

RESEARCH PAPER

Analysis of five novel putative constitutive gene promoters in transgenic rice plants

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Abstract

Novel constitutive gene promoters are essential components of crop biotechnology. Our analysis of five such promoters, *APX*, *SCP1*, *PGD1*, *R1G1B*, and *EIF5*, in transgenic rice plants is reported here. The five promoter regions were linked to the *gfp* reporter gene and transformed into rice. Using fluorescent microscopy and q-RT-PCR, promoter activities were analysed in comparison with *OsCcl1*, *Act1*, and *ZmUbi1*, previously characterized as strong constitutive promoters. The *APX* and *PGD1* promoters direct high levels of gene expression in all tissues and stages, producing GFP at levels of up to 1.3% of the total soluble protein. *PGD1* is particularly active in flowers and mature roots. The *R1G1B* is active in the whole grain including the embryo, endosperm, and aleurone layer, and thus represents a constitutive promoter with activity in whole seeds that has not been described previously. The *ZmUbi1* and *R1G1B* promoters are markedly less active in young roots and mature leaves whilst the *APX*, *PGD1*, *OsCcl1*, and *Act1* promoters are highly active in both vegetative and reproductive tissues. Overall, our results demonstrate that *APX*, *PGD1*, and *R1G1B* are novel gene promoters that are highly active at all stages of plant growth with distinct levels of activity.

Key words: *APX*, constitutive gene promoter, GFP, *PGD1*, *R1G1B*, transgenic rice, vegetative and reproductive tissues.

Introduction

Gene promoters that direct high levels of constitutive gene expression are important for crop biotechnology applications. In recent years, several well-characterized gene promoters have become available for transgene expression in plants. The cauliflower mosaic virus (CaMV) 35S gene promoter is the most commonly used to drive transgene expression in a constitutive manner (Odell *et al.*, 1985). However, although the 35S promoter and its derivatives can drive high levels of transgene expression in dicotyledonous plants (Battraw and Hall, 1990; Benfey *et al.*, 1990), their activities are substantially lower in monocotyledonous plants (Christensen *et al.*, 1992; Gupta *et al.*, 2001; Weeks *et al.*, 1993). Conversely, the activity of monocot-derived promoters is higher in monocots than in dicots (Cornejo *et al.*, 1993), necessitating the development of both monocot and dicot promoters.

Several gene promoters have been evaluated for their ability to drive the constitutively high expression of transgenes in monocots including *ZmUbi1* (Christensen *et al.*, 1992; Cornejo *et al.*, 1993) from maize (*Zea mays*), and *Act1* (McElroy *et al.*, 1990, 1991), *OsTubA1* (Jeon *et al.*, 2000), *OsCcl1* (Jang *et al.*, 2002), *RUBQ1* and 2 (Wang and Oard, 2003), *rubi3* (Lu *et al.*, 2008), and *OsAct2* (He *et al.*, 2009) from rice (*Oryza sativa*). These promoters are highly active in monocot crops including rice but are also distinct in various ways. The *ZmUbi1* promoter is widely used in monocot crops due to its ability to direct high levels of gene expression in virtually all tissues. This promoter is active in many, although not all, cell types and drives strong expression in young roots and leaves, but these expression levels decrease markedly as these organs mature (Cornejo *et al.*, 1993). The *OsCcl1* and *Act1* promoters are active in

both the vegetative and reproductive tissues of transgenic rice plants (McElroy *et al.*, 1990; Jang *et al.*, 2002). The activity profile of the *OsAct2* promoter is similar to that of *Act1* in leaves and roots of transgenic rice plants, but is slightly stronger (He *et al.*, 2009). The rice ubiquitin gene promoters, *RUBQ1* and *RUBQ2*, have been shown to drive higher GUS expression in transgenic rice plants by 8–35-fold, respectively, when compared with the 35S promoter (Wang and Oard, 2003). The rice polyubiquitin gene promoter, *rubi3*, has been tested in all tissues and at all growth stages of transgenic rice plants and found to drive a higher level of constitutive expression of reporter genes than the maize *ZmUbi1* promoter (Lu *et al.*, 2008). Further, polyubiquitin gene promoters have been isolated and characterized from soybean (Chiera *et al.*, 2007; Hernandez-Garcia *et al.*, 2009), potato (Garbarino *et al.*, 1995; Rockhold *et al.*, 2008), and sugarcane (Wei *et al.*, 2003). Despite such efforts to characterize constitutive gene promoters functionally, few of these have been analysed over the whole spectrum of transgenic plant growth. There is currently therefore a shortage of efficient promoters for high-level constitutive gene expression, particularly in monocot crops. In addition, stacking of several transgenes in a single transgenic plant requires a battery of different promoters, which may otherwise undergo homology-dependent gene silencing, a common occurrence in transgenic plants with multiple copies of the same promoter (Lessard *et al.*, 2002; Potenza *et al.*, 2004).

To characterize novel gene promoters that confer high levels of gene expression in rice, five genes, *APX*, *SCP1*, *PGD1*, *RIG1B*, and *EIF5*, were identified by microarray that are expressed at high levels throughout the whole plant. The activity of the corresponding constitutive gene promoters was then quantitatively analysed in transgenic rice plants using the *gfp* reporter. These promoter activities were analysed in different tissues and at various growth stages in comparison with those of the previously characterized strong constitutive promoters *OsCcl1*, *Act1*, and *ZmUbi1*.

Materials and methods

Plant materials

The rice (*Oryza sativa* L. var. Japonica) plants cv. Nipponbare and cv. Nakdong were used to isolate gene promoters and perform plant transformations, respectively. Rice genomic DNA was prepared from the leaves of 14-d-old Nipponbare plants using the DNazol plant genomic DNA isolation reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's instructions for the isolation of promoter regions by genomic PCR. The T₃ generation of three to six independent lines for each of the promoters was used for our subsequent detailed analysis.

Expression of endogenous genes in rice

Rice 3'-Tiling Microarray analysis was performed as reported previously (Kim *et al.*, 2009). RNA samples from drought-treated and untreated leaf tissues of 14-d-old rice (*O. sativa* L. cv. Nakdong) plants were used to generate Cy3-labelled complementary DNA probes, which were then hybridized to the microarray.

Each dataset was obtained from two biological repeats. Comparisons of the datasets from various tissues identified five genes for promoter analysis out of more than 20 000 on the microarray (see Supplementary Table S1 available at *JXB* online). For RNA extraction (Oh *et al.*, 2008), rice seeds were germinated in half-strength Murashige-Skoog (MS) (Murahige and Skoog, 1962) solid medium in a growth chamber in the dark at 28 °C for 3 d, followed by light exposure at 28 °C for 1 d, transplanted into soil, and grown in a greenhouse (16/8 h light/dark cycle) at 28–30 °C. Each plant was grown in a pot filled with rice nursery soil (Bio-media, Co., Kyongju, Korea) for the indicated number of days after germination (DAG) and in the flowering stage when the panicles were at <10 cm in size. Total RNA was extracted from dry seeds, leaves, roots, and flowers using the Qiagen RNeasy plant mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. For amplification of the corresponding genes, cDNA was synthesized with random primers using a First-strand cDNA synthesis kit (Fermentas, Burlington, ON) and subsequent RT-PCR was carried out with 40 ng of cDNA template and gene-specific primer pairs designed with Primer Designer 4 software v4.20 (Sci-Ed Software, Durham, NC). These primer pairs are listed in Supplementary Table S2 at *JXB* online. RT-PCR was performed at 95 °C for 10 min, followed by 28 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, with a final cycle at 4 °C for 10 min. The *OsUbi1* (AK121590) gene was used to verify RNA loading. The amplified PCR products were sequenced to ensure fidelity.

Plasmid construction and transformation of rice

The various promoter regions were amplified and isolated by PCR using promoter-specific primer pairs, *APX* (AK068430), *SCP1* (AK101133), *PGD1* (AK065920), *RIG1B* (AF503583), *EIF5* (AK060387), *OsCcl1* (AK060267), *Act1* (AK100267), and *ZmUbi1* (DQ141598), and the 2× *EF-Taq* DNA polymerase pre-mix (SolGent, Seoul). Genomic PCR was performed at 95 °C for 10 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, with a final cycle at 4 °C for 10 min using 50 ng of gDNA. The amplified DNAs were re-amplified using the adaptor primer pairs. All primer pairs are listed in Supplementary Table S2 at *JXB* online. The isolated promoters with adaptor sequences were individually inserted into the rice transformation vector using site-specific recombination. PCR reactions and *in vitro* recombination reactions were carried out according to the manufacturer's instructions (Gateway® Cloning Technology, Invitrogen, Carlsbad, CA). The rice transformation vector contained the *bar* gene under the control of the CaMV 35S promoter for use with herbicide-based plant selection. Vectors were introduced into *Agrobacterium tumefaciens* LBA4404 by tri-parental mating. Embryonic calli from the mature seeds of rice (*O. sativa* L. cv. Nakdong) were transformed by co-cultivation (Hiei *et al.*, 1994), selected with 7 mg l⁻¹ phosphinothricin, and used to regenerate transgenic plants as previously described by Jang *et al.* (2002).

Detection of GFP fluorescence

Rice plant leaves, roots, dry seeds, and flowers were observed using a research stereomicroscope (SZX9-3122, Olympus, Tokyo) equipped with an attachment for fluorescence observations. Images were captured using a C5060-ZOOM digital camera (Olympus, Tokyo). Observations under blue light were carried out using a specific filter set (460–480 nm excitation filters, dichroic mirrors of 485 nm and a 495–540 nm barrier filter). The detection of GFP fluorescence was carried out in transgenic plants and also non-transgenic controls.

Real-time PCR analysis

Total RNA was extracted with leaves, roots, dry seeds, and flowers of transgenic and nontransgenic (NT) rice plants using the TRI REAGENT® (Molecular Research Center, Inc., Cincinnati, OH).

Total RNAs from dry seeds and flowers were cleaned up by the Qiagen RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The First-strand cDNA synthesis kit with random primers for RT-PCR (Fermentas, Burlington, ON) was used for cDNA synthesis. Real-time PCR analysis was carried out using 2× real-time PCR Pre-mix with EvaGreen (SolGent, Seoul). The reaction was performed at 95 °C for 15 min followed by 40 cycles of 95 °C for 20 s, 58 °C for 40 s, 72 °C for 20 s in a 20 µl volume mix containing 1 µl of 20× EvaGreen™, 0.25 µM primers and 40 ng cDNA. Thermocycling and fluorescence detection were performed using a Stratagene Mx3000p Real-Time PCR machine and Mx3000P software v2.02 (Stratagene, La Jolla, CA). Melting curve analysis (55–95 °C at a heating rate of 0.1 °C s⁻¹) was performed to ensure that only the required PCR product at a specific melting temperature was measured (Ririe *et al.*, 1997). Real-time PCR reactions were performed in triplicate for each cDNA sample. Following amplification, the experiment was converted to a comparative quantification (calibrator) experiment type and analysed with the Mx3000P software v2.02 (Stratagene, La Jolla, CA). The *OsUbi1* (AK121590) gene was used as a reference in the real-time PCR reactions. All primer pairs are listed in Supplementary Table S2 at *JXB* online.

Immunoblotting analysis

Total soluble proteins were extracted from 30-d-old leaves, roots, and flowers. The extraction buffer consisted of 20 mM TRIS-Cl, pH 8.0, 10 mM EDTA pH 8.0, 30 mM NaCl, and 100 µM phenylmethylsulphonylfluoride (PMSF). The extracts were centrifuged at 9000 g at 4 °C for 30 min and protein concentrations were determined using the Bradford method (Bio-Rad, Hercules, CA). Protein extracts were then separated on 12% SDS polyacrylamide gels and blotted onto a polyvinylidenedifluoride (PVDF) membrane (Immobilon-P, Millipore Co., Billerica, MA) using a semi-dry transfer apparatus (Hofer, Inc., San Francisco, CA). The immunoreactive proteins were detected using primary antibodies against GFP (Nacalai Tesque, Inc., Kyoto). The chemiluminescence signals generated by the bound antibodies were detected using the Pierce Super Signal Substrate (Pierce, Rockford, IL) according to the manufacturer's protocol. Recombinant GFP proteins were used as a positive control (Abcam, Cambridge, UK).

Results

Identification of novel constitutively expressed genes in rice

To screen for genes that are constitutively expressed in rice, expression profiling was performed using RNAs from leaves, roots, and flowers and the Rice 3'-Tiling Microarray (Oh *et al.*, 2009). These analyses identified the genes *APX*, *SCPI*, *PGDI*, *RIG1B*, and *EIF5* as being expressed in all tissues at high levels (see Supplementary Table S1 at *JXB* online). Expression of these five genes was further analysed by RT-PCR using total RNAs from eight different tissues and/or stages of rice plant growth. These included dry seeds, leaves, and roots at the indicated days after germination (DAG), and also the flowers (Fig. 1A). The transcript levels of *APX* and *SCPI* were high in all tissues throughout the developmental stages except dry seeds and 60 DAG roots. The transcript levels of *PGDI*, *RIG1B*, and *EIF5* were comparable to those of the positive control genes, *OsCcl* (Jang *et al.*, 2002) and *Act1* (McElroy *et al.*, 1990) in all tissues and stages of plant growth in rice. It was found that expression levels of *RIG1B* in flower was the lowest in

microarray data (see Supplementary Table S1 at *JXB* online) but that the *gfp* mRNA levels (Fig. 1A) were comparable to other *promoter:gfp* plants. The discrepancy could be due to the difference in stages of flowers used for microarray and RT-PCR.

Approximately 2.0 kb of the region immediately upstream of a translation initiation codon (ATG) was predicted to be the promoter region in each case (Fig. 1B). The predicted promoter regions of the five constitutive genes were amplified by PCR using rice (*Oryza sativa* cv. Nipponbare) genomic DNA. The resulting PCR products were then inserted into a rice transformation vector via site-specific recombination (Fig. 1C), generating constructs with each promoter sequence linked to the green fluorescent protein gene *gfp*. Several independent transgenic lines were obtained for each gene promoter using the *Agrobacterium*-mediated transformation method (Hiei *et al.*, 1994) and were grown to maturity in a greenhouse. Through the self-pollination and selection of germinating transgenic seeds on phosphinothricin-containing MS media, T₃ homozygous lines were obtained. Six independent lines for each construct were chosen for further analysis.

Activity of the promoter:gfp constructs in various tissues of transgenic rice plants

To investigate promoter activity in the whole rice plant, GFP fluorescence in the vegetative and reproductive tissues of the *promoter:gfp* transgenic plants was visually detected with a fluorescence microscope. Transgenic plants harbouring the *APX*, *SCPI*, *PGDI*, *RIG1B*, and *EIF5* promoter constructs were analysed in comparison with those containing the *OsCcl* (Jang *et al.*, 2002), *Act1* (McElroy *et al.*, 1990), and *ZmUbi1* (Cornejo *et al.*, 1993), three known constitutive promoters. Images of non-transgenic (NT) tissues were used as negative controls. For the observation of GFP fluorescence, one transgenic line for each construct was chosen whose expression pattern was closest to that of the endogenous gene. To detect GFP fluorescence separately in embryos, endosperms, and aleurones of dry seeds, hand-cut vertical and horizontal sections of dry seeds were used for analysis by fluorescence microscopy (Fig. 2A). The *APX*, *SCPI*, and *EIF5* promoters were found to be active only in embryos, as were the *OsCcl* and *Act1* promoters. GFP fluorescence level of the *PGDI:gfp* seeds was similar to that of the *ZmUbi1:gfp* seeds, being positive in the aleurones as well as the embryos. Interestingly, the *RIG1B* promoter directed high levels of GFP fluorescence in the whole grain including the embryo, endosperm, and aleurone layer. GFP fluorescence was examined further in the leaves and roots of 30 DAG plants by microscopy (Fig. 2B). A tip of 7th leaf blade and a crown root around the node (denoted the 7th L and CR in Fig. 1A, respectively) were used for the detection of GFP fluorescence. In vegetative tissues, the *APX*, *PGDI*, and *RIG1B* promoters produced high levels of GFP fluorescence in a similar manner to the constitutive control promoters. The levels of GFP fluorescence in the leaves, roots and flowers (Fig. 2B, C) driven by

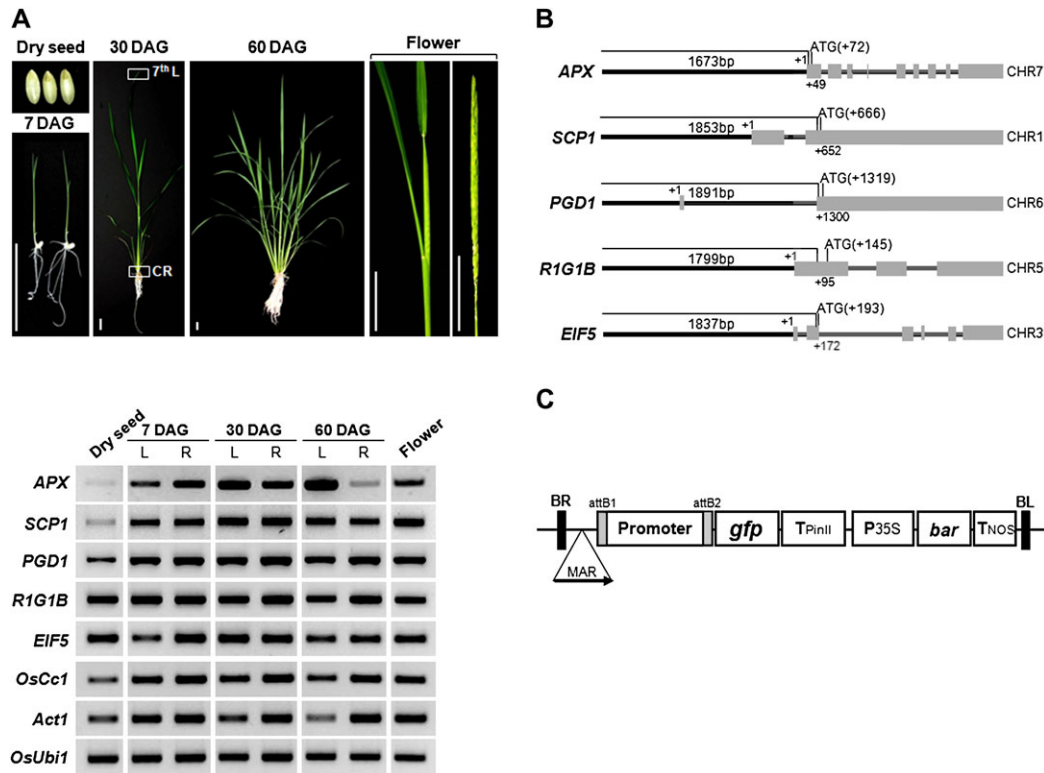


Fig. 1. Expression and genomic structures of five constitutively expressed rice genes. (A) The expression of five constitutive rice genes in eight different tissues and/or three stages of plant growth was analysed by RT-PCR. These include dry seeds, leaves (L), and roots (R) at the indicated days after germination (DAG), and also the panicles (flower; <10 cm in size). Bars, 3 cm. The genes *OsCc1* (Jang *et al.*, 2002), *Act1* (McElroy *et al.*, 1990), and *OsUbi1* (AK121590) were used as constitutive controls. 7th L, 7th leaf blade; CR, crown root. (B) Genomic structures of the five newly identified constitutive genes. Bold lines, 5' upstream region; filled boxes, exons; lines between boxes, introns. All boxes were drawn to scale and chromosome numbers (CHR) are shown. The transcriptional start site and the translational start codon are indicated by +1 and ATG, respectively. (C) Schematic representation the T-DNA of the rice transformation vector used in this study. Rice promoters were linked to the *gfp* reporter gene by site-specific recombination. Expression of the *gfp* reporter gene is then regulated by the promoter and the potato *Pin II* 3' region (T_{pinII}). The bacterial phosphinothricin acetyl transferase gene (*bar*), a selectable marker for rice transformation, was fused between the CaMV 35S promoter (P_{35S}) and the nopaline synthase polyadenylation region (T_{nos}). MAR, the 5'-matrix attachment region from the chicken lysozyme gene (Oh *et al.*, 2005), was inserted to produce stable expression of the transgene.

the *SCP1:gfp* and *EIF5:gfp* plants were markedly lower. GFP fluorescence was also examined in flowers (spikelets) at the meiosis stage (Fig. 2C). As observed in the vegetative tissues, the transgenic flowers all showed a high level of GFP fluorescence except for those harbouring *SCP1:gfp* and *EIF5:gfp*. More specifically, the *APX*, *PGD1*, and *R1G1B* promoters directed high levels of *gfp* expression in all floral organs including the lemma, palea, lodicule, anther, and filament, which is a similar pattern to that in the *OsCc1:gfp* and *ZmUbi1:gfp* flowers.

Quantitative analysis of promoter activity

To compare promoter activity levels, the *gfp* mRNA levels of six independent T₃ *promoter:gfp* transgenic lines was measured by qPCR (Fig. 3). Total RNA was extracted from dry seeds, leaves, and roots at the indicated days after germination (DAG), and also from the flowers. The levels of promoter-driven *gfp* transcripts were calculated relative to the *gfp* mRNA concentration in the *ZmUbi1:gfp* transgenic lines (Table 1). Overall, promoter-driven *gfp* mRNA

was found to be abundant at various levels in all of the vegetative tissues and flowers in most *promoter:gfp* transgenic lines. In particular, the *gfp* mRNA levels driven by the *APX*, *SCP1*, *PGD1*, and *R1G1B* promoters were found to be comparable to those produced by the *OsCc1*, *Act1*, and *ZmUbi1* constitutive control promoters. By contrast, the activity of the *EIF5* promoter was markedly lower in all vegetative tissues and flowers, although was still widely constitutive. Interestingly, the *PGD1* and *R1G1B* promoters were far more active than the others in 60 DAG roots and dry seeds, respectively. Conversely, the *R1G1B* and *ZmUbi1* promoters were found to have significantly lower activity than all others in 7 DAG roots and 30 DAG leaves.

Promoter activities are known to vary greatly among transgenic lines. To assess this variation, the *gfp* mRNA levels in the leaves, roots, and flowers of 9–15 transgenic lines were compared for each promoter. The transcript levels were measured by q-RT-PCR and plotted relative to those in the *EIF5* transgenic plants. As shown in Fig. 4, the promoter activity levels ranged from 6.0–13.7-fold in leaves

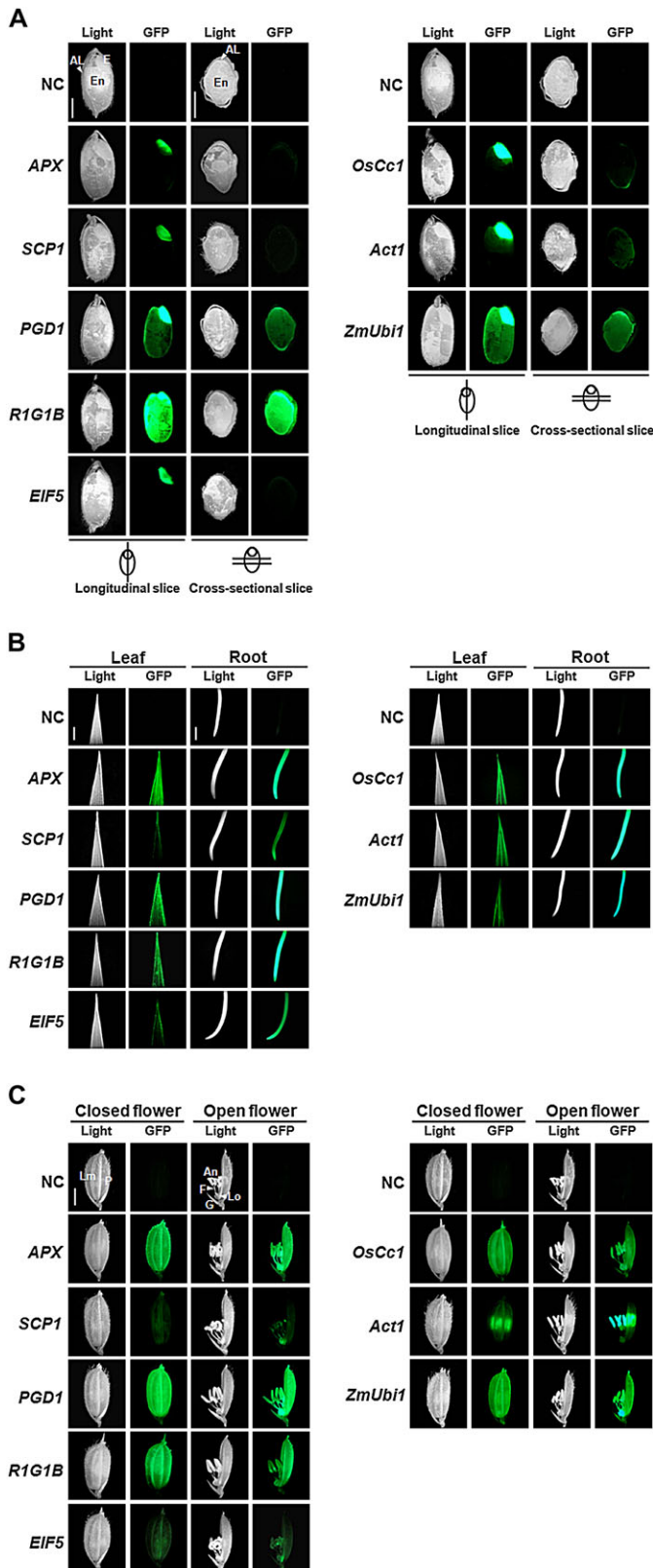


Fig. 2. Promoter activity in various tissues of the *promoter:gfp* transgenic plants. Transgenic plants harbouring the constitutive gene promoters, *APX*, *SCP1*, *PGD1*, *R1G1B*, and *EIF5*, were analysed in comparison with the transgenic plants containing the known constitutive gene promoters, *OsCc1* (Jang *et al.*, 2002), *Act1* (McElroy *et al.*, 1990), and *ZmUbi1* (Cornejo *et al.*, 1993).

and roots and from 2.2–18.2-fold in flowers in different *promoter:gfp* plants.

It was observed that the levels of GFP fluorescence do not always reflect the mRNA levels in *promoter:gfp* plants. It was found that the GFP fluorescence in *SCP1:gfp* plants was by far the lowest but that the *gfp* mRNA levels were comparable to other *promoter:gfp* plants. Therefore, the GFP protein levels among the *promoter:gfp* plants were determined by immunoblotting. As shown in Fig. 5, these levels were strikingly low in *SCP1:gfp* plants (0.02–0.06% of total soluble proteins) given the high levels of *gfp* mRNA. This observation prompted us to investigate the 5' UTR sequence of *SCP1*. As shown in Supplementary Fig. S1 at *JXB* online, *SCP1* contains a 489 bp 5'-UTR that contains three upstream ATGs (uATG) and ORFs (uORFs), which may have a negative impact upon the translation efficiency of the *SCP1:gfp* mRNA (Fig. 5). The GFP protein levels of *APX:gfp* and *PGD1:gfp* plants were high and comparable to those of *OsCc1:gfp*, *Act1:gfp*, and *ZmUbi1:gfp* plants (0.6–1.3% of the total soluble proteins; Fig. 5). The GFP protein levels of *R1G1B:gfp* and *EIF5:gfp* plants ranged from 0.11% to 0.34% and from 0.01% to 0.17% of the total soluble proteins, respectively, depending on the specific transgenic lines and/or tissues. Overall, our current results demonstrate that at least two out of our five gene promoters are constitutively active in a whole plant body. The other three have distinct levels of activity in different tissues and/or at different stages of plant growth.

Discussion

In our current study, the activity of five different gene promoters throughout the entire growth cycle of transgenic rice plants was quantitatively analysed. All of these promoters were found to be active in various tissues and/or stages of plant growth with different patterns of relative activity. The *APX* and *PGD1* promoters direct significantly higher levels of gene expression than any of the others tested in all the tissues and at all growth stages. In addition, *PGD1* was found to have outstanding levels of activity in flowers and roots at 60 DAG. Overall, the levels of *APX*, *PGD1* promoter activity were comparable to those of *OsCc1*, *Act1* and *ZmUbi1*, which are previously characterized strong constitutive promoters. Hence, the *APX* and *PGD1* promoters may provide useful alternatives that will facilitate strong constitutive expression of a transgene in the whole rice plant. *R1G1B* is another constitutive promoter found to have high levels of activity in all the tissues and

Light and GFP indicate images captured using light and fluorescence microscopy, respectively. Bars, 2 cm. (A) GFP fluorescence in hand-cut vertical and horizontal sections of dry seeds. AL, aleurone layer; E, embryo; En, endosperm. (B) GFP fluorescence in the leaves and roots of 30 DAG plants. (C) GFP fluorescence in flowers. An, anther; G, glume; Lm, lemma; Lo, lodicule; F, filament; P, palea.

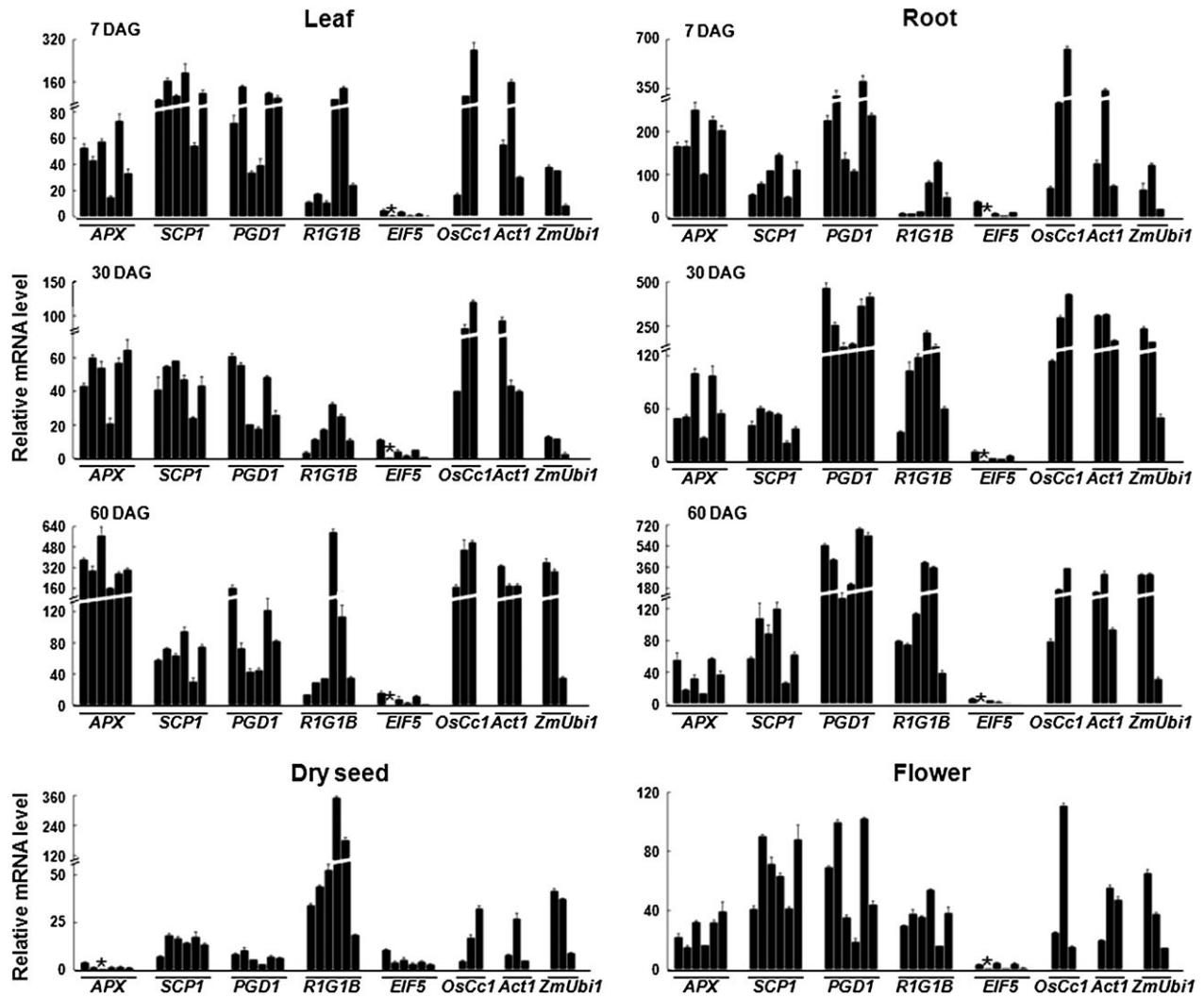


Fig. 3. Quantitative comparison of promoter activity in six independent lines of each of the *promoter:gfp* transgenic plants. Total RNA was extracted from the leaves and roots at the indicated days after germination (DAG), from dry seeds, and from the flowers of five independent T₃ lines of transgenic plants harbouring the *APX*, *SCP1*, *PGD1*, *R1G1B*, and *EIF5* gene promoters. Levels of *gfp* mRNAs were measured using real-time PCR, normalized with respect to that of the internal control gene *OsUbi1* (AK121590), and plotted relative to the level of *gfp* mRNAs in the lowest-expressing transgenic plants (indicated by the asterisk). Three independent lines from transgenic plants harbouring the *OsCc1* (Jang et al., 2002), *Act1* (McElroy et al., 1990), and *ZmUbi1* (Cornejo et al., 1993) gene promoters, were included as constitutive controls. Data bars represent the mean \pm SD of triplicate measurements. Note that the y-axis is discontinuous.

Table 1. Relative promoter activities in various tissues and stages of growth in rice plants

Levels of promoter-driven *gfp* mRNA in different transgenic lines were calculated relative to those of *gfp* mRNAs in *ZmUbi1:gfp* transgenic lines. Data are the means \pm SD of the *gfp* mRNA levels in six independent transgenic lines of *APX*, *SCP1*, *PGD1*, *R1G1B*, and *EIF5* or three independent transgenic lines of *OsCc1*, *Act1*, and *ZmUbi1*.

| Promoter | Dry seed | 7 DAG | | 30 DAG | | 60 DAG | | Flower |
|---------------|---------------|---------------|---------------|---------------|----------------|----------------|----------------|---------------|
| | | Leaf | Root | Leaf | Root | Leaf | Root | |
| <i>APX</i> | 0.1 \pm 0.0 | 1.7 \pm 0.7 | 2.6 \pm 0.7 | 5.3 \pm 1.7 | 0.4 \pm 0.2 | 1.4 \pm 0.6 | 0.2 \pm 0.1 | 0.7 \pm 0.2 |
| <i>SCP1</i> | 0.5 \pm 0.1 | 4.5 \pm 1.9 | 1.3 \pm 0.5 | 4.7 \pm 1.3 | 0.3 \pm 0.1 | 0.3 \pm 0.1 | 0.4 \pm 0.2 | 1.7 \pm 0.6 |
| <i>PGD1</i> | 0.2 \pm 0.1 | 3.1 \pm 1.6 | 3.3 \pm 1.5 | 4.0 \pm 2.0 | 2.0 \pm 0.9 | 0.4 \pm 0.2 | 2.1 \pm 1.1 | 1.6 \pm 0.9 |
| <i>R1G1B</i> | 3.9 \pm 4.5 | 1.8 \pm 2.0 | 0.7 \pm 0.7 | 1.8 \pm 1.1 | 0.7 \pm 0.4 | 0.6 \pm 1.0 | 0.8 \pm 0.8 | 0.9 \pm 0.3 |
| <i>EIF5</i> | 0.2 \pm 0.1 | 0.1 \pm 0.1 | 0.2 \pm 0.2 | 0.4 \pm 0.4 | 0.03 \pm 0.0 | 0.03 \pm 0.0 | 0.01 \pm 0.0 | 0.1 \pm 0.0 |
| <i>OsCc1</i> | 0.6 \pm 0.5 | 5.1 \pm 5.0 | 4.6 \pm 4.0 | 8.3 \pm 4.1 | 1.9 \pm 1.0 | 1.7 \pm 0.8 | 1.0 \pm 0.7 | 1.3 \pm 1.3 |
| <i>Act1</i> | 0.5 \pm 4 | 3.1 \pm 2.6 | 2.5 \pm 1.9 | 6.1 \pm 3.0 | 1.8 \pm 0.6 | 1.0 \pm 0.4 | 0.9 \pm 0.5 | 1.0 \pm 0.5 |
| <i>ZmUbi1</i> | 1.0 \pm 0.6 | 1.0 \pm 0.6 | 1.0 \pm 0.7 | 1.0 \pm 0.6 | 1.0 \pm 0.6 | 1.0 \pm 0.7 | 1.0 \pm 0.7 | 1.0 \pm 0.6 |

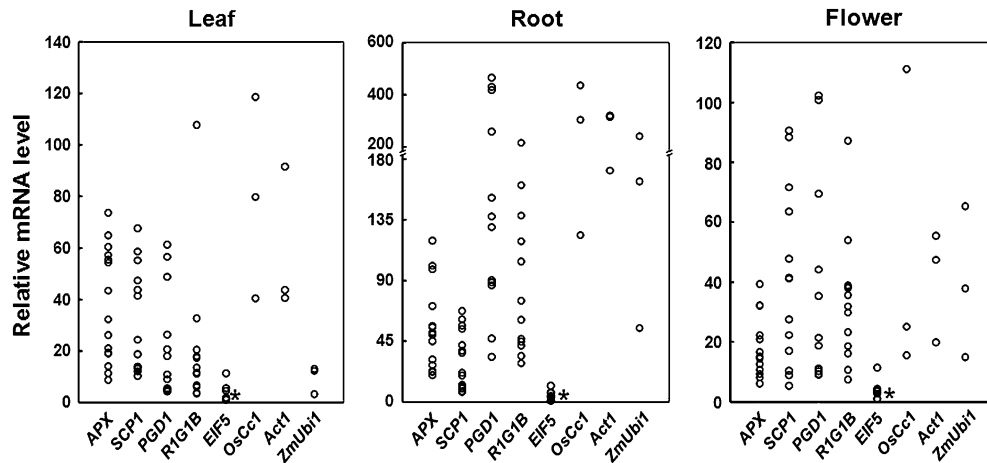


Fig. 4. Range of variation in the promoter activity in different transgenic lines. A total of 9–15 independent transgenic lines per promoter were analysed. Total RNA was isolated from the leaf and root tissues of 30 DAG plants and from flowers prior to the pollination stage. The levels of *gfp* mRNA were measured using real-time PCR and plotted relative to the *gfp* mRNA levels in *EIF5* transgenic plants (indicated by the asterisk). All mRNA levels were normalized with respect to that of the internal control gene *OsUbi1* (AK121590). Three independent lines of the transgenic plants harbouring the *OsCc1* (Jang *et al.*, 2002), *Act1* (McElroy *et al.*, 1990), and *ZmUbi1* (Cornejo *et al.*, 1993) gene promoters were used as constitutive controls. The data points represent the mean of triplicate measurements.



Fig. 5. Levels of GFP protein in the *promoter:gfp* transgenic rice plants. Protein extracts were prepared from the leaf and root tissues at 30 DAG and from flowers just before pollination. Three independent *promoter:gfp* transgenic plants and non-transgenic (NT) plants were analysed. One line of each of the transgenic plants harbouring the *OsCc1* (Jang *et al.*, 2002), *Act1* (McElroy *et al.*, 1990), and *ZmUbi1* (Cornejo *et al.*, 1993) gene promoters were included as constitutive controls. Ten μ g of total soluble proteins were separated on a SDS-polyacrylamide gel, transferred to a membrane, and then immunoblotted with an anti-GFP antibody. The amount of GFP protein in *promoter:gfp* transgenic plants were calculated by comparing band intensities with those of known amounts of recombinant GFP protein used as positive controls (PC).

stages except for 7 DAG roots and 30 DAG leaves (Table 1). It was interesting to learn also that the *R1G1B* promoter is very active in the whole grain including the embryo, endosperm, and aleurone layer. To our knowledge, a constitutive gene promoter that is also active in the whole grain has not been described previously. In addition, it was found that the activity of the *ZmUbi1* and *R1G1B* promoters is markedly reduced in 7 DAG roots and 30 DAG leaves whilst the *APX*, *PGD1*, *OsCc1*, and *Act1* promoters are active in both vegetative and reproductive tissues of transgenic rice plants (Fig. 3; Table 1). Interestingly, the *APX*, *PGD1*, *R1G1B*, *OsCc1*, and *ZmUbi1* promoters showed similar activity levels in all of the floral organs whereas the activity of the *Act1* promoter was observed to be predominant in the anthers. The *PGD1* and *ZmUbi1* promoters were similarly active in the embryos and aleurones yet distinct from the other promoters (Fig. 2A). *EIF5* was

found to be a constitutive promoter but to have low levels of activity in all tissues and at all growth stages. *EIF5* exhibited the lowest activity among the five novel promoters analysed despite the high expression levels of its endogenous gene on the microarray (see Supplementary Table S1 at *JXB* online) and evidenced by RT-PCR (Fig. 1A). It thus appears that *cis*-acting elements located at regions other than the promoter are important for high levels of endogenous gene expression. There is a demand for such weak constitutive promoters, for example, for the expression of transgenes that are toxic or otherwise inhibitory in abundance, and for which a very strong promoter would not be suitable.

It was found that the GFP protein levels of *SCP1:gfp* plants are much lower than those of the other *promoter:gfp* plants although their *gfp* mRNA levels are high. The presence of three upstream ATGs in the 5'-UTR of the

SCPI promoter may have a negative impact upon the translation efficiency of the *SCPI:gfp* mRNA (Fig. 5). Consistent with our current results, the *Arabidopsis ABSCL-SIC ACID-INSENSITIVE3* promoter was previously reported to contain three uORFs within its 405 bp 5'-UTR and the excision of this region dramatically increased GUS expression (Ng *et al.*, 2004). The UTRs within transcripts often determine gene expression by influencing mRNA stability and translation efficiency. About 30% of the genes in rice and *Arabidopsis* are likely to be influenced at the translational level by signals present in their 5'-UTR as these motifs possess uAUG/uORFs with sequence similarities to regulatory consensus sequences (Shashikanth *et al.*, 2008). A potential mechanism for uORFs to affect translational efficiency is therefore to act as a decoy from the principal ORF, and thereby potentially to cause premature termination, the triggering of mRNA decay via NMD (Nonsense-Mediated mRNA Decay), or a delay in the attachment of the ribosomal complex and thus the initiation of translation at the correct AUG start codon (Lawless *et al.*, 2009).

Using the PlantCare program (Lescot *et al.*, 2002), several *cis*-elements were identified in the promoter regions of the *APX*, *SCPI*, *PGD1*, *RIG1B*, and *EIF5* as well as *OsCcl1*, *Act1*, and *ZmUbi1* genes (see Supplementary Fig. S2 at *JXB* online). A putative TATA box sequence was located at a region approximately between -23 and -62 relative to the transcription initiation site, and two to three CAAT boxes and common *cis*-acting elements were found in all of these promoter regions. The G-box motif, a *cis*-acting element involved in light responsiveness, is also located in all of these promoter regions. The G-box element has been found to be essential for transcriptional activity in the *Arabidopsis rbcS-1A* (Donald and Cashmore, 1990), the spinach Rubisco activase (*Rca*) (Orozco and Ogren, 1993), and in the rice *OsAct2* (He *et al.*, 2009) genes. The TGACG motif, which is important for high-level gene expression of *Agrobacterium tumefaciens* T-DNA octopine synthase (*ocs*) (Ellis *et al.*, 1987), nopaline synthase (*nos*) (Mitra and An, 1989), CaMV 35S (Fang *et al.*, 1989), and rice *GOS2* (De Pater *et al.*, 1992), was also found to be present within the *APX*, *SCPI*, *PGD1*, *RIG1B*, *EIF5*, and *Act1* promoter regions. The rice endosperm-specific *cis*-acting elements, AACA and ACGT, were also identified (Wu *et al.*, 2000; Qu *et al.*, 2008) in the *PGD1* and *RIG1B* promoters that are active in dry seeds.

In summary, five different gene promoters in transgenic rice plants were analysed throughout all stages of plant growth. These promoters were each found to be active in the whole rice plant body including flowers and dry seeds with distinct patterns of activity. These elements therefore provide novel putative constitutive gene promoters with great potential for use in crop biotechnology applications. The use of alternative promoters with similar characteristics is essential for the stacking of several transgenes in order to avoid homology-dependent gene silencing, a phenomenon which often occurs in transgenic plants with multiple copies of the same promoter. Our novel rice promoters may also be useful for transgene expression in other monocot crops such as maize, barley, and wheat.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. Microarray data for seven constitutive rice genes in 14-d-old leaves and roots and early stage flowers.

Supplementary Table S2. Primers used in this study for RT-PCR and promoter isolation.

Supplementary Fig. S1. Structure of the *SCPI* 5'-UTR.

Supplementary Fig. S2. Potential regulatory elements in the promoter regions of the eight constitutive genes analysed in this study.

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