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Generation of a selectable marker free, highly expressed single copy locus as landing pad for transgene stacking in sugarcane

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Abstract

Key message **A selectable marker free, highly expressed single copy locus flanked by insulators was created as landing pad for transgene stacking in sugarcane. These events displayed superior transgene expression compared to single-copy transgenic lines lacking insulators. Excision of the selectable marker gene from transgenic sugarcane lines was supported by FLPe/*FRT* site-specific recombination.**

Abstract Sugarcane, a tropical C4 grass in the genus *Saccharum* (*Poaceae*), accounts for nearly 80% of sugar produced worldwide and is also an important feedstock for biofuel production. Generating transgenic sugarcane with predictable and stable transgene expression is critical for crop improvement. In this study, we generated a highly expressed single copy locus as landing pad for transgene stacking. Transgenic sugarcane lines with stable integration of a single copy *npt*II expression cassette flanked by insulators supported higher transgene expression along with reduced line to line variation when compared to single copy events without insulators by NPTII ELISA analysis. Subsequently, the *npt*II selectable marker gene was efficiently excised from the sugarcane genome by the FLPe/*FRT* site-specific recombination system to create selectable marker free plants. This study provides valuable resources for future gene stacking using site-specific recombination or genome editing tools.

Keywords Sugarcane · Selection marker removal · Site-specific recombination · Insulator · FLP · FLPe

Introduction

Sugarcane (*Saccharum* spp.) is a perennial C4 grass that is grown in approximately all tropical, semi-tropical, and subtropical countries of the world (Tew and Cobill 2008). It is an important crop supplying approximately 80% of the world's sugar, greatly exceeding sugar beet (Cordeiro et al. 2007; Henry and Cole 2010). Sugarcane is also one of the most important feedstock for commercial production of biofuel. In spite of success in improving sugar yield, disease resistance and ratooning ability, sugarcane breeding remains challenging due to its complex genome, poor fertility, and the long breeding/selection cycle (Altpeter and Oraby 2010). Transgenic technology, therefore, is expected to complement traditional breeding in the development of advanced cultivars and benefit the global sugar and cellulosic biofuel industries.

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Future transgenic strategies will aim at incorporating several traits or entire pathways using transformation vectors harboring multiple transgene expression cassettes (Altpeter et al. 2016). Prediction of transgene performance, however, remains difficult due to its random insertion into the genome and associated position effects, as well as variegated, ectopic, and silenced gene expression (Giraldo et al. 2003). In addition, enhancer–promoter interference may disturb the specificity and strength of promoters (Singer et al. 2011) in gene stacking applications. The generation of transgenic sugarcane with high level and stable transgene expression is particularly difficult compared to other plants due to its large, complex, polyploidy, and highly redundant genome (Ingelbrecht et al. 1999). A large number of transgenic events are needed in sugarcane to identify lines with the desired transgene expression level. Removing the selectable marker gene following transformation supports multiple cycles of re-transformation with the same selectable marker for stacking of multiple traits or multigenic pathways. Site-specific recombination (SSR) systems support both targeted integration into plant genomes as well as selectable marker gene removal. The heterologous recombinase catalyzes highly specific recombination between its corresponding recombination sites and can produce insertions, inversions, and deletions, depending on the orientation of the recombination sites (Lyznik et al. 2003; Grønlund et al. 2007; Gidoni et al. 2008; Thomson et al. 2009, 2010; Srivastava and Thomson 2016). In addition, 'off-target' recombination by SSR systems are generally undetectable in plant genomes. Establishing SSR technology for sugarcane, therefore, would streamline the development of elite transgenic sugarcane lines and facilitate gene stacking without position effects and may facilitate regulatory approval. To date, several SSR systems have been successfully used for site-specific integration of transgenes into crops including the bacteriophage P1 Cre-*lox*, yeast FLP-*FRT, Streptomyces* phage φC31-*att* system, and mycobacterio phage Bxb1large serine recombination system (Albert et al. 1995; De Paepe et al. 2013; Lutz et al. 2004; Srivastava and Ow 2001; Srivastava et al. 2004; Li et al. 2009, 2016; Fladung and Becker 2010; Nandy and Srivastava 2011; Hou et al. 2014).

Insulators can also contribute to reducing the position effects of transgenes. These DNA boundary elements prevent influences from adjacent chromatin domains by establishing genomic barriers and thereby protecting genes from the influence of neighboring heterochromatin regions. Insulators also block the activity of enhancers (West et al. 2002). Although many insulators have been identified in different eukaryotic systems, only a few insulators have been studied in plants. An 1 kilobase (kb) fragment from bacteriophage λ (EXOB) and 2 kb fragment from a petunia (TBS) matrix-attachment region were reported as effective insulators for enhancer blocking in transgenic plants (Hily et al. 2009; Singer et al. 2010; Yang et al. 2011). The properties of effective insulators make them desirable in transgenic vector construction to ensure the stable and high expression of transgenes by shielding them from neighboring enhancers.

In this study, we generated a landing pad for transgene stacking consisting of single-copy integration of a highly expressed selectable marker gene flanked by insulators. We also demonstrated removal of the selectable marker gene, which will facilitate retransformation for gene stacking.

Materials and methods

Plant material

The commercially important sugarcane cv. CP88-1762 was used for transformation in this study. The single node segments from mature stalks were transplanted into pots containing Fafard No. 2 mix (Conrad Fafard, Agawam, MA) in an air-conditioned greenhouse with natural photoperiod and temperature at 28 °C/22 °C (day/night). The tops including the shoot apex and the top visible node, were harvested when plants had 6 to 10 above ground nodes to obtain leaf whorl cross-section explants for induction of somatic embryogenesis.

Genetic constructs

Two genetic constructs pJKIS (Fig. 1a) and pJKIe (Fig. 1c), carrying FLP-*FRT* or FLPe-*FRT* systems respectively, were generated for gene transfer into sugarcane. FLPe is a thermostable variant of wild-type FLP generated by protein evolution for enhanced thermostability. The two constructs contain the same vector elements except for the vector backbone and for the recombinase genes. While pJKIS is a pUC57 derivative, which was used for biolistic gene transfer, pJKIe is a binary vector that was used for *Agrobacterium tumefaciens*-mediated transformation. In both vectors the expression cassettes were flanked by two insulator sequences (EXOB and TBS; Singer et al. 2011, 2012). The selectable marker gene *neomycin phosphotransferase* II (*npt*II) was driven by CaMV 35S promoter. *FLP* or *FLPe* were cloned under transcriptional control of heat shock promoter to catalyze the recombination between two *FRT* sites in the same orientation flanking the *FLP*/*FLPe* expression cassette and *npt*II expression cassette. Maize ubiquitin promoter (Ubi) was inserted 5′ of a mutated *lox76* site to support site-directed Cre/*lox* integration (Albert et al. 1995) in future experiments. A vector pJKNI (Fig. 1b) without insulator sequences was also constructed for comparison of transgene expression with and without insulators.

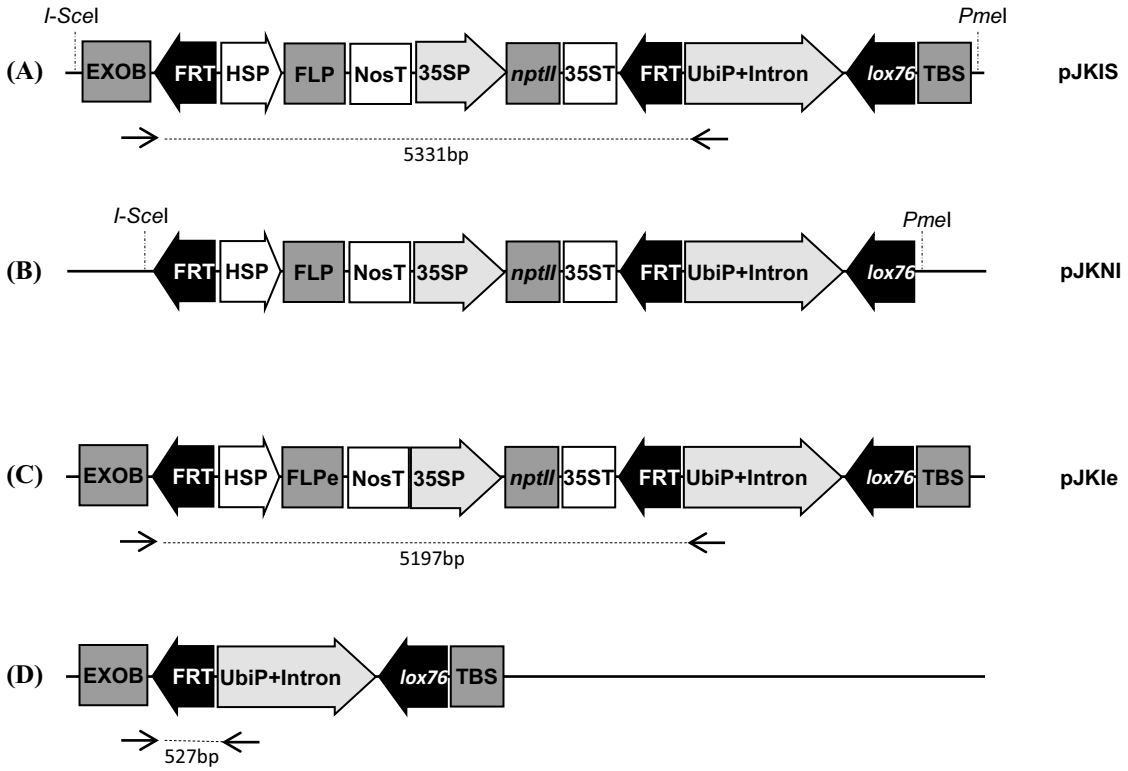


Fig. 1 Vector construction and strategies for FLP-*FRT* and FLPe-*FRT* mediated excision. **a** FLP-*FRT* system for excision (pJKIS). **b** FLP-*FRT* system for excision without insulator flanking (pJKNI). **c** FLPe-*FRT* system for excision (pJKIe). **d** Expected final genomic structure after excisions. The expression cassettes are flanked by insulator sequences (EXOB and TBS) to minimize position effects and block the influence from neighboring genes. *lox76*: right arm mutant *lox* site; ubiP: ubiquitin promoter and 1st intron from maize; the arrows represent the forward and reverse primers used for identifying the excision. *FRT*: wild-type *FRT* site; *npt*II: neomycin phosphotransferase gene; 35SP: CaMV 35S promoter; 35ST: CaMV 35S Terminator; Nos: Nopaline synthase terminator

Tissue culture and sugarcane transformation

The outermost leaf sheaths of the immature leaf whorl were wiped with 70% ethanol and removed under aseptic conditions. About 1–2 mm thick cross-sections were cut from the region (1–10 cm) above the apical meristem, and placed onto direct embryogenesis medium (DEM) or calli induction medium (CI3) (Taparia et al. 2012b; Chengalrayan and Gallo-Meagher 2001). The explants on DEM for direct somatic embryo induction were cultured at 28 °C under light of 30 µmol m^{-2} s^{-1} intensity with 16 h/8 h (light/dark) photoperiod and were sub-cultured weekly, while the explants on CI3 for callus induction were incubated in dark at 28 °C and were subcultured every 10 days.

Minimal expression cassettes (MC) for biolistic gene transfer were released from plasmids pJKIS and pJKNI by restriction enzyme digestion with *I-Sce*I and *Pme*I (Fig. 1a, b), purified by gel electrophoresis and extraction using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA). The minimal expression cassettes were quantified using Synergy H1 Hybrid Reader (Biotek, Winooski, VT) and then coated onto 1 µm diameter gold particles (Bio-Rad) as described by Altpeter et al. (2010). Biolistic gene transfer was conducted as described by Taparia et al. (2012a). The plasmid pJKIe was transformed into *Agrobacterium tumefaciens* strain *AGL1* under selection with 100 mg l^{-1} kanamycin. The *Agrobacterium*-mediated transformation method was described by Wu and Altpeter (2015a); and Wu et al. (2015b). Rooting plants were transferred to pots containing Fafard No. 2 mix (Conrad Fafard, Agawam, MA). Plants were grown in air-conditioned greenhouse with natural photoperiod and temperature at 28 °C/22 °C (day/night).

ELISA to quantify NPTII expression

The NPTII ELISA analysis was conducted using the kit from Agdia Inc. (Elkhart, IN). Fifty milligrams of leaf material was collected and extracted with protein extraction buffer (PEB1) provided with the kit in a Tissuelyzer II (Qiagen, Valencia, CA). The extracted total proteins were quantified with the Bradford Assay (Bradford 1976), using the Coomassie Plus Protein Assay reagent (Thermo Fisher Scientific Inc., Rockford, IL), on a Synergy H1 Hybrid Reader (Biotek, Winooski, VT). The ELISA plates containing 20 µg

total proteins in each well were read against NPTII positive control provided with the kit on the Synergy H1 Hybrid Reader at A650.

Taqman® qPCR analysis

About 200 mg of leaf material of selected pJKIS, pJKNI and pJKIe lines as well as wild-type plants were collected in four replicates for DNA isolation. The quantitative TaqMan® qPCR assay for determination of the copy number was performed using the method described by Ingham et al. (2001). Different probes targeting different vector components (*npt*II, EXOB, TBS, and *bla*) were used. In each well of the 384-well plates, 3 µl of genomic DNA (gDNA), or the DNA samples for the copy control were loaded into 3 µl master mix (2X JumpStart Taq ReadyMix, primer for the crop-specific endogenous gene (Glyceraldehyde 3-phosphate dehydrogenase), and 2X primer set stock assay target) (Wu et al. 2015b). Controls for one and two copies were obtained from transgenic sugarcane events that were previously confirmed by Southern blot analysis (Wu et al. 2015b).

PCR amplification of transgenic *npt*II expression cassette

The gDNA from 200 mg leaf material of single-copy pJKNI transgenic lines was extracted using a modified CTAB protocol (Murray and Thompson 1980) and quantified by Synergy H1 Hybrid Reader (Biotek, Winooski, VT). One hundred nanograms of gDNA was used for each PCR reaction. The primers (forward primer: 5′-AGGAT GAGAC TAATC CAATT GAGGA GTG-3′ and reverse primer: 5′-TCATT ATCTC TAGAG AGGGG CACGA C-3′) flanking the *npt*II expression cassette were used. The PCR reactions were performed using hot start Taq DNA polymerase (New England Biolabs Inc., Ipswich, MA) with the following conditions: 30 s at 95 °C, followed by 30 cycles of 95 °C for 20 s, 64 °C for 30 s, and 68 °C for 4 min, and final elongation at 68 °C for 5 min. No template control and plasmid positive control were included.

Southern blot analysis

Total genomic DNA was extracted from leaf tissue of single-copy pJKIS transgenic lines using a modified CTAB protocol (Murray and Thompson 1980). Twenty microgram of genomic DNA was fully digested with *Xho*I, *EcoR*I, or *Kpn*I (New England Biolabs, Ipswich, MA), respectively, for targeting EXOB, TBS, *npt*II and *FLP* region with different probes. The digestion products were separated by electrophoresis overnight on 0.8% agarose gel with 1X TAE buffer at low voltage, and transferred onto Hybond-N + nylon membranes (GE Healthcare Biosciences, Pittsburgh, PA) overnight with 10X SSC buffer. The membranes were rinsed with 6X SSC for 5 min, air-dried, and exposed to UV light in a crosslinker (Select™XLE-Series, Spectroline®, Westbury, NY). The following primers were designed to amplify the probes (EXOB forward primer: 5′-ACTTC TTTCC GGAGC GGGGT-3′, EXOB reverse primer: 5′-GCCTT AATCC GGTGG ACGAA AGA-3′, TBS forward primer: 5′-ACAAA ATGGT GTTGT GGAGA GAAAG-3′, TBS reverse primer: 5′-TGTCA CAAAG TCCTT GAGCC AGAT-3′, *npt*II forward primer: 5′-ATGGG GATTG AACAA GATGG ATT-3′, and *npt*II reverse primer: 5′-AACTC GTCAA GAAGG CGATA GAAG-3′, *FLP* forward primer: 5′-TGCTT GTTCG TCAGT TTGTG G-3′, *FLP* reverse primer: 5′-GTCAA CTCCG TTAGG CCCTT C-3′). The probes were labeled using ^{32}P-dCTP (Perkin Elmer Inc., Waltham, MA) with a Prime-It II Random Primer Labeling Kit (Stratagene Inc., La Jolla, CA). Pre-hybridization, hybridization and washing were performed according to the manufacturer's instructions. The membranes were exposed onto X-ray film for two days at − 80 °C and visualized by autoradiography. The single-copy pJKIe lines were also analyzed by southern blot for the *FLPe* gene copy numbers with *Kpn*I digestion and probe amplified by PCR (*FLPe* forward primer: 5′-TCTTC AGAGG AGGCC GATAA-3′, *FLPe* reverse primer: 5′-TTGAA GGACT TTGGG TCCAC-3′).

Sequencing of cloned PCR amplicons of the highly expressed, single copy locus

Total genomic DNA of single-copy pJKIS lines confirmed by Southern blots was extracted from leaf tissue using a modified CTAB protocol (Murray and Thompson 1980). PCR amplification products of the region corresponding to Ubiquitin promoter, intron, and *lox76* site (forward primers: 5′-CTGAC CGCTT CCTCG TGCTT TA-3′ and reverse primer: 5′-CCAAG AGAGG CCAGC AACTC ATTA-3′) and the region corresponding to the upstream 1 kb of the TBS insulator (forward primers: 5′-TATAC TTGGA TGATG GCATA TGCAG CAG-3′ and reverse primer: 5′-GGCCT TAAAC CTGTC AACTT CACCA TTA-3′) were separated by electrophoresis and purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA). The PCR products were generated with the following primers (Primers for sequencing the Ubiquitin promoter, intron, and *lox76* site: 5′-ACAGG CTGGC ATTAT CTACT CGAA-3′, 5′-TGAAC AGTTA GACAT GGTCT AAAGG ACA-3′, 5′-CAAGC GAACA AAAAG CATCT CTGT-3′, 5′-ACTTG TTTGT CGGGT CATCT TTTCA-3′; primers for sequencing the upstream 1 kb of the TBS insulator: 5′-TGAAA CTCTC TGAAA ACTAC ATCCC TGC-3′, 5′-CCCAG AGCCT TTGTT CAGTG TCACA-3′, 5′-ATTCC TGACA ATGTA GAGAC CAATG AGG-3′). The sequencing results were

analyzed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) with the default parameters.

TAIL-PCR was carried out to identify the genomic DNA flanking the single copy transgene insertion as described by Singer and Burke (2003) using the following nested, insertion-specific primers LS1 5′ CGATG TGAGA GCTGT CGAAC AG 3′, LS3 5′ GTCCT ACTTC CACAC CCTGC 3′, LS5 5′ TACGA GAACG ACGCC AGAAC 3′, RS1 5′ AGTGT CAACC CCAAT GGAAC AT 3′, RS3 5′ GGTGG GAAAG CTTCT CTACT TG 3′, RS5 5′ CTGGA AGCAG CATTG AGGGT TG 3′ together with the following arbitrary degenerate primers (AD primers) AD1 5′ NGTCG ASWGA NAWGA A 3′, AD2 5′ TGWGN AGSAN CASAG A 3′, AD3 5′ AGWGN AGWAN CAWAG G 3′, AD4 5′ STTGN TASTN CTNTG C 3′, AD5 5′ NTCGA STWTS GWGTT 3′, AD6 5′ WGTGN AGWAN CANAG A 3′. One cloned PCR amplicon which included both vector and genomic sequences was blasted against the monoploid sugarcane genome reference sequence (Garsmeur et al. 2018) after trimming off the vector sequence component.

Quantitative real-time RT-PCR to evaluate inducible expression of *FLP* and *FLPe*

Quantitative real-time RT-PCR was performed on heat-treated single-copy pJKIS (Ins20 and Ins62) and pJKIe (FLPe31, FLPe59 and FLPe92) lines as well as the non-heat-treated groups grown in soil. Two biologically-replicated plants were tested per line for both treated and untreated groups. The mid-section of the youngest fully expanded leaf was collected at four time points: before heat-treatment, 0 h after heat-treatment, 4 h after heat-treatment and 8 h after heat-treatment. Total RNA was extracted with Trizol reagent (Invitrogen) and treated with RNase-Free RQ1 DNase (Promega) according to the manufacturer's instruction. cDNA was synthesized from 1000 ng RNA with iScript cDNA Synthesis kit (Bio-Rad). Primers (forward primer: 5′-CACAT AACGG AACAG CAATC A-3′ and reverse primer: 5′-TTTAA ACTGC AGTGA CTTGT TGAC-3′) targeting *FLP* gene and primers (forward primer: 5′-AACGG CACAG CGATT AAGAG-3′ and reverse primer: 5′-CTGGG TCTTG TACTT GAACT GC-3′) targeting *FLPe* gene were designed to amplify the specific fragments. Another pair of primers (forward: 5′-CACGG CCACT GGAAG CA-3′ and reverse: 5′-TCCTC AGGGT TCCTG ATGCC-3′) were used to amplify the specific fragment of the sugarcane GAPDH reference gene for normalization. Quantitative real-time PCR was performed in a MyiQ cycler (Bio-Rad) with SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad) under the following conditions: 95 °C for 3 min, followed by 39 cycles of 95 °C for 10 s and 58 °C for 30 s. Amplicon specificity was confirmed by melt curve analysis from 65 °C to 95 °C. *FLP* and *FLPe* expression levels in treated and untreated plants relative to GAPDH gene were calculated using the $2^{-\Delta Ct}$ method.

Heat treatment and detection of selectable marker gene removal

Heat treatment at 40 °C for 4 h was performed on the selected single-copy pJKIS lines and single-copy pJKIe lines. The single-copy pJKIS lines Ins20, Ins62, and the single-copy pJKIe line FLPe59 as well as two-copy pJKIe line FLPe13 were heat treated in sterile petri dishes with rooting medium in four replicates. One single-copy pJKIe line FLPe31 in soil was also tested. Genomic DNA of the plants before and after heat treatments was extracted according to previously described method from tissue samples. Primers (forward primer: 5′-CAACG ATCAG TAATG CGATG AACTG-3′ and reverse primer: 5′-GTCTA TAAAA ACCAT TAACC CTAAA CC-3′) were designed to amplify the region between two *FRT* sites for detecting the excision. The PCR reactions were performed with the LongAmp® Taq DNA Polymerase (New England BioLabs Inc., Ipswich, MA) and with 94 °C for 30 s, followed by 30 cycles of 94 °C for 30 s, 60 °C for 60 s, and 65 °C for 5 min, and an additional 65 °C for 10 min. No template control and plasmid control were included. The 527-bp amplification products, which indicated the excision (Fig. 1d), were sequenced.

Statistical analysis

The Chi-square test with one degree of freedom was performed to compare the frequency of single copy integration, backbone integration, and truncations at 5% probability level. The *t* test was performed for comparing means and values are considered as significantly different if $P < 0.05$. All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA).

Results

Generation of transgenic sugarcane lines with site-specific integration sites

Three vectors, pJKIS (Fig. 1a), pJKNI (Fig. 1b), and pJKIe (Fig. 1c), were constructed for sugarcane transformation. The minimal expression cassettes of vectors pJKIS and pJKNI were bombarded into sugarcane direct somatic embryos 10 days after culture initiation (Fig. 2a). Regenerating plantlets were selected with 30 mgl^{-1} geneticin sulfate (Fig. 2b). Rooting plants (Fig. 2c) were transferred to soil (Fig. 2d). The pJKIe plasmid was transformed into *Agrobacterium* strain *AGL1* and introduced into sugarcane genome through *Agrobacterium*-mediated

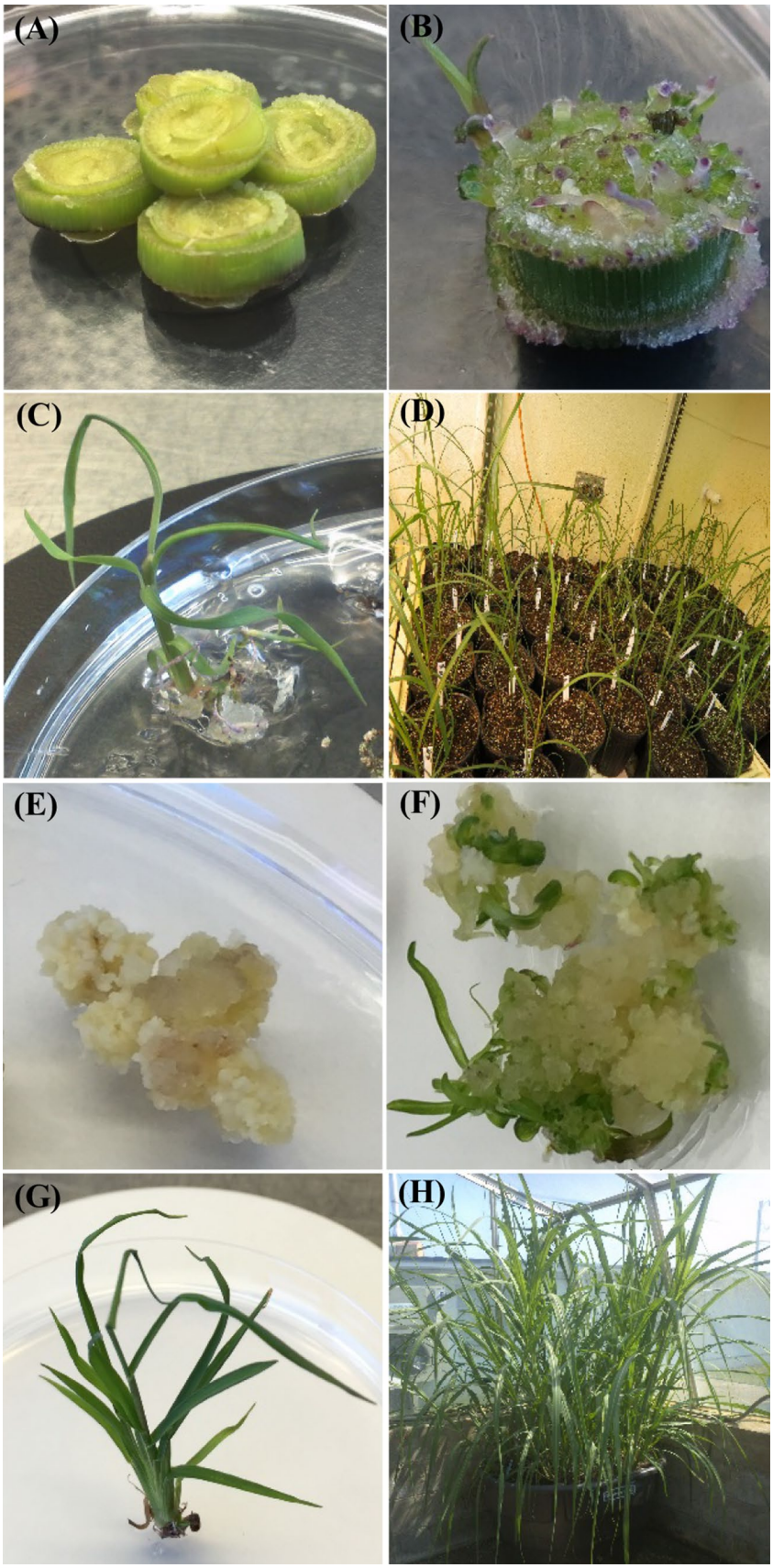


Fig. 2 Generation of transgenic plants: Biolistic transformation. **a** Cross sections prior bombardment. **b** Resistant shoot on 30 mg l^{-1} geneticin sulfate containing media. **c** Rooting of resistant plantlets. **d** Plants growing in soil. *Agrobacterium*-mediated transformation. **e** Selection of resistant callus on 30 mg l^{-1} geneticin sulfate containing media. **f** Regeneration of shoots on 30 mg l^{-1} geneticin sulfate containing media. **g** Rooting of resistant plantlets. **h** Propagation of elite single-copy target lines in large containers in green house

Table 1 Transgenic pJKNI and pJKIS lines confirmed by NPTII ELISA

Constructs	Biolistic gene transfer (shots)	Lines regenerated	NPTII-ELISA (positive)	Number of lines per shot
pJKIS	19	118	107	5.6
pJKNI	12	98	93	7.8
Total	31	216	200	6.5

Table 2 Taqman® qPCR analysis of transgene copy number of pJKNI lines

Copy numbers	Lines	% of total lines
1 copy *nptII*, no *bla* insertion	22	24.2
1 copy *nptII* with *bla* insertion	1	1.1
2 copies *nptII*, no *bla* insertion	23	25.3
2 copies *nptII* with *bla* insertion	2	2.2
> 2 copies *nptII*, no *bla* insertion	38	41.8
> 2 copies *nptII* with *bla* insertion	5	5.5
Total	91	

transformation (Fig. 2e). Regenerating plantlets were selected with 30 mgl^{-1} geneticin sulfate (Fig. 2f). Rooting plants (Fig. 2g) were transferred to soil and grown in the greenhouse (Fig. 2h). A total of 118 pJKIS, 98 pJKNI, and 97 pJKIe plants were obtained (Table 1) with an average transformation efficiency of 6.5 transgenic lines per bombardment. Transformation efficiency did not differ significantly between the insulator containing pJKIS and non-insulator containing pJKNI transgenic lines (Table 1).

Identification of single-copy transgenic lines

A total of 91 pJKNI, 86 pJKIS and 78 pJKIe lines were selected to perform Taqman® qPCR for determination of transgene copy number and backbone (*bla* gene) integration. Probes targeting the *npt*II gene, the insulators EXOB, TBS in the minimal cassette and the *bla* (*beta-lactamase* gene) on the backbone were used for testing pJKIS and pJKIe lines (Fig. 1a, c). Probes only targeting the *npt*II and *bla* genes were used for testing the non-insulator containing pJKNI lines. The evaluated lines were confirmed as positive for at least one of the *npt*II, EXOB or TBS sequences. Twenty-two pJKNI transgenic lines showed single-copy insertion of the *npt*II gene without *bla* sequence integration (Table 2). Seven pJKIS lines (Table 3) and six pJKIe lines (Table 4) showed a single-copy insertion of the *npt*II gene and a single-copy insertion of both insulators as well as the absence of the *bla* sequence. The frequency of these single copy integration events without backbone (*bla*) integration did not differ significantly ($P > 0.05$) between biolistic transfer of the pJKIS (8.1%) and *Agrobacterium tumefaciens*-mediated transfer of pJKIe (7.7%). However, the frequency of single copy integration following biolistic transfer of the shorter pJKNI (24.2%) was significantly higher than the longer pJKIS (8.1%) or pJKIe (7.7%) constructs delivered by biolistics or *Agrobacterium*, respectively. The frequency of backbone integration, estimated by detection of *bla* sequence amplicons was not significantly different ($P > 0.05$) between the two biolistic constructs (8.8% and 14.0%, respectively for pJKNI and pJKIS), but was significantly higher ($P < 0.01$) with *Agrobacterium tumefaciens*-mediated transfer (26.9%). The frequency of the truncation of the delivered constructs was estimated by different copy numbers for different vector elements and was significantly higher ($P < 0.01$) following biolistic transfer of pJKIS (88.4%) compared to

Table 3 Taqman® qPCR analysis of transgene copy number following biolistic delivery of vector pJKIS

Copy numbers	Lines	% of total lines
1 copy *npt*II, 1 copy EXOB, 1 copy TBS, no *bla* insertion	7	8.1
1 copy *npt*II, 1 copy of EXOB and/or TBS missing, no *bla* insertion#	15	17.4
1 copy *npt*II, 1 copy of EXOB and/or TBS missing with *bla* insertion #	3	3.5
1 copy *npt*II, > 1 copy of EXOB and/or TBS, no *bla* insertion#	1	1.2
2 copies *npt*II, 2 copies EXOB, 2 copies TBS, no *bla* insertion	1	1.2
2 copies *npt*II, 1 or 2 EXOB and/or TBS missing, no *bla* insertion#	17	19.8
2 copies *npt*II, 1 or 2 EXOB and/or TBS missing with *bla* insertion#	5	5.8
> 2 copies of *npt*II, EXOB and TBS, no *bla* insertion	2	2.3
> 2 copies with at least one copy missing of *npt*II, EXOB or TBS, no *bla* insertion#	30	34.9
> 2 copies with at least one copy missing of *npt*II, EXOB or TBS with *bla* insertion#	4	4.7
No *npt*II, 1 or 2 copies EXOB or TBS, no *bla* insertion#	1	1.2
Total	86	

Events (76/86) with evidence of truncation of the delivered vector

Table 4 Taqman® qPCR analysis of transgene copy number following *Agrobacterium*-mediated delivery of vector pJKIe

Copy numbers	Lines	% of total lines
1 copy *npt*II, 1 copy EXOB, 1 copy TBS, no *bla* insertion	6	7.7
1 copy *npt*II, 1 copy EXOB, 1 copy TBS with *bla* insertion	2	2.6
1 copy *npt*II, 1 copy of EXOB and/or TBS missing, no *bla* insertion [#]	6	7.7
1 copy *npt*II, > 1 copy of EXOB and/or TBS, no *bla* insertion [#]	4	5.1
1 copy *npt*II, > 1 copy of EXOB and/or TBS with *bla* insertion [#]	2	2.6
2 copies *npt*II, 2 copies EXOB, 2 copies TBS, no *bla* insertion	2	2.6
2 copies *npt*II, 2 copies EXOB, 2 copies TBS with *bla* insertion	1	1.3
2 copies *npt*II, 1 or 2 EXOB and/or TBS missing, no *bla* insertion [#]	5	6.4
2 copies *npt*II, 1 or 2 EXOB and/or TBS missing with *bla* insertion [#]	3	3.8
> 2 copies of *npt*II, EXOB and TBS, no *bla* insertion	6	7.7
> 2 copies with at least one copy missing of *npt*II, EXOB or TBS, no *bla* insertion [#]	27	34.6
> 2 copies with at least one copy missing of *npt*II, EXOB or TBS with *bla* insertion [#]	13	16.7
No *nptII*, 1 or more copies of EXOB and TBS, no *bla* insertion [#]	1	1.3
Total	78	

[#]Events (60/78) with evidence of truncation of the delivered vector

Agrobacterium tumefaciens-mediated transfer of pJKIe (76.9%).

The identification of single-copy pJKIS and pJKNI lines provided the possibility to compare transgene expression levels with the same copy number. Nevertheless the identification of single-copy pJKNI lines by Taqman® qPCR only targeted the *npt*II region. Therefore, further investigation if the full length cassette is present or not was warranted. PCR analysis using oligonucleotides supporting the amplification of the entire *npt*II cassette including the 35S promoter and terminator was performed. A 3217-bp amplification product should be detected if the full-length *npt*II expression cassette is present. The PCR analysis confirmed that 11 out of 22 single-copy pJKNI lines displaying the corresponding PCR product contain the full length *npt*II cassette (Fig. 3).

Comparison of NPTII protein levels between single-copy pJKIS (with insulators) and pJKNI (without insulators) lines

The identification of both single-copy pJKIS lines and pJKNI lines with full length *npt*II expression cassettes provided the candidate lines for comparing transgene expression levels. Quantitative NPTII ELISA analysis was conducted on 6 single-copy pJKIS lines and 11 single-copy pJKNI lines in two replicates. The single-copy pJKNI lines displayed an average NPTII level of 9.1 ng/10 µg soluble protein, ranging from 0.0 to 21.5 ng/10 µg soluble protein, while the single-copy pJKIS lines displayed an average NPTII level of 17.24 ng/10 µg soluble protein, ranging from 14.5 to 20.5 ng/10 µg soluble protein (Fig. 4). The line-to-line variation of NPTII protein levels was reduced in single-copy transgenic lines shielded with insulators (pJKIS) compared to the lines without insulators (pJKNI) (Fig. 4). The NPTII protein level of single-copy lines with insulators (pJKIS) was significantly higher ($P < 0.01$) than that of lines without insulators (pJKNI).

Comparison of NPTII expression levels in single-copy pJKIS and pJKNI lines between primary transgenic plants and their vegetative progeny

Quantitative NPTII ELISA analysis was conducted for comparing the NPTII protein levels between primary transgenic lines and their progeny. Six single-copy pJKIS and eight single-copy pJKNI primary transgenic and vegetative propagated lines were analyzed in two replicates (Table 5). The average NPTII protein level of pJKIS vegetative progeny

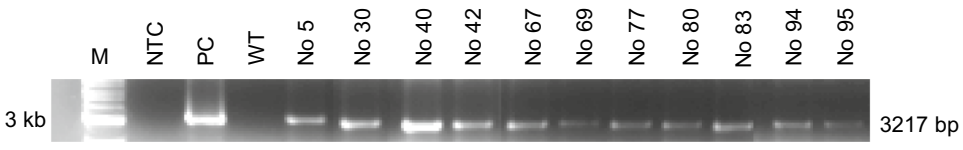


Fig. 3 PCR analysis of genomic DNA from single-copy pJKNI lines (lines without insulator) for presence of the full-length *npt*II expression cassette. M: 2-log DNA ladder; NTC: no template control; PC: pJKNI plasmid control; WT: wild-type control

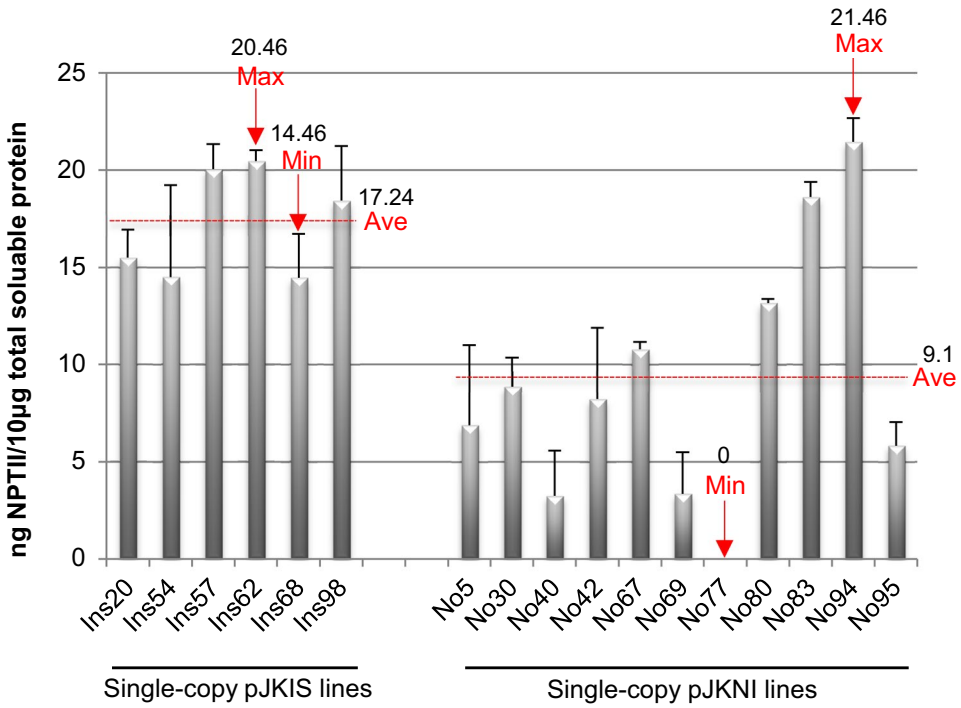


Fig. 4 NPTII expression levels for mature transgenic sugarcane leaves tested by NPTII ELISA. The mid sections of the first dewlap leaves from two mature tillers were sampled for replicated analysis. The standard error bars are shown. MAX: maximum NPTII expression level; MIN: minimum NPTII expression level; AVE: average NPTII expression level

(20.61 ng/10 µg soluble protein) were similar to the primary generation (17.24 ng/10 µg soluble protein). Among these propagated lines, one was significantly higher ($P < 0.05$) than their primary lines, while the other five lines didn't show significant difference ($P > 0.05$). The average NPTII protein level of pJKNI vegetative progeny (12.09 ng/10 µg soluble protein) did not differ significantly ($P > 0.05$) from the parental generation (10.8 ng/10 µg soluble protein). In addition, the line-to-line variation of the NPTII protein levels was consistently reduced in vegetatively propagated progenies of pJKIS lines in contrast to pJKNI lines.

Independent single-copy pJKIS lines were confirmed by Southern Blot

The Taqman® qPCR assays indicated that 7 out of 86 transgenic pJKIS lines were single copy events. Five pJKIS lines which displayed a growth vigor comparable to the non-transgenic sugarcane control were selected for Southern blot analysis with different probes for the different vector components (EXOB insulator, TBS insulator, *npt*II gene, and *FLP* gene). Independent events with a single copy of both EXOB and TBS insulator and a single copy insert of the *npt*II were verified from all five analyzed lines (Fig. 5a–c). Two selected pJKIS lines were further confirmed to carry one insert of the *FLP* gene (Fig. 5d). Southern blot with *FLPe* probe detected that four selected single-copy pJKIe lines have one copy of *FLPe* (FLPe31, FLPe59, FLPe83 and FLPe92) and another line showed two copies of *FLPe* (FLPe13) (Fig. 6). Wild-type plant was included to verify that an extra band was caused by off-target hybridization (Fig. 6).

Sequencing and propagation of elite single-copy pJKIS lines

The independent single-copy pJKIS sugarcane lines with stable and high level expression of *npt*II may be used for subsequent transgene stacking. Transgene stacking is facilitated in this highly expressed single copy locus as landing pad by the presence of the ubiquitin promoter upstream of the *lox76* site, supporting site-specific integration of a promoter-less selectable marker construct with additional transgenes into the *lox76* site. The presence of the promoter and insulator sequences flanking the *lox76* site in the transgenic locus was confirmed, with the region containing the ubiquitin promoter, intron, and *lox76* site as well as the upstream 1 kb TBS region sequenced following PCR amplification from genomic DNA of three transgenic lines. Alignments with the plasmid pJKIS revealed that pJKIS lines (Ins20, Ins54, and Ins62) contain the identical sequence (Supplementary A1). The presence of two *FRT* sites was also confirmed in Ins20 and Ins62 lines by sequencing (Supplementary A3). These independent single-copy pJKIS lines were selected to evaluate FLP/*FRT* mediated marker gene removal and as elite target lines for future transgene integration, propagated and maintained in the greenhouse (Fig. 2h).

Table 5 NPTII expression levels of first generation and propagated generation of single-copy pJKIS and pJKNI lines

Generations	NPTII expression levels (ng NPTII/10 µg soluble protein) (Mean ± SE)													
	pJKIS						pJKNI							
	Ins20	Ins54	Ins57	Ins62	Ins68	Ins98	No5	No30	No42	No69	No80	No83	No94	No95
Primary lines	15.51 ± 1.42	14.50 ± 4.72	20.05 ± 1.28	20.46 ± 0.55	14.46 ± 2.26	18.44 ± 2.78	6.88 ± 4.12	8.85 ± 1.49	8.23 ± 3.64	3.36 ± 2.11	13.16 ± 0.22	18.62 ± 0.75	21.46 ± 1.21	5.83 ± 1.21
Vegetative progeny	22.56 ± 1.05	17.98 ± 1.42	22.97 ± 0.47	21.96 ± 0.86	20.11 ± 1.00	18.09 ± 1.36	19.92 ± 1.03	14.43 ± 6.21	13.89 ± 2.74	4.91 ± 4.91	10.52 ± 3.91	0 ± 0	12.04 ± 2.15	21.03 ± 6.04
P value	0.03[#]	0.3	0.12	0.15	0.1	0.46	0..18	0.53	0.34	0.81	0.62	0.026[#]	0.089	0.23

Ins20, Ins54, Ins57, Ins62, Ins68, and Ins98: single-copy pJKIS lines. No5, No30, No42, No69, No80, No83, No94, and No95: single-copy pJKNI lines. Primary lines: first generation transgenic target lines. Vegetative progeny: propagated lines of first generation target lines

[#]Primary lines significantly different from vegetative progeny in NPTII expression levels

FLP and *FLPe* relative expression levels in single-copy lines

The *FLP* and *FLPe* relative expression levels in single-copy heat-treated and untreated plants were investigated with Quantitative real-time RT-PCR. The expression levels of *FLP* and *FLPe* of the treated plants, immediately tested after heat treatment, were significantly increased compared to the untreated groups ($P < 0.01$). Similar expression levels were observed in different FLP and FLPe lines with a trend to higher expression levels on average of all FLP lines. The expression levels gradually decreased after the heat treatment measured at different time points (4 h and 8 h after) (Supplementary A5a, A5b). The untreated plants didn't show significant variation ($P > 0.05$) of gene expression among the four time points (before heat treatment, 0 h after heat treatment, 4 h after heat treatment and 8 h after heat treatment) (Supplementary A5a, A5b). There was no significant difference between the expression levels of treated and untreated plants before the heat treatment ($P > 0.05$) for *FLP* and *FLPe*.

Removal of *npt*II selectable marker gene

Two single-copy pJKIS lines containing the *FLP* gene and one single-copy pJKIe line containing the *FLPe* gene were compared for removing the *npt*II expression cassette flanked by *FRT* sites. Plantlets in petri dishes were subjected to heat treatment for induction of the *FLP* and *FLPe* expression. A 5331-bp amplification product was obtained from the heat-treated and untreated single-copy pJKIS lines (Ins20 and Ins62, Fig. 7a, b, c). This indicates that the FLP recombinase-mediated recombination did not occur. The untreated single-copy pJKIe line FLPe59 displayed a 5197-bp PCR product (Fig. 7d), while the heat-treated FLPe59 line displayed a 527-bp PCR product, indicating the successful excision between the two *FRT* sites (Figs. 1d, 7e). The same result was obtained using root tissue for genomic DNA extraction and PCR analysis (Fig. 7f). Interestingly, another single-copy FLPe31 line which was heat-treated after establishment in soil showed both 5179-bp and 527-bp PCR products. This indicates that the cassette between two *FRT* sites was not completely excised in all cells of the plants (Fig. 7d). The FLPe13 line, which has been confirmed to contain two *npt*II cassettes, was selected to test the removal of *npt*II from its genome. Only the 527-bp amplification product could be detected after heat treatment, which most likely indicates the complete excision of two-copies of the selectable marker gene (Fig. 7g). The 527-bp amplicons from the single-copy and two-copy pJKIe lines were sequenced and analyzed. The sequencing results indicate that the cassette between the two *FRT* sites in single-copy as well as two-copy pJKIe lines

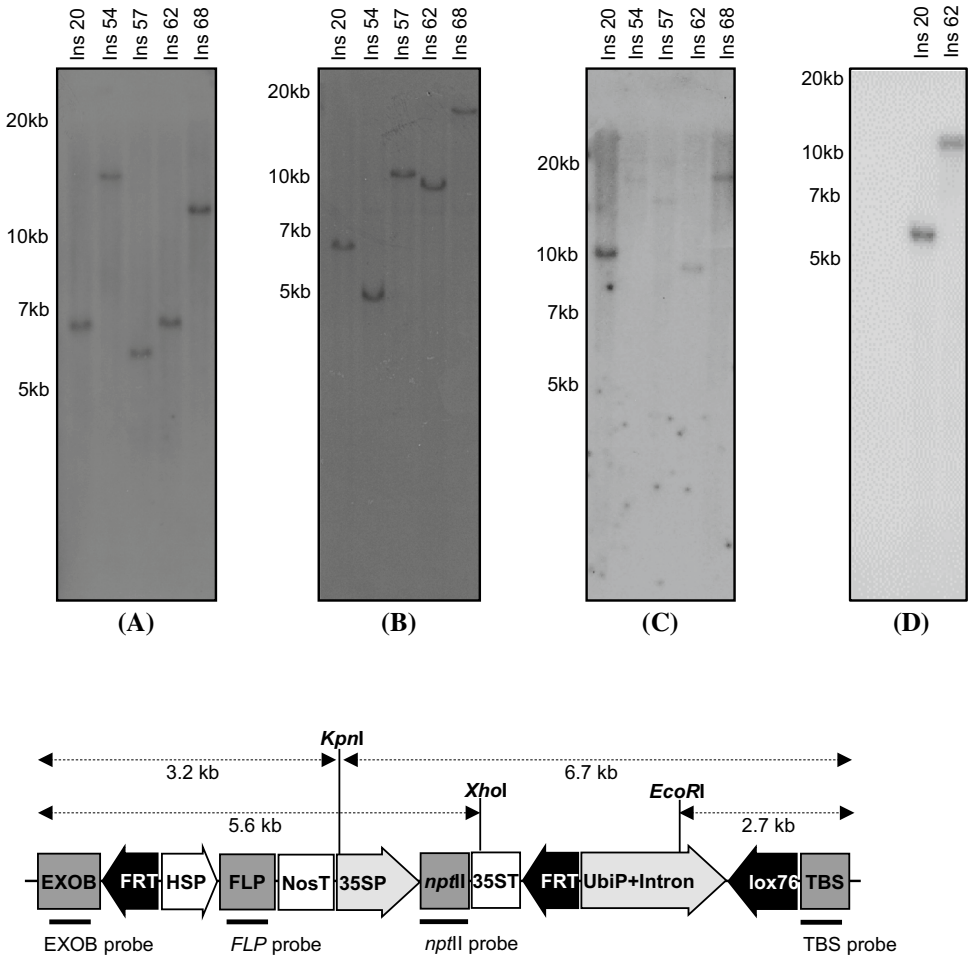


Fig. 5 Southern blot of selected pJKIS lines using different probes. **a** Southern blot with probe targeting EXOB (genomic DNA digested with *Xho*I). **b** Southern blot with probe targeting TBS (genomic DNA digested with *Eco*RI). **c** Southern blot with probe targeting *npt*II (genomic DNA digested with *Kpn*I). **d** Southern blot with probe targeting *FLP* (genomic DNA digested with *Kpn*I)

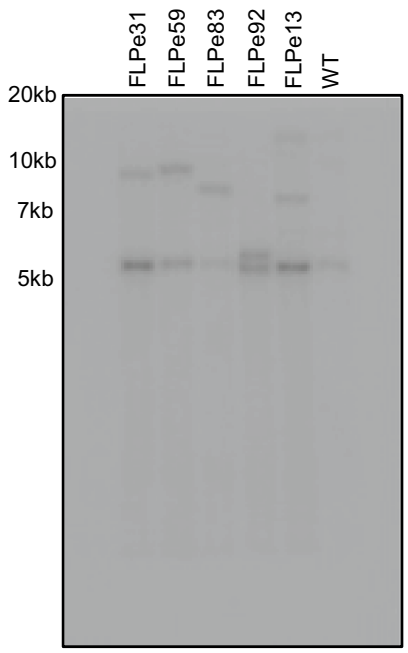
was successfully excised in the transformed sugarcane lines (Supplementary A2).

TAIL-PCR analysis to identify the genomic sequence flanking the single copy lines with insulator

One cloned PCR amplicon from line FLPe83 which included both vector and 1763nt of flanking genomic sequences was confirmed by Sanger sequencing (Supplementary A5) from a total of 130 colonies from 13 PCR amplicons from 4 different transgenic lines (Ins20, Ins62, FLPe83, FLPe92) following TAIL-PCR. After trimming off the vector sequence component the 1763nt genomic sequence flanking the single copy transgene was blasted against the monoploid sugarcane genome reference sequence, which generated 143 potential gene hits. The longest region of alignment of the 1763nt flanking sequence with these potential 143 gene hits was 335nt. Evaluation of the hits with the highest bit scores revealed alignment to an intron sequence which is similar across many genes and may also occur frequently in non-genic regions or pseudogenes. The aligned sequence was mapped to a distance of 143 or more nucleotides from the intron–exon boundaries and at a distance of more than 100 nt from the branch point.

Discussion

Prediction of transgene performance is complicated by the random insertion of transgenes into the plant genome and interaction among transgene copies with each other or the neighboring chromatin (Assaad et al. 1993; Ingelbrecht et al. 1994, 1999; Meyer and Saedler 1996; Angell and Baulcombe 1997; Cerutti et al. 1997; Kanno et al. 2000). To enhance the predictability of transgene performance we constructed and characterized a safe harbor transgene locus in sugarcane that is expected to support transgene stacking. The transgene expression cassettes and the *lox76* recombination site for targeted integration of additional transgenes were flanked with insulators for improving the stability of transgene expression. Removal of the selectable marker gene by using the FLPe/*FRT* site-specific recombination system was also demonstrated.



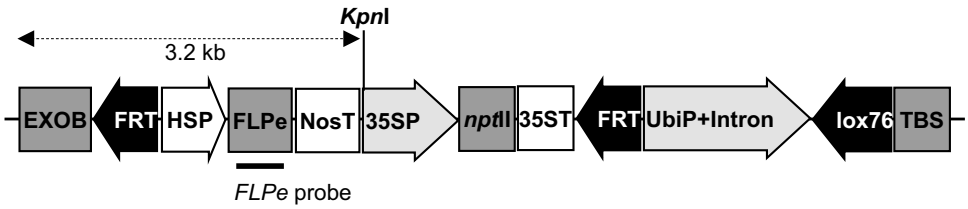


Fig. 6 Southern blot of selected pJKIe lines with probe targeting *FLPe* (genomic DNA digested with *Kpn*I), WT: wild-type control

Transgene stacking refers to the co-integration of multiple transgenes and supports metabolic engineering of crops (Karunanandaa et al. 2005; Naqvi et al. 2009; Zhu et al. 2008; Zale et al. 2016). Stacking of genes with different modes of action introduces durable insect or herbicide resistance as the resistance conferred by a single transgene can break down more rapidly (Moellenbeck et al. 2001; Gatehouse 2008; Storer et al. 2012; Jiang et al. 2016; Heap 2018). Further, stacking of multiple transgenic crop traits is accelerating crop improvement (Halpin 2005; Agapito-Tenfen et al. 2014). Targeted integration of transgenes into a safe harbor locus requires site-specific recombination or genome editing approaches (Albert et al. 1995; Srivastava and Ow 2001; Nandy and Srivastava 2011; Forsyth et al. 2016; Srivastava and Thomson 2016; Cermak et al. 2015). Recently, TALEN or CRISPR/Cas9-mediated targeted mutagenesis and precision nucleotide substitutions have been accomplished in sugarcane (Jung and Altpeter 2016; Kannan et al. 2018; Oz et al. 2019). A report demonstrating a site-specific recombination system in sugarcane is still missing. Targeted transgene integration technologies will not only facilitate gene stacking but should also streamline regulatory approval of transgenic events and enhance transgene performance. The genomic region adjacent to the transgene insertion site influences expression level and stability (Dobie et al. 1996; Iglesias et al. 1997; Ng and Bird 1999; Clark et al. 1994; Bestor 2000; Fiering et al. 2000). Random transgene integration can also lead to insertional mutagenesis of genic regions (Jeon et al. 2000) but is more of concern in diploid species than in the highly polyploid sugarcane genome, where multiple alleles may provide functional redundancy. Transgene performance is also influenced by the transgene copy number (Jorgensen et al. 1987, 1996; Hobbs et al. 1993; Depicher and Montagu 1997; Stam et al. 1997; Muskens et al. 2000; Wang and Waterhouse 2000; Kohli et al. 2003; Akbudak et al., 2010). This can be addressed by selecting events with single-copy insertion of transgenes (De Buck et al. 2007; Chawla et al. 2006; Srivastava and Gidoni 2010). In this study, we observed a similar frequency of single-copy and full-length integration events without backbone integration using biolistic (8.1%; 7 out of 86 events) or *Agrobacterium*-mediated transformation (7.7%; 6 out of 78). Simple transgene integration patterns following biolistic gene transfer are supported by the reduced amount of minimal expression cassette used during DNA coating of particles used for biolistic gene transfer as

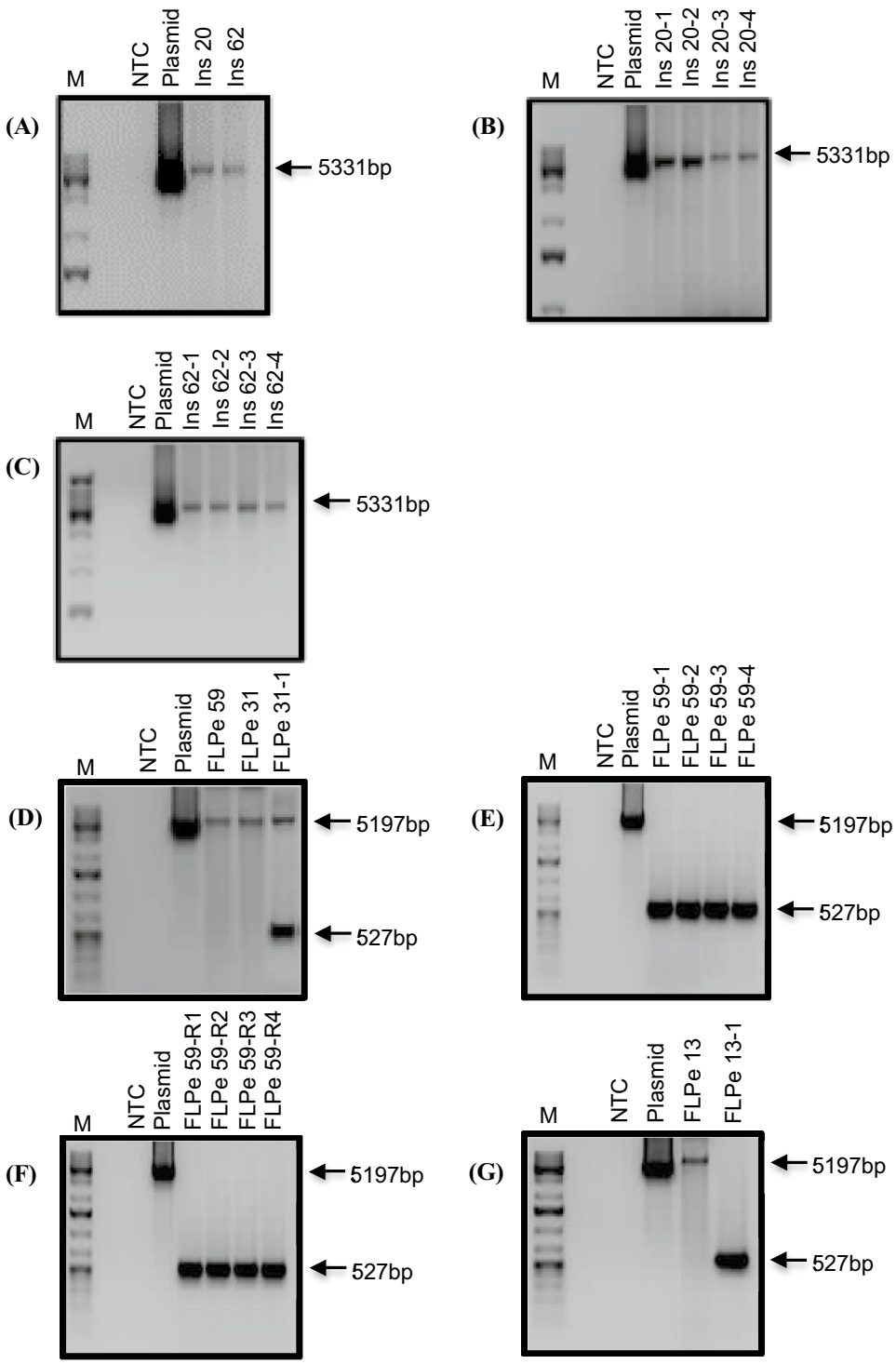


Fig. 7 PCR analysis of pJKIS (target lines with *FLP* recombinase gene) and pJKIe (target lines with *FLPe* recombinase gene) using genomic DNA extracted before and after heat treatment. **a** Single-copy pJKIS lines Ins20, Ins62 before heat treatment (leaf tissue). **b** Single-copy pJKIS line Ins20 after heat treatment (leaf tissue). 1, 2, 3, 4 represent the four replicates. **c** Single-copy pJKIS line Ins62 after heat treatment (leaf tissue). 1, 2, 3, 4 represent the four replicates. **d** Single-copy pJKIe line FLPe59 before heat treatment (leaf tissue) and single-copy pJKIe line FLPe31 in soil before (FLPe31) and after (FLPe31-1) heat treatment (leaf tissue). **e** Single-copy pJKIe line FLPe59 after heat treatment (leaf tissue). 1, 2, 3, 4 represent the four replicates. **f** Single-copy pJKIe line FLPe59 after heat treatment (root material). 1, 2, 3, 4 represent the four replicates. **g** Two-copy pJKIe line FLPe13 before (FLPe13) and after (FLPe13-1) heat treatment (leaf tissue). M: 1 kb plus DNA ladder; NTC: no template control

reported earlier for sugarcane (Jackson et al. 2013; Wu et al. 2015b) and other crops (Sandhu and Altpeter 2008; Lowe et al. 2009).

Agrobacterium-mediated transformation frequently causes undesired vector backbone integration (De Buck et al. 2000). We detected backbone integration in 26.9% of the sugarcane events that were generated with *Agrobacterium*. However, despite the excision of expression cassettes by gel electrophoresis, backbone integration was also detected for 14% of the biolistic gene transfer events. This surprising finding may be caused by the contamination with lagging backbone DNA fragments during gel electrophoresis. To overcome this problem, minicircle

technology can be applied as an alternative to plasmid derived minimal expression cassettes (Heinz et al. 2012).

Some of the earlier reports suggested that biolistic gene transfer results, in contrast to *Agrobacterium* mediated transformation, in frequent truncation of transgenes associated with gene silencing (Dai et al. 2001; Travella et al. 2005; Kohli et al. 1999). In our study, the frequency of truncation of the constructs following biolistic transfer (88.4%) was only slightly higher compared to *Agrobacterium tumefaciens*-mediated transfer (76.9%). Following *Agrobacterium*-mediated gene transfer in wheat, maize or barley, 44–97% of the events displayed T-DNA truncations (Cheng et al. 1997; Shou et al. 2004; Bartlett et al. 2008). Most of the truncations in *Agrobacterium*-mediated transformation occur at the left T-DNA border (Wu et al. 2006) due to the lack of protection of the left border by covalently attached VirD2 (Tinland et al. 1995; Windels et al. 2008). Buffer sequences, for example insulators sequences, may be used to protect the expression cassette from truncation and may also reduce the effect of the transgene integration site on gene expression. In this study, TAIL-PCR identified a short 48 bp truncation, including the left T-DNA border of line FLPe83. However, this short truncation left both the expression cassette and the flanking insulator sequence fully intact. An alignment of the genomic sequence flanking the transgene with the monoploid sugarcane genome reference sequence (Garsmeur et al. 2018) suggested the absence of insertional mutagenesis. Grosveld et al. (1987) utilized the strong locus control regions (LCRs) in their construct for stable transgene expression in transgenic mice (Grosveld et al. 1987). Peterson et al. (1993) and Porcu et al. (1997) attempted to reduce position effects by making large constructs including all the required elements to form a native epigenetic structure. Nevertheless, those attempts turned out less effective than expected. Insulators have been reported to eliminate gene silencing and position effects in mammalian cells (Chung et al. 1993). A few effective insulators have been characterized in plants (Hily et al. 2009; Singer et al. 2010, 2011, 2012; Yang et al. 2011; Zhang et al. 2012). A few insulators, including EXOB of bacteriophage λ and TBS, the transformation booster sequence from *Petunia hybrida* demonstrated enhancer blocking activity in plants, increased the transgene expression level and reduced the line-to-line variation in transgene expression (Singer et al. 2011, 2012). Here, the combination of EXOB and TBS were evaluated for their ability to improve transgene expression stability in sugarcane. The *npt*II expression cassette flanked with EXOB and TBS were compared to an identical construct missing the insulators. Similar to earlier reports (Singer et al. 2011, 2012) the insulator construct resulted in significantly reduced line-to-line variation of transgene expression in sugarcane when compared to lines without the insulators. In addition, a significant difference in the expression level between the lines with and without insulators was detected. The average transgene expression level of insulator-containing lines was nearly two times higher compared to the lines without insulators. This result was in accordance with that of Singer et al. (2012), and was consistent between the primary transgenic events and their vegetative progenies. Furthermore, only lines with a single-copy of the full length *npt*II expression cassette were compared to eliminate the influence of copy number or truncations. For future use, these lines with insulators could be used for site-directed stacking of transgenes into the *lox76* site via Cre-mediated site-specific recombination or CRISPR/Cas9 mediated homologous recombination.

Different types of site-specific recombinase proteins have been compared in rice (Akbudak and Srivastava 2011). In this study, we compared the efficiency of two different FLP variants, wild-type FLP and thermo-stable FLPe, to excise a selectable marker gene *npt*II between two *FRT* sites. We demonstrated that FLPe supported removal of the selectable marker gene in single and two-copy transgenic sugarcane lines in both leaf and root tissue of *in vitro* grown sugarcane plantlets. In contrast, wild-type FLP/*FRT*-mediated excision was not detectable in sugarcane lines. This is consistent with previous reports on rice, which found that FLPe is superior to wild-type FLP in both extra-chromosomal transient expression and consistent stable expression from the genomic locus by heat-shock or constitutive promoters in excising *FRT*-flanked transgene from the rice genome (Akbudak and Srivastava 2011; Nguyen et al. 2014). Although, inefficiency of FLPe in germline was noted, FLPe activity in somatic tissues was found to be robust in rice (Nguyen et al. 2014). Our study compared FLP and FLPe under heat-inducible promoter in the clonal segments of an asexually propagating plant species. As heat-induced expression levels were not significantly different in different lines, superior recombination efficiency of FLPe is likely resulting from its thermostability in the cells (Buchholz et al. 1998). Reports that describe the successful application of wild-type FLP for removal of a selectable marker have also been published, *e.g.*, in creeping bentgrass (Hu et al. 2006), rice (Hu et al. 2008), and dicots, such as tobacco (Davies et al. 1999; Gidoni et al. 2001; Woo et al. 2009), *Arabidopsis* (Kumar and Thompson 2009) and aspen (Fladung and Becker 2010).

In conclusion, we generated and characterized a landing pad for transgene stacking in sugarcane that consists of a highly and stably expressed single-copy transgene flanked by insulators. We also demonstrated marker gene removal using the FLPe/*FRT* site-specific recombination system, which will facilitate re-transformation.

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Author contributions F.A. and V.S. conceived and designed the experiments; Y.Z., J.Y.K. and J.H.J. constructed the plasmids; Y.Z., B.P. and B.W. generated the transgenic plants; Y.Z., B.K. and D.W. analyzed the transgenic plants; C.F., W.Y. and S.D. performed the TaqMan® qPCR assay; R.K. and Y.Z. carried out Southern Blot hybridization; Y.Z. and F.A. wrote the manuscript. All authors read and approved the final manuscript.

References

Agapito-Tenfen SZ, Vilperte V, Benevenuto RF, Rover CM, Traavik TI, Nodari RO (2014) Effect of stacking insecticidal cry and herbicide tolerance epsps transgenes on transgenic maize proteome. BMC Plant Biol 14:346

Akbudak MA, Srivastava V (2011) Improved FLP recombinase, FLPe, efficiently removes marker gene from transgene locus developed by Cre-*lox* mediated site-specific gene integration in rice. Mol Biotechnol 49:82–89

Albert H, Dale EC, Lee E, Ow DW (1995) Site-specific integration of DNA into wild-type and mutant *lox* sites placed in the plant genome. Plant J 7:649–659

Altpeter F, Oraby H (2010) Sugarcane. In: Kempken F, Jung C (eds) Genetic modification of plants. Springer, Berlin, pp 453–472

Altpeter F, Sandhu S, Davey MR, Anthony P (2010) Genetic transformation—biolistics. In: Davey MR, Anthony P (eds) Plant cell culture. Wiley, Chichester, pp 217–239

Altpeter F, Springer NM, Bartley LE, Blechl A, Brutnell TP, Citovsky V, Conrad L et al (2016) Advancing crop transformation in the era of genome editing. Plant Cell 28:1510–1520

Angell SM, Baulcombe DC (1997) Consistent gene silencing in transgenic plants expressing a replicating potato virus × RNA. EMBO J 16:3675–3684

Assaad FF, Tucker KL, Signer ER (1993) Epigenetic repeat-induced gene silencing (RIGS) in *Arabidopsis*. Plant Mol Biol 22:067–1085

Bartlett JG, Alves SC, Smedley M, Snape JW, Harwood WA (2008) High-throughput *Agrobacterium*-mediated barley transformation. Plant Methods 4:22

Bestor TH (2000) Gene silencing as a threat to the success of gene therapy. J Clin Investig 105:409–411

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Buchholz F, Angrand PO, Stewart AF (1998) Improved properties of FLP recombinase evolved by cycling mutagenesis. Nat Biotechnol 16:657–662

Cermak T, Baltes NJ, Cegan R, Zhang Y, Voytas DF (2015) High-frequency, precise modification of the tomato genome. Genome Biol 16:232

Cerutti H, Johnson AM, Gillham NW, Boynton JE (1997) Epigenetic silencing of a foreign gene in nuclear transformants of *Chlamydomonas*. Plant Cell 9:925–945

Chawla R, Ariza-Nieto M, Wilson AJ, Moore SK, Srivastava V (2006) Transgene expression produced by biolistic-mediated, site-specific gene integration is consistently inherited by the subsequent generations. Plant Biotechnol J 4:209–218

Cheng M, Frey JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997) Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol 115:971–980

Chengalrayan K, Gallo-Meagher M (2001) Effect of various growth regulators on shoot regeneration of sugarcane. In Vitro Cell Dev Biol Plant 37:434–439

Chung JH, Whiteley M, Felsenfeld G (1993) A 5′ element of the chicken beta-globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. Cell 74:505–514

Clark AJ, Bissinger P, Bullock DW, Damak S, Wallace R, Whitelaw CB, Yull F (1994) Chromosomal position effects and the modulation of transgene expression. Reprod Fertil Dev 6:589–598

Cordeiro GM, Amouyal O, Eloitt F, Henry RJ (2007) Sugarcane. In: Kole C (ed) Genome mapping and molecular breeding in plants, vol 3. Pulses, sugar and tuber crops. Springer, Berlin, pp 175–204

Dai S, Zheng P, Marmey P, Zhang S, Tian W, Chen S, Beachy RN, Fauquet C (2001) Comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardment. Mol Breed 7:25–33

Davies GJ, Kilby NJ, Riou-Khamlichi C, Murray JAH (1999) Somatic and germinal inheritance of an FLP-mediated deletion in transgenic tobacco. J Exp Bot 50:1447–1456

De Buck S, De Wilde C, Van Montagu M, Depicker A (2000) T-DNA vector backbone sequences are frequently integrated into the genome of transgenic plants obtained by Agrobacterium-mediated transformation. Mol Breed 6:459–468

De Buck S, Peck I, De Wilde C, Marjanac G, Nolf J, De Paepe A, Depicker A (2007) Generation of single-copy T-DNA transformants in *Arabidopsis* by the CRE/*loxP* recombination-mediated resolution system. Plant Physiol 145:1171–1182

De Paepe A, De Buck S, Nolf J, Van Lerberge E, Depicker A (2013) Site-specific T–DNA integration in *Arabidopsis thaliana* mediated by the combined action of CRE recombinase and ϕC31 integrase. Plant J 75:172–184

Depicher A, Montagu MV (1997) Post-transcriptional gene silencing in plants. Curr Opin Cell Biol 9:373–382

Dobie KW, Lee M, Fantes JA, Graham E, Clark AJ, Springbett A, Lathe R, McClenaghan M (1996) Variegated transgene expression in mouse mammary gland is determined by the transgene integration locus. Proc Natl Acad Sci USA 93:6659–6664

Fiering S, Whitelaw E, Martin DI (2000) To be or not to be active: the stochastic nature of enhancer action. Bioessays 22:381–387

Fladung M, Becker D (2010) Targeted integration and removal of transgenes in hybrid aspen (*Populus tremula* L. X *P. tremuloides* Michx.) using site-specific recombination systems. Plant Biol 12:334–340

Forsyth A, Weeks T, Richael C, Duan H (2016) Transcription Activator-Like Effector Nucleases (TALEN)-mediated targeted DNA insertion in potato plants. Front Plant Sci 7:1572. eCollection

Garsmeur O, Droc G, Antonise R, Grimwood J, Potier B, Aitken K, Jenkins J, Martin G, Charron C, Hervouet C, Costet L (2018) A mosaic monoploid reference sequence for the highly complex genome of sugarcane. Nat Commun 9:2638

Gatehouse JA (2008) Biotechnological prospects for engineering insect-resistant plants. Plant Physiol 146:881–887

Gidoni D, Bar M, Gilboa N (2001) FLP/*FRT*-mediated restoration of normal phenotypes and clonal sectors formation in *rol*C transgenic tobacco. Transgenic Res 10:317–328

Gidoni D, Srivastava V, Carmi N (2008) Site-specific excisional recombination strategies for elimination of undesirable transgenes from crop plants. In Vitro Cell Dev Biol Plant 44:457–467

Giraldo P, Rival-Gervier S, Houdebine LM, Montoliu L (2003) The potential benefits of insulators on heterologous constructs in transgenic animals. Transgenic Res 12:751–755

Grønlund JT, Stemmer C, Lichota J, Merkle T, Grasser KD (2007) Functionality of the beta/*six* site-specific recombination system in tobacco and *Arabidopsis*: a novel tool for genetic engineering of plant genomes. Plant Mol Biol 63:545–556

Grosveld F, van Assendelft GB, Greaves DR, Kollias G (1987) Position-independent, high-level expression of the human beta-globin gene in transgenic mice. Cell 51:975–985

Halpin C (2005) Gene stacking in transgenic plants—the challenge for 21st century plant biotechnology. Plant Biotechnol J 3:141–155

Heap I (2018) International survey of herbicide resistant weeds (Online). http://www.weedscience.org/Summary/SOASummary.aspx

Heinz N, Broll S, Schleef M, Baum C, Bode J (2012) Filling a gap: S/MAR-based replicating minicircles. CliniBook— Nonviral Platform; Clinigene Network,pp 271–277

Henry R, Cole C (2010) Genetics, genomics and breeding of sugarcane. CRC Press, Boca Raton

Hily JM, Singer SD, Yang Y, Liu Z (2009) A transformation booster sequence (TBS) from *Petunia hybrid* functions as an enhancer-blocking insulator in *Arabidopsis thaliana*. Plant Cell Rep 28:1095–1104

Hobbs SL, Warketin TD, Delong CM (1993) Transgene copy number can be positively or negatively associated with transgene expression. Plant Mol Biol 21:17–26

Hou L, Yau YY, Wei J, Han Z, Dong Z, Ow DW (2014) An open-source system for in planta gene stacking by Bxb1 and Cre recombinases. Mol Plant 7:1756–1765

Hu Q, Nelson K, Luo H (2006) FLP-mediated site-specific recombination for genome modification in turfgrass. Biotechnol Lett 28:1793–1804

Hu Q, Kononowicz-Hodges H, Nelson-Vasilchik K, Viola D, Zeng P, Liu H, Kausch AP, Chandlee JM, Hodges TK, Luo H (2008) FLP recombinase-mediated site-specific recombination in rice. Plant Biotechnol J 6:176–188

Iglesias VA, Moscone EA, Papp I, Neuhuber F, Michalowski S, Phelan T, Spiker S, Matzke M, Matzke AJ (1997) Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. Plant Cell 9:1251–1264

Ingelbrecht IL, Van HH, Van MM, Depicker A (1994) Post-transcriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proc Natl Acad Sci USA 91:10502–10506

Ingelbrecht IL, Irvine JE, Mirkov E (1999) Posttranscriptional gene silencing in transgenic sugarcane. Dissection of homology-dependent virus resistance in a monocot that has a complex polyploidy genome. Plant Physiol 119:1187–1197

Ingham DJ, Beer S, Money S, Hansen G (2001) Quantitative real-time PCR assay for determining transgene copy number in transformed plants. Biotechniques 31:132–141

Jackson MA, Anderson DJ, Birch RG (2013) Comparison of Agrobacterium and particle bombardment using whole plasmid or minimal cassette for production of high-expressing, low-copy transgenic plants. Transgenic Res 22:143–151

Jeon JS, Lee S, Jung KH, Jun SH, Jeong DH, Lee J, Kim C, Jang S, Yang K, Nam J, An K, Han MJ, Sung RJ, Choi HS, Yu JH, Choi JH, Cho SY, Cha SS, Kim SI, An G (2000) T-DNA insertional mutagenesis for functional genomics in rice. Plant J 22:561–570

Jiang F, Zhang T, Bai S, Wang Z, He K (2016) Evaluation of Bt corn with pyramided genes on efficacy and insect resistance management for the Asian corn borer in China. PLoS ONE 11:e0168442

Jorgensen R, Snyder C, Jones JDG (1987) T-DNA is organized predominantly in inverted repeat structures in plants transformed with *Agrobacterium tumefaciens* C58 derivatives. Mol Gen Genet 207:471–477

Jorgensen RA, Cluster PD, English J, Que Q, Napoli CA (1996) Chalcone synthase co suppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. Plant Mol Biol 31:957–973

Jung JH, Altpeter F (2016) TALEN mediated targeted mutagenesis of the caffeic acid O-methyltransferase in highly polyploid sugarcane improves cell wall composition for production of bioethanol. Plant Mol Biol 92:131–142

Kannan B, Jung JH, Moxley GW, Lee SM, Altpeter F (2018) TALEN-mediated targeted mutagenesis of more than 100 COMT copies/alleles in highly polyploid sugarcane improves saccharification efficiency without compromising biomass yield. Plant Biotechnol J 16:856–866

Kanno T, Naito S, Shimamoto G (2000) Post-transcriptional gene silencing in cultured rice cells. Plant Cell Physiol 41:321–326

Karunanandaa B, Qi Q, Hao M, Baszis SR, Jensen PK, Wong YH, Jiang J, Venkatramesh M, Gruys KJ, Moshiri F, Post-Beittenmiller D, Weiss JD, Valentin HE (2005) Metabolically engineered oilseed crops with enhanced seed tocopherol. Metab Eng 7:384–400

Kohli A, Gahakwa D, Vain P, Laurie DA, Christou P (1999) Transgene expression in rice engineered through particle bombardment: Molecular factors controlling stable expression and transgene silencing. Planta 208:88–97

Kohli A, Twyman RM, Abranches R, Wegel E, Stoger E, Christou P (2003) Transgene integration, organization and interaction in plants. Plant Mol Biol 52:247–258

Kumar S, Thompson WF (2009) Simultaneous excision of two transgene flanking sequences and resolution of complex integration loci. Plant Mol Biol 69:23–32

Li Z, Xing A, Moon BP, McCardell RP, Mills K, Falco SC (2009) Site-specific integration of transgenes in soybean via recombinase-mediated DNA cassette exchange. Plant Physiol 151:1087–1095

Li R, Han Z, Hou L, Kaur G, Qian Y, Ow DW (2016) Method for biolistic site-specific integration in plants catalyzed by Bxb1 integrase. Methods Mol Biol 1469:15–30

Lowe BA, Prakash NS, Way M, Mann MT, Spencer TM, Boddupalli RS (2009) Enhanced single copy integration events in corn via particle bombardment using low quantities of DNA. Transgenic Res 18:831–840

Lutz KA, Corneille S, Azhagiri AK, Svab Z, Maliga P (2004) A novel approach to plastid transformation utilizes the phiC31 phage integrase. Plant J 37:906–913

Lyznik L, Gordon-Kamm W, Tao Y (2003) Site-specific recombination for genetic engineering in plants. Plant cell Rep 21:925–932

Meyer P, Saedler H (1996) Homology-dependent gene silencing in plants. Annu Rev Plant Physiol Plant Mol Biol 47:23–48

Moellenbeck DJ, Peters ML, Bing JW, Rouse JR, Higgins LS, Sims L, Nevshemal T, Marshall L, Ellis RT, Bystrak PG, Lang BA, Stewart JL, Kouba K, Sondag V, Gustafson V, Nour K, Xu D, Swenson J, Zhang J, Czapla T, Schwab G, Jayne S, Stockhoff BA, Narva K, Schnepf HE, Stelman SJ, Poutre C, Koziel M, Duck N (2001) Insecticidal proteins from *Bacillus thuringiensis* protect corn from corn rootworms. Nat Biotechnol 19:668–672

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4326

Muskens MW, Vissers AP, Mol JN, Kooter JM (2000) Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing. Plant Mol Biol 43:243–260

Nandy S, Srivastava V (2011) Site-specific gene integration in rice genome mediated by the FLP-*FRT* recombination system. Plant Biotechnol J 9:713–721

Naqvi S, Farre G, Sanahuja G, Capell T, Zhu C, Christou P (2009) When more is better: multigene engineering in plants. Trends Plant Sci 15:48–56

Ng HH, Bird AP (1999) DNA Methylation and chromatin modification. Curr Opin Genet Dev 9:158–163

Nguyen LD, Underwood JL, Nandy S, Akbudak MA, Srivastava V (2014) Strong activity of FLPe recombinase in rice plants does not correlate with the transmission of the recombined locus to the progeny. Plant Biotechnol Rep 8:455–462

Oz T, Karan R, Merotto A, Altpeter F (2019) CRISPR/Cas9 and template-mediated multi-allelic homologous recombination of the acetolactate synthase gene in sugarcane confers herbicide resistance (submitted for publication)

Peterson KR, Clegg CH, Huxley C, Josephson BM, Haugen HS, Furukawa T (1993) Transgenic mice containing a 248-kb yeast artificial chromosome carrying the human beta-globin locus display proper developmental control of human globin genes. Proc Natl Acad Sci USA 90:7593–7597

Porcu S, Kitamura M, Witkowska E, Zhang Z, Mutero A, Lin C, Chang J, Gaensler KML (1997) The human beta globin locus introduced by YAC transfer exhibits a specific and reproducible pattern of developmental regulation in transgenic mice. Blood 90:4602–4609

Sandhu S, Altpeter F (2008) Co-integration, co-expression and inheritance of unlinked minimal transgene expression cassettes in an apomictic turf and forage grass (*Paspalum notatum* Flugge). Plant Cell Rep 27:1755–1765

Shou H, Frame BR, Whitham SA, Wang K (2004) Assessment of transgenic maize events produced by particle bombardment or *Agrobacterium*-mediated transformation. Mol Breed 13:201–208

Singer T, Burke E (2003) High-throughput TAIL-PCR as a tool to identify DNA flanking insertions. Methods Mol Biol 236:241–272

Singer SD, Hily JM, Liu Z (2010) A 1-kb bacteriophage lamda fragment functions as an insulator to effectively block enhancer-promoter interactions in *Arabidopsis thaliana*. Plant Mol Biol Rep 28:69–76

Singer SD, Cox KD, Liu Z (2011) Enhancer-promoter interference and its prevention in transgenic plants. Plant Cell Rep 30:723–731

Singer SD, Liu Z, Cox KD (2012) Minimizing the unpredictability of transgene expression in plants: the role of genetic insulators. Plant Cell Rep 31:13–25

Srivastava V, Gidoni D (2010) Site-specific gene integration technologies for crop improvement. In Vitro Cell Dev Biol Plant 46(3):219–232

Srivastava V, Ow DW (2001) Biolistic mediated site-specific integration in rice. Mol Breed 8:345–350

Srivastava V, Thomson J (2016) Gene stacking by recombinases. Plant Biotechnol J 14:471–482

Srivastava V, Ariza-Nieto M, Wilson AJ (2004) Cre-mediated site-specific gene integration for consistent transgene expression in rice. Plant Biotechnol J 2:169–179

Stam M, De Bruin R, Kenter S, Van Der Hoorn RAL, Van Blokland R, Mol JNM, Kooter JM (1997) Post-transcriptional silencing of chalcone synthase in *Petunia* by inverted transgene repeats. Plant J 1:63–82

Storer NP, Thompson GD, Head GP (2012) Application of pyramided traits against Lepidoptera in insect resistance management for Bt crops. GM Crops Food 3:154–162

Taparia Y, Fouad W, Gallo M, Altpeter F (2012a) Rapid production of transgenic sugarcane with the introduction of simple loci following biolistic transfer of a minimal expression cassette and direct embryogenesis. In Vitro Cell Dev Biol Plant 48:15–22

Taparia Y, Gallo M, Altpeter F (2012b) Comparison of direct and indirect embryogenesis protocols, biolistic gene transfer and selection parameters for efficient genetic transformation of sugarcane. Plant Cell Tissue Organ Cult 111:131–141

Tew TL, Cobill RM (2008) Genetic improvement of sugarcane (*Saccharum* spp.) as an energy crop. In: Vermerris W (ed) Genetic improvement of bioenergy crops. Springer, New York, pp 273–294

Thomson JG, Yau YY, Blanvillain R, Chiniquy D, Thilmony R, Ow DW (2009) ParA resolvase catalyzes site-specific excision of DNA from the *Arabidopsis* genome. Transgenic Res 18:237–248

Thomson JG, Chan R, Thilmony R, Yau YY, Ow DW (2010) PhiC31 recombination system demonstrates heritable germinal transmission of site-specific excision from the *Arabidopsis* genome. BMC Biotechnol 10:17

Tinland B, Schoumacher F, Gloeckler V, Bravo-Angel AM, Hohn B (1995) The *Agrobacterium tumefaciens* virulence D2 protein is responsible for precise integration of T-DNA into the plant genome. EMBO J 14:3585–3595

Travella S, Ross SM, Harden J, Everett C, Snape JW, Harwood WA (2005) A comparison of transgenic barley lines produced by particle bombardment and *Agrobacterium*-mediated techniques. Plant Cell Rep 23:780–789

Wang MB, Waterhouse PM (2000) High-efficiency silencing of a beta-glucuronidase gene in rice is correlated with repetitive transgene structure but is independent of DNA methylation. Plant Mol Biol 43:67–82

West AG, Gaszner M, Felsenfeld G (2002) Insulators: many functions, many mechanisms. Gene Dev 16:271–288

Windels P, De Buck S, Depicker A (2008) *Agrobacterium tumefaciens*-mediated transformation: patterns of T-DNA integration into the host genome. In: *Agrobacterium*: from biology to biotechnology. Springer, New York, pp 442–483

Woo H, Cho H, Lim S, Shin K, Lee S, Lee K, Kim D, Cho Y (2009) Auto-excision of selectable marker genes from transgenic tobacco via a stress inducible FLP/*FRT* site-specific recombination system. Transgenic Res 18(3):455–465

Wu H, Altpeter F (2015a) Sugarcane (*Saccharum* spp. hybrids). In: Wang K (ed) *Agrobacterium* protocols. Springer, Heidelberg, pp 307–316

Wu H, Sparks CA, Jones HD (2006) Characterisation of T-DNA loci and vector backbone sequences in transgenic wheat produced by *Agrobacterium*-mediated transformation. Mol Breed 18:195–208

Wu H, Awan FS, Vilarinho A, Zeng Q, Kannan B, Phipps T, McCuiston J, Wang W, Caffall K, Altpeter F (2015b) Transgene integration complexity and expression stability following biolistic or *Agrobacterium*-mediated transformation of sugarcane. In Vitro Cell Dev Biol –Plant. https://doi.org/10.1007/s11627-015-9710-0

Yang Y, Singer SD, Liu Z (2011) Evaluation and comparison of the insulation efficiency of three enhancer-blocking insulators in plants. Plant Cell Tiss Org 105:405–414

Yoder JI, Goldsbrough AP (1994) Transformation systems for generating marker-free transgenic plants. Bio Technology 12:263–267

Zale J, Jung JH, Kim JY, Pathak B, Karan R, Liu H, Chen X, Wu H, Candreva J, Zhai Z, Shanklin J, Altpeter F (2016) Metabolic engineering of sugarcane to accumulate energy-dense triacylglycerols in vegetative biomass. Plant Biotechnol J 14:661–669

Zhang Y, Zheng Y, Xiao N, Wang L, Zhang Z, Fang R, Chen X (2012) Functional analysis of the HS185 regulatory element in the rice HSP70 promotor. Mol Biol Rep 39:1649–1657

Zhu C, Naqvi S, Breitenbach J, Sandmann G, Christou P, Capell T (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. Proc Natl Acad Sci USA 105:18232–18237