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Isolation and characterization of two novel root-specific promoters in rice (*Oryza sativa* L.)

Q1 Yuanya Li[a,b], Shaojun Liu[a], Zhiming Yu[c], Yu Liu[a], Ping Wu[a,*]

[a] State Key Laboratory of Plant Physiology and Biochemistry, College of Life Science, Zhejiang University, Hangzhou 310058, China
[b] College of Life Science, Yunnan University, Kunming 650091, China
[c] Research Centre for Plant RNA Signaling, College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, China

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ABSTRACT

Novel root-specific promoters are important for developing methods to drive root-specific gene expression for nutrient and water absorption. RT-PCR (reverse transcription polymerase chain reaction) analysis identified high expression levels of *Os03g01700* and *Os02g37190* in root tissues across developmental stages in comparison with the constitutive genes *OsAct1* (rice Actin1 gene), *OsUbi1* (rice polyubiquitin rubi1 gene), and *OsCc1* (rice cytochrome c gene). The copy numbers of *Os03g01700* and *Os02g37190* were evaluated by qRT-PCR. The results showed that *Os03g01700* and *Os02g37190* transcripts were highly accumulated in the examined root tissues but were not detected in young embryos or leaves at the indicated days after germination or in the panicle, in contrast to the ubiquitous expression of *OsAct1*, *OsUbi1*, and *OsCc1*. Additionally, the promoter regions of these two genes were linked to the *GUSplus* reporter gene and transformed into rice. GUS staining of the transgenic plants showed that the *Os03g01700* and *Os02g37190* promoters were active in primary and secondary roots throughout the developmental stages, except in root hairs. The *GUSPlus* transcript levels were also highly root-specific in the transgenic rice. Overall, the two promoters are highly active in the root tissues of rice and can be useful for the root-specific enhancement of target gene(s).

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1. Introduction

The use of plant-specific promoters suitable for a specific target transgene is important for fine-tuning the control of transgene expression. A few, well-characterized constitutive promoters have become valuable tools for controlling transgenes in plants, including the cauliflower mosaic virus (CaMV) *35S* [1], *OsAct1* [2], the maize polyubiquitin genes (*ZmUbi1*) [3,4], the rice α-tubulin gene (*OsTubA1*) [5], *OsCc1* [6], the rice polyubiquitin rubi1 and 2 genes (*RUBQ1* and 2) [7], the rice polyubiquitin rubi3 gene (*rubi3*) [8], the rice Actin2 gene (*OsAct2*) [9] and *PGD1* [10]. Although such constitutive promoters can provide high levels of transgenic expression in both dicotyledonous and monocotyledonous plants, if the gene product is detrimental, ubiquitous gene expression is often associated with undesirable phenotypes, including growth retardation, delayed flowering, and even lethality or sterility [11–16]. The development of tissue-specific promoters to drive foreign gene expression in a spatially controlled manner is important to avoid the potential harmful effects of overexpression of the target gene under control of constitutive promoters in non-targeted organs of transgenic plants.

Most studies on such tissue-specific promoters focus on the aerial organs of the plant, whereas roots are only rarely considered [17]. However, plant roots perform many essential functions, such as nutrient and water absorption, storage, and transportation. Root-specific promoters are important to drive root-specific gene expression and are used to improve nutrient and water uptake, root growth, drought tolerance, and resistance to multiple pathogens in *Arabidopsis*, tobacco, and rice plants [18–20].

A few root-specific genes have been isolated from a wide variety of plants, and their promoters have also been used to drive root-specific gene expression in transgenic plants [21–30]. The *rolD* promoter [21] derived from the Ri (root-inducing) plasmid of *Agrobacterium rhizogenes* has been used for the root-directed transformation and expression of gene products, including root-directed glutamine synthetase overexpression [31] and overexpression of a high-affinity nitrate transporter [32], which exhibits motifs similar to root-specific elements (RSEs) in plant root-specific promoters [33,34]. Domain A (290 bp upstream) of the CaMV *35S* promoter is a second important, non-plant-specific promoter used for

Abbreviations: *OsAct1*, rice Actin1 gene; *OsUbi1*, rice polyubiquitin rubi1 gene; *OsCc1*, rice cytochrome c gene; CaMV, cauliflower mosaic virus; *ZmUbi1*, maize polyubiquitin genes; *OsTubA1*, rice α-tubulin gene; *RUBQ1* and *2*, rice polyubiquitin rubi1 and 2 genes; *rubi3*, rice polyubiquitin rubi3 gene; *OsAct2*, rice Actin2 gene.

* Corresponding author. Tel.: +86 571 88206412; fax: +86 571 88206613.
E-mail address: clspwu@zju.edu.cn (P. Wu).

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root-specific expression [35]. In comparison to the *rolD* promoter, GUS expression from the CaMV *35S* Domain A *promoter:GUS* fusion construct was 5–15-fold less than that from the *rolD*:*GUS* fusion constructs, and the GUS expression was significantly localized in root tips [33]. The *TobRB7* promoter from tobacco is a plant-valuable root-specific promoter that has been used for many genetic engineering objectives [22]. More recent reports state that the hormone cytokinin, which is regulated by the *PYK10* promoter [23], promotes a large root system, the whereas growth and development of the shoots are similar in *Arabidopsis* and tobacco [19]. Overexpression of *OsNAC10* in rice under the control of the root-specific promoter *RCc3* [29] increases plant tolerance to drought, high salinity, and low temperatures at the vegetative stage in rice [20]. Although efforts have been made to characterize root-specific genes and their promoters, few have analyzed the entire plant growth cycle. Therefore, root-specific promoters for gene expression are lacking, particularly in monocot crops. Additionally, differences in the transformation activities driven by the same promoter in different species also indicate the need to develop endogenous root-specific promoters for plant transformation [17].

To characterize novel promoters that cause root-specific expression in rice, we examined the expression patterns of *Os03g01700* and *Os02g37190* throughout the entire rice plant, as well as their promoter activities in transgenic rice. The cDNA copy number, which reflects the expression levels of these two root-specific genes, was evaluated using real-time PCR in different tissues at various growth stages and compared with the copy numbers of the previously characterized, strong constitutive promoters *OsCc1*, *OsAct1*, and *OsUbi1*. The activities of the corresponding root-specific promoters were subsequently analyzed in transgenic rice plants using the *GUSplus* reporter. *Os02g37190* and *Os03g01700* promoter activities in rice root tissues demonstrate that these promoters may be suitable for driving root-specific expression in transgenic plants and provide useful alternatives for root-specific transformations of rice and other cereals.

2. Materials and methods

2.1. Plant materials and growth conditions

Rice (*Oryza sativa* L. cv. Nipponbare) was used to examine the expression of endogenous genes, isolate gene promoters, and perform plant transformations. For RNA extraction, the germinating seeds were collected by soaking dry seeds in water (changed every 12 h) for 72 h at 37 °C. The germinated seeds were transplanted onto moist, sterilized filter screens in a greenhouse (16/8 h light–dark cycle) at 28–30 °C. Each plant was grown in normal rice culture solution [36] for the indicated number of days after germination (DAG), and then, growth continued until the panicles were <10 cm in size. The young embryos were collected at 12–15 days after fertilization.

Rice genomic DNA was prepared from leaves of 10-d-old rice plants using the CTAB method [37]. The 3500-bp promoter regions upstream from the ATG of *Os03g01700* and *Os02g37190* were isolated by genomic PCR. The T_3 generation of three to six independent lines for each of the promoters was used for our subsequent detailed analysis.

2.2. Reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR analysis

Total RNA was extracted from young embryos, leaves, roots, and flowers using the Qiagen RNeasy plant mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. To amplify the corresponding genes, 4 μg of total RNA was converted into cDNA using the Reverse Transcription System (Promega, Madison, WI, USA) primed with oligo-dT, and subsequent RT-PCR was performed with gene-specific primer pairs designed with the Roche Primer Designer. These primer pairs are listed in Supplementary Table 1. RT-PCR was initiated at 95 °C for 3 min, followed by 26 amplification cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with a final cycle at 72 °C for 5 min. The amplified PCR products were sequenced to ensure fidelity.

A quantitative real-time PCR (qRT-PCR) assay using the SYBR® Green fluorescent intercalation dye (Roche Diagnostics, Basel, Switzerland) was employed in this study. Each amplification trial was performed in a 384-well PCR plate covered with optical tape in the LightCycler 480 real-time PCR system (Roche Diagnostics, Basel, Switzerland). The reaction mixture was subjected to denaturation at 94 °C for 3 min, followed by 45 cycles of denaturation at 94 °C for 10 s, annealing at 58 °C for 15 s, and elongation at 72 °C for 20 s. Triplicate quantitative assays were performed on each cDNA sample. The fluorescence resulting from the binding (intercalation) of SYBR® Green into the double-stranded DNA was recorded at the end of the elongation step of every cycle. The relative expression level was calculated using the formula $2^{-\Delta(\Delta Cp)}$. Quantitative real-time PCR data were analyzed using LightCycler 480 Software.

2.3. Preparation of plasmid DNA standards

The five cDNA standard curves were obtained by cloning the 385-bp, 298-bp, 294-bp, 187-bp, and 127-bp segments of *OsAct1*, *Os03g01700*, *Os02g37190*, *OsUbi1*, and *OsCc1*, respectively, into the pUCm-T vector (2773 bp) using the pUCm-T TA cloning kit (Sangon Biotech, Shanghai, China). The primer pairs are listed in Supplemental Table 1. The plasmid DNA concentration was determined in triplicate by UV spectrophotometry. The mass of a single pDNA molecule was calculated using the formula MW = 660 × number of bp of the recombinant plasmid (1 bp ≈ 660 g/mol) [38].

2.4. Copy number analysis

The recombinant plasmids were used to generate the standard curve. Briefly, the PCR products of *OsAct1* (385 bp), *Os03g01700* (298 bp), *Os02g37190* (294 bp), *OsUbi1* (187 bp), and *OsCc1* (127 bp) were amplified from rice root cDNA using gene-specific primers (see Supplemental Table 1). The obtained gene fragments were purified and then inserted into the pUCm-T vector (2773 bp) (Sangon Biotech, Shanghai, China). These recombinant plasmids containing the known gene-specific sequences can be detected using gene-specific primers. These plasmids were designated as the standard plasmids, and their DNA concentrations were determined in triplicate.

Tenfold serial dilutions of the recombinant plasmids, which corresponding to copy numbers ranging from 1×10^{-2} to 1×10^{-6} per reaction mixture, were used for real-time PCR to obtain the standard curve, and a linear regression model was used to fit the C_T values to variables representing the natural log of the copy number. Each standard dilution was tested in triplicate to ensure reproducibility. Equal volumes of standard and sample DNAs were used for PCR amplification.

Each amplification trial was performed in a 384-well PCR plate covered with optical tape in the LightCycler 480 real-time PCR system, following the method in the ABI manual for absolute real-time RT-PCR quantification [39]. To maintain consistency, the baseline was set automatically by the software. Based on these values, a linear regression line was generated, and the resulting equation was used to calculate the log starting quantities and copy numbers of the cDNAs for the unknown samples. With the molecular weights

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