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Transient reporter gene (GUS) expression in creeping bentgrass (*Agrostis palustris*) is affected by *in vivo* nucleolytic activity

Chhandak Basu[1], Hong Luo[2], Albert P. Kausch[2] & Joel M. Chandlee[3,*]
[1]*Department of Plant Sciences, University of Rhode Island, 9 East Alumni Avenue, Suite 7, Kingston, Rhode Island 02881, USA*
[2]*HybriGene, Inc., 530 Liberty Lane, West Kingston, Rhode Island 02892, USA*
[3]*Department of Cell and Molecular Biology, University of Rhode Island, Morrill Science Center, Kingston, Rhode Island 02881, USA*
**Author for correspondence (Fax: 401-874-4256; E-mail: joel_chandlee@uri.edu)*

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Abstract

Leaf and callus tissues of a creeping bentgrass cultivar (Penn A4) had high nuclease activities that degraded exogenously added plasmid DNA. When callus tissue was incubated for 24 h with heparin, spermidine, aurintricarboxylic acid or polyethylene glycol, only heparin and spermidine were effective as *in vitro* nuclease inhibitors, protecting exogenously added plasmid DNA from degradation. When β-glucuronidase (GUS) reporter gene activity was evaluated in heparin-treated (0.6%), 14-month old callus following microprojectile bombardment, GUS activity increased 1000-fold compared to equivalent aged untreated Penn A4 callus. Similar enhancement from heparin pretreatment (0.6% or 1.2%) was not observed in 6-month old callus. This is likely due to much higher activities of nuclease in the younger callus.

Introduction

Turfgrass, an economically important horticultural crop (Lee 1996) in the USA, is a target for trait modification and enhancement using the modern tools of biotechnology for gene transfer (Chai & Sticklen 1998). Although the first reported production of transgenic turfgrass was about ten years ago (Zhong *et al.* 1993), there have been no reports on the effect of *in vivo* nuclease activity on transformation efficiency when using the biolistics approach for gene transfer. The action of nucleases has been investigated in pollen (Booy *et al.* 1989, Brogila & Corona 1995, Van Wert & Saunders 1992), microspores (Jardinaud *et al.* 1993, Vischi & Marchetti 1997, Simon & Foroughi 2000) and protoplasts (Folling *et al.* 1998) used for plant transformation purposes in various plant species. Nuclease activity has also been found in pepper leaves as a defense mechanism against the pathogen *Xanthomonas campestris* pv. (Buonaurio *et al.* 2001). Nuclease activity is also associated with the senescence process in plants [e.g., LeNUC1, a nuclease associated with leaf senescence of tomato (Lers *et al.* 2001), BFN1, a nuclease induced during leaf and stem senescence of *Arabidopsis* (Perez *et al.* 2000), and single strand preferring nuclease activity during leaf senescence of barley (Wood *et al.* 1998)]. Muramoto *et al.* (1999) reported higher nuclease activity in barley leaves under salt stress. Here we report the characterization and analysis of nuclease activity and its effect on transient β-glucuronidase (GUS) reporter gene (Jefferson 1987) expression following microprojectile bombardment (Sanford 1988) of turfgrass.

Materials and methods

Tissue culture and plant materials

Two cultivars of creeping bentgrass (*Agrostis palustris*) namely, Providence and Penn A4, were investigated. Penn A4 creeping bentgrass plants were grown from seeds in pathogen-free soil at room temperature under a 12 h d photoperiod and periodically watered. For callus induction with Providence and Penn A4, seeds were first soaked in 70% (v/v) ethanol for 2 min followed by a solution consisting of 10% (v/v) $NaClO_4$ and 4 drops of Tween 20 with continuous shaking for about 45 min. The seeds were then rinsed 3 times in sterile water in a laminar flow hood and plated on turfgrass tissue culture medium (referred to as MMSG medium) consisting of: 4.33 g MS (Murashige and Skoog) salts, 30 g sucrose, 1 ml MS vitamins (1000×), 0.5 g casein hydrolysate, 1 ml 60 mM dicamba (dichloro-*o*-anisic acid) and 500 μl 4 mM 6-BAP (6-benzylaminopurine) in 1 l of water. The pH was adjusted to 5.6–5.8 with 1 M KOH. Phytagel (2.4 g; Sigma Chemicals, USA) was added prior to autoclaving. Embryogenic callus was cultured on this media for either 6 or 14 months and transferred to osmoticum medium (1 l MMSG medium plus 45.6 g sorbitol and mannitol each) 4–6 h prior to particle bombardment. Six month old callus was defined as 'young' and 14-month old callus was defined as 'old'. The callus was routinely subcultured to fresh MMSG medium every four weeks. Liquid MMSG and osmoticum media were prepared in the same way described above without the addition of Phytagel before autoclaving.

Nuclease activity determination

Nuclease activity in various turfgrass tissues was determined following Barandiaran *et al.* (1998) with modifications. Young Penn A4 (4-weeks old) leaves were first rinsed with sterile water, then 70% ethanol, and finally again with sterile water to reduce any nuclease activity in the leaves derived from microbes. Nuclease activity determination mix was prepared as follows: 10 mg leaves of Penn A4 were ground in 1 ml liquid MMSG medium in a sterile microfuge tube using a sterile mini-pestle. Ten μg $pSKII^+$ plasmid DNA (1 $\mu g\ \mu l^{-1}$; Stratagene, USA) was then added. The mix was briefly vortexed, centrifuged for 1 min and incubated at 23 °C for 10 min. The control DNA was processed in the same manner with the exception that no tissue extract was added to the mix. To determine a time course for nuclease activity, 20 μl samples were collected periodically and run in a 1% agarose gel.

Nuclease inhibition

Microfuge tubes containing 5 μl nuclease activity determination mix (for both leaf and callus) and 10 μl sterile deionized water were prepared. Then 4.2 μl of each of the four different nuclease inhibitors [the stock concentrations were: 0.6% heparin salt solution in water, 2 mM aurintricarboxylic acid (ATA), 10% polyethylene glycol (PEG) and 1 M spermidine] were added separately in each microfuge tube (for both leaf and callus). Ten μg (1 $\mu g\ \mu l^{-1}$) of exogenous $pSKII^+$ plasmid DNA was added to each of the microfuge tubes and incubated at 23 °C for 10 min. The control DNA was not treated with any nuclease inhibitors. Twenty μl of this reaction mix was run in a 1% agarose gel. Samples containing of each of the four nuclease inhibitors and plasmid DNA were mixed individually and also run in the same gel without any tissue extract to determine whether nuclease inhibitors had any adverse effects on DNA.

Microprojectile bombardment

A plasmid (1 $\mu g\ \mu l^{-1}$) carrying a GUS reporter gene construct driven by the ubiquitin rice promoter (Huq *et al.* 2000) was coated on the surface of 0.6 μm gold particles and delivered into callus using the PDS-1000/He (Bio-Rad, USA) biolistic gene delivery device. Seven μl of the plasmid DNA coated gold particles were placed in the center of a macrocarrier (Sanford 1988). The particle delivery system was adjusted to 3 mm gap distance, 10 mm macrocarrier flight distance, 1100 psi (77.34 kg cm^{-2}) of helium pressure for delivery and 27 mm Hg vacuum inside the chamber.

14-month old callus tissue of Penn A4 was submerged overnight in four separate solutions as follows: 8.33 ml 2 mM ATA in 10 ml liquid MMSG medium or 10 ml liquid osmoticum medium, and 8.33 ml 0.6% heparin salt in 10 ml liquid MMSG medium or 10 ml liquid osmoticum medium. On the next day, the tissues were vacuum infiltrated for 15 min and then arranged in the center of a petri dish (covering an area of 19.64 cm^2) on solid osmoticum medium about four hours prior to bombardment. 0.6% heparin salt in combination with liquid osmoticum medium was able to trigger the highest GUS activity and therefore used for all subsequent experiments investigating the inter-

action of nuclease activity with biolistically delivered plasmids.

Tissues were kept in the dark at room temperature after bombardment. GUS hits or TEUs (Transient Expression Units) (Moore *et al.* 1994) were counted manually under a dissecting scope 48 h after bombardment after GUS staining.

Young (6-month old) and old (12-month old) callus tissue of Providence and young (6-month old) callus tissue of Penn A4 (3 plates each) were treated with 0.6% heparin salt plus osmoticum medium overnight and bombarded following the same experimental procedure described above. Young callus tissue of Providence and Penn A4 (3 plates each) were also treated with 1.2% heparin salt plus liquid osmoticum medium overnight and bombarded following the same experimental procedure described above. The control callus was transferred to osmoticum medium 4–6 h prior to bombardment and bombarded without any treatment.

Reporter gene assay

GUS staining solution consisted of the following: 50 mM potassium ferrocyanide, 50 mM potassium ferricyanide, 5 ml 0.2 M sodium phosphate buffer, 0.5 M sodium EDTA and 10% Triton X and water. X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronide) (working concentration of 25 mg X-Gluc ml^{-1} of *N*,*N* dimethyl formamide; Gold Biotechnology, USA) was added to the above reaction mix at 352 μl reaction mix to 48 μl X-Gluc solution. Tissues were incubated in the GUS solution at 37 °C for 16 h. The GUS solution was discarded and the tissues were rinsed with water.

Results and discussion

Strong nuclease activity was detected in both young leaf tissue (Figure 1) and 6-month old callus (Figure 2A) of creeping bentgrass cultivar Penn A4. Nuclease in callus tissue of Penn A4 degraded the foreign DNA within 1 min after addition of the DNA (Figure 2A; lane 3). Barandiaran *et al.* (1998) also reported high nuclease activity in leaf, bulb and callus tissues of garlic. They reported that nuclease activity in garlic callus was sufficient to completely degrade the added plasmid DNA. Vischi & Marchetti (1997) reported immediate nucleolytic activity after addition of foreign DNA in barley microspores. The extent of DNA degradation reported by them within a minute is less drastic than what we observed for leaf extracts (Figure 1). Jardinaud *et al.* (1993) reported that nuclease activity in *Bassica napus* microspores degraded exogenous DNA within 5 min of incubation.

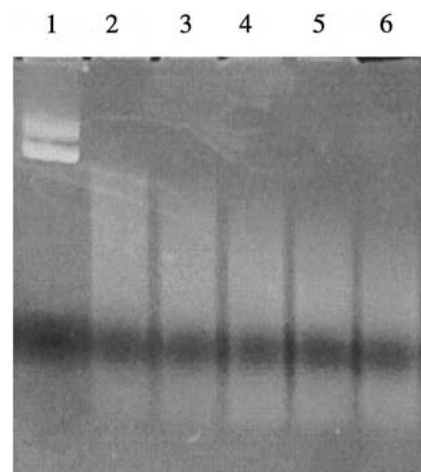

Fig. 1. Time course of nuclease activity in young leaf tissue extracts of Penn A4; lane 1: control DNA; lanes 2–6: samples (plasmid DNA + leaf tissue extract) taken 1, 3, 5, 7, 10 min, respectively, after addition of plasmid DNA.

Heparin salt (0.6%) effectively inhibited nuclease activity in both 6-month old callus (Figure 2A; lane 4) and mature leaf (Figure 2A; lane 10) tissue extracts since the plasmid DNA had almost the same integrity compared to control or untreated plasmid DNA (Figure 2A; lane 1). Spermidine (Figure 2A; lanes 5 and 11) was also an effective nuclease inhibitor. ATA (Figure 2A; lanes 6 and 12) and PEG were less effective (Figure 2A; lanes 7 and 13) in protecting exogenously added plasmid DNA. Barandiaran *et al.* (1998) reported that ATA was the best nuclease inhibitor in protecting exogenous DNA in garlic callus tissue extracts. Folling *et al.* (1998) showed that Ca^{2+} most effectively decreased nuclease activity in *Lolium perenne* L. protoplasts. Vischi & Marchetti (1997) reported that Mn^{2+} inhibited nucleolytic activity in barley microspores.

Nucleolytic activity in callus (Figure 2A; lane 3) was much greater than in the mature leaf tissue (Figure 2A; lane 9). For callus, the exogenously added plasmid DNA was almost completely degraded, whereas the control DNA was intact (Figure 2A; lane 1) after 10 min. The results also indicate that nuclease activity in young leaves after only one minute of incubation (Figure 1; lane 6) was much stronger than in mature leaves (Figure 2A; lane 9). Different tissues of turfgrass had different nuclease activities. Barandiaran

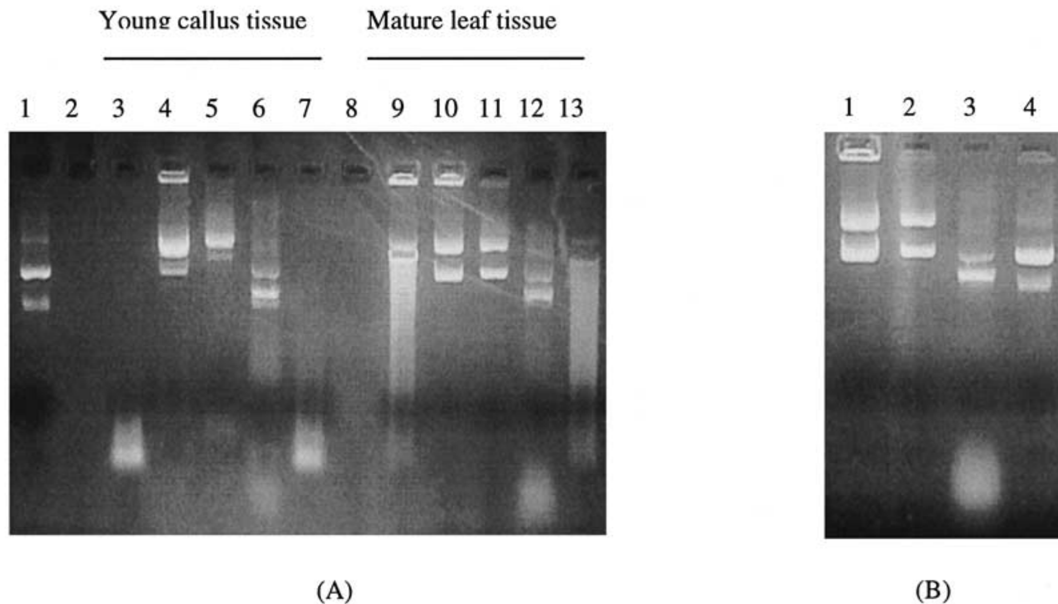

Fig. 2. Ability of different nuclease inhibitors to protect the integrity of plasmid DNA in 6-month old callus and mature leaf tissue extracts of Penn A4 after 10 min of incubation in nuclease activity determination mix. Note: nuclease activity in callus (A, lane 3) degraded the plasmid DNA completely whereas nuclease activity in leaf (A, lane 9) was less effective. (A) callus or mature leaf tissue extract + nuclease inhibitors + plasmid DNA; lane 1: control DNA, lane 3: plasmid DNA + callus tissue extract with no nuclease inhibitor, lane 4: plasmid DNA + callus tissue extract + 0.6% heparin, lane 5: plasmid DNA + callus tissue extract + 1 M spermidine, lane 6: plasmid DNA + callus tissue extract + 2 mM ATA, lane 7: plasmid DNA + callus tissue extract + 10% PEG, lane 9: plasmid DNA + leaf tissue extract with no nuclease inhibitor, lane 10: plasmid DNA + leaf tissue extract + 0.6% heparin, lane 11: plasmid DNA + leaf tissue extract + 1 M spermidine, lane 12: plasmid DNA + leaf tissue extract + 2 mM ATA, lane 13: plasmid DNA + leaf tissue extract + 10% PEG. (B) plasmid DNA + nuclease inhibitors only (no tissue extract); lane 1: 0.6% heparin + plasmid DNA, lane 2: 1 M spermidine + plasmid DNA, lane 3: 2 mM ATA + plasmid DNA, lane 4: 10% PEG + plasmid DNA.

et al. (1998) also reported that nuclease activity in callus tissues of garlic were much more higher compared to those in bulb or leaf.

To determine whether the added nuclease inhibitors themselves had any adverse effects on exogenous DNA, nucleases inhibitors and plasmid DNA were mixed without any added tissue extracts. While ATA affected plasmid mobility (Figure 2B; lane 3), no plasmid degradation was observed. When tissue extract was added with ATA (Figure 2A; lanes 6 and 12), plasmid degradation did occur to some extent.

Following microprojectile bombardment, we observed a dramatic increase (more than 1000-fold compared to the untreated control) in TEUs (Transient Expression Units; Moore *et al.* 1994) for GUS in 14-month old callus of Penn A4 treated with heparin salt in osmoticum medium (Figure 3). ATA (either in combination with liquid MMSG or osmoticum medium) did not enhance TEUs to this extent and neither did heparin in combination with liquid MMSG medium only (Figure 3). It appears that osmoticum medium, which plays an important role in cell plasmolysis before bombardment, enhances TEUs in combination with heparin salt.

Although Barandiaran *et al.* (1998) reported no TEUs in garlic tissues unless the tissues were treated with nuclease inhibitors, we observed a high number of TEUs in untreated young callus tissue of two cultivars of creeping bentgrass (Providence and Penn A4) (Figure 4). Barandiaran *et al.* (1998) also reported that nuclease inhibition treatment prior to bombardment enhanced TEUs in various tissues of garlic. But, Ferrer *et al.* (2000) reported nuclease inhibition treatment was unnecessary prior to bombardment for enhancement of TEUs in garlic tissues. While, these two reports are at odds, it should be noted that each group used different garlic cultivars. We observed that nuclease inhibition by heparin was not equally effective in enhancing GUS TEUs for different genotypes of creeping bentgrass or for all developmental stages of the callus (Figure 4). Nuclease inhibition treatment with 0.6% heparin salt was not able to enhance TEUs in old Providence callus as it did for Penn A4 (Figure 4). We did not see any enhancement of TEUs

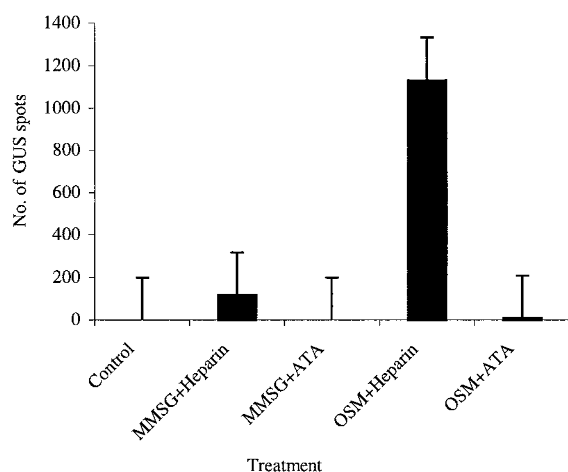

Fig. 3. Number of GUS spots (Transient Expression Units or TEUs) following microprojectile bombardment in 14-month old callus tissue of Penn A4 after treatment with two different nuclease inhibitors (2 mM aurintricarboxylic acid or ATA and 0.6% heparin). MMSG: regular turfgrass tissue culture medium, OSM: 45.6 g sorbitol and mannitol each l^{-1} of MMSG. Data are the average of 3 plates.

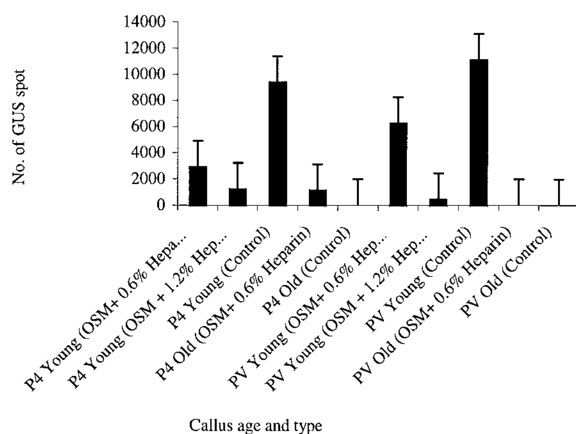

Fig. 4. Number of GUS spots (Transient Expression Units or TEUs) following microprojectile bombardment of old callus tissue (14-month old) of Providence and Penn A4 and young callus tissue (6-month old) of Providence and Penn A4 after treatment with different nuclease inhibitors (0.6% and 1.2% heparin). Data are the average of 3 plates. PV: Providence, P4: Penn A4, MMSG: turfgrass tissue culture medium, OSM: 45.6 g sorbitol and mannitol each l^{-1} of MMSG. Note: Old control callus did not express GUS spots at any time.

when we treated Providence or Penn A4 callus with either 0.6% or 1.2% heparin compared to the untreated control (Figure 4). In fact, heparin treatment had a negative effect on young Penn A4 and Providence callus compared to the untreated control (Figure 4) and we observed that the number of TEUs went down as heparin concentration was increased from 0.6% to 1.2% (Figure 4). To investigate this phenomenon

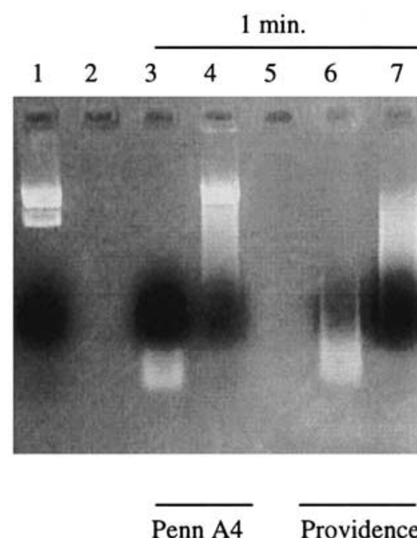

Fig. 5. Nuclease activity in Providence and Penn A4 callus extracts of varying age. Lane 1: control plasmid DNA; lane 3: Penn A4 young callus extract + plasmid DNA; lane 4: Penn A4 old callus extract + plasmid DNA; lane 6: Providence young callus extract + plasmid DNA; lane 7: Providence old callus extract + plasmid DNA.

further (inability of heparin to enhance TEUs after bombardment of young and old callus of Providence and young callus of Penn A4), we determined that old Penn A4 callus had the least nuclease activity compared to all other types of callus (old Providence, young Providence and young Penn A4) (Figure 5). It is clear that within 1 min, old Providence and young Providence and young Penn A4 callus degraded the exogenously added plasmid DNA to a greater extent than old Penn A4 callus (Figure 5). We conclude that because of high nuclease activity in old Providence callus and young Providence and Penn A4 callus (Figure 5), heparin treatment was not effective in inhibiting nuclease activity in those callus tissues (Figure 4). On the other hand, we were able to reduce nuclease activity to a great extent in old Penn A4 callus (Figure 3) as there was comparatively lower nuclease activity present (Figure 5). This observation also supports our reasoning for the presence of higher TEUs following nuclease inhibition treatment in old Penn A4 callus compared to all other types of callus (Figures 3 and 4). In other words, less nuclease activity in old Penn A4 callus is more easily inhibited by the heparin concentration used, thus leading to enhanced TEUs in this treated tissue. It may be that heparin is an effective inhibitor when nuclease activities are below a threshold

level and ineffective when above the threshold level as would be present in young callus.

It is not uncommon for different genotypes to express different frequencies of TEUs (e.g. in the case of onions) (Eady *et al.* 1996). We have also previously seen that Providence and Penn A4 callus have different abilities to express TEUs following microprojectile bombardment (data not shown). The age of the callus can also play a prominent role in transient reporter gene expression following microprojectile bombardment (Takumi *et al.* 1996). We have shown that nuclease activities in callus tissues of turfgrass are dependent on age and on genotype (Figure 5). The variation in GUS expression with different genotypes at different developmental stages (Figure 4) may be due to 'other inhibitory compounds' (Ferrer *et al.* 2000). Vainstein *et al.* (1993) reported that some phenolic compounds repressed TEUs in carnation leaf following microprojectile bombardment. They also reported that treating the leaf extract with polyvinylpolypyrrolidone enhanced GUS activity as measured by a fluorometric assay.

This is the first report of effect of nuclease inhibitors on transient reporter gene (GUS) expression following microprojectile bombardment in turfgrass. More research needs to be performed to determine whether heparin may have any negative (or positive) effect on regeneration frequencies in old Penn A4 callus. We are in the process of analyzing stable GUS expression in Penn A4 young and old callus after heparin treatment.

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