

Promoter analysis in transient assays using a GUS reporter gene construct in creeping bentgrass (*Agrostis palustris*)

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Summary

Transient expression profiles for several chimeric β -glucuronidase (GUS) gene constructs were determined in tissues (young leaves, mature leaves and roots) of creeping bentgrass (*Agrostis palustris*, cv. Penn A4) following microprojectile bombardment. The constructs analyzed consisted of the *uidA* (GUS) reporter gene driven by four different promoters (ubiquitin 3-potato, ubiquitin corn, ubiquitin rice and CaMV 35S). The total number of GUS hits (or transient expression units; TEUs) were determined manually under a dissecting scope after histochemical staining for GUS. Results suggest that the ubiquitin rice promoter is most active in cells of turfgrass, regardless of the developmental stage or tissue-type. The ubiquitin corn promoter was the next best. Of the four promoter used, except for ubiquitin 3-potato, reporter gene activity was dramatically higher in mature leaves compared to young leaves. The relative efficiency of each promoter was about the same in roots and leaves. We have also analyzed *uidA* (GUS) reporter gene activity following microprojectile bombardment in transient expression assays with callus from two cultivars (Providence or Penn A4) of creeping bentgrass. Differences in the frequency of GUS positive hits were observed between cultivars up to 72 hours post-bombardment. However, this difference between cultivars disappeared after 72 hours post-bombardment. This information describing promoter functionality in bentgrass will be important when designing gene constructs for trait modification and when choosing appropriate cultivars for improvement through gene transfer experiments. This is the first in depth report on organ-specific and developmental gene expression profiles for transgenes in a turfgrass species.

Key words: GUS – microprojectile bombardment – promoter analysis – turfgrass transformation

Abbreviations: GUS = β -glucuronidase. – TEU = Transient expression unit

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Introduction

Turfgrass, an important member of the monocotyledonous plant family, is used widely in lawns, golf course fairways and athletic fields all over the world. The turfgrass seed market is the second largest in the USA just behind hybrid corn. The annual retail sale of turfgrass seeds in the USA is over \$580 million (Lee 1996). Although agribiotech firms have largely neglected research on turfgrass biotechnology (Kidd 1993) in the past, its rising economic importance has led to increased efforts over the past few years in applying biotechnology strategies for turfgrass genetic improvement. In particular, trait improvement through gene transfer technology has become feasible (Chai and Sticklen 1998). In order to drive efficient transgene expression in turfgrass, it is necessary to have available effective promoter elements that control sufficient levels of gene expression and to understand the specific characteristics of promoter functionality in the transgenic plants.

The *E. coli uidA* gene encoding β -glucuronidase (GUS) (Jefferson 1987) is one of the most popular reporter genes used in plant transformation for assessing promoter activity. It has been used in only a limited number of studies with turfgrass species. Penmetsa and Ha (1994) studied various factors and parameters that influence transient gene (GUS) expression in tall fescue (*Festuca arundinacea*) protoplasts and observed highest GUS activity 24 hours after electroporation. Bettany et al. (1998) observed GUS expression at different vegetative developmental stages of transgenic fescue plants (derived from stably transformed protoplasts) and found unstable GUS expression during early stages of tillering. Zhong et al. (1993) analyzed transgenic creeping bentgrass (*Agrostis palustris*) for GUS gene expression (after microprojectile bombardment) under the control of the rice actin promoter and observed GUS activities in all the tissues tested from transgenic plants.

Plant researchers have bombarded both embryogenic and non-embryogenic tissue-cultured cells and used chimeric GUS reporter gene fusions for transient expression studies in order to better understand promoter-specified developmental gene expression patterns, for promoter functional analysis, to optimize the bombardment parameters and to optimize the tissue culture conditions most suitable for the plant material under study. Previous experiments have shown that transient GUS expression patterns can be variable. Cowpea (*Vigna unguiculata*) seed-derived embryos can take up and transiently express GUS up to 7 days after electroporation (Penza et al. 1992). Transient GUS expression in pollen embryoids of wheat was observed to drop dramatically after 10 weeks in culture (Loeb and Reynolds 1994). It has been shown that the developmental stage of suspension culture cells of tall fescue (*Festuca arundinacea*) can influence GUS expression in the cell protoplasts using a polyethylene glycol-mediated transformation system (Kuai and Morris 1995). Protoplasts isolated from cell cultures 5–20 weeks after initiation expressed

higher levels of GUS than those from cell cultures 25 weeks after initiation (Kuai and Morris 1995).

Herein we report on our analysis of the functioning of a reporter gene, β -glucuronidase (GUS), driven by four different promoters in different tissues (leaf, young and fully mature, and root) of creeping bentgrass (*A. palustris*). We also investigated the effect of genotypic differences on the activity of the ubiquitin rice-GUS construct in two creeping bentgrass cultivars, Providence and Penn A4. These two cultivars were chosen for this study because they are the targets for our broader effort in developing improved turfgrass germplasm through biotechnology applications.

Our experiments were designed specifically for: (1) the identification of an optimum promoter to be used for genetic engineering of turfgrass; (2) the analysis of tissue-specific and developmental-specific patterns of gene expression conferred by these promoters; and, (3) the identification of genotypic effects on patterns of reporter gene expression. To the best of our knowledge, this is the first reported use of the ubiquitin rice-GUS promoter in transient reporter gene (GUS) expression studies in turfgrass.

Materials and Methods

Plant materials

Creeping bentgrass (*Agrostis palustris* L., cv. Penn A4) plants were grown under a 12-hour day photoperiod in sterile soil and in a pathogen-free environment and periodically watered with sterile water. Four-week old, non-expanded leaves were defined as 'young' and twelve-week old fully expanded leaves as 'mature'. On the day of bombardment the leaves were excised from the plants, cleaned with sterile water and arranged in parallel on sterile filter paper in a sterile petri dish and moistened with sterile water. The young and mature leaves were spread to cover the entire area of the petri plate. Some leaves needed to be trimmed at the apexes to make them fit on the circular petri plate. All the leaves were excised at the point of junction between leaf blade and leaf sheath. A total of 5 petri plates per promoter construct per developmental stage (young and mature) were bombarded (a total of 20 plates were bombarded with all four promoters using young leaves and another 20 plates using mature leaves).

Seeds of Penn A4 were also grown in perlite immersed in 1.2 cm of water in rectangular flat pots. The water was changed every other day. On the day of bombardment, roots were excised from the plants, washed and cleaned with sterile water and placed on petri plates as above for leaves. Afterwards, the roots were scanned and total root area was determined using the Delta T-Scan[®] software package (Delta T Services Ltd., Cambridge, UK). A total of 5 plates per promoter construct were bombed (a total of 20 plates for all four promoters).

Tissue culture

Two cultivars of creeping bentgrass, Providence and Penn A4, were used in these experiments. Seeds were first soaked in 70% ethanol for 2 minutes. Then, seeds were sterilized in a solution consisting of 10% bleach and 4 drops of Tween-20 with continuous shaking for 45

minutes. The seeds were then rinsed 3 times in sterile water in a laminar flow hood. Afterwards, the seeds were plated on MMSG media consisting of: 4.33 g MS Salts, 30.0 g of sucrose, 1 mL MS vitamins (1000X), 0.5 g casein hydrolysate, 1 mL 0.06 mol/L dicamba (dichloro-o-anisic acid) and 500 µL 0.004 mol/L 6-BAP (6-benzylaminopurine) in 1 L of water. The pH was adjusted to 5.6–5.8 (with 1 mol/L KOH) and 2.4 g of Phytigel (Sigma Chemicals, USA) was added prior to autoclaving. Embryogenic callus was cultured on this medium for about 12 weeks and transferred to osmoticum medium (MMSG plus 45.6 g of sorbitol and mannitol each) 4–6 hours prior to particle bombardment.

Plasmids

Four plasmids carrying the GUS reporter gene driven by the ubiquitin rice promoter (Huq et al. 1997), the ubiquitin corn promoter (Christensen and Quail 1996), the ubiquitin 3-potato promoter (Garbarino and Belknap 1994) and the CaMV 35S promoter were kindly provided by Dr. Hong Luo of HybriGene L.L.C., West Kingston, Rhode Island, USA.

Microprojectile bombardment

The biolistic gene delivery device PDS-1000/He (Bio-Rad, USA) was used for transgene delivery via microprojectile bombardment. Plasmid DNA (at the concentration of 1 µg/µL) was coated on the surface of 1.0 µm gold particles and bombarded onto root and leaf tissues. Seven µL of the plasmid DNA coated gold particles were placed in a center of a macrocarrier. The particle delivery system was adjusted to 1100 psi of helium pressure and 27 mm Hg of vacuum pressure inside the chamber. After bombardment, leaves (incubated in a 16:8 photoperiod cycle) and root (in dark) tissues were kept moist at room temperature for 48 hours prior to histochemical staining for GUS activity.

Callus tissue of Providence and Penn A4 were bombarded after 24, 48, 72, 168 and 336 hours. Three plates per cultivar were randomly chosen and analyzed for GUS expression. A total of 15 plates per cultivar were bombarded. The calli were arranged at the center of the petri dish covering an area of about 19.64 cm² and bombarded using the PDS-1000/He (Bio-Rad, USA) biolistic gene delivery device.

Reporter gene (GUS) assay

The GUS reaction (Jefferson 1987) mix consisted of the following: 50 mmol/L potassium ferrocyanide, 50 mmol/L potassium ferricyanide, 5 mL 0.2 mol/L sodium phosphate buffer, 0.5 mol/L sodium EDTA and 10 % Triton X-100 and water. A separate solution of X-Gluc (5-Bromo-4-Chloro-3-Indolyl-Beta-D-Glucuronide) (Gold Biotechnology, USA) at a concentration of 25 mg X-Gluc/mL of N-N dimethyl formamide was added to the above reaction mix at a ratio of 352 µL of reaction mix to 48 µL of X-Gluc solution. Tissues were incubated in the GUS solution at 37 °C for 16 hours. The GUS solution was discarded and the tissues were rinsed with water. Then the leaf tissues were bleached sequentially with 25 %, 50 % and 75 % ethyl alcohol and finally kept in 95 % ethyl alcohol. GUS hits were counted manually under a dissecting scope when the leaves were fully bleached and white. This bleaching procedure was not necessary for the roots and callus tissues.

Data analysis

A two sample (independent) student's t-test ($P < 0.05$) was performed with the data.

Results

Transient GUS expression in young leaves

Analysis of relative promoter strength suggests that ubiquitin corn and ubiquitin rice are the two best promoters for driving GUS expression in turfgrass cells (Fig. 1 A and Fig. 1 B). 26.2 % of young leaves bombarded with the ubiquitin corn construct expressed GUS and 25.1 % of young leaves bombarded with the ubiquitin rice construct expressed GUS (Fig. 1 A). The average number of TEUs (an average number of blue spots/GUS expressing leaf or Transient Expression Unit, Moore et al. 1994) per GUS expressing leaf was higher for the ubiquitin rice (34.6) promoter than for the ubiquitin corn (22.2) promoter (Fig. 1 B). On the other hand, the dicot-derived ubiquitin 3-potato promoter performed poorly with only 2.1 % of the total leaves bombarded with this promoter expressing GUS (Fig. 1 A) and an average number of blue spots/ GUS expressing leaf of only 4.3 (Fig. 1 B). The transient expression activity of the widely used CaMV 35S promoter was also poor. Only 2 % of the total leaves bombarded with the CaMV 35S construct expressed GUS TEUs (Fig. 1 A) and of those there was only 14.3 average TEUs/ GUS expressing leaf in the young leaves of creeping bentgrass (Fig. 1 B). The TEUs were evenly spread across the young leaves.

Transient GUS expression in mature leaves

When the four different promoter-GUS constructs were bombarded onto mature leaves, the relative efficacies of the promoters were about the same as observed for young leaves (i.e. ubiquitin rice expressed the most GUS TEUs followed by ubiquitin corn, ubiquitin 3-potato and CaMV 35S) although GUS TEUs were more numerous overall for each promoter compared with young leaves (Fig. 1 B). The TEUs were evenly spread out across the mature leaves. In general, for all the

Table 1. Comparative activities of the GUS reporter gene driven by four different promoters.

Promoter Driving GUS Expression	Total number of leaves bombarded		Total number of GUS spots	
	Young	Mature	Young	Mature
Ubiquitin 3-potato	142	170	13	35
Ubiquitin corn	137	176	801	4256
Ubiquitin rice	143	165	1248	7994
CaMV 35S	150	162	43	330

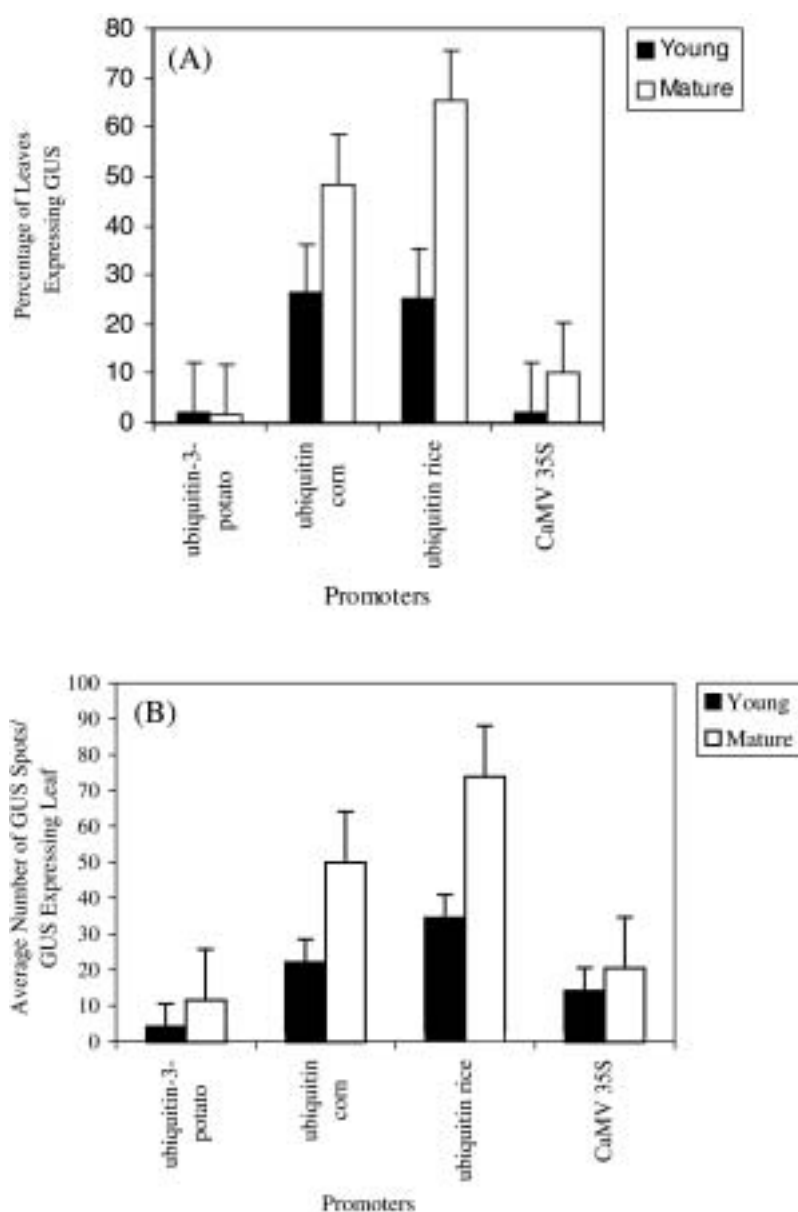


Figure 1. Assessment of relative promoter strengths. (A) Activity of four different promoters with respect to the number of leaves expressing the reporter gene (GUS) vs. the total number of leaves bombarded. (B) Relative activity of four different promoters in young vs. mature bentgrass leaves following microprojectile bombardment. Error bars represent standard error.

promoters, the percentage of leaves expressing GUS activity was much higher in mature leaves compared to young leaves (Table 1 and Fig. 1A). About 48.2% of the mature leaves expressed GUS TEUs with the ubiquitin corn promoter and 65.4% of the mature leaves expressed GUS TEUs with the ubiquitin rice promoter, although these numbers were 26.2% and 25.1%, respectively, with the young leaves (Fig. 1A).

A more than six-fold enhancement of GUS expression was observed with the ubiquitin rice promoter (Table 1) in mature leaves compared to young leaves. Also, with the ubiquitin rice promoter, the average number of GUS TEUs/ GUS expressing leaf was more than double (Fig. 1B) with mature leaves compared to young leaves. The promoter, ubiquitin corn, also performed better in mature leaves when compared to young

leaves (Table 1 and Fig. 1B). The TEUs of the ubiquitin rice and ubiquitin corn promoters are significantly different in mature leaves ($t = 2.42$). This difference does not occur in young leaves ($t = 1.15$). The average number of TEUs/ GUS expressing leaf was more than double in mature leaves compared to young leaves (Table 1). It was observed (Fig. 1A) that only 2% of the young leaves expressed GUS TEUs with CaMV 35S, but this number went up to 10.1% with mature leaves. This difference is statistically significant ($t = 2.67$).

Finally, the average number of GUS spots for all promoters was also higher when using mature leaves (Fig. 1B). There were an average of 74 TEUs/ GUS expressing leaf with the ubiquitin rice promoter (Fig. 1B) in mature leaves. This value is almost double (Fig. 1B), and statistically, significantly

higher ($t = 5.89$) when compared to young leaves. There were an average number of 50.0 TEUs/ GUS expressing leaf with the ubiquitin corn promoter (Fig. 1B). This value is also almost double (Fig. 1B), and statistically, significantly higher ($t = 2.43$) when compared to young leaves. There were an average number of 20.6 TEUs/ GUS expressing leaf with the CaMV 35S promoter (Fig. 1B) in mature leaves, whereas there were an average number of 14.3 TEUs/ GUS expressing leaf in young leaves. This value is statistically, significantly higher ($t = 2.85$) when compared to young leaves. TEUs produced by ubiquitin corn on young leaves is significantly different from TEUs produced by CaMV 35S (Fig. 1B, $t = 3.22$). Average TEUs produced by the CaMV 35S promoter and ubiquitin corn promoter are significantly different from each other. The calculated t values are as follows: $t = 3.58$ (when percentage of mature leaves expressing TEUs were compared between CaMV 35S and ubiquitin corn) and $t = 5.11$ (when percentage of young leaves expressing TEUs were compared between CaMV 35S and ubiquitin corn).

For the other promoter, ubiquitin 3-potato, its average number of GUS TEUs/ GUS expressing leaf were not statistically significantly higher ($t = 0.84$) compared to young leaves.

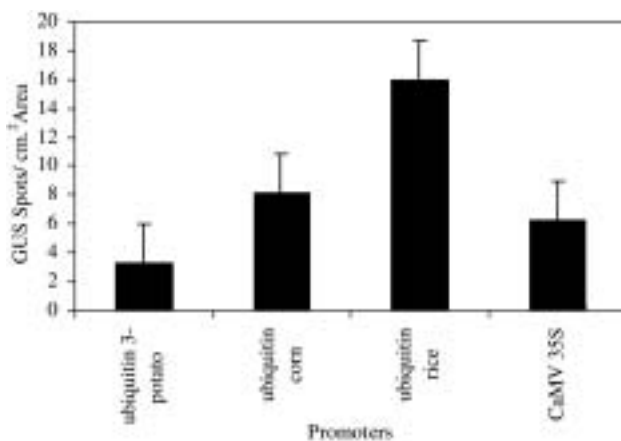


Figure 2. GUS reporter gene expression in bentgrass roots after bombardment with the four different promoter-GUS constructs. Error bars represent standard error.

Transient GUS expression in roots

The ubiquitin rice promoter performed most efficiently in producing GUS TEUs in Penn A4 roots followed next by the ubiquitin corn promoter (Fig. 2). There were almost double the number of GUS TEUs (15.9 spots/cm² area of root) with the ubiquitin rice promoter compared to the ubiquitin corn promoter (8.2 spots/cm² area of root). Surprisingly, the CaMV 35S promoter performed quite well (6.2 spots/cm² area of root), also. There were only 3.3 spots/cm² area of root with the ubiquitin 3-potato promoter.

Genotypic affect on reporter gene expression in callus

Friable, embryogenic callus (Fig. 3A) of two important bentgrass cultivars (Providence and Penn A4) was chosen for transient assays of post-bombardment GUS reporter gene expression. Other types of non-embryogenic callus (Figs. 3B, 3C and 3D) produced under our tissue culture conditions were discarded. At 24-hours post-bombardment there were greater than 2.5 times more GUS TEUs in Penn A4 than in Providence (Fig. 4). After 48 hours, there were almost 3 times more TEUs in the Penn A4 callus compared to Providence. But, after 72 hours both cultivars exhibited about the same number of GUS TEUs. GUS TEUs were found to steadily decrease after 72 hours for Providence and GUS TEUs in Penn A4 dropped slightly after 48 hours and dropped sharply after 168 hours in this 14-day observation period (Fig. 4).

Discussion

The primary objective of this study was to assess the activity of various promoters that may be useful for driving high levels of gene activity in turfgrass transformation studies. Those promoters capable of driving a high level of gene activity could then be used to design constructs with various genes that confer beneficial traits to turfgrass. As part of this analysis we were interested to study the transient expression patterns of these promoters in different tissue types and at different developmental stages of the turfgrass plant.

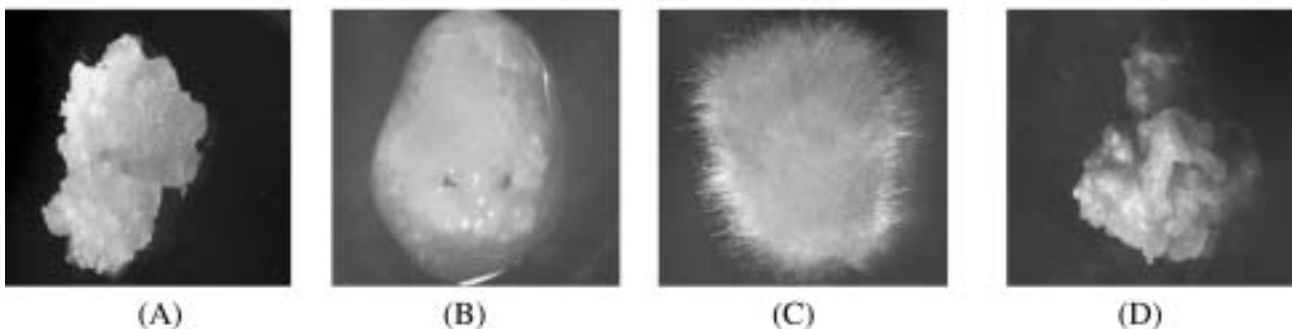


Figure 3. (A) Friable, embryogenic callus of Penn A4, suitable for transformation through particle bombardment. (B), (C) and (D) represent non-embryogenic gelatinous callus, hairy callus and hard callus of Penn A4, respectively.

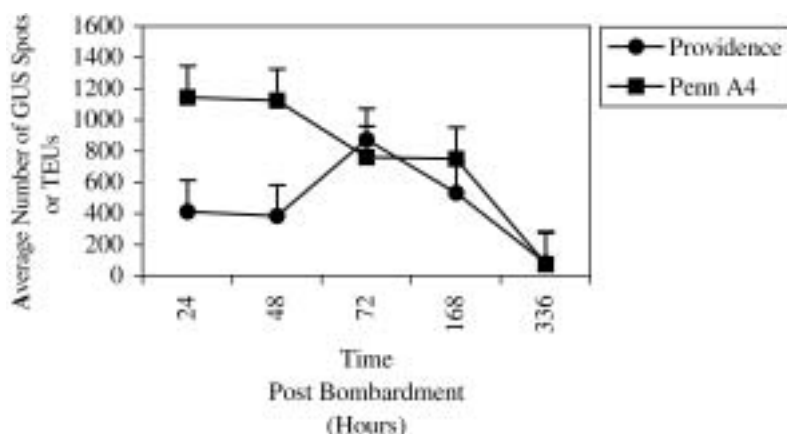


Figure 4. Comparative analysis of GUS expression in two cultivars of bentgrass following microprojectile bombardment. Data points shown are the average of 3 separate plates for each cultivar after 24, 48, 72 and after 168 and 336 hours post bombardment. Error bars represent the standard error.

We conclude that the ubiquitin rice promoter is the best for driving high levels of gene expression in bentgrass followed by the ubiquitin corn promoter. Both these promoters are monocot-specific promoters. On the other hand, the dicot-specific promoter (ubiquitin 3-potato) and CaMV 35S did not perform that well in the monocot used for these studies. Able et al. (2001) observed similar results when they bombarded sorghum tissues with both monocot- and dicot-specific promoters. They concluded that monocot-specific promoters are much more suitable for transformation of monocot species. Cornejo et al. (1993) also reported that transient expression of the maize ubiquitin promoter (monocot-specific) in rice was 7 to 10.3 times more active than other dicot-specific (Adh 1, Actin1) and CaMV 35S promoters. Chowdhury et al. (1997) also got similar results and concluded that the maize ubiquitin promoter is more effective in driving reporter gene expression in oil palm than other dicot-specific promoters.

It is evident from our studies that, for all four promoters tested, ubiquitin rice ($t = 5.89$), ubiquitin corn ($t = 2.43$) and CaMV 35S ($t = 2.85$) performed better (statistically significantly different when compared TEUs with young leaves) with mature leaves than young leaves (Fig. 1). Even the percentages of leaves expressing GUS were much higher in mature leaves compared to young leaves (Fig. 1A). The total number of GUS TEUs for the ubiquitin rice construct were more than six times and for the ubiquitin corn construct, more than five times higher in mature leaves when compared to young leaves (Table 1). From Table 1, it is also evident that the total number of GUS TEUs with the CaMV 35S promoter were more than 2.5 times greater in mature leaves compared to young leaves. The relative efficiencies of the four promoters were almost the same in leaves and roots and ubiquitin rice performed best in root tissues followed by ubiquitin corn (Fig. 2).

Previous reports in other plant species have investigated both short-term and long-term GUS expression patterns in embryogenic callus cells. It has been reported that GUS expression can sharply decrease over a 3-day period in sorghum callus after bombardment (Able et al. 2001). On the other hand, GUS expression can also increase over a 3-day period

in embryogenic callus of alfalfa following microprojectile bombardment (Tian et al. 2000). These two reports indicate completely opposite responses depending on the type of plant callus tissue bombarded. It is evident that the efficiency of GUS expression can change significantly over a short period of time following bombardment. Results reported here indicate that the two different cultivars of creeping bentgrass differ in their abilities to express TEUs up to 72 hours post-bombardment (Fig. 4). After 72 hours this difference no longer existed. Instead, TEUs in Providence began to decline sharply after 72 hours. The TEUs in Penn A4 remains quite high until 48 hours and there was a slight drop after that. After 168 hours GUS TEUs sharply declined (Fig. 4). There is also the possibility that the protein produced by *uidA* (GUS) might have degraded over time.

Different genotypes of soybean were found to express different levels of GUS TEUs when immature zygotic cotyledons were bombarded. The genotype McCall was found to express the highest number of GUS TEUs out of six genotypes of soybeans bombarded with the CaMV 35S promoter driving *uidA* (GUS) (Moore et al. 1994). Moore et al. (1994) also reported that increasing the bombardment pressure also could not increase transient GUS TEUs expressions in the constitutively low GUS TEUs expressing soybean genotypes Fayette and Peking. Arias-Garzon and Sayre (1993) concluded that high levels of DNase activity were responsible for the very low levels of transient GUS TEUs in cassava roots, compared to leaves, following microprojectile bombardment. Zhou et al. (1996) showed that different levels of GUS reporter gene expression occurred between calli and somatic embryos and both tissues exhibited an increase in TEUs when treated with 5-azacytidine (DNA hypomethylating agent) post-bombardment. They suggest that different degrees of methylation of the transgene in these two tissues may be responsible for the different activity levels. Tian et al. (2000) observed that alfalfa embryogenic cells had a poor ability to transiently express GUS TEUs during the cell proliferation stage following microprojectile bombardment. On the other hand, they observed high levels of GUS TEUs in actively dividing non-embryo-

genic suspension cultures of alfalfa. From these examples it is clear that there are many complex physiological and biochemical phenomena responsible for variable transient GUS TEUs expression in different tissues, at different developmental stages of the plant, and in different genotypic backgrounds. Any or all of these possibilities may be responsible for our observed patterns of expression in Penn A4 and Providence.

The results presented (Fig. 4) suggest that transient expression of a reporter gene (GUS) does change during early embryogenic cell development of creeping bentgrass cultivars. Experiments are underway to compare GUS expression in stably transformed bentgrass cultivars (i.e. Penn A4 and Providence) and compare the expression patterns to the transient assay data. The transient expression analysis data presented here provides unique and valuable information about the function and expression patterns of a strong promoter (ubiquitin rice) in short term embryogenic cell development of creeping bentgrass in different genetic backgrounds. Knowledge of differential reporter gene expression patterns between cultivars will help plant breeders and plant transformation specialists to choose appropriate target material for plant transformation and germplasm enhancement.

The experiments described herein are the first report of promoter analysis in a turfgrass species. The variable responses observed among the promoters suggest that more research is needed to better understand the mechanisms behind the variable responses. A very important observation from our study was the determination that the relative strengths of the ubiquitin rice and ubiquitin corn monocot-specific promoters compared to each other are essentially the same regardless of the tissue type or developmental stage. From these transient expression studies we can conclude that both of these promoters can be effective in future turfgrass transformation studies, with ubiquitin rice to be the best promoter followed by ubiquitin corn.

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