Expression of a Rice Chlorophyll a/b Binding Protein Promoter in Sweetpotato

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Abstract. Leaves are usually the target tissue for expressing transgenes conferring resistances to herbicides, pests, and diseases. To achieve leaf-specific expression, a light-harvest chlorophyll a/b binding protein (CAB) of photosystem-II (CAB2) promoter (CAB2-p) from rice (Oryza sativa L.) and the cauliflower mosaic virus 35S promoter were fused to the ß-glucuronidase (GUS) reporter and subsequently evaluated in transgenic sweetpotato (Ipomoea batatas L. (Lam.)). The 35S promoter-directed GUS activities varied from 46.0 to 61.2 nmol 4-methyl-umbelliferyl-ß-D-glucuronide (4-MU) per minute per milligram of protein in leaf, stem, primary, and storage roots. In contrast, the CAB2-p directed an uneven distribution of GUS activities (4-MU at 1.1 to 12.6 nmol min⁻¹ mg⁻¹ protein); GUS activity in mature leaves was ~12-fold as high as that in storage roots. In addition, GUS assay in leaf tissues revealed that CAB2-p enabled a developmentally controlled and light-regulated GUS expression. These results indicate that the rice CAB2-p could be used to drive leaf-specific expression of linked genes in sweetpotato.

The aim of this study was to evaluate the rice CAB2-p for directing leaf-specific expression of model transgenes in hexaploid sweetpotato plants.

Materials and Methods

Vector and Agrobacterium strain. The rice CAB2-p (Xbal-BamHI fragment), 848 base pairs (Sakamoto et al., 1991), was fused with ß-glucuronidase (GUS) in the binary vector backbone of pBI-H1 whose T-DNA region contains a hygromycin phosphotransferase gene (hpt) and a neomycin phosphotransferase gene (nptII), to give pBH1CG containing CAB2-p-GUS (Fig. 1). Agrobacterium tumefaciens (Smith and Towns) Conn. strain EHA105:pBH1CG was used for transformation. The binary vector pG121Hm containing a GUS expression cassette under the 35S promoter (35S-GUS) was previously introduced into sweetpotato plants (Song et al., 2004).

Transformation. Transformation using EHA105: pBH1CG was performed according to Song et al. (2004). In vitro cultures of sweetpotato ‘Beniazuma’ were maintained in Magenta GA7 (Magenta Corp., Chicago) boxes each containing 50 mL of medium (GM [Linsmaier and Skoog (1965) medium (LS) + 2.85 µM indole-3-acetic acid]). Unless otherwise mentioned, all media contained 0.32% gellan gum and all in vitro cultures were maintained at 26 °C under a 16-h photoperiod of 30 µmol-m⁻²-s⁻¹ using cool white fluorescent tubes. Stems, from 6-week-old stock cultures, and all leaves removed, were cut transversely into explants 6 to 10 mm in length and sliced in half along the axis were the explants. EHA105:pBH1CG was suspended in liquid embryogenic...
callus induction medium [ECIM (LS + 6.49 mm 4-fluorophenoxycetic acid)] containing 100 μM acetosyringone (AS) (Song et al., 2004). Explants were incubated with suspension cells (OD_{600} = 0.8) for 20 min at 30 °C. Cocultivation was carried out on ECIM + 100 μM AS in 100 × 20-mm petri dishes for 3 d in the dark. Selection of transformed embryogenic calluses first began with culture on ECIM + 50 mg·L^{-1} kanamycin (Km) + 250 mg·L^{-1} cefotaxime for 6 weeks and then transfer to ECIM + 30 mg·L^{-1} hygromycin + 250 mg·L^{-1} cefotaxime for 9 weeks both in the dark. Regeneration of transgenic plants from selected friable calluses was performed on regeneration medium (LS + 15.13 μM acesulfame acid + 2.89 μM gibberellic acid) for 4 weeks under a 16-h photoperiod of 30 μmol·m^{-2}·s^{-1}. Selected transgenic plants each with six to 10 leaves were planted in 20 × 30-cm (diameter × height) plastic pots using planting medium and placed in a growth chamber at 26 °C under a 16-h photoperiod at a light intensity of 50 μmol·m^{-2}·s^{-1} and 50% relative humidity.

Transgenic plants each with a single copy of 35S-GUS (Song et al., 2004) as well as nontransgenic plants were used as controls. They were all maintained under the same environmental conditions as those of the transgenic plants with CAB2-p-GUS.

Southern blot analysis. DNA samples were isolated from leaves of in vitro cultured plants according to the method described by Rogers and Bendich (1985). Twenty micrograms of DNA was digested with HindIII or PstI + EcoRI, electrophoresed in 0.8% agarose gel, and then blotted onto a Hybond N\(^{-}\)-nylon membrane (Amersham Biosciences, Piscataway, NJ). A 1.9-kb Smal-SacI fragment containing the GUS coding region was used as a probe. Labeling, hybridization, and detection were performed using the Alkphos Direct Kit (Amersham Biosciences) following the manufacturer’s instructions.

β-glucuronidase assays. Location of GUS activity in plant tissues was determined histochemically according to the procedure of Jefferson et al. (1987). All tissues for GUS staining were vacuum-infiltrated for 20 s, fixed for 30 min in 4% formaldehyde solution at room temperature, and incubated for 12 h at 37 °C in assay buffer containing 1 μM X-Gluc. The whole procedure was carried out in the dark. Chlorophyll was removed from the tissues using 70% ethanol rinses. Quantitative measurement of GUS activity was performed as described by Stomp (1992) with protein concentration measured according to Bradford (1976). GUS activities in different tissues were analyzed for significance by analysis of variance (ANOVA) with mean separation by standard deviation; PROC GENMOD (version 8.2; SAS Institute, Cary, NC) was used.

For in vitro plant tissues, GUS expression was assayed in the whole plantlet (4-week-old) and leaves excised from 8-week-old plantlets. To investigate the light response of CAB2-p-GUS expression, in vitro shoot tips or internode sections, 1 to 2 cm in length, were cultured in Magenta GA7 boxes containing each 50 mL GM + 30 mg·L^{-1} hygromycin for 7 d in darkness. Half of the obtained materials were submitted for GUS assays immediately and the remainder cultured for 2 d or more under a 16-h photoperiod at 30 μmol·m^{-2}·s^{-1} before GUS staining. GUS expression of potted plants was assayed using leaves, stems, storage, and primary roots. To enable more staining solution to enter the cells, the surface of the mature leaves was gently scored perpendicular to the midrib with a surgical blade, stem sections, 2 to 3 cm in length, were cut in half along the axis, and storage roots were either scored along the axis or sliced into disks perpendicular to the axis.

Results

Selection, regeneration, and molecular analysis of transgenic plants. Most inoculated stem explants, 56 of 90, produced Km-resistant calluses from the wounded edges in ≈6 weeks. After selection with 30 mg·L^{-1} hygromycin, 22 of 56 explants had bright yellow and friable callus clusters from which numerous regenerants formed through somatic embryogenesis. The 22 explants gave rise to 110 morphologically normal plants obtained after 20 weeks also had GUS-positive leaves, indicating that the two-step kanamycin–hygromycin selection method enables efficient production of transgenic sweetpotato plants (Song et al., 2004).

Southern hybridization showed that the expected 3.0-kb EcoRI-PstI fragment was detected in each of the eight randomly selected transgenic lines and was absent in the nontransformed control (Fig. 2A). When HindIII-digested DNA samples were hybridized to the 1.9-kb probe, the expected fragments were observed in all eight transgenic lines; six had a single band and two had two discrete bands; there was no signal in nontransformed plants (Fig. 2B). HindIII cuts outside of the

Fig. 1. Schematic representation of the T-DNA region of the binary vector pBIH175. P-nos = nopaline synthase (nos) promoter; NPTII = neomycin phosphotransferase coding region (kanamycin resistance); T-nos = nos gene terminator; P-cab2 = rice CAB2 promoter; GUS = β-glucuronidase coding region; P-3SS = CaMV 35S promoter; HPT = hygromycin phosphotransferase coding region (hygromycin resistance); RB = right T-DNA border; LB = left T-DNA border; PstI, EcoRI, and HindIII = unique restriction endonucleases at T-DNA region; CAB2 = chlorophyll a/b binding protein of photosystem-II; GUS = β-glucuronidase.

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**Table 1.** Southern hybridization analysis of the GUS reporter gene in sweetpotato. DNA samples were digested by PstI and EcoRI (A) and HindIII (B) and hybridized with a 1.9-kb probe containing the GUS coding region. Lanes 1, 3, 5, 8, 10, 12, 13, and 16, and transgenic lines CG1, CG3, CG5, CG8, CG10, CG12, CG13, and CG16; lane N, nontransformed wild type (N); lane M, DNA 1-kb marker. GUS = β-glucuronidase.
GUS gene and an uncertain site in the genomic DNA. Therefore, the differences in the patterns of the hybridization bands most likely represent the copy numbers as well as random integration of the GUS gene. These results confirmed stable integration of \( \text{CAB2-p-GUS} \). The six transgenic lines each with a single T-DNA insert were subsequently used for GUS assay.

**Leaf specificity of chlorophyll a/b binding protein of photosystem-II promoter—\( \beta \)-glucuronidase expression.**
A typical pattern of \( \text{CAB2-p-GUS} \) expression was present in all six transgenic lines. In 4-week-old plantlets grown in vitro under 16 h of 30 \( \mu \)mol-m\(^{-2}\)-s\(^{-1}\) light, blue staining indicating that the \( \text{CAB2-p-GUS} \) activity was detected and varied not only in green tissues (leaves, petioles, and stems), but also in roots containing no visible chlorenchyma (Fig. 3A). Similarly, blue staining in soil-grown plants, 16-h photoperiod, 50 \( \mu \)mol-m\(^{-2}\)-s\(^{-1}\) light, was stronger and not limited to the green tissues as leaves, petioles, and stems (Fig. 3B, C). Faint blue staining, indicating weak expression of \( \text{CAB2-p-GUS} \), was also observed in primary and storage roots (Fig. 3D, E). In addition, intense blue staining indicated more activity of \( \text{CAB2-p-GUS} \) in mesophyll, stem vascular, and the cortex and vascular cylinder of the developing storage root. Unlike the expression of \( \text{CAB2-p-GUS} \), the 35S directed a constitutive expression of GUS in all six transgenic lines each containing a single copy of the 35S-GUS. In vitro plants displayed high GUS activity (dark blue staining) in the whole plant (Fig. 3F). Similarly, a high level of expression of the 35S-GUS (intense blue staining) was present in leaves, stems, and primary and storage roots from potted plants (Fig. 3G–J). No blue staining was observed in nontransformed plant tissues.

GUS activities directed by the \( \text{CAB2-p} \) were consistent with histochemical GUS staining and showed variability for different plant organs. The highest GUS activity, 4 MU at 12.6 nmol-min\(^{-1}\)-mg\(^{-1}\) protein, was observed in the mature leaves. This contrasts with an 11-fold decrease in GUS activity in developing storage roots (Table 1). In comparison, GUS activity directed by the 35S promoter was similar in leaves, stems, and roots (Table 1). These results showed that use of the rice \( \text{CAB2-p} \) enabled a leaf-specific directed expression of linked genes in the green tissues of sweetpotato.

**Developmental regulation of chlorophyll a/b binding protein of photosystem-II promoter—\( \beta \)-glucuronidase expression.** Variation in \( \text{CAB2-p-GUS} \) expression was observed in stained in vitro leaves. Leaves at the apex stained less intensively in comparison with older leaves from the apical meristem as depicted in Figure 3A; however, staining diminished in old and yellow leaves. Similarly, expression of \( \text{CAB2-p-GUS} \) resulted in intensive blue staining in mature leaves and less in old leaves of the potted plants. In contrast to \( \text{CAB2-p-GUS} \), expression of the 35S-GUS yielded intense blue staining in all leaves from both the in vitro-cultured plants (Fig. 3F) and the potted plants (data not shown).

Developmental regulation of \( \text{CAB2-p} \) was assessed by assaying GUS activity in four leaf samples from the apical meristem for each of the six independent transgenic lines from in vitro-grown plants (Fig. 4A). \( \text{CAB2-p-GUS} \) activity was 5 to 6-fold higher in leaves 5 and 6 in comparison with leaves 9 and 10 (Fig. 4B). In contrast, the activity of the 35S promoter did not vary significantly among leaves (Fig. 4B).

**Light-regulated expression of chlorophyll a/b binding protein of photosystem-II promoter—\( \beta \)-glucuronidase activity.** Activity of the \( \text{CAB2-p-GUS} \) was also evaluated on etiolated in vitro tissue. Plant tissue forming in the absence of light was free of visible blue pigmentation (Fig. 5). However, after a 2-d exposure of the etiolated shoots to light conditions (16-h...
photoperiod, 30 μmol·m⁻²·s⁻¹), faint blue staining was detected (Fig. 5). In etiolated shoots with the 35S-GUS, intense blue staining was not obviously influenced by light conditions (Fig. 5).

**Discussion**

**Leaf-specific responses for the rice chlorophyll a/b binding protein of photosystem-II promoter.** Expression patterns of the rice CAB2-p had been previously investigated in transgenic tobacco (Sakamoto et al., 1991) and rice (Tada et al., 1991), respectively. In tobacco, the rice CAB2-p was expected to be active only in chlorenchyma cells. However, the GUS activity directed by the CAB2-p was not completely restricted to chlorenchyma tissues, and the activity in leaves was ≈2-fold higher than that in roots (Sakamoto et al., 1991). The imperfect recognition of a monocot gene promoter in dicot systems was postulated to be responsible for the limited expression of the rice CAB2-p in nonchlorenchyma cells of tobacco. In rice, expression of the rice CAB2-p was tissue-specific, although the CAB2-p-GUS activity was also observed in nongreen organs such as anther, pollen, stigma, and roots of transgenic rice. The rice CAB2-p-GUS activity in leaves was ≈120-fold higher than that in roots (Tada et al., 1991). In sweetpotato, we found that the rice CAB2-p-GUS activity was significantly greater in leaves in comparison with primary roots (≈2-fold) (Table 1). Our results are consistent with previous research on tobacco (Sakamoto et al., 1991).

The detectable expression of rice CAB2-p in nonchlorenchyma cells of tobacco, rice, and sweetpotato plants reflects the leaf-specific characteristic of the rice CAB2-p. In higher plants CAB products, which bind chlorophyll a/b and assemble it into the light-harvest complex, it is controlled by a complex of regulatory networks related to phytochrome (Bischoff et al., 1997; Karlin-Neumann et al., 1988; Piechulla et al., 1991; Thompson and White, 1991), development (Brusslan and Tobin, 1992; Chang and Walling, 1992; Kretsch et al., 1995), and circadian rhythms (Kaldis et al., 2003; Millar and Kay, 1991; Sugiyama et al., 2001). Native CAB expression in dark-grown seedlings has also been reported in Arabidopsis thaliana (L.) Heynh. (Brusslan and Tobin, 1992) and soybeans [Glycine max (L.) Merr.] (Chang and Walling, 1992). Our results are consistent with theoretical expectations.
of previous research, which suggest the CAB promoter is only active in tissues containing chloroplasts.

Storage root is usually the harvest target for most sweetpotato cultivars. Our results showed that the CAB2-p-GUS in leaves was greater than activity in roots by a factor of 12, thereby indicating the potential use of the rice CAB2-p for devising leaf-specific gene expression in sweetpotato.

**STRENGTH OF THE RICE CHLOROPHYLL A/B BINDING PROTEIN OF PHOTOSYSTEM-II PROMOTER.** Expression levels of linked genes are generally related to promoters as well as plant species (Ni et al., 1995; Wilmink et al., 1995). GUS activity with the rice CAB2-p in leaves of transgenic rice was approximately five times as high as that of rice CAB2-p. However, the expression level of the CAB2-p-GUS in sweetpotato leaves is much higher than reported in tobacco leaves (Sakamoto et al., 1991). Thus, both the rice CAB2-p and the 3SS promoter have great potential to direct high-level expression of linked genes in sweetpotato. In addition, further studies are still required to determine if the activity of the rice CAB2-p is sufficiently active for directing transgene expression in sweetpotato leaves.

**REGULATION OF THE RICE CHLOROPHYLL A/B BINDING PROTEIN OF PHOTOSYSTEM-II PROMOTER.** We have demonstrated that the rice CAB2-p is developmentally regulated in sweetpotato plants with a higher level of CAB2-p-GUS in developing leaves versus old ones. This suggests that the rice CAB2-p can be used to express herbicide- or disease-resistant genes targeted to sweetpotato leaves. Light regulation of the rice CAB2-p in sweetpotato is similar to what has been observed in *A. thaliana* transformed with various light-regulated promoters of CAB or rbcS genes (Gao and Kaufman, 1994).

In conclusion, the rice CAB2-p directed a leaf-specific, development-controlled, and light-regulated GUS expression in sweetpotato. Although the activity of the CAB2-p was approximately 20% the activity of the 3SS promoter in mature leaves of sweetpotato, the leaf-specific manner of the CAB2-p enables its potential application for driving leaf-specific expression of transgenes in sweetpotato plant.

**Literature Cited**


