GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Cloning and characterization of the drought-resistance *OsRCI2-5* gene in rice (*Oryza sativa* L.)

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Genet. Mol. Res. 13 (2): 4022-4035 (2014)
Received September 6, 2013
Accepted December 10, 2013
Published May 23, 2014
DOI http://dx.doi.org/10.4238/2014.May.23.13

ABSTRACT. The genomic expression profile of the super-hybrid rice Liangyoupeijiu female parent Pei'ai 64S in different tissues at different developmental stages under low temperature, drought, and high temperature stresses were detected using an Affymetrix GeneChip Rice Genome Array to screen upregulated and downregulated genes. In this study, we screened the drought-resistant gene *OsRCI2-5*, after which a constitutive *OsRCI2-5* construct was created and transferred into Nipponbare. After polyethylene glycol-6000 and drought treatment, we found that the *OsRCI2-5* gene improved the drought resistance of Nipponbare. Gene expression profiling showed that the *OsRCI2-5* gene was expressed in the rice leaves, stems, and flower organs. Subcellular localization revealed that the gene was located in the membranes, and hence, we can deduce that a membrane signal peptide was responsible

for signal transduction.

Key words: Rice (*Oryza sativa* L.); Drought resistance; Drought-resistance mechanisms; *OsRCI2-5*

INTRODUCTION

Drought, which is one of the most important abiotic factors that threatens crop growth, has become a worldwide problem. Currently, one-third of the world's land area is arid or semi-arid (Liu et al., 2008). Simple modifications to conventional breeding methods cannot efficiently improve crop drought resistance. As such, current research hotspots have focused on improving crop drought resistance via drought-related genes (Rohila et al., 2002; Liu et al., 2005).

As an important food and large water-consumption crop, rice production is restricted by irrigation. It is increasingly important to improve rice drought resistance, and drought-related rice genes have been cloned. Takahashi et al. (1994) cloned the *wsi18* cDNA drought-resistance gene from rice, and Joshee et al. (1998) isolated the *pwsi18* gene, which was verified to be related to drought stress. One study showed that in crops with general stress tolerance, a single physiological mechanism could respond to different adverse conditions (Zhang, 2000). Two DREB transcription factors, *DREB2A-B* and *DREB1A-C*, were cloned from materials under drought treatment (Liu et al., 2000) that proved the general stress tolerance of crops at the molecular level.

Drought-resistance genes can be divided into two classes according to their different roles: functional and regulatory. The functional genes encode proteins that directly protect plants from drought stress, while the regulatory genes regulate gene expression and signal transduction. Xu et al. (1996) used the microprojectile bombardment method to transfer a barley late embryogenesis abundant (*LEA*) gene into rice and detected a significant increase in LEA protein as well as obviously improved rice drought tolerance, which suggested that the LEA protein could protect rice against drought stress-related injury. In the presence of the overexpression of *P5CS* and *δ-OAT*, two key genes involved in the proline biosynthesis process, proline content was increased and rice drought resistance was significantly enhanced (Ingram et al., 1997; Wu et al., 2003). Igarashi et al. (1997) isolated *P5C* cDNA (*cosP5CS*) from Akibare and found that *P5CS* could be induced by all stress factors including drought. Chinnusamy et al. (2004) overexpressed *P5CS* using an ABA-specific promoter and detected a 2.5-fold increase in the *P5CS* expression level and increased fresh weights of roots and stems of T2 transgenic plants under drought conditions. Su and Wu (2004) examined the *P5CS* expression under the drought-inducible *P5CS* promoter and the constitutive *Act1* promoter and found a seven-fold increased proline content under drought treatment for 72 h. Wu et al. (2003) transferred the *AtOAT* gene into rice and detected a 4-14-fold increased proline content under drought stress. DREB transcription factors are DNA-binding proteins that specifically bind the cis-acting element of the promoter region. In recent years, DREB transcription factors have been extensively studied and were found to be inducible under stress conditions and control stress response gene expression. Dubouzet et al. (2003) cloned the *OsDREB1A* gene, which could specifically bind to DRE in rice protoplasm and activate the expression of the *GUS* reporter gene, and they found that its overexpression increased rice drought resistance. Tian et al. (2005) cloned three DREB transcription factors from rice - *OsDREB4-1*, *OsDREB4-2*, and *OsDREB1-1* - and found that *OsDREB4-1* was expressed only under drought treatment; therefore, it might be responsible for drought stress.

With the development of the Human Genome Project, microarray analysis has be-

come more readily available. This technique integrates microelectronics, biology, physics, chemistry, and computer science into one biotechnology and has the distinguished merits of being efficient, scalable, and capable of high throughput. Gene chip technology has been widely used in gene expression profiles, disease diagnosis, drug screening, genetic mapping, gene mutation, and other areas (Yu, 2009). The rice drought-resistance gene, *OsRCI2-5*, was identified using microarray technology. This study investigated the function of *OsRCI2-5* in the rice drought resistance and aimed to improve rice drought resistance by exploring new drought-resistance genes and providing further reference information for the future breeding of new rice germplasms with strong drought tolerance.

MATERIAL AND METHODS

Plant growth and treatments

PA64S rice seeds were surface-sterilized with 0.1% (w/v) $HgCl_2$ for 10 min and washed three times with distilled water. The sterilized seeds were soaked in distilled water at 25°C in the dark for 72 h for imbibition. Fully imbibed seeds were germinated at 37°C in the dark for 48-72 h, and the germinated seeds were sown in plastic pots (20 cm tall and 10 cm in diameter) that were filled with soil in the greenhouse at 28°/22°C (day/night) with a 16-h photoperiod. Rice plants at the five-leaf, booting, and heading stages were subjected to environmental stress treatments. For cold and hot treatments, the plants were kept at 12°C for 16 h and 45°C for 2 h, respectively. For drought treatment, the plants were kept out of water until the leaves began to roll. The controls were grown under normal conditions at 28°C.

Sampling and preparation of total RNA

After treatment, the leaves and panicles were collected from six individual rice plants at the same development stage and immediately frozen in liquid nitrogen. Total RNA was extracted using Trizol reagent. The total RNA was treated with DNase I (Fermentas, Canada) to remove the DNA that was co-extracted with the RNA at 37°C for 15 min.

Microarray analysis

Total RNA from the leaves and panicles of rice cultivar PA64S were used as the experimental materials for microarray analysis. Microarray analysis was performed using a GeneChip Rice Genome Array according to the GeneChip® Expression Analysis Technical Manual (2005 version) provided by Affymetrix. The main operation procedures were as follows: 1) extraction and purification of total RNA, 2) synthesis and purification of cDNA, 3) synthesis and segmentation of cRNA, 4) array hybridization and washing, 5) array scanning, and 6) data analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Primers were designed using the Primer Express 3.0 software (Applied Biosystems, USA). The primer sequences used for the gene expression analysis are OsRCI2-5RT-F 5'-TACGGCTTGGGTATTGAGTTCTGG-3'andOsRCI2-5RT-R5'-CCGACGCTGGCTCTGC

TC-3'. The levels accumulated of the target transcripts were analyzed using qRT-PCR with an ABI 7900HT Sequence Detection System (Applied Biosystems) by monitoring amplification with SYBR Green dye. A QuantiTect SYBR Green RT-PCR Kit (QIAGEN) was used in this study. The parameters for the thermocycling conditions were as follows: 48°C for 30 min; 95°C for 10 min; 40 cycles of 95°C for 15 s and 58°C for 1 min; 95°C for 15 s; 60°C for 20 s; and 95°C for 15 s. The 18S rRNA gene was used as the internal control, and the primers are 18S-F 5'-CGTCCCTGCCCTTTGTACAC-3' and 18S-R 5'-CGAACACTTCACCGGATCATT-3'. The comparative cycle threshold ($2^{-\Delta\Delta}$Ct) method (Livak and Schimittgen, 2001) was used to analyze the expression levels of the 10 abiotic stress-related genes. The data were analyzed using repeated-measures analysis of variance.

Isolation and sequence analysis of the *OsRCI2-5* gene

An *OsRCI2-5* clone was obtained from the rice leaf cDNA. The nucleotide and deduced amino acid sequences were analyzed by the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) and the soft Vector NTI Advance® 11.5 program (Invitrogen). Sequence comparisons were conducted using Clustal X.

Phylogenetic analysis

RCI2 family proteins were screened by BLAST of the NCBI protein databases (http://www.ncbi.nlm.nih.gov/). The phylogenetic tree of the RCI2 family was aligned and analyzed using MEGA version 5.1 (http://www.megasoftware.net/).

Construction of the plant expression vector and generation of the transgenic plants

To construct the plant transformation vector, the cDNA fragment with the whole open reading frame of *OsRCI2-5* was amplified from total PA64S RNA using primers OsRCI2-5F, 5'-AAGCTTGCTACGGCTTGGGTATTGAGTTC-3', and OsRCI2-5R, 5'-GGATCCCCGACG CTGGCTCTGCTC-3' (the underlined bases indicate the restriction sites for *Hin*dIII and *Bam*HI, respectively). After sequence verification, the PCR fragment was digested by *Hin*dIII and *Bam*HI and ligated into the intermediate vector pJIT163, thus, allowing the gene to be driven by the *CaMV35S* promoter. The CaMV35S-OsRCI2-5 fragment was then digested by *Kpn*I and *Xho*I and inserted into pCAMBIA1300 that was digested with *Kpn*I and *Sal*I (*Xho*I and *Sal*I are isocaudomers). The *OsRCI2-5* promoter region (1560-bp upstream of the start codon) was cloned to be recombined into the pCAMBIA1301 vector upstream of the *GUS* gene for expression profile analysis. *Agrobacterium*-mediated transformation of the rice callus (Nipponbare) was performed as published by Xiao et al. (2008). Transformed calli were selected by hygromycin resistance, and the transgenic plants were regenerated from the transformed calli. The regenerated transgenic rice plants were grown in a greenhouse, and the seeds were collected.

Drought treatment and drought stress simulation using polyethylene glycol (PEG)-6000

For phenotyping, T-13, T-26, and T-34 transgenic and wild-type (WT) seeds were grown in Petri dishes with water at 28°C. Once they had grown three leaves, analogous drought

and drought-resistance experiments were performed. The T-13, T-26, and T-34 transgenic and WT seedlings were treated with different concentrations of PEG-6000 (5, 10, and 15%) for 10 days, and their morphological phenotypes were recorded by a camera each day. The T-13 transgenic and WT seedlings were kept out of water for drought treatment in Petri dishes.

Subcellular localization of the OsRCI2-5 protein in onion epidermal cells

To construct the vector for transient expression analysis, the coding sequences of *OsRCI2-5* were first amplified using the primer pairs OsRCI2-5-GFP, F, 5'-AAGTCCCTGAGAAGAGGGAGAATGGCGT-3', and OsRCI2-5-GFP, R, 5'-CCATGGCCACCAGCACATACACCG-3' (the underlined bases indicate the restriction sites for *Hin*dIII and *Noc*I, respectively). The intactness of the PCR fragment was verified by sequencing. It was then digested with *Hin*dIII and *Noc*I and ligated into the vector pJIT163-GFP, thus allowing the gene to be driven by the *CaMV35S* promoter. The empty pJIT163-GFP vector was used as a control. The constructs were delivered into onion epidermal cells by particle bombardment as described by Varagona et al. (1992). Twenty-four hours after bombardment, green fluorescent protein (GFP) fluorescence was observed using a Leica MZ16FA fluorescent stereomicroscope (Leica Microsystems, Germany).

Histological analysis

In different developmental stages of rice, different samples (leaf, stem spikelet, seed, and root) were prepared and incubated in β-glucuronidase (GUS) assay buffer (10 mM phosphate buffer, pH 7, 0.5% Triton X-100, 1 mg/mL 5-bromo-4-chloro-3indolyl-β-D-glucuronic acid, and 2 mM potassium ferricyanide) at 37°C for 2 days in the dark. After staining, the tissues were dehydrated in a 30-70% graded ethanol series. To obtain tissue sections, GUS-stained samples were dehydrated through a graded ethanol series followed by a *tert*-butanol series. Samples were embedded in paraffin for 3 days in a 58°C oven and sectioned at a thickness of 10 μm. Sections were photographed using a light microscope.

RESULTS

Expression levels of stress-responsive genes under different stress treatments

The genomic expression levels in the leaves and spikes of the PA64S line at the seedling, booting, and heading stages under low temperature, high temperature, and drought stresses were detected using the Affymetrix GeneChip Rice GenomeArray (containing 51,279 rice transcripts) to screen genes with increased or decreased expression levels. *OsRCI2-5* was chosen for this study because it is a drought- and low temperature-inducible gene whose expression levels were upregulated in all growth stages by 2.2-81.6-fold under drought stress and 2.4-3.6-fold under low temperature stress (Figure 1).

The results were verified using qRT-PCR, which showed a similar *OsRCI2-5* expression change trend in the different tissues at each growing stage under all stress conditions (Figure 1). However, slight differences were noted that might have been caused by methods or sampling error.

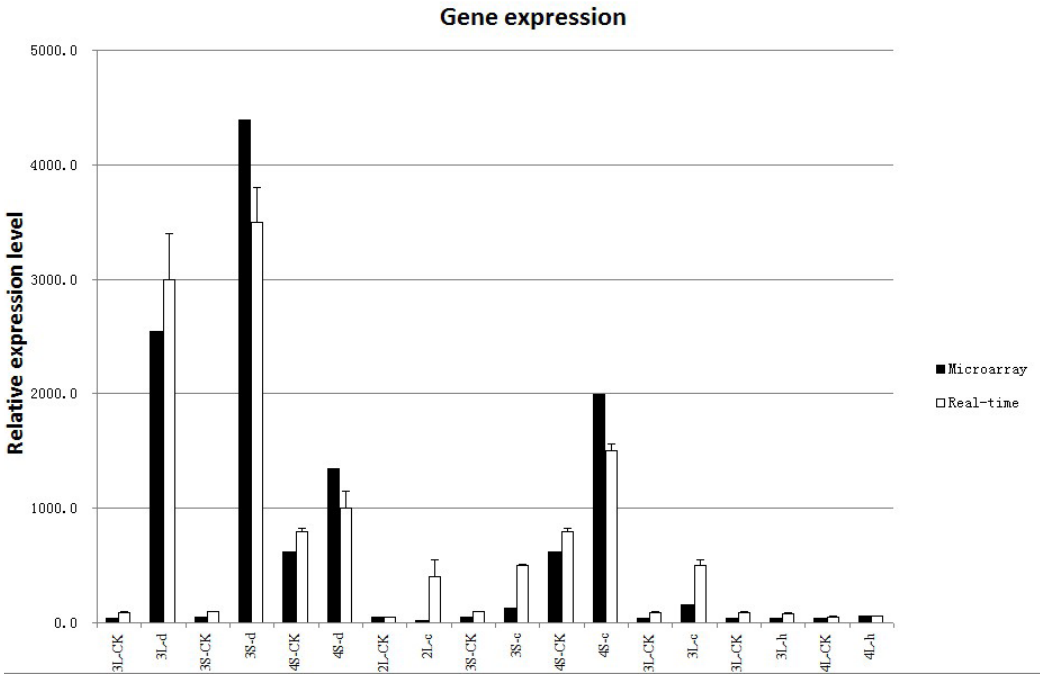

Figure 1. Relative *OsRCI2-5* expression levels. 2 = seedling stage; 3 = booting stage; 4 = heading stage. L = leaf; S = spike. CK = control; c = cold; d = drought; h = heat.

Analysis of the *OsRCI2-5* gene sequence, encoded protein, and evolution

Sequence analysis of *OsRCI2-5* showed that it was localized to chromosome 3 and contained an intron, two exons (Figure 2), and a 165-bp open reading frame that encoded a 55-amino acid sequence and contained two conservative hydrophobic domains. The protein function prediction revealed it to be low temperature-inducible. A homologous protein was identified in *Arabidopsis thaliana*, LTI6A, which is a low temperature- and salt-inducible protein. This suggested that *OsRCI2-5* might play a significant role in the cold stress-signaling pathway. The protein sequence was predicted to encode two transmembrane domains.

The phylogenic tree of the RCI2 plant proteins was constructed using the neighbor-joining method (Saitou and Nei, 1987). Sequence comparisons were performed between the OsRCI2 and OsRCI2-related proteins (Figure 3). These sequences were divided into three clusters (Figure 4). OsRCI2-5 belonged to the second cluster, which also contained RCI2 sequences from maize, sorghum, and grape. According to the analysis results, the RCI2 protein sequences were conservative, and all sequences were predicted to be related to stress treatment. The *XP002311114* and *XP002316342* genes from *Populus trichocarpa* were predicted to encode a hydrophobic stress-responsive peptide, the *NP565897.1* gene from *Arabidopsis thaliana* encoded a low temperature- and low salinity-responsive protein, the *P68178.1* gene from *Agropyron elongatum* encoded a hydrophobic salt-responsive peptide, and the *Q9ARD5.1* gene from *Hordeum vulgare* encoded a temperature-responsive protein. A homolog

also exists in eukaryotes such as yeasts and worms. The homologous protein in yeast, PMP3, maintains the sodium ion balance (Navarre and Goffeau, 2000).

Figure 2. Structure of the *OsRCI2-5* gene. Exons and introns are indicated by the white and black boxes, respectively.

Figure 3. Sequence comparison of OsRCI2 and OsRCI2-related proteins. Black arrows mean identity and gray arrow means conserved. XP_001764858.1 (*Physcomitrella patens* subsp. *patens*), EFJ05790.1 (*Selaginella moellendorffii*), EFJ34491.1 (*Selaginella moellendorffii*), XP_002277985.1 (*Vitis vinifera*), AK070872, ACG26760.1 (*Zea mays*), XP_002465426 (*Sorghum bicolor*), Q9ARD5.1 (*Hordeum vulgare*), P68178.1 (*Lophopyrum elongatum*), XP_002440508 (*Sorghum bicolor*), ACG48459.1 (*Z. mays*), XP_002278351.1 (*V. vinifera*), NP_565897.1 (*Arabidopsis thaliana*), XP_002316342.1 (*Populus trichocarpa*), ADC67798.1 (*Populus balsamifera*), and XP_002311114.1 (*P. trichocarpa*).

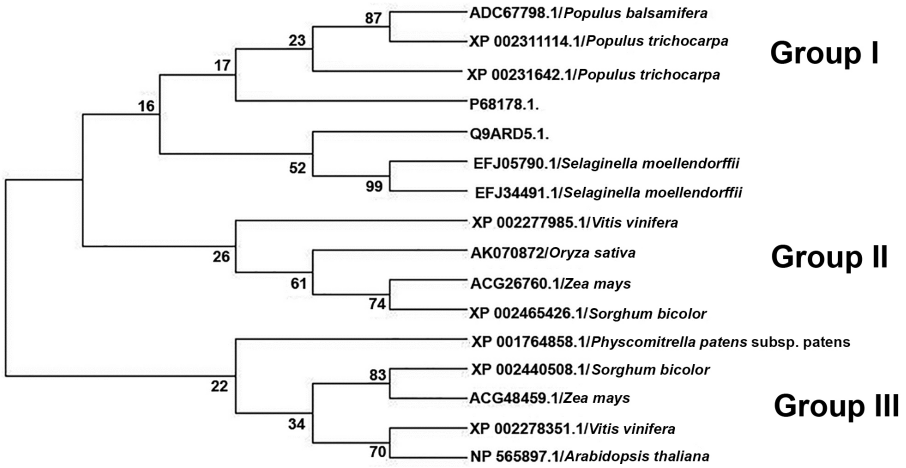

Figure 4. Phylogenetic tree of OsRCI2 and representative OsRCI2-related proteins from different organisms. The number for each interior branch shows the percentage of the bootstrap value.

Drought tolerance with *OsRCI2-5* overexpression in rice

To understand the drought tolerance of *OsRCI2-5*, we used overexpression. Twelve independent T0 generation plants were obtained. To further identify that the plant was positive, we used PCR and Southern blot analysis (data not shown).

Treatment with 5% PEG had a very small impact on all of the rice seedlings; however, treatment with 15% PEG severely influenced all of the seedlings. When treated with 10% PEG, the transgenic rice group and the WT group reacted differently. With 10% PEG, most rice leaves turned yellow and began to curl on the third day, and there were no significant differences between the overexpressed *OsRCI2-5* transgenic rice group (T13, T26, and T34) and the WT group (Figure 5A). On the tenth day, the transgenic rice group began to demonstrate restored growth that was evidenced by expanded and green leaves, while all plants in the WT group died (Figure 5B). This result suggested that *OsRCI2-5* transgenic plants have stronger drought tolerance capacities than WT plants.

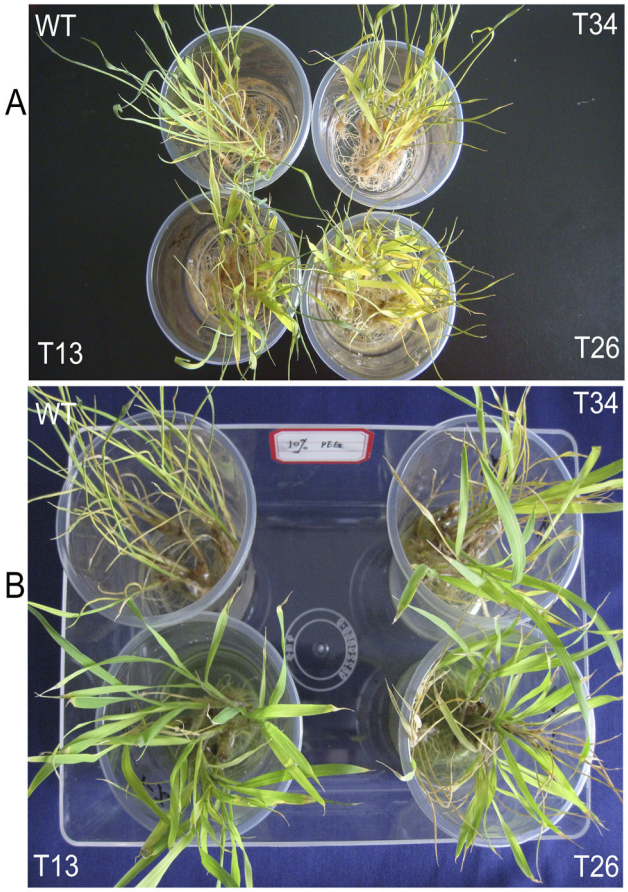

Figure 5. Analog drought experiment. Phenotype comparison of wild-type (WT) and *OsRCI2*-overexpressing (T13, T26, and T34) plants treated with 10% polyethylene glycol for 3 days (**A**) and 10 days (**B**).

In order to further prove the drought tolerance of the transgenic plant, the "No water" test was designed. After being deprived of water for three days, the leaves of the WT rice turned yellow and began to curl, whereas those of the *OsRCI2-5* transgenic rice T13 line grew normally (Figure 6A). On the fourth day, when the transgenic rice exerted the same phenotype as the WT rice, the water was restored (Figure 6B). On the second day after restored water, most of the *OsRCI2-5* transgenic plants began to grow, and their leaves expanded and turned green; in contrast, the plants in the WT group all died (Figure 6C). The *OsRCI2-5* expression levels were detected, and transgenic rice exerted significantly higher expression levels than the WT plants, which indicated that constitutive overexpression of *OsRCI2-5* could dramatically increase tolerance to drought in rice.

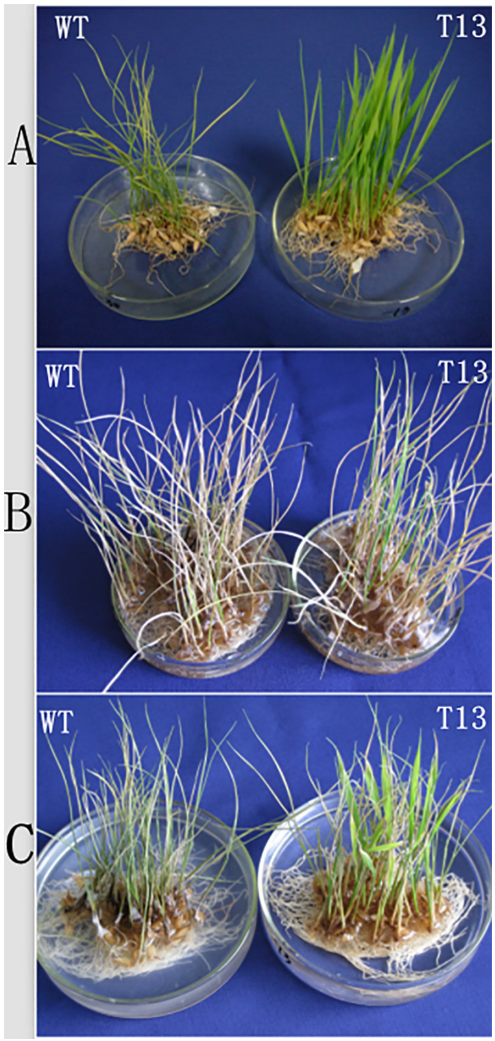

Figure 6. Drought resistance experiment. WT and *OsRCI2*-overexpressing (T13) plants were deprived of water for 3 days (**A**), deprived of water for 4 days and recovered with water (**B**), and recovered with water for 2 days (**C**).

Expression profile analysis of *OsRCI2-5*

We detected *OsRCI2-5* expression in the leaves of seedling-stage rice via GUS activity detection, and we found that it was mainly expressed in the veins. It was also expressed in the internodes of the tillering stage and the spikelets of the booting stage, especially the anthers and lemmas (Figure 7). To better study the tissue specificity and cell specificity of *OsRCI2-5* expression, GUS materials were prepared in paraffin and sectioned for examination under the microscope. We observed that *OsRCI2-5* was expressed in the following tissues: 1) stem, mechanical tissue, and vascular bundle; 2) vascular bundle of the palea and lemma; 3) anther wall; 4) pollen grain; 5) leaf vein phloem; and 6) mesophyll cell (Figure 8).

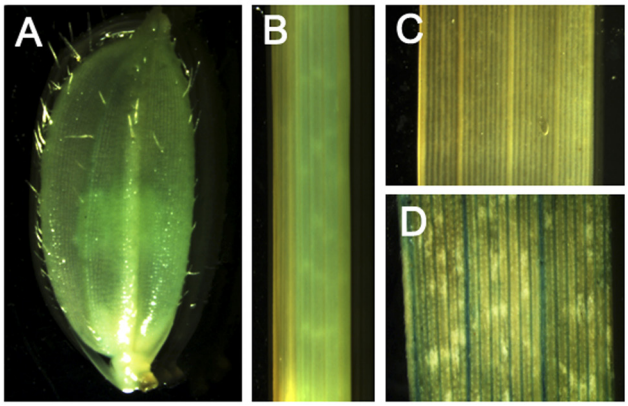

Figure 7. Expression of the *OsRCI2*-5 gene by GUS Assay in the spikelet (**A**), stem (**B**), leaf contrast (**C**), and leaf (**D**).

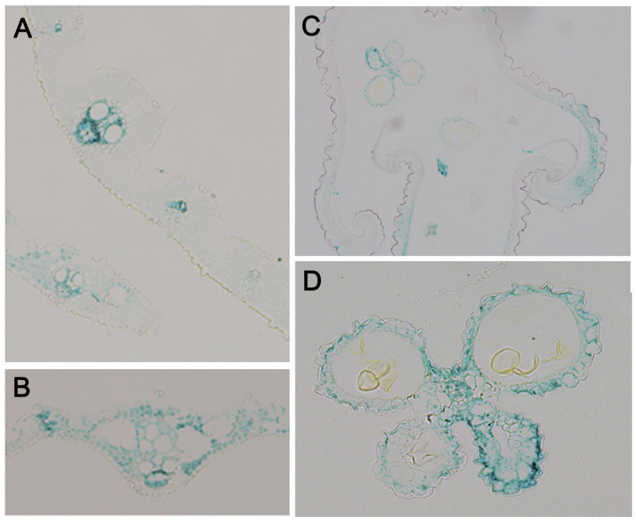

Figure 8. Paraffin sectioning of a transverse leaf section (**A**, **B**), transverse spikelet (**C**), and transverse anther section (**D**).

Subcellular localization analysis of the OsRCI2-5 protein

Using the particle bombardment method, the fusion expression construct of *OsRCI2-5* with GFP was transferred into onion epithelial cells. After culturing for 16 h, it was observed under the microscope, and the OsRCI2-5-GFP fusion protein was found to concentrate in the cell membrane (Figure 9).

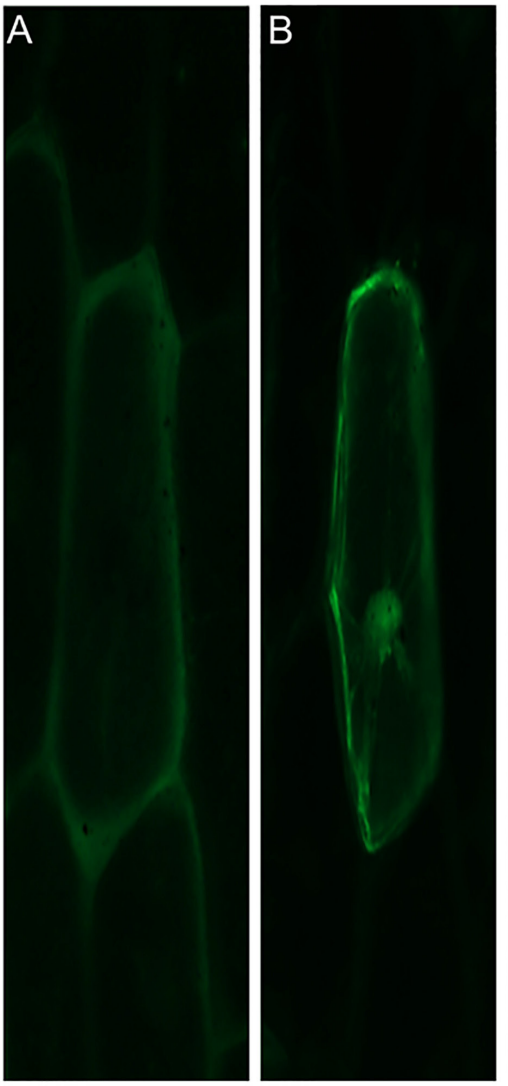

Figure 9. Subcellular localization of the OsRCI2-5 protein. **A.** Onion epithelial cell expressing the OsRCI2-5-GFP fusion protein. **B.** Control cell. Green fluorescence was observed in the cell membrane and nucleus of the control onion epidermal cells. In A, only the cell membrane showed the fluorescence signal, which suggested that OsRCI2-5 was located in the cell membrane.

DISCUSSION

We obtained a drought stress-related gene, *OsRCI2-5*, gene using microarray technology and studied its biological function via overexpression, expression profile analysis, and subcellular localization.

In recent years, research has been done on plant responses to adverse environments, and many stress-related gene families have been discovered and analyzed (Yokoi et al., 2002; Tran et al., 2004; Shigaki et al., 2006). Many low temperature-, drought-, and high salt-related genes were discovered from the transcriptome analysis of *Arabidopsis thaliana*, and many different signaling pathways were found to be involved in these stress responses (Fowler and Thomashow, 2002; Jiang and Deyholos, 2006; Oono et al., 2006).

The *RCI2* gene has been reported in many plants, including barley, wheat, *Arabidopsis thaliana*, rice, wheatgrass, and maize (Goddard et al., 1993; Gulick et al., 1994; Hughes and Dunn, 1996; Inada et al., 2005; Koike et al., 2005; Morsy et al., 2005). *Arabidopsis thaliana* contained eight *RCI2* genes, while rice contained the following seven genes: *OsRCI2-3*, *OsRCI2-5*, *OsRCI2-6* (Oslti6B), *OsRCI2-8*, *OsRCI2-9*, *OsRCI2-10* (Oslti6A), and *OsRCI2-11*. The *RCI2* family has a highly conserved gene structure, of which, most had two exons and one intron and encoded two transmembrane domains. Protein analysis showed that the OsRCI2-5 protein also contained two transmembrane domains. Subcellular localization analysis indicated that it was localized to the plasma membrane, which suggested that it might be a membrane protein or a signal peptide that functions in signal transduction. The *OsRCI2-5* gene was detected in many tissues, including stems and flowers, via expression profile analysis, which indicated that it had no evident organ-specific expression. However, the microarray data showed that *OsRCI2-5* presented higher expression levels in leaves under drought stress and in spikes under low temperature stress, which suggested that its expression had organ specificity in response to different stress environments.

RCI2 family genes were induced and upregulated by low temperature, drought, and salt stress. It was recently reported that *ZmPMP3* expression improved the stress tolerance of maize by maintaining its ionic equilibrium. In rice, *OsLti6a* and *OsLti6b*, with two conservative domains, were induced under low temperature and salt conditions (Morsy et al., 2005). In this study, the overexpression of *OsRCI2-5* remarkably enhanced rice drought resistance. Therefore, we can deduce that *OsRCI2-5* might function as other *RCI2* genes do, by controlling the same regulatory factors and safeguarding cells against dehydration and death by maintaining the intracellular fluid balance. In a future study, we will perform the drought tolerance ability test of *OsRCI2-5*-overexpressing plants in field conditions, identify the *OsRCI2-5* upstream regulator genes and the interacting proteins to investigate the *OsRCI2-5* regulation pathway, and elucidate the molecular mechanism of the OsRCI2-5 protein in enhancing the drought resistance of rice.

ACKNOWLEDGMENTS

We thank Dr. Wang Manling for providing technical assistance for the GeneChip analysis. Research supported by the National Natural Science Foundation of China (#31201184), the Natural Science Foundation of Hunan Province, China (#14JJ2138), the National Key Programs for Transgenic Crops (#2011ZX08001-004), and the Program of Breeding and Application of Hybrid Rice Witer Strong Heterosis (#2011AA10A101).

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