Interaction between programmed cell death 5 and calcineurin B-like interacting protein kinase 23 in *Oryza sativa*

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Abstract

Programmed cell death (PCD) is crucial for plants during development and stress survival. *OsPDCD5*, an ortholog to mammalian-programmed cell death 5, was previously cloned from rice (*Oryza sativa*, cv Zhenxian 97A), and its overexpression can induce PCD in transgenic rice. In the present study, immunoblotting analysis revealed that the *OsPDCD5* protein was widely expressed in the tassel, leaf, leaf sheath, and different parts of the stem but not in the anther. RT-PCR analysis showed that *OsPDCD5* was related to the senescence of leaf and root tissues as well as the development of stem tissues. Furthermore, *OsPDCD5* was up-regulated by UV-B irradiation. Calcineurin B-like interacting protein kinase 23 (*OsCIPK23*), which is involved in the calcineurin B-like proteins (CLBs)/CBL-interacting protein kinases (CIPKs) signaling network, was identified as interacting with *OsPDCD5* by yeast two-hybrid screening and subsequently confirmed by pull-down assay in vitro. Present findings may shed light on the investigation of the biochemical function of *OsPDCD5* in rice.

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1. Introduction

Programmed cell death (PCD) is a genetically controlled process that functions in the development of multicellular organisms and in their responses to biotic and abiotic stresses [1,2]. Many genes inducing or inhibiting PCD have been found in animals, but cloning of their homologues has rarely been reported in plants. Programmed cell death 5 (PDCD5), formerly known as TF-1 cell apoptosis-related gene 19 (TFAR19), is well conserved in yeast and mammals [3]. PDCD5 was significantly induced and rapidly translocated to the nucleus in the cells undergoing apoptosis, preceding chromosome DNA fragmentation and phosphatidylerine externalization [4,5]. Suppressed expression of PDCD5 using short interfering RNA (siRNA) or an antibody against PDCD5 can inhibit apoptosis, demonstrating that PDCD5 is crucial for mammalian apoptosis [6,7]. Overexpression of PDCD5 can enhance TAJ/TROY-induced parapoptotic cell death, a death pathway distinct from apoptosis, suggesting PDCD5 protein is an important regulator in both apoptotic and non-apoptotic programmed cell death [8].

Previously we identified the *OsPDCD5* gene from *Oryza sativa*, which was highly homologous with human PDCD5. We found that its expression was up-regulated under low temperature and NaCl treatments [9]. Recently, we also found that overexpression of *OsPDCD5* can induce PCD in transgenic rice plants, including fragmentation of genomic DNA into a DNA ladder and the early death of transgenic plants [10].

In the present work, we investigated the distribution of *OsPDCD5* protein in various tissues and the expression profiles of *OsPDCD5* during development and under UV-B treatment. We also identified CBL-interacting protein kinase 23 (CIPK23) as an interacting protein of *OsPDCD5* by yeast two-hybrid screening and pull-down analysis.

2. Materials and methods

2.1. Plant materials and growth conditions

Rice (*Oryza sativa*, cv Zhenxian 97A) was grown under white fluorescent light (wavelength 390–500 nm, 150 μeinstein m⁻² s⁻¹, 12-h photoperiod) at 25 °C and 70% relative humidity.
Leaf sheath, young leaf, and root were prepared from 2-week-old seedlings, and mature leaf, mature root, tassel, anther, and stem were taken from mature plants growing in the field. For UV-B treatment, seedlings in a closed chamber were exposed to 1500 μJ/m² UV-B irradiation [11], and the leaf samples were collected at 0, 1, 2, 4 and 8 h of treatment. All materials were frozen in liquid nitrogen and stored at −80 °C.

2.2. Generation of anti-recombinant OsPDCD5 antibody

To generate antibodies against OsPDCD5, the full-length encoding region of OsPDCD5 was cloned into a pGEX 4T-1 expression vector (Amersham Pharmacia Biotech, Piscataway, NJ) using the primers PDCD1F (5'-CGCAATTCATGGCGACCGAGTTG-3') and PDCD1R (5'-CGACTCGAGCTATCGATCGCCGCAAG-3') and confirmed by sequencing. The recombining GST-OsPDCD5 fusion protein was expressed in Escherichia coli BL21 and purified using glutathione–Sepharose 4B MicroSpin columns (Amersham Pharmacia Biotech), then immunized rabbit as described previously [12]. The antibodies were further purified using protein A Sepharose (Amersham Pharmacia Biotech).

2.3. Preparation of protein extracts and immunoblotting analysis

Total protein was extracted from multiple rice tissues and leaves treated by UV-B irradiation. Rice tissues were ground under liquid N2 gas and re-suspended in extraction buffer (0.1 M Tris–HCl, pH 7.6, and 0.25 M sucrose and 1 mM PMSF) at 4 °C. After centrifugation (500 × g, 15 min), the supernatant was used as a soluble protein fraction for immunoblotting analysis [13]. The protein in the supernatant was resolved by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with phosphate-buffered saline (PBS) containing 0.2% Tween 20 and 5% non-fat dry milk for 1 h, the membrane was probed with anti-OsPDCD5 antibody (1:500) for 2 h and then washed and exposed to horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (1:5000) for 1 h. The bound antibodies were visualized with a Photoprobe HRP Western detection kit (Cell Signaling Technology, USA).

2.4. Semi-quantitative RT-PCR analysis

Total RNAs were extracted from young root, old root, young leaf, old leaf, and different parts of stem and leaf treated by UV-B using TRIZOL reagent (GIBCO BRL, USA). First-strand cDNA was generated using Superscript II reverse transcriptase (Invitrogen). The primers PDCDF (5'-GGATCCGGATCTCC-GGCC-3') and PDCDR (5'-CAAGGACGGCTCCGGCGC-3') were designed for RT-PCR analysis. The templates were amplified at 95 °C for 5 min, followed by amplification (94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s) for 27 cycles, and prolonged at 72 °C for 10 min. The RT-PCR reaction for the rice Actin gene (Genbank Accession No. X16280) using specific primers ActinF (5'-CCATTGCTGACGTGGC-3') and ActinR (5'-TAGGAGTGGAAGCGGCGG-3') was also performed as a control as described above. PCR products were analyzed on 1% agarose gels.

2.5. Yeast two-hybridization analysis

The yeast two-hybrid library construction and screening were performed with BD Matchmaker™ library construction and screening kits (Clontech). The OsPDCD5 coding region was fused in-frame with the GAL4 DNA binding domain in the pGBKTT7 vector to generate the bait. The primers were PDCD2F (5'-CGGATCCATGGCGACCGAGTTG-3') and PDCD2R (5'-CGGGATCCGGGTAGCGTAGCTCCGGCGC-3'). The rice ds-cDNA, the pGAD-T7-Rec2 vector, and the pGBKTT7-OsPDCD5 bait were cotransformed into the yeast strain AH109 then plated directly onto medium lacking Ade, Leu, Try, and His and incubated at 30 °C for 4 days. Screening of positive clones was performed according to the manufacturer’s protocol. The OsCIPK23 coding region was cloned into the pGADT7 vector in yeast two-hybrid tests using the primers CIPK1F (5'-GCCGATCCGGATCGCCTTCCGGGTTGTCGAGCTCCGGCGC-3') and CIPK1R (5'-GCCGATCCGGATCGCCTTCCGGGTTGTCGAGCTCCGGCGC-3'). Yeast cells harboring both pGBKTT7-OsPDCD5 and pGADT7-OsCIPK23 were selected on high stringency nutrition -Ade/-His/-Leu/-Trp plates.

2.6. GST pull-down assay

The OsCIPK23 coding region was obtained by PCR amplification using the primers CIPK2F (5'-GCCTTGACGCGAGCGTCCGGGCGC-3') and CIPK2R (5'-GCCTTGACGCGAGCGTCCGGGCGC-3') and cloned into the eukaryotic expression vector pCMV-Myc with an N-terminal c-Myc tag. HEK293 cells were maintained in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin sulfate. Cells were transiently transfected using Lipofectamine reagent according to the manufacturer’s instructions (Invitrogen) and cultured for 36 h. The cells were washed twice with PBS (pH 7.4) and then lysed with PBS containing 0.2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1.0% Triton X-100. Lysates were clarified by centrifugation (13,000 × g, 20 min) at 4 °C.

An equal amount of GST or GST-OsPDCD5 fusion protein was added into glutathione–Sepharose 4B MicroSpin Columns (Amersham Pharmacia BioTech) and incubated for 3 h at 4 °C. After washing (four times, 5 min each time) in PBS containing 1.0% Triton X-100, soluble extracts (100 μg protein) prepared from HEK293 cells transiently transfected with Myc-OsCIPK23 were added into each reaction and incubated at 4 °C for 4 h. The beads were washed (four times, 5 min each time) in PBS containing 1.0% Triton X-100. The bound protein was mixed with 50 μl SDS sample buffer then resolved on a SDS-PAGE gel before it being used for western blot assay using anti-Myc antibodies.
3. Results and discussion

3.1. Distribution of OsPDCD5 protein in various rice organs

We succeeded in obtaining a recombinant protein of GST-OsPDCD5 (∼43 kDa, see Fig. 1A) and generating a polyclonal antibody. To determine whether OsPDCD5 has detectable protein levels and to identify the organs where the protein is expressed, the crude extracts from various tissues at the mature stage were analyzed by western blotting using anti-OsPDCD5 polyclonal antibody. The molecular weight of the detected protein was about 17 kDa, matching the size of the amino acid sequence deduced from OsPDCD5 (Fig. 1B). The OsPDCD5 protein was expressed widely in the tassel, leaf, leaf sheath, and different parts of stem (near the tassel, near the root) but was absent in anther tissues. A recombinant protein of Myc-OsPDCD5 was used as a positive control (Fig. 1B, upper panel). A Coomassie-stained SDS-PAGE gel was used as the total protein loading control (Fig. 1B, lower panel).

3.2. Semi-quantitative RT-PCR analysis

Total RNAs extracted from young root, old root, young leaf, old leaf, and different parts of the stem (according to the content of the pith in the center of stem termed S1–S3, respectively, see Fig. 2B) were used to investigate developmental expression profiles of OsPDCD5 by semi-quantitative RT-PCR analysis. The result showed that OsPDCD5 was highly expressed in old roots and leaves but weakly expressed in young roots and leaves (Fig. 2A), suggesting that OsPDCD5 was up-regulated by the senescence of both leaf and root. OsPDCD5 declined gradually from S1 to S3 (Fig. 2B), suggesting that the expression of OsPDCD5 was down-regulated along with stem development and pith cell death. Organ senescence and vascular bundle formation are involved in PCD [14]. As a whole, these results indicated that OsPDCD5 gene may play an important role in PCD regulation and that its expression is induced by developmental cues.

3.3. OsPDCD5 was induced by UV-B irradiation

To determine whether the amounts of OsPDCD5 transcript and protein change in response to UV-B treatment, seedlings in a closed chamber were exposed to 1500 μJ/m² UV-B irradiation for 0, 1, 2, 4 and 8 h. RT-PCR analysis showed OsPDCD5 was induced by UV-B treatment (Fig. 3A), and western blotting analysis using anti-OsPDCD5 polyclonal antibody revealed the expression level of OsPDCD5 showed a tendency to increase after UV-B exposure (Fig. 3B), which was consistent with the RT-PCR result. Our previous studies showed that the expression level of OsPDCD5 is up-regulated by low temperature and NaCl [9]. These data suggested that OsPDCD5 expression was enhanced in response to various abiotic stresses such as UV-B irradiation, low temperature, and salt treatment.

3.4. Identification of OsCIKP23 as an OsPDCD5 interacting protein by yeast two-hybrid screening

To elucidate the roles of OsPDCD5, we performed yeast two-hybrid screening to detect the proteins with which it interacts, and we identified six potential interacting molecules...
as a result. Nucleotide sequence analysis revealed that one of the cDNA fragments encoded the C-terminal residues 265–450 of CBL-interacting protein kinase 23 (termed OsCIPK23-C). OsCIPK23 (Genebank Accession No. AK069726) has been identified as a target of calcium signals transduced by CBL-type calcium sensors [15]. It contains an N-terminal S_TKc domain and a C-terminal NAF domain, and the NAF domain is highly conserved in all CIPK-type kinases and responsible for the interaction with the CBL proteins [16,17]. OsCIPK23-C contains the NAF domain and the junction region between the S_TKc and the NAF domain of OsCIPK23 (Fig. 4A). To confirm the direct interaction between OsPDCD5 and OsCIPK23, we performed a yeast-two-hybrid assay using co-transformation of the pGBK77-OsPDCD5 bait construct and the full-length pGADT7-OsCIPK23, or the N-terminal truncated pGADT7-OsCIPK23-C. As expected, Ade, Leu, Try, and His prototrophy were observed in yeast harboring both OsPDCD5 and OsCIPK23, though the interaction was weaker than with OsCIPK23-C, based on the percentage of colonies growing on quadruple dropout medium (Fig. 4B). This result implied that the C-terminal of OsCIPK23 might

Fig. 2. Expression profiles of OsPDCD5 during rice development by RT-PCR analysis. Expression of OsPDCD5 (A) in young leaf, old leaf, young root, and old root; (B) in stem near the tassel (S1), stem near the root (S3), and stem between S1 and S3 (S2). The rice Actin gene was used in RT-PCR amplification as the control. Relative mRNA levels were analyzed using Scion image and Excel software.

Fig. 3. Expression profiles and protein gel blot of OsPDCD5 under UV-B treatment. (A) Expression of OsPDCD5 under UV-B treatment. The rice Actin gene was used in RT-PCR amplification as the control. Relative mRNA levels were analyzed using Scion image and Excel software. (B) Western analysis of OsPDCD5 under UV-B treatment. Total soluble proteins isolated from the rice leaves treated by UV-B irradiation were separated by 12% SDS-PAGE and immuno-detected by anti-OsPDCD5 antibodies (upper panel). A recombinant protein of Myc-OsPDCD5 was used as a positive control. The CBB-stained gel used as a loading control is shown in the lower panel.
be responsible for the interaction between OsCIPK23 and OsPDCD5 and that the N-terminal of OsCIPK23 was likely to impair this interaction.

3.5. OsPDCD5 interacts with OsCIPK23 directly in vitro

To further elucidate the interaction between OsCIPK23 and OsPDCD5 in vitro, we performed a GST pull-down assay. As described above, the molecular mass of GST-OsPDCD5 was about 43 kDa, and the GST protein with a molecular mass of 26 kDa was also expressed as a control (Fig. 5B). The Myc-OsCIPK23 fusion protein expressed in HEK293 cells was detected using anti-Myc antibody and was about 50 kDa. As shown in Fig. 5A, Myc-OsCIPK23 bound to GST-OsPDCD5, but not to GST alone, which was consistent with the yeast two-hybrid assay results. Recent studies have shown that the CBL and CIPK network responds to developmental needs and environmental changes [15]. These findings suggest that external signals might regulate the function of OsPDCD5 protein via the CBLs/CIPKs network. We speculate that OsPDCD5 may be a phosphorylation substrate of OsCIPK23. Further studies, such as analyses of OsPDCD5 and OsCIPK23 loss-of-function mutants, will elucidate whether OsCIPK23 relates to cell death or not and help to understand the physiological meaning of its interaction with OsPDCD5 protein.

In conclusion, we found that the OsPDCD5 expression was induced by developmental and environmental cues and identified an interaction between OsPDCD5 and OsCIPK23. All of these findings will aid in further investigations of the biochemical function of OsPDCD5 in rice.

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References