

High-Level and Ubiquitous Expression of the Rice Cytochrome *c* Gene *OsCc1* and Its Promoter Activity in Transgenic Plants Provides a Useful Promoter for Transgenesis of Monocots¹

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Expression patterns of a rice (*Oryza sativa*) cytochrome *c* gene *OsCc1* and its promoter activity were characterized in transgenic rice plants. *OsCc1* transcripts accumulate in most cell types, but to varying levels. Large amounts of *OsCc1* transcripts are found in the roots, calli, and suspension cells, but relatively lower in mature leaves, demonstrating its higher levels of expression in non-photosynthetic tissues. Unlike the human cytochrome *c* gene, which is responsive to cAMP, *OsCc1* expression is not enhanced in various rice tissues after dibutyryl cAMP treatments. *OsCc1* promoter was linked to the *sgfp* gene and its activities in different tissues and cell types of transgenic rice plants were analyzed in comparison with the *Act1* and *RbcS* promoters. *OsCc1* promoter directs expression in virtually all organs of transgenic plants including roots, leaves, calli, embryos, and suspension cells, showing a particularly high activity in calli and roots. Activity of the *OsCc1* promoter was 3-fold higher than *Act1* in calli and roots and comparable with *RbcS* in leaves, representing a useful alternative to the maize (*Zea mays*) *Ubi1* and the rice *Act1* promoters for transgene expression in monocots.

Several factors increase the expression of chimeric genes in transgenic plants. The choice of promoters affects transgene transcription, resulting in changes not only in concentration, but also in the stage, tissue, and cell specificity of its expression. Over the years, several well-characterized promoters have been made available for transgene expression in plants. However, most of these promoters are from dicot plants. Presently, the cauliflower mosaic virus (CaMV) 35S promoter and its derivatives are among the most commonly used. The CaMV 35S promoter is active in monocots, but its relative strength is substantially lower in monocot than in dicot cells. In addition, it is inactive in some cell types; for example, pollen (Bruce et al., 1989; Christensen et al., 1992; McElroy and Brettell, 1994). Other dicot promoters have also been used for monocot transformation, but activity tends to be lower than for monocot promoters (Wilmink et al., 1995). For example, Kyo-zuka et al. (1993) reported that expression of the *gusA* gene driven by a tomato (*Lycopersicon esculentum*) *RbcS* promoter is induced by light. However, the expression of the rice (*Oryza sativa*) *RbcS::gusA* gene is sig-

nificantly higher than expression of the tomato *RbcS::gusA* gene. Conversely, the use of monocot promoters has resulted in a high degree of gene expression in monocots, including rice (McElroy et al., 1991; Cornejo et al., 1993; Kyo-zuka et al., 1993). Several promoters have been investigated as useful alternatives to drive a high level of expression in monocot transgenesis; for example, the rice *Act1* promoter (McElroy et al., 1991), the rice *RbcS* promoter (Kyo-zuka et al., 1993; Jang et al., 1999), and the maize (*Zea mays*) *Ubi1* promoter (Uchimiya et al., 1993). In many cases, introns strongly enhance transgene expression in transgenic plants (Wilmink et al., 1995). In the case of the rice *Act1* and maize *Ubi1* promoters, the first introns in the 5'-untranslated region of the genes (McElroy et al., 1991; Christensen et al., 1992) were included for their enhanced promoter activities (Christensen and Quail, 1996). The last two are the constitutive promoters that are commonly used in monocot transformation (McElroy and Brettell, 1994). Thus, there is currently a shortage of efficient promoters for ubiquitous gene expression in monocots.

Cytochrome *c* is a small, peripheral, nuclear-encoded membrane protein located in the intermembrane space of mitochondria. It functions in the catalytic transfer of electrons between respiratory complexes III and IV in eukaryotes. The cytochrome *c* molecule has been investigated for its functional importance and unique intra-organelle position. Cytochrome *c* proteins and their nucleotide sequences are highly conserved in organisms as distantly related as yeast (*Saccharomyces cerevisiae*), mammals, and plants. Therefore, these proteins have been used

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for many evolutionary comparisons at the molecular level (Syvanen et al., 1989; Kemmerer et al., 1991a, 1991b). In animals, the cytochrome *c* gene promoter is reported to contain cAMP response element (CRE) and nuclear respiratory factor (NRF)-binding sites (Gopalakrishnan and Scarpulla, 1994). CRE is involved in cAMP-dependent expression, and NRF is involved in the coordination of nuclear and mitochondrial gene activities (Evans and Scarpulla, 1990). Recent focus on the cytochrome *c* protein has been in relation to its release into the cytosol, which is an indication of apoptotic processes in human cells. In plants, however, little is known about the cytochrome *c* gene, except that it has been cloned and sequenced in Arabidopsis and rice. This study examined the regulation of a rice cytochrome *c* gene *OsCc1* (accession no. M63704) and its promoter activities in transgenic rice. Our data demonstrate that *OsCc1* is expressed in most tissues, and that its expression is particularly high in the non-photosynthetic parts of plants, including the roots, calli, and suspension cells. To evaluate the *OsCc1* promoter, it was linked to the *sgfp* gene and introduced into rice. Using confocal laser scanning microscopy (CLSM) and RNA-blot hybridization, activity of the *OsCc1* promoter in various tissues and cell types of transgenic plants was analyzed in comparison with *Act1* and *RbcS* promoters.

RESULTS

Sequence Analysis of the *OsCc1* Promoter Region

The physical map of the genomic clone *OsCc1* is shown in Figure 1A and the nucleotide sequence of its promoter region was deposited in GenBank under the accession number AF399666. We assigned the transcription start site because the sequence at positions -6 to $+4$ (CTCGGAATCG) resembles the plant transcription initiation sequence CTCATCA (Joshi, 1987). Several potential regulatory elements were identified in a human cytochrome *c* promoter (Gopalakrishnan and Scarpulla, 1994). Of particular interest in the *OsCc1* promoter are the sequences at positions -218 to -225 (GCACGTGG; I in Fig. 1A) and -180 to -189 (CCGAATGGGC; II in Fig. 1A). These are similar to the sequences of CRE-1 (TGACGTCA) and NRF-1 (CCGCATGCGC), which are binding sites of a human cytochrome *c* gene promoter (Gopalakrishnan and Scarpulla, 1994). In animal systems, CRE-1 is involved in the cAMP-dependent expression of the cytochrome *c* gene, and NRF-1 is implicated in a mechanism for coordinating nuclear and mitochondrial genetic systems (Evans and Scarpulla, 1990).

High-Level Expression of the *OsCc1* Gene in Non-Photosynthetic Tissues

Genomic Southern analysis showed that a single copy of *OsCc1* is present in the genome of rice cv

Nakdong (data not shown). To investigate the expression pattern of the gene, northern-blot hybridization was carried out for different tissues and cells using total RNA prepared with a cDNA fragment (cyt in Fig. 1A). The cyt probe is *OsCc1*-specific, corresponding to the second and third exons, and the 3'-untranslated region of the gene. The cyt probe detected a single mRNA band of approximately 0.7 kb. As shown in Figure 1B, *OsCc1* transcripts accumulated in high concentrations in the roots, calli, and suspension cells, but were present at relatively lower levels in mature leaves, demonstrating higher levels of expression in non-photosynthetic tissues.

The *OsCc1* promoter contains a sequence at positions -218 to -225 (GCACGTGG), which is similar to that of CRE-1 (TGACGTCA) in the human cytochrome *c* gene. This finding prompted us to determine whether *OsCc1* is regulated by cAMP in the leaves, roots, and suspension cells. Total RNA was prepared at various time intervals after treatment with Bt₂cAMP (Gopalakrishnan and Scarpulla, 1994), and assayed for mRNA levels using RNA-blot hybridization (Fig. 1C). The Bt₂cAMP treatment did not result in an increase in *OsCc1* mRNA levels at any time interval in any of the tissues, but rather caused a decrease for the 6-h time point for both suspension culture and root cells.

The fact that *OsCc1* transcript levels were relatively lower in mature leaves than in non-photosynthetic tissues implies that *OsCc1* may be negatively regulated by light during the growth of young seedlings. To determine whether *OsCc1* is regulated by light or not, total RNA was prepared from etiolated young seedlings at various times after exposure to light. In contrast to the increased mRNA levels in *RbcS*, *OsCc1* mRNA levels were not changed significantly by light (Fig. 2), suggesting its light-independent expression. The lower level of *OsCc1* expression in leaves appears to be due to tissue specificity.

Analysis of *OsCc1* Promoter Activity in Different Transgenic Tissues in Comparison with Other Rice Promoters

Figure 3 shows the plasmid components used in rice transformation. The 1.8-kb promoter region of *OsCc1* from $-1,700$ to $+100$ (Fig. 1A) was linked to *sgfp* (Chiu et al., 1996), which encodes a modified green fluorescent protein (sGFP). The chimeric *OsCc1::sgfp* gene was then ligated to the expression cassette that carries the coding region of the phosphinothricin acetyl transferase gene (*bar*) under the control of the 35S promoter. This procedure generated a pSB-CG (*OsCc1::sgfp*) plasmid. For comparison, two additional constructs were made: pSB-RG (*RbcS::sgfp*) and pSBG700 (*Act1::sgfp*). *RbcS::sgfp* contains promoter regions of a small ribulose biphosphate carboxylase/oxygenase subunit (*RbcS*) from rice (Kyojuka et al., 1993; Jang et al., 1999), whereas

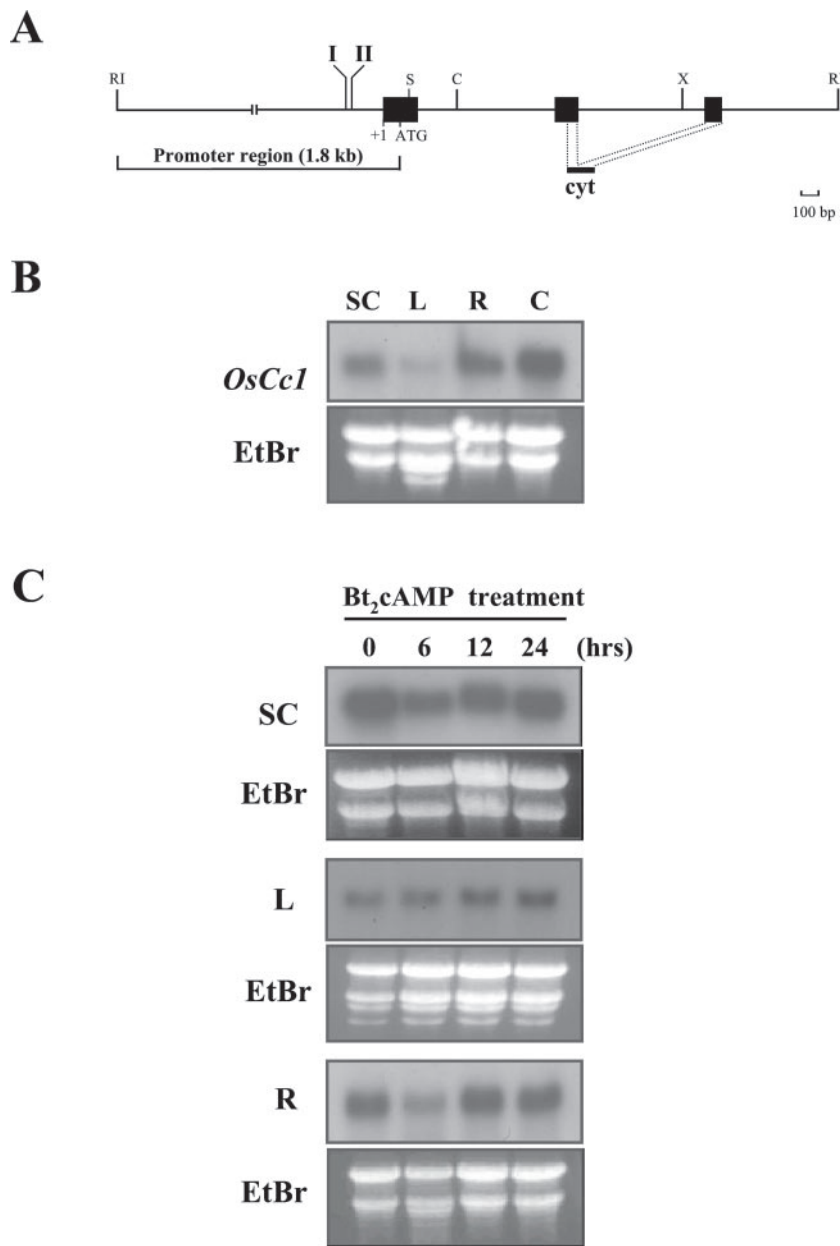


Figure 1. A, Physical map of the genomic clone (*OsCc1*) encoding a rice cytochrome *c*. The solid boxes represent exons. A partial cDNA (*cyt*) that was used as a probe for RNA-blot experiments is indicated. The transcriptional start site and the translational start codon are marked with +1 and ATG, respectively. Sequences that are similar to those of CRE and NRF1-binding sites are indicated by I and II, respectively. Important restriction enzyme sites are abbreviated as follows: C, *Clal*; RI, *EcoRI*; S, *Sall*; and X, *Xbal*. B, The expression of *OsCc1* in different tissues of rice. RNA-blot analysis was carried out using total RNA from rice suspension cells (SC), leaves (L), roots (R), and calli (C). C, The response of *OsCc1* mRNA expression to the Bt_2cAMP treatment. Suspension cells (SC), leaves (L), and roots (R) were treated with 1 mM dibutyryl cAMP (Bt_2cAMP) for the time periods indicated, and total RNA was extracted. Total RNA was fractionated on a denaturing agarose gel, blotted to a nylon membrane, and hybridized with a [^{32}P]-labeled *cyt* cDNA probe, shown in A (*OsCc1*). The loading of an equal amount of total RNA in each lane was verified by ethidium bromide staining (EtBr).

Act1::sgfp contains promoter regions from the *actin1* (*Act1*) gene of rice (McElroy et al., 1991). All three of these plasmids contain the *bar* gene as a selectable marker for transformation under the control of the CaMV 35S promoter. Several independent lines of transgenic rice plants (21 for *OsCc1::sgfp*, 55 for *RbcS::sgfp*, and 16 for *Act1::sgfp*) were obtained by the *Agrobacterium tumefaciens*-mediated method, and grown in a greenhouse. Phosphinothricin acetyl transferase encoded by the *bar* gene can detoxify phosphinothricin-based herbicides (Kim et al., 1999). All the transformants were herbicide resistant, as tested by painting leaves with the commercial herbicide Basta (Jang et al., 1999). For each promoter, three independent lines were randomly chosen for further

analysis. Genomic Southern blots were used to determine the number of copies and integration events of transgene in the lines. The strategy for Southern-blot analysis is depicted in Figure 3. The genomic DNAs from *OsCc1::sgfp*-transformed plants were digested either with *NcoI*, which excised the intact size of *sgfp*, or with *EcoRV*, which cut a unique site of the plasmid. The 0.7-kb fragment corresponding to "*NcoI* digest" (Fig. 3) appeared in all the transformants, indicating that they contained a full-length *sgfp* gene (Fig. 4A). The three lines showed distinct band patterns in "*EcoRV* digest," suggesting that each line was generated by an independent integration event. The genomic DNAs from *Act1::sgfp*- or *RbcS::sgfp*-transformed plants were similarly ana-

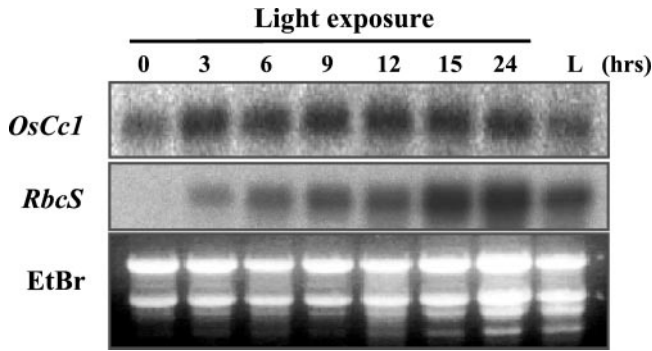


Figure 2. The response of *OsCc1* mRNA expression in etiolated seedlings to light exposure. Etiolated seedlings were treated with light at $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ for the time periods indicated, and total RNA was extracted. Total RNA was fractionated on a denaturing agarose gel, blotted to a nylon membrane, and hybridized with either a [^{32}P]-labeled *cyt* cDNA probe, shown in Figure 1A (*OsCc1*), or the coding region for a small subunit of ribulose-1,5-bisphosphate carboxylase (*RbcS*). The loading of an equal amount of total RNA in each lane was confirmed by ethidium bromide staining (EtBr). Lane L shows total RNA extracted from mature leaves.

lyzed, consequently validating that all the lines are independent and copy numbers of corresponding transgene are either one or two (Fig. 4).

To examine *OsCc1* promoter activity at a whole-plant level, sGFP fluorescence in *OsCc1::sgfp*-transformed plants was examined visually with a digital video imaging system. Non-transformed etiolated seedlings did not show any fluorescence, whereas all tissues of the transgenic etiolated seedlings fluoresced bright green (Fig. 5A, left). The degree and pattern of green fluorescence in the *OsCc1::sgfp* plant was similar to that of the *Act1::sgfp* plant. The expression of sGFP was also observed in dry seeds, with the embryos fluorescing strongly (Fig. 5A, right). Intensity of fluorescence in embryos of *OsCc1::sgfp* plants than in those of *Act1::sgfp*, or *RbcS::sgfp* plants. Thus, the *OsCc1* promoter is highly active in driving gene expression in most tissues of transgenic rice plants.

OsCc1 promoter activity in different tissues was compared with those of the *Act1* and *rbcS* promoters in transgenic rice plants. Using CLSM, we investigated the distribution of sGFP fluorescence in the cells of leaf and root tissues (Fig. 5B). In transformed cells, fluorescence emitted from sGFP was detected in a green channel, whereas fluorescence was not detected in untransformed cells under the same conditions. In leaf cells, *OsCc1::sgfp* plants had higher levels of *sgfp* expression than *Act1::sgfp* plants and comparable levels with *RbcS::sgfp* plants. In particular, *OsCc1::sgfp* and *Act1::sgfp* plants displayed brighter fluorescence in guard and mesophyll cells (Fig. 5B). In contrast, sGFP fluorescence in root cells was much higher in *OsCc1::sgfp* and *Act1::sgfp* plants than in *RbcS::sgfp* plants. Interestingly, *OsCc1::sgfp* plants showed the greatest GFP fluorescence in the root cap region (RC on Fig. 5B). The *OsCc1* and *Act1* promoters also directed high levels of *sgfp* expression

in the root hair protuberances of both primary and secondary roots.

For a quantitative comparison of activities of the promoters, total RNAs were extracted from leaf, root, and callus of three independent lines of *OsCc1::sgfp*, *Act1::sgfp*, and *RbcS::sgfp* plants (shown in Fig. 4) and hybridized with a 0.7-kb DNA fragment containing the *sgfp* coding region (Fig. 6A). The total RNAs were reprobbed with the rice *RL5*, which encodes the 5S rRNA-binding protein (Kim and Wu, 1993), for equal RNA loading. As shown in Figure 6B, relative levels of *sgfp* transcripts were calculated and normalized using *RL5* as a reference. In leaf tissues, the *sgfp* transcript levels of *OsCc1::sgfp* plants were comparable with those of *RbcS::sgfp* plants, but 2-fold higher than those of *Act1::sgfp* plants. In roots and calli, *sgfp* transcript levels of *OsCc1::sgfp* plants were 3- and 10-fold higher than those of *Act1::sgfp* and *RbcS::sgfp* plants, respectively.

Our CLSM and RNA gel-blot data demonstrated that the *OsCc1* promoter is similar to the rice *Act1* in driving high levels of expression of *sgfp* in cells that are rapidly dividing or have high metabolic activity. However, the *OsCc1* promoter activity was much higher than that of *Act1* in leaves, roots, embryos, and calli (Fig. 5C). The *OsCc1* promoter is particularly active in guard cells, root cap, apex regions, root hair protuberances, and calli. Thus, *OsCc1* is useful as a promoter for ubiquitous expression of transgene in monocots.

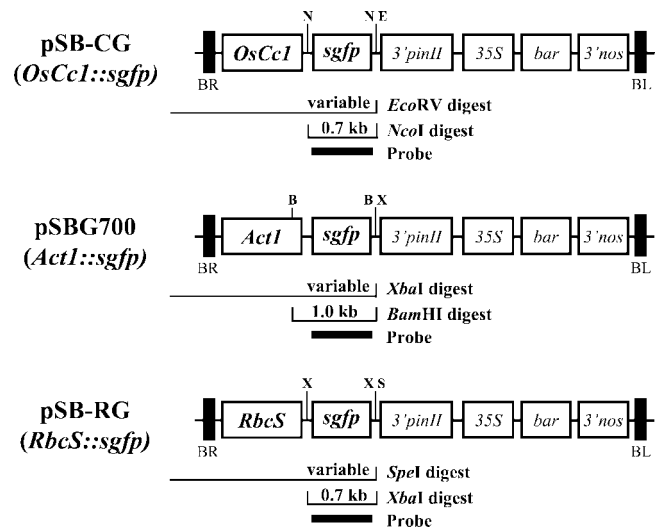


Figure 3. The expression vectors used for rice transformation. pSB-CG (*OsCc1::sgfp*) consists of the *OsCc1* promoter linked to the *sgfp* coding region, the 3' region of the potato proteinase inhibitor II gene (3'pinII), and the *bar* gene expression cassette that contains a 35S promoter/*bar* coding region/3' region of the nopaline synthase gene (3'nos). pSBG700 (*Act1::sgfp*) and pSB-RG (*RbcS::sgfp*) are identical to pSB-CG except that the rice *Act1* and *RbcS* promoters, respectively, are fused to the *sgfp*-coding region. Important restriction sites are indicated: *EcoRV* (E), *NcoI* (N), *XbaI* (X), *BamHI* (B), and *SpeI* (S). Restriction enzymes followed by the expected fragments and hybridization probe (probe) used for genomic DNA-blot analyses are shown below the map.

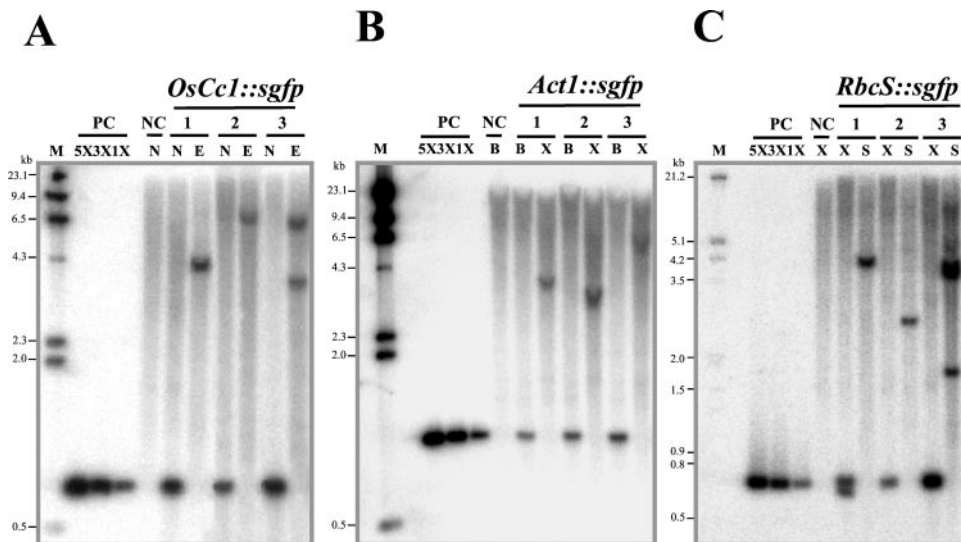


Figure 4. Genomic DNA-blot analysis of transgenic rice plants. A, Genomic DNAs from the leaf tissues of three (1–3) independent lines of *OsCc1::sgfp*-transformed plants were digested with *NcoI* (N), *EcoRV* (E), and hybridized with a 0.7-kb DNA fragment containing the *sgfp* coding region (see Fig. 3). PC contains *NcoI*-digested pSB-CG. B, Genomic DNAs from the leaf tissues of three (1–3) independent lines of *Act1::sgfp*-transformed plants were digested with *BamHI* (B), *XbaI* (X), and hybridized with the same probe described in A. PC contains *BamHI*-digested pSBG700. C, Genomic DNAs from the leaf tissues of three (1–3) independent lines of *RbcS::sgfp*-transformed plants were digested with *XbaI* (X), *Spel* (S), and hybridized with the same probe described in A. PC contains *XbaI*-digested pSB-RG. NC, Genomic DNAs from an untransformed control plant; 1X, 3X, and 5X in PC represent one, three, and five genome equivalents of pSB-CG (A), pSBG700 (B), or pSB-RG (C), relative to 5 μ g of rice genomic DNA, respectively. The DNA molecular size markers (M) are indicated.

DISCUSSION

Cytochrome *c* is the product of a nuclear gene, and resides in the intermembrane space of mitochondria. It functions in the catalytic transfer of electrons between respiratory complexes III and IV. The amino acid and nucleotide sequence of cytochrome *c* has been extensively used for studies of molecular evolution because it is present in all eukaryotes, and sequences are strikingly similar among organisms (Syvanen et al., 1989; Kemmerer et al., 1991). With its unique subcellular location, cytochrome *c* has been a molecule of scientific interest for protein targeting in organelles (von Heijne et al., 1989). In addition, the human cytochrome *c* gene is regulated by cAMP (Gopalakrishnan and Scarpulla, 1994), and is involved in a mechanism for coordination of nuclear and mitochondrial genetic systems (Evans and Scarpulla, 1990). In contrast to the rapid progress in other organisms, little attention has been given to cytochrome *c* in plants. It is generally accepted that the main mitochondrial function of generating ATP by oxidative phosphorylation is regulated according to differing requirements during plant development (e.g. the requirements of photosynthetic leaves, roots, or other types of cells). However, gene expression patterns, promoter activity, and the responses of the plant cytochrome *c* gene to cAMP remain largely unexplored. A rice genomic clone, *OsCc1*, encoding cytochrome *c*, was previously sequenced (Kemmerer et al., 1991). Rice contains a single copy of *OsCc1* in its genome, as demonstrated by our genomic DNA hy-

bridization. Northern-blot hybridization indicated that high concentrations of *OsCc1* transcripts accumulated in the roots, calli, and suspension cells, whereas levels were lower in mature leaves. This suggests that *OsCc1* is highly expressed in non-photosynthetic tissues, which is consistent with the fact that chloroplasts function as the main energy supply in photosynthetic tissues, whereas mitochondria are responsible for supplying energy in non-photosynthetic tissues. Undifferentiated plastids are known to cause complex alterations to nuclear gene expression (Hess et al., 1994, 1997; Hedtke et al., 1999). Calli, cultured suspension cells, and roots do not have differentiated chloroplasts, resulting in higher levels of *OsCc1* expression than in leaf cells. Because the decrease in *OsCc1* mRNA levels in leaves was not due to light exposure (Fig. 4), it is likely that there is specific repression of the gene in leaf tissues.

cAMP is an important signaling molecule in all organisms except plants. In higher plants, cAMP is barely detectable (Trewavas, 1997), so its function has not been elucidated. The *OsCc1* promoter contains the sequence GCACGTGG (at positions –218 to –225), which is similar to the TGACGTCA motif. The latter functions as a CRE in a number of cAMP-responsive promoters of animal systems, including a human cytochrome *c* gene (Gopalakrishnan and Scarpulla, 1994). In our experiments, however, no increase in *OsCc1* mRNA levels was detected after Bt_2cAMP treatment (Fig. 3B). This result was consistent at all time intervals measured and for all tissues

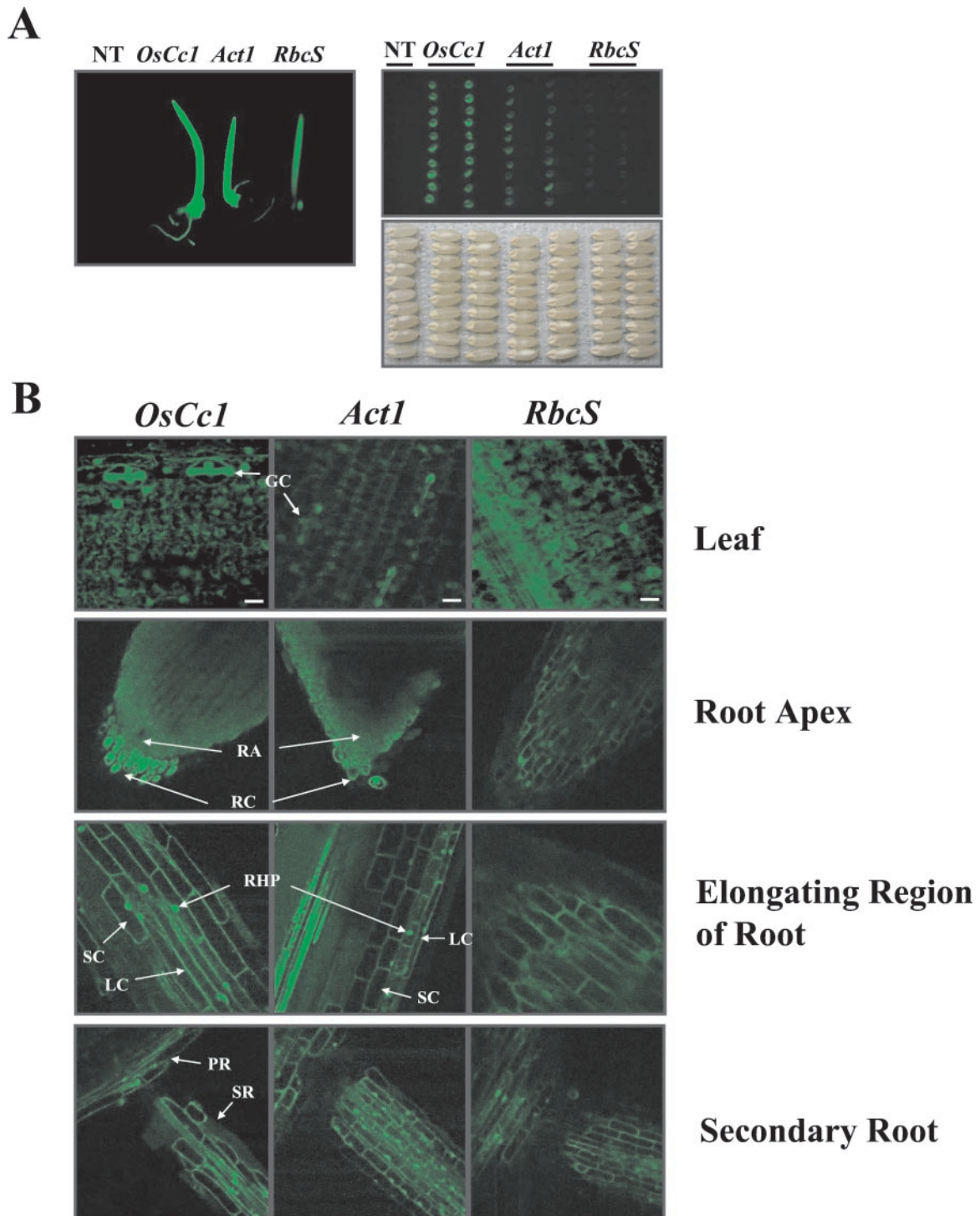


Figure 5. Images of sGFP fluorescence in transgenic rice plants expressing *OsCc1::sgfp* (*OsCc1*), *Act1::sgfp* (*Act1*), and *RbcS::sgfp* (*RbcS*) chimeric genes. A, sGFP fluorescence in transgenic rice seedlings (left) and uncoated dry seeds (right) taken by a digital video imaging system. NT represents an untransformed rice seedling (right) and seeds (left). B, Confocal microscopic image of sGFP fluorescence in a leaf, a root apex, elongating region of a root, and secondary root of transgenic rice plants. GC, Guard cells; RA, root apex; RC, root cap; RHP, root hair protuberances; LC, long cells; SC, short cells; PR, primary root; SR, secondary root. Scale bars = 30 μ m.

examined, including suspension cells, leaves, and roots. This led us to speculate that *OsCc1* expression does not seem to be responsive to cAMP levels in rice

cells. However, it remains to be determined whether cAMP levels in the rice cells were actually enhanced by the Bt₂cAMP treatment before drawing any con-

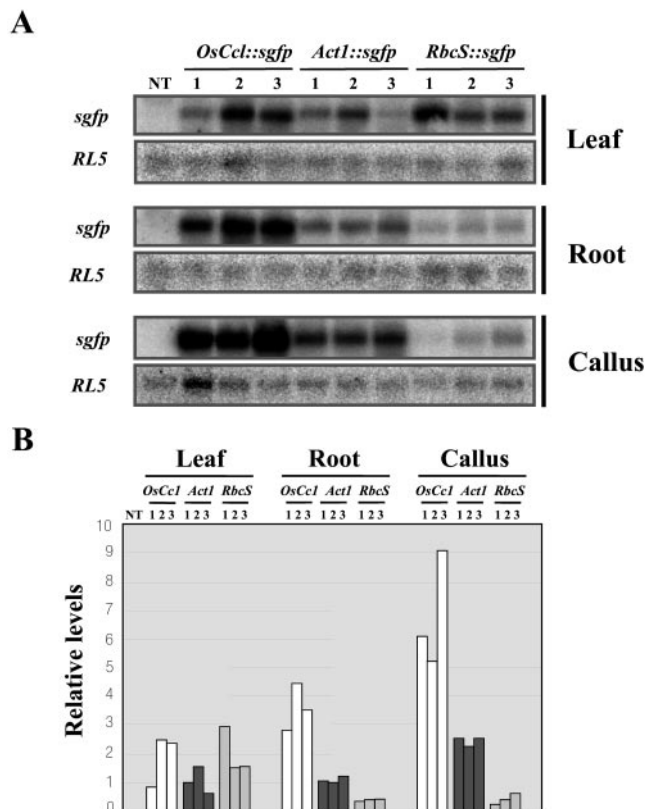


Figure 6. RNA gel-blot analysis and relative levels of *sgfp* transcripts in transgenic rice plants shown in Figure 4. A, Total RNAs extracted from leaf, root and callus of three (1–3) independent lines of *OsCc1::sgfp*-, *Act1::sgfp*-, and *RbcS::sgfp*-transformed plants and from an untransformed control plant (NT) were hybridized with a 0.7-kb DNA fragment containing the *sgfp* coding region (see Fig. 3). Hybridizations with the rice *RL5* encoding the 5S rRNA-binding protein (Kim and Wu, 1993) were used for equal RNA loading. B, Transcript levels of *sgfp* shown in A were calculated using those of corresponding *RL5* as a reference and the resultant values were then normalized to 1 for that from leaf tissues of *Act1::sgfp*-transgenic line 1.

clusion. It is also possible that *OsCc1* may be more sensitive to Bt₂cAMP treatment in other types of plant cells, or in combination with a plant hormone. This is supported by the observation in animal systems that the cytochrome *c* gene was not induced in COS-1 cells, but was highly induced in BALB/3T3 fibroblasts after Bt₂cAMP treatment (Gopalakrishnan and Scarpulla, 1994).

Despite isolating numerous promoters from a wide variety of plants, only a few of them are commonly used in plant transformation. The CaMV 35S promoter is active in dicot tissues, but has much lower activity in monocots (McElroy et al., 1991; Cornejo et al., 1993; Schledzewski and Mendel, 1994; Wilmink et al., 1995). The rice *Act1* (McElroy et al., 1990) and maize *Ubi1* (Christensen et al., 1992) promoters are commonly used in monocot transformation because they are significantly more active than the CaMV 35S promoter in these cells (McElroy et al., 1990; Christensen et al., 1992; Cornejo et al., 1993; McElroy and

Brettell, 1994; Schledzewski and Mendel, 1994; Wilmink et al., 1995; Christensen and Quail, 1996). Our results demonstrated that the *OsCc1* promoter was 2- and 3-fold more active in transgenic rice leaves and roots, respectively, than the *Act1* promoter (Figs. 5 and 6). Although *OsCc1::sgfp* expression varies depending on the plant tissue, it is clear that the *OsCc1* promoter is active in most cell types with particular preference in non-photosynthetic tissues, including calli and roots. The feature of the *OsCc1* promoter that makes it useful for rice transformation is its ability to drive high levels of expression in calli, which are typically used for the selection and screening of transformed cells. The high activity of the *OsCc1* promoter in rapidly dividing cells should allow the clear identification of stable transformants.

The distribution of sGFP fluorescence in transgenic rice plants reveals several other features of the *OsCc1* promoter. Young roots showed strong sGFP expression, particularly in the root cap and apex regions. Strong sGFP fluorescence was also observed in the mesophyll and guard cells of leaves. The *OsCc1* pattern of expression suggests that this promoter is most active in cells with high metabolic activity, and that it is distinct from other promoters characterized from transgenic rice. CaMV 35S promoter expression is widespread, exhibiting activity in the vascular tissue (Terada and Shimamoto, 1990), and in most root and leaf cell types (Battraw and Hall, 1990). *Ubi1* is expressed in many, but not all, cell types. This promoter drives strong expression in young roots, but expression levels decrease drastically as the roots mature. *Ubi1* is also active in the vascular system and guard cells of transgenic rice leaves (Cornejo et al., 1993). The *Act1* promoter in rice is active in both vegetative and reproductive tissues of transgenic rice plants (McElroy et al., 1990). Thus, the *OsCc1* pattern of expression is similar to that of the rice *Act1* and CaMV 35S promoters, but is, in fact, stronger than either of these promoters.

In summary, a detailed examination of the expression patterns of the *OsCc1::sgfp* construct indicates that the gene is expressed in most organs of transgenic rice, consistent with the potential for targeting a wide spectrum of cells. Therefore, the *OsCc1* promoter represents a useful alternative to the maize *Ubi1* and rice *Act1* promoters for the high-level expression of genes in monocots.

MATERIALS AND METHODS

Plant Materials

Transgenic and nontransgenic rice (*Oryza sativa*) plants were grown in a greenhouse. Embryogenic calli were initiated from mature rice cv Nakdong embryos, and were maintained on solid Murashige and Skoog medium (pH 5.8), containing 1% (w/v) agarose, 30 g L⁻¹ Suc, and 2.5 mg L⁻¹ 2,4-dichlorophenoxyacetic acid. Suspension cultures were started from embryogenic calli in liquid AA medium, containing 30 g L⁻¹ Suc, 2.0 mg L⁻¹ 2,4-dichlorophenoxyacetic acid, and 0.2 mg L⁻¹ kinetin. The cultures were kept on an incubator that rotated while it shook (120 rpm) in the dark at 26°C.

RNA Isolation and Analysis

Rice plants, cultured cells, and calli were subjected to various treatments. Each sample was soaked in 1 mM Bt₂cAMP (Sigma, St. Louis), or was exposed to light at 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for the time periods indicated. After treatment, all plant materials were frozen in liquid nitrogen and stored at -80°C . Total RNA was extracted from different tissues of rice by the guanidinium/LiCl method. About 0.1 g of leaves, roots, calli, and etiolated seedlings was homogenized in 1 mL of an extraction buffer, containing 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% (w/v) sarkosyl, and 0.1 M β -mercaptoethanol. After homogenization, 0.1 mL of 2 M sodium acetate (pH 4.0), 1 mL of water-saturated phenol (pH 4.5), and 0.2 mL of chloroform:isoamyl alcohol (1:1, v/v) were added. The mixture was vortexed for 30 s, and centrifuged at room temperature for 10 min at 5,000g. The supernatant was removed to a new tube, and an equal volume of isopropanol was added to it. The sample was then incubated at -20°C for 1 h, and centrifuged at 4°C for 10 min at 10,000g. The RNA pellet was dissolved in 0.5 mL of diethyl pyrocarbonate-treated water, and was precipitated with an equal volume of 4 M lithium chloride, followed by incubation at 4°C for at least 1 h. The precipitated RNA was subsequently centrifuged at 4°C for 10 min at 10,000g, washed with 70% (v/v) ethanol, and dried. The RNA pellet was dissolved in 40 mL of diethyl pyrocarbonate-treated water and its concentration was calculated from A_{260} . For the northern-blot analysis, 20 μg of total RNA was electrophoresed on a 1.2% (w/v) agarose gel containing iodoacetamide, and was then blotted onto a nylon membrane (Hybond N⁺, Amersham, Buckinghamshire, UK) following standard procedures (Sambrook et al., 1989). The membrane was hybridized overnight at 65°C with a [³²P]labeled DNA probe, which was prepared according to the manufacturer's instructions using a random primer labeling kit (Takara, Kyoto). Hybridization was carried out overnight in a mixture containing 1 M sodium phosphate buffer and 1 mg of salmon sperm DNA. After hybridization, the membrane was washed once in 2 \times SSC (0.3 M NaCl and 50 mM sodium citrate, pH 7.0) and 0.1% (w/v) SDS solution at 65°C for 15 min, and then once in 1 \times SSC and 0.5% (w/v) SDS at 65°C for 15 min, and finally in 0.5 \times SSC and 0.5% (w/v) SDS at 65°C . The membrane was then exposed on an intensifying plate, and analyzed with a phosphor imager analyzer (FLA 3000, Fuji, Tokyo).

Vector Construction

The *OsCc1* promoter sequence (1.8 kb) was PCR amplified using an upstream primer (5'-AACTGGAGGAATTCGGATCTCGAAGGTAGGC-3', *XhoI* site underlined), a downstream primer (5'-AACCATGCGCCGCGCCGCGGAGAACG-3', *NcoI* site underlined), and the plasmid pOsCc1 (Kemmerer et al., 1991a) as template. PCR-amplified DNA was digested with *XhoI* and *NcoI*, and ligated with *XhoI/NcoI*-linearized pBlue-script KSII containing the *sgfp* gene (Chiu et al., 1996). This process produced the plasmid pKSCG. Once sequencing verified the regions spanning the junction, the 2.5-kb DNA fragment containing the *OsCc1* promoter-*sgfp* was obtained by digesting pKSCG with *XhoI* and *NotI*, and ligating it into *XhoI/NotI*-linearized pSB105 (Jang et al., 1999). This produced the plasmid pSB-CG. pSB105 contains the potato protease inhibitor II terminator/35S promoter/bar/nopaline synthase terminator between the right and left border sequences of pSB11 (Komari et al., 1996). To construct pSB-RG, the pSK-RG plasmid containing the *rbcS* promoter linked to the *sgfp* gene (Chiu et al., 1996) was digested with *BamHI* and *NotI*, and was then ligated into *BamHI/NotI*-linearized pSB105. The *rbcS* promoter in pSB-RG was replaced by the *Act1* promoter of rice (McElroy et al., 1991), producing pSBG700. Finally, the three plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 by triparental mating (Jang et al., 1999).

A. *tumefaciens*-Mediated Rice Transformation

For *A. tumefaciens*-mediated transformation, about 200 mature seeds of rice cv Nakdong were husked and sterilized with 70% (v/v) ethanol for 1 min using gentle shaking. The ethanol was discarded and the seeds were sterilized further with 100 mL of 20% (v/v) commercial bleach for 1 h with gentle shaking. The sterilized seeds were rinsed several times with sterile water. Callus induction, cocultivation with *A. tumefaciens*, and the selection of transformed calli were carried out as previously described by Jang et al. (1999).

Video Imaging of sGFP Fluorescence

sGFP fluorescence in etiolated seedlings and dry seeds was captured on a digital video imaging system (Chung et al., 2000). The excitation light came from a 250-W halogen lamp. A blue band pass filter with a peak at 480 nm (Edmund, Barrington, NJ) was used with the lamp. The light beam was focused on the sample at an angle of approximately 45° from the vertical, illuminating an area of 4 cm^2 with a maximum photon flux density of 2 $\mu\text{mol m}^{-2} \text{s}^{-1}$. A high-resolution CCD color video camera (model Cool-SNAP, Roper Scientific Inc., Trenton, NJ) took images of the sample from a vertical position. Using a zoom lens (Nikon, Tokyo) attached to the CCD, an image was taken of a 20- \times 20-mm area of the sample. A green-transmitting band pass filter with a peak at 510 nm (Edmund) was then positioned in front of the lens to transmit only a green fluorescence beam. A personal computer (686-500 MHz; Intel Corp., Seoul, Korea) controlled the CCD camera through an interface board (Roper Scientific Inc.), and collected the image files on the hard drive.

CLSM

The leaves, roots, and calli of the rice plants were observed using a confocal laser scanning microscope (Carl Zeiss LSM510, Zeiss, Jena, Germany) with a standard filter set. The microscope was located at the Korean Basic Science Institute (Daejeon). Pseudocolor, similar to the color observed with a fluorescence microscope (Olympus, Tokyo), was added to the images by importing data collected in the green and red channels of the confocal microscope. Sections were taken along the optical axis and were projected into a single image.

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