# Generation of low copy number and stably expressing transgenic creeping bentgrass plants using minimal gene cassette bombardment

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## Abstract

A minimal gene cassette comprised of the ubiquitin (*Ubi*) promoter + green fluorescent protein (*Gfp*) gene + *Nos* terminator DNA sequences, derived from the plasmid vector *pPZP201-Gfp* was utilized for transformation of creeping bentgrass using particle bombardment. Bentgrass calli bombarded individually with equivalent amounts of the cassette or whole plasmid DNA were compared for *Gfp* expression and the GFP-positive calli were subsequently regenerated into plants. Percentage of GFP expressing calli and the number of GFP spots/calli were significantly higher in calli that were bombarded with the minimal gene cassette when compared to the whole plasmid. The *Gfp* expression was stable up to the $T_2$ generation in minimal gene cassette transformants and there was a lower degree of gene silencing. Southern blot analysis of transgenic plants derived from minimum gene cassette bombardment revealed the presence of single or few copy of the transgene and fairly simple integration patterns. In comparison, whole plasmid transformants had multiple copies and complex integration patterns of the transgene. These results illustrate the advantages of using simple gene cassette for stable plant transformation in bentgrass with possible applications to other plant species.

*Additional key words*: gene integration, green fluorescent protein expression, plant transformation.

## Introduction

Plant transformation protocols routinely involve the use of whole plasmid constructs that contain the target gene, bacterial antibiotic resistance gene and vector backbone. This results in integration of vector backbone sequences and the antibiotic resistance selection gene into the plant genome, which is undesirable. Use of whole plasmid DNA is obligatory in the case of *Agrobacterium*-mediated transformation with cointegrative vectors *i.e.* those with the *vir* genes linked to the T-DNA. In contrast, the vector backbone serves no purpose in direct DNA transfer procedures such as particle bombardment. Even in *Agrobacterium*-mediated transformations, the random integration of vector backbone sequences does occur (Ramanathan and Veluthambi 1995, Tingay *et al*. 1997). When integrated into plant DNA, excess vector sequences can spontaneously acquire extensive methylation that can spread to neighboring transgenes (Jakowitsch *et al.* 1999). Vector backbone sequences may also cause undesirable effects in *cis* (Artelt *et al.* 1991) including proneness to transgene rearrangement and recombination events, which can lead to complex plasmid multimerization events (Muller *et al.* 1999) and cointegration of bacterial antibiotic selection marker genes. Furthermore, the presence of very large transgenic loci would render the transgene meiotically unstable, leading to excision of the locus and loss of transgene expression in subsequent generations (Stöger *et al.* 1998). The use of minimal cassettes (containing the promoter, coding sequence and polyadenylation signal of the transgene) eliminates this problem and results in "clean DNA transformation" (Fu *et al.* 2000, Breitler *et al.* 2002). In animal transformation systems, it is a routine practice that all vector sequences be removed before microinjecting foreign DNA into eggs or embryos (Palmiter and Brinster 1986), since extensive stretches of prokaryotic vector sequences are not well-tolerated by higher eukaryotic genomes (Matzke *et al.* 2000).

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*Abbreviations*: CIM - calli induction medium; 2,4-D - 2,4-dichlorophenoxyacetic acid; GFP - green fluorescent protein; WP - whole plasmid; MGC - minimum gene cassette; MS - Murashige and Skoog's medium.
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The use of minimal cassettes (containing the promoter, coding sequence and polyadenylation signal of the transgene) eliminates this problem and results in "clean DNA transformation" (Fu *et al.* 2000, Breitler *et al.* 2002). Fu *et al*. (2000) found that the use of such minimum cassettes also increased the proportion of low-copy-number events and structurally intact transgene loci compared to whole plasmid transformations, and this resulted in improved stability of expression. There are very few published reports on the utility of minimal gene cassettes in transformation of other crop plants.

Creeping bentgrass (*Agrostis palustris*) is an important cool-season turfgrass extensively used in golf courses. Tissue culture and transformation systems are well characterized for creeping bentgrass (Yu *et al*. 2000) and microprojectile bombardment was first used to produce stable transformants (Zhong *et al.* 1993). GFP has been used as an efficient and non-destructive selectable marker in plant tissues and can be detected visually soon after transformation, allowing the identification and selection of transgenic calli. Regenerated transgenic plantlets also can be identified by the presence of green fluorescence in organs such as emerging leaves and roots (Yu *et al.* 2000). Recently, we have used the *Gfp* gene as a sole selectable marker to transform embryo-derived calli of sorghum and select and regenerate transgenic sorghum plants, which co-harbored a rice thaumatin-like protein gene (Gao *et al.* 2005). In the present study, we compared the use of minimum gene cassette comprising *Ubi*-promoter + *Gfp* + *Nos* terminator versus the whole plasmid construct containing the above expression cassette in creeping bentgrass calli and also monitored the expression efficiency of minimum gene cassette over several generations.

## Materials and methods

**Plasmid construction:** The plasmid vector *pPZP201-Gfp* containing the *sgfp* gene (Chiu *et al*. 1996) and the maize ubiquitin promoter (Christensen *et al*. 1992) were used for the construction of plasmid vector. The new construct was made in *pCAMBIA1300* (*CAMBIA*, Canberra, Australia) wherein the *Ubi* promoter + *Gfp* + *Nos* were subcloned into the *EcoRI* site (Fig. 1). The resulting plasmid DNA was used directly or digested with *EcoRI* to release the gene cassette. The gene cassette DNA fragment was gel-purified in 1 % low melting agarose and concentrated by phenol-chloroform extraction and ethanol precipitation (Sambrook and Russel 2001).

**Tissue culture and transformation:** Seeds of creeping bentgrass (*Agrostis palustris* Huds.) cultivar Crenshaw were surface-sterilized with 0.1 % $HgCl_2$ for 15 min and cultured in callus induction medium (CIM) containing Murashige and Skoog (MS) medium + 2 g $dm^{-3}$ 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg $dm^{-3}$ kinetin, 30 g $dm^{-3}$ sucrose, and 2.5 g $dm^{-3}$ *Phytagel*, pH 5.8.

Fifty-day-old embryogenic calli were incubated in callus induction medium containing 0.4 M mannitol for 6 h prior to bombardment.

Whole plasmid DNA (WP; 6 μg) or minimal gene cassette (MGC; 2 μg) DNA were used for particle bombardment in a *Helium PDS 1000HE* gene gun (*BioRad*, Hercules, CA, USA) (Sanford *et al.* 1993). The bombarded calli (500) were observed under a *Nikon SMZ800* (*Nikon Instruments*, Melville, NY, USA) stereo epifluorescence microscope with GFP 2 and GFP 3 filter sets. The green fluorescent spots/patches on the calli were detected and recorded at 5-d intervals from the 2nd day onwards up to 60 d. One set of calli was also bombarded with *pCAMBIA1300* (without *Gfp*) which served as a control.

Forty-day-old GFP positive calli or sectors of calli were subcultured in CIM in darkness. The embryogenic calli were subcultured on regeneration medium (MS + 1 mg 2,4-D). Identifiable shoots and (or) roots with strong GFP fluorescence were physically separated and cultured onto half-strength MS medium until plantlets were obtained. Regenerated plants were placed in a humid chamber, potted in sterilized potting mix and grown in a greenhouse (22 - 28 °C; 70 - 85 % relative humidity; irradiance 600 - 1000 μmol $m^{-2}$ $s^{-1}$; 12-h photoperiod) to maturity. The regenerated plants were self-pollinated and $T_0$, $T_1$ and $T_2$ generation seeds of selected lines were germinated on half-strength MS medium and the seedlings (20-d-old) were scored for *Gfp* expression as mentioned above.

**PCR and Southern blotting:** Total genomic DNA was extracted from young leaves by using the maize miniprep method (Dellaporta *et al*. 1983). 300 ng of DNA was subjected to polymerase chain reaction (PCR) analysis using the *Red-Amp* PCR kit (*Sigma*, St Louis, MO, USA) employing *Gfp* gene specific primers (forward primer: 5'-TGAAGTTCA TCTGCACCACC-3'; reverse primer: 5'-GAACTCCAG CAGGACCATGT-3') in a thermocycler (denaturation at 95 °C for 30 s, annealing at 56.5 °C for 1 min and extension at 72 °C for 1 min for 25 cycles) to amplify a 620 bp fragment in the *Gfp* coding region. For Southern blot analysis, 10 μg of total genomic DNA was digested with *EcoRI*/*HindIII* restriction enzymes, fractionated by 0.8 % agarose gel electrophoresis, alkaline blotted onto *Hybond* $N^+$ (*Amersham*, Piscataway, NJ, USA) membrane and probed using α-32P (dCTP) labelled *Gfp* probe. Prehybridization and hybridization were carried out employing the *PerfectHyb-Plus* hybridization buffer (*Sigma*) and probed by following standard procedures (Sambrook and Russel, 2001). Probed membranes were exposed to X-ray film (*Kodak*) using two intensifying screens for 24 h at -80 °C.

**Northern blotting:** Total RNA was isolated from bentgrass leaves using *Trizol* reagent (*Invitrogen*, Carlsbad, CA, USA) and 10 μg of the total RNA was resolved on a denaturing agarose gel and blotted onto a nylon membrane. Northern analysis was carried out as described by Sambrook and Russel (2001) utilizing a 0.62 kb PCR amplified *Gfp* coding fragment labelled with α-32P (dCTP) as probe. Probed membranes were exposed to X-ray film (*Kodak*) using two intensifying screens for 24 h at -80 °C.

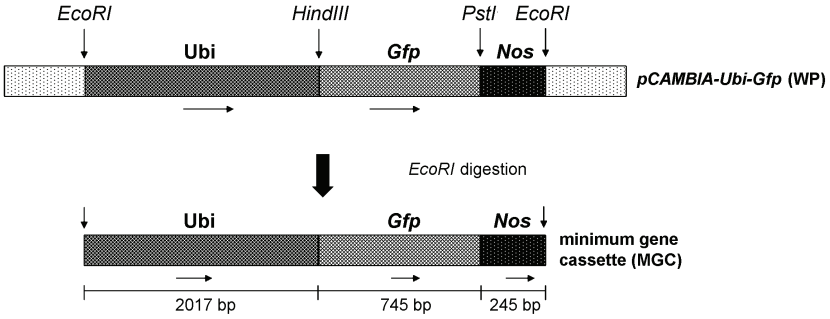


Fig. 1. Plasmid constructs/cassette used for transformation.

## Results and discussion

**GFP expression in calli:** GFP-fluorescence was clearly seen in calli bombarded with both types of DNA (MGC and WP). The number of calli showing GFP spots and the number of GFP spots/calli were higher in MGC bombardment (Table 1). However, fluorescent spots gradually decreased in number after 10 d of bombardment. The individual stable spots began to coalesce and turn into fluorescent patches, which continued to grow over time (Fig. 2). These fluorescent patches were carefully dissected and propagated further in the nutrient medium. At the end of culture, either the whole callus or most of the callus tissue was fully fluorescent. This developmental change was similar in both MGC and WP-derived calli except for the number of calli, which was greater in the former. The significant decrease in the number of GFP spots during culture for embryogenic calli development suggests that DNA degradation as well as gene silencing mechanisms, may be occurring leading to a reduction in the number of observable stable expression events. It is possible that callus tissues receiving multiple copies of transgene are more prone to transgene silencing and this might be the reason for the higher number of calli with diminished fluorescence in WP bombarded calli (Jayaraj *et al*. 2005). In neither whole calli nor thin sections of such calli in which GFP expression was abolished any reversion of green fluorescence in the subsequent stages of growth was observed.

**Regeneration of GFP-positive plants and molecular characterization:** The GFP positive calli were continuously monitored for fluorescence and propagated to regenerate plants. Regenerating calli and the emerging plants were selected based on GFP expression. A total of 190 and 160 independent GFP-positive plants were regenerated from MGC and WP bombarded calli, respectively. DNA extracted from all the individual transgenic plants was analyzed by PCR employing gene-specific primers. A 620 bp band was amplified from DNA isolated from in all of *Gfp*-positive plants, which corresponds to the coding region of the *Gfp* gene sequence. The above band was not amplified from DNA from control plants. GFP fluorescence was clearly visible at every stage of plant growth, especially in young and achlorophyllous tissues. Expression of green fluorescence was masked somewhat by chlorophyll in mature leaves as green portions showed less fluorescence than non-green parts of the plant. We clearly observed GFP fluorescence in the achlorophylous leaf bases of the core leaf, stigma, anthers, pollen, germinating seeds and roots. However, the intensity of green fluorescence varied considerably among the lines and their progenies.

Southern blots were performed on DNAs extracted from 100 MGC and WP lines: A representative sample of lines is presented in Fig. 3. Analysis of genomic DNA from MGC-derived plants after *HindIII* digestion showed that a majority (75 %) of the lines had a single copy of

Table 1. GFP expression in bentgrass calli [%] at days following bombardment using two DNA types (MGC - Ubi-Gfp-Nos, WP - pCambia-Ubi-Gfp). In each column, means followed by the same letter are not significantly different ($P$ = 0.05) according to Fisher's protected LSD test.

| DNA type | 2 d | 5 d | 10 d | 20 d | 30 d | 40 d | 50 d | 60 d |
|---|---|---|---|---|---|---|---|---|
| MGC | 38.1a | 46.5a | 61.0a | 65.8a | 55.1a | 51.2a | 47.5a | 42.0a |
| WP | 35.5a | 40.7b | 48.0b | 55.2b | 49.5b | 42.0b | 37.0b | 30.8b |

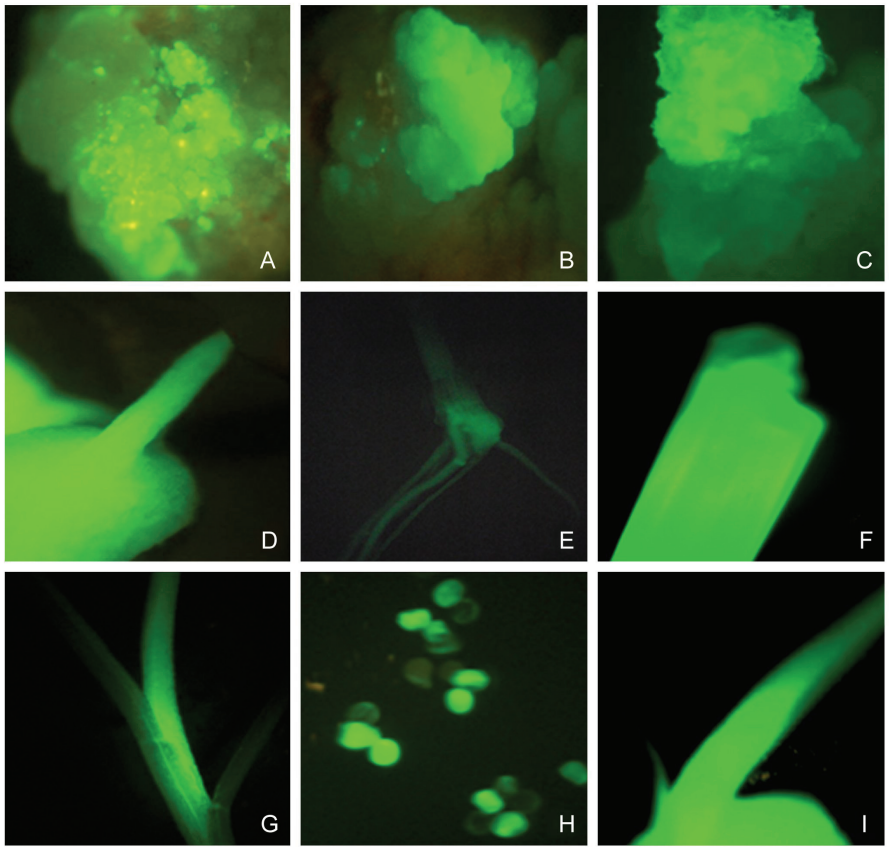


Fig. 2. GFP expression in MGC derived bentgrass calli/plants. *A* to *C* - calli at different stages of growth, *D* - regenerating callus, *E* - achlorophylous stem base, *F* - leaf base, *G* - leaf sheath, *H* - in pollen grains, *I* - germinating seed.

the gene while a few plants (25 %) had two copies (Fig. 3*A*). In contrast, the majority of the plants (75 %) derived from WP-bombarded calli had multiple copies of the transgene (3 - 9 copies) and only a few plants had 2 copies (16 %) or a single copy (9 %) (Fig. 3*C*). Probably as a consequence of this simple integration pattern, we observed more stable and heritable *Gfp* expression through subsequent generations in our experiments compared to WP derived plants. Fu *et al*. (2000) reported a similar trend when transforming rice utilizing a minimum gene cassette and whole plasmid DNA containing the *bar* gene. They found that most of the rice lines derived from MGC (80 %) had simple integration patterns (one or two copies of the transgene).

When genomic DNA is digested using appropriate restriction enzymes, a minimum of one band of the expected size was anticipated in GFP-positive lines. We found that 98 % of the GFP expressing lines derived from WP bombarded calli yielded the band of the expected size after *EcoRI* digestion (Fig. 3*D*). In comparison, only 19.5 % of the MGC derived lines had the expected 3 kb band. The majority (80.5 %) of MGC derived plants following *EcoRI* digestion did not release the expected 3 kb band following *EcoRI* digestion (Fig. 3*B*). Fu *et al*. (2000) reported a similar phenomenon in their studies with rice plants. They found that only 14 % of the lines derived from MGC bombardment showed a correct band size. When cassette-genomic DNA junctions were sequenced, they found that a single copy of *bar* cassette has integrated with a loss of five nucleotides at the 5' end. We suggest that similar events may have occurred in our plant lines to result in loss of enzyme restriction sites.

The very distinct DNA banding patterns observed among the MGC and WP lines indicates that individual molecules of foreign DNA can have different patterns of integration. Fu *et al*. (2000) proposed two different mechanisms by which this can occur. First, the vector backbone could promote high copy number by providing quantitatively extensive homologous regions, so the removal of vector backbone would limit quantitatively the available homologous sites. Second, the removal of vector backbone may have reduced the influence of such recombinogenic elements on the process of integration. Earlier reports provide evidence that a substantial proportion of transgene loci derived using microprojectile bombardment exhibit interspersion with genomic DNA, with scrambled and ectopic genomic DNA found flanking the transgene locus and the integration target site (Kohli *et al.* 1998, Pawlowski *et al.* 1998, Sawasaki *et al.* 1998). Studies of DNA integration in mouse cells suggest that the transgenic DNA fragments delivered into the cell are

Table 2. GFP-expression at different plant generations. Values in parenthesis indicate the percentage of non-expressing (silenced) plants. In each column, means followed by the same letter are not significantly different ($P$ = 0.05) according to Fisher's protected LSD test.

| DNA type | $T_0$ total number of plants | number of GFP expressing plants | $T_1$ total number of plants | number of GFP expressing plants | $T_2$ total number of plants | number of GFP expressing plants |
|---|---|---|---|---|---|---|
| MGC | 190a | 185a (2.6) | 115b | 110a (4.3) | 150a | 134a (10.7) |
| WP | 160b | 129b (19.4) | 145a | 100b (31.0) | 131b | 80b (38.9) |

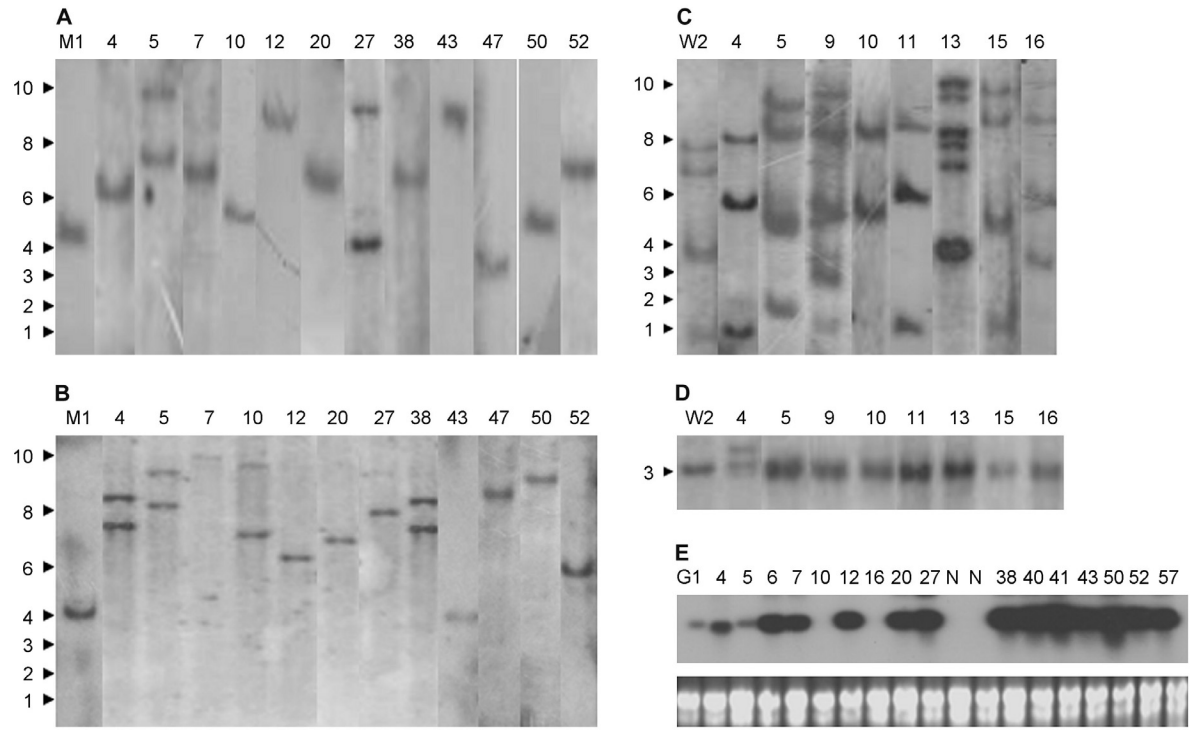


Fig. 3. *A* - Southern blot of genomic DNA from *Gfp*-transgenic bentgrass plant lines (M1 - M52) derived from MGC (minimal gene cassette bombardment) probed with *Gfp*; DNA digested with *HindIII* which cuts once in the transgene. Numbers on the left margin represent sizes of standard markers; *B* - Southern blot of genomic DNAs of *Gfp*-transgenic bentgrass plant lines (M1 - M52) derived from MGC digested with *EcoRI,* which cuts twice the transgene (expected fragment size 3.0 kb); *C* - Southern blot of genomic DNAs of *Gfp*-transgenic bentgrass plant lines (W2 - W16) derived from WP (whole plasmid bombardment), DNAs digested with *HindIII* which cuts once in the transgene; *D* - Southern blot of genomic DNAs of *Gfp*-transgenic bentgrass plant lines (W2 - W16) derived from WP digested with *EcoRI* which cuts twice within the transgene releasing a 3 kb band (expected fragment size 3.0 kb); *E* - Northern blot of total RNAs of *Gfp*-transgenic representative bentgrass plant lines ($T_2$) (G1 - G57) derived from MGC bombardment. NC - RNA from negative control plants. *Below*: Picture of the ethidium bromide stained RNA gel with rRNA bands.

engaged in end-to-end ligation before they are integrated into the genome, forming multiple transgene concatamers (Anderson *et al.* 1982 and Folger *et al.* 1982). In plant transformation experiments, head-to-head or head-to-tail concatamerization of the introduced transgenes also have been reported (Finer and McMullen 1990), supporting the concept that contiguous arrays of transgenic DNA are integrated into a single genomic site. However, from the report of Kohli *et al*. (1998), it appears that transgene loci in rice plants transformed by microprojectile bombardment may have host DNA separating closely linked transgene sequences. Therefore, utilizing MGC might minimize all these unfavourable phenomena from occurring and contribute towards effective integration. Occurrence of relatively simpler banding patterns in the Southern blots of MGC plants proves that rearrangements occurred less frequently than with circular plasmid DNA.

**Stability of transgene expression:** In order to show the stability of transgenes over generations, we selfed the selected plants from representative, MGC and WP lines. Seeds of ten representative $T_0$, $T_1$ and $T_2$ lines were grown in half-strength MS medium and tested for Gfp expression. Most of the MGC-progenies showed stable

expression but at varying levels, with very low frequency (2.6, 4.3 and 10.7 % in $T_0$, $T_1$, $T_2$ generations, respectively) of plants lacking expression, which points to stable expression with less frequent silencing of the transgene through generations (Table 2). The WP-progenies showed a moderate to high degree of silencing in $T_0$ (19.4 %), $T_1$ (31.0 %) and $T_2$ (38.9 %) generations. An earlier report by Fu *et al*. (2000) indicates the absolute stability of transgene in MGC-derived rice plants exemplified by the complete absence of silencing. We also observed a higher degree of stability of transgene expression through generations. However, we did observe a low degree of silencing among the MGC-derived progenies through generations. Northern analysis of $T_2$ generation MGC plants clearly showed the absence of a transcript band (Fig. 3*E*) in the silenced plants (#M10 and 16) and also indicated slightly varying levels of transcript accumulation among the plant population. Our preliminary investigation indicated that transgene silencing is often associated with methylation of transgene (data not shown). Breitler *et al*. (2002) also observed a loss of expression of the *Yfp* transgene in rice transformants, which occurred two-fold more frequently among plants transformed with whole plasmid DNA than among those transformed with gene cassettes. Fu *et al*. (2000) proposed that structurally intact transgene loci coupled with a low copy number were the possible reasons for improved stability of expression of transgenes in minimum cassettes compared to whole plasmid transformations. It is not clear how the use of short cassettes has such a profound effect on locus organization and expression. Perhaps the foreign DNA is in a relaxed configuration and therefore is less likely to succumb to shear forces and prokaryotic vector-borne sequences are typically absent in this method (Kohli *et al*. 2003).

One of the most useful applications of particle bombardment is the ease of the method and opportunity for simultaneous delivery of multiple genes (Skinner *et al*. 2004). The inherent constraints associated with bombardment are derivation of plants with varying copy numbers of transgenes, introduction of vector backbone sequences in conventional plasmid DNA and unnecessary insertion of elements including bacterial origin of replication and a selectable bacterial gene, which are not essential for plant transformation. Persistence of these sequences in field-released commercial transformants is considered undesirable from the stand-point of biosafety. The use of minimum gene cassettes can further simplify the bombardment protocol and maximize its efficiency and improve its selectivity to regenerate high expressing low copy number plants.

Our study demonstrates the utility of MGC for transformation of bentgrass as reported earlier in rice (Fu *et al.* 2000, Breitler *et al*. 2002). Further experiments are being conducted to evaluate the effectiveness of this approach for transformation of other crop plants using agronomically important transgenes.

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