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BIOTECHNOLOGY

Transgene integration complexity and expression stability following biolistic or *Agrobacterium*-mediated transformation of sugarcane

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Abstract Sugarcane (*Saccharum* spp. hybrids) accounts for 80% of the table sugar produced worldwide and is also a prime feedstock for biofuel production. However, very few studies are available for directly comparing *Agrobacterium tumefaciens*-mediated transfer of T-DNA (AMT) and biolistic transfer of minimal expression cassettes (BLT MC) regarding transgene complexity and expression stability. In this study, the transformation efficiency, transgene integration pattern, expression level, and expression stability were compared in the commercially important sugarcane cultivar CP88-1762. A total of 312 transgenic lines derived from AMT and 250 lines derived from BLT MC were identified by PCR from genomic DNA using *npt*II-specific primers. Lines were analyzed with both qPCR (TaqMan®) and NPTII ELISA to determine the *npt*II transgene copy number and expression level. The results of Southern blot analysis on selected lines were highly correlated to the qPCR results. There were no significant differences between the two transformation systems for transformation efficiency, frequency of single copy integration, or level and stability of transgene expression when carried out with the same expression cassette, tissue culture, and selection procedure in 12 independent experiments. These findings suggested that both BLT MC and AMT provide suitable platforms for generation of elite sugarcane events.

Keywords Sugarcane · *Saccharum* · Biolistic transformation · *Agrobacterium tumefaciens*-mediated transformation · Transgene integration · Transgene expression level · Transgene expression stability

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Introduction

Commercialization of transgenic sugarcane requires reliable transgene performance. Biolistic gene transfer (BLT) is the most common method used for transgene delivery to sugarcane because of its applicability to a wide range of genotypes (Altpeter and Oraby 2010). *Agrobacterium tumefaciens*-mediated gene transfer (AMT) is typically limited to few genotypes (Jackson *et al.* 2013; Joyce *et al.* 2014). BLT and AMT to sugarcane were first reported by Bower and Birch (1992) and Arencibia *et al.* (1998), respectively. Both methods result in variable transgene integration complexities with subsequent consequences for the transgene performance. Transgenes which are inserted in multiple copies are more likely to be silenced (Meyer 1995; Schubert *et al.* 2004; Meng *et al.* 2006). But even single-copy transgenic events can undergo silencing depending on where in the genome they are inserted (Stoger *et al.* 1998; Kohli *et al.* 1999). Single-copy integration of transgenes into the plant genome also facilitates structural characterization (Que *et al.* 2014).

AMT has traditionally been the preferred method to generate events with low transgene copy number. Standard BLT protocols, in which large quantities of whole plasmid constructs are introduced, typically result in the integration of

multiple transgene copies as well as vector backbone sequences into the plant genome (Jayaraj *et al.* 2008). BLT protocols were improved by removal of the vector backbone prior to gene transfer (Fu *et al.* 2000; Breitler *et al.* 2002) and delivery of drastically reduced quantities of such minimal expression cassettes (MC) (Lowe *et al.* 2009). Compared with delivery of whole plasmids in large quantities, delivery of lower quantities of MC eliminated the vector backbone integration and increased the proportion of low-copy, structurally-intact transgene loci. This resulted in improved transgene performance (Fu *et al.* 2000; Lowe *et al.* 2009). To date, MC technology has been used to introduce genes of interest into several plant species such as rice (*Oryza sativa*; Fu *et al.* 2000; Breitler *et al.* 2002; Loc *et al.* 2002; Agrawal *et al.* 2005; Zhao *et al.* 2007), corn (*Zea mays*; Lowe *et al.* 2009; Prakash *et al.* 2009), wheat (*Triticum aestivum*; Yao *et al.* 2006, 2007), creeping bentgrass (*Agrostis stolonifera*; Jayaraj *et al.* 2008), bahiagrass (*Paspalum notatum*, Sandhu and Altpeter 2008), sugarcane (*Saccharum* spp. hybrids, Taparia *et al.* 2012a; Taparia *et al.* 2012b; Jackson *et al.* 2013), soybean (*Glycine max*; Vianna *et al.* 2004; Gao *et al.* 2008; Liu *et al.* 2009), grapevine (*Vitis vinifera*; Vidal *et al.* 2006), potato (*Solanum tuberosum*; Romano *et al.* 2003), and common bean (*Phaseolus vulgaris*; Vianna *et al.* 2004).

Although both AMT and BLT are the main gene delivery systems for many plant species, few comparisons of these methods have been made for transformation efficiency, transgene copy number, and transgene expression. The first comparison was described in barley by Travella *et al.* (2005). Recently, a comparison of the two gene transfer methods was carried out using sugarcane cultivar Q117, which is efficiently transformed but, unfortunately, is susceptible to smut (Jackson *et al.* 2013; Joyce *et al.* 2014). Here, a comparison of AMT and BLT is made for transformation efficiency, number of integrated transgene copies, transgene expression, and stability of transgene expression in the commercially important sugarcane cultivar CP88-1762.

Materials and Methods

*Plant material***.** Sugarcane tops, including the shoot apex and the top visible node, were harvested from field grown cultivar CP88-1762 at the Everglades Research and Education Center, University of Florida, Belle Glade, Florida.

DNA for gene transfer. For both AMT and BLT of minimal expression cassettes (BLT MC), the pPZP 200 binary vector (Hajdukiewicz *et al.* 1994) was used which carried *npt*II driven by maize ubiquitin 1 promoter, the ubiquitin 1st intron (Christensen *et al.* 1992), and the 3′ UTR of nopaline synthase gene (Fig. 1*a*). For BLT, this expression cassette was released by digestion with *Pme*I and *Ssp*I, electrophoresed (70 V, 180 min) on agarose gel (0.8% *w/v*), and gel-elution using QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA). The purified fragment was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE).

Tissue culture and transformation. The outermost leaf sheaths were removed from immature leaf whorls. Immature leaf whorls were wiped with 70% ethanol in a laminar flow bench and 2-mm cross-sections were transferred to CI3 medium (Chengalrayan and Gallo-Meagher 2001). Tissue cultures were incubated in the dark at 28°C and subcultured at weekly intervals. After 6 to 8 weeks, embryogenic calluses emerging from leaf whorl cross-sections were used as targets for gene transfer. AMT was conducted as described by Wu and Altpeter (2015). Briefly, *Agrobacterium tumefaciens* strain AGL1 harboring the binary vector was grown to a density OD_{600}=0.6 and diluted 50-fold with CI3 medium. Calluses were immersed in the inoculum for 25 min. The calluses were then blotted with sterile filter paper to remove excess *A. tumefaciens* and transferred to co-cultivation medium for 3 d at 19°C in the dark. After co-cultivation, calluses were cultured on CI3 medium with 100 mg l^{-1} cefotaxime and 100 mg l^{-1} timentin (Cat #s C380, T869, respectively, PhytoTechnology Laboratories®, Shawnee Mission, KS) without geneticin for 4 d in darkness. The calluses were then transferred to CI3 medium supplemented with 30 mg l^{-1} geneticin (Cat# G810 PhytoTechnology Laboratories®), 100 mg l^{-1} cefotaxime, and 100 mg l^{-1} timentin for three biweekly subcultures in light (30 μE/m^2/s, 16 h photoperiod, F17T8/TL841, Philips, Amsterdam, The Netherlands). Geneticin resistant calluses were transferred to regeneration medium with 0.1 mg l^{-1} BAP, 1.86 mg l^{-1} NAA, 30 mg l^{-1} paromomycin (Cat# P710, PhytoTechnology Laboratories®), 100 mg l^{-1} cefotaxime, and 100 mg l^{-1} timentin, and cultured at 28°C under a light density of 150 μE/m^2/s. After reaching ~2 cm in length, regenerating shoots were transferred for rooting to hormone free MS medium (PhytoTechnology Laboratories® Murashige and Skoog 1962) with 30 mg l^{-1} paromomycin, 100 mg l^{-1} cefotaxime, and 100 mg l^{-1} timentin, and cultured at 28°C under a light intensity of 150 μE/m^2/s.

BLT was performed as described by Altpeter and Sandhu (2010). The MC was precipitated onto gold particles as described previously (Taparia *et al.* 2012a) at a concentration of 54.6 ng per 100 μl of the final particle suspension, resulting in 2.73 ng of MC per shot.

PCR amplification of transgenic nptII. Genomic DNA was extracted from 200 mg young leaf tissue following a modified CTAB protocol (Porebski *et al.* 1997). DNA was quantified

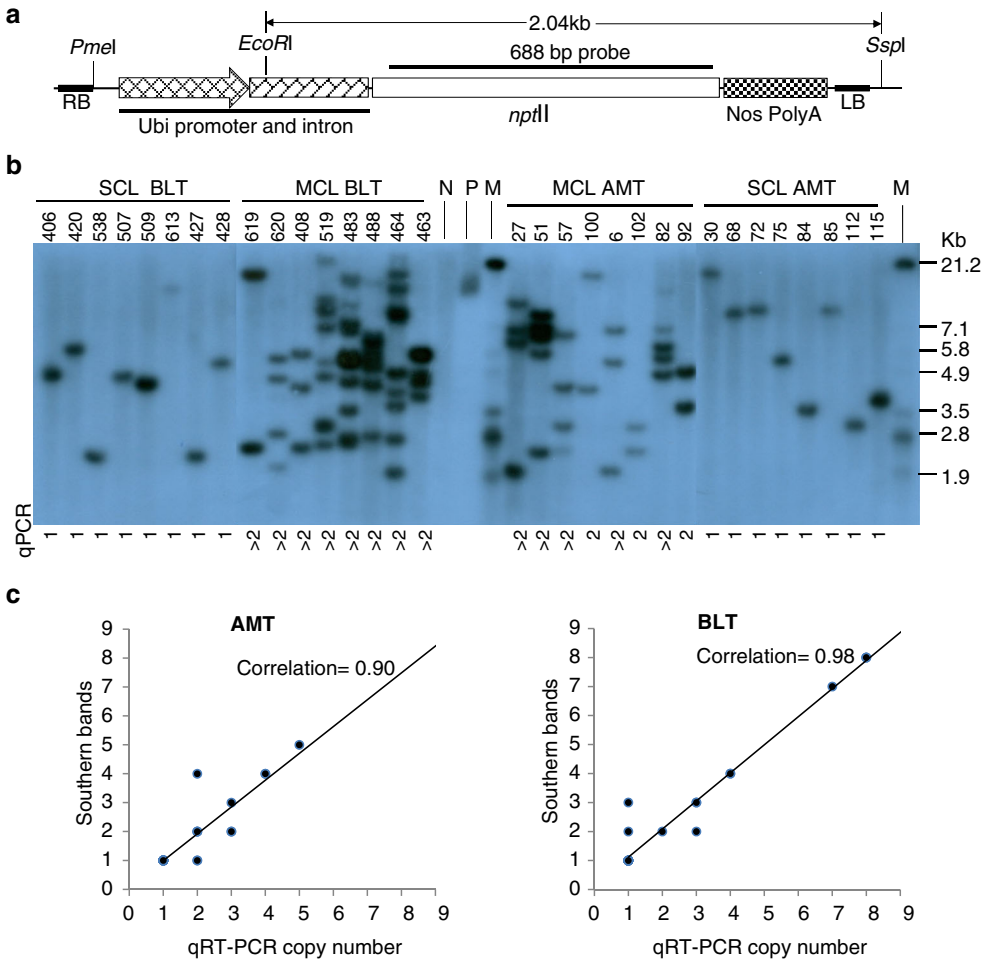

Figure 1. Analysis of *npt*II transgene copy number. (*a*) *npt*II expression cassette transferred into sugarcane genome by AMT or BLT. Vector components are not drawn to scale. (*b*) Southern blot analysis of *npt*II transgenic sugarcane lines. Genomic DNA was digested with *Eco*RI which cut once in the expression cassette at maize ubiquitin first intron and is 1790 bp upstream of T-DNA left border and 2044 bp upstream of S*sp* I site. A 688 bp probe from the *npt*II coding region was used for Southern hybridization. Transgenic line number is indicated above each lane, and the qPCR result is indicated below each lane. *N* negative control (DNA from non-transgenic sugarcane); *P* plasmid DNA digested with *Pme*I which cuts only once in the construct; *M* molecular marker; *SCL BLT* single copy transgenic lines from biolistic transformation; *MCL BLT* multiple copy transgenic lines from biolistic transformation; *MCL AMT* multiple copy transgenic lines from *Agrobacterium*-mediated transformation; *SCL AMT* single copy transgenic lines from *Agrobacterium*-mediated transformation. (*c*) Correlation between qPCR and Southern blot: 34 AMT-derived lines and 28 BLT-derived lines underwent both types of copy number determination. qPCR results are displayed as 1, 2, and >2 transgene copies.

using a NanoDrop (Thermo Scientific Inc., Wilmington, DE) spectrophotometer, and 50 ng genomic DNA was used as template for PCR with 30 cycles of 95°C 20 s, 57°C 30 s, and 72°C 30 s using the following *npt*II-specific primers: forward primer 5′-tgctcctgccgagaaagtat-3′ and reverse primer 5′-catgtgtcacgacgagatcc-3′.

nptII Immuno-chromatography (Immunostrip®) assay. Young leaves (60 mg) of putative transgenic lines were ground in 1 ml of the extraction buffer provided with the NPTII ImmunoStrip® kit (Cat# STX 73000, Agdia Inc., Elkhart, IN). Samples were centrifuged at 16,000*g* at room temperature for 2 min. The supernatant was transferred into a clean microfuge tube where it was absorbed by ImmunoStrip®. A positive reaction for NPTII was indicated by the development of two purple lines.

qRT-PCR analysis of transgene copy number. DNA was isolated as reported by Murray and Thompson (1980) with modifications. The pelleted DNA was resuspended in Tris-EDTA, pH 8.0 for TaqMan copy call estimation. The quantitative TaqMan assay for copy number was adapted from methods previously described (Ingham *et al.* 2001). Master mix (3 μl/well; 2× JumpStart Taq ReadyMix, primer for the crop-specific endogenous gene (Glyceraldehyde 3-phosphate dehydrogenase), and 2× primer set stock assay target (*npt*II) were combined in 384-well plates with 3 μl genomic DNA, or with the DNA samples for the copy control. The copy control is a

transgenic sugarcane event that was previously confirmed by Southern blot for carrying a single copy of the *npt*II gene.

NPTII expression analysis. The NPTII ELISA kit (Cat# PSP73000, Agdia Inc., Elkhart, IN) was used to evaluate NPTII expression. Protein was extracted from the midsection of the first dewlap leaf following the manufacturer's instructions. Extracted protein samples were quantified by the Bradford assay (Bradford 1976), utilizing Coomassie Plus Protein Assay reagent (Cat# 23238, Thermo Fisher Scientific Inc., Rockford, IL). A total of 20 μg of soluble protein per plant extract were loaded into wells of ELISA plates. ELISA was performed following the manufacturer's instructions. Absorbance readings were recorded with a Synergy™ H1 Hybrid multi-mode microplate reader (BioTek, Winooski, VT).

Southern blot analysis of transgene copy number. Total genomic DNA was extracted from 2 g leaf tissue following a modified CTAB protocol (Porebski *et al.* 1997). DNA (20 μg) was digested with *Eco*RI (Cat# R0101M, New England Biolabs, Ipswich, MA), electrophoresed overnight on an agarose gel (agarose 0.8% [*w/v*]; 1× TAE), and blotted onto Hybond-N+ nylon membranes (Cat# RPN87B, Amersham Biosciences, Piscataway, NJ) by capillary transfer overnight in 10× SSC. After air drying, the membranes were exposed to UV light in a crosslinker (Select™ XLE-Series, Spectroline®, Westbury, NY) and prehybridized in 6× SSC, 1× Denhardt's Solution with 100 mg l^{-1} denatured herring sperm DNA, and 1% SDS at 42°C for 3 h. A 688-bp PCR product amplified from the *npt*II coding region using forward primer 5′-ggctattcggctatgactgg-3′ and reverse primer 5′-gcgataccgtaaagcacgag-3′(PCR conditions: 30 cycles of 95°C 20 s, 58°C 30 s, and 72°C 60 s) was then labeled for use as a probe using (α-^{32}P) dCTP (Cat# NEG013H250UC, Perkin Elmer Inc., Waltham, MA) with a Prime-It II Random Primer Labeling Kit (Cat#300385, Stratagene Inc., La Jolla, CA). The membranes were hybridized with the denatured probe in 6× SSC, 50% formamide, 500 mg l^{-1} denatured herring sperm DNA, and 1% SDS at 42°C overnight; rinsed once in 0.1× SSC and 0.1% SDS for 30 s; and then washed twice with 50 ml of 0.1× SSC and 0.1% SDS at 65°C for 20 min each wash. Hybridization signals were visualized by autoradiography on x-ray film following a 2-d exposure to the membranes at −80°C.

Statistical analysis. For the analysis of transgene copy number, the chi-square test was performed with one degree of freedom at the 5% probability level. All other statistical analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC). Means were compared by the *t* test, and values are considered as significantly different if $P<0.05$.

Results

Transformation efficiency. PCR analysis of genomic DNA using *npt*II-specific primers identified 312 transgenic lines following AMT and 250 lines derived from BLT in 12 independent experiments (Table 1). The transformation efficiency was not significantly different ($P<0.05$) between AMT and BLT with 1.82±0.09 lines/g callus for AMT and 1.76±0.25 lines/g callus for BLT (Table 1). The frequency of non-transgenic regenerated lines escaping the selection was not significantly different ($P>0.05$) between AMT (1%) and BLT (4%). Expression of the *npt*II gene was confirmed by ELISA in 99% of the lines generated by AMT and 100% of the lines generated by BLT that showed positive PCR reactions.

Transgene integration complexity. qPCR Taqman® assays for determination of transgene copy number were carried out on 513 transgenic lines, including 279 lines from AMT and 234 lines from BLT. There was no significant difference between the two methods for the frequency of single-copy transgene integration events. A single-copy event was detected in 49.2% of the BLT-derived lines and 35.5% of the AMT-derived lines (Table 2). Southern blot analysis was carried out on 34 AMT-derived lines (22 single-copy and 12 multiple-copy lines) and 28 BLT-derived lines (18 single-copy and 10 multiple-copy lines). Figure 1*b* shows representative Southern blots. The correlations between copy number estimates based on Southern blot and qPCR analysis were 0.90 (AMT-derived lines) and 0.98 (BLT-derived lines) (Fig. 1*c*).

Transgene expression analysis. NPTII ELISA for quantification of transgene expression was carried out with 243 AMT-derived and 221 BLT-derived lines. Of the 243 analyzed AMT-derived lines, 94 carried single-copy transgenes and 149 carried multiple-copy transgenes. The 221 BLT lines included 114 lines with single-copy inserts and 107 lines with multiple-copy inserts. There was no significant difference ($P>0.05$) in the frequency of transgene expression between the two transformation methods among single-copy lines or among multiple-copy lines. However, a highly significant difference ($P<0.01$) was found for the frequency of transgene expression between single- vs. multiple-copy lines irrespective of the transformation method (Table 3). For AMT-derived lines, mean NPTII for multiple-copy lines was 11.17 ng/20 μg soluble protein, 37% higher than observed for single-copy lines (7.01 ng/20 μg soluble protein). Similarly, for BLT-derived lines, mean NPTII for multiple-copy lines was 13.21 ng/20 μg soluble protein, 48.4% higher than observed for single-copy lines (6.82 ng/20 μg soluble protein).

Table 1. Summary of transgenic lines and transformation efficiencies following AMT or BLT

Exp.	Lines tested	Number of *npt*II PCR-positive events	Number of events expressing NPTII	Total callus used (g)	Escapes[a]	Transformation efficiency (Mean±SE)[b]
AMT 1	18	18	18	14	0	1.29
AMT 2	40	40	39	27	0	1.48
AMT 3	23	22	21	14	1	1.57
AMT 4	31	31	30	18	0	1.72
AMT 5	22	22	21	12	0	1.83
AMT 6	25	24	24	13	1	1.85
AMT 7	19	19	19	12	0	1.58
AMT 8	29	29	29	13	0	2.23
AMT 9	27	26	26	12	1	2.17
AMT 10	21	21	21	10	0	2.10
AMT 11	32	32	32	15	0	2.13
AMT 12	28	28	28	15	0	1.87
AMT total	315	312 (99%)	308 (99%)	175	3 (1%)	1.82±0.09
BLT 1	48	48	48	15	0	3.20
BLT 2	44	44	44	14	0	3.14
BLT 3	17	17	17	10	0	1.70
BLT 4	20	20	20	14	0	1.43
BLT 5	10	10	10	10	0	1.00
BLT 6	8	8	8	12	0	0.67
BLT 7	17	17	17	12	0	1.42
BLT 8	12	12	12	10	0	1.20
BLT 9	10	10	10	10	0	1.00
BLT 10	26	21	21	10	5	2.10
BLT 11	28	28	28	10	0	2.80
BLT 12	21	15	15	10	6	1.50
BLT total	261	250 (96%)	250 (100%)	137	11 (4.2%)	1.76±0.25
AMT vs BLT	*P* value for escapes is 0.6622; *P* value for transformation efficiencies is 0.6057					

[a] Escapes were based on number of lines without PCR amplification product using *npt*II-specific primers.

[b] Transformation efficiency was calculated as number of independent, PCR-positive lines per gram callus. BLT or AMT transformation efficiencies are calculated as mean of 12 independent experiments.

AMT Agrobacterium-mediated gene transfer, *BLT* biolistic gene transfer, *SE* standard error.

Analysis of transgene expression stability. ELISA was used to compare NPTII levels in primary transgenic plants and their vegetative progeny lines. Three biological replicates representing three individual progeny plants were analyzed for vegetative progeny lines for each primary transgenic plant. For primary transgenic plants, two replicates, each from different tillers of the same plant, were tested. A total of 120 vegetative progeny lines were tested, representing 20 primary transgenic lines from AMT and 20 primary transgenic lines from BLT. The results are shown in Fig. 2. The majority of transgenic lines displayed similar levels of NPTII expression between primary transgenic plants and their vegetative progeny lines. Overall, there was no significant difference ($P>0.05$) in expression levels between primary transgenic plants and their respective vegetative progeny lines for both transformation methods or single-copy vs. multiple-copy lines (Table 4). However, there were exceptions. For two single-copy lines (line #110 from AMT and line #428 from BLT) and two multiple-copy lines (both from AMT, line #27 and

Table 2. qPCR analysis for transgene copy number

qPCR copy numbers	AMT	BLT	*P* value
1 copy	99	115	
1 or 2 copies	9	5	
2 copies	71	45	
>2 copies	100	69	
Number of tested lines	279	234	
% single integration events	35.5%	49.2%	0.1585

AMT Agrobacterium-mediated gene transfer; *BLT* biolistic gene transfer. *P* values are based on the chi-square test.

Table 3. NPTII expression analysis of transgenic lines

Comparison	Mean (ng NPTII/ 20 μg soluble protein)	Standard error	Maximum (ng NPTII/ 20 μg soluble protein)	*P* value
AMT vs. BLT	9.50 vs. 9.90	0.43 vs. 0.44	115.1 vs. 122.2	0.5130
AMT SCL vs. AMT MCL	7.01 vs. 11.17	0.34 vs. 0.67	22.9 vs. 115.1	<0.0001**
BLT SCL vs. BLT MCL	6.82 vs. 13.21	0.21 vs. 0.84	15.5 vs. 122.2	<0.0001**
AMT SCL vs. BLT SCL	7.01 vs. 6.82	0.34 vs. 0.21	22.9 vs. 15.5	0.6386
AMT MCL vs. BLT MCL	11.17 vs. 13.21	0.67 vs. 0.84	115.1 vs. 122.2	0.0556

AMT Agrobacterium-mediated gene transfer; *BLT* biolistic gene transfer; *SCL* single copy transgenic lines; *MCL* multiple copy transgenic lines. *P* values are based on the *t* test.

line #82), NPTII expression in primary transgenic plants was more than twice as high as that of their respective vegetative progeny lines. For three single-copy lines (line #75 from AMT, and lines # 406 and #588 from BLT), NPTII expression in vegetative progeny lines was approximately twice as high as that of their respective primary transgenic plants.

Discussion

Here, we directly compared the transformation efficiency, number of integrated transgene copies, and transgene expression stability following AMT and BLT MC in the commercially important sugarcane cultivar CP88-1762. Very few studies have directly compared integration complexity and expression levels following AMT vs. BLT in crops. Most of these studies have compared both methods side-by-side using genotypes that were amenable to both gene transfer systems but lacking commercial importance and/or based the comparison on a small number of transgenic lines (Snyder *et al.* 1999; Dai *et al.* 2001; Shou *et al.* 2004; Travella *et al.* 2005; Zalewski *et al.* 2012; Jackson *et al.* 2013; Joyce *et al.* 2014).

The transformation efficiency following AMT or BLT MC did not significantly differ in 12 independent experiments that generated 562 transgenic plants from the sugarcane cultivar CP88-1762. This is in agreement with the results of Jackson *et al.* (2013) for the cultivar Q117, suggesting that the genotype is not a major factor for this outcome. Travella *et al.*

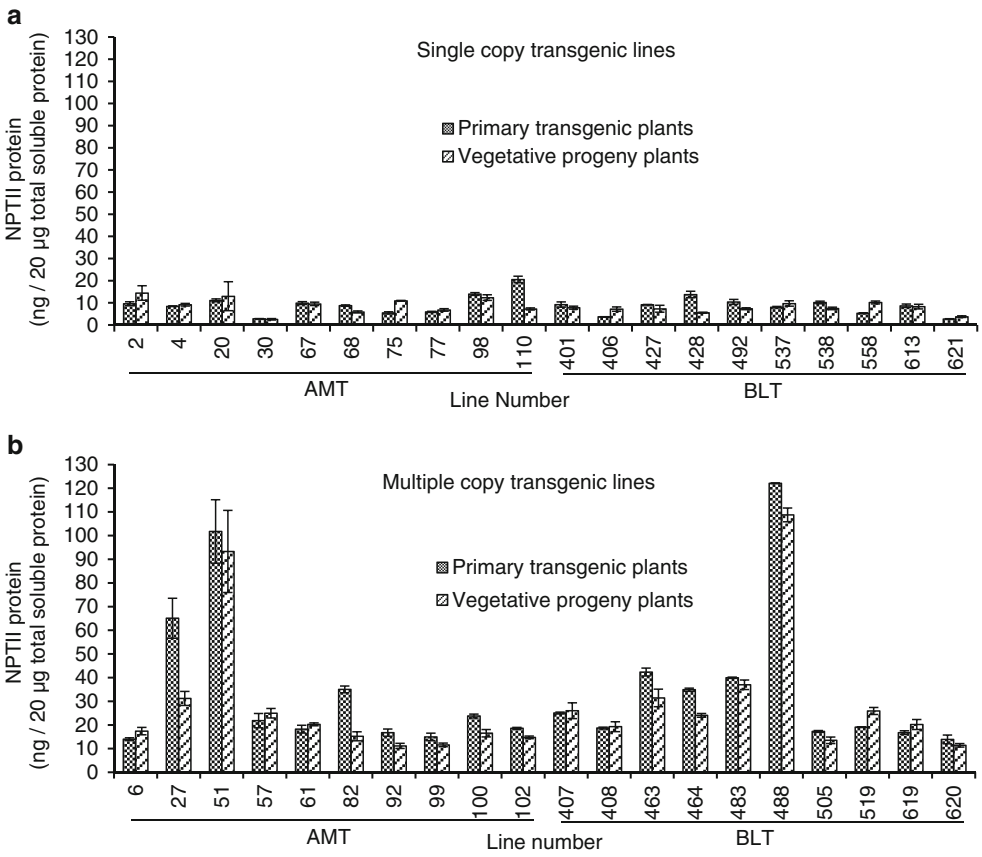

Figure 2. NPTII expression analysis of primary transgenic plants and vegetative progenies. (*a*) NPT II expression of single copy transgenic lines. Lines with number below 400 were from *Agrobacterium*-mediated gene transfer (AMT) and above 400 from biolistic gene transfer (BLT). (*b*) NPT II expression of multiple copy transgenic lines. Lines with number below 400 were from *Agrobacterium*-mediated gene transfer (AMT) and above 400 from biolistic gene transfer (BLT).

Table 4. NPTII expression in primary transgenic plants and vegetative progenies

Generations	ASC (ng NPTII/20 μg soluble protein) (Mean±SE) [a]	BSC (ng NPTII/20 μg soluble protein) (Mean±SE) [a]	AMC (ng NPTII/20 μg soluble protein) (Mean±SE) [a]	BMC (ng NPTII/20 μg soluble protein) (Mean±SE) [a]
Primary transgenic plant	9.7±1.1	8.1±0.8	33.0±6.4	35.0±7.0
Vegetative progeny	9.2±0.9	7.5±0.4	25.6±4.6	31.8±5.0
P value	0.9126	0.4495	0.3391	0.7001

[a] The values in this table are mean of NPTII expression from 10 independent lines. *P* values are based on the *t* test

ASC single copy transgenic lines derived from *Agrobacterium*-mediated gene transfer (AMT); *BSC* single copy transgenic lines derived from biolistic gene transfer (BLT); *AMC* multiple copy transgenic lines from AMT; *BMC* multiple copy transgenic lines from BLT.

(2005) and Khanna and Raina (2002) observed, for barley and rice, respectively, that transformation efficiency of AMT was twice as high as BLT. Others reported that BLT was 2.2- to 3.1-fold more efficient than AMT for maize (Shou *et al.* 2004) and rice (Dai *et al.* 2001), respectively. Beside tissue culture and selection parameters, *A. tumefaciens* strains, co-cultivation conditions, attenuation of plant defense responses, and control of *A. tumefaciens* overgrowth determine the efficiency of AMT (Gelvin 2003; Zhang *et al.* 2013). For the efficiency of BLT, osmotic treatment prior to gene transfer (Vain *et al.* 1993; Altpeter *et al.* 1996; Kemper *et al.* 1996) and the biolistic gene transfer parameters play important roles (Altpeter *et al.* 2005; Taparia *et al.* 2012b). Higher DNA concentrations during particle coating increase the transformation efficiency but also result in complex transgene integration patterns (Lowe *et al.* 2009).

Earlier reports in sugarcane (Jackson *et al.* 2013; Joyce *et al.* 2014) and other crops (Kohli *et al.* 1999; Loc *et al.* 2002; Beltrán *et al.* 2009) described a lack of correlation between copy number and transgene expression level. In contrast, our results indicated that transgenic sugarcane with multiple transgene copies displayed significantly higher transgene expression than those with a single transgene copy. These discrepancies can be caused by a smaller number of lines evaluated in the earlier reports or by truncated expression cassettes in a higher proportion of the multiple copy lines in the earlier reports. To avoid the bias of small sample sizes, we analyzed 216 single-copy and 169 multiple-copy lines for transgene expression. Following selection and regeneration, the transgenic lines displayed a wide range of transgene expression. This suggest that only events with no or very low transgene expression were eliminated due to the selection process. Our study evaluated the expression of the selectable marker *npt*II instead of a non-selected transgene. The selection process with geneticin for expression of *npt*II ensured that events with truncations in the transgene constructs or gene silencing during the tissue culture process were not considered for further analysis. For practical applications, elite events identified after tissue culture need to have a consistent and predictable performance. Therefore, it is most relevant to evaluate gene silencing after the tissue culture process and not during the tissue culture process. Such evaluation is facilitated for a large number of events with a selectable transgene.

The transgenic sugarcane lines from both AMT and BLT MC did not differ significantly for the level of transgene expression. This is consistent with the recent findings of Jackson *et al.* (2013) with cultivar Q117. However, earlier results in rice (Dai *et al.* 2001; Breitler *et al.* 2004), barley (Travella *et al.* 2005; Zalewski *et al.* 2012), maize (Shou *et al.* 2004), and fescue (Gao *et al.* 2008) showed that AMT transformants displayed higher expression than those of BLT. The main reason for the conflicting reports appears to be the use of small amounts of minimal expression cassettes by Jackson *et al.* (2013), which dramatically increased the frequency of simple integration events. We observed single-copy events in 49.2% of the BLT MC-derived lines and 35.5% of the AMT-derived lines. Jackson *et al.* (2013) did not evaluate transgene expression stability, and our data indicated no significant difference in transgene expression stability between AMT- and BLT MC-derived vegetative progenies. This was consistent with the findings of Joyce *et al.* (2014), who also used small amounts of MC for BLT, and in contrast to earlier reports using large amounts of full plasmids for BLT (Dai *et al.* 2001; Shou *et al.* 2004; Travella *et al.* 2005).

We conclude that both BLT MC and AMT represent alternative means to generate transgenic sugarcane with simple transgene integration pattern and stable transgene expression.

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