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Roles of calcineurin B-like protein-interacting protein kinases in innate immunity in rice

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Cytosolic free Ca^{2+} mobilization induced by microbe/pathogen-asssociated molecular patterns (MAMPs/PAMPs) plays key roles in plant innate immunity. However, components involved in Ca^{2+} signaling pathways still remain to be identified and possible involvement of the CBL (calcineurin B-like proteins)-CIPK (CBL-interacting protein kinases) system in biotic defense signaling have yet to be clarified. Recently we identified two *CIPKs*, *OsCIPK14* and *OsCIPK15*, which are rapidly induced by MAMPs, involved in various MAMP-induced immune responses including defense-related gene expression, phytoalexin biosynthesis and hypersensitive cell death. MAMP-induced production of reactive oxygen species as well as cell browning were also suppressed in *OsCIPK14/15-RNAi* transgenic cell lines. Possible molecular mechanisms and physiological functions of the CIPKs in plant innate immunity are discussed.

Ca^{2+} plays an essential role as an intracellular second messenger in plants as well as in animals. Several families of Ca^{2+} sensor proteins have been identified in higher plants, which decode spatiotemporal patterns of intracellular Ca^{2+} concentration.[1,2] Calcineurin B-Like Proteins (CBLs) comprise a family of Ca^{2+} sensor proteins similar to both the regulatory β-subunit of calcineurin and neuronal Ca^{2+} sensors of animals.[3,4] Unlike calcineurin B that regulates protein phosphatases, CBLs specifically target a family of protein kinases referred to as CIPKs (CBL-Interacting Protein Kinases).[5] The CBL-CIPK system has been shown to be involved in a wide range of signaling pathways, including abiotic stress responses such as drought and salt, plant hormone responses and K^+ channel regulation.[6,7]

Following the recognition of pathogenic signals, plant cells initiate the activation of a widespread signal transduction network that trigger inducible defense responses, including the production of reactive oxygen species (ROS), biosynthesis of phytoalexins, expression of pathogenesis-related (PR) genes and reorganization of cytoskeletons and the vacuole,[8] followed by a form of programmed cell death known as hypersensitive response (HR).[9,10] Because complexed spatiotemporal patterns of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) have been suggested to play pivotal roles in defense signaling,[1,9] multiple Ca^{2+} sensor proteins and their effectors should function in defense signaling pathways. Although possible involvement of some calmodulin isoforms[11-13] and the calmodulin-domain/calcium-dependent protein kinases (CDPKs)[14-19] has been suggested, other Ca^{2+}-regulated signaling components still remain to be identified. No CBLs or CIPKs had so far been implicated as signaling components in innate immunity.

The Role of OsCIPK14/15 in MAMP-Triggered Immunity

We have been establishing a model system to analyze a variety of defense responses including hypersensitive cell death in rice cultured cells by using TvX/EIX (xylanase from *Trichoderma viride*/ethylene-inducing xylanase) as an elicitor or a MAMP.[20] We surveyed the expression

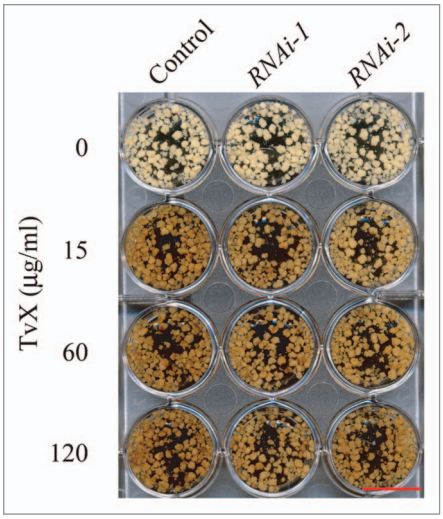

Figure 1. Effects of *OsCIPK14/15* suppression on TvX/EIX-induced cell browning. Rice cells of five days after subculture were treated with the TvX/EIX elicitor. Cells 24 h after elicitation are shown as representative of three experiments. Scale bar: 2 cm.

patterns of several *CIPK* genes in response to several MAMPs, including TvX/EIX and *N*-acetylchitooligosaccharides, and identified two MAMP-inducible *CIPKs*, *OsCIPK14* and *OsCIPK15*, which are duplicated genes in the rice genome. OsCIPK14/15 interacted with several OsCBLs through the FISL/NAF-motif in yeast cells and showed the strongest interaction with OsCBL4, whose expression was also induced by the MAMP. The recombinant OsCIPK14/15 proteins showed Mn^{2+}-dependent protein kinase activity, which was enhanced both by deletion of their FISL/NAF-motifs and by combination with OsCBL4.[21]

Functional characterization of the *OsCIPK14/15-RNAi* lines, as well as the overexpressing lines, suggested that these CIPKs are involved in the regulation of various TvX/EIX-induced defense responses, including mitochondrial dysfunction, hypersensitive cell death, biosynthesis of phytoalexins and PR gene expression.[21] During the induction of TvX/EIX-induced defense responses, cell browning is also triggered,[20] which was also suppressed in the *OsCIPK14/15*-knockdown cell lines (**Fig. 1**). Cell browning has been shown to be accompanied by accumulation of phenolic compounds and lignification in the cell wall during defense reactions against pathogen infection.[22] These results imply that OsCIPK14/15 may play an important role in the TvX/EIX-induced reprogramming of secondary metabolism. These consequences of knockdown/overexpression of the CIPKs were similar at least in part with those of a putative voltage-dependent Ca^{2+} channel, OsTPC1,[20] suggesting that these components may play roles in a common defense signaling pathway.

Possible Involvement of OsCIPK14/15 in the Regulation of MAMP-Induced ROS Production

TvX/EIX-induced ROS production was partially impaired (**Fig. 2A**) in *OsCIPK14/15-RNAi*-lines. Moreover, ROS production triggered by *N*-acetylchitooligosaccharides was also substantially reduced in the *OsCIPK14/15-RNAi* lines (**Fig. 2B**), suggesting possible involvement of OsCIPK14/15 on MAMPs-induced ROS production in rice cultured cells. External Ca^{2+} is required not only for hypersensitive cell death but also for NADPH oxidase-mediated ROS generation, which is prerequisite for TvX/EIX-induced hypersensitive cell death in rice.[20]

Rbohs (Respiratory Burst Oxidase Homologues) have been suggested to be involved in oxidative burst and the regulation of hypersensitive cell death in Arabidopsis and rice.[23-25] Some rboh proteins have recently been shown to possess ROS-producing NADPH oxidase activity and are synergistically activated by the direct binding of Ca^{2+} to their EF-hand motifs and protein phosphorylation.[26,27] Potato CDPK4 and 5, representative Ca^{2+} sensor proteins, have been shown to be involved in the regulation of rboh-mediated ROS prodution.[17] OsCIPK14/15 may also somehow play roles to activate rboh-mediated ROS prodution directly or indirectly to regulate various defense responses including the hypersensitive cell death.

Concluding Remarks

Some OsCBLs, including OsCBL4, or other unidentified factors that interact with OsCIPK15 via the FISL/NAF-motif may regulate the activity and localization of OsCIPK14/15 in the MAMP-triggered signal transduction pathway. Searches for the in vivo substrates of these CIPKs are currently underway to further elucidate the Ca^{2+} signaling pathways regulating hypersensitive cell death and innate immunity. These findings should shed further light on our understanding of defense signaling pathways.

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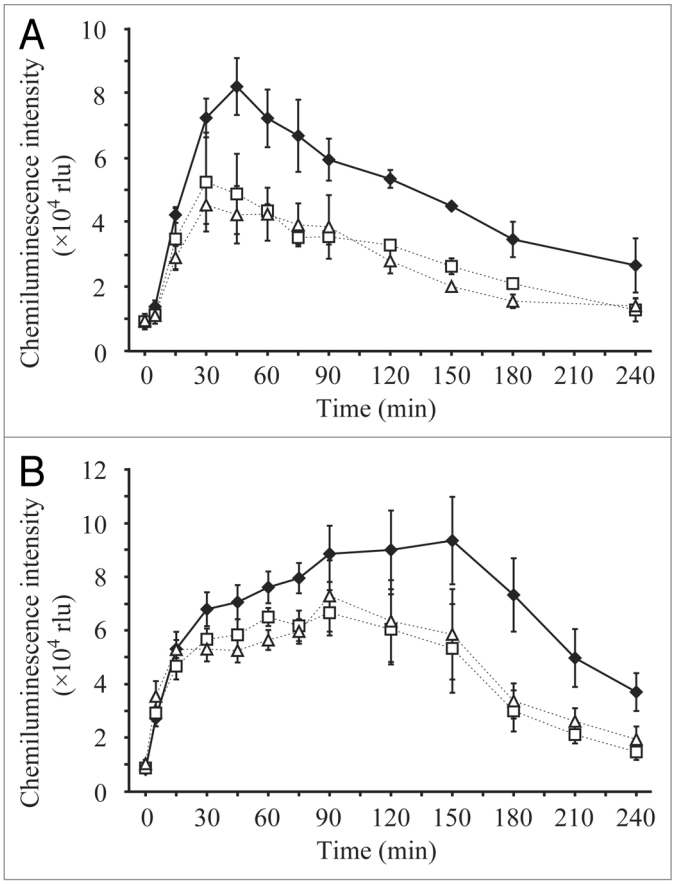

Figure 2. Effects of *OsCIPK14/15* suppression on MAMP-induced ROS generation. Time course of ROS ($\cdot O_2^-$) generation in the cell suspension after TvX/EIX treatment (120 μg mL^{-1}) (A) and *N*-acetylchitoheptaose (10 μM) (B). Cells of five days after subculture were washed and resuspended in fresh growth medium 30 min before measurement. A 250 μl aliquot of cells was collected at the indicated time and treated with 2 μM MCLA (Molecular Probes, Eugene, OR). The $\cdot O_2^-$-dependent chemiluminescence was measured with a Lumicounter 2500 (Microtech Nition, Chiba, Japan) with continuous aeration by shaking of the vial. Average values and standard errors of three independent experiments for the control line (black diamond) and two independent *RNAi* lines (white square for *RNAi-1* and white triangle for *RNAi-2*) are shown.

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