Comparative expression analysis of two sugarcane polyubiquitin promoters and flanking sequences in transgenic plants

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Summary

GUS (uidA) reporter gene expression for two sugarcane polyubiquitin promoters, ubi4 and ubi9, was compared to expression from the maize Ubi-1 promoter in stable transgenic rice (only ubi9) and sugarcane (ubi4 and ubi9). Ubi9 drove high-level GUS expression, comparable to the maize Ubi-1 promoter, in both callus and regenerated plants of rice transformed by Agrobacterium. This high level expression was inherited in R1 plants. Expression from ubi4 and ubi9 was quite high in sugarcane callus transformed via particle bombardment. Expression dropped to very low or undetectable levels in the resulting plants; this drop in expression resulted from PTGS. PTGS in regenerated sugarcane plants also occurred with the maize Ubi-1 promoter. In sugarcane callus, ubi4 was HS inducible, but ubi9 was not. This physiological difference corresponds to a MITE insertion that is present in the putative HSEs of ubi9 but not present in ubi4.

Key words: gene expression – monocot – promoter – PTGS – rice – sugarcane

Abbreviations: GUS = β-glucuronidase. – HS = heat shock. – HSE = heat shock element. – MARs = nuclear matrix attachment regions. – MITE = miniature inverted-repeat transposable element. – NOS = nopaline synthase terminator. – nptII = neomycin phosphotransferase II. – PTGS = post-transcriptional gene silencing. – UTR = untranslated region. – WT = wild-type

Introduction

We previously reported the isolation of two polyubiquitin genes, ubi4 and ubi9, from sugarcane and characterization of these promoters in transient expression assays in tobacco, sugarcane (Wei et al. 1999), maize, sorghum, banana, pineapple, and garlic (Wei 2001). Our purpose was to increase the number of constitutive promoters available for monocot transformation. By constitutive we mean high levels of expression in most or all plant organs in the absence of an inducing stimulus, not necessarily equal expression in every organ. We now report further characterization of these promoters in stable transgenic sugarcane and rice.

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The maize polyubiquitin promoter Ubi-1 (Christensen et al. 1992) has been tested with transient assays in a wide range of monocot species (Wilmink et al. 1995, Christensen and Quail 1996), including rice (Cornejo et al. 1993) and sugarcane (Gallo-Meagher and Irvine 1993). In both rice and sugarcane, these assays found that Ubi-1 produced more expression foci than other tested promoters, including the rice actin Act1 promoter (McElroy et al. 1990, McElroy et al. 1991), the pEmu promoter (Last et al. 1991, Chamberlain et al. 1994), and some configurations of the Cauliflower Mosaic Virus 35S promoter. Additionally, Ubi-1 has been used to produce stable transgenic rice (Cornejo et al. 1993) and sugarcane (Gallo-Meagher and Irvine 1996, Ma et al. 2000). For these reasons, Ubi-1 is often used as a standard of comparison when characterizing new monocot promoters (Schenk et al. 1999, Wang et al. 2000, Schenk et al. 2001). We also chose the maize Ubi-1 promoter as a standard of comparison.

In rice the sugarcane ubi9 promoter and the maize Ubi-1 promoter produced numerous callus lines that expressed β-glucoronidase (GUS) at high levels. These high levels were maintained (or increased) in regenerated plants and through the R1 generation. In sugarcane both of the sugarcane ubi promoters and the maize Ubi-1 promoter produced numerous callus lines that expressed GUS at high levels. In regenerated sugarcane plants, however, all of the GUS reporter constructs were silenced. To determine what type of silencing had occurred, nuclear run-off experiments were conducted to assay transcription of GUS and several other genetic elements.

The maize Ubi-1 promoter is induced by heat shock (HS); however, the sugarcane gene sub-family containing the ubi4 and ubi9 genes appears not to respond to HS (Wei et al. 1999). Transgenic sugarcane callus lines containing all three of these promoters were tested for induction of GUS expression by HS.

Analysis of the nucleotide sequence flanking the ubi4 gene revealed the presence of numerous motifs associated with nuclear matrix attachment regions (MARs). To test whether these elements had any effect on transgene expression, GUS expression constructs with or without upstream and downstream putative MARs were compared in transgenic rice lines.

**Materials and Methods**

**Plasmid construction**

Expression constructs publi4-GUS and publi9-GUS contain the indicated promoter (including its 5′ untranslated region [UTR] and intron) driving a GUS reporter gene (Jefferson et al. 1986) followed by a nopalin synthase (NOS) terminator in pUC19, as previously described (Wei et al. 1999). Intron deletion cassettes ubi4ΔI-GUS and ubi9ΔI-GUS were made by replacing the HindIII-XbaI fragments containing the promoter, 5′UTR, and intron of ubi4-GUS and ubi9-GUS, respectively, with PCR fragments containing the same promoter and 5′UTR, followed immediately by an XbaI site. Intron deletion constructs were not used for plant expression experiments reported here, but fragments of them were used as probes in the nuclear run-off experiments (see below). pAH227 (Ubi-1-GUS) is a similar GUS expression construct that uses the maize Ubi-1 promoter (also including its 5′UTR and intron) (Christensen and Quail 1996).

Binary plasmids pCAM-ubi9-GUS and pCAM-Ubi-1-GUS were made by inserting HindIII-EcoRI fragments containing the ubi9-GUS and Ubi-1-GUS expression cassettes into pCAMBIA1300 (www.cambia.org).

pCAM-5′M-ubi9-GUS-3′M adds putative MARs from the ubi4 gene to the ubi9-GUS cassette in pCAM-ubi9-GUS (see Fig. 1 for expression cassettes). A 2 kb fragment beginning just downstream of the stop codon and including the putative 3′MAR was amplified (upstream primer: GCACGTCGACAGCTGTCCTTCCAGGTTCAC, downstream primer: ATTTAATCCCGCGCTACACTGGCTGTTCC). The PCR product was blunt-end cloned into the SrfI site of pCRScript (Stratagene) and orientation confirmed by PCR. This fragment was excised as a SacI-EcoRI fragment and used to replace the SacI-EcoRI fragment containing the NOS terminator of pCAM-ubi9-GUS, forming pCAM-ubi9-GUS-3′M. A 2.3 kb fragment containing a putative MAR upstream of the ubi4 gene was inserted into the HindIII and EcoRI sites of pBluescript II KS+ (Stratagene). This fragment was then released with KpnI and XbaI and cloned into the same sites of pGEM-4Z (Promega). The insert was then released as a HindIII-HindIII fragment and cloned into the HindIII site 5′ of the ubi9 promoter in pCAM-ubi9-GUS-3′M, forming pCAM-5′M-ubi9-GUS-3′M. Orientation of the 5′MAR containing insert was confirmed by PCR.

PCR for expression constructs was performed with the Expand High Fidelity PCR (Boehringer) mix of polymerases per manufacturer’s instructions to reduce the likelihood of errors in amplification.

**Plant transformation and growth**

**Rice**

*Agrobacterium* (EHA 105) -mediated transformation of rice (*Oryza sativa* cv. Taipei 309) was performed with mature seed, scutellum-derived callus as described (Hiei et al. 1994). Selection was performed on 2N6 media (Hiei et al. 1994) containing 50 µg/mL hygromycin B (Sigma) and 250µg/mL cefotaxime (Agri-Bio) to eliminate Agrobacterium. After regeneration, plants were transferred to pots immersed in tubs of water in a greenhouse.

**Sugarcane**

Embryogenic sugarcane calli (*Saccharum* hybrid cv. H62-4671) were bombarded as described (Ma et al. 2000). GUS reporter constructs were co-bombarded with pHAB, a selection plasmid that contains the neomycinphosphotransferase II (*nptII*) gene driven by the maize Ubi-1 promoter. Bombarded calli were initially placed on MS1 (Ma et al. 2000) plates without selection. After 5–7 days, calli were transferred to MS1 plates with 50µg/mL G418 (Agri-Bio). After four weeks, calli were transferred to MS1 plates with 100 µg/mL G418. G418 resistant calli were proliferated on MS1 media and then placed on MS0 (Ma et al. 2000) media for regeneration. Regenerated plants were transferred to pots in a greenhouse and then to a field test plot.
**Histochemical GUS staining**

Plant tissue was incubated in GUS staining solution containing 50 mmol/L sodium phosphate buffer, pH 7.4, 10 mmol/L EDTA, 0.1 % Triton X-100, and 2 mmol/L X-Gluc (Clontech, #8080–2) for 12–16 h at 37°C. Leaf tissue in staining buffer was briefly placed under vacuum several times to aid substrate penetration. Chlorophyll was extracted from green tissues in 70 % ethanol after incubation in GUS staining solution.

**GUS activity assays**

Quantitative analysis of GUS activity was performed using the GUS-Light kit (Tropix) according to the manufacturer’s protocol. Purified *E. coli* GUS (Sigma, cat. # G7396) was used as a standard. Chemiluminescence was quantified on an MLX microplate luminometer (DYNEX). Protein quantification (Bradford 1976) was performed using the Bio-Rad Protein Assay Kit II (500-0002) with BSA as a standard. Average values, from multiple samples and replicate measurements, and standard deviations were calculated with Microsoft Excel. Data for expression after HS were presented as percent change. Absolute expression values before and after HS were analysed by Student's t test to determine if the observed changes were significant at P<0.05.

**Quantitation of NPTII protein**

Crude protein extracts were prepared by grinding approximately 100 mg of leaf tissue in liquid nitrogen and mixing with 0.5 mL of extraction buffer containing 10 mmol/L sodium sulfite, 2 % (w/v) polyvinylpyrrolidone (average mol. wt. 40,000), 3 mmol/L sodium azide, and 2 % Tween–20 in 1X PBS-T (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L NaHPO4, and 2 mmol/L KH2PO4). The samples were centrifuged at 20,000 g for 15 min at 4°C. Concentration of NPTII protein in the supernatant was determined by ELISA using the PathoScreen kit for NPTII (Agdia psp7300) according to manufacturer’s protocol. Protein quantification (Bradford 1976) was performed using the Bio-Rad Protein Assay Kit II (500-0002) with BSA as a standard.
Nuclear run-off assays

Nuclei were isolated from the leaves of field-grown sugarcane plants and run-offs performed as previously described (Ingelbrecht and de Carvalho 1992). Approximately 2×10⁶ nuclei were used per run-off assay. Concentration of nuclei was estimated by purifying DNA from an aliquot of nuclei and determining the OD₂₆₀. Each sugarcane nucleus was assumed to contain 8 pg DNA (Bennett and Smith 1976). Slot blots were prepared by applying purified (GeneClean spin, Bio 101) DNA fragments (1 µg per slot) to Hybond-N+ membranes (Amer-sham) following the manufacturer’s recommendations. Probe DNA was gel purified for the sequence of interest. The ubi4 promoter probe was a 0.4 kb HindIII-Xbal fragment from ubi4-Δ1-GUS (Fig. 1); the ubi9 promoter probe was a 2.3 kb HindIII-Xbal fragment from ubi9-Δ1-GUS (Fig. 1). The ubi4 and ubi9 probes did not include intron sequence but did include the 64 bp 5′ UTR. The Ubi-1 promoter probe was a 0.7 kb HindIII-Xhol fragment from pAH27 (see Ubi-1-GUS, Fig. 1), which contains only upstream sequence with no 5′ UTR or intron. The GUS probe was a 1.9 kb BamHI-BamHI fragment from ubi9-GUS (Fig. 1); the nptI probe was a 0.8 kb SalI-SalI fragment containing only the nptII gene from pHA9 (see above); the pUC probe was linearized pUC19, the nos 3′ probe was a 0.26 kb PCR product amplified from ubi4-GUS. A 1.2 kb EcoRI-EcoRI insert from scubi561, a sugarcane polyubiquitin cDNA (Albert et al. 1995), was used as an endogenous control. Ubiquitin coding sequence is highly conserved so the scubi561 probe is expected to hybridize to transcripts from all members of the polyubiquitin gene family. After hybridization and stringency washes, blots were exposed to Marsh Blue Sensitive autoradio-graph film (Marsh Bioproducts) with 2 Lightning Plus intensifying screens (DuPont 200224) for 48 to 72 h at –80°C.

Results

Expression in transgenic rice

GUS reporter constructs pCAM-ubi9-GUS and pCAM-Ubi-1-GUS were introduced into rice calli by co-cultivation with Agrobacterium. Nine hygromycinR callus lines harboring ubi9 (seven lines) and Ubi-1 (two lines) were chosen for GUS expression analysis. With the exception of four lines (ubi9 lines 6, 9, 16, and 30, which were not tested), these lines were judged to be independent transformation events, based on Southern analysis (data not shown). Quantitative analysis of GUS expression in these callus lines showed that the strongest line accumulated GUS protein to approximately 0.1 % of soluble protein, with several lines accumulating 0.05 % or greater (Fig. 2A).

Not all rice callus lines were successfully regenerated. GUS expression in leaves, stems, and roots was analyzed when regenerated plants grew to 3 months old (Fig. 2B). Comparison of the relative expression levels revealed similar ranking among calli and plant lines; however, GUS expression in differentiated tissues was more than ten-fold higher than that in calli when normalized to total soluble protein. On a protein basis, roots accumulated more GUS than leaves or stems; however, the majority of this difference results from the lower levels of total protein in roots.

Several lines were grown to maturity, and seeds were grown to assay GUS expression in the R₁ generation. GUS expression in all these lines remained high, with one line (ubi9 line #30) producing 2.6 %, 0.7 %, and 0.8 % of soluble protein in roots, stems, and leaves, respectively.

Expression in transgenic sugarcane

GUS reporter constructs publi4-GUS, publi9-GUS, and pAH27 were introduced into embryogenic sugarcane callus by particle bombardment. The great majority of calli surviving selection showed GUS activity detectable by histochemical staining. Many of these produced uniform dark blue staining. Seventeen GUS+ callus lines for each publi4-GUS and publi9-GUS construct and nine GUS+ callus lines with pAH27 were chosen for expression analysis. These lines are thought to be independent; however, because of the large number of unresolved bands on genomic Southern (data not shown), this was not confirmed for all lines. Quantitative expression of the GUS reporter gene in each transgenic callus line was determined soon after recovery (Fig. 3). The strongest line accumulated GUS protein to approximately 0.5 % of soluble protein, with numerous lines accumulating greater than 0.1 %.

Plants were successfully regenerated from the majority of the transgenic sugarcane lines. Histochemical staining was then employed to monitor expression of the GUS reporter gene during plant development. GUS activity was detectable in the young seedlings grown in vitro but progressively decreased to very low levels after the seedlings were planted in soil and transferred to the greenhouse. Subsequent examinations during 9 months of greenhouse growth and more than 6 months of field growth found that GUS activity remained at very low or undetectable levels, with the highest line accumulating GUS at 0.0003 % of total protein. Apparently some form of transgene silencing was triggered in all of these sugarcane lines during or after plant regeneration.

Accumulation of neomycin phosphotransferase II protein (NPTII) was determined in leaf tissues of 38 mature, field-grown sugarcane lines, including 21 lines produced for the present study. For each of these lines, maize Ubi-1 was used to drive the nptII gene. NPTII accumulation was below 0.1 % of total protein for all of these lines, with only two lines averaging more than 0.05 %, and many lines below the limits of detection. Individual leaf samples from three lines did exceed 0.1 % (the highest being 0.3 %); however, these relatively high levels were not reproduced in other samples from the same line. Each sugarcane plant (or stool) consists of numerous stems (or culms). Variation between samples from the same plant may represent sectors of expression/silencing or may be a non-systemic stress response, as has been previously observed with the Ubi-1 promoter (Takimoto et al. 1994).

Overall, accumulation of NPTII was quite low in mature plants. Quantitative assays of NPTII accumulation were not done while these lines were in callus stage, so we do not know
whether the low levels observed in plants represent a decrease from callus expression, as was observed for GUS.

Nuclear run-off assays in sugarcane

Nuclear run-off assays showed that the GUS and nptII transgenes were being transcribed in all five tested, silenced, transgenic lines (Fig. 4). As transgene transcription is occurring at a significant rate when compared to the endogenous polyubiquitin genes, the low level or absence of transgene protein indicates that the silenced plant lines have undergone post-transcriptional gene silencing (PTGS). In addition to the GUS gene, pUC vector sequences and in some lines the Ubi-1 promoter (present in the selection cassette of all lines) were also transcribed. Because the ubi4 and ubi9 promoter probes contained the 5′ UTR sequence, we cannot determine whether the hybridization to these probes may represent transcription of promoter sequences. This is consistent with complex and/or rearranged transgene loci, wherein sequences not intended to be transcribed become inserted downstream of transgene or endogenous promoters. Transcription of the nos 3′ region, present on both the GUS and nptII genes, appeared to be very low or undetectable. This may be due to the short length of this fragment or unknown factors causing poor hybridization. Transcription of vector and promoter se-
Effect of HS on transgene expression

Transgenic sugarcane calli growing at 26 °C were moved to 37 °C or 42 °C for 2 h, and then assayed for GUS activity. Fig. 5A shows the relative change of GUS activity following HS treatment. In sugarcane calli, GUS expression driven by the ubi4 promoter increased substantially, with one line increased 650% and an average of 185%, after HS. In three of the five tested ubi4 lines and in three of the four tested Ubi-I lines, the increase in expression after HS was significant (Student’s t test, P < 0.05). In contrast the ubi9 promoter did not produce increased GUS expression after HS, but rather showed a small decrease; two lines showed no significant change and two lines showed a significant decrease. The HS induction of GUS expression in ubi4 lines was similar to the induction seen in lines containing Ubi-I, which has been shown to be HS inducible (Christensen et al. 1989, 1992).

To test if this HS response occurs in rice plants, four ubi9 lines were moved from 26 °C to 42 °C for 2 h. Average GUS expression was substantially reduced after HS— in some cases the decrease was significant, in others it was not; however, none of the lines showed an increase in GUS expression after HS (Fig. 5B). This supports the hypothesis that the ubi9 promoter is not HS inducible.

Previously, we determined that the mRNA pool from the polyubiquitin gene sub-family that includes ubi4 and ubi9 is not significantly altered by HS (Wei et al. 1999). At first glance this appears to contradict the HS expression results of the present experiments. However, if some members like ubi4 of the sub-family are up-regulated while others like ubi9 are down-regulated, the result could be little net change in the combined mRNA pool.

The maize Ubi-I promoter is HS inducible and is described as having two overlapping HS element (HSE) consensus sequences (Christensen et al. 1992). This determination of the HSE consensus sequence (Pelham 1982) is 15 bp long. Later work characterized functional HSEs as consisting of 5 bp modules in alternating palindromic orientation, with a minimum of three modules required for activity, and an «invariant» G in position 2 of the module. In some cases spacing between modules was found critical for function (Scharf et al.
Sugarcane polyubiquitin promoters

Figure 5. Percent change in GUS expression after HS treatment in sugarcane callus lines (A) containing GUS controlled by the indicated promoter, and rice plant lines (B). * indicates significant change at $P<0.05$. All rice plant lines contain ubi9-GUS. $^a$contains pCAM-5'M-ubi9-GUS-3'M, see Methods.

Figure 6. A. Alignments of putative overlapping HSEs in Ubi-1, ubi4 and ubi9. Bold letters indicate the putative HSEs, invariant G/Cs are underlined.

B

By aligning ubi4 with Ubi-1, a good match to the overlapping HSEs can be found (Fig. 6). In the Ubi-1 HSEs, all four of the «invariant» G/C residues are present; three are present in ubi4 and ubi9. Because this region is 100% identical in ubi4 and ubi9, it seems inconsistent with the different HS response from the two promoters in transgenic calli. Further comparison of the two sugarcane promoters revealed two additional possible HSEs further upstream in ubi4. These potential HSEs show higher identity (67% and 80% vs. 47% and 47%) to the HSE consensus sequence as defined by (Pelham 1982). Again three invariant G/C residues are present. Unlike Ubi-1, the two HSEs are not overlapping, with 11 intervening nucleotides. Comparison of this region of ubi4 to a corresponding region of ubi9 finds 92% identity for the down-
stream HSE and the downstream part of the 11 intervening bases. Upstream from this point, however, the nucleotide identity ceases abruptly. This point marks the downstream end of an approximately 200 bp miniature inverted-repeat transposable element (MITE) previously described in the ubi9 promoter (Wei et al. 1999). Immediately upstream from this MITE is another possible HSE, 80% identical to the upstream ubi4 HSE. However, this HSE contains only one of the invariant G/C residues. The combination of this point mutation and the significantly altered spacing between the HSEs resulting from the MITE insertion may explain the loss of HS induction in the ubi9 promoter.

**Effect of putative MARs on transgene expression**

Analysis of the ubi4 genomic sequence with «MAR-Finder» (Singh et al. 1997, http://www.futuresoft.org/MAR-Wiz/) revealed regions both upstream and downstream of the gene containing numerous motifs associated with MARs. An expression construct, pCAM-5‘M-ubi9-GUS-3’M (see Methods and Fig. 1), flanking the ubi9-GUS cassette with these putative MARs was assembled and used to transform rice.

Quantitative assays of GUS expression were performed on callus (Fig. 7A) and R0 plants from the four lines that were regenerated (Fig. 7B) and compared to rice lines expressing
the ubi9-GUS construct without MARs. The average concentration of GUS protein in callus lines containing MARs was 102 % greater than in lines lacking MARs. However, because of the wide overlapping range in levels of expression, the difference was not significant at $P = 0.05$. The average GUS concentrations in the MARs-containing regenerated plant lines were also higher than in lines without MARs, but the difference was smaller than that seen in callus. In callus, variation between independent lines was not significantly affected by use of the putative MARs, with the coefficient of variation (cv) for lines without MARs at 0.82 and for those with MARs at 0.78. In differentiated plants, cv. values for lines with and without MARs were 0.99 and 0.15 in leaves, 0.97 and 0.47 in stems, and 0.40 and 0.30 in roots, respectively. Even with this small population size, the difference in cv. values for leaves was significant at $P = 0.01$. For callus, stems, and roots the differences were not significant, even at $P = 0.05$. It is possible that the MAR-like sequences flanking the ubi4 gene may provide some increase in expression and/or reduced variation between independent lines; however, our data do not clearly support this.

Discussion

In summary, these sugarcane polyubiquitin promoters were found to produce high levels of transgene expression in rice and sugarcane callus lines. In rice, where transformation was performed by Agrobacterium, this was also true in regenerated plants, and this high level of expression was inherited and remained stable through the R1 generation. In sugarcane plants, transgenes driven by these promoters underwent PTGS; however, this was also observed with the maize Ubi-1 promoter.

Hansom et al. (1999) found that transgenic sugarcane lines using the maize Ubi-1 promoter undergo PTGS less frequently than lines using other promoters; however, the measure of silencing they used is different from that used in our analysis. Using firefly luciferase as a reporter gene, they reported a range of luciferase expression from approximately 1,500 fg/µg protein to 50,000 fg/µg protein in plant lines scored as not silenced. This range means these lines accumulate luciferase at 0.00015 % to 0.005 % of total protein. This range is for the highest expressing organs in each line, with some organs in some lines at near zero.

While some of our sugarcane lines accumulated no detectable GUS protein after plant regeneration, others accumulated GUS up to 0.0003 % of total protein. Because this level was such a significant decrease from the levels observed in callus, we considered all the sugarcane plant lines to be silenced. However, the range for accumulation of GUS in our silenced lines overlapped with the range of accumulation of luciferase in the sugarcane plant lines scored as «not silenced» by Hansom et al. (1999). In our small sample, Ubi-1-GUS (pAHC27) lines frequently underwent silencing, in the sense of a significant decrease in GUS expression from callus levels («developmental»), and also in the sense that these plant lines accumulated very low levels of GUS even though the GUS gene was transcribed at a significant rate. Because we did not assay NPTII accumulation in sugarcane callus, we cannot say that we have observed silencing for Ubi-1-nptII genes in the «developmental» sense. However, we did find low levels of expression in mature plants: 36 of 38 tested lines accumulated NPTII protein to less than 0.05 % of TSP. Numerous lines were below our detection threshold for NPTII protein. As these lines were originally selected as callus on G418 antibiotic, we think it likely that at least some of these lines did undergo a reduction in NPTII accumulation after plant regeneration. And for four of five tested lines (4T1, 4T5, 9T28, MT2, Fig. 4) transcription occurred at a significant rate when compared to the endogenous control, suggesting that low levels of NPTII accumulation are due to PTGS. While accumulation of both GUS and NPTII protein was low in mature sugarcane plants, the highest level of NPTII was approximately 100-fold higher than the highest level of GUS. It is possible that this reflects a difference between the promoters, but as there was no significant difference in GUS expression between the promoters, we feel it more likely the GUS vs. NPTII difference reflects some difference in the genes themselves (e.g., susceptibility to PTGS) or relative stability of the proteins.

We are aware that sugarcane lines that produce high levels of foreign protein (0.5–1.0 % of total protein) have been obtained (Robert Birch, T. Erik Mirkov, personal communications); however, in some cases it has been necessary to screen hundreds of independent lines to recover one superior line that produces these high levels. Our data indicate that transgenes introduced to sugarcane by particle bombardment frequently undergo a major decrease in expression after plant regeneration. In many cases, transgene expression remains detectable but at a level far lower than the same line produced at the callus stage. We observed this expression decrease similarly with sugarcane ubi4, ubi9, or maize Ubi-1 promoters.

What accounts for the difference in frequency of PTGS between our rice and sugarcane lines? The rice and sugarcane lines differ in three major respects: species, whether the promoters are endogenous or foreign to the species, and method of transgene introduction. We are not aware of differences in PTGS between rice and sugarcane, but it is certainly possible that diploid rice may have evolved different mechanisms for responding to homologous DNA sequences than has highly polyploid sugarcane. As to the endogenous/foreign character of the promoters, other sugarcane promoters that became silenced when re-introduced into sugarcane have been reported (Birch et al. 1995, Hansom et al. 1999), however, there are also reports of endogenous sugarcane promoters that did not become silenced when re-introduced into sugarcane (Birch et al. 1995, Hansom et al. 1999). Additionally, the rice Act1 and cytochrome c (OscCc1) promoters have been used in transgenic rice without incurring silencing


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