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The mechanism of substrate recognition of Ca^{2+}-dependent protein kinases

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Abbreviations: CDPK, Ca^{2+}-dependent protein kinase; RSG, repression of shoot growth; CAD, CDPK activation domain

Ca^{2+}-dependent protein kinases (CDPKs) are encoded by a multigene family and are thought to play central roles in Ca^{2+} signaling in plants. Although the primary structures of CDPK isoforms are highly conserved, several studies suggested a distinct physiological function for each CDPK isoform in plants. Hence, there should be mechanisms by which individual CDPK specifically recognizes its substrate. Recently, the variable N-terminal domain of NtCDPK1 was shown to play an essential role in the specific recognition of the substrate. Because the variable N-terminal domain of other CDPKs may also be involved in the substrate recognition, the search for interacting proteins of the variable N-terminal domain would provide important clues to identify the physiological substrates of each CDPK. Additionally, manipulation of the variable N-terminal domain may enable us to engineer the substrate specificity of CDPK, leading a rational rewiring of cellular signaling pathways.

Ca^{2+} ions play a vital role as second messengers in plant cells during various developmental processes and in response to environmental stimuli.[1] Ca^{2+}-dependent protein kinases (CDPKs) are thought to play central roles in the Ca^{2+} signaling pathway in plants and some protozoans.[2-5] There are 34 genes encoding CDPKs in Arabidopsis[6] and 29 genes in rice.[7] CDPK proteins are Ser/Thr protein kinases that are composed of a variable N-terminal domain, a catalytic domain, a junction domain and a calmodulin-like domain.[2] Recently, structural analysis of CDPK showed that Ca^{2+} binding triggers the reorganization of the CAD (CDPK activation domain), which is composed of the junction domain and the calmodulin-like domain, into a highly intricate fold, leading to the relocation of the CAD around the base of the catalytic domain to a site remote from the substrate binding site.[8]

A tobacco transcription factor RSG (REPRESSION OF SHOOT GROWTH), which is involved in the gibberellin homeostasis, is negatively regulated by 14-3-3 signaling proteins.[9-11] The 14-3-3 proteins bind to RSG depending on the RSG phosphorylation of Ser-114 and thereby sequester RSG in the cytoplasm so that it is unable to regulate its target genes in the nucleus.[12] NtCDPK1 was identified as an RSG kinase that promotes 14-3-3 binding to RSG by phosphorylation of Ser-114 of RSG. NtCDPK1 interacts with RSG in vivo and in vitro and specifically phosphorylates Ser-114 of RSG in vitro. Knockdown of NtCDPK1 by RNAi repressed the GA-induced phosphorylation of Ser-114 of RSG, while overexpression of NtCDPK1 in transgenic plants promoted phosphorylation of Ser-114.[13] These results showed that RSG is a direct target of NtCDPK1.

Cells must somehow maintain the specificity of distinct Ca^{2+} signaling and avoid unwanted crosstalk. The functional specificity of individual CDPKs may be determined by Ca^{2+} and lipid sensitivity, expression pattern, posttranslational regulation, targeted subcellular compartmentalization and substrate recognition.[14,15] The first level of substrate specificity arises from the interaction between the active site of the kinase and the amino acid sequences surrounding the phosphorylation site of the substrate.[16] Additional conserved docking motifs on the substrate that interacts with specific regions of the catalytic domain may increase the selectivity of the kinase substrate.[17] However, because the sequences of phosphorylation sites and docking motifs are rather simple and ambiguous, they are insufficient to account for the substrate specificity. Other molecular mechanisms are required to select the functional targets among potential phosphorylation sites. Scaffold proteins or targeting subunits, which help to enhance substrate specificity, were not found for CDPKs.

The primary structures of CDPK isoforms are highly conserved, especially within their catalytic domains. Thus, it had been considered unlikely that CDPKs would have distinguishable substrate specificities. However, several studies using loss-of-function mutants and knockdown plants suggested that each CDPK isoform in plants possesses a distinct physiological function, such as stress response,[18,19] oxidative burst,[20] GA response,[13] pollen tube growth,[21] and stomatal regulation.[22] There should be a mechanism by which the substrates are specifically recognized by CDPKs.

We found that the variable N-terminal domain of NtCDPK1 plays an essential role in the specific recognition of substrate RSG.[23] The recognition by the variable N-terminal domain of NtCDPK1 may strictly determine the substrate specificity, in concert with the interaction between the catalytic domain

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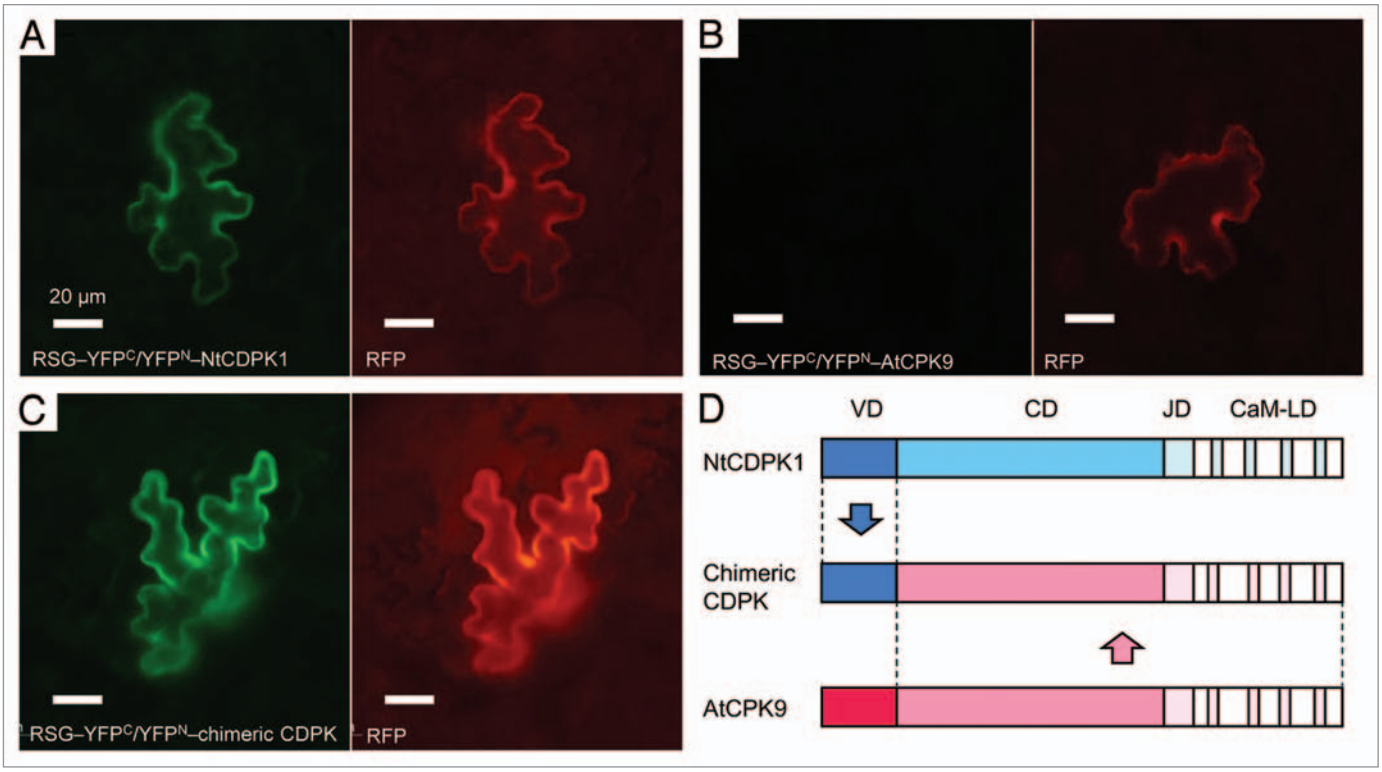

Figure 1. Visualization of the interactions between RSG and CDPKs in plant cells using BiFC (bimolecular fluorescence complementation) analysis. (A) NtCDPK1 interacts with RSG. YFP^N and YFP^C represent N-terminal and C-terminal portion of a yellow fluorescent protein, respectively. RFP (red fluorescent protein) was used as a control for transfection efficiency. (B and C) The chimeric CDPK in which the variable N-terminal domain of AtCPK9 was substituted with that of NtCDPK1 interacts with RSG, although AtCPK9 does not. (D) Schematic diagram of the chimeric CDPK. VD, variable N-terminal domain; CD, catalytic domain; JD, junction domain; CaM-LD, calmodulin-like domain.

of NtCDPK1 and the phosphorylation site of RSG. The variable N-terminal domain of other CDPKs may be involved in the substrate recognition. Yeast two-hybrid analysis suggested that the variable N-terminal domain of an Arabidopsis CDPK, AtCPK32, participates in the interaction with transcription factor ABF4.[24] AtCPK11 and a *Mesembryanthemum crystallinum* CDPK, McCDPK1, also interact with their substrate.[25,26] They may need the variable N-terminal domain for the substrate recognition. Although CDPKs have been reported to be involved in diverse physiological processes, very limited information is available about the direct substrates in vivo. The search for interacting proteins of the variable N-terminal domain by yeast two-hybrid screen or the tandem affinity purification technique would provide important clues to identify the physiological substrates of each CDPK. Such comprehensive studies would improve our understanding of both the physiological roles of each CDPK and the complicated network of Ca^{2+} signaling in plants.

The redesign of the substrate specificity of kinases is a major challenge of protein engineering.[27] The variable N-terminal domain of NtCDPK1 conferred sufficient activities as RSG kinase to an Arabidopsis CDPK, AtCPK9, that only poorly phosphorylates RSG (**Fig. 1**).[23] Substitutions of amino acids near the active center in the catalytic domain might affect the substrate specificity but often simultaneously decrease the kinase activity.[28] This is major obstacle in the specificity engineering of kinases. The finding that the substrate recognition of CDPK is separable from the catalytic activity opens the possibility of engineering the substrate specificity of CDPK by manipulations of the variable N-terminal domain, which would provide an approach for the rewiring the signaling pathways.

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