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 (PTSBI) and a phytochrome B gene (PPHYB) as alternatives to the 35S RNA promoter (P35S) of Cauliflower mosaic virus. Characteristics of the Soybean chrototic mottle virus promoter (PNCR) were also studied for comparison. In transgenic calli, GUS gene fused with PTSBI, 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 PTSBI 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 PTSBI 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 chrototic mosaic virus, transgenic tobacco, tryptophan synthase.

Abbreviations

PTSBI, 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 chrototic mosaic 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; Tml, the terminator sequence of the tumor morphology large (tml) gene in A. tumefaciens; Kmβ, kanamycin resistant; Hygβ, 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 chlorotic 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 \( \beta \) subunit gene (PTSB1, Berlin et al., 1989; Pruitt and Last, 1993). The Trp synthase protein \( \beta \) 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 Ouail, 1995a, b) which was reported to be expressed throughout the plant. To evaluate the usefulness of the two Arabidopsis promoters in a heterogenous 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'–CAGTAAAGCTTCAGCCTCGCATCCTCCTAGAAGT–3' (corresponding to nucleotide positions 1 to 26; accession number M23872) and 5'–TAGGGATCTTGGATTACGCTCTCCTAGT–3' (1571 to 1550) containing HindIII and BamHI 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'–CAGTAAAGCTTGGCCGCGTGCAGCATTGCCAGCCTCGGT–3' (corresponding to HindIII and NotI sites and the sequence corresponding to nucleotide positions 1 to 22 (accession number L90262) and 5'–TGACGGATCTGACGAGTTCCTCTCGG–3' (2370 to 2349) containing BamHI site in the 5' flanking region. The reactions were run at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for 30 cycles. The PCR products were ligated to TA cloning vector pCR\(^{\text{TM}}\)2.1 (Invitrogen), and verified by DNA sequencing. Each fragment excised from pCR\(^{\text{TM}}\)2.1 by HindIII and BamHI digestion was subcloned into the binary vector pMLH (Mochizuki et al., 1999), which is a derivative of pBI121 (Clontech), previously digested with HindIII and BamHI, 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 BamHI and SacI sites of pCR\(^{\text{TM}}\)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'–GCAAGCTTGGTAAATCTCCAGC–3' corresponding to the GUS coding region and an antisense primer sequence 5'–TTATTGGCAAATGTTGAACG–3' corresponding to the nopaline synthase gene terminator region. For introduction of promoter::NPTII fusion constructs, kanamycin-resistant (Km\(^{\text{R}}\)) shoots were selected on the medium containing 50, 150, or 300 mg l\(^{-1}\) kanamycin and transferred to hormone–free
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°C 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 PTBS1 and PPHYB in kanamycin-resistant tobacco calli and transgenic plants

DNA fragments of PTBS1 (Prutil 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 PTBS1 (pMLH-PTBS1-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-
crated kanamycin-resistant (KmⅠ) 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 PTBS1 was slightly lower than that conferred by P3SS 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 PTBS1 and PPHYB was 240% and 150% of that of the line with P3SS, respectively (Fig. 3). Thus, both PTBS1 and PPHYB conferred at least comparable levels of GUS activity to that of P3SS in mature leaves. The activity of PPHYB was greater than that of PNCR.

**Tissue-specific expression of PTBS1 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. PTBS1 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 PTBS1::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 PTBS1::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 PTBS1::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 PTBS1::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 PTBS1 plants (Fig. 5A–e, f). In comparison with the GUS staining profiles in PPHYB::GUS and PTBS1::GUS plants, GUS activity in P3SS::GUS plants was ubiquitous in mesophyll (mes) and epidermal cells (epi) but weak in small veins (sv) of mature leaves (Fig. 5A–
**PSTB1::GUS plants**

(A, C) Fluorometric GUS analysis in several organs in PSTB1::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.

Fig. 4 Tissue-specific and developmental GUS expression in PSTB1::GUS and PPHYB::GUS tobacco plants.

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

(A, C) Fluorometric GUS analysis in several organs in PSTB1::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.

The localization of GUS staining in the midrib of P35S::GUS plants was similar to that of PSTB1::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 PSTB1, PPHYB, PNCR or P35S (Fig. 5B). When five Km<sup>R</sup> 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 PSTB1::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 pTRAbased binary vectors containing NPTII under the control of PTSB1 or PPHYB (Fig. 1B). These constructs were introduced into tobacco plants by Agrobacteriummediated transformation. pTRA415(R) with P35S::NPTII (Ohshima et al., 1990) and pTN1 which contains PNCR::NPTII (Fukuhara et al., 2000) were used for control vectors. At 50 mg l⁻¹ of kanamycin, the number of regenerated Kmₐ 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 Kmₐ 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>
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<th>No. of Kmₐ shoots (B)</th>
<th>No. of Kmₐ plants (C)</th>
<th>C/B</th>
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Fig. 5  Histochemical GUS analysis of transgenic tobacco plants.

(A) Cross sections from the fully expanded leaves of 2-month-old self-pollinated progeny (T1) of the regenerated transformants were subjected to GUS staining for 8 h at 37°C.

a-c, \textit{PTSBI::GUS} plant (100 \(\mu\text{m}\) thick); d-f, \textit{PHHYB::GUS} plant (100 \(\mu\text{m}\) thick); g-i, \textit{P35S::GUS} plant (80 \(\mu\text{m}\) thick). c, f and i are the magnification of the indicated square in b, e and h, respectively. Bar = 200 \(\mu\text{m}\), sv, small veins of leaves; mes, mesophyll cells; epi, epidermal cells; phl, phloems; xp, xylem parenchyma cells; end, external endodermis.

(B) The seeds (T1) of transgenic plants were germinated on wet filter paper and incubated at 28°C in 16 h light and 8 h dark. Intact sprouts, 4 days after inhibition, were stained at 37°C for period indicated.

a and d, \textit{PTSBI::GUS} plant; b and e, \textit{PHHYB::GUS} plant; c, \textit{PNCR::GUS} plant; f, \textit{P35S::GUS} plant. Bar = 500 \(\mu\text{m}\).
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 Quail, 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<sup>e</sup> 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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