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A CHASE domain containing protein kinase *OsCRL*4, represents a new *AtCRE*1-like gene family in rice*

HAN Qiu-min (韩秋敏)[1,2], JIANG Hua-wu (姜华武)[1], QI Xiao-peng (齐晓朋)[1],
YU Jie (于 洁)[1], WU Ping (吴 平)[†1]

([1]*State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, Hangzhou 310029, China*)
([2]*Wotson Institute of Genomics, Zhejiang University, Hangzhou 310029, China*)
[†]E-mail: docpwu@zju.edu.cn

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Abstract: *AtCRE*1 is known to be a cytokinin receptor in *Arabidopsis*. The *AtCRE*1 protein contains CHASE domain at the N-terminal part, followed by a transmitter (histidine kinase) domain and two receiver domains. The N-terminal CHASE domain of *AtCRE*1 contains putative recognition sites for cytokinin. Five CHASE domains containing proteins were found in rice, *OsCRL*1*a*, *OsCRL*1*b*, *OsCRL*2, *OsCRL*3, and *OsCRL*4. *OsCRL*1*a*, *OsCRL*1*b*, *OsCRL*2 and *OsCRL*3 contain the four domains existing in *CRE*1, whereas *OsCRL*4 only contains the CHASE domain and a putative Ser/Thr protein kinase domain. The authors cloned the encoding gene *OsCRL*4 and found that it represents a new member of the cytokinin receptor protein in rice.

Key words: *Oryza sativa* L, *OsCRL*4, CHASE domain
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INTRODUCTION

Cytokinins play a major role in many different developmental and physiological plant processes, such as cell division, regulation of root and shoot growth and branching, chloroplast development, leaf senescence, stress response and pathogen resistance (Mok and Mok, 2001). Significant advances have been achieved in our understanding of cytokinin signaling, providing important insights into the molecular partners involved (Hutchison and Kieber, 2002; Sheen, 2002). It had been proposed that cytokinins are detected by a two-component system, because over-expression of the histidine kinase gene *CKI*1, induces typical cytokinin responses (Kakimoto, 1996) and that a set of response regulators of two-component systems can be induced by cytokinin (Brandstatter and Kieber, 1998; Sakakibara *et al.*, 1998). Two-component systems use a histidine kinase as an environmental sensor and rely on a phosphorelay for signal transduction. They are common in microorganisms, and are also emerging as important signal detection routes in plants. At present, three cytokinin receptors such as *CRE*1, *AHK*2 and *AHK*3 have been found in *Arabidopsis*. The primary structure of all three cytokinin receptors displays two to three transmembrane domains at the N-terminal part, followed by a transmitter (histidine kinase) domain and two receiver domains. The predicted extracellular ligand-binding regions, which are ~270 amino

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acids long, have drawn special at tention, as they are the putative recognition sites for cytokinin. *CRE*1/*AHK*4 mutation of Thr 278 to Ile in this domain leads to a loss of function (Mahonen *et al*., 2000). Sequence comparison showed the presence of this domain in other receptor-like protein in both prokaryotes and lower eukaryotes. Because of its presence in a variety of functionally diverse membrane receptor proteins that recognize cytokinin-like adenine derivatives or peptide ligands and that have intracellular histidine-kinase or nucleotide cyclase domain, the domain has been named the CHASE domain (cyclases/histidine-kinase-associated sensory extracellular) (Alexander and Thomas, 2003). In *Arabidopsis*, the CHASE domain is specific for *AHK*2, *AHK*3 and *CRE*1/*AHK*4 (Alexander and Thomas, 2003). In rice, less is known about cytokinin signaling and the CHASE domain containing proteins. Blast search in NCBI database, five CHASE domain containing proteins were found in rice genomic sequences. According to the homology with histidine kinase of Zea mays, the five genes were named *OsCRL*1*a*, *OsCRL*1*b*, *OsCRL*2, *OsCRL*3 and *OsCRL*4. *OsCRE*1*a*, *OsCRE*1*b*, *OsCRE*2 and *OsCRL*3 and contained structure similar to that of *AtCRE*1, which contains four conserved domains, and they may act as cytokinin receptors in rice. The transmitter (histidine kinase) domain and the receiver domain, however, were not found in *OsCRL*4. To know whether *OsCRL*4 acts as cytokinin receptor in rice, the authors cloned the *OsCRL*4 gene and analyzed its function in *Arabidopsis*.

MATHERIALS AND METHODS

Plant materials and conditions

Rice plants (*Oryza sativa L ssp.* Japonica*, Nipponbare*) were used for the analyses. The plants were greenhouse grown at 30 ºC/24 ºC (day/night). *Arabidopsis cre*1-1 mutant (Ler genetic background) was kindly provided by Tatsuo Kahimoto. The wild and mutant plants of *Arabidopsis* (Ler) were grown at 24 ºC/22 ºC (day/night).

Cloning and sequencing analysis of *OsCEL*4 gene

A rice BAC clone (OSJNBa0080D17) containing a CHASE domain was obtained by Blast in NCBI. Rice GAAS was used to predict the *OsCRL*4 open reading frame in RGP. cDNA of *OsCRL*4 was cloned using RT-PCR with the primers of 5'-ATGGCGCTACTGCTCTGGGTGTTCAG-3' and 5'-ATAAGCCTGTTCTTGCATGGACCGGATG-3' from a rice root cDNA library constructed in previous work (Xia *et al*., 2002). PCR conditions were 94 ºC for 5 min, followed by 35 cycles, 94 ºC for 30 s, 58 ºC for 30 s, 72 ºC for 2 min plus 30 s. The PCR product was sequenced using MegaBACE^{TM} 100 DNA sequencer (Amersham Pharmacia Biotech).

Complementation analysis for *Atcre*1-1

The *OsCRL*4 cDNA was inserted into the binary plant vector pCAMBIA1301 by (*Xba* I/*Pst* I). The resulting plasmid was named 35*S::OsCRL*4, and mobilized to *Agrobacterium tumefaciens* strain GV3101 for plant transformation. Five weeks old wild type and *cre*1-1 *Arabidopsis* plants were infected with the *A. tumefaciens* by floral dipping method (Clough and Bent, 1998) and grown in greenhouse. The seeds were collected and screened in MS (Murashige and Skoog) medium supplemented with 20 μg/ml Hygromycin.

GUS staining analysis

The rice genomic DNA was used as the template to amplify the *OsCRL*4 promoter sequence, using 5'-ATAGTCGACTCCAGGGCACACGAAAAGACACAAGTCA-3' and 5'-ATAGAATTCAACGGTAGCAGCAGCAGCCACAACCAG-3' as upstream and downstream primers, respectively. The promoter fragment was approximately 2.1 kb. The *OsCRL*4 promoter sequence was inserted into pCAMBIA1391Z plant vector at the site of *Sal*I and *Ecor*I, which carries the structural gene for GUS. *OsCRL*4-GUS was mobilized to *Agrobacterium tumefaciens* strain GV3101 and used for plant transformation. The transformation and regeneration procedures were carried out as described previously (Hiei *et al*., 1994).

The samples were collected in 1.5 ml Eppendorf tubes containing 1 ml GUS staining solution (100 mmol/L NaH_2PO_4 buffer pH 7.0, 0.5% Trition

X-100, 0.5 mg/ml X-Gluc and 20% methanol). After a brief period of vacuum infiltration, the samples were incubated at 37 ºC up to 24 hours. After staining, tissues were fixed in FAA solution and examined under a dissection microscope. For GUS localization, 4-day old seedlings were infiltrated with 5-Bromo-4-chloro-3-indolyl-β-glucuronic for 10 min, followed by an overnight incubation in the dark at 37 ºC, as described (Brocard *et al*., 2002). Seedlings were then fixed for 12 h at 4 ºC in FAA buffer and then dehydrated in an acetone, gradually infiltrated with Spur's resin, and finally embedded in 100% resin and incubated at 70 ºC for one day. Ten micrometer-thick sections were cut using KCQ-2. 8500 (Netherlands) and attached to glass slides with heat and viewed on an Axiovert 200 microscope (German). Images were taken with an Axiocam and processed with Adobe photoshop.

Semiquantitative RT-PCR analysis for *OsCRL4* expression

Total RNA was extracted from root, stem, leaf, and spikelet of the wild plant using the previously described method. After Dnase I treatment, 5 μg of the total RNA was used to synthesize the first strand of cDNA with an oligo (dT) and RT Supperscript II as recommended by the manufacturer (Invitrogen, Carlsbad, CA). Two microliters of the first strand of cDNA was used as a template for semiquantitative RT-PCR. The PCR reaction containing cDNA 1× polymerase buffer (Promega), 2.5 mmol/L $MgCl_2$, 200 μmol/L of each of dNTP, 0.1 μmol/L of each primer, and 2 units of *Taq* DNA polymerase (Promega) was performed in a final volume of 20 μl. Primers specific for *OsCRL4* 5'-CTGACGGAGCGTGGTTACTCATTC-3' and 5'-GCTAGGAGCAAGGCAGTGATCTTC-3' were used. The PCR reactions were carried out for 34 cycles.

RESULTS AND DISCUSSSION

CHASE domain containing proteins in rice

Five CHASE domains containing proteins were detected in rice genomic sequences with the blast in NCBI. *OsCRL*1*a* (Ch2, AP005112) had 13 exons and 12 introns; *OsCRL*1*b* (Ch3, AC091532) had 11 exons and 10 introns; *OsCRL*2 (Ch1, AP004672) had 9 exons and 8 introns; *OsCRL*3 (Ch10, AC092548) has 13 exons and 12 introns; *OsCRL*4 (Ch12, AL954854) had no intron (Fig.1a). *OsCRL*1*a*, *OsCRL*1*b*, *OsCRL*2 and *OsCRL*3 contained the structure to that of *AtCRE*1. All of them had CHASE domain, transmitter domain, Rec domain and H, N, GI, F, G2 conserved blocks characteristic of functional histidine kinase. *OsCRL*4 had only a CHASE domain and a serine/threonine protein kinase domain (Fig.1a). Phylogenetic alignment analysis indicated that *OsCRL*1*a* and *OsCRL*1*b* had close relationship with *AtCRE*1 and that *OsCRL*4 had the low homology with *AtCRE*1 of CHASE domain family in rice (Fig.1b).

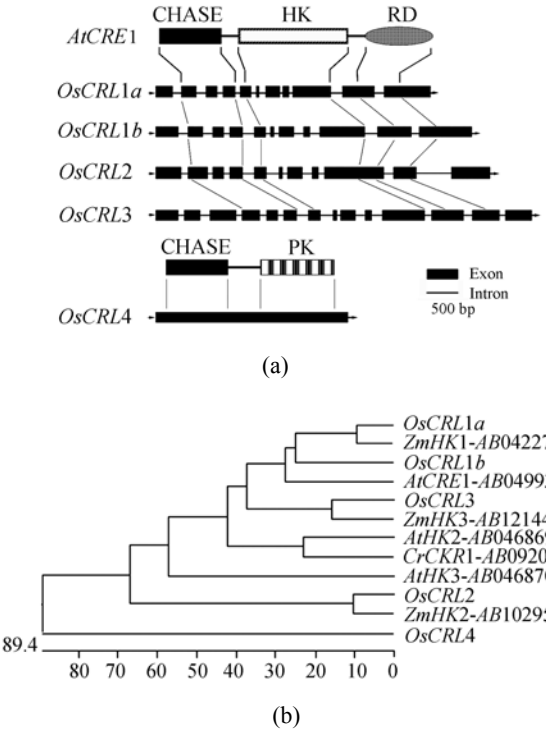

Fig.1 The CHASE domain containing protein family in rice

(a) Intron/exon structure of rice CHASE domain containing family genes. CHASE, cyclases/histidine kinases associated sensory extracellular; HK, histidine kinase; RD, receiver domain; PK, Protein kinase domain; (b) The phylogenetic analysis of the CHASE domain family in plants. The proposed rectangular cladogram was generated by ClustalW. Zm, zea mays; At, arabidopsis; Cr, catharanthus roseus

*OsCRL*4 expression pattern

The results of RT-PCR analysis showed that the expression of *OsCRL*4 was mainly expressed in root and developed spikelets, but not in stems and leaves (Fig.2). Transgenic plant with *OsCRL*4-*β-glucuronidase* (*GUS*) gene, in 4-day seeding and developed spikelets was detailedly examined. *OsCRL*4*:GUS* staining was observed clearly in lateral roots and tip of developed spikelets. Transverse and longitudinal sections of lateral roots of the transgenic plants showed that *OsCRL*4 was expressed in the phloem and periderm of the lateral roots. The pattern of expression suggested that *OsCRL*4 might be involved in proliferation of vascular tissue.

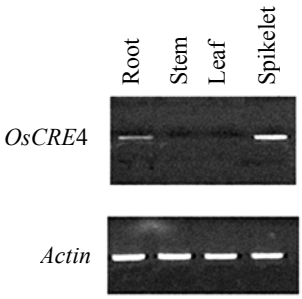

Fig.2 Semiquantitative RT-PCR analysis for *OsCRL*4 expression. Total RNA was extracted from root, stem leaf and spikelet of wild-type rice. Transcript levels of an actin gene were used as an internal amplification control

*OsCRL*4 complements the phenotype of *Arabidopsis cre*1-1

To analyze the function of *OsCRL*4, 35*S::OsCRL*4 was constructed and *Atcre*1-1 mutant transformation. Six transgenic lines were obtained. Three transgenic lines for 10-day-old were detected by RT-PCR (Fig.3a).

In order to know the function of *OsCRL*4, wild-type *Arabidopsis* plants and transgenic *Arabidopsis* plants with 35*S::OsCRL*4, *OsCRL*4 and *Atcre*1-1 mutants were grown on standard 1/2 MS medium for 7 days and grown in 1/2 MS medium containing 0.05 μmol/L kinetin for another 7 days. Fig.3b shows that root elongation of wild-type plants was notably inhibited in 1/2 MS medium

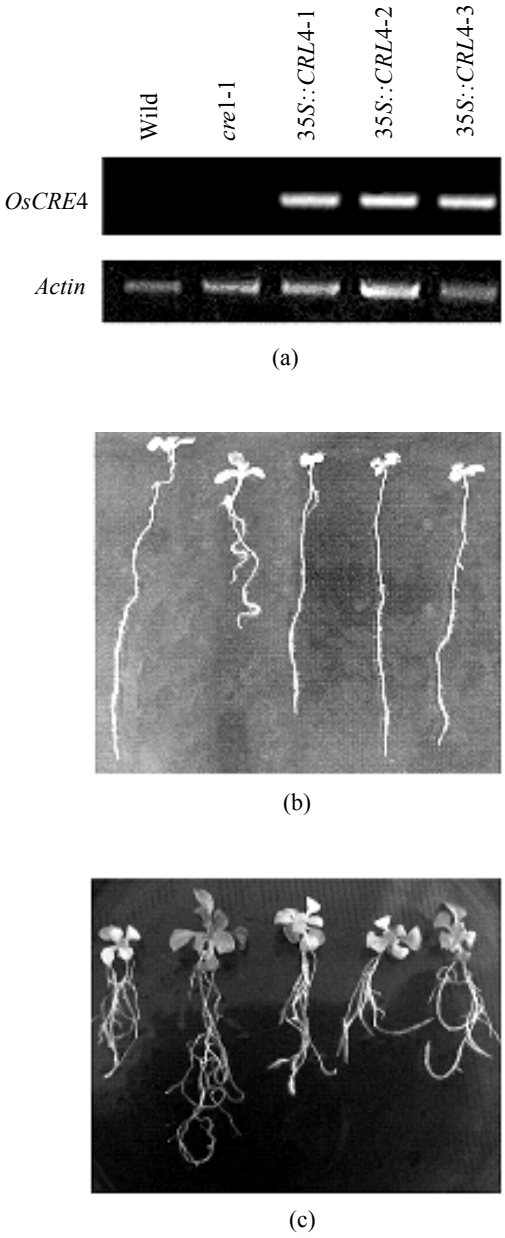

Fig.3 *OsCRL*4 complement the phenotype of *Arabidopsis Atcre*1-1 mutation

(a) Expression of 35*S::OsCRL*4 Transgenes by Semiquantitative RT-PCR analysis, for the expression of 35*S::OsCRL*4 in 3 transgenic lines. Total RNA was extracted from 35*S::OsCRL*4 transgenic *Arabidopsis* and wild-type *Arabidopsis* and *Atcre*1-1 *Arabidopsis* mutant. Transcript levels of an actin gene were used as an internal amplification control; (b) 7-days, grown in *Arabidopsis* standard 1/2 MS medium. 35*SCRL*4-1; 35*SCRL*4-2; 35*SCRL*4-3, represent different transgenic; (c) The seedlings for 15 days on vertical plate, were grown in 1/2 MS medium that containing 0.05 mmol/L 6-BA

containing 0.05 μmol/L kinetin and that *Atcre*1-1 mutants were short root phenotypes in both standard 1/2 MS medium and 1/2 MS medium containing 0.05 μmol/L kinetin (Fig.3c). Roots elongation of three transgenic lines with 35*S::OsCRL*4 was notably inhibited in 1/2 MS medium containing 0.05 μmol/L kinetin, as the phenotype of wild-type plant. The results showed that *OsCRL*4 complement the phenotype of *Atcre*1-1 mutation and that *OsCRL*4 probably acted as a cytokinin receptor like protein in rice. The protein structure of *OsCRL*4 was different with *AtCRE*1. *AtCRE*1 is perceived and transmitted by a multi-step phosphorelay system through a complex form of the two-component signaling pathway (Hwang and Sheen, 2001). In this signaling system, a membrane-located receptor kinase with an extracellular ligand-recognition domain (sensor) dimerises upon binding to a ligand and autophosphorylates a histidine within its cytoplasmic transmitter domain. The phosphoryl group is first transferred to an aspartate residue within the receiver domain at the C terminus of the receptor and then from there to a histidine phosphotransfer protein (HPT), which ultimately phosphorylates and thus activates response regulation at a central Asp residue (Sheen, 2002). *OsCRL*4 does not contain the histidine kinase domain and receiver domain, so the signaling pathway of *OsCRL*4 should not be two-component signaling pathway. The CHASE domain is always followed by other intracellular tail housing diverse enyzmatic signaling domains such as adenylyl cyclase, GGDEF-type nucleotide cyclase and EAL-type phosphodiesterase domains (Anantharaman and Aravind, 2001) as well as non-enzymatic domains such PAS, GAF, and phosphohistidine and receiver domains. *OsCRL*4 does not pass through these pathways, but can complement the phenotype of *Atcre*1-1 mutation. The signal pathway of *OsCRL*4 probably cross-talk with *CRE*1 two-component signaling pathway. The enzymology characteric of the Ser/Thr kinases, signaling pathway and function need further study.

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