ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science 2018 Vol. 28. No. 10. 1132~1139 DOI : https://doi.org/10.5352/JLS.2018.28.10.1132

Expression Study on the Scaffold Gene of CRL4 Complex in Rice (*Oryza sativa* L.)

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Received June 5, 2018 /Revised August 7, 2018 /Accepted August 21, 2018

The stability of diverse cellular proteins in eukaryotes is regulated via ubiquitination. Moreover, E3 ligase plays a crucial role in determining substrate specificity and transfers ubiquitins into the substrates during the ubiquitination process. As a type of multi-subunit E3 ligase, cullin4 (CUL4)-based E3 ligase (CRL4) complex is involved in a variety of cellular processes, such as hormonal and stress responses in plants. In spite of several reports on the versatile roles of CRL4 in various signalings in *Arabidopsis*, CRL4's function in rice has been poorly known. To learn about CRL4-mediated cellular processes in rice in more detail, *OsCUL4* that exhibits the highest homology with *Arabidopsis CUL4* was isolated, and its expression patterns in various tissues and in response to plant hormones and abiotic stresses were monitored. Exogenous application of ABA or cytokinin increased the transcript levels of the *OsCUL4* gene. Moreover, *OsCUL4* was significantly upregulated in response to drought and salt stresses. These findings imply that OsCUL4 may be functionally related to ABA- and/or cytokinin-mediated cellular responses. OsCUL4 directly interacted with OsDDB1, an adaptor protein of CRL4, indicating that OsCUL4 can act as a scaffold protein of CRL4. An expression study on the *OsCUL4* gene from this report could be used as a starting point to elucidate cellular responses in which a CRL4-mediated ubiquitination process is involved in rice.

Key words : CRL4, cullin4, hormones, rice, stresses

Introduction

To properly respond to developmental and environmental cues, eukaryotes have developed a specific mechanism to control populations of various signaling proteins and enzymes. Ubiquitination, a representative post-translational modification, mediates attachment of ubiquitins to an appropriate target protein (substrate). Since ubiquitinated proteins are eventually degraded via ubiquitin-proteasome system (UPS), ubiquitination process serves as a powerful tool to efficiently control the stability of regulatory proteins [3]. In plants, ubiquitination impinges on a diversity of biological processes such as developments, stress responses and hormone responses [17]. The process is sequentially conducted by three kinds of enzymes such as E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase). Of them, E3 plays a crucial role to determine the substrate specificity of ubiquitination [12]. Among several types of E3 ubiquitin ligases, cullin-RING E3 ubiquitin ligase (CRL) is the most prominent one and exists as a complex form. In the case of plants, CRL is largely categorized into CRL1, CRL3 and CRL4, according to the type of cullin proteins as scaffold proteins [10, 11, 17, 24]. All CRLs commonly possesses RBX1, a RING finger protein for E2 recruitment. CRL1 is associated with CUL1 as a scaffold, SKP1 as an adaptor, and F-box protein (FBP) as a substrate receptor. Unlike CRL1, CRL3 utilizes CUL3 as a scaffold and BTB/POZ protein as a substrate receptor, without a separate adaptor. On the other hand, CRL4 has CUL4 as a scaffold, DDB1 as an adaptor, and DCAF as a substrate receptor. The substrate specificity of CRLs are dependent on their substrate receptors such as FBPs, BTB/POZ proteins and DCAF proteins. In *Arabidopsis*, there are 692 *Arabidopsis* FBP, 80 BTB/POZ protein and 119 DCAF proteins [6, 7, 18, 30, 33]. As shown in the report by Lee et al. (2008), rice possesses 110 putative DCAF proteins as substrate receptors for CRL4 which shares the conserved WDxR motif within the WD40 repeats, like *Arabidopsis*, implying that the structure and biological role of rice CRL4 is functionally similar with

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that of *Arabidopsis* CRL4 [18].

Since elucidation of CRL4's role has been performed focusing on its substrate receptors, the study on CUL4's biological role has been poorly known in plants. Nevertheless, several reports with loss-of-function mutants of CUL4 in *Arabidopsis* and tomato enable to predict its function related to various plant signaling pathways and developmental processes [1, 4, 29]. Chen et al. (2006) showed that *CUL4* RNAi (*cul4i*) and *CUL4* cosuppression (*cul4cs*) lines displayed constitutive photomorphogenesis, along with upregulation of light-inducible genes in dark condition, indicating its functional relevance to light signal transduction pathway. Moreover, genetic studies revealed that CUL4 acts together with COP10 and DET1 for repressing photomorphogenesis [4]. Another report by Bernhardt et al. (2006) also showed that reduced *CUL4* expression resulted in developmental changes for lateral roots, vascular tissues and stomata [1]. In the case of tomato, reduced expression of *CUL4* led to phenotypic alteration during development of fruit, trichome and flower, and to inhibition of plant stature [29]. Collectively, these results indicate functional versatility of CUL4 during the life cycle of a plant.

To more clarify CRL4-mediated cellular responses in rice, here we investigated rice CUL4's possible involvement in various events related to phytohormone- and stress-triggered processes. In detail, the expression pattern of rice *CUL4* gene in response to diverse phytohormones and abiotic stresses was monitored and analyzed. Furthermore, its expression in various rice tissues was also checked. These researches will serves as a useful base for further studies on understanding the biological function of CUL4 and CRL4 in rice plants.

Materials and Methods

Plant materials and growth conditions

Rice seeds (*Oryza sativa* L. cv. Dongjin) were incubated for 1 minute with 70% ethanol and subsequently treated with 50% bleach supplemented with 2 drop of Tween 20 for 30 minutes. After rinses with autoclaved distilled water several times, seeds were placed on 1× MS medium supplemented with 1% sucrose and 0.8% bactoagar (pH 5.8). Seeds were then moved into a plant growth chamber and grown at 28℃. For adult plants, 4-day-old seedlings were moved into soil and kept in a plant growth chamber at 28℃.

Generation of phylogenetic tree

OsCUL4 in *Oryza sativa* and its homologues from several plant species (*Arabidopsis thaliana*, *Capsicum annuum*, *Vigna radiata*, *Zea mays* and *Glycine max*) were aligned with ClustalW in MEGA6.0. A rooted phylogenetic tree was generated with MEGA6.0 by the neighbor-joining method and the p-distance model [27]. The bootstrap method was used to assess statistical significance of the tree using 1,000 replicates.

Yeast two hybrid assay

Coding sequence regions of OsDDB1 and OsCUL4 were inserted into pDEST32 and pDEST22 vectors to generate GAL4 DBD- OsDDB1 and GAL4 AD-OsCUL4, respectively (Invitrogen, CA, USA). Yeast two-hybrid assays were conducted according to the ProQuest™ Two-Hybrid System manual (Invitrogen, CA, USA). The constructs were inserted into yeast strain MaV203, and then the resulting yeasts were incubated on SD/-Leu/-Trp plates at 30℃ for 7 days. Interactions were monitored on SD/-Leu/-Trp/-His plates including 25 or 50 mM 3-amino-1,2,4-triazole (3-AT).

Treatments of various phytohormones and abiotic stresses

For hormone treatments, 3-day-old seedlings were kept for 6 hr in MS liquid media including 50 μM abscisic acid (ABA), 10 μM indole-3-acetic acid (IAA), 10 μM 1-aminocyclopropane-1-carboxylic acid (ACC), 30 μM GA_3, 50 μM jasmonic acid (JA), 1 μM epibrassinolide (EBL) or 50 μM kinetin. For cold and heat stresses, 4-day-old seedlings were kept at 4℃ and 42℃ for 3 hr, respectively. In the case of UV stress treatment, 4-day-old seedlings were treated with UV-B at 20 μmol m^{-2} s^{-1} irradiance for 3 hr. For drought stress, 17-day-old plants were kept without water until loss of approximately 10% of total fresh weight. In the case of salt stress, 3-day-old seedlings were incubated for 6 hours in MS liquid media including 250 mM NaCl. The collected samples were then kept at -70℃ for further RNA isolation process.

RNA isolation and real-time qRT-PCR analyses

Total RNA from seedlings and adult plants was retrieved using TRIsure reagent (Bioline, MA, USA), while total RNA from seeds was isolated as described by Oñate-Sánchez and Vicente-Carbajosa (2008)[23], followed by DNase I (Promega, WI, USA) treatment to eliminate genomic DNA con-

tamination. The first-strand cDNA was synthesized from 1 μg total RNA using RevertAid H Minus Reverse Transcriptase (Thermo Scientific, MA, USA). Real-time qRT-PCR was carried out using a Rotor-Gene SYBR Green PCR Kit (Qiagen, CA, USA) according to the manufacturer's instructions and the Rotor-Gene Q system (Qiagen, CA, USA). Transcript levels of *OsCUL4* were determined using the comparative CT method and normalized to that of *OsACTIN1* (LOC_Os03g 50885) from the corresponding sample. All experiments were conducted with biological replicates at least three times. The information of *OsCUL4* primers used for qPCR analysis are as follows: *OsCUL4* forward primer (5′-GGATGCTGCCATA GTTCGAATA-3′), *OsCUL4* reverse primer (5′-TCAAGCCA GGTAATTGTAGATCTG-3′). *OsACTIN1* primers used for qPCR analysis are the same ones as those from Kim et al. (2018) [16].

Results and Discussion

Identification of *OsCUL4* gene and its deduced protein

For further understanding of CRL4-mediated cellular responses in rice, rice *cullin4* (*OsCUL4*) gene was identified. To isolate rice putative *cullin4*, *Arabidopsis CUL4* (*AtCUL4*) was selected as a query to find *OsCUL1* using the Rice Genome Annotation Project BLAST Search. One protein (LOC_Os03g57290.1) exhibited 81.9% similarity in amino acid sequences when compared to AtCUL4, based on EMBOSS Pairwise Alignment Algorithms at EMBL-EBI [25]. Since AtCUL4 protein exists as two different forms (CUL4-L and CUL4-S) which generate identical proteins, except that CUL4-L possesses additional 50 amino acids compared to CUL4-S at the N-terminal region, we cannot exclude the possibility that OsCUL4 also exist as multiple isoforms [4]. *OsCUL4* gene possesses 16 exons and 15 introns, and its coding region is composed of 2,508 bp encoding 836 amino acids. Like other cullin proteins, OsCUL4 has a large 'Cullin', and a 'Nedd8' domain that is responsible for neddylation process at its C-terminal region (Fig. 1A, Fig. 1B). The neddylation (attachment of Nedd8 into cullin) is known to increase CRL activity by promoting E2 recruitment [10]. OsCUL4 homologues from several plant species such as *Zea mays*, *Vigna*

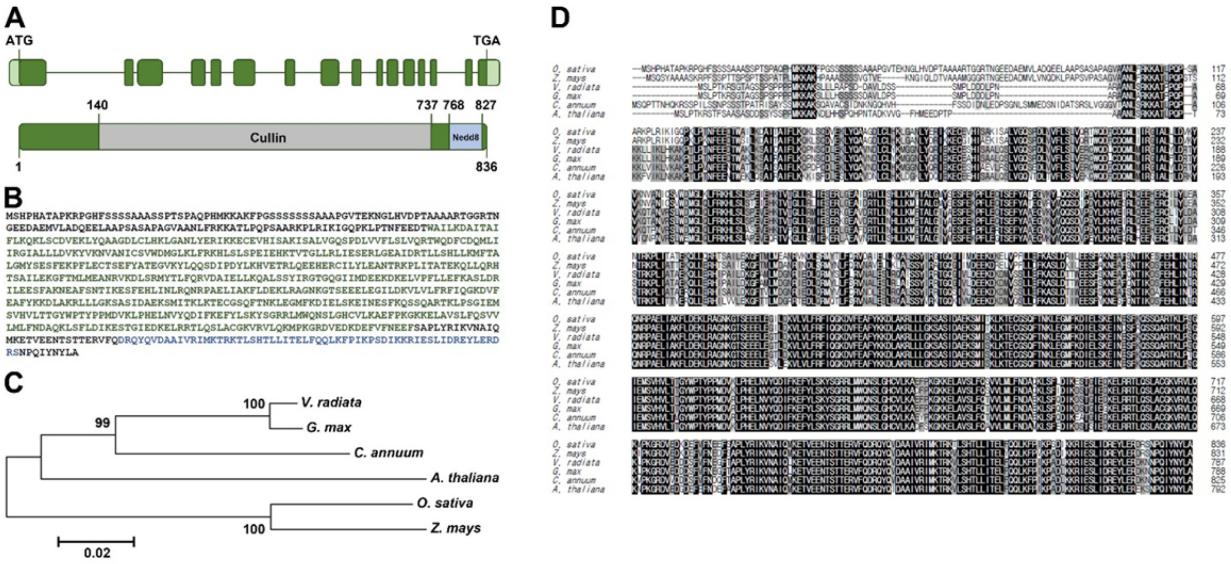

Fig. 1. Identification of rice *CUL4* gene. (A) Schematic structure of *OsCUL4*. Closed boxes represent exons. Light green and dark green boxes indicate UTR (untranslated region) and CDS (coding sequence) regions, respectively. Lines between closed boxes represent introns. 'Cullin' and 'Nedd8' domains were designated based on the information from the pfam database. (B) Deduced amino acid sequence of OsCUL4. The regions with green and blue letters indicate 'Cullin' and 'Nedd8' domains, respectively. (C) Phylogenetic tree with OsCUL4 protein and its homologues from other plant species. The tree was generated with OsCUL4 protein and the proteins that displayed the highest homology with OsCUL4 from other species. The selected proteins are as follows: *O. sativa* CUL4 (XP_015632421.1), *Z. mays* CUL4 (XP_008644442.1), *V. radiata* CUL4 (XP_014518580.1), *G. max* CUL4 (XP_003546083.1), *C. annuum* CUL4 (XP_016558560.1) and *A. thaliana* CUL4 (NP_568658.1). Numbers represent the percentage of replicate trees after 1,000 replications. (D) Alignment of OsCUL4 protein and its homologues from (C). The regions with a high-level of conservation are shown with black shade (100%), a middle-level with dark gray (67~83%) and a low level with light gray (50%).

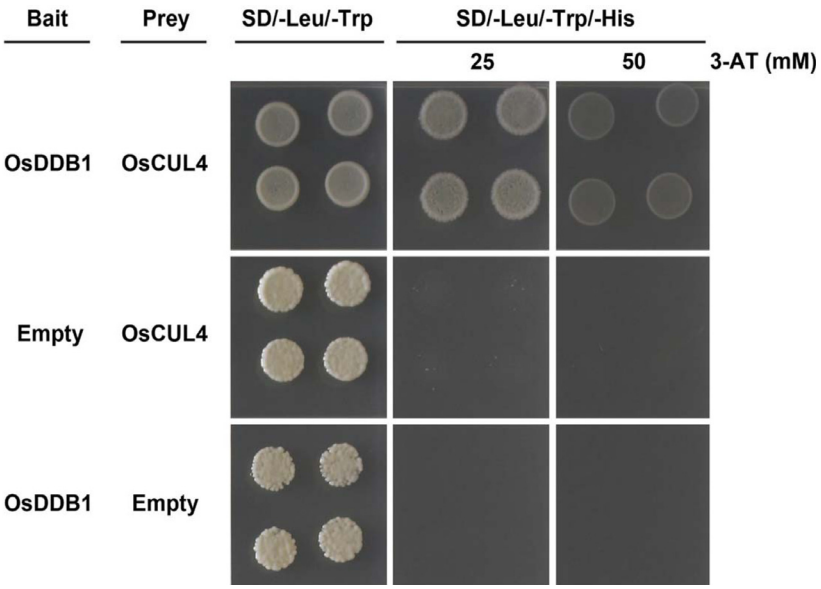

Fig. 2. Direct interaction of OsCUL4 with OsDDB1. Yeast two hybrid assays were performed to check the physical interactions between OsCUL4 and OsDDB1. Combinations of empty bait vector-OsCUL4 and OsDDB1-empty prey vector were used as negative controls.

radiata, *Glycine max*, *Capsicum annuum* and *Arabidopsis thaliana* were selected using the BLAST search of the GenBank database, and their phylogenetic analysis and amino acid alignment were performed with OsCUL4 (Fig. 1C, Fig. 1D). Homologous proteins from other species displayed more than 81.9% similarity in amino acid sequences when compared to OsCUL4. Especially, OsCUL4 showed 94.5% similarity in amino acid sequences with CUL4 homologue in *Zea mays* as a monocot, while 81.9-84.8% with those in other dicots. These findings imply that CUL4 proteins have been evolutionarily conserved and CRL4's biological function is similar among plant species.

Selected OsCUL4 acts as a scaffold protein of CRL4 complex

CUL4 protein has been known to function as a scaffold protein of CRL4 complex. In the complex, CUL4 directly interacts with RBX1 as a RING finger domain protein for E2 recruitment, and DDB1 as an adaptor from the complex [10, 11, 21, 28]. To confirm whether the selected OsCUL4 functions as a cullin protein in rice CRL4 complex, we performed a yeast two hybrid assay to check the physical interaction between OsCUL4 and OsDDB1 (LOC_Os05g51480.1). As shown in Fig. 2, OsCUL4 strongly interacted with OsDDB1 in the presence of 25 or 50 mM 3-AT, while any meaningful binding activity was not detected in the combinations of empty vector-OsCUL4 and OsDDB1-empty vector as negative controls under the same condition. Collectively, these results show that OsCUL4 plays its role as a scaffold protein of CRL4 complex in rice.

Accumulation pattern of *OsCUL4* transcripts in various rice tissues

To understand the biological role of OsCUL4 in detail, we firstly monitored the expression pattern of *OsCUL4* in various rice tissues such as seeds, whole seedlings, coleoptiles, roots and leaves. As compared to its expressions in seedlings and coleoptiles, those in seeds, roots and leaves were relatively high (Fig. 3). Relatively high expression of *OsCUL4* in roots and leaves is in collusion with a previous report that GUS expression was mainly detected in young leaves of transgenic pCUL4::GUS plants and CUL4 is in-

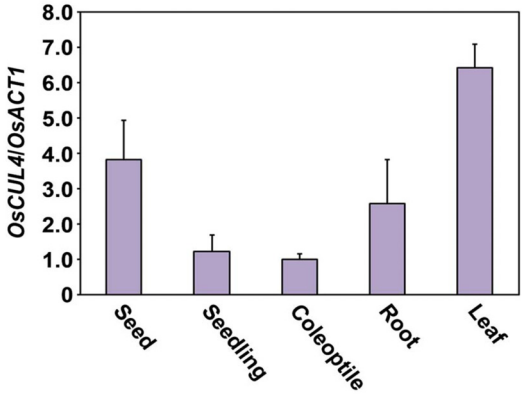

Fig. 3. *OsCUL4* expression in various tissues. Seeds, 3-day-old seedlings, and coleoptile, root and leaf tissues from 5-day-old plants were used to monitor *OsCUL4* expression patterns. Accumulations of *OsCUL4* transcripts were normalized to those of *OsACT1* transcripts from the same sample. The average expression value of *OsCUL4* in coleoptile tissue was considered to be 1.0. Values are the means ± SD (n=6).

volved in leaf and root development in *Arabidopsis* [1]. It has been reported that *cul4cs* (*CUL4* cosuppression line), which possesses reduced levels of *CUL4*, exhibited hypersensitivity to 0.1 μM ABA, indicating CUL4's role in ABA-mediated cellular responses [4, 18]. This result may explain high expression of *OsCUL4* in seeds in which ABA is abundant (Fig. 3).

Accumulation pattern of *OsCUL4* transcripts by diverse phytohormone treatments

We next investigated the expression pattern of *OsCUL4* by the treatment of various phytohormones. In the case of kinetin, a kind of cytokinin, its treatment resulted in marked increase of *OsCUL4* transcripts (Fig. 4). The upregulation of *OsCUL4* in response to cytokinin is in keeping with the result from Lee et al. (2008) that functional defect of *Arabidopsis* CUL4 (AtCUL4) led to increased sensitivity in response to cytokinin and hyper-induction of cytokinin-responsive genes, suggesting the functional relationship between AtCUL4 and cytokinin signaling [18]. In agreement with high expression in seeds, *OsCUL4* was strongly upregulated by exogenous ABA application (Fig. 4). In *Arabidopsis*, there have been several evidences on possible involvement of CRL4 in ABA-mediated processes. A subset of DCAF proteins (substrate receptors of CRL4 complex) such as DWA1, DWA2, DWA3, ABD1, ASG2 and AtRAE1 were reported as a negative regulator in ABA signal transduction pathway [5, 19, 20, 22, 26]. Although CRL4 complex is typically composed of DDB1, RBX1 and DCAF, CUL4 is also able to associate with another type of complex, COP10-DET1-DDB1 (CDD) that has been proposed to promote target recognition by CRL4 [4]. A recent report shows that DDA1, a substrate receptor for CRL4-CDD complexes, is responsible for degradation of ABA receptor and therefore, plays a repressive role in ABA signaling [13]. Furthermore, OsDET1, an *Arabidopsis* DET1 homolog as a component of CRL4-CDD complexes is known to regulates ABA signaling/biosynthesis in rice [31]. Collectively, these reports reveal that a large fraction of CRL4 complexes participates in ABA signaling pathway, reflecting the functional connection between CUL4 and ABA response, which is in collusion with *OsCUL4* upregulation by ABA in our study (Fig. 4).

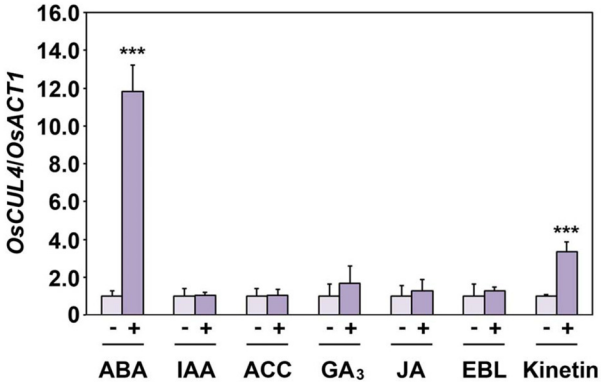

Fig. 4. *OsCUL4* expression in response to various phytohormones. Three-day-old seedlings were kept for 6 hr in MS liquid media including 50 μM ABA, 10 μM (IAA), 10 μM ACC, 30 μM GA_3, 50 μM JA, 1 μM EBL or 50 μM kinetin. Accumulations of *OsCUL4* transcripts were normalized to those of *OsACT1* transcripts from the same sample. The average expression value of *OsCUL4* from each mock-treated sample was considered to be 1.0. Values are the means ± SD ($n \geq 6$). Significant differences between mock-treated and hormone-treated samples were identified by a Student's t test; $^{*}p<0.05$; $^{***}p<0.001$.

Accumulation pattern of *OsCUL4* transcripts by diverse abiotic stresses

The expression pattern of *OsCUL4* in response to various abiotic stresses is also elucidated to further understand its function in rice. As shown in Fig. 5, *OsCUL4* was upregulated in response to drought and 250 mM NaCl treatments. Since drought and high salt stresses trigger increase of endogenous ABA level, this result confirms ABA-inducibility of *OsCUL4* gene. On the other hand, UV-B significantly inhibits the expression of *OsCUL4*, suggesting that CUL4-mediated ubiquitination process is involved in UV-B response as well as ABA (Fig. 5). Similar with the case in ABA signaling, it has been reported that several CRL4 substrate receptors such as RUP1/2, DHU1, CSAat1A/B and DDB2 play their roles in UV-B signaling in *Arabidopsis*. RUP1 and RUP2 function as negative regulators of the UV-B specific response by repressing UVR8-COP1 interaction via interaction with UVR8 as UV-B receptor [8, 9]. DHU1 also acts as a negative regulator of UV-B response via possibly sequestering COP1 from the active UVR8-COP1 complex [14, 15]. CSAat1A and CSAat1B proteins form heterotetramers and associate with CRL4 complex to play their role in tolerance process against UV-B-triggered DNA damage [32]. CSAat1A also acts together with DDB2 for UV-B-triggered DNA damage repair [2]. Taken together, these results indicate that a part of CUL4-based E3 ligase complexes mediated by a subset of DCAF proteins is important for UV-B response, which is in collusion with altered expression of

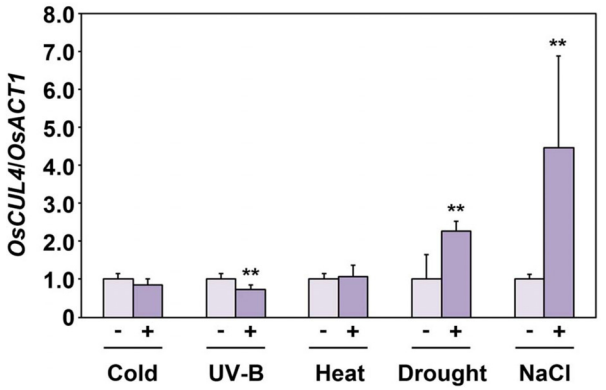

Fig. 5. *OsCUL4* expression by the treatment of various abiotic stresses such as cold, heat, UV-B, drought and salt stresses. Accumulations of *OsCUL4* transcripts were normalized to those of *OsACT1* transcripts from the same sample. The average expression value of *OsCUL4* from each mock-treated sample was considered to be 1.0. Values are the means ± SD ($n \geq 6$). Significant differences between mock-treated and stress-treated samples were identified by a Student's t test; $^{**}p<0.01$.

OsCUL4 in response to UV-B, in our data (Fig. 5).

Expression analysis of *OsCUL4* gene from this study is thought to serve as a jumping-off point to further understand CUL4's detailed action mode and CRL4-mediated cellular processes in rice. Based on several reports with loss-of-function mutants of *Arabidopsis CUL4* gene, functional defect of OsCUL4 is expected to give rise to alteration of sensitivities in response to diverse hormones and abiotic stresses in rice. Therefore, to elucidate the biological role of rice CUL4 in more detail, a phenotype study using transgenic plants with altered expression level of *OsCUL4* gene would be required, which enables to predict the involvement of OsCUL4 in specific hormones and/or abiotic stresses signaling.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2016R1D1A1B03930213), and by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through Agri-Bio industry Technology Development Program funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (115081-2).

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초록 : 벼에 존재하는 CRL4 복합체 scaffold 유전자의 발현 양상에 대한 연구

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진핵생물에서 유비퀴틴화 과정을 통해 단백질 안정성이 조절되며, E3 ligase는 유비퀴틴화 과정 동안 분해 대상 기질의 결정 및 기질로의 유비퀴틴 전달을 위한 주효소로 작용한다. Multi-subunit E3 ligase의 일종인 cullin4 (CUL4)-based E3 ligase (CRL4) 복합체는 식물의 다양한 호르몬, 스트레스와 관련된 세포 내 과정에서 중요한 역할을 하는 것으로 알려져 있다. 호르몬, 스트레스 신호 전달 과정에서 CRL4의 다양한 역할에 대한 보고가 애기장대에서 이루어져 왔음에도 불구하고, 주요 식량 작물인 벼에서의 CRL4 기능에 대한 연구는 매우 미흡한 실정이다. 이에 벼에서 CRL4에 의해 매개되는 세포 내 반응들을 상세히 이해하기 위해, 본 연구에서는 애기장대 *cullin4* (*CUL4*)의 상동 유전자를 벼에서 동정하고, 조직별 벼 *CUL4* 유전자의 발현 양상과 다양한 식물 호르몬 및 환경 스트레스 처리에 의한 해당 유전자의 발현 양상을 탐색하였다. 벼 *CUL4* 유전자인 *OsCUL4*는 앱시스산, 사이토키닌과 같은 식물 호르몬과 가뭄, 고염 스트레스에 의해 발현량이 급격히 상형 조절되는 양상을 보였는데 이는 해당 단백질이 앱시스산 및 사이토키닌에 의해 매개되는 세포 내 반응과 기능적으로 연계되어 있음을 암시한다. 또한, OsCUL4는 CRL4 복합체의 어댑터로 작용하는 OsDDB1과 직접적으로 결합하였는데, 이는 본 연구를 통해 동정한 OsCUL4가 벼에서 실질적으로 CRL4의 scaffold 단백질로 기능할 수 있음을 보여준다. 본 연구를 통해 수행된 *OsCUL4* 유전자의 발현 양상에 대한 연구는, 벼에서 CRL4 매개 유비퀴틴화 과정이 관여하는 세포 내 반응을 규명하기 위한 시작점으로 활용될 수 있을 것이라 사료된다.