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Development and validation of promoters expressed specifically in root tips and bases of lateral roots in rice and tobacco

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Abstract

The root system plays important functions in water and nutrient absorption, significantly associated with crop yield. Development of promoters of expression vector specifically expressed in root system are indispensable in biotechnological breeding for cultivars with high yield and stress resistance. In this study, two promoters highly and specifically expressed in root tip tissues according to Rice Expression Profile Database, designated Root4 and Root6, were isolated, fused with reporter gene GUS, constructed with the vector pARS3m GUSPlus, and then the fused vector pARS3m GUSPlus-Root4 and GUSPlus-Root6 were transformed in rice and tobacco, respectively. Transgenic plants were detected by GUS staining, observation of tissue slices, ddPCR and Southern blotting. The results showed that the promoter Root4 drove GUS being highly expressed in the elongation zone of root tips and bases of lateral roots of both transgenic rice and tobacco plants, whereas Root6 could not drive GUS expression in any tissues of transgenic tobacco, but in the epidermis cell of root tips of transgenic rice. These results provide new vector types for use in plant genetic engineering and lay a foundation for enhancing the function of water and nutrient absorption and tolerance to abiotic stress in the roots of rice and tobacco.

Key message

We characterized two promoters *Root4* and *Root6* in rice and tobacco; *Root4* drove GUS expression in root tips and bases of lateral roots both in rice and tobacco, whereas *Root6* drove GUS expression only in the root tips of rice.

Introduction

Biotechnological breeding has the potential to improve crop productivity and utilization properties significantly, and to facilitate production of new products. Numerous transgenic cultivars with biotic and abiotic tolerance were created by biotechnology (Jeong et al., 2010; Guan et al., 2016; Zhang et al., 2019; Oladosu et al., 2019).

Promoters are important elements for gene expression of the introduced genes, and play important roles in the improvement of crop traits by gene transformation. Usually, three types of promoters were used in gene transformation, including constitutive promoter, inducible promoter and organ- or tissue-specific promoters (Thilmony et al., 2009).

The CaMV35S promoter was initially used driving constitutive expression of genes in transgenic dicots (Odell et al., 1985). Some constitutive promoters were frequently used in earlier years, for example, the maize *Ubi1* promoter was proved to be more suitable for gene constitutive expression in transgenic monocots (Christensen and Quail, 1996), and the *Act1* promoter was applied in transgenic rice (McElroy et al., 1990; Zhang et al., 1991). Recent years, several new constitutive promoters, such as *Oscon1*, *ARP1*, *H3F3*, *HSP* and *H2BF3* in rice (Li et al., 2014; Bang et al., 2015), *PEAMT* in maize (Niu et al., 2018) have been characterized. Although these constitutive promoters are desirable in certain cases, for example, establishment of resistance to insect pest or herbicides in transgenic crops, they usually have negative effects, including co-suppression of homologous genes, increasing the metabolic burden, or even leading to infertility (Chavez-Barcenas *et al.*, 2000; Zhao et al., 2009). Therefore, some inducible promoters, such as *GSLT2* (Hu et al., 2011), *HsfB2c*, *PM19P* and *Hsp90* (Rerksiri et al., 2013), *XPB2* (Raikwar *et al*., 2014) and *RGLP2* (Rehman et al., 2018), were newly characterized from rice. Inducible promoters that differ from the constitutive promoters drive gene expression only in specific tissues exposed to certain stresses or bioreactors. Therefore, they cause no metabolic burden on transgenic plants in normal living condition.

In plant life cycle, the formation of certain organs is determined by expression of a set of genes at a given time and in a certain type of cells. Such genes with spatiotemporal specific expression are regulated precisely by the promoter interaction with corresponding transcription factors. These promoters are termed as organ- or tissue-specific promoters. Root-specific promoters drive the target genes *OsWox11* and *OsNAC*10 to express specifically in rice roots to significantly increase drought resistance (Zhao et al., 2009; Jeong et al., 2010), whereas a constitutive promoter drives constitutive expression of the target genes in rice, leading to the infertility of transgenic plants (Zhao et al., 2009) or no expected traits (Jeong et al., 2010).

In order to achieve precise regulation of introduced genes, a modest number of promoters with organ- or tissue-specific expression have been thoroughly examined in transgenic rice. These promoters include *OsIAGP* (Anand and Tuagi, 2010), *OsIPA* (Swapna et al., 2011), *OsLTP6* (Liu et al., 2013), *OsIPP3* (Malimaran *et al.*, 2015), *OsPLP1*, *OsPLP2* (Yan et al., 2015) and *OsSUT3* (Li et al., 2020), driving the expression of target genes specifically in pollen, *OsHd3a* (Pasriga, et al., 2018) and *PvPfn2* (Xu et al., 2018) specifically in vascular tissue, and *OsLP2 (*Thilmony et al., 2009), *OsSNFL1* (He et al., 2018), *OrGSE* (Xue et al., 2018) and *PvMYB4* (Liu et al., 2018) specifically in green tissue. In addition, six promoters of rice *Glutelin* (*GluA-1*, *GluA-2*, *GluA-3*, *GluB-3*, *GluA-5* and *GluC*) and one promoter of *α-zein* were endosperm-specific (Qu et al., 2008; Joshi et al., 2015). The promoter of *1Cys-Prx* was proved to be in embryo and aleurone layer expressed

specifically (Kim et al., 2011). Therefore, the availability of a series of organ-specific promoters with diverse specificity would allow precise spatial expression of target genes for crop improvement by transformation.

The root system is a crucial determinant for plant growth, whose major functions are water up-taking, nutrients absorbing, structural supporting and interaction with symbiotic organisms (Takehisa et al., 2012), directly associated with crop yield (Carvalhais et al., 2011). Improvement of crop root system to make it tailored to environment by biotechnology will increase yield notably. Significant improvement of nitrogen absorption efficiency was identified in *AlaAT* transgenic rice lines, which was driven by a root-specific promoter (Shraw at *et al.*, 2008), and 30% higher grain yield was observed in transgenic rice than that in wild-type under 50% reduced nitrogen content (Selvaraj et al., 2017). Besides nitrogen deficiency, the root systems usually suffered from other unfavorable soil conditions such as phosphorus deficiency, potassium deficiency, drought, saline and alkaline, and nematode attack. To overcome these abiotic and biotic stresses, multiple genes under the control of different root-specific promoters required to be simultaneously introduced into crop genome. In recent years, promoters with root-specificity were isolated both from dicots (Chen et al., 2014; Yu et al., 2015; Wu et al., 2018) and monocots (Li et al., 2013;Wang et al., 2014; Liu et al., 2015; Xu *et al.*, 2016; Yamamoto et al., 2018; Li *et al.*, 2019). Most of these root-specific promoters drive reporter gene expression universally in whole root systems. While *GmPRP2* from soybean, encoding a proline-rich protein, can promote GUS specifically express in hairy roots (Chen et al., 2014).

Root tip is a functional part of plant root system which is not only essential for its development but also accounts for a majority of absorption of water and other nutrients. Few promoters have been characterized as root tip-specific expression. In this study, we found two rice genes, *LOC_Os09g24370* and *LOC_Os10g31640*, based on publicly available gene expression data. *LOC_Os09g24370* is expressed specifically in root epidermal cell, while *LOC_Os10g31640* is specifically expressed in root tip. The function of the promoters of *LOC_Os09g24370* and *LOC_Os10g31640* was validated, which can be used to improve the vector types and lay a foundation for enhancing the absorption of water and nutrients and abiotic stress tolerance in roots of monocots and dicots.

Results

Identification and validation of candidate genes highly and specifically expressed in rice root

In order to select promoters highly and specifically expressed in root tissue for using in crop biotechnology, expression profile analysis was performed to examine the expression of tissue-specific genes (Supplementary Fig. 1). Two genes, *LOC_Os09g24370* (no intron) and *LOC_Os Os10g31640*, were identified, which are predominantly expressed in roots according to Rice Expression Profile Database, RiceXPro (www.ricexpro.dna.affrc.go.jp) (Supplementary Fig. 2 and Supplementary Fig. 3). In other tissues, including leaves, florets or seeds, the transcripts of *LOC_Os09g24370* and *LOC_Os Os10g31640* were not detected on agarose gel (Figs. 1a, 1b). Moreover, although both *LOC_Os09g24370* and *LOC_Os10g31640* predominantly expressed in root, the transcripts of *LOC_Os09g24370* can be detected from elongation zone to maturation zone (Supplementary Fig. 2), and *LOC_Os10g31640* is restricted mainly in root cap and division zone (Supplementary Fig. 3). It is interesting to find a common area, in which transcripts of *LOC_Os09g24370* and *LOC_Os10g31640* were observed in the whole zone of root tip.

To further validate the organ-specific expression patterns of *LOC_Os09g24370* and *LOC_Os10g31640*, PCR and semi-quantitative RT-PCR were performed using a specific primer set OsCC1mRNAF/R (Table 1). *Cytochrome c1* (*CC1*) was selected as a reference gene that can be amplified in a 1189 bp fragment by PCR, and a 473 bp fragment by RT-PCR in all test samples in rice cultivar Nipponbare. Moreover, a 542 bp PCR fragment for *LOC_Os09g24370* using a specific primer set Root4mRNAF/R, and a 969 bp PCR fragment for *LOC_Os10g31640* using a specific primer set Root6mRNAF/R, *Root4* and *Root6* were both detected in roots, leaves, florets and seeds (Figs. 1a and 1b). Moreover, the 542 bp RT-PCR fragment of *LOC_Os09g24370*, was only detected in roots, whereas a 680 bp mis-spliced fragment of *LOC_Os10g31640* was detected in roots, and no RT-PCR product was detected in leaves, florets and seeds (Figs. 1a and 1b). These results indicated that the transcripts of *Root4* and *Root6* were tissue-specifically expressed in rice.

Table 1
The primer sets and probes used in this study

Primer sets	Forward primer (5'-3')	Reverse primer (5'-3')	Fragment size (bp)
Root4F/R	ataaggcgcgccATGAGAGTGTTCTGCAACAGTTGAC	aattccatggTTAATTACTTAGCTTGGTGGGTGCG	1865
Root6F/R	ttttggcgcgcc CGGTGTATTGGCTATTTGGCTCG	ttttccatgg TGTGGACTTACTGGCTAGCTAGG	1913
HygroF/R	CTTGACATTGGGGAGTTTAGC	CTATTTCTTTGCCCTCGGAC	1464
Root4mRNAF/R	CATCAATGGCTTCCGCCGTG	CGATAACCGAGACACAGCCGA	542
Root6mRNAF/R	GAATGCTGAAGGAAATGGCTCGG	ATGGTGAGGAGAATTATGGGCTGG	680 (cDNA), 969 (gDNA)
OsCC1mRNAF/R	TGGTCTGTTTGGAAGGCAGTCA	CCAAAAGGGATGCAATCCAGTC	473 (cDNA),1189 (gDNA)

Supplementary Fig. 1 Organ-specific expression genes in Rice Expression Profile Database

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Supplementary Fig. 2 Screening of candidate gene Os09g24370 (Root4) with root-specific expression and cell-specific expression in silico genes in Rice Expression Profile Database.

Isolation of promoters of *LOC_Os09g24370* and *LOC_Os10g31640* **and construction of recombinant vectors fused with reporter gene GUS**

To further verify the function of promoters of *LOC_Os09g24370* and *LOC_Os10g31640* driving target genes, the fragments of 1867 bp (from − 1872 to -5) and 1913 bp (from − 1914 to -1) from *LOC_Os09g24370* and *LOC_Os10g31640*, designated as *Root4* and *Root6*, respectively, were obtained from rice cultivar Nipponbare, and these two fragments were double digested by restriction endonucleases, then cloned into vector pARS3m GUSPlus (Fig. 2a).

To confirm whether right sequences of *Root4* and *Root6* promoters were obtained, PCR was performed using the specific primers (Table 1), and the PCR products were sequenced and blasted. The results indicated that the right sequences of *Root4* and *Root6* promoters were recombined into the pARS3mGUSPlus vector, demonstrating that the plant binary vectors were constructed successfully using the promoters of *LOC_Os09g24370* and *LOC_Os10g31640*, which were named as pARS3 Root4-GUSPlus and pARS3 Root6-GUSPlus, respectively (Figs. 2c and 2d). In the binary vector pARS3mGUSPlus, the selective gene hygromycin phosphotransferase (*hpt II*) is under the control of *RUBQ2* promoter, resulting in highly expressed *hpt II* in monocot, and improving the efficiency of selection for transgenic seedlings.

Previously, we constructed a binary vector, pCTAG2-GUSPlus, with *GUS* driven by CaMV 35S promoter (Figs. 2b, 2e and 2f), which was designed for establishment of transgenic dicots, embraced neomycin phosphotransferase II (*npt II*) driven by UBQ10 promoter. In this study, in order to explore the function of *Root4* and *Root6* promoters driving GUS expression in dicots, each of them was sub-cloned into pCTAG2-GUSPlus and recombinant vectors pCTAG2-Root4-GP and pCTAG2-Root6-GP (Figs. 2d and 2e) to generate transgenic tobacco plants.

Generation of pARS3 Root4-GUSPlus and pARS3 Root6-GUSPlus transgenic rice and tobacco lines

Transgenic rice lines were generated using the constructs of pARS3 Root4-GUSPlus and pARS3 Root6-GUSPlus via *Agrobacterium tumefaciens* transformation. For pARS3 Root4-GUSPlus, 32 individual rice seedlings were generated; among them, 20 seedlings showed the GUS activities in root tissue. For the pARS3 Root6-GUSPlus, 45 individual rice seedlings were generated, in which 33 seedlings showed the GUS activities in root tissue. Compared with pARS3 Root4-GUSPlus, pARS3 Root6-GUSPlus showed much higher transformation ratio.

To check whether *Root4* and *Root6* promoters worked in dicots, transgenic pCTAG2-Root4-GP and pCTAG2-Root6-GP tobacco plants, respectively, were also created via *Agrobacterium tumefaciens* transformation. The GUS staining in pCTAG2-Root4-GP and pARS3 Root4-GUSPlus transgenic lines showed that GUS signals were all detected in root tips and junction of primary root and lateral roots (Figs. 4a and 6a), indicating that the regulation mechanism of *Root4* was conserved both in rice and tobacco, or in monocot and dicot. But for pCTAG2-Root6 transgenic tobacco lines, there was no GUS signal detected, implying that *Root6* expression in rice must require some particular transcription factors which are absent in tobacco.

Determination of copy number of pARS3 Root4-GUSPlus and pARS3 Root6-GUSPlus transgenic rice lines by ddPCR and Southern blotting

For transgenic plants, copy numbers of integrated gene affects its expression patterns and targeting traits. Therefore, the hptII (hygromycin phosphotransferase II) transgene copy numbers were determined in transgenic plants using droplet digital PCR (ddPCR) and OsUBC

reference gene with probe labels of FAMTM and VICTM (Table 2 from Feng *et al.*, 2022). The quality of ddPCR data was good as indicated by the significantly different fluorescence values between negative and positive droplets, and no positive droplets were observed in the negative control samples. Three positive lines from pARS3 Root4-GUSPlus transgenic rice and pARS3 Root6-GUSPlus transgenic tobacco, respectively, were selected to identify the copy number by ddPCR. The results showed that l pARS3 Root4-GUSPlus transgenic rice lines L1, L37 and L79 had one, two and three copies of the *hptII* gene, respectively (Fig. 3a), and pARS3 Root6-GUSPlus transgenic rice lines L5, L9 and L14 each had two copies of the *hptII* gene (Fig. 3b). Moreover, Southern blotting was performed in pARS3 Root4-GUSPlus and pARS3 Root6-GUSPlus transgenic rice lines. Three lines L1, L37 and L79, with GUS signal from pARS3 Root4-GUSPlus transgenic plants, were selected for Southern blotting. The results showed that L79 had only one-copy insertion, and L1 had two copies, while L37 had more than three copies of insertions (Fig. 3c). Three pARS3 Root6-GUSPlus transgenic rice lines with GUS signal, L5, L9 and L14, were selected for Southern blotting. The result showed that all the three lines, which were randomly selected, had two copies of introduced gene (Fig. 3d).

pARS3 Root4-GUSPlus transgenic lines, L1, L79 and L37, with two, one and three copies of target gene, respectively, were detected by ddPCR and Southern blotting, respectively (Figs. 3a and 3c). For the three pARS3 Root6-GUSPlus transgenic lines, L5, L9 and L14, each had two copies of target gene determined by ddPCR and Southern blotting (Figs. 3b and 3d). For pARS3 Root4-GUSPlus and pARS3 Root6-GUSPlus transgenic rice lines, the results of ddPCR and Southern blotting were consistent (Fig. 3).

Root4 promoter driving GUS expression in the elongation zone of root tips and junction of primary and lateral roots

The GUS expression of whole seedlings, mature plant roots, leaves, florets and seeds in pARS3 Root4-GUSPlus transgenic T1 rice lines were observed with an optical microscope after treatment with dye and decolorizer solution of GUS. The results showed no GUS signal in leaves, florets and seeds (Fig. 4d-f), whereas strong GUS signal was detected in root tips as well as in the bases of lateral roots (Fig. 4a-c), which was consistent with the GUS signal in whole seedlings (Fig. 4g). These indicated that the promoter of *Root4* was expressed specifically in root tip. Moreover, there was much stronger GUS signal in the elongation zone of root tips, and junction of primary and lateral roots, especially at the vascular area of primary roots where lateral roots emerged (Fig. 4a-c). In addition, the observation of optical microscope showed that the GUS signals in root cap area and epithelial cells of meristematic area were very strong (Fig. 4c), and the epidermis of junction of primary and lateral roots with GUS signals formed a circular area where lateral roots emerged (Fig. 4b).

Root6 promoter driving GUS expression in the division zone of root tips

Different organ samples including whole seedlings, young roots, leaves, florets and seeds from T1 lines of pARS3 Root6-GUSPlus transgenic rice, were collected, and then the GUS expression was observed with an optical microscope after treatment with dye and decolorizer solution of GUS. The GUS signal was only detected in young roots; there was very strong GUS signal in the area of young root tips (Figs. 5a and 5e).

To better characterize the cell-specific GUS staining in pARS3 Root6-GUSPlus transgenic lines, mature plant roots were stained by GUS (Fig. 5f), and root tips were detected after decolorizer solution of GUS. The result showed that GUS signal in root tips was not only detected in seeding roots, but also in mature plant roots, implying that the root tip-specific pARS3 Root4-GUSPlus was independent on root development. In addition, both longitude sections and cross sections of maturing plant roots stained by GUS were prepared (Figs. 5g and 5h). The observation of optical microscope showed that the GUS signals in root caps and division zones were very strong in longitude section (Fig. 7g), and the GUS signals decreased gradually from division zone to maturing zones (Fig. 5f). These results are coherent with *Root6* expression pattern annotated in Rice Expression Profile Database. At the same time, cross sections from adjacent division zone and maturing zone, where GUS signals were much stronger in epidermis than other cells (Fig. 5h). These results showed the promoter of *Root6* driving GUS expressed specifically in the area of root tips, and much higher expression level was detected in epidermis cells.

The promoter of Root4 has the same expression mechanism between rice and tobacco

Another binary vector pCTAG2-GUSPlus was designed for creation of transgenic tobacco lines (Fig. 2b). The promoters of *Root4* and *Root6* were recombined with pCTAG2-GUSPlus to construct the dicot expression vectors pCTAG2-Root4-GP and pCTAG2-Root6-GP (Figs. 2e and 2f), and generate the pCTAG2-Root4-GP and pCTAG2-Root6-GP transgenic tobacco lines. The seedlings of pCTAG2-Root4-GP and pCTAG2-Root6-GP transgenic tobacco lines, respectively, were stained by GUS. The optical microscope observation showed that GUS signals of pCTAG2-Root4-GP transgenic tobacco lines, similar with pARS3 Root4-GUSPlus transgenic rice (Fig. 4), were only detected in the elongation region of root tips, as well as in the bases of lateral roots (Figs. 6a and 6b). While for pCTAG2-Root6-GP transgenic tobacco seedlings, no GUS signals were detected in any tissue, indicating that the promoter of *Root6* could not drive the GUS expression in tobacco. These data indicated the same expression mechanism between rice and tobacco for the promoter *Root4* rather than *Root6*. Therefore, the promoter of *Root4* could not only be applied to establish expression vectors in rice, but also in tobacco.

Discussion

The promoter Root4 driving GUS expression in root tips in the elongation zone and junction of primary and lateral roots.

Based on Rice Expression Profile Database, promoter *Root4* expresses in cell-specificity, i.e., in epidermal cells in the root-tip elongation zone, whereas in this study, the GUS signals were not only identified in the elongation zone of root tips, but also in the junction of primary and lateral roots, especially at the vascular area of primary roots where lateral roots emerged in pARS3 Root4-GUSPlus transgenic rice lines (Fig. 4 and Fig. 6). This new discovery is very interesting, because lateral roots are important parts of plant root systems, which play an important role in acquiring water and nutrients, and aiding in anchoring both young and established plants to their growth substrate (Rory, 2024). The lateral root formation is so important, in fact, that it is a defining feature of all extant euphyllophytes (Hetherington et al., 2020). It is a worthy task to develop vectors expressed specifically in root tips and the vascular of primary root where lateral root is present for improving the molecular mechanism of lateral root formation.

The possible reasons of Root6 driving GUS expression specifically in rice root tips, but not in tobacco

Development of vectors expressed specifically in root tissue is the first step to make clear the molecular mechanism on root growth for improvement of root system and field crops. In this study, it was proven that the promoter *Root4* was not only expressed in root tips of rice (monocot), but also in root tips of tobacco (dicot). Therefore, *Root4* expressed in roots will be used universally in moncots and dicots. Moreover, *LOC_Os09g24370* with promoter *Root4*, was annotated as a conserved protein in Rice Expression Profile Database; it may be also conserved in tobacco like in rice, and this protein probably implicated in root development, especially in lateral root development. Whereas for pCTAG-Root6-GP transgenic tobacco seedlings, no GUS signals were detected in any tissues, implying that the *LOC_Os Os10g31640* protein in tobacco may differ from that in rice, leading to differences in GUS protein temporal and spatial expression. Because in eukaryotes, the temporal and spatial expression of a gene is precisely regulated by a series of trans factors encoded by appropriate regulatory genes interacted with their specific cis elements localized in the given gene promoter region (Christ et al., 2013), and promoter sequences were different between *Root4* and *Root6*. But in tobacco, the special trans factors for promoter *Root6* might be absent, leading to no GUS signal in pCTAG-Root6-GP transgenic tobacco lines. Additionally, the glycine-rich cell wall protein (GRPs) is mainly present in monocots (Lei and Wu, 1991), but not in dicots, which might lead to a default GUS expression driven by promoter *Root6* in tobacco.

TATA-box and CAAT box of promoters Root4 and Root6 might lead to tissue- or organ-specific expression

PlantCARE (Plant Cis-acting Regulatory Element) was used to search for cis elements of *Root4* and *Root6*. TATA box and CAAT box, included in the promoters *Root4* and *Root6*, were analyzed in silico, which was termed core or common promoter elements in the promoter regions of eukaryotic protein-coding genes (Corden et al., 1980, Dierks et al., 1981). These core promoter elements TATA and/or CAAT boxes were present in the promoters of a few protein-coding genes in eukaryote. For example, in *Saccharomyces cerevisiae*, approximately 19% of protein-coding genes have promoters containing TATA box (Basehoar et al., 2004). Comparative genomics analysis of plant gene coding proteins revealed that genes containing TATA box was from 13 to 22% both in dicots and monocots (Kumari and Ware, 2013). The genes with core elements of TATA and CAAT boxes were strictly regulated and were response to biotic or stress stimuli (Bae et al., 2015, Song et al., 2018, Xu et al., 2019), and they were typically tissue- or organ-specific expression (Zhang et al., 2016, Xue et al., 2018, Gao et al., 2019, Liu et al., 2019, Dasgupta et al., 2020, Zhong et al., 2021).

In addition, G-box, GA-motif, GT1-moti, MRE and CAT-box, were also found in *Root6* promoter, among them, CAT box is a cis-acting regulatory element related to meristem expression (Zhang et al., 2013). The presence of CAT box in promoter *Root6* probably explained why *Root6* can drive GUS expression in root tips of pARS3 Root6-GUSPlus transgenic rice lines (Fig. 5), predominantly in meristem zone of root tips (Fig. 5g).

The vector pARS3 Root4-GUSPlus might have totipotency for root-specific expression in monocots and dicots

In the field of plant biotechnology, organ-specific promoters highly driving introduced gene expression in monocots and dicots are rare except *Sad1* (Saponin-deficient 1) promoter isolated from oat (*Avena strigosa*), which can drive GUS expression in monocots and dicots (Kemen et al., 2014). In this study, pARS3 Root4-GUSPlus expressed highly and specifically in rice (monocots) and tobacco (dicots) roots. The promoter *Root4* in pARS3 Root4-GUSPlus has the capacity of enhancing the expression of target or selective genes, which was similar to the constitutive cauliflower mosaic virus 35S promoter (CaMV 35S). The successful construction of the vector pARS3 Root4-GUSPlus has laid a solid foundation for the industrial service of transgenic plants in crop field.

Materials and methods

Plant materials, growth conditions and transformation

Agrobacterium tumefaciens strain EHA105 was used to genetically transform embryonic calli of rice with previously described recombinant vectors (Feng et al., 2019). Rice plants were grown in greenhouse under 28–30°C temperature and supplemented with 16 h/day of sodium lamplight. Regenerated T0 plants were grown in soil in greenhouse as described above. Harvested T1 transgenic rice seeds were dried for 5 d at 50°C, de-hulled and then surface sterilized prior to sowing. The seeds were then sown in sterilized soil, and seedlings were scored for antibiotic resistance after 2–3 weeks of growth.

Tobacco (*Nicotiana tabacum* SR1) were grown in greenhouse under 25–28°C temperature and supplemented with 16 h /day of sodium lamplight.

Agrobacterium tumefaciens GV3101 transformation of tobacco was performed according to Dasgupta et al. (2020).

Plasmid construction

The pARS3m-GUSPlus and pCTAG2 vectors were kindly provided by Professor Kasturi Dasgupta, Western Regional Research Center, Crop Improvement and Genetics Research Unit (USDA-ARS). The *Root4* promoter was amplified using the specific primer sets Root4F/R (Table 1). The 1865 bp promoter fragment of *Root4* (from − 1871 to -7bp of LOC_Os10g24370) was digested and cloned into the sites (between AscI and NcoI) of binary vector pARS3m-GUSPlus. This recombination vector was termed as pARS3 Root4-GUSPlus. Then, the promoter *Root4* was digested with *Sma*I and *Nco*I from the pARS3 Root4-GUSPlus and sub-cloned into pCTAG2-GP between *Nco*I and *Pme*I sites. The recombined binary vector pCTAG2-Root4-GP was confirmed by *Nco*I and *Sma*I digestion. Using the same method, the *Root6* promoter was amplified with specific primer sets Root6F/R (Table 1). Then, the 1913 bp promoter fragment of *Root6* (from − 1914 to -1 bp of LOC_Os10g31640) was cloned into the site (between *Asc*I and *Nco*I) of pARS3m-GUSPlus and the site (*Nco*I and PmeI) of pCTAG2, respectively. The four vectors, including pARS3 Root4-GUSPlus, pCTAG2-Root4-GP, pARS3 Root6-GUSPlus and pCTAG2-Root6-GP, were constructed.

RNA extraction and reverse transcription semi-qRT-PCR analysis

Total RNA was extracted from samples of seeds, leaves, roots, and florets using the TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa). Five hundred nanograms of total RNA of each tissue sample was used as template for the reverse transcription (RT) reaction by Superscript III reverse transcriptase (Invitrogen). In all experiments, cDNA was synthesized using an oligo dT primer in the RT reaction. After RT, 1 μL of cDNA, 1 unit of *Taq* DNA polymerase (New England Biolabs) and gene-specific primers were added for 20 μL PCR reaction. Primers used for PCR are listed in Table 1. Except the primers for *Root4* which has no intron, each set of primers spanned an intron to allow for differentiation of PCR products which were amplified with the templates of genomic DNA and cDNA, respectively. For reaction of semi-qRT-PCR, 30 cycles were performed using specific primers, with *CC1* as an internal control in each reaction to normalize the expression of target genes (Jang et al. 2002).

Analyses of Southern blotting

Approximately 10 μg genomic DNA from individual transgenic rice lines was digested by *Bam*HI and *Hin*dIII. In pARS3M-GUSPlus vectors, *Bam*HI site localized just at the beginning of *hptII* gene. Between the left border (LB) of T-DNA and the end of *hptII* gene, there are around 300 bp. Therefore, after *Bam*HI digestion, using labeled *hptII* gene as probe, the detected DNA band larger than 1.3kb represented one copy of the transformed gene. This reason was also applicable for *Hin*dIII digestion; it would generate a much larger DNA band that was more than 3 kb. After complete digestion, the DNA samples were separated on a 0.8% agarose gel and then transferred to a positively charged nylon membrane in an alkaline transfer solution (0.4 M NaOH, 0.6 M NaCl). In situ, the DNA samples were cross-linked to the membrane using the UV Stratalinker 2400 (Agilent Technologies, Santa Clara, CA).

Gene-specific fragment of *hptII* amplified from pARS3m-GUSPlus using primer sets HygroF/R (Table 1) was used to synthesize the radiolabeled probes using αP32-dCTP with the NEBlot kit (New England Biolabs, Ipswich, MA) and purified through ProbeQuant G-50 microcolumns from Amersham Biosciences (Piscataway, NJ). Blot hybridizations were performed using the Sigma Perfect HybTM Plus hybridization buffer (Sigma-Aldrich, St. Louis, MO) as recommended by the manufacturer. Hybridized blots were washed to 1× SSC 0.1% SDS at 50°C and exposed to X-ray film.

Observation of microscope, sectioning and photography

The expression of GUS reporter gene was visualized under bright field conditions using an Olympus BX51 microscope system, and images were documented using the attached DP70 digital camera (Olympus, Melville, NY, USA). Microscopic images between 2X and 10X were documented using a Leica MZ16F stereomicroscope (Leica Microsystems, Bannockburn, IL, USA) with attached Retiga 2000R FAST Cooled Color 12-bit digital camera (Q Imaging, Pleasanton, CA, USA).

Author contribution statement Yanping Xing performed the experiments, Yang Han, Siyi Wang, Xiaolong Li and Shasha Zhu performed some experimental validation., Bing Han and Yan Yang provided funding for the experiments and completed the manuscript writing.

Declarations

Conflict of interest The authors declare no conflicts of interest in regard to this manuscript.

Ethical standards

We declare that these experiments comply with the ethical standards in China where they were performed.

Author Contribution

Yanping Xing performed the experiments, Yang Han, Siyi Wang, Xiaolong Li and Shasha Zhu performed some experimental validation., Bing Han and Yan Yang provided funding for the experiments and completed the manuscript writing.

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Figures

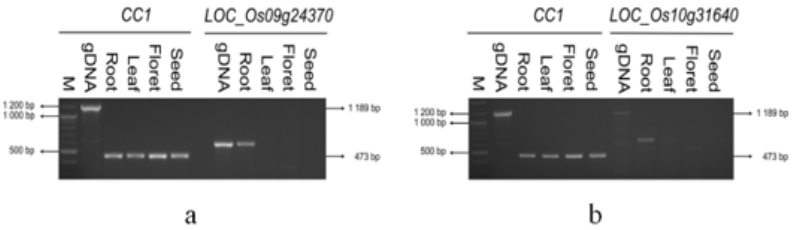


Figure 1

Screening of candidate genes with root-specific expression in silico and validation of *Root4*and *Root6* by semi-quantitative RT-PCR and PCR.

a and b, Validation of organ-specific expression of *Root4*and *Root6*, respectively, by semi-quantitative RT-PCR and PCR.

Note: The samples of root, leaf, floret and seed means the cDNA of root, leaf, floret and seed, respectively, added as template in the PCR system; M, 100 bp DNA ladder.



Figure 2

The plant binary vectors used in this work for characterization of *Root4* and *Root6*promoter activity.

a and b: The plant binary vectors pARS3mGUSPlus and pCTAG2-GUSPlus;

c and d: The pARS3mGUSPlus recombinants with *Root*4 and *Root6* promoters, respectively;

e and f: The pCTAG2GUSPlus recombinants with *Root4*and *Root6* promoters, respectively.

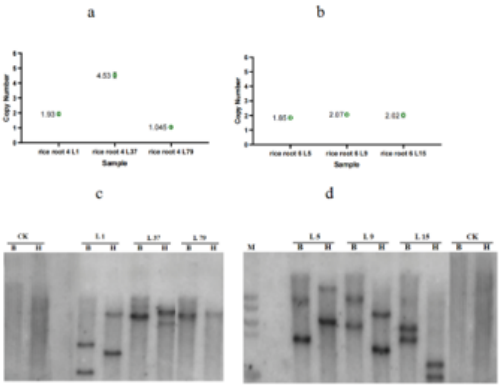


Figure 3

The copy number of transformed genes detected by Southern Blotting and ddPCR.

a: The copy number of three pARS3 Root4-GUSPlus transgenic rice lines (L1, L37 and L79) were checked by ddPCR.

b: The copy number of three pARS3 Root6-GUSPlus transgenic rice lines (L5, L9 and L15) were checked by ddPCR.

c: Southern Blotting for pARS3 Root4-GUSPlus transgenic rice lines (L1, L37 and L79).

d: Southern Blotting for pARS3 Root4-GUSPlus transgenic rice lines (L5, L9 and L15).

Note: CK means Nipponbare, as a negative control; M, 10K DNA Ladder. B, genomic DNA digested by BamHI hybridized with HPTII probe, and H, genomic DNA digested by HindIII hybridized with HPTII probe.

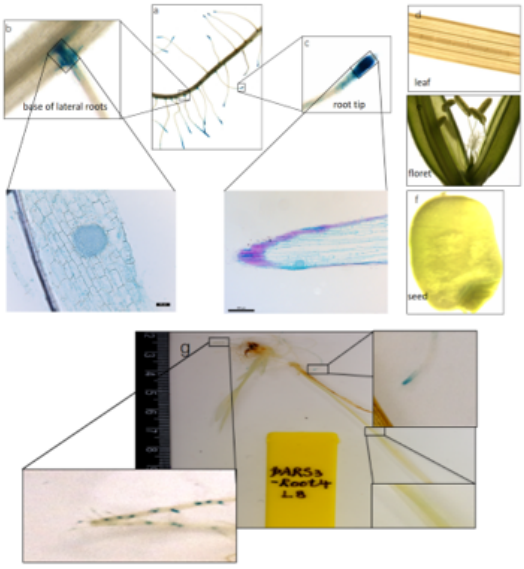


Figure 4

GUS staining for pARS3 Root4-GUSPlus transgenic rice lines.

a, b and c: GUS staining for mature roots of pARS3 Root4-GUSPlus transgenic rice line, GUS signals detected both in root tips and the base of lateral roots;

a, The mature plantroots;

b and c, were base of lateral root and the root tip, respectively, which are marked by black boxes in picture a, and were enlarged and exhibited under a higher magnification of microscope (16×).

d, e and f: no GUS signal was detected in leaves, florets and seeds of pARS3 Root4-GUSPlus transgenic rice lines.

g, GUS staining for pARS3 Root4-GUSPlus transgenic rice seedling.

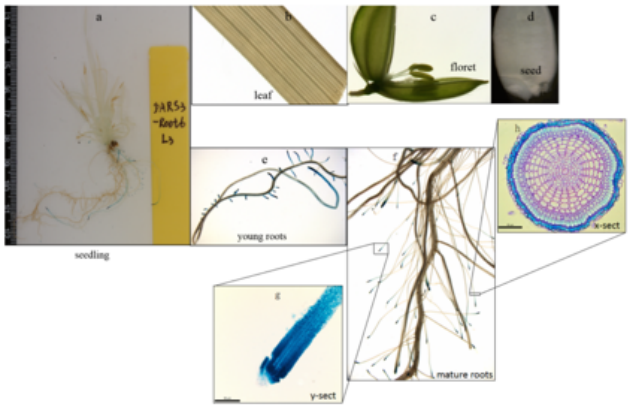


Figure 5

GUS staining for pARS3 Root6-GUSPlus transgenic rice lines.

a, GUS staining for seedling of pARS3 Root6-GUSPlus transgenic rice line.

b, c, d, e and f, GUS staining for leaf, floret, seed, young root and mature plant root, and the GUS signals only detected in root tips.

g, the longitude sections (y-Sect) of the division zone of the root tip stained by GUS.

h, the cross sections (x-Sect) between division zone and maturing zone of the root tip stained by GUS, much more GUS signal were observed in epidermis cell than in other cells.

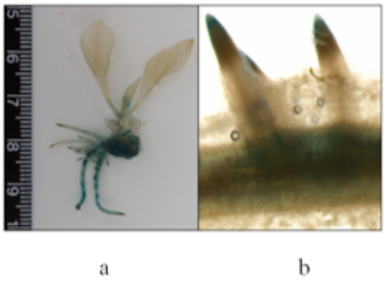


Figure 6

GUS staining of pCTAG2 Root4-GUSPlus transgenic tobacco lines.

a. GUS staining of seedling showed the GUS signal in root area only.

b. GUS signal was detected in root tips and the base of lateral roots under a high magnification of microscope (16×)

Supplementary Files

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- SupplementaryFigures.docx