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Constitutive Promoters Available for Transgene Expression Instead of *CaMV 35S RNA* Promoter : *Arabidopsis* Promoters of Tryptophan Synthase Protein β Subunit and Phytochrome B

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Abstract

To search for strong promoters that confer constitutive expression of transgenes, we evaluated the promoters of an *Arabidopsis* tryptophan synthase protein β subunit gene (*PTSB1*) and a phytochrome B gene (*PPHYB*) as alternatives to the *35S RNA* promoter (*P35S*) of *Cauliflower mosaic virus*. Characteristics of the *Soybean chrolotic mottle virus* promoter (*PNCR*) were also studied for comparison. In transgenic calli, *GUS* gene fused with *PTSB1*, *PPHYB* and *PNCR* showed 50% or more of the activity of *P35S*. To drive the *NPTII* marker gene, the four promoters were similarly useful. In generated transgenic tobacco plants, both *PTSB1* and *PPHYB* were active in all tissues tested, and superior to *P35S* in the leaves. The four promoters differed slightly in their tissue-specific expression, but were expressed constitutively, indicating that *PTSB1* and *PPHYB* as well as *PNCR* are useful as strong and constitutive promoters as alternatives to *P35S* for genetic manipulation of plants.

Keywords: *Arabidopsis*, constitutive expression, GUS assay, kanamycin resistance, phytochrome B, promoter cassette, 35S promoter, *Soybean chrolotic mosaic virus*, transgenic tobacco, tryptophan synthase.

Abbreviations

PTSB1, the promoter of the *Arabidopsis* tryptophan synthase protein β subunit gene; *PPHYB*, the promoter of the *Arabidopsis* phytochrome B gene; *PNCR*, the promoter of a large noncoding region of *Soybean chrolotic mottle virus*; *P35S*, the *35S RNA* promoter of *Cauliflower mosaic virus*; GUS, β-glucuronidase; NPTII, neomycin phosphotransferase II; *Tnos*, the terminator sequence of the nopaline synthase gene in *Agrobacterium tumefaciens*; *Ttml*, the terminator sequence of the tumor morphology large (*tml*) gene in *A. tumefaciens*; Km^R, kanamycin resistant; Hyg^R, hygromycin resistant; 4-MU, 4-methylumbelliferone.

Introduction

Genetic engineering is a promising strategy to improve crops in a short period without the need for time-consuming crosses. For the expression of a foreign gene, the choice of a promoter suitable for the experimental purpose is critical to generate useful transgenic plants with desirable phenotypes. The *35S RNA* promoter (*P35S*) of the *Cauliflower mosaic virus* (Benfey and Chua, 1990) has been used as a strong and constitutive promoter for the introduction of foreign genes in many plant species. There are only a limited number of promoters that are known to provide a strong and constitutive expression. The promoter of the nopaline synthase gene (*Pnos*) from *Agrobacterium tumefaciens* has been widely used, and the promoter of a large

noncoding region of *Soybean chrolotic mottle virus* (*PNCR*) was recently reported as a constitutive promoter available for the selection of rice and tobacco transformants (Fukuoka *et al.*, 2000). However, the activity of *Pnos* was reported to be weaker than that of *P35S* (Sanders *et al.*, 1987; Harpster *et al.*, 1988), and the expression levels and profile of *PNCR* have not been studied in detail in transgenic plants. Thus the sequence of *P35S* has been used repeatedly for the expression of both marker genes and genes of interest in the same binary vector (Mitsuhara *et al.*, 1996). However, the introduction of repeated DNA sequences was reported to induce inactivation of the introduced gene, a phenomenon known as "homology-dependent transgene silencing" (Vaucheret, 1993; Park *et al.*, 1996).

To obtain alternatives comparable to *P35S*, we selected two *Arabidopsis* promoters from among hundreds of candidates which were reported to be able to work as the active promoters in plants. One is the promoter of the tryptophan (Trp) synthase protein β subunit gene (*PTSB1*, Berlin *et al.*, 1989; Pruitt and Last, 1993). The Trp synthase protein β subunit catalyzes the conversion of indole plus serine to Trp at the last step of the Trp biosynthetic pathway. The other is the promoter of the phytochrome B gene (*PPHYB*, Somers and Quail, 1995a, b) which was reported to be expressed throughout the plant. To evaluate the usefulness of the two *Arabidopsis* promoters in a heterogeneous system, we studied the expression profile in tobacco plants. For the control, we used *P35S*, a well characterized strong promoter in pBI121 (Jefferson *et al.*, 1987). At the same time, the activity of *PNCR* was studied to compare the usefulness with other promoters. Furthermore, the four promoters were tested for their ability to drive a selectable marker gene, *NPTII*. We describe here that *PTSB1* and *PPHYB* are powerful promoters comparable to *P35S*, and *PNCR* is similarly useful as a constitutive promoter for basal and practical studies.

Materials and Methods

Vector construction

The region of *PTSB1* was amplified by polymerase chain reaction (PCR) using genomic DNA of *Arabidopsis thaliana* ecotype Columbia as the template and primers 5'-CAGTAAGCTTGAATTCTTTCATATCTCCTGCAAAGT-3' (corresponding to nucleotide positions 1 to 26; accession number M23872) and 5'-TAGCGGATCCTACTGAATGAATCTCTCTCTGA-3' (1571 to 1550) containing *Hin*dIII and *Bam*HI sites, respectively, in the 5' flanking regions. The region of *PPHYB* was amplified by PCR using genomic DNA of *A. thaliana* ecotype Columbia as the template and primers 5'-CAGTAAGCTTGCGGCCGCGTCGACTTGTGCACCACCGTCT-3' which contains *Hin*dIII and *Not*I sites and the sequence corresponding to nucleotide positions 1 to 22 (accession number L09262) and 5'-TGACGGATCCTGACGACGGTTCTTCTTCTCCG-3' (2370 to 2349) containing *Bam*HI site in the 5' flanking region. The reactions were run at 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 30 s for 30 cycles. The PCR products were ligated to TA cloning vector pCR(R)2.1 (Invitrogen), and verified by DNA sequencing. Each fragment excised from pCR(R)2.1 by *Hin*dIII and *Bam*HI digestion was subcloned into the binary vector pMLH (Mochizuki *et al.*, 1999), which is a derivative of pBI121 (Clontech), previously digested with *Hin*dIII and *Bam*HI, thus resulting in the replacement of the promoter with *PTSB1* or *PPHYB*. The resulting constructs, pMLH-TSB1-GUS (*PTSB1::GUS*) and pMLH-PHYB-GUS (*PPHYB::GUS*), are shown in **Fig. 1A**. The promoter from a large noncoding region of *Soybean chlorotic mottle virus* (*PNCR*; Conci *et al.*, 1993) was subcloned into pMLH (*PNCR::GUS*).

The *NPTII* gene from pTRA415(R) (Fukuoka *et al.*, 2000) was subcloned into the *Bam*HI and *Sac*I sites of pCR(R)2.1 containing *PTSB1* or *PPHYB* with *Tml* (terminator sequence of the tumor morphology large gene of *Agrobacterium tumefaciens*), and the resulting constructs were used to excise *PTSB1-NPTII-Tml* and *PPHYB-NPTII-Tml* fragments. These two fragments were inserted into pTRA415(R)-delNPT (Fukuoka *et al.*, 2000) to obtain pTRA-TSB1-NPTII (*PTSB1::NPTII*) and pTRA-PHYB-NPTII (*PPHYB::NPTII*), respectively (**Fig. 1B**).

Transformation of tobacco

The promoter::*GUS* fusion constructs were introduced into tobacco (*Nicotiana tabacum* cv. Samsun NN) plants using the *Agrobacterium* infection method (Horsch *et al.*, 1985) and regenerated shoots were selected on the medium containing 100 mg l^{-1} kanamycin. Rooted plants were confirmed the integration of the transgene by detecting a 1.6-kb PCR product, using a sense primer 5'-GCAACGTCTGGTATCAGC-3' corresponding to the GUS coding region and an antisense primer sequence 5'-TTTATTGCCAAATGTTTGAACG-3' corresponding to the nopaline synthase gene terminator region. For introduction of promoter::*NPTII* fusion constructs, kanamycin-resistant (Km^R) shoots were selected on the medium containing 50, 150, or 300 mg l^{-1} kanamycin and transferred to hormone-free

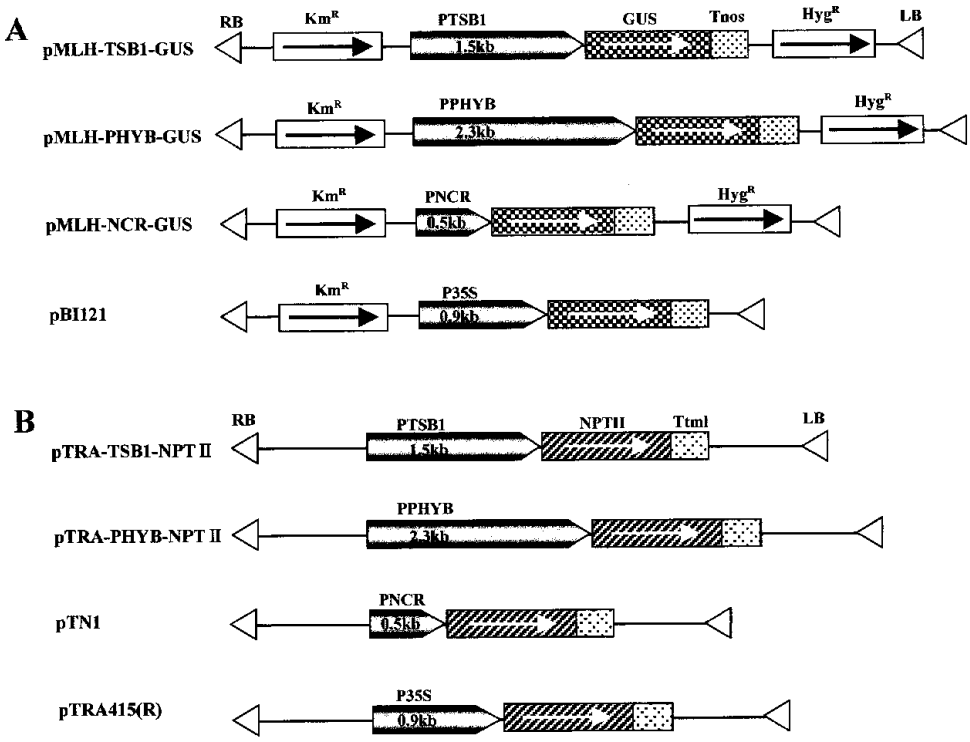


Fig. 1 Structure of the introduced genes.

(A) Schematic diagram of *GUS* fusion gene constructs.

pMLH, the binary vector which is a derivative from pBI121; RB and LB, right and left borders of T-DNA of *Agrobacterium tumefaciens* Ti plasmid, respectively; *PTSB1*, the promoter sequence of the *Arabidopsis TSB1* gene; *PPHYB*, the promoter sequence of the *Arabidopsis PHYB* gene; *PNCR*, the promoter sequence of the large non-coding region of the *Soybean chlorotic mottle virus*; GUS, the coding sequence of β-glucuronidase; *Tnos*, the terminator sequence of the nopaline synthase gene; Km^R, kanamycin-resistance gene driven by *Pnos*; Hyg^R, hygromysin-resistance gene driven by a modified *P35S*.

(B) Schematic diagram of *NPTII* fusion gene constructs.

NPTII, the coding sequence of the neomycin phosphotransferase II gene; *Ttml*, the terminator sequence of the tumor morphology large (*tml*) gene in *A. tumefaciens*.

selection medium containing 50 mg l^{-1} kanamycin to allow rooting. The number of rooted plants was counted as Km^R plants.

Fluorometric and histochemical GUS assays

GUS activity in crude extracts of leaf discs or callus was assayed by fluorometric quantification of 4-methylumbelliferone (4-MU) produced from the glucuronide precursor as reported (Kosugi *et al.*, 1990). A histochemical GUS assay was performed at 37 ℃ as described previously (Ohshima *et al.*, 1990) with a modified reaction mixture; 50 mM phosphate buffer (pH7.0) containing 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), 5% methanol, 10 μg ml^{-1} cycloheximide and 1 mM dithiothreitol. For the structure of tobacco leaf, we referred to the report of Avery (1932).

Results

Activities of PTSB1 and PPHYB in kanamycin-resistant tobacco calli and transgenic plants

DNA fragments of *PTSB1* (Pruitt and Last, 1993) and *PPHYB* (Somers and Quail, 1995a) were amplified by PCR using *Arabidopsis* genomic DNA as the template. After confirmation of the sequences, we constructed *GUS* genes driven by *PTSB1* (pMLH-TSB1-GUS) and *PPHYB* (pMLH-PHYB-GUS) (**Fig. 1A**). These vectors were introduced into tobacco plants (*Nicotiana tabacum* cv. Samsun NN) by *Agrobacterium*-mediated transformation. As the control, *P35S::GUS* (pBI121) and *PNCR::GUS* (pMLH-NCR-GUS) which contains a plant DNA virus-originated promoter (Conci *et al.*, 1993) conferring sufficient activity to drive selection marker genes for generation of transgenic rice and tobacco plants (Fukuoka *et al.*, 2000), were used for transformation at the same time. Regen-

erated kanamycin-resistant (Km^R) calli were further selected in the medium containing 100 mg l^{-1} kanamycin, and the GUS activity in eight individual transgenic calli at 20 days after the selection was assayed by the fluorometric quantification method. The level of GUS activity in the calli with *PTSB1* was slightly lower than that conferred by *P35S* and higher than that conferred by *PPHYB*. The GUS activity of *PPHYB* was almost the same as that of *PNCR* (**Fig. 2**).

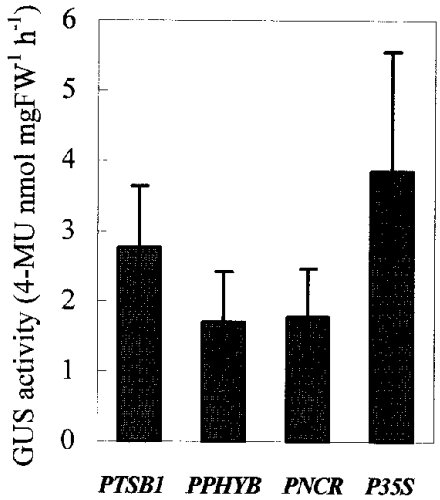
Kanamycin-resistant tobacco plants were smoothly regenerated. Introduction of individual transgene was confirmed by PCR. The level of GUS activity in mature leaves of one-month-old transgenic lines with *PTSB1* and *PPHYB* was 240% and 150% of that of the line with *P35S*, respectively (**Fig. 3**). Thus, both *PTSB1* and *PPHYB* conferred at least comparable levels of GUS activity to that of *P35S* in mature leaves. The activity of *PPHYB* was greater than that of *PNCR*.

Tissue-specific expression of PTSB1 and PPHYB in tobacco plants

We further studied the expression profiles of the introduced *Arabidopsis* promoters in tobacco plants by both fluorometric and histochemical GUS assays. *PTSB1* and *PPHYB* exhibited very similar characteristics. They were active in all tissues tested, with the level of GUS activity highest in leaves and lowest in roots per mg fresh weight (**Fig. 4A, C**). In two-month-old *PTSB1::GUS*, the level of GUS activity in the leaves varied with positions (**Fig. 4B**); it was low in upper leaf and increased as leaf position lowered. However, for the GUS activity conferred by *PPHYB*, there was no clear difference between leaf positions (**Fig. 4D**).

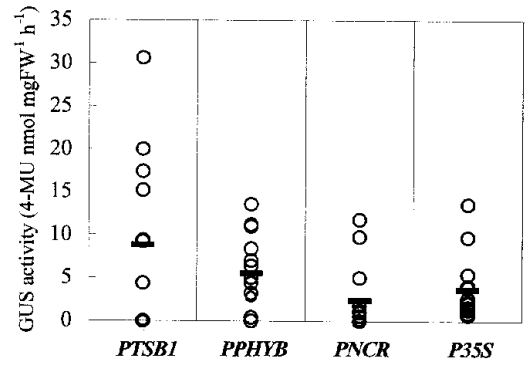
Fig. 5A shows cross sections of mature leaves after GUS staining. In a representative *PTSB1::GUS* transgenic line, intense GUS activity was found in the small veins of leaves (sv) and in middle mesophyll cells (mes), and weak staining in epidermal cells (epi) containing trichomes (**Fig. 5A-a**). In the midrib, GUS activity was predominant in both adaxial and abaxial phloems (phl), xylem parenchyma (xp) and external endodermis (end) (**Fig. 5A-b, c**). The GUS staining profile in the transgenic line was almost the same as that in the two other independent *PTSB1::GUS* lines tested (data not shown).

In mature leaves of *PPHYB::GUS* plants, GUS activity was localized intensively to small veins (sv) similarly to in *PTSB1::GUS* plant (**Fig. 5A-d**). In the vascular system, the activity in xylem parenchyma (xp) in the midrib was quite low, while it was strong in phloem (phl) as in *PTSB1* plants (**Fig. 5A-e, f**). In comparison with the GUS staining profiles in *PPHYB::GUS* and *PTSB1::GUS* plants, GUS activity in *P35S::GUS* plants was ubiquitous in mesophyll (mes) and epidermal cells (epi) but weak in small veins (sv) of mature leaves (**Fig. 5A-**



	PTSB1	*PPHYB*	*PNCR*	*P35S*
Ratio to *P35S*	0.72	0.44	0.46	1.00

Fig. 2 Levels of GUS activity conferred by four different promoters in transgenic tobacco calli. The level of GUS activity in transgenic calli containing each promoter::*GUS* fusion construct was measured as described in Materials and Methods. Means and standard deviations from eight independent experiments are shown. Ratio of mean value conferred by each promoter to that by *P35S* was shown at the bottom.



	PTSB1	*PPHYB*	*PNCR*	*P35S*
Mean value	8.8	5.5	2.4	3.6
Ratio to *P35S*	2.4	1.5	0.67	1.0
Number of plants	13	15	13	15

Fig. 3 Levels of GUS activity conferred by four different promoters in transgenic tobacco leaves. Leaf discs were cut from the fully expanded upper leaves of one-month-old regenerated transformants. Mean GUS activity, ratio of mean value conferred by each promoter to that by *P35S* and the numbers of plants used are shown at the bottom of the figure. The bar indicates the mean value. Each open circle shows the GUS value of an individual transgenic plant.

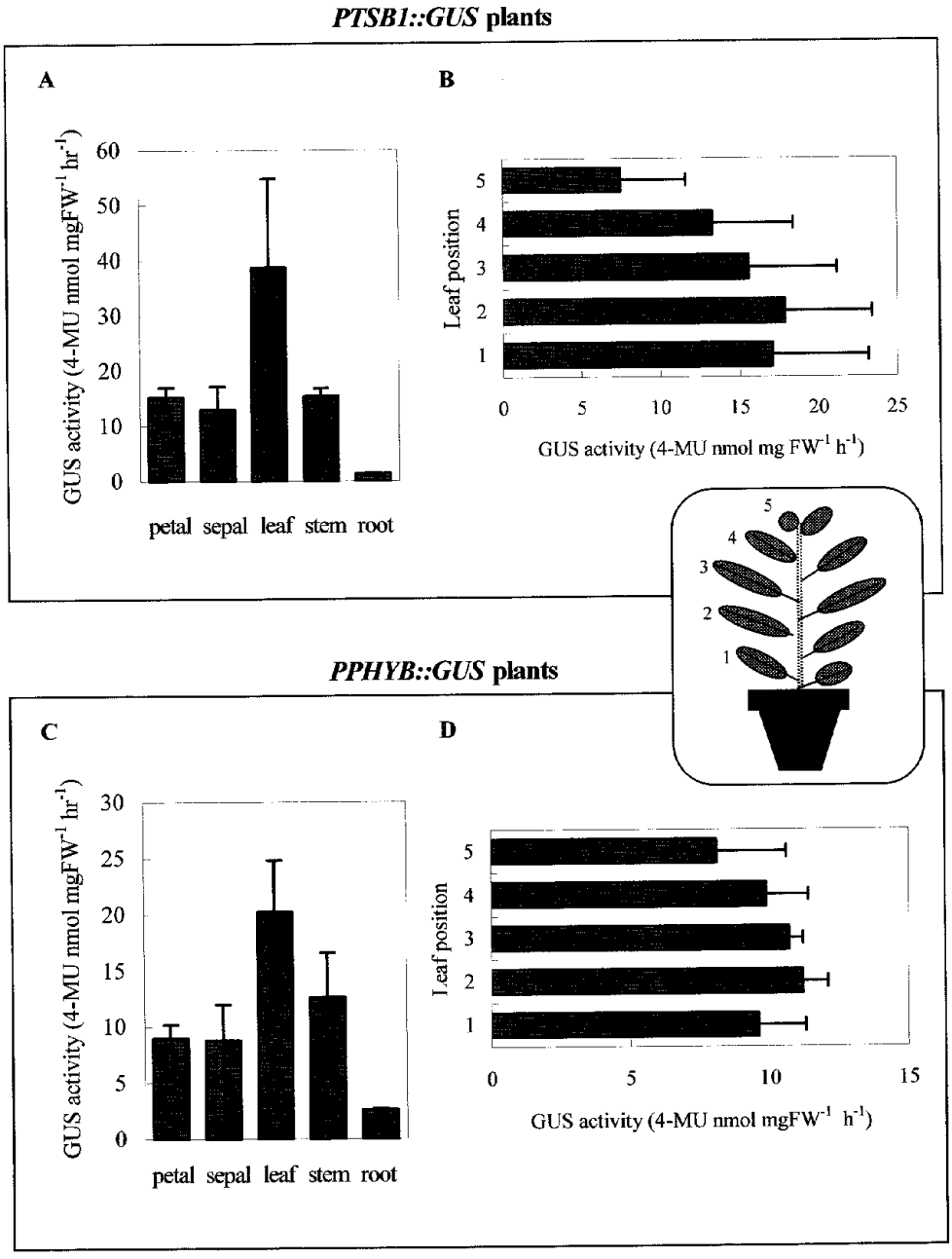


Fig. 4 Tissue-specific and developmental GUS expression in *PTSB1::GUS* and *PPHYB::GUS* tobacco plants.

All transgenic plants were grown in a growth chamber (16 h light/8 h dark at 28 ℃).

(A, C) Fluorometric GUS analysis in several organs in *PTSB1::GUS* plants and *PPHYB::GUS* plants at the flowering stage. Means and standard deviations from three independent experiments are shown.

(B, D) Effect of leaf position on GUS activity. Leaf discs were cut from 2-month-old self-pollinated progenies (T1) of the regenerated transformants. Means and standard deviations from six independent experiments are shown.

g). The localization of GUS staining in the midrib of *P35S::GUS* plants was similar to that of *PTSB1::GUS* plants, however, the activity was likely higher in xylem parenchyma (xp) and lower in phloem (phl).

Furthermore, the GUS activity in four-day-old whole sprouts was studied using selfed second-generation progeny containing *GUS* fused with *PTSB1*, *PPHYB*, *PNCR* or *P35S* (**Fig. 5B**). When five Km^R progenies of individual transgenic lines were subjected to the GUS reaction for 2 h, a high level of GUS activity was found in cotyledons in all five sprouts in *PTSB1::GUS* (a representative is shown in **Fig. 5B-a**), *PPHYB::GUS* (**Fig. 5B-b**) and *P35S::GUS* (**Fig. 5B-f**) plants, whereas a low level was detected in *PNCR::GUS* (**Fig. 5B-c**). When the reaction was prolonged to 8 h, GUS activity was additively increased in stele of hypo-

cotyls in *PTSB1::GUS* plants (**Fig. 5B-d**) and in the upper region of the roots of *PPHYB::GUS* plants (**Fig. 5B-e**). The GUS reaction for 2 h resulted in a blue color in the upper region of roots and stele of hypocotyls of *P35S::GUS* plants (**Fig. 5B-f**). Prolonging the reaction period to 8 h did not enhance the GUS staining in hypocotyls and roots in *PNCR::GUS* plants (data not shown).

Evaluation of PTSB1 and PPHYB as promoters of selectable marker genes

To evaluate *PTSB1* and *PPHYB* as promoters for driving selectable marker genes, we constructed pTRA-based binary vectors containing *NPTII* under the control of *PTSB1* or *PPHYB* (**Fig. 1B**). These constructs were introduced into tobacco plants by *Agrobacterium*-mediated transformation. pTRA415(R) with *P35S::NPTII* (Ohshima *et al.*, 1990) and pTN1 which contains *PNCR::NPTII* (Fukuoka *et al.*, 2000) were used for control vectors. At 50 mg l^{-1} of kanamycin, the number of regenerated Km^R plants per leaf section was almost the same among the three constructs although *PNCR::NPTII* was slightly less effective 80 days after *Agrobacterium* infection (**Table 1**). Even at a higher concentration of kanamycin such as 150 or 300 mg l^{-1}, all four promoters were useful for selection of Km^R plants.

Discussion

For the genetic engineering of plants, a suitable promoter available for a strong and constitutive expression of a foreign gene is required. *P35S* and *Pnos* have been widely used as constitutive and well-characterized promoters. However, the number of other promoters available for such a purpose is limited. Here we evaluated the ability of *Arabidopsis* PTSB1 and PPHYB to drive both a foreign gene and a selectable marker gene in a heterogeneous plant system, and found that the two promoters are expressed strongly and constitutively, comparable to *P35S* in tobacco plants. Furthermore, we confirmed that *PNCR* is also useful for constitutive expression with a moderate level of activity. *PTSB1* and *PPHYB* as well as *PNCR* are not protected by patent in our search, thus these promoters have advantage in practical use.

The vectors used here have a structure suited to the construction of new vectors. Unique restriction sites that located at the ends of each of the promoters allow for the replacement of any part of the fusion gene with other functional sequences.

Avoiding the repeated use of the same promoters could prevent homology-dependent gene silencing which has been frequently observed in transgenic plants containing multi-copies of transgenes. In this context, the availability of the three constitutive promoters characterized here would provide choice. Recently, Al-Kaff *et al.* (2000) reported that *Cauliflower mosaic virus* infection in transgenic oilseed rape plants results in a suppressed expression of the introduced herbicide tolerance gene regulated by *P35S*, indicating that this promoter derived from virus genome could trigger suppression of the transgene in the host plants. *PTSB1* and *PPHYB*, promoters of *Arabidopsis* housekeeping genes, would be useful in other plant species. We found that *PTSB1* and *PPHYB* functioned in tobacco plants as did *P35S*, and thus these promot-

Table 1 Transformation efficiency for various selectable marker genes in tobacco at 80 days after *Agrobacterium* infection

Km concentration	Promoter	No. of infected leaf sections (A)	No. of Km^R shoots (B)	No. of Km^R plants (C)	C/B	C/A
50 mgl^{-1}	TSB1	45	111	66	0.59	1.5
	PHYB	45	118	65	0.55	1.4
	NCR	45	71	45	0.63	1.0
	35S	45	106	68	0.64	1.5
150 mgl^{-1}	TSB1	45	59	47	0.80	1.0
	PHYB	45	45	36	0.80	0.80
	NCR	45	43	37	0.86	0.82
	35S	45	45	42	0.93	0.93
300 mgl^{-1}	TSB1	45	54	49	0.91	1.1
	PHYB	45	30	26	0.87	0.58
	NCR	45	36	33	0.92	0.73
	35S	45	38	36	0.94	0.80

ers would be widely available, at least in dicotyledonous plants. Actually we introduced *PTSB1::GUS* into carnations (*Dianthus* spp.) and detected a high level of GUS activity in leaves and roots (data not shown).

Although *P35S* has been described as a constitutive and strong promoter, it conferred different tissue specificities among plant species (Benfey and Chua, 1989; Terada and Shimamoto, 1990). Actually, *PTSB1* expression observed here was likely in this case. In transgenic *Arabidopsis* plants with *PTSB1::GUS*, the level of GUS activity is high in rosette leaves and roots, moderate in flower buds, and low in immature seed pods (Pruitt and Last, 1993). In contrast, our quantitative GUS assay demonstrated that the activity of *PTSB1* was strong in leaves, moderate in stems, sepals and petals, and weak in roots. In transgenic *Arabidopsis* sprouts, *PTSB1* was expressed only in the vasculature of hypocotyls and petioles of cotyledons and it seemed to be not induced by wounding or stress (Pruitt and Last, 1993). In our observation, strong expression of PTSB1 was detected in whole cotyledons (**Fig. 5B**). It has been reported that *PPHYB* is induced by far-red light and wounding and expressed extensively throughout *Arabidopsis* plants, including roots, shoots and flowers during the entire life cycle (Somers and Quails, 1995). However, our result showed that *PPHYB* was most active in leaves independent of development and also active in stems, petals and roots in tobacco plants.

Because of the possible enhanced effect of a modified *P35S* which drives Hyg^R at the down stream of the GUS fusion gene, GUS activity originated from promoter::*GUS* gene in pMLH could not be strictly compared to that from *P35S::GUS* gene in pBI121 in transgenic plants. However, the distance between the two promoters is far as 2.4 kb so that transcription of the promoter::*GUS* gene might be not significantly affected by the presence of a modified *P35S*. Moreover, actually *PTSB1* and *PPHYB* would confer higher expression, as considered by the fact that even *PNCR* was capable of driving a selectable marker gene on generating transgenic rice plants (Fukuoka *et al.*, 2000). Thus, our results showed that the three constitutive promoters *PTSB1*, *PPHYB* and *PNCR* differ slightly in tissue-specific expression, but they would be widely available to drive foreign genes for both basic science and practical use.

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