

Efficient Sugarcane Transformation via *bar* Gene Selection

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Abstract Genetic engineering provides new opportunities for improving economically important traits in sugarcane cultivars. In this study, an efficient *Agrobacterium*-mediated transformation system that uses the *bar* gene (a herbicide resistance gene that is used in conjunction with the herbicide Basta) as a selection marker was developed. Using this transformation selection system, all of the resistant plants after selection were nearly 100% polymerase chain reaction (PCR) detection positive and showed herbicide resistance. Each gram of sugarcane calli used for transformation produced approximately 12 transgenic lines. It took approximately 4 months to generate transgenic plants that measured 10 cm in height for greenhouse transplantation.

Keywords Sugarcane · Transgenic plant · *Agrobacterium* · *Bar* gene · Herbicide resistance

Abbreviations

MS	Murashige and Skoog medium
GFP	Green fluorescent protein
2,4-D	2,4-Dichlorophenoxyacetic acid
6-BA	6-Benzylaminopurine
NAA	1-Naphthaleneacetic acid
AD2	Synthetic <i>Medicago truncatula</i> Defensins4
AD3	Native <i>Medicago truncatula</i> Defensins4 with KDEL

AD4	Nynthetic <i>Medicago truncatula</i> Defensins4 with KDEL
Ppase	Inorganic Pyrophosphatase Gene
SST	Sucrose:sucrose 1-fructosyl transferase gene

Introduction

Sugarcane is one of the most important commercial crops and is an important bio- energy crop. Sugarcane is cultivated in large areas of Indonesia and other tropical and subtropical countries, and it contributes to 60 to 70% of the annual sugar consumption worldwide (Manickavasagam et al. 2004; Suprasanna et al. 2011). In addition, it is used as raw material for ethanol production in Brazil, which is also the largest producer of cane sugar in the world (Snyman 2004). Similar to other food crops, the production of sugarcane is affected by biotic and abiotic stresses, such as disease, insects, and drought (Viswanathan and Rao 2011; Srikanth et al. 2011; Priji and Hemaprabha 2015). Most modern sugarcane cultivars are inter-specific hybrids and have complex genetic characteristics and low fertility, thus rendering genetic improvement through traditional breeding difficult. These cultivars are primary candidates for improvement through genetic engineering (Ingelbrecht et al. 1999). Transformation and transgene expression are key biotechnologies for sugarcane improvement (Dong et al. 2014). Electroporation (Arencibia et al. 1995), particle bombardment (Falco et al. 2000), and *Agrobacterium*-mediated transformation (Enríquez-Obregón et al. 1998) are the three main methods of successful sugarcane transformation. Particle bombardment has been used in more than 60% of the existing sugarcane transformation studies (Arvinth et al. 2010; Basnayake et al. 2011; Zhu et al. 2011; Rani et al. 2012; Xiong et al. 2012). Direct transformation procedures, such as particle bombardment and electroporation, have several disadvantages,

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including poor reproducibility, variable transgene copy number, and instability of the gene construct in the new host (Santosa et al. 2004). *Agrobacterium*-mediated transformation, by contrast, has advantages over other gene-delivery technologies. Successful sugarcane transformation via *Agrobacterium* has been reported but exhibited low efficiency (Zhangsun et al. 2007; Joyce et al. 2010; Mayavan et al. 2013). However, Dong et al. (2014) achieved high-efficiency *Agrobacterium*-mediated transformation for sugarcane. Desiccation treatment during co-cultivation played a critical role in improving sugarcane transformation efficiency for sugarcane transformation efficiency using *Agrobacterium*. However, the selection system that they used for the transformation was the *pmi*/mannose system. The *pmi* gene was thought to be a safety gene, but it was out of use after transformation. Several studies reported transgene silencing in sugarcane (Wei et al. 2003; Mudge et al. 2009). Some studies also reported the use of antibiotic-resistant genes, such as *hptII* and *nptII* (Zhangsun et al. 2007; Joyce et al. 2010; Kalunke et al. 2009), but these selection markers were also useless after transformation.

In this study, an efficient *Agrobacterium*-mediated transformation system that uses the *bar* gene as a selection agent for sugarcane was developed for use on an industrial scale. A *bar*/Basta selection system was successfully used in this transformation system. The *bar* gene is a herbicide resistance gene, and Basta is a herbicide. By using this transformation system, the detection rate of resistant plants following transformation was almost 100%. Each gram of sugarcane calli used during the transformation produced more than 12 transgenic lines. It took less than 4 months from callus infection to produce greenhouse-ready transgenic plants that measured 10 cm in height.

Material and Methods

Media Composition

- M1: MS +30 g/L sucrose + 8 g/L agar +2.0 mg/L 2,4-D
- M2: MS +30 g/L sucrose + 8 g/L agar +2.0 mg/L 6-BA
- M3: MS +30 g/L sucrose + 8 g/L agar
- M-Initiating: 1/5 strength MS medium + 30 g/L sucrose + 30 g/L glucose + 100 mM acetosyringone
- M-Resting: M1+300 mg/L Timentin
- M-Selection: M1+ 300 mg/L Timentin + 2.0 mg/L glufosinate-ammonium
- M-Regeneration: M2+300 mg/L Timentin + 2.0 mg/L glufosinate-ammonium
- M-Elongation: M3+300 mg/L Timentin + 2.0 mg/L glufosinate-ammonium
- M-Rooting: M3+1 mg/L NAA+300 mg/L Timentin + 2.0 mg/L glufosinate-ammonium

Plant Material and Callus Induction

Plant material of the sugarcane variety ROC22 was obtained from a field in Hainan, China. Tops and tillers containing the immature leaf whorl were used as source material for embryogenic callus induction, which was initiated within 24 h of cutting and collection. Transverse sections of immature sugarcane leaf whorls were prepared essentially as described by Bower and Brich (1992). Transverse, 1-mm-thick sections were obtained from just above the meristem and placed on callus induction medium (M1) (Murashige and Skoog 1962). Callus cultures were maintained in the dark at 28 °C and subcultured on fresh medium every 2 weeks, for a total culture duration of approximately 45 days. Light yellow, compact calli were selected and fragmented before transformation.

Determination of Minimum Inhibitory Concentration of Basta

Transverse sections that were prepared as above for callus induction were inoculated in M1 medium modified with different concentrations (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/L) of Basta (200 g/L glufosinate-ammonium, Bayer, Germany) (Mayavan et al. 2013). The cultures were maintained in the dark at 28 °C for 14 days and then transferred to regeneration medium (M2) modified with different concentrations (0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) of Basta. The Basta concentration with an inhibitory effect on secondary leaf development was chosen as the minimum inhibitory concentration and adopted for transformation selection. Every treatment contained 48 pieces of transverse sections in duplicates of five.

Binary Vectors and *Agrobacterium* Strain

All of the binary vectors comprised a range of target genes and a selectable marker gene of the *bar* gene. The *bar* gene promoted by CaMV 35S promoter was introduced between the two XhoI sites of pCAMBIA3300. The binary vector pCAMBIA3300 contained the *npt II* gene for bacterial selection. The reporter gene green fluorescent protein gene (GFP) promoted by Ubi1 was introduced between the BamHI and SacI sites of pCAMBIA3300 (Fig. 1). The recombinant binary vectors were mobilized into *Agrobacterium* EHA105 via freeze-thawing with liquid nitrogen. The *Agrobacterium* strain was cultured on YEP (yeast extract 10 g/L, peptone 10 g/L, NaCl 5 g/L) medium containing the appropriate antibiotics (kanamycin 50 mg/L, streptomycin 50 mg/L, and rifampicin 10 mg/L). Five of the other vectors had the same construction as the *bar*-GFP vector, but the GFP gene was replaced by five other

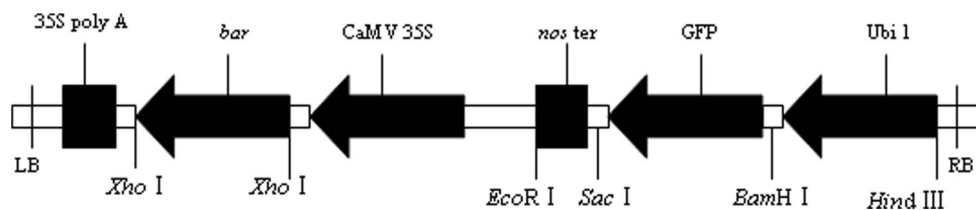


Fig. 1 Schematic representation of the binary vector used in sugarcane transformation. Bar gene promoted by CaMV 35S promoter and GFP gene or five other target genes (AD2, AD3, AD4, SST, Ppase) promoted by Ubi 1 promoter. CaMV 35S P: cauliflower mosaic virus

35S promoter; 35S poly A: cauliflower mosaic virus 35S poly A terminator; nos ter: nopaline synthase terminator; Ubi 1 P: maize Ubi 1 promoter

genes (synthetic *Medicago truncatula* defensins 4 (AD2), native *Medicago truncatula* defensins 4 with KDEL (AD3), synthetic *Medicago truncatula* defensins 4 with KDEL (AD4), inorganic pyrophosphatase gene (Ppase), sucrose:sucrose 1-fructosyl transferase gene (SST)).

Initiation of Agrobacterium

Agrobacterium EHA105 cultures that harbored a vector comprising the visual gene GFP (Ubi1 promoter with an intron) and the selection marker gene *bar* (CaMV 35S promoter) were streaked on YEP medium containing antibiotics, as described above, and grown at 28 °C for 2 days. A single colony was selected and sub-cultured overnight on fresh YEP medium at 28 °C. Bacteria were collected after centrifugation, resuspended in a starter culture of liquid initiating medium (M-Initiating) and diluted to an optical density of approximately 0.6 at 600 nm. The starter culture was vortexed at 100 rpm for 2 h at 28 °C before infection.

Infection and co-Cultivation

The calli used for transformation were weighed before infection, placed in Petri dishes, air-dried for 1 h on a clean laboratory bench, and then transferred to an Erlenmeyer flask. All of the calli were heat-shocked in an incubator at 45 °C for 5 min in sufficient pre-warmed (45 °C) M-Initiating medium without *Agrobacterium* to cover the calli. The M-initiating medium was pipetted out, and sufficient infection medium with *Agrobacterium* starter culture was added and shaken gently (less than 100 revolutions) for 10 min at 28 °C. The calli/*Agrobacterium* mixture was vacuumed for 5 min at -27.5 mmHg of vacuum and then sonicated for 2 min in an ultrasonic cleaner. The mixture was shaken gently (fewer than 100 revolutions) for 10 min again in the dark. The *Agrobacterium* suspension was then pipetted out, and the calli were transferred to a Petri dish, blotted dry with filter paper to remove excess *Agrobacterium* suspension, and air-dried for approximately 30 min on the clean bench. The calli were

then transferred to a new empty Petri dish, sealed with paraffin film, and then incubated in the dark at 22 °C for 3 days (Dong et al. 2014).

Selection of Infected Calli

After co-cultivation, the calli were transferred to resting medium (M-Resting) and stored in the dark at 28 °C for 7 days. Subsequently, all calli were transferred to selection medium (M-Selection) for 30 days in the dark at 28 °C. The selection medium was renewed once every 2 weeks during the 30 days of selection. Each Petri dish contained approximately 10 pieces of callus.

Regeneration, Elongation, and Rooting

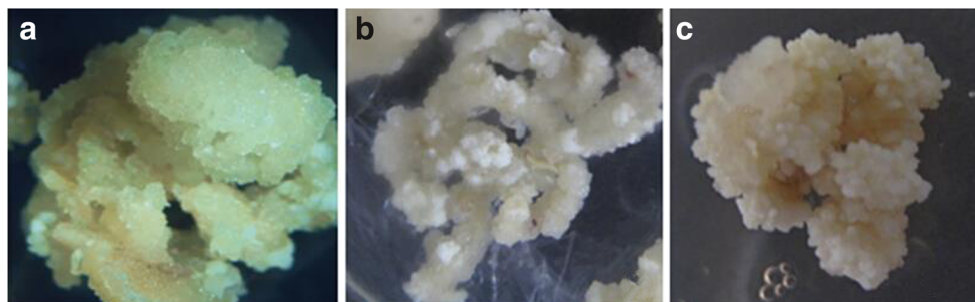
At least two or three calli continued to grow well in each Petri dish after 30 days of selection. These resistant calli were transferred to regeneration medium (M-Regeneration) and cultivated in an illuminated incubator for 14 days at 30 ± 1 °C in 14 h of light each day. Transgenic healthy calli sprouted green buds during these 14 d. The green buds were then transferred to elongation medium (M-Elongation) and cultivated in an illuminated incubator for 20 days at 30 ± 1 °C in 14 h of light each day. After 20 days of elongation, buds grew to approximately 3 to 4 cm. A single shoot from each bud was

Table 1 Primer sequences for PCR analysis

Gene	Primers	
<i>bar</i>	Forward	CGGATGAGCCCAGAACGACGCC
	Reverse	CGGTCAGATCTCGGTGACGGGCAG
GFP	Forward	GTACCACGAGTCCAAGTTCTACG
	Reverse	TTGTGCTGGATGAAGTGCC
VirG	Forward	TTCGTTCCGATGCTCTATGA
	Reverse	AGGTCGTCTTTCTGCTTTCC

All of these primers for PCR analysis were designed with Primer Premier version 5.0 and amplified a 558-bp DNA fragment for the *bar* gene, a 249-bp DNA fragment for the GFP gene, and a 332-bp DNA fragment for VirG gene.

Fig. 2 Embryogenic callus induction of cultivar ROC22 for transformation. **a.** 15 days after induction: no embryogenic cells. **b.** 30 days after induction: tiny outgrowths of embryogenic callus. **c.** 45 days after induction: embryogenic callus suitable for transformation



transferred to rooting medium (M-Rooting) and cultivated in an illuminated incubator for 20 days at $30\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ in 14 h of light each day. These shoots then grew to 6 to 7 cm in height and were sampled for molecular analysis and transferred to soil.

Visualization of GFP Expression

The resistant calli, shoots, and leaves were observed for GFP gene expression using fluorescence microscope equipment. A GFP 510-nm long-pass emission filter with a 480/40-nm excitation filter was used. Fluorescence images were captured with a Canon digital camera.

Total Genomic DNA Extraction and PCR Assay

A PCR assay was performed to detect the integration of the *bar* gene and to confirm the absence of *Agrobacterium* contamination in the transformed lines as the resistant plants grew to 8–10 cm in height. The PCR reactions were conducted using the primers specific to the *bar*, GFP, and *VirG* genes (Table 1). Vector plasmid was used as a positive control, and

the total genomic DNA extracted from wild-type plants was used as a negative control.

Detection of PAT/*bar* Protein by QuickStix Strips

All of the resistant plants were sampled and tested by QuickStix strips (QuickStix Kit for Libertylink (*bar*) Cotton Leaf & Seed, Envirologix, USA). Approximately 10 mg of leaf tissue was taken from each resistant plant and pushed into the tapered bottom of a 1.5-ml Eppendorf tube. Wild-type plant tissue samples served as a negative control. A pestle was inserted into the tube, and the tissue was ground by rotating the pestle against the sides of the tube with twisting motions. This process was continued for 20–30 s or until the leaf tissue was well grounded, at which point 0.5 ml of extraction buffer was added. The grinding step was repeated to mix the tissue thoroughly with extraction buffer. The pestle was discarded, and QuickStix strips were inserted into the extraction tube. The strips were allowed to develop for 10 min before final assay interpretations were made.

Fig. 3 Minimum inhibitory concentration of Basta. **a.** No-Basta medium; **b.** 0.5 mg/L Basta; **c.** 1.0 mg/L Basta; **d.** 1.5 mg/L Basta; **e.** 2.0 mg/L Basta; **f.** 2.5 mg/L Basta. All of the transverse sections grew well, and the secondary leaf emerged quickly after regeneration on 0 mg/L Basta medium. Callus growth and bud formation were inhibited in direct proportion to the increases in the concentration of Basta from 0.5 mg/L to 1.5 mg/L. At 2.0 mg/L or above, callus growth and secondary leaf formation were completely inhibited

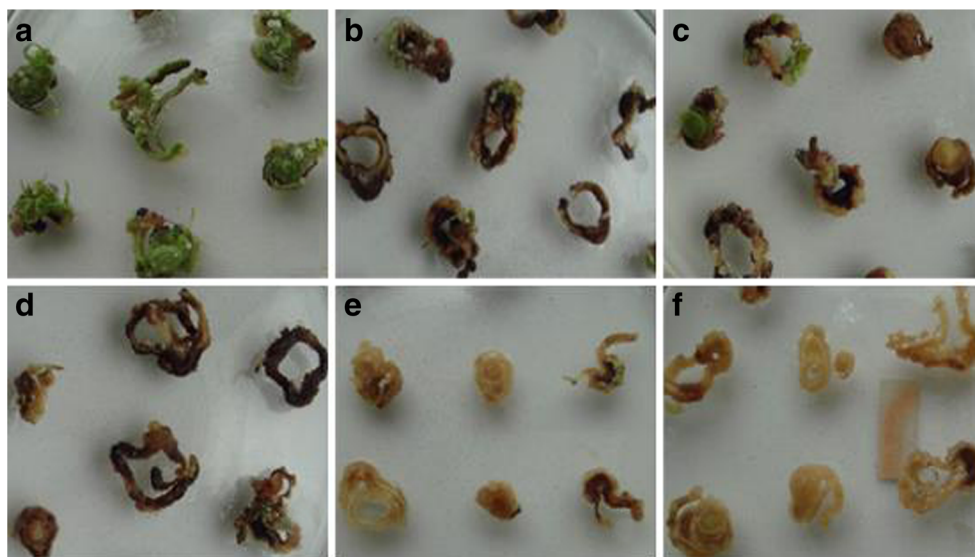
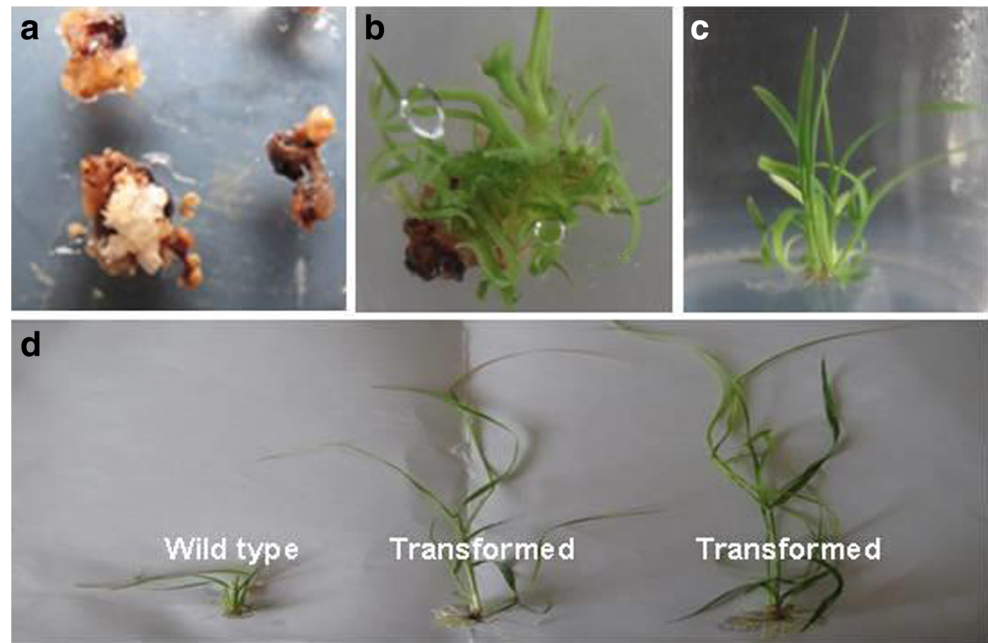


Fig. 4 Selection of transformed sugarcane plants. **a.** After 30 days of dark selection, some resistant calli were healthy on the selection medium; **b.** after 14 days of regeneration, cultivation-resistant calli emerged quickly and were healthy; **c.** after 20 days of elongation, cultivation-resistant, dark-green shoots grew quickly; **d.** after 20 days of rooting cultivation, several branched roots emerged; the resistant shoots elongated to 10-cm in height and were sampled for DNA extraction and PCR analysis. All media used were supplemented with 2.0 mg/L of Basta



Southern Blot Analysis

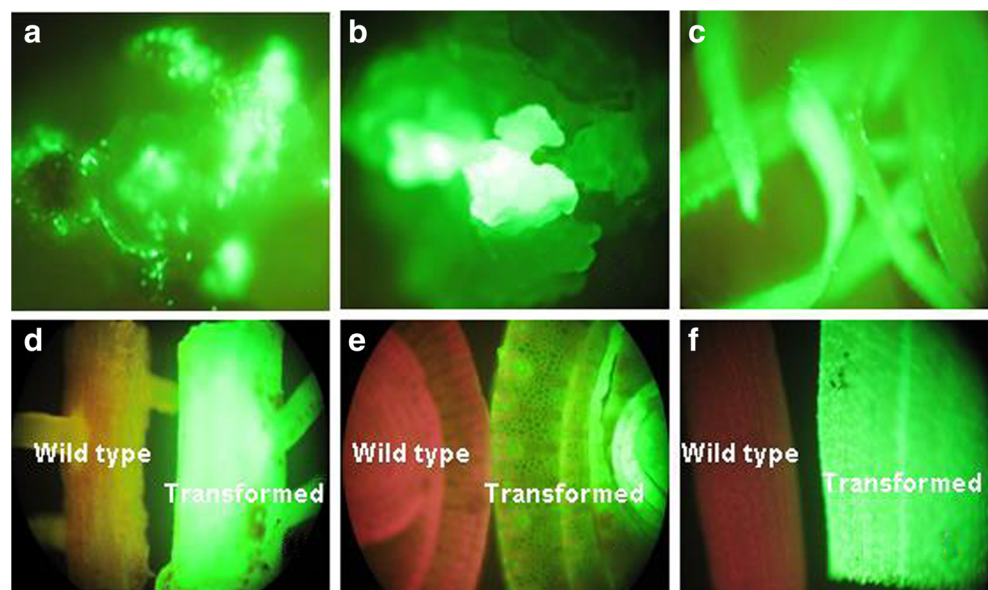
Southern blot analysis was performed to determine the integration and copy number of the *bar* gene. Approximately 10 μ g of total genomic DNA that was extracted from transformed sugarcane plants was digested with EcoRI restriction enzyme, separated by electrophoresis agarose gel, and transferred onto a Hybond N+ membrane. A 558-bp digoxin-labeled DNA fragment corresponding to the *bar* gene was used as a probe for hybridization according to the instruction manual (DIG High Prime DNA Labeling and Detection Starter Kit

I, Roche). The results were documented with a Canon color digital camera system.

Herbicide Sensitivity Assay

Herbicide (Basta, 200 g/L glufosinate-ammonium, YONON Biosciences Co., Ltd., Zhejiang, China) sensitivity assays were performed on transformed sugarcane plants containing single *bar* gene insertions under greenhouse conditions. According to the instruction manual for this herbicide, 0.8 g/L to 2.0 g/L was used in the sugarcane fields. Hence,

Fig. 5 Observation of GFP gene expression. **a.** Infected callus after 3 days of co-cultivation showing transient GFP expression; **b.** resistant callus after 30 days of callus selection showing stable GFP expression; **c.** resistant buds after 14 days of regeneration showing stable GFP expression; **d.** root of transformed plants from greenhouse showing stable GFP expression; **e.** hand-cut section of transformed plants from greenhouse showing stable GFP expression; **f.** leaf of transformed plants from greenhouse showing stable GFP expression



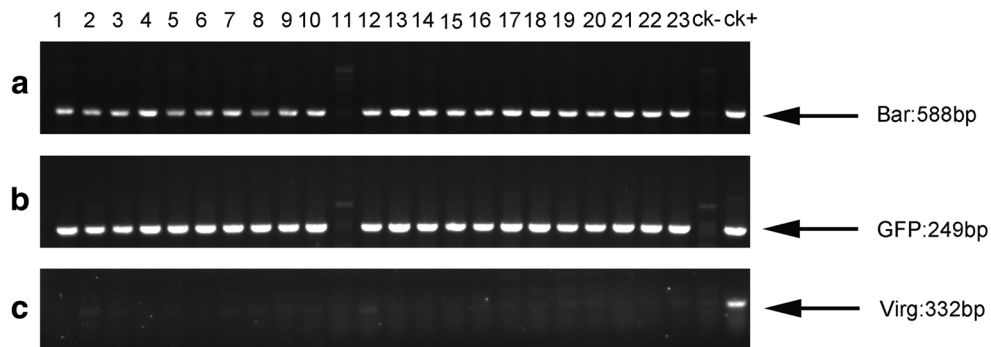


Fig. 6 PCR assay of transformed sugarcane plants. Lanes 1–23 contained genomic DNA of resistant plants; CK-: wild-type sugarcane genomic DNA; CK+: vector plasmid DNA. **a.** PCR amplification of *bar* gene from the genomic DNA of resistant plants; **b.** PCR amplification of the GFP

gene from the genomic DNA of resistant plants; **c.** identification of *Agrobacterium* contamination from resistant plants via VirG gene amplification

2.0 g/L was chosen to screen the resistance of the transformed lines in the greenhouse.

Estimated Transformation Efficiency

Cultivar ROC22 and six vectors with the *bar* selection marker gene with different target genes (GFP, Ppase, SST, AD2, AD3, AD4) were used to test the efficiency of the transformation system. The transformation process and medium used were as described above. Selection Efficiency (SE) and Transformation Efficiency (TE) formulas were used to estimate the transformation efficiency. SE = number of *bar* gene-PCR-positive shoots / number of resistant shoots obtained after rooting selection, or number of *bar* gene-PCR-positive shoots / weight (gram) of calli used for transformation. TE = number of both *bar* gene and target gene-PCR-positive shoots / number of resistant shoots or number of both *bar* gene and target gene-

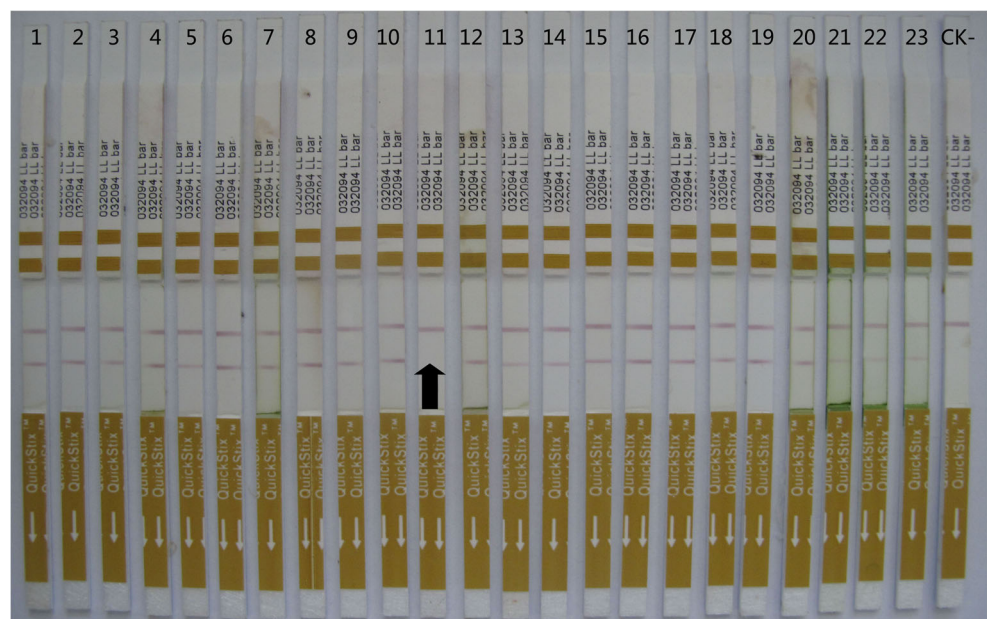
PCR-positive shoots / weight (gram) of the calli used for transformation.

Results

Plant Material and Callus Induction

ROC22 represents the largest acreage of sugarcane cultivars in China and is affected by serious diseases and insect pests. Therefore, considerable genetic improvement has been achieved in this cultivar. Young embryogenic calli (Fig. 2) of this cultivar were chosen as explants for genetic transformation because of their year-round availability, ease of handling, and efficiency of transformant selection. After 45 days of induction, light yellow, hard and compact calli (Fig. 2c) were obtained that were suitable for transformation.

Fig. 7 Detection of PAT/*bar* protein by QuickStix strips. 1–23 resistant plant leaf tissue extraction samples; CK- wild-type leaf tissue extraction sample



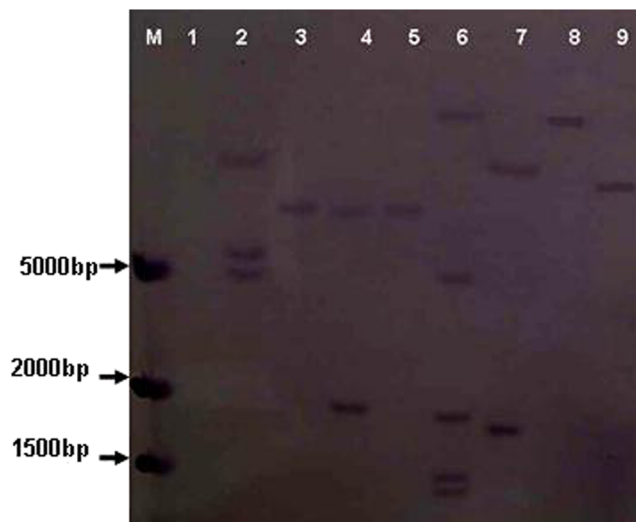


Fig. 8 Southern blot analysis of integration sites in transgenic sugarcane. Lane M: DNA marker ladder, which had been labeled (DSTM5000, DONGSHENG BIOTECH, Guangzhou, China); lane 1: wild-type; lanes 2–8: transformed sugarcane plant DNA samples; lane 9: EcoRI-digested plasmid DNA as positive control

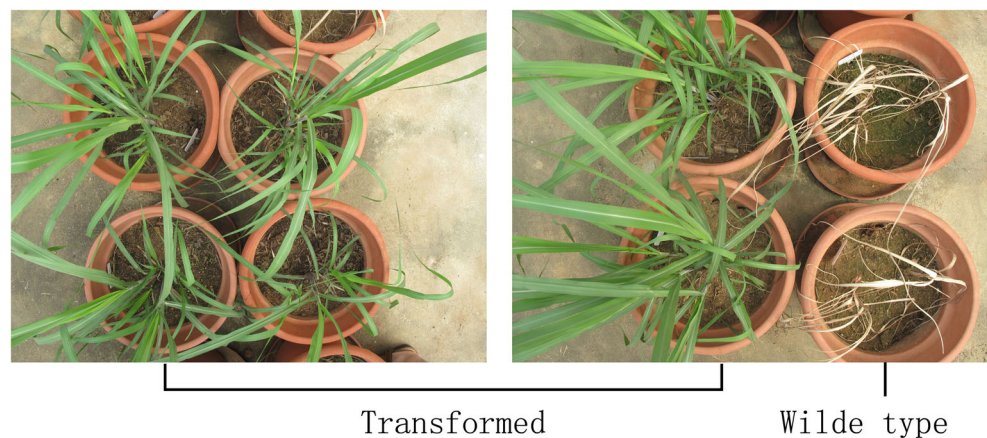
Determination of Minimum Inhibitory Concentration of Basta

Basta has been identified as an efficient selective agent in monocot species (Fromm et al. 1990). At a concentration of 2.0 mg/L, Basta completely inhibited callus growth and secondary leaf formation (Fig. 3). Hence, 2.0 mg/L of Basta was chosen as the minimum inhibitory concentration to select transformed calli and plants.

Post-Transformation and Selection

To decrease the percentage of chimeras and escapes in plant transformation, stringent selection pressure must be employed (Darbani et al. 2007). Hence, we added 2.0 mg/L of Basta to all of the transformation media used, including the callus selection, regeneration, elongation, and rooting media.

Fig. 9 Herbicide sensitivity assay. Transformed sugarcane shoots stayed healthy 2 weeks after Basta spraying, but the wild-type sugarcane plants died off



After 30 days of callus selection in the dark, 2–3 or more pieces of new calli sprouted and showed significant resistance to 2.0 mg/L of Basta in each Petri dish (Fig. 4a). These resistant calli regenerated quickly and stayed healthy on the regeneration medium supplemented with 2.0 mg/L of Basta (Fig. 4b). All of the regenerated buds were transferred to elongation medium (Fig. 4c). After 20 days of elongation, a single shoot was selected from each of the buds and transferred to rooting medium. Transformed shoots grew fast and germinated healthy roots (Fig. 4d).

Visual Observation of GFP Gene Expression

The infected, resistant calli and resistant shoots were evaluated for GFP gene expression under a microscope during the transformation process (Fig. 5a–c). Roots, transected sections of shoot, and leaves of 1-y-old transformed plants from the greenhouse were evaluated for GFP gene expression (Fig. 5d–f). The GFP gene expression level was tested in all tissue types. Bright green fluorescence was observed in the transformed calli, leaves, and roots. Roots, transverse sections, and leaves of the wild type showed red auto fluorescence.

PCR Assay of Potential Transformed Shoots

All resistant shoots were sampled and evaluated for *bar* gene and GFP gene integration by PCR following rooting cultivation. *Bar* gene-specific primers amplified a 558 bp fragment in the vector plasmid and resistant plants (Fig. 6a), and the GFP gene-specific primers amplified a 249-bp fragment in the vector plasmid and resistant shoots (Fig. 6b). Both the *bar* gene and the GFP gene were negative for wild-type sugarcane genomic DNA. Figure 6 shows 23 resistant plants, with only one (Fig. 6, lane 11) that was PCR negative for both the *bar* gene and the GFP gene.

To determine the absence of *Agrobacterium* in the resistant plants, VirG gene PCR detection was done using VirG gene-specific primers. No amplification was found in

Table 2 Sugarcane transformation efficiency

Tr. NO.	Vector	Calli (gram)	Resistant shoots (Line)	<i>bar</i> Gene PCR positive (Line)	Target Gene PCR positive (Line)
1	<i>bar</i> -GFP	2	23	22	22
2	<i>bar</i> -Ppase	2	23	20	17
3	<i>bar</i> -SST	2	14	14	12
4	<i>bar</i> -AD2	4	80	78	71
5	<i>bar</i> -AD3	4	51	51	46
6	<i>bar</i> -AD4	4	40	40	37
Total		18	231	225(SE = 97.4%) (12.5 each gram) ^a	205(TE = 88.7%) (11.4 each gram) ^b

^a SE (Selection Efficiency) = *bar* gene PCR-positive shoots/resistant shoots; each gram of calli used produced 12.5 lines of *bar* gene PCR-positive transformed plants

^b TE (Transformation Efficiency) = both *bar* gene and target gene PCR-positive shoots/resistant shoots; each gram of calli produced 11.4 lines of both *bar* and target gene PCR-positive transformed plants

the resistant plants (Fig. 6c), which suggested that the resistant sugarcane plants from the rooting medium were free from *Agrobacterium*. This result also indicates that the PCR-amplified fragments emerged from the integrated *bar* gene and GFP gene in the plant genome. Therefore, only the PCR-positive resistant plants were transplanted to the greenhouse. The PCR-negative plants were discarded.

Detection of PAT/*bar* Protein by Quick Stix Strips

All resistant plants were sampled and tested with QuickStix strips (QuickStix Kit for Libertylink (*bar*) Cotton Leaf & Seed, Envirologix, USA). If the sample extraction contained the PAT/*bar* protein, a test line would develop on the membrane strip between the control line and the protective arrow tape. As Fig. 7 shows, most of the resistant plant sample extractions developed a test line; this QuickStix strip testing result was in complete concordance with the *bar* gene PCR analysis results. Thus, if the resistant plant was *bar* gene PCR-positive, the QuickStix strip testing was also positive.

Southern Blot Assay

Leaves of transformed shoots were sampled to determine the copy number of the integrated *bar* gene in transformed plant genomes by Southern blot. The DNA extracted from transformed sugarcane plants was digested with EcoRI, and a 558-bp digoxin-labeled DNA fragment corresponding to the *bar* gene was used as a probe. The transformed sugarcane shoots showed one to five integrated copies (Fig. 8).

Herbicide Sensitivity Assay

We sprayed 2.0 g/L of Basta (200 g/L glufosinate-ammonium, YONON Biosciences Co., Ltd., Zhejiang, China) on transformed and wild-type sugarcane plants under greenhouse

conditions. Ten days later, wild-type sugarcane plants were growth-arrested, which resulted in death, but the transformed plants were not affected by Basta and grew healthily (Fig. 9).

Transformation Efficiency

As shown in Table 2, a high level of transformation efficiency was achieved. Each gram of calli used for transformation produced approximately 12 lines of transformed sugarcane plants. Our results showed 97.4% of the resistant shoots were *bar* gene PCR-positive and 88.7% of the resistant shoots were PCR-positive for the *bar* and target genes. Target genes were lost more often than the *bar* gene was during the process of integration into the sugarcane genome, which led to a lower percentage of target gene PCR-positive plants compared with the *bar* gene-positive transformants.

Discussion

Increased sugarcane transformation efficiency was achieved using the *bar*/Basta transformation selection system in this study. Referring to relevant literature and discussing with some other experts, we knew that utilizing a suitable formation of calli was very important to increase the transformation efficiency. The embryonic calli were the best formation for transformation in our previous studies. Thus, after approximately 45 days of induction, the light yellow and compact embryonic calli were chosen for the initiation of transformation. Each gram of calli used for transformation produced approximately 12 lines of transformed sugarcane plants. The most suitable Basta concentration was chosen and supplemented in the media during the dark, regeneration, and rooting selection steps, thereby inducing high selection efficiency. Almost all of the resistant plants in the rooting medium were PCR-positive for the selection marker *bar* gene. Inadequate Basta concentration (< 1 mg/L) in the selection

medium may induce a low PCR-positive percentage of the selection marker *bar* gene. An excessive Basta concentration (> 3 mg/L) may result in fewer resistant plants obtained and higher copy numbers of transformed shoots at the end. The production of transgenic plants takes fewer than 4 months from callus formation to greenhouse transplantation. This sugarcane transformation protocol resulted in a high proportion of low-copy-number (Manickavasagam et al. 2004; Suprasanna et al. 2011; Snyman 2004) transformed lines and approximately 15% high-copy-number (≥ 4) transformed lines. The selection marker *bar* genes were expressed stably and conferred excellent herbicide resistance in our later studies on transformed sugarcane ratoon generations. The high transformation efficiency and herbicide resistance demonstrate the broad utility of our transformation selection system in the application of biotechnology for sugarcane variety improvement.

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