Field performance of transgenic sugarcane produced using *Agrobacterium* and biolistics methods

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Summary

Future genetic improvement of sugarcane depends, in part, on the ability to produce high-yielding transgenic cultivars with improved traits such as herbicide and insect resistance. Here, transgenic sugarcane plants generated by different transformation methods were assessed for field performance over 3 years. *Agrobacterium*-mediated (Agro) transgenic events (35) were produced using four different *Agrobacterium tumefaciens* strains, while biolistic (Biol) transgenic events (48) were produced using either minimal linearized DNA (LDNA) transgene cassettes with 5′, 3′ or blunt ends or whole circular plasmid (PDNA) vectors containing the same transgenes. A combined analysis showed a reduction in growth and cane yield in Biol, Agro as well as untransformed tissue culture (TC) events, compared with the parent clone (PC) Q117 (no transformation or tissue culture) in the plant, first ratoon and second ratoon crops. However, when individual events were analysed separately, yields of some transgenic events from both Agro and Biol were comparable to PC, suggesting that either transformation method can produce commercially suitable clones. Interestingly, a greater percentage of Biol transformants were similar to PC for growth and yield than Agro clones. Crop ratoonability and sugar yield components (Brix%, Pol%, and commercial cane sugar (CCS)) were unaffected by transformation or tissue culture. Transgene expression remained stable over different crop cycles and increased with plant maturity. Transgene copy number did not influence transgene expression, and both transformation methods produced low transgene copy number events. No consistent pattern of genetic changes was detected in the test population using three DNA fingerprinting techniques.

Introduction

Worldwide sugar industries and multinational agribusinesses are now heavily investing in transgenic sugarcane development to capture the expanding sugar and biofuel market (Matsuoka *et al.*, 2011; Minol and Sinemus, 2011). The first transgenic traits in commercially released sugarcane will likely be drought tolerance (Hautea, 2011) and resistance to herbicides (glyphosate and possibly glufosinate or dicamba), insects (stalk borer), and viral diseases (Minol and Sinemus, 2011). Commercial release of any transgenic event will depend on the stable and uniform expression of introduced trait(s) and their comparable agronomic performance to elite commercial cultivars. Given the large genetic variability inherent in transgenic sugarcane populations (Gilbert *et al.*, 2005; Joyce *et al.*, 1998; Pribil *et al.*, 2007; Vickers *et al.*, 2005b), identification of elite, commercially valuable and deregulatable events requires extensive field assessment of a large population of independent transgenic events. Field evaluations of transgenic sugarcane, however, have been limited to a few studies (Arencibia *et al.*, 1999; Basnayake *et al.*, 2012; Gilbert *et al.*, 2009; Lakshmanan *et al.*, 2005; Leibbrandt and Snyman, 2003; Vickers *et al.*, 2005b; Weng *et al.*, 2011), with most of them focusing on a few selected events. The above field trials were of events produced using biolistics, except for Arencibia *et al.* (1999) who used cell electroporation methods. To the best of our knowledge, there has been no report on field trials of *Agrobacterium*-derived transgenic sugarcane.

Biolistics remains the dominant technology for transgenic sugarcane production (Altpeter and Oraby, 2010), and conse-

quently, events derived using this transformation method were employed for the vast majority of field studies reported to date. Although *Agrobacterium*-mediated transformation is considered to be more desirable for generating commercially useful events due to simpler transgene integration patterns and a propensity for single transgene insertions, it has been successfully applied only to a limited number of sugarcane germplasm (Joyce *et al.*, 2010). More recently, biolistic transformation using minimal DNA cassettes (containing only promoter, gene of interest, and terminator) has been reported in sugarcane (Jackson *et al.*, 2013; Kim *et al.*, 2012). However, for sugarcane, any of these methods of transformation involve a considerable period in tissue culture, which may cause varying degrees of somaclonal variation (Burner and Grisham, 1995). Thus, a rigorous comparison of biolistic (using whole plasmid and minimal vector) as well as *Agrobacterium*-derived transgenic events in crop production conditions is needed to determine the relative merit of these methods for the development of commercial transgenic sugarcane.

Gallo-Meagher and Irvine (1996) were the first to report on stable expression of a transgenic trait, herbicide resistance, in successive generations of sugarcane following vegetative propagation. Since then, stable transgene expression has been reported in glasshouse and field studies on virus and insect resistance (Gilbert *et al.*, 2009; Joyce *et al.*, 1998; Weng *et al.*, 2011), herbicide resistance (Leibbrandt and Snyman, 2003), polyphenol oxidase activity (Vickers *et al.*, 2005a), shoot architecture (Pribil *et al.*, 2007), sorbitol (Chong *et al.*, 2007), and isomaltulose accumulation (Basnayake *et al.*, 2012).

Published analyses of agronomic performance of transgenic sugarcane are few and contradictory. Arencibia *et al.* (1999) were the first to test the field performance of five transgenic sugarcane events resistant to stalk borers. They concluded that most of the transgenic events had agronomic traits similar to that of the untransformed parent clone; however, agronomic performance of the transgenic clones was not compared to the parent clone in the absence of borers. Leibbrandt and Snyman (2003) and Gilbert *et al.* (2005) performed field trials over 3 years and compared one and four transgenic events respectively to parent clones. Both reports concluded that stable expression of the transgene and agronomic performance equivalent to parent clones were achievable. In contrast, Vickers *et al.* (2005b) and Gilbert *et al.* (2009) showed that most transgenic events suffered a significant yield reduction (tonnes of cane/ha) in comparison with the parent clone. Although the reports on field performance of transgenic sugarcane for yield and agronomic characteristics are contradictory, the sugar content, %Brix, %Pol and %purity measurements have consistently been reported as unaffected by the tissue culture and transformation process.

The large variation in agronomic performance of transgenic plants in field studies has been attributed to factors originating from tissue culture and/or the introduction of the transgene itself into the plant genome. Some of the tissue culture-induced variations appear to be epigenetic, as they are present in the plant crop and gradually disappear in subsequent crop cycles (ratoon crops), whereas some others appear to be stable (Burner and Grisham, 1995; Hoy *et al.*, 2003; Lourens and Martin, 1987).

Despite the progress to date, caution should be exercised when drawing conclusions from these field trial reports for the following reasons: (i) differences in transformation methods and genotypes, (ii) different transgenes with varied impact on plant metabolism, (iii) the tissue culture and propagation history of transgenic events used in the field trials, and (iv) the small numbers of transgenic events and genotypes evaluated in each of these studies.

In this study, we investigated the field performance of tissue cultured and transgenic sugarcane events produced using four different strains of *Agrobacterium tumefaciens* and biolistics (using linearized minimal DNA cassettes or whole plasmid DNA vectors) for transgene expression stability, agronomic performance, and yield characteristics over several years, with the objective of determining the most appropriate transformation technology for commercial transgenic sugarcane development.

Results

Agronomic and yield trait assessment

Field trial 1 (FT1)

Impact of transformation methods on agronomic traits. A group comparison shows that stalk weight, stalk diameter, and tonnes of cane per hectare (TCH) were significantly higher in the PC than in the other three groups (TC, Agro, and Biol) in all three crop classes (P, 1R, and 2R; Figure 1a,b and d) with the P crop showing the biggest difference. Stalk height was significantly higher in PC in the P crop only (Figure 1c), while the number of stools and stalks per stool did not differ between any groups across all crop classes (Figure 1e and f). The TC, Agro, and Biol groups did not differ significantly for all traits measured in all crop classes. This group analysis of the data containing a large

number of transgenic events (35 Agro and 48 Biol) within each group suggests that PC was superior to the other three groups.

Sugar yield was much less affected by transformation than cane yield. The P crop showed that PC had the highest, and the Biol events the lowest, values of brix (% soluble solids), pol (apparent sucrose in juice), and CCS (Table 1). In the 1R crop, these differences were smaller (Table 1). Purity% was not different in either P or 1R.

Transformation method had no interaction with crop class. The interaction between crop class and the method of transformation was not significant for any of the measured traits (data not shown). The P crop consistently showed highest values for all traits when crop classes were compared, and the 2R showed lowest values for all traits except for the number of stools and stalks per stool. The number of stalks per stool were higher in the P crop, but not affected by the method of transformation.

Comparisons of sugar yield determinants showed that P had greater values than 1R for all traits. At harvest, the P crop was 16 months old compared with the 1R, which was 11 months old. Thus, the estimated CCS (calculated from Brix, pol, and purity) was also higher as expected (Table 1).

Growth variation in transgenic events diminished in successive crops. As both Agro and Biol groups consisted of a number of events arising from either different *Agrobacterium* strains or transformation vectors, events were recategorized and analysed in smaller subgroups according to the vector used for transformation. The reason for using this method of analysis was twofold: (i) to understand the extent of trait variation within subgroups, and (ii) to identify whether any of these transformation methods was superior to the others (which may have been masked when analysed in four main (larger) groups). In each crop class, all subgroups showed a large variation in stalk weight, with PC having the largest stalk weight (Figure 2). This difference was again most pronounced in the P crop. Stalk diameter and height showed a similar trend while stalk numbers were equivalent in all subgroups and across all three crops (data not shown). The difference in stalk traits was reduced in subsequent crops with some Agro and Biol events showing equivalence to PC clones (Figure 2). Within the Agro group, the AGL1 events showed a higher median stalk weight than the rest in all three crop classes, while no Biol subgroup was superior to others. Both stool count and stalk numbers were not different between PC and other categories, within and between crop classes, indicating that ratoonability is not affected by the transformation methods used.

Field trial 2 (FT2)

Cane, but not sugar, yield parameters declined in the first ratoon crop (1R) in FT2. Stalk diameter, plot weight, and TCH were higher ($P \leq 0.01$, ≤ 0.05, and ≤ 0.05, respectively), while stalk number was lower ($P \leq 0.01$) in the P compared with the 1R crop (Figures 3 and 4). In the 1R, all three traits were reduced to 87% of that of P. In contrast, stalk numbers were 13% higher in the 1R in all four groups compared with the P crop (Figure 3).

All sugar yield traits except purity were not different within and between crop classes. Brix, Pol, and CCS recorded no significant difference between the transformation groups in either P or 1R crop class. Juice purity was significantly lower in PC and TC compared with Agro and Biol groups of P crop. The reason for this difference remains unclear (Table 2). Statistical analysis
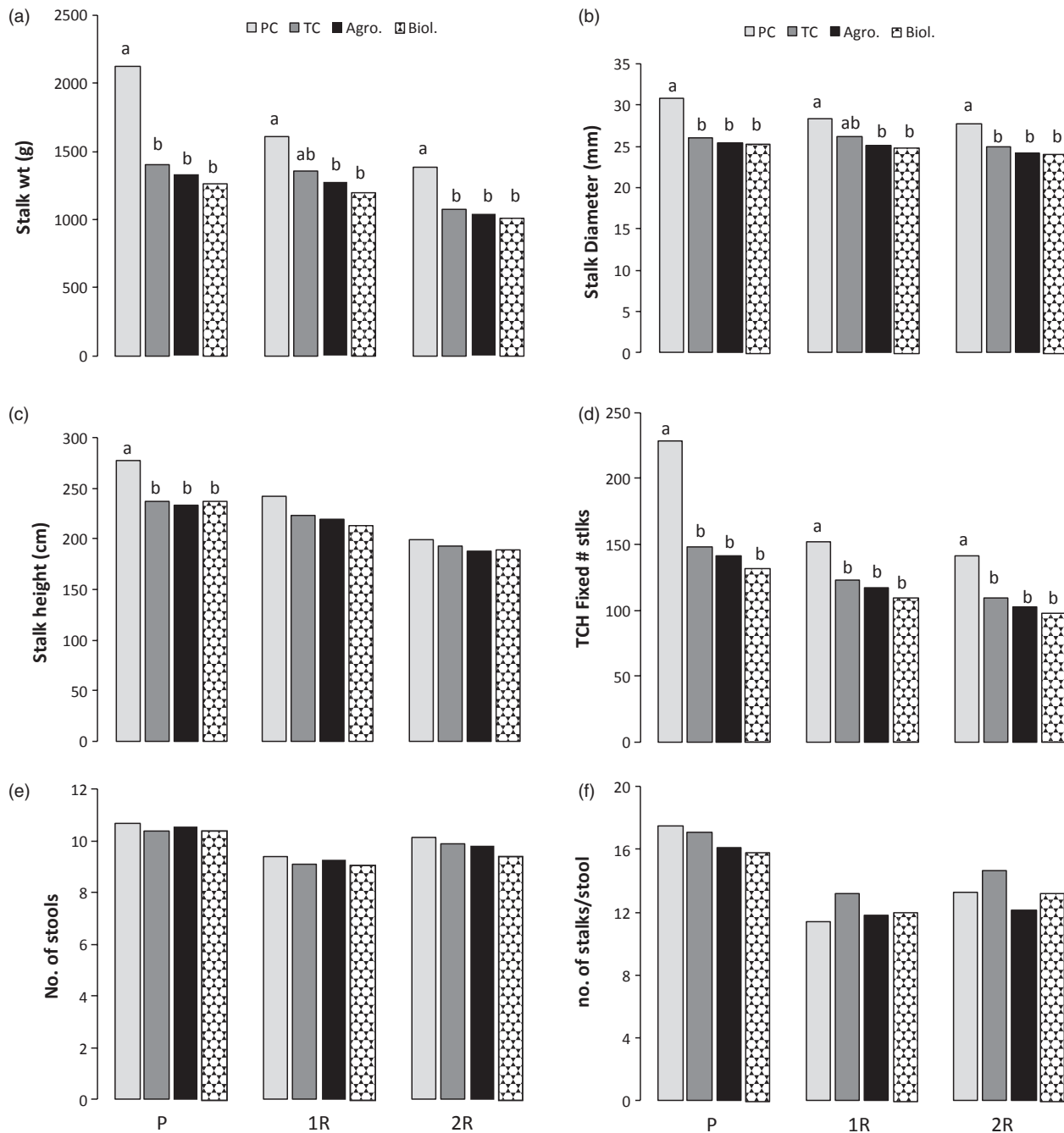
Figure 1 Comparison of yield and agronomic characteristics of different transformation groups both within and between crop class (P, 1R, and 2R) of the FT1 trials. (a) Stalk weight, (b) stalk diameter, (c) stalk height, (d) TCH, (e) number of stools per plot, (f) number of stalks per stool. Different coloured bars represent the four event categories: PC, TC, Agro, and Biol. Columns with different letters within a crop class are significantly different ($P \leq 0.05$).

showed no interaction for any of the agronomic traits between crop class and method of transformation.

Transformation-induced yield reduction and its disappearance in the following crop were confirmed in FT2. In the FT2 P crop, stalk diameter and plot weight varied between the four groups with the PC clones having the largest weight and diameter and the Agro group the smallest (Figure 3a and b). Both stalk height and number were not different ($P \leq 0.05$) between groups (Figure 3c and d). In the 1R crop, none of the measured cane yield traits showed any significant difference (Figure 3).

Comparisons within individual lines. All transformation methods produced lines with no yield penalty, with biolistics producing more than that by *Agrobacterium*. In each group, TCH and plot weight of many individual clones were similar to that of PC in both P and 1R crops (Figure 4, Table 3a and b). Agro groups, however, had fewer events equivalent to PC in P and 1R (27% and 73% respectively) compared with Biol (65% and 94%, respectively) and TC (63% and 100%, respectively).

All clones with significantly low plot weight had smaller stalk diameter, whereas stalk height was not always low (Table 3). In EHA and Biol groups, there were some events with reduced stalk

Table 1 Comparison of sugar yield characteristics between crop classes and within each group in the FT1

	Brix (%)	Pol (%)	Purity (%)	CCS
FT1-P				
PC	21.32a	85.37a	96.25	16.22a
TC	21.31a	82.99ab	94.89	15.56ab
Agro	20.83ab	81.07ab	93.58	15.20ab
Biol	20.33b	78.58b	92.63	14.69b
FT1-1R				
PC	19.60	75.23	92.50	13.38
TC	19.33	72.09	90.45	12.66
Agro	19.34	70.79	88.57	12.29
Biol	19.04	70.12	89.09	12.23

Different letters following the mean indicate significant difference within the crop class.
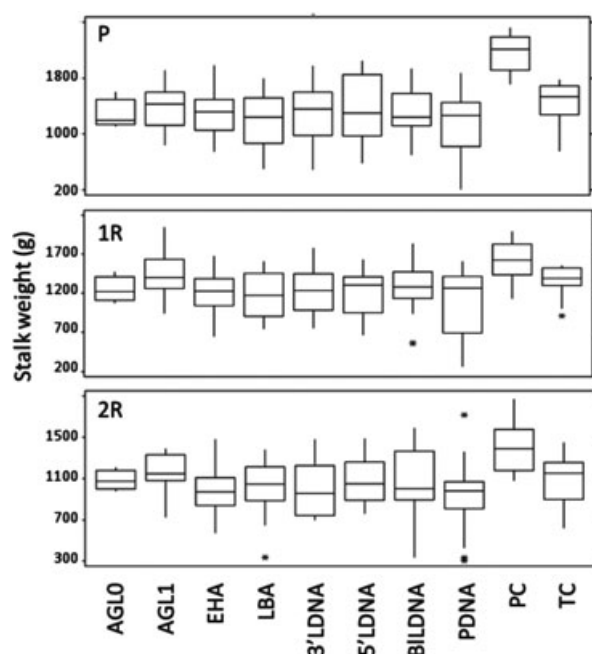
Figure 2 Stalk weights in FT1 trials. Box and whiskers plots of stalk weight in FT1 trials (P, 1R, and 2R) for each of the Agro (AGLO, AGL1, EHA, and LBA), Biol LDNA (3′, 5′, and Bl), and PDNA subgroups compared with PC and TC groups. Asterisk indicates outliers.

diameter but increased stalk number, resulting in higher plot weight. One PDNA event (PDNA-363), which exceeded the PC plot weight in both P and 1R crops, was significantly taller (240 versus 165 cm, respectively).

A correlation matrix comparing plot weight, stalk diameter, stalk height, and stalk number in P and 1R in the FT2 showed high correlation between plot weight, stalk height, and stalk diameter [$R^2 = 0.85$ (pwt-P vs pwt-1R) and 0.87 (ht-P vs ht-1R) and 0.75 (dia-P vs dia-1R), respectively]. Among the agronomic traits, stalk height had the highest correlation with plot weight ($R^2 = 0.84$ and 0.75 in P and 1R respectively), and total number of stalks (sno) had the least ($R^2 = 0.54$ sno-P vs sno-1R) (Figure 5). When clones were ranked for TCH in the FT2, the PC clone ranked 4th in the P crop but only 10th in the 1R, indicating that yield reduction due to

growth under suboptimal environments (tissue culture and glasshouse) will be eliminated within a few cycles of field cultivation.

Six of the 36 transgenic events in the field had significantly lower plot weight than PC in the P crop, while in the 1R this number was reduced to 1 at $P \leq 0.05$. A similar analysis at a higher α of $P \geq 0.85$ showed that plot weights of 53% and 89% of P and 1R transgenic clones respectively were similar to PC (Tables 3a and b and S2). Agro events performed poorer than the Biol and TC events, having lower plot weight in the FT2 P crop. Both TC and Biol events performed equally well (>60% having an adjusted P value of ≥ 0.85). Transgenic stalks were generally thinner than PC in the P crop (>40% of all events), while in the 1R, only 5% were thinner than PC. Six events showed equivalent or thicker stalks when compared to PC.

Comparisons between FT1 and FT2 crops. Comparison of stalk weight of clones present in both FT1 and FT2 crop trials showed a correlation coefficient R^2 value of 0.61. These differences were observed within all subgroups irrespective of the method of transformation (Figure 6).

Transgene expression is stable across crop classes

NPTII protein expression was present in all transgenic events across all crop classes from FT1-P through to FT2-1R (data not shown). NPTII expression increased significantly with plant maturity ($P \leq 0.001$, Figure 7), and the relative increase in NPTII expression varied between individual events. The increase in NPTII protein in mature plant samples may be the result of either increased rate of NPTII protein synthesis, reduced breakdown, or both in mature plant parts. Consequently, all comparisons of NPTII expression were made between plants of equivalent maturity.

Relative NPTII expression for each transgenic event was stable across all crop classes (Figure 8 shows a comparison between FT1-1R, 2R, and FT2-P). For example high-NPTII-expressing events had consistently high relative NPTII protein concentration in all crop classes, while low-NPTII-expressing events maintained low relative NPTII protein levels at all times. There was no correlation between cane yield (plot weight) and NPTII expression for events in the FT2 (Figure 9).

## Transgene expression was not affected by transformation method

The transgenic events with the highest concentrations of NPTII protein were found among the plasmid-transformed Biol events in the FT1-2R (Figure 10) and *Agrobacterium* strain EHA-transformed events in the FT2. However, due to the large variability in NPTII protein concentration between individual events, no transformation group was identified as producing consistently higher mean expression levels across all crop classes ($P \leq 0.05$).

## Transgene copy number does not influence transgene expression

Transgene copy number was determined by Southern analysis for all events in the FT2. Agro events had significantly lower mean transgene copy number (2.0) than the Biol events (7.1) with a P value ≤ 0.001. However, no correlation was found between transgene copy number and transgene expression (Figure 11).

Genotypic analysis

A total of 389 loci were generated with five AFLP primer sets used for fingerprinting using genomic DNA from leaf samples of 89 events grown in the FT1 P crop. Only two polymorphic bands
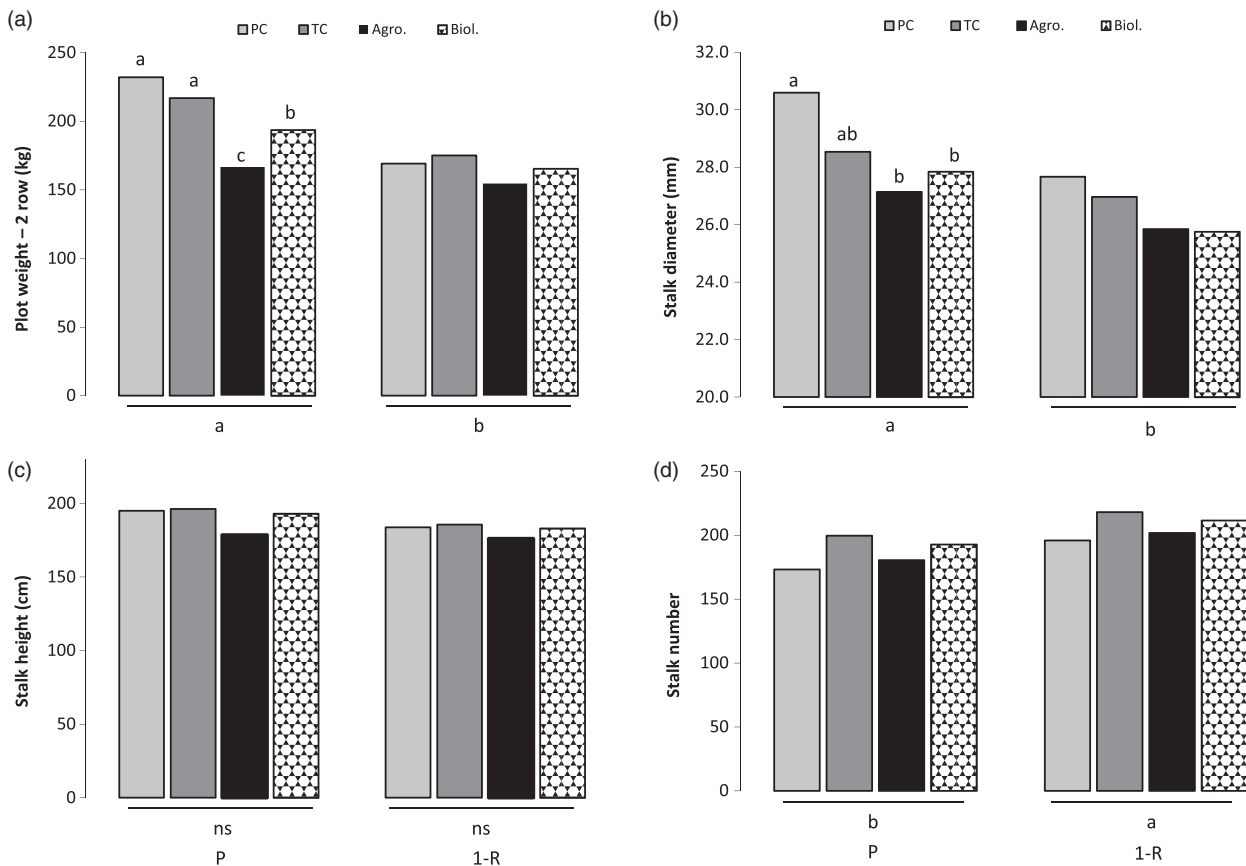
Figure 3 Comparison of yield characteristics of different transformation groups both within and between crop class (P and 1R) of the FT2. (a) Plot weight, (b) stalk diameter, (c) stalk height, and (d) stalk numbers in the four categories: PC, TC, Agro, and Biol. Columns with different letters within a crop class are significantly different ($P \leq 0.05$).
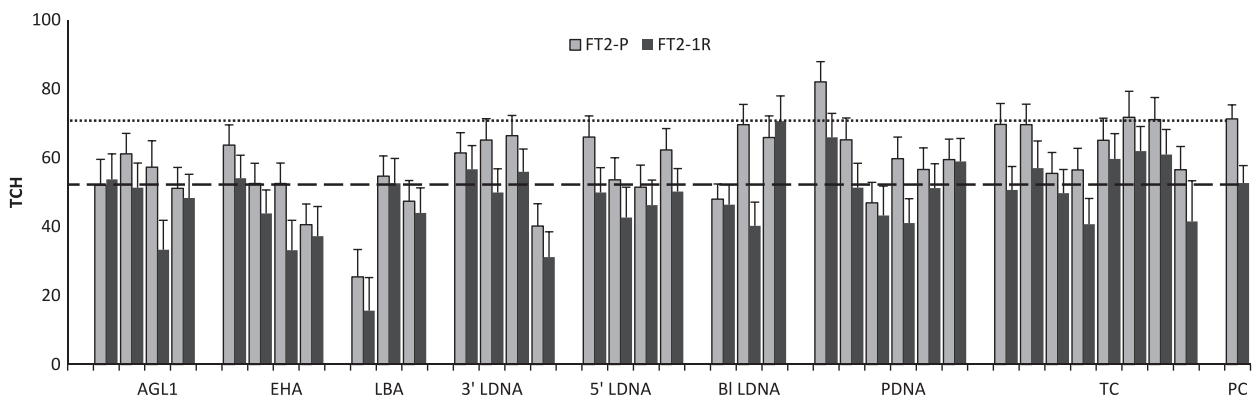
Figure 4 Comparison of tonnes of cane per hectare (TCH) between P and 1R crop of the FT2 trial. All clones are grouped into one of nine subgroups. Dotted lines represent the yield from the PC in the FT2-P (grey bars) and 1R crops (dark grey bars).

were detected: one in a Biol and the other in an Agro event (results not shown). In an attempt to further investigate transformation-induced genome changes, methylation-sensitive AFLP (MS-AFLP) was performed. The same 89 events were subjected to MS-AFLP using eight different primer combinations and the percentage of nonmethylated (type 1), fully methylated (type 2), and hemi-methylated (type 3) sites in the entire population. No clear trend differentiating transformed and PC events was evident (Table 4a). The data were analysed within polymorphism type 1,

2, or 3 using mixed models and was shown to be not significantly different between groups (P = 0.64, 0.59 and 0.89, respectively). The third approach using MSDarT method targeted three TC and four Biol clones showing distinct phenotypic somaclonal variation (short and/or highly tillered) compared with PC clones. Despite being able to score a total of 15360 loci using this technique (although only 10% could be given discrete scores), consistent differences between the PC and the seven events compared were not identified (Table 4b).

Table 2 Comparison of sugar yield characteristics between crop classes and within each group in the FT2

	Brix (%)	Pol (%)	Purity (%)	CCS
FT2-P				
PC	19.95	74.71	78.73c	13.78
TC	19.78	74.93	83.69b	13.87
Agro	19.68	74.16	90.94a	13.75
Biol	20.01	75.79	91.22a	14.05
FT2-1R				
PC	19.89	77.06	93.65	14.52
TC	19.93	75.10	90.42	13.84
Agro	19.98	75.63	91.41	14.04
Biol	19.87	75.28	91.33	13.96

Different letters following the mean indicate significant difference within the crop class.

Discussion

This is the first report of a comparative analysis of field performance of a large number of transgenic sugarcane events produced using either *Agrobacterium* or biolistic transformation methods over a 3-year growing period using commercial sugarcane crop production and harvest practices. Additionally, this study provides new knowledge on performance of transgenic sugarcane events produced using four different strains of *Agrobacterium tumefaciens* as well as biolistic transformation using two types of vectors (minimal, no backbone LDNA and whole plasmid). Our results showed that both agronomic and yield performance were affected by tissue culture as well as transgenesis. This was evident in both field trials (FT1 and FT2), especially in the plant crop. This difference, however, decreased in successive crops, suggesting that the growth and morphological variation observed was transient and epigenetic in nature. Studies by Taylor *et al.* (1995) and Basnayake *et al.* (2012) also concluded that morphological and epigenetic effects in transgenic sugarcane can be eliminated within three propagation cycles and thus enable selection of vigorous lines. Furthermore, individual event-based analysis revealed that all transformation methods could produce events with yield equivalent to the nontransformed PC albeit at varying efficiencies. Yield trials in barley (Kobayashi *et al.*, 2008), wheat (Shewry *et al.*, 2006), and rice (Travella *et al.*, 2005) also concluded that equivalence in transgenic plants was achievable. In field studies of transgenic potato (which, like sugarcane, also has a highly heterozygous genome), the authors concluded that although higher somaclonal variation existed in transgenic plants, many of them showed equivalence to PC, thus enabling selection of plants with desirable characteristics with no yield penalty (Dale and McPartlan, 1992). In sugarcane, much of this variation is possibly caused by protracted tissue culture manipulations (Arencibia *et al.*, 1999; Carmona *et al.*, 2005; Zucchi *et al.*, 2002). Interestingly, the sugar yield characteristics were not influenced by either tissue culture or transformation, indicating that the sucrose accumulation and storage are more resilient to transgenesis than the agronomic attributes. Other reports on transgenic sugarcane came to a similar conclusion on sugar yield characteristics (Arencibia *et al.*, 1999; Leibbrandt and Snyman, 2003; Vickers *et al.*, 2005b).

In crops with simple homozygous genome, undesirable side effects due to tissue culture and transgenesis can be eradicated by back-crossing (Bregitzer *et al.*, 2008) or cross-breeding (Horvath *et al.*, 2001) of promising T0 primary transformants and subsequent selection of crosses. However, in sugarcane such an approach is very difficult due to its complex genome with varying chromosome numbers (D'Hont *et al.*, 1996) and the long duration required for breeding new cultivars (over 10–12 years). Thus, transgenic sugarcane clones developed for commercial release must perform as good as or better than its parent. Because of this imperative, the work reported here is of particular significance as it provides a rigorous objective analysis of the relative merit of two main sugarcane transformation technologies for the production of commercially useful events.

Publications on other crops report differences in the two transformation methods. Biolistics is genotype independent and more efficient (Altpeter *et al.*, 2005), while *Agrobacterium*-mediated transformation produces low transgene copy events with complete multigene inserts and more stable trait expression (Dai *et al.*, 2001; Shou *et al.*, 2004; Travella *et al.*, 2005). In contrast, our results show that both methods will deliver events equivalent to parent clones in field performance, but at different efficiencies (27% and 73% in Agro versus 63% and 91% in Biol in the P and 1R crops, respectively).

Transgene expression studies

Both stability and consistency of transgene expression are important criteria for future commercialization of transgenic plants. We have used *nptII* instead of genes for herbicide or pest tolerance as it does not affect primary metabolism, allowing comparisons of all transgenic events under similar growing conditions. For instance, in potato GUS expression had a significant negative effect while NPTII had no impact on agronomic and yield performance (Dale and McPartlan, 1992). Our results for NPTII expression in the youngest fully expanded leaf showed high correlation with maturity of the plant, with 11-month-old plants consistently outperforming 5-month-old ones, irrespective of the transformation method. A similar result for two other transgene products—polyhydroxybutyrate (Purnell *et al.*, 2007) and isomaltulose (Basnayake *et al.*, 2012)—was reported in sugarcane. However, that was not the case with sorbitol, which accumulated more in younger leaves in mature sugarcane plants grown in the glasshouse (Chong *et al.*, 2007).

Transgene expression was not correlated with either copy number or transformation method. While the lack of correlation between copy number and transgene expression has been reported in both monocots and dicots (Beltran *et al.*, 2009; Kohli *et al.*, 1999; Loc *et al.*, 2002), variation in expression level is affected by integration site in both Agro and Biol. Our results showed similar expression in Biol and Agro transgenics and concurred with that reported by Jackson *et al.* (2013) in sugarcane, but contrasted that reported in other monocots: rice (Breitler *et al.*, 2004; Dai *et al.*, 2001), barley (Travella *et al.*, 2005), and maize (Dai *et al.*, 2001; Shou *et al.*, 2004), where Agro transformants had higher transgene expression and stability compared with Biol ones. NPTII expression remained stable in different crop classes with high-NPTII expressors continuing to express high amounts of the protein in the different crop classes and plantings (correlation coefficients R^2 greater than 0.68). NPTII protein varied from 0.005 to 0.05% of total soluble protein in different events, which is similar to that reported in other sugarcane transgenics (Wang *et al.*, 2005). Basnayake *et al.*

Table 3 Yield and stalk measurements taken at harvest of the FT2-P (a) and 1R (b) crop. Stalk agronomic measurements were taken at harvest and is the mean of eight individual stalks

(a) FT2-P	Plot Wt (kg) (2 row)	Diameter (mm)	Height (cm)	Stalk number (2 row)	(b) FT2-1R	Plot Wt (kg) (2 row)	Diameter (mm)	Height (cm)	Stalk number (2 row)
PC	237.7	30.8	164.5	203.09	PC	175.53	28.6	189.9	187.60
AGL1-27	173.6	27.8	178.2	163.67*	AGL1-27	179.02	27.1	191.2	244.78
AGL1-31	203.7	29.7	182.8	182.19	AGL1-31	171.15	28.5	177.2	191.57
AGL1-54	191.0	28.9	142.9	207.55	AGL1-54	110.97	27.1	199.6	160.37
AGL1-57	170.5	26.6**	178.9	177.39	AGL1-57	161.03	26.9	177.2	188.26
EHA-73	212.2	25.4***	218.8*	194.26	EHA-73	180.30	26.7	196.8	233.18
EHA-80	175.0	28.0	167.1	187.68	EHA-80	146.20	26.1	174.2	202.70
EHA-83	175.0	25.7***	223.7**	191.33	EHA-83	110.58	24.3	174.9	191.27
EHA-87	135.3***	24.4***	211.8	139.87***	EHA-87	124.25	22.6**	138.3	271.67
LBA-1	84.7***	25.8**	132.5	89.04***	LBA-1	52.17**	24.0	101.3***	158.14
LBA-10	182.3	30.3	158.3	212.65	LBA-10	175.38	26.2	193.8	202.25
LBA-22	157.9*	25.2***	173.7	189.52	LBA-22	146.57	24.2	192.7	228.76
3'LDNA-314	204.7	30.7	171.8	224.11	3'LDNA-314	188.83	27.6	193.3	181.35
3'LDNA-395	217.2	28.6	213.4	184.11	3'LDNA-395	166.50	26.7	190.2	223.79
3'LDNA-399	221.4	25.7***	192.6	219.70	3'LDNA-399	186.34	25.2	205.5	215.53
3'LDNA-794	134.0**	27.0*	178.7	163.67	3'LDNA-794	103.89	25.5	150.3*	147.35
5'LDNA-263	220.1	30.0	205.5	202.10	5'LDNA-263	166.46	27.2	170.1	233.98
5'LDNA-268	178.7	25.3***	210.7	173.12	5'LDNA-268	142.27	21.7**	167.1	265.56
5'LDNA-284	171.5	23.8***	224.5**	193.32	5'LDNA-284	154.13	25.7	194. 5	252.17
5'LDNA-297	207.5	29.7	168.4	199.60	5'LDNA-297	167.31	27.3	191.1	184.24
Bl LDNA-214	160.0**	25.9***	212.7**	167.05**	Bl LDNA-214	154.73	27.2	176.8	240.16
Bl LDNA-217	232.0	28.6	201.2	196.78	Bl LDNA-217	134.27	26.5	183.6	216.39
Bl LDNA-678	219.7	30.2	202.4	220.97	Bl LDNA-678	235.25	30.7	224.1	221.31
PDNA-363	273.3	28.3	240.4***	208.68	PDNA-363	219.76	26.8	199.6	200.65
PDNA-570	217.4	31.5	151.2	231.54	PDNA-570	171.01	27.2	184.5	186.77
PDNA-579	156.5**	26.6**	205.3	148.23***	PDNA-579	144.10	24.5	140.7***	210.02
PDNA-717	199.1	28.2	174.4	216.79	PDNA-717	136.85	26.6	211.8	198.91
PDNA-S34	188.7	26.6***	210.0	194.46	PDNA-S34	170.58	24.0	175.3	216.32
PDNA-S76	198.3	25.5***	255.4***	172.70	PDNA-S76	196.47	24.6	171.3	262.70*
TC-LBA-1	232.4	27.7	202.8	204.23	TC-LBA-1	168.79	25.7	192.6	218.85
TC-LBA-2	232.0	30.6	170.6	210.47	TC-LBA-2	189.90	26.4	184.0	196.81
TC-AGL1-1	184.9	27.6	190.4	182.28	TC-AGL1-1	165.72	26.4	174.1	217.92
TC-AGL1-2	188.2	26.8**	228.7	202.73	TC-AGL1-2	135.74	26.8	181.2	220.68
TC-AGL1-3	216.9	29.0	183.4	201.95	TC-AGL1-3	198.84	26.4	181.3	226.18
TC-351	239.1	30.6	191.9	189.24	TC-351	206.40	30.1	192.8	208.66
TC-356	236.8	30.3	196.8	210.29	TC-356	203.21	27.4	198.0	219.75
TC-8	188.5	26.8**	216.9	154.16**	TC-8	138.39	24.0	163.4	267.80

Individual clones which were significantly different from the PC using the linear mixed model SAS program are shown as *$P \le 0.05$, **$P \le 0.01$, and ***$P \le 0.001$.

(2012) draw a similar conclusion in their field study of transgenic sugarcane containing the isomaltulose synthase gene.

DNA analysis

Earlier work in transgenic sugarcane using 17 RAPD primers showed that PCR amplification products were identical in 11 transgenic plants compared with the nontransgenic parent line leading the authors to conclude that RAPD analyses lack the sensitivity to reliably detect small genetic changes due to somaclonal variation (Taylor *et al.*, 1995). However, fingerprinting of 89 independent events using AFLP analysis also yielded only two polymorphic bands. Further genomic analysis on DNA methylation patterns was able to identify more polymorphism, but failed to show a trend specific to transgenic sugarcane plants.

MS-DArT analysis, which scored over 15360 loci, however, showed no consistent genome differences between clones with visually distinct phenotypic variation. This is because the genome of sugarcane is naturally highly variable and the resolution of this technique is still insufficient given the large size of the sugarcane genome. A similar lack of extensive variation in sugarcane plants transformed with *Agrobacterium* was reported by Carmona *et al.* (2005).

Our results conclusively show that transgenic sugarcane plants with no yield penalty can be generated using either biolistics or *Agrobacterium*-mediated transformation method. Efficiency and genotypic independence and the use of minimal expression cassettes make biolistics more attractive for commercial transgenic sugarcane production.
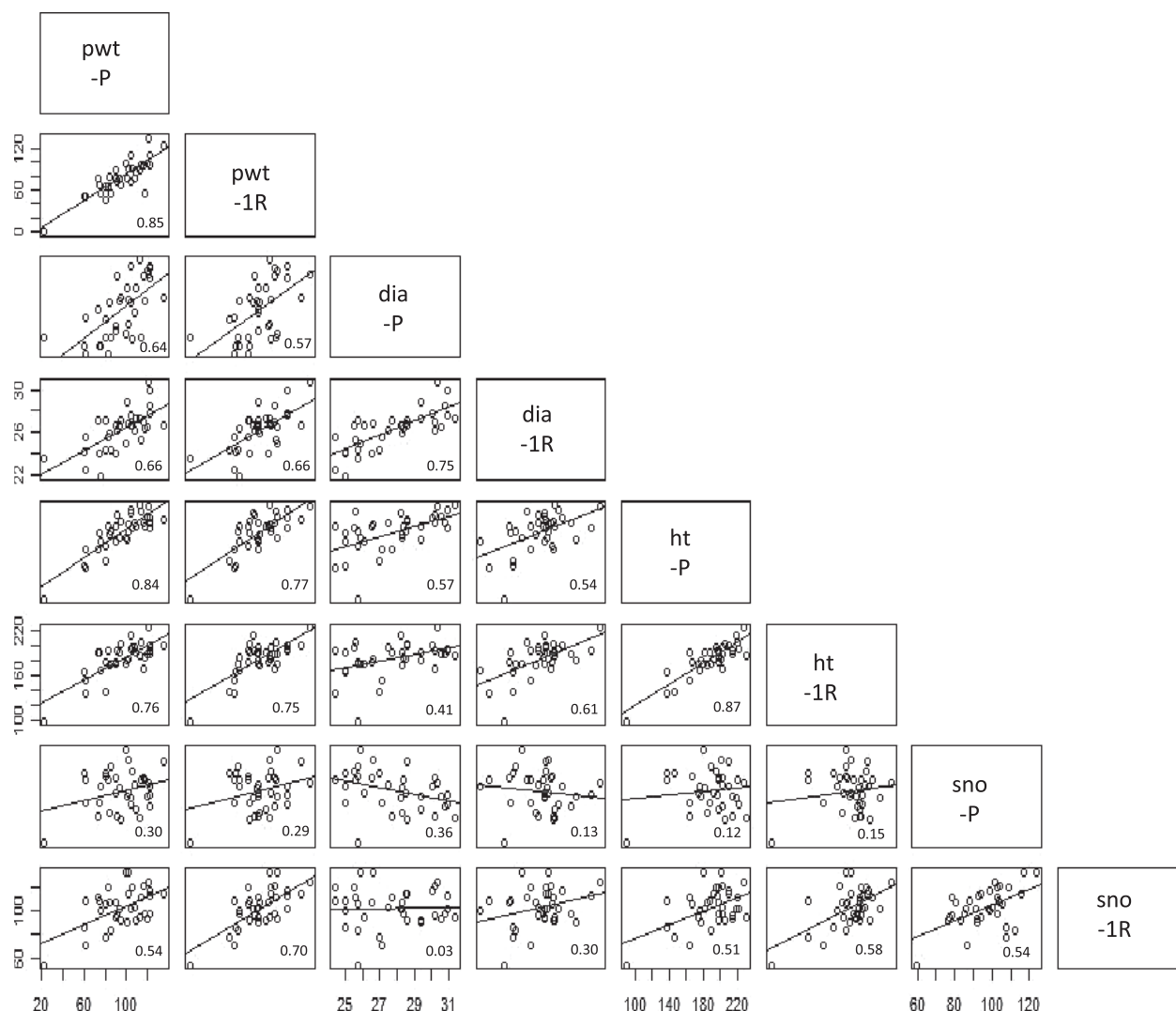
Figure 5 Correlation between plot weight (pwt), stalk diameter (dia), stalk height (ht), and stalk number (sno) in the FT2 trials (P and 1R). Pairwise comparisons are shown for individual events across different crop classes. This includes six PDNA events, 11 LDNA events, and 11 Agro events. Correlation coefficients are shown in the corner of each graph (pwt = 2-row plot weight (kg), dia = stalk diameter (mm), sno = stalk number/2-row plot, ht = stalk height (cm), P = plant crop, 1R = 1st ratoon crop).

Experimental Procedures

Plant material

The commercial sugarcane variety Q117 was used as the parent germplasm. Q117 plants were transformed using either biolistics (McQualter *et al.*, 2005) or *Agrobacterium tumefaciens* (Joyce *et al.*, 2010). Embryogenic callus (8–12 weeks old) produced from immature leaf whorls using an established protocol (Bower and Birch, 1992) was the target tissue.

DNA for transformation

The plasmid used for particle bombardment contained the maize *ubiquitin* promoter with intron, gene of interest (*neomycin phosphotransferase* (*nptII*) or *Malus domestica* sorbitol-6-phosphate dehydrogenase gene (*mds6pdh*)) (Chong *et al.*, 2007) and *nos* terminator. Linear DNA was produced by digesting the above plasmids with restriction enzymes to release a linear fragment containing only the expression cassette (promoter,

gene of interest, and terminator). The linear *nptII* gene cassette contained blunt ends only, while the linearized *mds6pdh* gene cassette contained either blunt ends or 3′ or 5′ overhangs at both ends of the fragment (referred to as bl, 3′ or 5′ LDNA, respectively).

Transformation

Biolistic transformation was performed using 1 μm gold particles coated with either a) whole circular plasmid containing the expression cassette along with the vector backbone (ampicillin resistance gene, *ori* gene, and other bacterial genes) referred to as PDNA (plasmid DNA) in this paper, or b) a linearized minimal DNA vector cassette (LDNA).

For biolistic experiments, embryogenic callus was co-transformed with *mds6pdh* and *nptII*, selected on MS medium (Murashige and Skoog, 1962) with 3 mg/L 2,4-D and 50 mg/L geneticin (Sigma-Aldrich, Castle Hill, NSW, Australia) in the dark for 4 weeks and then transferred to a 16-h photoperiod for plant regeneration (medium with no growth regulators but with the
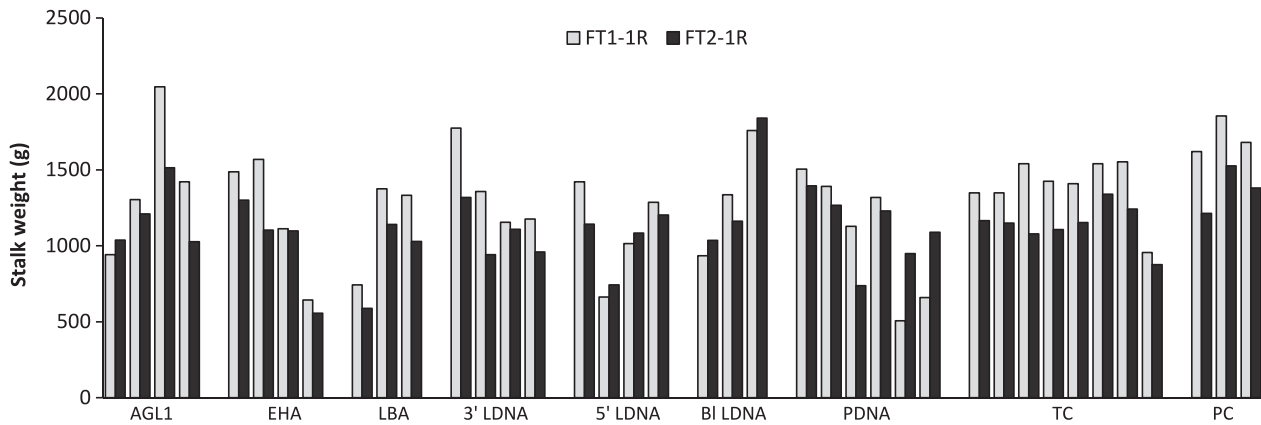
Figure 6 Graph of mean stalk weights (g) in FT1-1R and FT2-1R at harvest of individual events in each of the nine subgroups. FT1-1R is represented by grey bars, and FT2-1R by black bars. The estimated correlation coefficient (R^2) is 0.61.
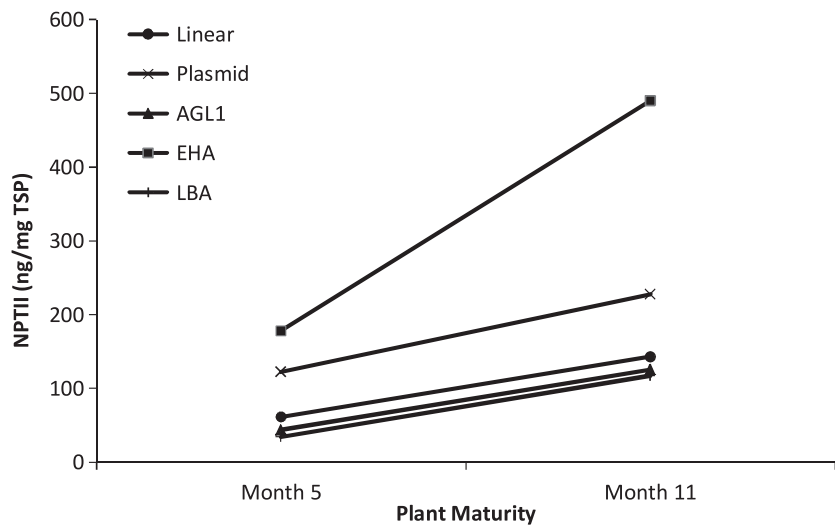
Figure 7 Effect of plant maturity on NPTII concentration in Biol and Agro transformants in FT2-P crop. NPTII protein was measured in leaf samples from plants after 5 and 11 months of growth in the field and expressed as ng of NPTII per mg total soluble protein (TSP). All events from the FT2 trial were included in this analysis. This includes six PDNA events, 11 LDNA events, and 11 Agro events (EHA, LBA, and AGL1).

same geneticin concentration). Shoots were regenerated after 8–12 weeks. Plantlets with roots were transferred to 15-cm pots containing sand, peat moss, and vermiculite (1 : 1 : 1 by volume) and grown in a glasshouse for 1 year, which served as a source for planting material for the first field trial (FT1).

Agrobacterium-mediated transformation was conducted by co-cultivating 8-week-old embryogenic callus with LBA4404, AGL1, AGL0, or EHA105 strains of *Agrobacterium tumefaciens* as described by Joyce *et al.* (2010). All the *Agrobacterium*-derived events had *nptII* as the selectable marker gene and beta glucuronidase (*gusA*) as the reporter gene. The selection and plant regeneration method was similar to that of biolistics, except that Timentin (200 mg/L, GlaxoSmithKline, Boronia, Vic., Australia) was added to prevent *Agrobacterium* overgrowth and paromomycin sulphate (150 mg/L, MP Biomedicals Australasia Pty Ltd., Seven Hills, NSW, Australia) was used instead of geneticin.

Tissue culture controls (TC) consisted of regenerated plants that had undergone a similar treatment as their transgenic counterparts, except for the omission of the vector in the transformation process and regeneration on culture medium containing no antibiotics.

Stalks of glasshouse-grown transgenic and TC events as well as field-grown nontransgenic Q117 parent clone (PC) were cut into single node setts, planted in Jiffy pots, and germinated in the

glasshouse. After 2 months, similar-sized plants were planted directly in the field.

Field trials and phenotyping

Transgenic events were evaluated in two field trials established at Woodford Sugar Experiment Station, Queensland, Australia. The FT1 consisted of 94 events randomly selected from a large population of transgenic events maintained in the glasshouse (>500 biolistics- and >100 *Agrobacterium*-derived independent events). The trial included 35 *Agrobacterium* (Agro) events, 48 biolistic (Biol) events, ten tissue culture (TC) events, and the parent clone (PC) (Table 5a). Each clonal plot consisted of a single row 6 m long with 1.5-m row spacing and had 11 plants from a single transgenic event planted with 0.6-m spacing between the plants. The trial was designed as described by Smith *et al.* (2005) with two blocks having 25% replication across them.

The second field trial, FT2, contained a subset of 36 events (Table 5a, selected for high-NPTII protein) from FT1 and followed a randomized complete block design with three separate blocks. Each clonal plot consisted of four rows planted using the entire stalk and was 10 m long with 1.2-m row spacing. A plant (P), first (1R) and second ratoon (2R) crops of the FT1 and a P and 1R of the FT2 were mechanically harvested at approximately 12 month intervals.
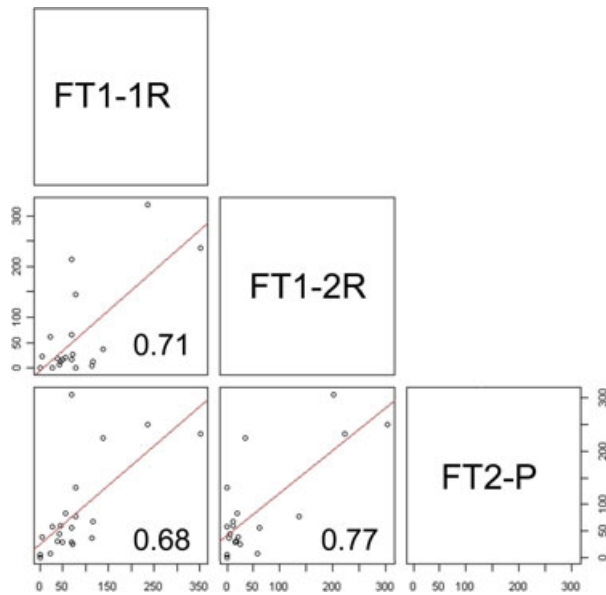
Figure 8 Relative NPTII expression of individual events across different crop classes. Pairwise comparisons are shown for individual events across different crop classes and correlation coefficients shown in the corner of each graph. Transgene expression is shown on both axes as ng of NPTII per mg of total soluble protein. All events from the FT2 trial were included in this analysis.

The trials were fertilized with Complete Mix 1 (Incitec Pivot Fertilisers, Melbourne, Vic., Australia; 290 kg/ha) soon after planting and Nitra K (Incitec Pivot Fertilisers; 290 kg/ha) after 8 months. The 6-month-old ratoon crop was fertilized once with Nitra K (440 kg/ha). Weeds were controlled with Stomp Xtra (a.i. pendimethalin; Crop Care Australasia Pty Ltd., Morningside, QLD, Australia; 3 L/ha) and Atrazine 600 (Farmalinx, Bondi Junction, NSW, Australia; 4 L/ha) at one and 9 weeks after planting, as well as Asulox (United Phosphurs, Baulkham Hills

BC, NSW, Australia; 8.5 L/ha) and Actril DS (Bayer CropScience, Hawthorn East, Vic., Australia; 1.5 L/ha) 5 weeks after harvest in the ratoon crop. Irrigation was provided every 2–3 weeks during the dry season. Limited incidence of smut and Fiji disease was observed (<1%) in some of the plots. The diseased stalks were removed and destroyed to minimize spreading.

Phenotypic characteristics (number of stools and stalks) were measured on the entire crop, 1 month prior to harvest. This provided information on ratoonability of the events.

Harvest data

At harvest, stalk height (from the base of the stalk to the first visible dewlap), stalk diameter (at the node, 1 m above the base of the stalk), stalk weight, and stalk-expressed juice parameters from eight stalks taken randomly from each FT1 plot or from the middle two rows of each replicate of FT2 were measured. Tonnes cane per hectare (TCH) for the FT1 was calculated as the product of stalk number, stalk weight, and plot area. In FT2, plot weight of the middle two rows was weighed at harvest, and TCH estimated from the plot weight and plot area.

Sugar analyses

Measurements were made within 24 h of harvest on expressed juice from stalks using a three-roller small mill, and commercial cane sugar (CCS), Brix, pol and purity were calculated (Chapman and Haysom, 1984). Ratoonability was assessed by the number of stalks produced in subsequent crops (1R and 2R) compared with that in the P crop.
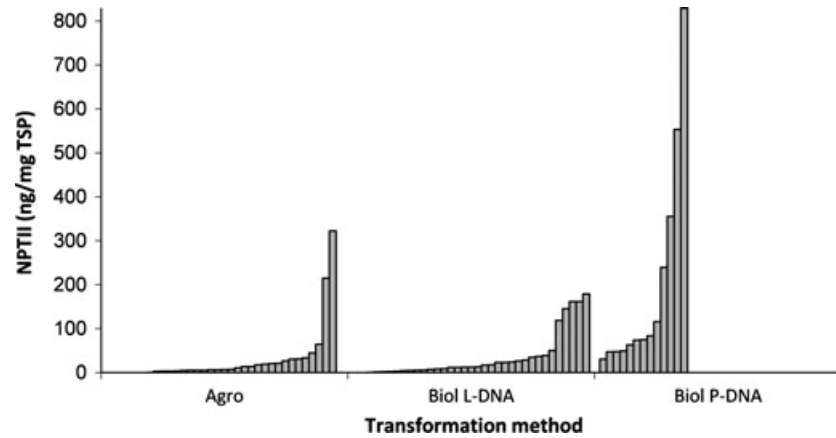
NPTII expression analysis

Three leaf samples from the second fully expanded leaf of three randomly selected stalks of each transgenic event from each of the plant and ratoon crops were collected at approximately 4 months and eleven months after germination. Tissue was frozen immediately after harvest and stored at −80 °C. *NptII* gene expression was determined by measuring NPTII protein with a commercial NPTII ELISA kit (Agdia; TASAG Elisa and Pathogen
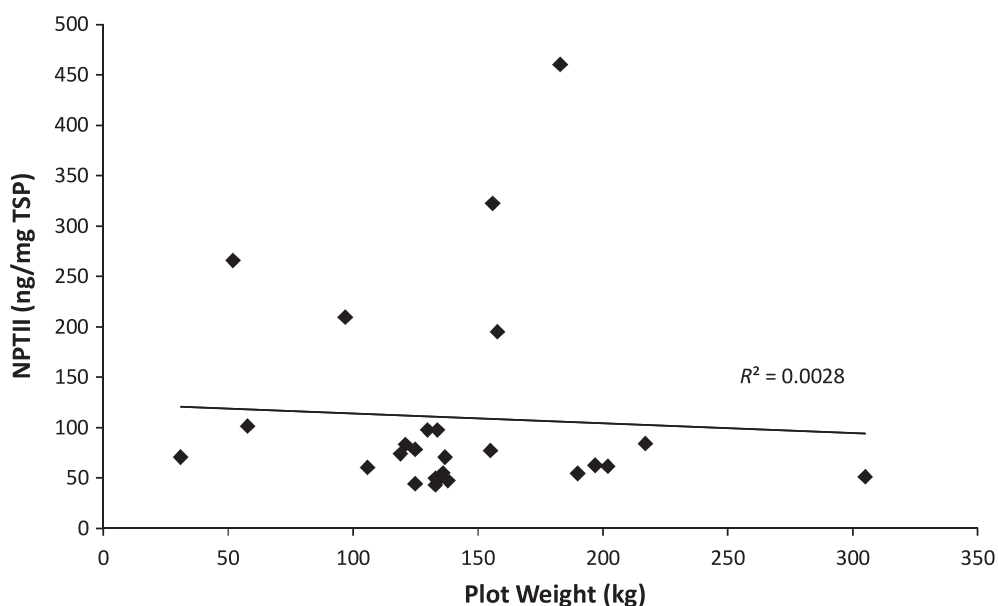

Figure 9 Correlation of NPTII expression with yield. Data shown are from the FT2-1R crop of all transgenic events. NPTII is expressed as ng per mg of total soluble protein. Plot weight shown is for the two middle rows of each four row plot only.

Figure 10 NPTII expression for individual transgenic events in the FT1-2R plants. Plants have been grouped into the transformation method and in ascending order of NPTII expression. NPTII is expressed as ng per mg of total soluble protein.

Figure 11 Correlation of NPTII expression and transgene copy number. Data shown are from all transgenic events in the FT2-P trial. Copy number was determined by Southern blot analysis. NPTII is expressed as ng per mg of total soluble protein.
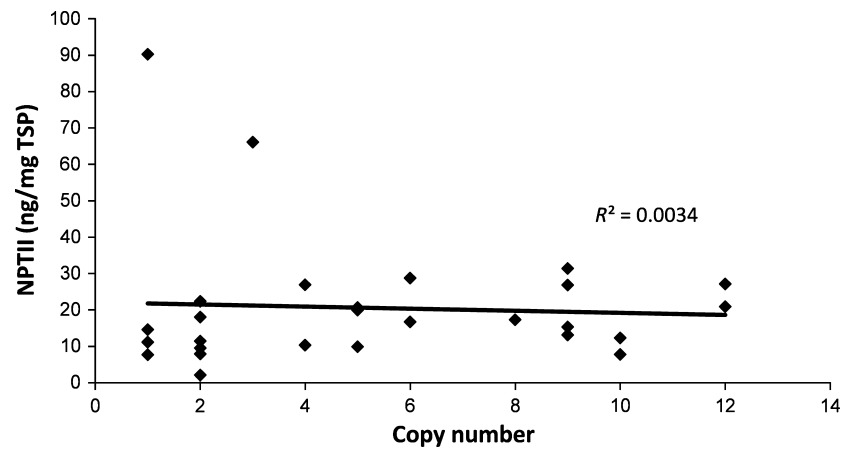
Table 4 Polymorphism detected by (a) AFLP (b) MS-DArT

| Group | Events tested | Loci/event | Non-methylated CCGC sites (%) (Type 1) | Methylated CCGC sites (%) | | |
				Fully methylated (Type 2)	Hemi-methylated (Type 3)	Total
(a)						
PC	12	86.9	85.8	9.8	4.4	14.2
TC	nd	nd	nd	nd	nd	nd
Agro	36	86.3	88.5	8.8	3.6	12.4
Biol	41	86.8	87.5	9	3.5	12.5
(b)						
PC	6	236.7	97.1	1.2	1.7	2.9
TC	3	248.0	98.3	1.1	0.7	1.8
Agro	nd	nd	nd	nd	nd	nd
Biol	4	244.5	97.5	0.9	1.5	2.4

Testing Service, New Town, Tas., Australia). Total soluble protein was extracted by homogenizing 100 mg of tissue in 400 μL of proprietary protein extraction buffer (supplied with the ELISA kit) and centrifuging the homogenate at 10 000 × g, 4 °C for 15 min. The resulting supernatant was used for the ELISA. Total soluble protein concentration in the supernatant was determined by the Bradford method (Bradford, 1976). The mean reading

Table 5 (a) Clones used in FT1 and FT2 field trials and (b) Planting and harvest dates for the FT1 and FT2 field trials

Method groups type		FT1 clones	FT2 clones
(a)			
Conventionally propagated wild-type Q117	PC	1	1
Tissue cultured Q117	TC	10	8
Agrobacterium transformed	Agro-EHA	17	4
	Agro-LBA	7	3
	Agro-Agl0	2	0
	Agro-Agl1	9	4
Agrobacterium total	Agro	35	11
Biolistics transformed	PDNA	14	6
	3'LDNA	8	4
	5'LDNA	8	4
	Bl LDNA	18	3
Biolistics total	Biol	48	17
Total		94	37

Trial type	Planting date	Plant crop (P) harvest	First ratoon (1R) harvest	Second ratoon (2R) harvest
(b)				
FT1	12/04/07	12/08/08	14/07/09	13/07/10
FT2	14/08/08	14/07/09	14/07/10	N/A

from two independent Bradford assays was used to normalize NPTII protein measurements against total soluble protein concentration for each sample.

The effect of plant maturity and transformation method on NPTII expression level was analysed using a mixed model analysis (see Statistical Analysis section). Transgene copy number was determined by Southern hybridization (Joyce *et al.*, 2010), and the impact of transformation method on transgene copy number was analysed using a *t*-test with unequal variances assumed.

Genotypic analysis

AFLP analysis was carried out as described by Carmona *et al.* (2005) to identify point mutations or indels (insertion and deletions) likely to occur with tissue culture and the transformation process. DNA was isolated from freeze-dried young leaves of FT1-1R plants using a modified cetyltrimethylammonium bromide (CTAB) extraction method (Rogers and Bendich, 1994). DNA was quantified and then digested using *Eco*RI and *Mse*I. Adapters for *Eco*RI and *Mse*I were prepared and ligated by mixing their respective adapters I and II (Sigma-Aldrich). Primers for the adapters containing one additional specific nucleotide, A and C, respectively, were used for the pre-amplification, and this was in turn used as template for five combinations (*Eco*RI-AAC/*Mse*I-CAT, *Eco*RI-ACA/*Mse*-CTC, *Eco*RI-ACG/*Mse*I-CAA, *Eco*RI-AGC/*Mse*I-CAC, and *Eco*RI-AAC/*Mse*I-CAA) of selective AFLP primers. Reaction products were separated using polyacrylimide gel electrophoresis (PAGE), and silver stained gels were scored for presence (1) or absence (0) of bands.

Methylation-sensitive AFLPs (MS-AFLP) were used to determine the methylation patterns in transgenic events. The MS-AFLP assay was carried out as described Xu and Korban (2002).

A small number of transgenic events with visually clear phenotypic variation were also analysed by DArT (Diversity Arrays Technology Pty Ltd, Yarralumla, ACT, Australia) genotyping to detect all types of DNA variation including SNP, indel, CNV, and methylation (Wei *et al.*, 2010). In total, four transgenic events (with two events replicated), three TC events (with one event replicated), and six PC clones were tested. A modified DArT technique was used to capture methylation differences by incorporating the methylation-sensitive enzymes *PstI/TaqI/HpaII* and *PstI/TaqI/MspI*. The former enzyme combination was also used for cloning and genotyping while the latter was used for genotyping only. The experiment included 6,528 standard DNA clones (Wei *et al.*, 2010), and a further 1,152 clones were generated for this experiment (from *PstI/TaqI/HpaII* digestion). Polymorphic DArT markers were scored as 'present' (1) and 'absent' (0) and analysed statistically (see Statistical Analysis section).

Statistical analysis

Field trials

Two separate analyses were performed on the harvest data. First, the data were pooled by the method of transformation (PC, TC, Agro, Biol) to determine their effect for each crop class separately, and then data of crop classes were combined and re-analysed to determine the interaction between crop class and method of transformation (Littell, 2002). For TCH in FT2, plot length was used as a covariate to normalize plot length variation due to gaps resulting from whole stick planting.

Box and whiskers plots for stalk weight were generated for each subgroup within each transformation method. Agro (AGL0,

AGL1, EHA, and LBA subgroups), LDNA (3′, 5′ and bl subgroups), and PDNA were compared to TC and PC. In the FT2, additional analysis between individual clones was also performed to identify clones that performed similar to the parent clone, thus enabling comparison between the clone means instead of the method means. Finally, each clone was compared to the PC for each trait, and adjusted P values generated using a two-tailed Dunnett to compare the estimated means. The adjusted P value for plot weight of individual clones was also compared with PC at two levels of significance: $\alpha > 0.85$ and <0.1.

NPTII expression

FT1 and FT2 data were analysed separately. Replicate was treated as a random effect, while event and plant maturity at time of sampling were the fixed effects.

Genotypic data

Data were analysed by treating classes of transformants as fixed and poly-acrylamide gel number or DArT plate number as random. Type 1 and 3 data were arcsine-transformed, and type 2 data were square-root-transformed. To summarize the data, it was grouped into (i) nonmethylated (type 1), (ii) fully methylated (type 2 and type 3 bands were shared). and (iii) hemi-methylated (type 2 and type 3 bands were not shared). A summary of the statistical methods is given in Table S1.

Acknowledgements

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Supporting information

Additional Supporting information may be found in the online version of this article:

Table S1 Summary of statistical methods used.

Table S2 Comparison of plot weights of events in different subgroups compared to PC in the FT2-P and 1R crop.