrobacterium tumefaciens* LBA4404 by triparental mating, and the *Agrobacterium* was used to transform leaf strips of *Nicotiana tabacum* (cv. Xanthi).

Results and Discussion

We have isolated a cDNA encoding a calcium-dependent protein kinase (CDPK) in *Nicotiana tabacum*, which was designated *NtCDPK1*(Figure 1). The deduced amino acid sequence of NtCDPK1 suggests that this protein contains the kinase domain at the amino terminus and the autoregulatory and calmodulin-like domains with four putative Ca^{++}-binding EF hands at the carboxy terminus. The protein sequence of NtCDPK1 shows homology to other

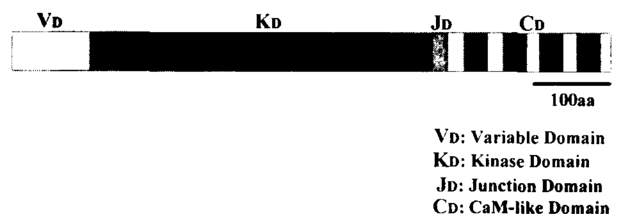

Figure 1. Schematic representation of the NtCDPK1 protein

CDPKs, including DcCPK1 (previous name, DcPK 431) in carrot (76.5% identity), MZECDPK2 in corn (75.8% identity), OSCPK2 in rice (74.3% identity), OSCPK11 (52.4% identity), and SK5 in soybean (57. 3% idenity). The variable domain of NtCDPK1 in the extreme amino-terminus is highly different from those of other CDPKs in the sequence and length, and it contains many proline and glutamine residues.

NtCDPK1 transcripts were present in roots, stems and flowers, but were almost undetectable in leaves. In leaves, *NtCDPK1* mRNA accumulation was stimulated by phytohormones (ABA, GA and cytokinin) and $CaCl_2$ treatment, while auxin treatment resulted in much less visible induction. When tobacco BY2 cells were exposed to methyl jasmonate, wounding, fungal elicitiors, chitosan, and NaCl, the level of the NtCDPK transcripts increased substantially. However, with dehydration and cold treatment, no change in transcript levels was observed.

To test if *NtCDPK1* encodes an active protein kinase which is regulated by Ca^{++}-binding, the full length NtCDPK1 and the truncated form (residues 1-394) containing only the amino-terminal variable and the kinase domain were expressed in *E. coli* as fusion proteins. The recombinant full length NtCDPK1 protein was catalytically active and highly stimulated by Ca^{++}. A truncated recombinant NtCDPK1 which lacks the C-terminal calmodulin-homologous domain also underwent autophosphorylation, but the kinase activity was not stimulated by Ca^{++}. Phosphoaminoacid analysis showed that NtCDPK1 possesses serine/threonine kinase activity.

Polyclonal antiserum raised against the N-terminal variable domain (Met1 through Lys80) of carrot DcCPK1 (Suen and Choi, 1991) cross-reacted with NtCDPK1. To detect NtCDPK1 in plant cells, we carried out immunoblotting of soluble and membrane proteins extracted from tobacco BY2 cells using the antiserum. The antiserum detected a protein of about 60 kDa, which is consistent with the size of NtCDPK1. To examine the phosphorylation activity of the extracted proteins, *in vitro* phosphorylation with immunoprecipitation was performed using soluble and membrane proteins. When *in vitro* phosphorylation was carried out with immunoprecipitated proteins with DcCPK1 antiserum, the phosphoprotein bands of 60 kDa and 38 kDa were found only in the membrane fraction. It is likely that the 60 kD protein band in the membrane fraction represents NtCDPK1, suggesting that NtCDPK1 is preferentially associated with membranes. Considering that the antiserum was raised against the variable domain which differs greatly among CDPK isoforms, the smaller 38 kDa phosphoprotein band may represent a proteolytic fragment of NtCDPK1 or other protein which carries a similar epitope, or a protein which coimmunoprecipitates with NtCDPK1 and is phosphorylated by NtCDPK1. These results suggest that NtCDPK1 is associated with membrane.

In addition, expression of NtCDPK1-GFP fusion protein in tobacco protoplasts was examined under a fluorescent microscope after PEG-mediated transfection of the fusion construct. The NtCDPK1-GFP protein was localized in membrane but it was also visualized in nucleus as well. Nuclear localization of NtCDPK1 was confirmed by immunodetection of the 60 kDa protein in the nuclear fraction using the antibody that reacts with NtCDPK1.

To test cellular function of NtCDPK1, we have generated transgenic tobacco plants carrying the *NtCDPK1* sense constructs, either the full cDNA (OF line) or the cDNA corresponding to the kinase domain of NtCDPK1 (KD line), fused to the 35S promoter. The transgene transcripts were detected at high level in the transgenic plants of both OF and KD lines by RNA gel blot analysis. In the KD lines, the endogenous *NtCDPK1* mRNA level was significantly reduced, indicating that the *NtCDPK1* gene was downregulated by expression of the sense transgene. The KD transgenic plants appeared normal except that the size of the plants was smaller than that of the control plants. Closer examination revealed that the lateral root development was significantly inhibited in the KD seedlings. When the KD transgenic plants became mature, they obtained smaller root masses than did the control plants, which is likely responsible for the reduced size of the plants. Thus silencing gene expression of *NtCDPK1* resulted in inhibition of lateral root development in transgenic tobacco plants. However, the length of primary and lateral roots of the KD plants was not different from that of the control plants. The KD plants exhibited no differences in their responses to high concentration of NaCl, auxin (NAA), cytokinin (Kinetin), or low calcium concentration from the control plants. The responses of the KD plants to other stimuli are currently under investigation. The OF transgenic plants did not show any visible phenotypes in plant growth and development under normal conditions. Responses of the OF plants to various physiological and environmental stimuli are under investigation.

It has been previously demonstrated that auxin regulates lateral root development in *Arabidopsis*, which was demonstrated by phenotypes of auxin signaling mutants. However, lateral root inhibition phenotype of the KD plants is not apparently related to disturbed auxin signaling, because the induction patterns of the auxin-inducible *IAA1* and *IAA2* genes in the KD plants were identical to those of the con-

trol plants upon auxin treatments of germinated seedlings.

To identify interacting proteins of NtCDPK1 *in vivo*, we have carried out yeast two hybrid screening using the kinase domain of NtCDPK1 as a bait. The screening resulted in isolation of a cDNA encoding a tobacco homologue of proteasome regulatory subunit 21D7, which was designated Nt21D7. The yeast mutant which carries mutation in *RPN3* gene, the *21D7* homologue of yeast, exhibited lethal phenotype. It was previously reported that the 21D7 mRNA is predominantly expressed in plant tissues that contain actively dividing cells, and the mRNA levels change significantly during cell cycle. The 21D7 protein was localized in the nucleus and nucleolus. Protein association between NtCDPK1 and Nt21D7 was confirmed *in vitro* by binding assays of the recombinant MBP-NtCDPK1 and GST-Nt21D7 proteins. In addition, the recombinant NtCDPK1 protein could phosphorylate the Nt21D7 protein *in vitro* in the presence of calcium. Interestingly, reduction of autophosphorylating activity of NtCDPK1 coincided with phosphorylation of Nt21D7. These results indicate that Nt21D7 protein may be a natural substrate of NtCDPK1 in plant cells. In animals and yeast, the ubiquitin/26S proteasome pathway plays a role in a number of cellular processes, primarily by controlling degradation of short-lived enzymes and regulators. Based on the interaction of NtCDPK1 with Nt21D7 and the phenotype of the transgenic plants, NtCDPK1 may be involved in regulation of plant development via controlling specificity and/or activity of proteasome-mediated protein degradation pathway.

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