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A Sterol Biosynthetic Gene *AtCYP51A2* Promoter for Constitutive and Ectopic Expression of a Transgene in Plants

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***Arabidopsis CYP51A2* (*AtCYP51A2*) mediates the sterol 14α-demethylation step in *de novo* sterol biosynthesis, and is constitutively and highly expressed in all plant tissues (Kim et al., 2005). We exploited the molecular features of its expression and the fundamental role of sterol biosynthesis in cells to develop a plant-derived promoter. Our GUS expression analysis between transgenic *Arabidopsis* lines for *AtCYP51A2::GUS* and *35S::GUS* revealed that activity of the *AtCYP51A2* promoter was comparable to that of the *35S* promoter, based on enzymatic activities and protein levels. The *AtCYP51A2* promoter was also constitutively active in transgenic tobacco, indicating that 5′ regulatory elements could be conserved among *CYP51* promoters in dicot plants. A homologue of *AtCYP51A2* was identified from rape seed, a crop species closely related to *Arabidopsis*. Its constitutive tissue expression pattern implies that the application of this *AtCYP51A2* promoter is possible for that species. Based on these results, we present a new binary vector system with the plant-derived *AtCYP51A2* promoter, which is able to constitutively and ectopically drive a transgene in various dicotyledonous plants.**

Keywords: *Arabidopsis CYP51A2* promoter, β-glucuronidase, binary vector, *CaMV 35S* promoter, constitutive overexpression, sterol biosynthesis

Since the promoter sequences for polyadenylated 35S transcripts of cauliflower mosaic virus (CaMV) were identified in the 1980s (Guilley et al., 1982; Odell et al., 1985), those sequences have been widely used for studying the constitutive overexpression of diverse transgenes in monocots and dicots (Potenza et al., 2004). They have also been modified to enhance the expression level of downstream genes (Kay et al., 1987). Although many other promoters of plant viral origin also are now being utilized (Potenza et al., 2004), there are numerous concerns about them. The 35S promoter used in the expression of selection marker genes can often affect the expression level and patterns of neighboring genes in transgenic plants (Yoo et al., 2005; Zheng et al., 2007). Elmayan and Vaucheret (1996) have reported that a high level of transgene expression by the foreign 35S promoter could trigger a mechanism for post-transcriptional gene-silencing through a dose effect. In addition, there are passionate debates about the potential risk of the virally derived 35S promoter to human health (Ewen and Pusztai, 1999; Ho et al., 1999; Hodgson, 2000; Hull et al., 2000; Paparini and Romano-Spica, 2006).

In an effort to replace viral origin promoters with those of plant origin for efficient transgene expression, scientists have developed several constitutive promoters from plants, and are using them in basic plant biology and agricultural biotechnology areas. Among them, actin and ubiquitin are representative housekeeping genes constitutively expressed in all plant tissues. The former encodes a fundamental cytoskeletal component that is expressed in almost every cell. Studies with RT-PCR and a GUS reporter gene have revealed that *Arabidopsis actin 2* is strongly expressed in leaves, roots, stems, flowers, pollen, and siliques (An et al., 1996). The rice actin gene promoter is also being used for transformation of monocot plants (Zhang et al., 1991; Xu et al., 1996). Ubiquitin is a small-sized protein (76 amino acids) that is highly conserved in all eukaryotes and plays critical roles in plant development by regulating various biological processes, including senescence and hormone signaling (Moon et al., 2004). Some ubiquitin genes show constitutive patterns, with increased expression in young tissues. For example, the maize ubiquitin 1 promoter is one of the most well-known for constitutive overexpression of transgenes in cereal monocots; however, it is ineffective in dicot plants (Christensen et al., 1992; Cornejo et al., 1993; Christensen and Quail, 1996). The rice ubiquitin promoter has approximately two-fold higher expression than its maize counterpart (Sivamani and Qu, 2006).

As essential membrane components, sterols not only regulate membrane fluidity and permeability, but also modulate the activity and distribution of membrane proteins (Hartmann, 1998). Therefore, all living plant cells synthesize sterols to support their vitality. *CYP51* is considered an essential gene in all organisms because it controls the biosynthesis of membrane sterols. CYP51, catalyzing the 14α-demethylation step in sterol biosynthesis, is also an evolutionarily conserved gene across biological kingdoms, from bacteria to protozoa, fungi, animals, and plants. *CYP51* genes have been found in 82 organisms from all biological kingdoms

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that synthesize sterols *de novo* (Lepesheva et al., 2006; Lepesheva and Waterman, 2007). Several sterol biosynthetic genes, including *Arabidopsis CYP51A2* (*AtCYP51A2*), are constitutively and highly expressed in plant tissues (Diener et al., 2000; Carland et al., 2002; Kim et al., 2005).

We previously showed that *AtCYP51A2* is constitutively expressed in all *Arabidopsis* tissues investigated (Kim et al., 2005). Here, we compared GUS expression patterns and activities between transgenic *Arabidopsis* lines harboring *AtCYP51A2::GUS* and those harboring *35S::GUS*. The comparative GUS expression analysis revealed that the activity of *AtCYP51A2* promoter was comparable to that of 35S promoter. Furthermore, *AtCYP51A2* promoter was also active in transgenic tobacco. Our data suggest that *AtCYP51A2* promoter could be useful for the constitutive and ectopic expression of transgenes at least in dicot plants. Finally, we generated a new binary vector system with *AtCYP51A2* promoter that is capable of constitutively driving transgene in plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Nicotiana tabacum cv. nc was used for transformation with *Agrobacterium tumefaciens* strain GV3101. Total RNA was extracted from various plant tissues of *Oryza sativa* cv. Dongjin and *Brassica napus* cv. Halla. Transgenic *Arabidopsis* expressing *AtCYP51A2::GUS/GFP* (hereafter *AtCYP51A2::GUS*) had been generated previously (Kim et al., 2005). *Arabidopsis thaliana* ecotype Col-0 was transformed with the binary vector pBI121 to generate *CaMV 35S::GUS* lines (hereafter *35S::GUS*), according to the floral dip method (Clough and Bent, 1998). Those *Arabidopsis* plants were cultured on MS solid media or soil as described previously (Kim et al., 2005).

GUS Histochemical Analysis

GUS histochemical analysis was performed as described by Stomp (1992). Briefly, plant materials were vacuum-infiltrated for 3 min in a GUS staining solution [0.1 M sodium phosphate buffer (pH 7.0), 0.1% (v/v)Triton X-100, 10 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, and 1 mM X-gluc] and incubated at 37°C for 12 h. Afterward, the tissues were cleared through a graded ethanol series. Images were obtained by an Olympus SZX9 stereo microscope equipped with an Olympus digital camera (Model C5060).

Measurement of GUS Enzyme Activity

Fluorometric analysis of GUS activity was performed as described by Jefferson (1987). Total soluble proteins were extracted from 10-day-old *Arabidopsis* seedlings and aerial parts of 3-week-old plants. They were then mixed with GUS assay buffer [1 mM 4-methylumbelliferyl-β-D-glucuronide, 50 mM sodium phosphate buffer (pH 7.0), 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% (v/v) sodium lauryl sarcosine, and 0.1% (v/v) Triton X-100]. The reaction was stopped by adding a stop buffer (0.2 M Na_2CO_3). GUS activity was determined by Victor 2 (Perkin-Elmer), and activity values were expressed as pmol 4-methylumbelliferone (4-MU) min^{-1} mg $protein^{-1}$.

Western Analysis of GUS Expression

Protein samples were extracted with RIPA buffer (Biosaesang, Korea) containing an appropriate amount of protease inhibitor cocktail (P9599; Sigma, Canada), and 20 μg of proteins were separated by SDS-PAGE. They were then transferred to a PVDF membrane and blocked in 5% (v/v) skim milk powder in PBS [1.5 mM KH_2PO_4, 8 mM Na_2HPO_4 (pH 7.4), 137 mM NaCl, 2.7 mM KCl, and 0.05% (v/v) Tween 20]. A polyclonal anti-GUS rabbit IgG (Molecular Probes, USA) was used at a 1:4000 dilution in PBS. Proteins were detected with a 1:15,000 dilution of goat antirabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch, USA), using the ECL Plus detection system (Amersham, UK) according to the manufacturer's instructions.

RNA Isolation and Reverse Transcription-PCR (RT-PCR)

Total RNA was isolated from plant tissues with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Purified total RNA (5 μg) was used for first-strand cDNA synthesis with M-MLV reverse transcriptase (Invitrogen). The conditions for PCR amplification were as follows: 96°C, 5 min for initial denaturation; followed by 25 to 30 cycles of 94°C for 15 sec, 50°C for 30 sec, and 72°C for 1 min; then 5 min of final extension at 72°C. Primers for RT-PCR are listed in Table 1. *Ubiquitin* and *GAPDH* transcripts were amplified as positive controls for PCR.

Callus and Shoot Induction from Root Explants of *Arabidopsis*

Arabidopsis seedlings were aseptically grown on 0.8% (v/v) agar-solidified MS media for 10 d under a 16-h photoperiod. Root segments (5 to 10 mm long) were excised and transferred to a callus-induction medium (CIM) containing 2,4-D (0.5 mg L^{-1}) and kinetin (0.1 mg L^{-1}). These segments were cultured for three weeks under the same photoperiod.

Table 1. Primers used for RT-PCR in this experiment.

Gene products	PCR primers (5′ → 3′)	
	Forward	Reverse
BnCYP51H (*B. napus* homolog of AtCYP51A2)	ACTAATCTTCTCCTTCTTCACATC	CTTTCAACTTGTTAACCCTTAGAG
OsCYP51H (rice homolog of AtCYP51A2)	TCTTCTTTTCCCTTTCTGTCAGTATT	CAACTCACATTGCCATAGTTTTATTC
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (GAPDH)	AGAGAAACAAGATCTCGAATATGAT	ATAAAAGTAGACTTGTGTATCAAAGG
Ubiquitin	GACTACAACATCCAGAAGGAGTC	TCATCTAATAACCAGTTCGATTTC

Shoots were induced by incubating the callus on a shoot-induction medium (SIM) containing IAA (0.15 mg L^{-1}) and 2-ip (0.5 mg L^{-1}) for 9 d (Ozawa et al., 1998).

Tobacco Transformation

Agrobacterium tumefaciens strain GV3101 carrying the vector construct was cultured on a YEP medium (An, 1987) supplemented with 50 mg L^{-1} kanamycin and 50 mg L^{-1} gentamycin. The *Agrobacterium*-mediated transformation of *Nicotiana tabacum* cv. nc was conducted by the method of Horsch et al. (1985). Leaves were cut into approx. 0.5 cm discs. Explants were inoculated with the *A. tumefaciens* suspension for 5 to 10 min, then transferred to an agar-solidified MS medium supplemented with 1 mg L^{-1} benzyladenine (BA) and 0.01 mg L^{-1} naphthylacetic acid (NAA). After 2 d of cocultivation, the explants were transferred to a selective regeneration medium, i.e., an MS medium with 1 mg L^{-1} BA, 0.01 mg L^{-1} NAA, 30 mg L^{-1} hygromycin, and 500 mg L^{-1} cefotaxime. The developing shoots were transferred to an MS medium with 30 mg L^{-1} hygromycin and 500 mg L^{-1} cefotaxime for rooting. Rooted plants (T0) were transferred to soil and grown in a growth chamber.

Binary Vector Construction for Constitutive and Ectopic Expression of the Transgene

A genomic fragment (1555 bp) containing the promoter region of *CYP51A2* was amplified by Pyrobest DNA polymerase (Takara, Japan) with a pair of primers. A restriction enzyme *Eco*R I site was introduced into the primers for cloning of the PCR product. Primer sequences were as follows: 51A2PF, 5′-GGAATTCTGTTGAAAATTCTCAACATGATAATCCA-3′; and 51A2PR, 5′-GGAATTCTGTTTTTCAACTGTCACACA-CACACAAA-3′. The PCR product was cloned into the *Eco*R I site of the pUC19 vector. Complete nucleotide sequences of that PCR product were then determined to check PCR errors. Finally, the *Eco*R I fragment of the PCR product was sub-cloned into the *Eco*R I site of the pCAMBIA1380 binary vector (CAMBIA, Canberra, Australia).

RESULTS AND DISCUSSION

Comparative Analysis between Transgenic *Arabidopsis* Lines for *35S::GUS* and *AtCYP51A2::GUS*

AtCYP51A2 plays a key role in the plant sterol biosynthetic pathway by mediating the 14α-demethylation step. We earlier reported that *AtCYP51A2* is highly and constitutively expressed in all *Arabidopsis* tissues that we had investigated, which implies a ubiquitous role for sterols as membrane components (Kim et al., 2005). As the first step in developing an *AtCYP51A2* promoter for the generation of a plant-derived constitutive promoter, we have now analyzed in detail the GUS expression patterns and activities of transgenic *Arabidopsis* harboring an *AtCYP51A2::GUS* construct, and also compared it with those of *35S::GUS* lines. Two independent and homozygous lines were selected for each construct. Tissue expression patterns and activities were very similar between the transgenic lines for each construct (data not shown). GUS expression was strongly detected in light-grown seedlings and all *Arabidopsis* tissues of both *AtCYP51A2::GUS* lines and *35S::GUS* lines, including the floral bud, flower, embryo, stem, silique, axillary bud, cauline leaf, rosette leaf, and root (Fig. 1). This ubiquitous pattern is very similar to that observed for *Arabidopsis* actin genes (*ACT2* and *ACT8*; An et al., 1996). Moreover, GUS activity of the *AtCYP51A2::GUS* lines was strongly detected in the callus and shoots regenerating from that cal-

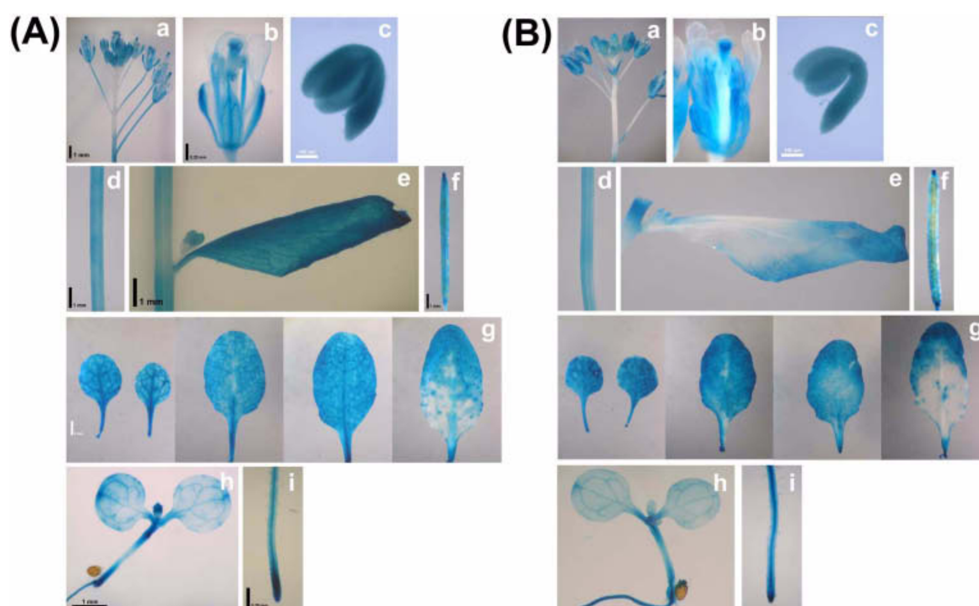

Figure 1. Comparative GUS histochemical analysis of transgenic *Arabidopsis* lines expressing *AtCYP51A2::GUS* **(A)** versus *35S::GUS* **(B)**. Tissues from 35-day-old plants and 9-day-old seedlings were used for histochemical GUS staining.
(a) floral bud, (b) opened flower, (c) embryo, (d) stem, (e) axillary bud and cauline leaf, (f) silique, (g) rosette leaves, (h) cotyledon and hypocotyl from 9-day-old seedling, (i) root from 9-day-old seedling. Bars = 1 mm for (a), (d), (e), (f), and (h); 0.25 µm for (b) and (i); 2 mm for (g); 100 µm for (c).

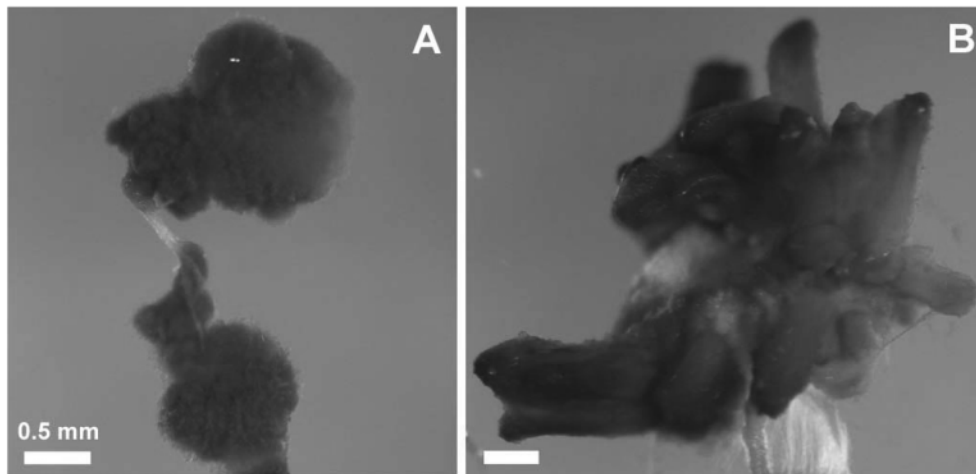

Figure 2. Histochemical GUS analysis for callus and shoot regenerated from callus of transgenic *Arabidopsis* harboring *AtCYP51A2::GUS*. **(A)** Callus induced on CIM for 3 weeks using root explants from 10-day-old seedlings. **(B)** Regenerating shoot induced from callus on SIM for 9 d. Bars = 0.5 mm

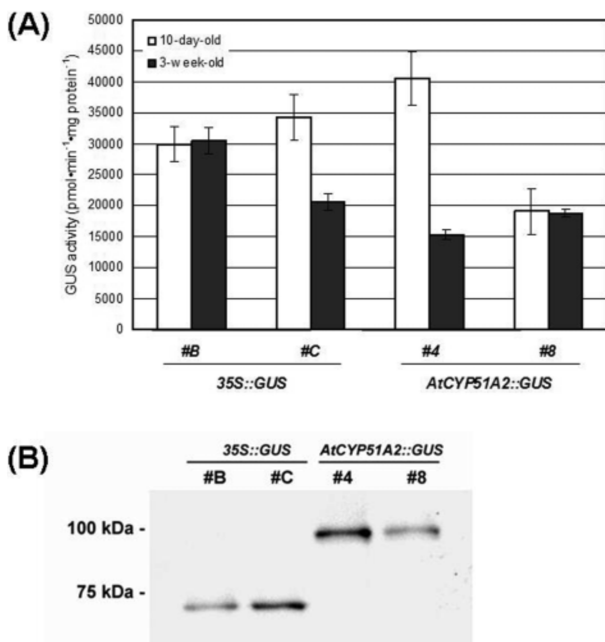

Figure 3. Comparative analysis of GUS expression levels for transgenic *Arabidopsis* lines expressing *AtCYP51A2::GUS* versus *35S::GUS*. **(A)** Fluorometric analysis of GUS enzyme activity. Total soluble protein was extracted from 10-day-old seedlings and aerial parts of 3-week-old plants. **(B)** Western blot analysis of GUS expression in transgenic seedlings. *35S::GUS* lines and *AtCYP51A2::GUS* lines expressed 69.6 kDa of GUS protein and 96.8 kDa of GUS/GFP fusion protein, respectively.

lus (Fig. 2). Zheng et al. (2007) have reported that the 35S promoter can convert an adjacent tissue-specific promoter into a globally active promoter. Therefore, as an alternative to the 35S promoter, the strong activity of this *AtCYP51A2* promoter in calli and regenerating shoots also can be applied to drive selection marker genes during tissue culture.

We also performed a comparative analysis between the *35S::GUS* and *AtCYP51A2::GUS* lines, measuring GUS enzyme activities and GUS protein levels in seedlings and the aerial parts of 3-week-old plants. At the seedling stage, activity in Line 4 of *AtCYP51A2::GUS* was comparable to that of Lines B and C from *35S::GUS*. At 3-week-old stage, that activity was relatively lower in the *AtCYP51A2::GUS* than in the *35S::GUS* lines (Fig. 3A). Our GUS antibody detected 69.6 kDa of GUS protein in the latter and 96.8 kDa of GUS/GFP fusion protein in the former (Fig. 3B). Protein levels were in good accordance with the enzyme activity data (Fig. 3B).

Expression of *AtCYP51A2::GUS* in Transgenic Tobacco

To examine if the *AtCYP51A2* promoter could drive GUS expression in other dicot species, we generated transgenic tobacco lines harboring an *AtCYP51A2::GUS* construct and performed GUS histochemical analysis. Activity was strongly detected in the leaves, roots, and stems (Fig. 4), thereby showing that this new promoter is active in tobacco as well. GUS expression was also detected in all tissue types of the transverse stem section, e.g., the pith, vascular system, cortex, and epidermis (Fig. 4D). *CYP51* is thought to be the most ancient and conserved cytochrome P450 gene across kingdoms (Lepesheva et al., 2006). Therefore, our data further suggest that 5′ regulatory elements conferring a constitutive pattern of expression by that gene also could be highly conserved among dicotyledonous plants.

Tissue Expression Patterns of *CYP51* Homologues from *Brassica napus* and Rice

Brassica species are economically important crops as fresh vegetables or oil sources. Recent comparative genomics approaches have revealed very close molecular relationships between them and the model plant *Arabidopsis* (Lysak et al., 2005; Kim et al., 2007). Before applying our *AtCYP51A2* promoter to the transformation of a crop species such as rape seed (*Brassica napus*), we identified a full-length cDNA (*BnCYP51H*) homologous to *AtCYP51A2* from rape seed (URL http://compbio.dfci.harvard.edu/tgi/, Gene Index ID

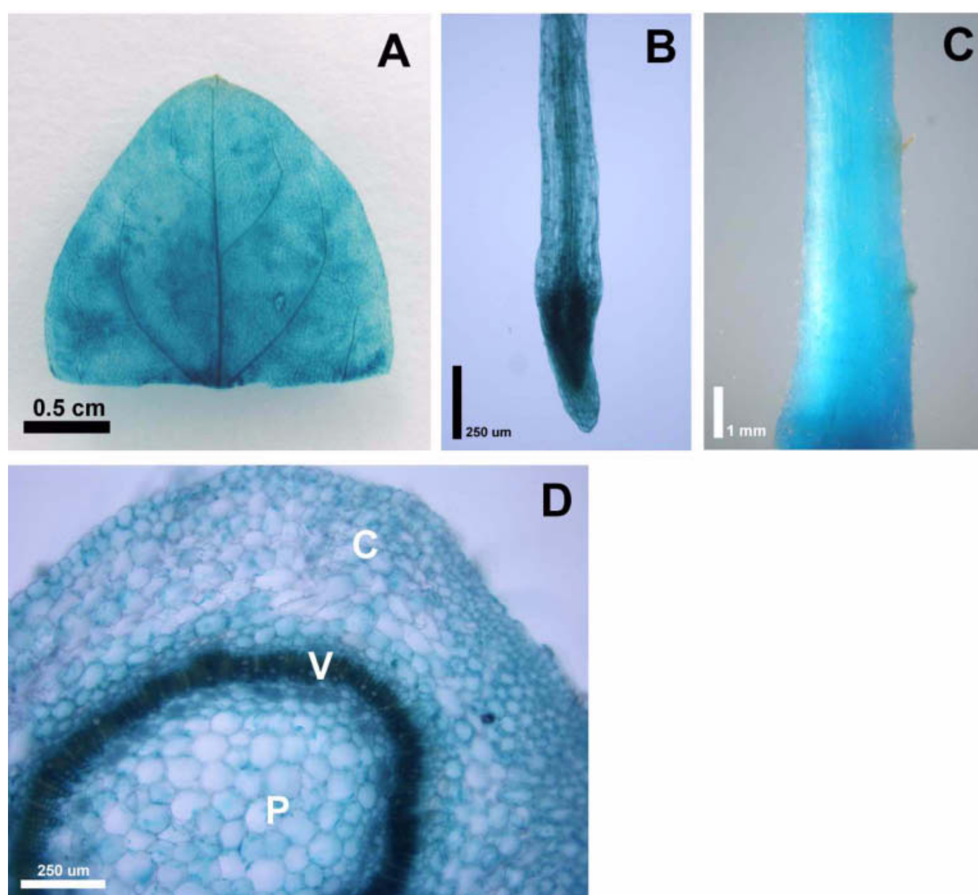

Figure 4. GUS histochemical analysis of transgenic tobacco lines harboring *AtCYP51A2::GUS* construct.
(A) Leaf. **(B)** Root. **(C)** Stem. **(D)** Transverse section of stem. P, pith; V, vascular system; C, cortex. Bars = 0.5 cm for **(A)**, 250 um for **(B)** and **(D)**, 1 mm for **(C)**.

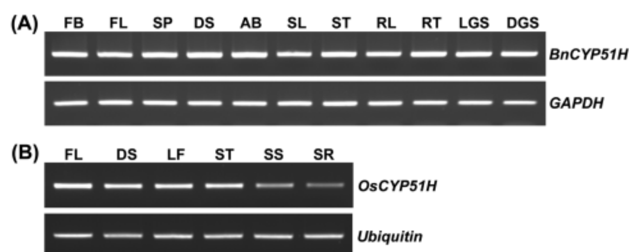

Figure 5. Tissue expression patterns of *CYP51* homologues from rape seed and rice.
(A) Expression pattern of *BnCYP51H* in different tissues of rape seed. FB, floral bud; FL, flower: SP, seed pod; DS, developing seed; AB, axillary bud; SL, stem leaf; ST, stem; RL, rosette leaf; RT, root; LGS, light-grown seedling; DGS, dark-grown seedling. Cytosolic GAPDH transcripts were amplified as positive control for PCR. **(B)** Expression pattern of *OsCYP51H* in different tissues of rice. FL, flower; DS, developing seed; LF, mature leaf; ST, stem; SS, seedling shoot; SR, seedling root. Ubiquitin transcripts were amplified as positive control for PCR.

for *B. napus*-TC57558), and analyzed its tissue expression pattern. RT-PCR analysis demonstrated that *BnCYP51H* was constitutively and highly expressed in the floral bud, flower, seed pod, developing seed, axillary bud, stem leaf, stem, rosette leaf, root, and seedlings (Fig. 5A). This constitutive expression pattern is very similar to that observed in *Arabidopsis* (Kim et al., 2005), indicating that 5′ regulatory elements also could be highly conserved between the *AtCYP51A2* and *BnCYP51H* promoters. These results further suggest that the *AtCYP51A2* promoter could be useful for the constitutive and ectopic expression of a transgene in *Brassica* species.

We also identified a *CYP51* homologue (*OsCYP51H*) from rice (KOME, URL http://cdna01.dna.affrc.go.jp/cDNA/, AK121491), which also showed a ubiquitous tissue expression pattern (Fig. 5B) that resembled that of the genes from *Arabidopsis* and rape seed (Figs. 1, 5A). Only a limited number of promoters derived from plants or plant viruses, including the 35S promoter, are so far known to be active for transgene expression in both monocots and dicots (Ouellet et al., 1998; Schenk et al., 2001; Shirasawa-Seo et al., 2005). For the constitutive and ectopic expression of a transgene in a wide range of species, it would be useful to know if the *AtCYP51A2* promoter is also active in such monocot plants as rice.

Generation of a New Binary Vector System for Constitutive and Ectopic Expression of a Transgene by the *AtCYP51A2* Promoter

In order to develop a new binary vector system constitutively driving downstream transgene by *AtCYP51A2* pro-

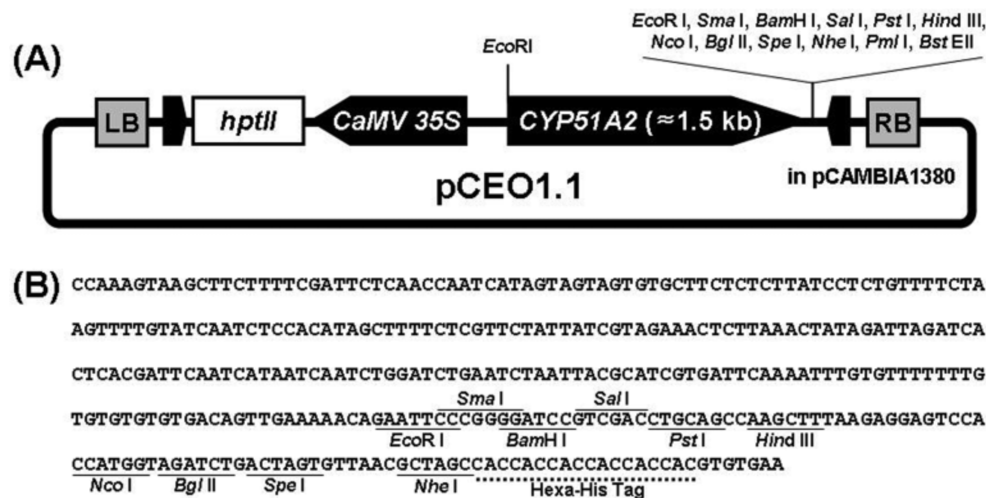

Figure 6. New binary vector system for constitutive and ectopic expression of transgene by *AtCYP51A2* promoter.
(A) Schematic diagram of a new binary vector pCEO1.1. **(B)** Nucleotide sequences of partial promoter region and multiple cloning site of pCEO1.1. LB and RB indicate left border and right border of T-DNA, respectively. Black pentagons close to LB or RB indicate CaMV35S poly-A or Nos poly-A, respectively. *hptII*, hygromycin phosphotransferase.

Table 2. Restriction enzymes that cut either the *AtCYP51A2* promoter region or MCS in binary vector pCEO1.1.

Restriction sites in *AtCYP51A2* promoter	Restriction sites in MCS of pCAMBIA 1380
***Hind* III**[a] (4 sites), ***Spe* I**[a] (1 site)	*Eco*R I, *Sma* I, *Bam*H I, *Sal* I, *Pst* I, ***Hind* III**[a], *Nco* I, *Bgl* II, ***Spe* I**[a], *Nhe* I, *Pml* I, *Bst* EII

[a]Bold restriction enzymes cut both promoter region and MCS of pCAMBIA1380 vector.

moter in dicotyledonous plants, we PCR-amplified 1555 bp of its 5′ flanking region, not containing the start codon, using Pfu DNA polymerase and sub-cloned the PCR product into the *Eco*R I position of a pCAMBIA 1380 binary vector (URL http:://www.cambia.org), which contains diverse restriction enzyme sites on its multiple cloning site (MCS) and hexa histidine-tag (Fig. 6). The resultant binary vector was named pCEO1.1 (Constitutive and Ectopic Overexpression). The *AtCYP51A2* promoter has two restriction enzyme sites (*Hind* III and *Spe* I) that are also present on the MCS of the binary vector. Nevertheless, a number of restriction sites are still unique, thereby facilitating the subcloning of transgenes (Table 2).

In conclusion, GUS activity driven by the *AtCYP51A2* promoter was constitutively and highly detected in all *Arabidopsis* tissues, which was also comparable to that of 35S promoter. In addition, the *AtCYP51A2* promoter was able to constitutively drive the *GUS* reporter gene in transgenic tobacco. Based on these results, we have now developed a new binary vector system harboring the plant-derived *AtCYP51A2* promoter, which could be a useful tool for the constitutive and ectopic expression of transgenes in dicotyledonous plants.

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