Promoter of *Arabidopsis thaliana* Phosphate Transporter Gene Drives Root-Specific Expression of Transgene in Rice

TAKAYOSHI KOYAMA, 1 TOSHIRO ONO, 1 MASAMI SHIMIZU, 1 TETSURO JINBO, 1 RIE MIZUNO, 2 KEIJI TOMITA, 2 NORIHIRO MITSUKAWA, 3 TETSU KAWAZU, 2 TETSUYA KIMURA, 1* KUNIO OHMIYA, 1 and KAZUO SAKKA 1

Faculty of Bioresources, Mie University, 1515 Kamihama, Tsu 514-8507, Japan and Forestry Research Institute, Oji Paper Co. Ltd., 24-9 Nobono, Kameyama 519-0212, Japan

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The *PHT1* promoter::GUS fusion gene was constructed and introduced into *Arabidopsis* and rice by *Agrobacterium*-mediated transformation. Strong β-glucuronidase (GUS) activity was detected in roots and showed phosphate starvation induction both in *Arabidopsis* and rice. In contrast, GUS activity in aerial tissues such as those of the leaf and stem was low. *In situ* GUS staining of root tissue indicated that *PHT1* was expressed in root hairs and the outer layer of the main roots, but not in root tips. The *PHT1* promoter has a desirable character for biotechnological transgene expression in monocot rice plants.

[Key words: *Arabidopsis thaliana*, heterologous expression, *PHT1*, rice, root-specific promoter]

The generation of transgenic plants is now becoming increasingly routine for agriculturally important plants using the *Agrobacterium*-mediated method and the particle bombardment method. Plant improvement by genetic engineering depends on efficient transformation methods and on a transgene expression system in desired tissues at the desired level. For efficient gene expression in transgenic plants with a desired phenotype, the choice of promoter is a crucial factor that affects not only the transgene transcription level, but also the stage, tissue, and cell specificity of its expression. Over the years, the regulatory sequences of many promoters from various plant species have been studied; thereby, much information has been accumulated. The cauliflower mosaic virus (*CaMV*) 35S promoter and its derivatives are the most commonly used for transgene expression in dicot plants. Their expression is constitutive in almost all plant cells even though the expression level is not so high in several cell types (1). Nevertheless, the relative strength of the *CaMV* 35S promoter and its derivatives is lower in monocots than in dicots (2, 3). Several constitutive and ubiquitous promoters, the rice Rubisco small subunit (*rbcS*) (4), Actin (*Act1*) (2), Ubiquitin (*UBQ1* and *UBQ2*) (5), and cytochrome c (*OsCc1*) (6) genes are commonly used in monocot plants to express transgenes. Notwithstanding, their expression levels are insufficient in root tissue.

Plant roots contain very important tissues that take up soil nutrients, interact with soil microorganisms, and secrete anti-pathogenic compounds to defend the plant against numerous pathogens. Moreover, they protect the upper ground tissues against heavy metals, drying, and acidic conditions. Expression of genes for stress resistance and nutrition uptake in root cells, especially root epidermis cells, by genetic engineering will allow the host plant to grow in stressed and nutrient-limited conditions. The members of the phosphate transporter family, which take up phosphate at the root-soil interface, have been well characterized and the genes encoding phosphate transporters have been described. The *PHT1* gene (*ARAth; Pht1; 1*) that encodes the phosphate transporter of *Arabidopsis thaliana* is a well-characterized gene (7–12). The *PHT1* gene is expressed predominantly in root tissues in response to phosphate deprivation. Recently, promoter fusions to β-glucuronidase (GUS) and green fluorescent protein (GFP) reporter genes showed that *PHT1* is active in root epidermis cells under phosphate starvation conditions (11, 12). The expression pattern of the *PHT1* gene showed that it can be used as a promoter to drive the expression of transgenes in root epidermis cells and root hairs for stress resistance, nutrient uptake, and phytoremediation. This study evaluated the *PHT1* promoter as a root-specific expression promoter for plant recombinant DNA technology in an agriculturally important rice plant.

**MATERIALS AND METHODS**

**Plasmid construction** A 3891-bp fragment containing the putative promoter region of *A. thaliana* *PHT1* was amplified by PCR with primers PPH11F (5′-GGGCCGGCCCGAAGAGGG CTAATGTGTAAAAGGGC-3′) and PPH11R (5′-GGGGATCCGGCCATTTCTAGAAGCTCTTATTAATCATACAACG-3′) using Pyrobest DNA polymerase (Takara, Otsu). Restriction sites *NdeI* and *BamHI* were incorporated into the primers to facilitate cloning. The amplified fragment was cloned into pGUS-nos between the

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NotI and BamHI sites to generate the pP1PG vector. The pP1PG vector was digested with NotI/HindIII and a HindIII linker was ligated to the vector after blunting by Klenow DNA polymerase. The pP1PG vector was digested with HindIII and cloned into the HindIII site of a Ti binary vector pGAH (Fig. 1). The constructed plasmid pGAH-PHT1::GUS was transformed into A. tumefaciens EHA101 by electroporation.

Transformation of Arabidopsis and rice A. thaliana (ecotype Columbia) was transformed by a floral dip procedure (13) and transgenic seedlings were selected on half-strength MS medium (14) containing 15 μg/ml hygromycin. Ten independent primary transgenic lines (T1) were generated; then seeds (T2) from each line were used for analysis of reporter gene expression. Rice plants (Oryza sativa L. subsp. japonica) were transformed by Agrobacterium-mediated transformation as described by Hiei et al. (15). More than 10 independent primary transgenic lines (T0) were generated and seeds (T1) from each line were used for analysis of reporter gene expression.

Histochemical GUS staining Histochemical GUS staining was performed using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as a substrate as described (16). For the transgenic A. thaliana, 7-day-old T2 seedlings grown in sterilized water at 22°C with continuous light were examined. Green tissues were detached with ethanol prior to observation. For sectioning, the GUS stained root segments were fixed and embedded in Quetol 812 (Nissin EM, Tokyo) according to the protocol described by Luff (17), with minor modifications. Embedded Arabidopsis tissue was cut into 5-μm-thick sections using a rotary microtome.

Quantitative GUS assay Quantitative GUS assay was done using 4-methylumbelliferyl-β-glucuronide as substrate as described by Jefferson et al. (16). For the transgenic A. thaliana, the T3 homozygous plants were cultivated vertically for 14 d on half-strength MGRL medium (18) containing 200 μM Pi, half-strength MS vitamins, 2.5 mM MES and 1.6% agar, and then transferred to half-strength MGRL medium containing various concentrations of Pi, half-strength MS vitamins, 2.5 mM MES and 1.6% agar. After cultivation for an additional 7 d, roots and shoots were harvested separately and the GUS activities in the cell-free extracts were analyzed.

For the transgenic rice, the nutrient solution used in this examination was the same as that described for histochemical staining. After 3 weeks of growth, the clonal plant sets were transferred to fresh nutrient solution with or without Pi. Plants were grown for another week and the GUS activities in the cell-free extracts were analyzed.

RESULTS

Histochemical analysis of GUS expression driven by the PHT1 promoter in the transgenic Arabidopsis and rice A 3891-bp upstream region of the PHT1 gene was linked to the E. coli GUS gene and introduced into Arabidopsis to analyze its promoter activity (Fig. 1). At least 10 independent lines, except for those shown in Fig. 2E–H, were used for histochemical analysis, and one transgenic line with representative results is shown in Fig. 2. Seven-day-old hydroponically cultured PHT1::GUS Arabidopsis plants were stained overnight in X-gluc (Fig. 2A). No expression was observed in the root meristematic zone, but 9 of 10 T2 transgenic lines showed strong GUS staining in primary and secondary roots (Fig. 2C, D). Weak GUS staining was detected in the hypocotyl and cotyledon (Fig. 2A, B). In the cotyledon, a GUS expression pattern was found in both primary and secondary veins (Fig. 2B). One T3 homozygous line was generated from the T2 transgenic line. Further analysis of this line revealed a 3:1 segregation for hygromycin resistance. The T3 transgenic plant, cultured on one strength MS plate for 26 d, was stained overnight in X-gluc (Fig. 2E–H). Strong GUS staining was detected mainly in the roots and weak GUS staining was detected in the cotyledon (Fig. 2E, G). The expression pattern of the T3 line was identical to that of T2 transgenic plants cultivated for 7 d. In contrast, in rosette and cauline leaves, GUS expression was detected in the hydathodes (arrowhead in Fig. 2F). In the root, the strongest GUS expression, relative to other cell types, was observed in trichoblasts (asterisk in Fig. 2H), which generate root hairs.

The PHT1::GUS fusion gene was introduced into rice to investigate the activity of the Arabidopsis PHT1 promoter in rice and histochemical GUS assay was conducted using at least 10 independent T0 and T1 lines, each in different stages of growth or different growth conditions. In all cases, intense GUS expression was observed in the root, except for the root tip, of transgenic rice plants. Further histochemical analysis using representative T1 lines showed that the PHT1 promoter directed expression of the GUS gene mainly in root tissues (Fig. 3A–C), while weak expression levels were observed in aerial tissues (Fig. 3A, B). Strong reporter gene

![Diagram](https://example.com/diagram.png)
expression was observed in epidermal cells of roots and root hairs throughout the root hair zone; no expression was observed in root tips (Fig. 3C–E). The expression pattern of GUS in rice plants was identical to that observed in Arabidopsis. In both PHT1::GUS Arabidopsis and rice plants grown in Pi-deficient medium, the expression pattern was the same as that of seedlings (E). GUS activity was detected in the hydathodes of leaves (indicated by arrowhead) (F). After germination of the leaf, the GUS expression pattern of the cotyledon (indicated by arrow) (E) was identical to that of the seedling (G). A transverse section of root showing that strong GUS signalling was found in trichoblasts (indicated by asterisk).

Quantitative analysis of GUS in the transgenic Arabidopsis To analyze GUS expression of the PHT1::GUS transgenic lines in response to phosphate concentration, a quantitative GUS assay was performed for the progeny of a homozygous T3 line of A. thaliana grown on a medium containing various levels of inorganic phosphate (Pi) (Fig. 4). GUS activity in root tissue was increased in response to a low Pi concentration, indicating that the PHT1 promoter up-regulated GUS expression in roots when cultivated under low Pi conditions. The GUS activity of the transgenic roots of plants grown on 0 μM Pi medium was found to be about four times higher than those of plants grown on 200 μM and 1000 μM Pi. On the contrary, the GUS activity of shoots did not respond to low Pi concentration.

The GUS gene driven by the PHT1 promoter was highly expressed in rice root A quantitative GUS assay was conducted using several rice transgenic T1 lines, indicating the inducibility of the promoter when plants were grown in liquid medium containing a low level of Pi (Fig. 5). GUS expression in the low Pi medium (386 ± 246 nmol MU/mg protein/min [mean ± SD], n = 31) was approximately seven times higher than that under the high Pi condition (57.7 ± 19.6 nmol MU/mg protein/min, n = 31).

We examined the promoter activity of PHT1 in rice roots of plants cultivated in commercially available soil since expression of transgenes in roots in soil is important for prac-
E7131::GUS activity between transgenic rice plants harboring the GUS gene expression cassette driven by the PHT1 promoter grown hydroponically at 28°C with a 16 h light period. Each bar represents the mean±SD (n=31).

In numerous studies of gene expression, analysis has been performed using microarrays, Northern hybridization, and reporter genes in Arabidopsis as a model plant. Producing a wide choice of promoters for genetic manipulation is important for the expression of transgenes in target cells of host plants. However, most dicot promoters are known to be inactive in monocot plants such as agronomically important rice. To date, only a few promoters are available for rice transformation. Although the modified CaMV35S promoter, rice Act1, and rice OsCc1 promoters are active in most cell types of rice with preference for rapidly dividing cells, their expression in root tissues, especially in root epidermal cells is insufficient. Previously, we reported that Arabidopsis PHT1 was expressed predominantly in root tissue. Therefore, we selected the PHT1 promoter as a candidate root-specific promoter to drive the expression of heterologous genes.

Our results demonstrated that the Arabidopsis PHT1 promoter is highly active in transgenic root epidermal cells that are at the root/soil interface. The PHT1 promoter activity introduced into Arabidopsis was at least one order of magnitude higher than that reported elsewhere without altering the expression pattern (11, 12). This difference may result from the difference in promoter length used in promoter activity analysis. In our study, 3891-bp of PHT1 the promoter (~3888 to +3 relative to the translational start site) were fused to the GUS gene. In contrast, they used shorter regions of about 2 kb in length (11, 12). The 5’ upstream region used in our study may contain an enhancer-like sequence for the PHT1 promoter.

Specific GUS activity in cell extracts of PHT1::GUS transgenic rice roots was almost identical to that in Arabidopsis, suggesting that the promoter of PHT1 is active in both dicot Arabidopsis and monocot rice. The inducibility of the PHT1 promoter at a low level of Pi in the transgenic rice suggests that cis elements for Pi starvation can function in both Arabidopsis and rice cells. In addition, specific GUS activity in cell extracts of the transgenic rice roots was comparable to that in Arabidopsis roots. It is interesting that a dicot promoter can function efficiently in a monocot plant. Although promoter activity is repressed in the presence of high Pi in hydroponic cultivation, the amount of heterologous protein produced by the PHT1 promoter is higher than that by the enhanced CaMV35S promoter E7131 when plants are grown in normally fertilized soil. The translational efficiency of GUS mRNA may result in higher GUS production since the PHT1 promoter region linked to the translational start site of the PHT1 ORF was fused to the GUS ORF. Our results showed that the PHT1 promoter is a practical promoter for root-specific expression of heterologous genes both in dicots and monocots.

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