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 β -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- β -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 $\approx$ 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.

Sweetpotato is one of the most important food crops in many developing countries as a result of its nutritional value, ease of cultivation, and high productivity of storage roots. It is also an important industrial material for producing starch, sugar, and alcohol (Otani and Shimada, 2002). Transformation provides a powerful tool for genetic manipulation of sweetpotato by efficiently introducing genes of interest for either improvement of crop traits or obtaining novel materials for industry (Otani and Shimada, 2002; Otani et al., 2003; Wakita et al., 2001). To obtain sufficient expression levels of transgenes in target tissues of genetically engineered sweetpotato plants, the selection of promoters is crucial. To date, few tissue-specific promoters have been evaluated in sweetpotato as well as constitutive promoters (Gama et al., 1996; Kimura et al., 2001; Morán et al., 1998; Newell et al., 1995; Song et al., 2004; Wakita et al., 2001). Leaf-specific promoters are preferable to constitutive promoters when transgenes, including most of those conferring resistances to herbicides, insects, and diseases, are targeted principally in green tissues of sweetpotato. This is because leaf-specific instead of constitutive expression of target genes can help to minimize the public concerns about the impact of transgene expression on food safety in nontargeted storage roots.

Light-harvesting chlorophyll a/b binding protein promoters are an important source for leaf-specific promoters (Churin et al., 2003; Lamppa et al., 1985; Luan and Bogorad, 1992; Nagy et al., 1986; Piechulla et al., 1991; Simpson et al., 1986; Sugiyama et al., 2001). Fourteen genes encoding members of the tomato (*Solanum lycopersicum* L.) chlorophyll a/b binding protein family were found to be expressed in leaf tissue (Piechulla et al., 1991). Rice phytochrome was found biolog-

ically active in transgenic tobacco (*Nicotiana tabacum* L.) (Kay et al., 1989). The chlorophyll a/b binding protein of photosystem-II promoter (*CAB2*-p) isolated from photosystem-II of rice displayed high activity in green tissues of transgenic rice (Tada et al., 1991; Tsuchida et al., 2001) and was also found active in tobacco (Sakamoto et al., 1991). Therefore, rice *CAB2*-p is likely an effective promoter for directing efficient expression of linked genes in green tissues of 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 (*Xba*I-*Bam*HI fragment), 848 base pairs (Sakamoto et al., 1991), was fused with β -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 pBIH1CG containing *CAB2*-p-GUS (Fig. 1). *Agrobacterium tumefaciens* (Smith and Towns) Conn. strain EHA105:pBIH1CG was used for transformation. The binary vector pLG121Hm 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:pBIH1CG 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 μ M 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 μ mol·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:pBIH1CG was suspended in liquid embryogenic

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Fig. 1. Schematic representation of the T-DNA region of the binary vector pBIH1CG. 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-35S = 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.

callus induction medium [ECIM (LS + 6.49 mM 4-fluorophenoxyacetic acid)] containing 100 μ M acetosyringone (AS) (Song et al., 2004). Explants were incubated with suspension cells ($OD_{600} = 0.8$) for 20 min at 30 $^{\circ}$ C. Cocultivation was carried out on ECIM + 100 μ M AS in 100 $\times$ 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 μ M abscisic acid + 2.89 μ M gibberellic acid) for 4 weeks under a 16-h photoperiod of 30 μ mol·m⁻²·s⁻¹. Selected transgenic plants each with six to 10 leaves were planted in 20 $\times$ 30-cm (diameter $\times$ height) plastic pots using planting medium and placed in a growth chamber at 26 $^{\circ}$ C under a 16-h photoperiod at a light intensity of 50 μ mol·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 *SmaI*-*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 $^{\circ}$ C in assay buffer containing 1 μ M 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; PROCGENMOD (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 μ mol·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 $\approx$ 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 non-transformed 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. 2. 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, λ DNA 1-kb marker. GUS = β -glucuronidase.

Organs from soil-grown plants

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 *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— β -GLUCURONIDASE EXPRESSION. A typical pattern of *CAB2*-p-GUS expression was present in all six transgenic lines. In 4-week-old plantlets grown in vitro under 16 h of $30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light, blue staining indicating that the *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, $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ 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 *CAB2*-p-GUS, was also observed in primary and storage roots (Fig. 3D, E). In addition, intense blue staining indicated more activity of *CAB2*-p-GUS in mesophyll, stem vascular, and the cortex and vascular cylinder of the developing storage root. Unlike the expression of *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 *CAB2*-p were consistent with histochemical GUS staining and showed variability for different plant organs. The highest GUS activity, 4 MU at $12.6 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ 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 *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— β -GLUCURONIDASE EXPRESSION. Variation in *CAB2*-p-GUS expression was observed in stained in vitro leaves. Leaves at the apex stained

Fig. 3. Expression of *CAB2*-p-GUS and 35S-GUS in transgenic sweetpotato CG10 (A–E) and S8 (F–J), respectively. All tissues were stained at 37 °C for 12 h in the dark. The circle in the upper corner of 3E is a stained root section. Bars = 1 cm, except in (C) and (H). *CAB2*-p-GUS = chlorophyll a/b binding protein of photosystem-II promoter— β -glucuronidase.

Table 1. Expression of *CAB2*-p-GUS in different tissues of transgenic sweetpotato plants.

Plant tissue	4-methyl-umbelliferyl- β -D-glucuronide [mean $\pm$ SD (nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ protein)]		
	Nontransformed control ^a	<i>CAB2</i> -p-GUS ^b	35S-GUS ^c
Leaf	0.3 $\pm$ 0.1	12.6 $\pm$ 1.8	61.2 $\pm$ 15.5
Stem	0.3 $\pm$ 0.1	9.2 $\pm$ 0.4	54.6 $\pm$ 11.5
Primary root	0.3 $\pm$ 0.1	4.3 $\pm$ 1.4	57.3 $\pm$ 15.4
Storage root	0.2 $\pm$ 0.1	1.1 $\pm$ 0.2	46.0 $\pm$ 7.8

^aMean value of six nontransgenic plants.

^bMean value of six independent transgenic events each containing a single copy of *CAB2*-p-GUS.

^cMean value of six independent transgenic events each containing a single copy of 35S-GUS.

CAB2-p-GUS = chlorophyll a/b binding protein of photosystem-II promoter— β -glucuronidase.

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 *CAB2*-p-GUS resulted in intensive blue staining in mature leaves and less in old leaves of the potted plants. In contrast to *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 *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). *CAB2*-p-GUS activity was $\approx$ 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— β -GLUCURONIDASE. Activity of the *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

Fig. 4. Effect of leaf age on GUS expression in sweetpotato plants. (A) Leaf positions (the fifth or sixth leaf is usually the first fully expanded leaf cultured in vitro; the ninth and 10th leaves are senescing). (B) Expression of *CAB2*-p-GUS (single copy lines: CG1, CG3, CG5, CG8, CG10, and CG12) and 35S-GUS (single copy lines: S1, S2, S3, S6, S7, and S8) in sweetpotato leaves from different positions. 4-MU = 4-methyl-umbelliferyl- β -D-glucuronide. Vertical bars indicate the sds. *CAB2*-p-GUS = chlorophyll a/b binding protein of photosystem-II promoter- β -glucuronidase.

Fig. 5. Expression of *CAB2*-p-GUS (line CG10) and 35S-GUS (line S8) in sweetpotato tissues cultured under different light conditions. In vitro shoot tips or internode sections were cultured 7 d in darkness and subsequently submitted for GUS assays either immediately or after a 2-d culture under a 16-h photoperiod at $30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ staining. Bars = 1 cm. *CAB2*-p-GUS = chlorophyll a/b binding protein of photosystem-II promoter- β -glucuronidase.

photoperiod, $30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), 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).

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

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 assembles 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

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; Wilmsink et al., 1995). GUS activity with the rice *CAB2*-p in leaves of transgenic rice was ≈ 10 times higher than with the 35S promoter. In sweetpotato mature leaves, GUS activity directed by the constitutive 35S promoter 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 35S 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 $\approx 20\%$ the activity of the 35S 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.

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