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SHORT COMMUNICATION

Tissue-specific expression pattern of calcium-dependent protein kinases-related kinases (CRKs) in rice

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ABSTRACT

Calcium-dependent protein kinases-related kinases (CDPK-related kinases; CRKs) are Ser/Thr kinases that bind with Ca^{2+}/Calmodulin and play crucial roles in signal transduction pathways during plant growth, development, and responses to multiple stresses. In this study, we have studied detailed organ and tissue-specific expression patterns of rice CRK genes. Our organ-specific RT-PCR analyzes show the differential expression pattern of these genes in various organs of rice. Moreover, our RNA-RNA *in situ* hybridization study in rice stem base containing developing crown root primordia demonstrates that the expression of CRK genes is spatially restricted to the developing crown root primordia, suggesting their putative role in protein phosphorylation-dependent cellular signaling during rice crown root development. Furthermore, organ-specific differentially expression pattern of CRK genes during floral organogenesis further support for the organ-specific cell signaling during organogenesis. Thus, our study provides a developmentally regulated expression pattern of rice CRK genes, though they are broadly expressed and a basic foundation for functional characterizations of CRK gene members to unravel their specific functions during plant growth and development.

Introduction

Plants have developed a sophisticated signal transduction pathway to adapt for climate change. Calcium (Ca^{2+}) plays a vital role in signal transduction as a secondary messenger during various biological processes including growth and development.[1] Transient fluctuations in Ca^{2+} concentrations in the cytoplasm can be sensed by members of four kinases superfamilies whose activities are regulated by calcium and/or calmodulin (CaM). These are Ca^{2+}-dependent protein kinases (CDPKs), Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), calcium and calmodulin-dependent protein kinases (CCaMKs) and CDPK-related protein kinases (CRKs).[2–5] CDPK-related kinases (CRKs) basically belong to a Ser/Thr protein kinase group while some of the *AtCRKs* have Tyr kinase activity but they are closely related to CDPKs.[6] They possess a CDPK-related catalytic domain and a C-terminal CaM domain-containing degenerated EF-hand Ca^{2+} binding motifs.[7,8] Members of CDPKs/CRKs superfamilies contain N-terminal myristoylation sites that target them to get localized in the plasma membrane and endomembrane system.[9] Unlike CDPKs, plant CRKs are not well characterized. A few studies in dicot plants have shown their role in plant development and stress tolerance[10–14] but they are not studied in monocot plants, particularly in crop species. In *Arabidopsis*, *AtCRK1* positively regulates heat and salt stresses in the plant whereas *AtCRK3* controls the remobilization of nitrogen during leaf senescence.[11,12] Importantly, a plasma membrane-localized *AtCRK5* is required for proper root growth, gravity responses, embryogenesis, and hypocotyls hook formation during skotomorphogenesis.[13,15,16] Its inactivation results in the inhibition of the root growth, enhanced lateral root formation, and defective gravitism in *Arabidopsis* due to defect in polar auxin distribution, strongly suggesting functions of CRKs in the regulation of hormonal signaling during plant growth and development.[9,13] Similarly, tomato *LeCRK1* has an important role in the fruit ripening process.[10] In rice, interestingly *OsCBK* (*OsCRK1*) binds to calmodulin in a calcium-dependent manner but its enzymatic activity is independent of calcium and calmodulin, indicating that its interaction with Ca^{2+}/calmodulin may more relevant for the regulation and may not be crucial for its direct kinase function.[17]

Here, we have studied the expression pattern of five predicted rice CRK genes in various organs. We show the differential expression levels of these genes in different organs. We reveal the spatial transcript distribution pattern of these genes in the stem base (crown tissue) which contains developing crown root primordia at different developmental stages and during floral organogenesis using RNA-RNA *in-situ* hybridization. Our results provide a base for detailed functional characterization of rice candidate CRK genes for revealing their tissue-specific roles during growth and development.

Materials and methods

Plant material and growth condition

Rice (*Oryza sativa* subsp. indica cv. IR64) seeds were germinated on ½ MS media and grown in a controlled condition at 27°C with a 16/8 h light/dark daily cycle as

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ⓘ Supplemental data for this article can be accessed on the publisher's website.

described earlier.[18] For organ-specific expression analysis, stem base (about 2 mm), crown root, primary root, leaf blade, and whole seedling were collected from 6d old rice plants, while 0.5–1.0 cm panicles were collected from the growing rice plants.

Expression analysis by RT-PCR

For reverse transcription-polymerase chain reaction (RT-PCR), total RNAs from seedling, crown tissue (stem base), crown root, primary root, leaf blade, and panicles were extracted using TRI reagent (Sigma, Missouri, USA). The RNAs were quantified using nanodrop (Thermo Fisher Scientific, Massachusetts, USA) and the quality of RNA samples was ensured by running total RNAs on 1% agarose gel. For reverse transcription, 1 μg total RNAs were used for first-strand cDNA synthesis using the iScript cDNA synthesis kit (BioRad, California, USA). Gene-specific primers for each gene were designed by PrimerQuest Tool (https://eu.idtdna.com/PrimerQuest/Home/Index) and rice *Ubq5* gene (GenBank accession number: AK061988) was used as an internal control (Supplementary Table 1). The relative expression level was calculated by quantifying band intensity and normalizing with an internal control using Image Lab Software (BioRad, California, USA). Data were depicted as mean value ±SD of three independent biological replicates. The data were analyzed by one-way analysis of variance (ANOVA) at ($P \leq 0.05$) using SPSS v.16.0 (SPSS, Chicago, USA). Statistical analysis and column diagrams have been prepared by using Prism 8, Graphpad software (https://www.graphpad.com). Data obtained from semi-quantitative-PCR in three independent biological replicates were used to produce heatmap through Bioconductor R (http://www.bioconductor.org).

RNA-RNA in situ hybridization

For RNA-RNA *in situ* hybridization analysis, as described previously by Neogy et al., (2019),[18] a rice stem base and young panicles undergoing floret organogenesis were fixed in FAA (10% formaldehyde, 5% glacial acetic acid and 50% ethanol) fixative, dehydrated through ethanol-xylene series and embedded in paraffin (Sigma, Missouri, USA). An 8 μm cross-sections of stem base and longitudinal sections of panicles were made using a microtome (Thermo Fisher Scientific, Massachusetts, USA) and collected on poly-L-lysine coated slides. For generating RNA probes, 110 to 130-bp gene-specific regions of CRK genes were PCR amplified using cDNA (Supplementary Table 1) and were cloned into *p*Bluescript SK⁺ vector as a blunt in *Eco*RV restriction site. The orientation of the fragments for T7/T3 promoters has verified the sequencing. *OsCRK1*, *OsCRK3*, and *OsCRK4* genes were cloned in anti-sense direction while *OsCRK2* and *OsCRK5* genes were cloned in the sense direction. The anti-sense probe for *OsCRK1, OsCRK3*, and *OsCRK4* was transcribed with T7 RNA Polymerase (Sigma-Aldrich, USA) using *Eco*RI linearized *p*Bluescript SK⁺ clones. However, the sense probe for *OsCRK2* and *OsCRK5* were transcribed with T3 RNA Polymerase (NEB, USA) using *Hind*III linearized

*p*Bluescript SK⁺ clones. Slides were treated and hybridized as described previously.[19,20] A color signal was developed performing enzymatic reaction for alkaline phosphatase (AP) which is conjugated with anti-DIG antibodies (Sigma-Aldrich, USA) using NBT/BCIP substrate (Sigma-Aldrich, USA) for the reaction (Sigma-Aldrich, USA). Entellan (Merck- Millipore, Germany) mounted sections were imaged under a bright-field microscope with a device camera (Carl Zeiss AG, Germany).

Results and discussion

Protein phosphorylation and dephosphorylation provide a key post-translational regulatory switch for regulating conformational changes, sub-cellular localization, and molecular interactions of proteins during several fundamental biological processes including growth and development. For example, protein phosphorylation is important in asymmetric localization of PIN proteins required for polar auxin transport during plant growth and development.[13,21,22] Similarly, phosphorylation is a crucial event for two-component cytokinin signaling in plants.[23] In *Arabidopsis, AtCRK5* has been shown to play a crucial role during various stages of plant growth and development.[9,13,15,16] *AtCRK5* is specifically required for proper PIN2 localization in the transition zone of the primary root.[13] It also controls auxin-gibberellic acid cross-talk during embryogenesis and auxin-ethylene-gibberellic acid crosstalk during hypocotyl hook development during skotomorphogenesis.[15,16]

Toward investigating functions of CRK genes during growth and development in rice, we investigated expression patterns of five putative rice CRK genes in six tissues. We performed RT-PCR analysis for these genes using RNAs from rice crown tissues at the stem base, emerged crown root, seedling, leaf blade, early panicle, and primary root. Our analysis showed that CRK genes were broadly expressed in all these rice tissues (Figure 1(a)) but transcript abundance is differential across these tissues. The expression level of *OsCRK1* is highest in the emerged crown roots and lowest in the panicles (Figure 1(a,b)). Similarly, *OsCRK3* is also most abundant in the crown roots but minimum in the crown tissues (Figure 1(a,b)). On the other hand, the expression level of *OsCRK4* and
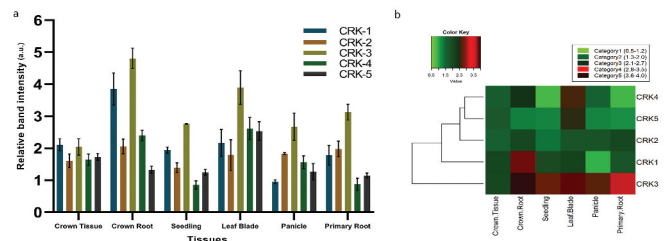

Figure 1. Organ-specific expression pattern of rice CRK genes. (a) Expression levels based on relative band intensity of CRK genes in different tissue of rice. (b) Heatmap analysis of CRK genes in different tissue. This figure combines a "heatmap" with a dendrogram. The rows represent genes while the type of tissues is shown in the columns. The expression levels are mapped on the color scale with low, intermediate, and high expression represented by green, red, and black color respectively.

OsCRK5 was maximum in the leaf blades (Figure 1(a,b)). Interestingly, the expression levels of *OsCRK1, OsCRK3,* and *OsCRK4* in primary roots are lower than the crown roots, suggesting root-type differential expression of these genes. However, we do not observe much difference in the expression level of *OsCRK2* across these tissues. A complete linkage algorithm and a euclidean distance matrix were used to construct a heat map by clustering of expressed genes for the different tissues (Figure 1(b)). The clustering tree is showing the division of genes into two broad groups; group 1 comprising of *OsCRK1, 2, 4, 5,* and the second group with only *OsCRK3* (Figure 1(b)). Based on this expression pattern, *OsCRK1, 3,* and *4* may have differential functions between primary and crown roots (Figure 1(b)).

Since the expression of some of the CRK genes was lower in the stem base containing developing crown root primordia as compared with emerged crown roots, we analyzed their spatial expression pattern in the internal tissues of the stem base. RNA *in situ* hybridization was performed on the cross-sections of the stem base using antisense RNA probes for each gene. Important to note that the expression of all these CRK genes was mostly restricted to the developing crown root primordia in the stem base and no expression above the background was observed in other tissue (Figure 2(aj)). The CRK genes are activated in the initiating crown root primordia and continue to express during outgrowth of the primordia (Figure 2(aj)). These observations together with the RT-PCR data suggest that rice CRK genes are expressed since the early stages of the crown root primordia and continue to express in the growing crown roots, post-emergence of the crown root primordia.

Similarly, we also studied the spatial expression pattern of rice CRK genes during rice floret development. Young panicle (0.5–1 cm) containing florets undergoing different stages of floral organogenesis were sectioned longitudinally and hybridized with DIG-UTP labeled anti-sense RNA probes against all five CRK genes. We observed that expression of *OsCRK1* and *OsCRK2* was relatively higher in the floret meristem as compared to *OsCRK3-5* (Figure 3(a,c,e,g,i)). They are expressed throughout the floret meristem including developing lemma and palea but during a later stage of floret organogenesis, their expression is restricted to the inner floret organs and is significantly decreased in the lemma and palea (Figure 3(ad)). *OsCRK1, OsCRK3,* and *OsCRK4* are expressed in developing lodicules, stamens, and carpel (Figure 3(b,f,h)) whereas

Figure 3. Spatial expression pattern of CRK genes during rice floret development. (a-j) Tissue-specific expression pattern of *OsCRK1* (a, b), *OsCRK2* (c, d), *OsCRK3* (e, f), *OsCRK4* (g, h), and *OsCRK5* (i, j) in rice floret meristem and during floret organogenesis, hybridized with anti-sense DIG-RNA probe. Bars: 20 µm.

OsCRK2 has relatively stronger expression in lodicules and stamens as compared to carpel tissues (Figure 3(d)). However, the expression of *OsCRK5* is not significantly higher than the background level (Figure 3(ij)). All these data together suggest that rice CRK genes display tissue and organ-specific differential expression pattern.

Conclusion

In the present study, our data showed that though all five rice CRK genes are expressed broadly but they display differential expression levels in different organs, suggesting their organ-specific regulation. This also suggests that they might have specific functions in different organs/tissues. Moreover, spatially regulated expression of these genes in the stem base and floret organs further supports toward their tissue-specific differential functions during organ growth and development. The functions of these CRKs remain elusive and this work can provide a foundation for the further functional characterization of these genes in rice as well as in other plant species.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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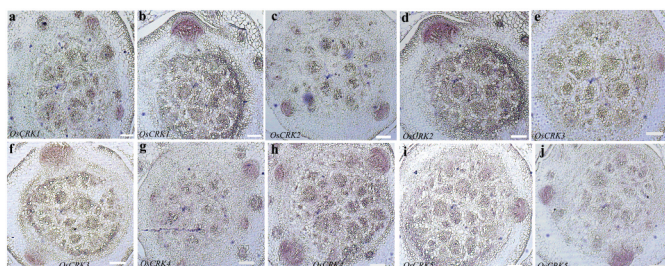Akhilesh Yadav 🆔 http://orcid.org/0000-0001-9168-7968

Figure 2. Spatial expression pattern of CRK genes during rice crown root primordia development. (aj) Tissue-specific expression pattern of *OsCRK1* (a,b), *OsCRK2* (c,d), *OsCRK3* (e,f), *OsCRK4* (g,h), and *OsCRK5* (i,j) during CRP development, hybridized with anti-sense DIG-RNA probe. Bars: 20 µm.
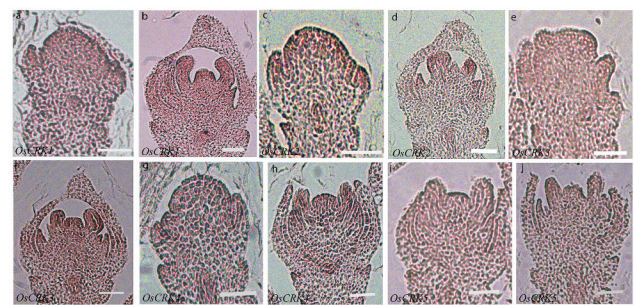
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