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Molecular Analysis of Rice *CIPKs* Involved in Both Biotic and Abiotic Stress Responses

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Abstract: Plant calcineurin B-like (CBL) proteins have been proposed as important Ca^{2+} sensors and specifically interact with CBL-interacting protein kinases (CIPKs) in plant-specific calcium signaling. Here, we identified and isolated 15 *CIPK* genes in a japonica rice variety Nipponbare based on the predicted sequences of rice *CIPK* gene family. Gene structure analysis showed that these 15 genes were divided into intron-less and intron-rich groups, and *OsCIPK3* and *OsCIPK24* exhibited alternative splicing in their mature process. The phylogenetic analyses indicated that rice *CIPKs* shared an ancestor with *Arabidopsis* and poplar *CIPKs*. Analyses of gene expression showed that these *OsCIPK* genes were differentially induced by biotic stresses such as bacterial blight and abiotic stresses (heavy metal such as Hg^{2+}, high salinity, cold and ABA). Interestingly, five *OsCIPK* genes, *OsCIPK1*, *2*, *10*, *11* and *12*, were transcriptionally up-regulated after bacterial blight infection whereas four *OsCIPK* genes, *OsCIPK2*, *10*, *11* and *14*, were induced by all treatments, indicating that some of *OsCIPK* genes are involved in multiple stress response pathways in plants. Our finding suggests that CIPKs play a key role in both biotic and abiotic stress responses.

Key words: rice; CBL-interacting protein kinase family; gene expression; biotic and abiotic stress; bacterial blight

Calcium, a universal second messenger, plays a crucial role in stress signaling pathways in plants (Reddy, 2001). Most stresses such as heat, low temperature, osmotic and oxidative stresses, as well as pathogen infection, stimulate plant cells to change the concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) and cause spatial and temporal Ca^{2+} signals (White and Broadley, 2003). Stress-induced Ca^{2+} signals are decoded by many Ca^{2+}-sensors, including calmodulins (Yang and Poovaiah, 2003), calmodulin-binding proteins (Jeong et al, 2007), calcineurin B-like proteins (CBLs) (Luan et al, 2002), calcium-dependent protein kinases (CDPKs) (Harmon et al, 2000) and CDPK-related kinases (Leclercq et al, 2005), and in turn downstream cascades are activated, finally leading to alter the expressions of stress-induced genes. The CBLs are plant-specific calcium binding proteins harboring EF-hand motifs as the structural basis for calcium binding and interact specifically with a group of protein kinases designated as CBL-interacting protein kinases (CIPKs) in Ca^{2+} signaling (Luan et al, 2002). The CIPKs, Ca^{2+}-dependent serine/threonine kinases with a highly conserved SNF kinase domain and a NAF amino-acid motif (Asn-Ala-Phe), belong to SnRK3 (sucrose non-fermenting-1 related kinases) protein family in plants (Hrabak et al, 2003). In the *Arabidopsis* genome, 10 AtCBLs and 25 AtCIPKs have been reported and the CBL-CIPK complex formation was proposed to contribute to specificity of their signals (Kolukisaoglu et al, 2004).

In the rice genome, 30 putative rice homologues of *AtCIPK* genes were bioinformatically predicted and designated as *OsCIPK1* to *OsCIPK30* (Kolukisaoglu et al, 2004). Recent study indicated that 20 *OsCIPK* genes were transcriptionally induced by some of abiotic stresses including drought, salinity, cold, polyethylene glycol, or abscisic acid (ABA) treatments (Xiang et al, 2007). Over-expressions of three *OsCIPK* genes, *OsCIPK3*, *12* and *15*, significantly improved tolerance of transgenic plants to cold, drought and salt stress, respectively (Xiang et al, 2007). However, whether *CIPK* genes respond to biotic stresses and participate in disease resistance in plants is still unclear.

Here, 15 *OsCIPK* genes were molecularly analyzed and their responses to biotic and abiotic stresses were examined in a japonica rice variety Nipponbare. Our results showed that the expressions

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of 15 *OsCIPK* genes were differentially induced by bacterial blight (BB), heavy metal (Hg^{2+}), high salinity, cold and ABA. Interestingly, five *OsCIPK* genes (*OsCIPK1*, *2*, *10*, *11* and *12*) were transcriptionally up-regulated after BB infection whereas four *OsCIPK* genes (*OsCIPK2*, *10*, *11* and *14*) were induced by all treatments, suggesting that these *OsCIPK* genes were involved in multiple stress response pathways and/or disease-resistance pathways in plants.

MATERIALS AND METHODS

Plant growth conditions and stress treatments

Seeds of the rice variety Nipponbare (*Oryza sativa* L. ssp. *japonica*) were sterilized by 0.1% $HgCl_2$, and germinated in a incubator at 37°C and then cultured in Yoshida's solution (Yoshida et al, 1976) in a growth chamber with 28°C/24°C day/night temperature, 14 h light/10 h dark photoperiod, and 250 μmol/(m^2·s) light intensity. Seedlings at the three-leaf stage were treated with ABA or other stresses. For ABA treatment, 0.1 mmol/L ABA solution was sprayed on the seedling leaves (Mahajan et al, 2006) followed by sampling at 0, 6, 12, and 24 h after treatment, respectively. For high salinity and Hg^{2+} treatments, the seedlings were transferred to the nutrition solution supplemented with 200 mmol/L NaCl and 0.15 mmol/L $HgCl_2$, respectively, and sampled after 0, 6, 12, and 24 h treatment. For cold treatment, the seedlings were chilled at 4°C in a growth chamber and sampled after 0, 6, 12, and 24 h, respectively. For infection with BB, the seedlings were inoculated with the Philippine race PXO99 (*Xanthomonas oryzae* pv. *oryzae*) by the leaf-clipping method (Kauffman et al, 1973), and sampled along the inoculated sites after 0, 12, 24, and 48 h, respectively. After time course treatments, the leaves were harvested from cold-, ABA-, and BB-treated plants, respectively, whereas the roots and shoots were separately harvested in high salinity- and Hg^{2+}-treated plants, respectively, since Hg^{2+} and salt are mainly taken up by roots. All rice materials were stored at -80°C for later processing.

Database search and sequence analysis

The protein sequences of *Arabidopsis* CIPKs and the reports on the rice CIPKs (Kolukisaoglu et al, 2004; Xiang et al, 2007) were used to search for homologous genes in japonica rice genome database using tBLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The results were further verified with updated rice expressed sequence tag (EST) (http://www.ncbi.nlm.nih.gov/dbEST) and full-length cDNA database (http://cdna01.dna.affrc.go.jp/cDNA/). To determine the gene structures of *OsCIPKs*, their genomic sequences were aligned with the cDNAs by the Clustal V program (http://fibernet.vanderbilt.edu/fiber/workbench/clustal.html) and further confirmed by the rule of GT-AG intron splicing donor-acceptor sequence in the genomic sequences between exons (Mount, 1982). Multiple sequence alignment of *OsCIPKs* was performed using the Clustal X program (Thompson et al, 1997) and edited by the software GeneDoc (Nicholas et al, 1997). A phylogenetic tree was presented using the TreeView (Version1.6.6) software (Page, 2002).

First strand cDNA synthesis

Total RNA was isolated from rice seedlings using Rneasy Plant Mini Kit (QIAGEN) according to the manufacture's instructions. The first-strand synthesis of cDNA from the total RNA was carried out by the reverse transcription-polymerase chain reaction (RT-PCR) system (Promega). Briefly, the RT was performed in a 25-μL volume containing 2 μg of total RNA (pre-treated with DNase I), 1 μg of oligo $(dT)_{18}$ primer, 200 U of molony murine leukemia virus (M-MLV) reverse transcriptase, 1×M-MLV buffer, 20 U of ribonuclease inhibitor and 0.15 mmol/L dNTPs at 42°C for 1 h. Then, RT was terminated by incubating at 70°C for 15 min and the reaction mixture was stored at -20°C.

Molecular cloning

A PCR-based strategy was used for cDNA cloning of *OsCIPK* genes. The primers were designed by the PRIMER3 program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) based on the sequences of 5′ untranslated region (UTR) and 3′ UTR of the target genes (Table 1). Distinct cDNAs from the seedlings treated by various stresses were mixed for use as PCR DNA templates. PCR was performed in a 50-μL volume containing 100 ng of cDNA mixture, 5

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