Sugar Tech (Oct-Dec 2014) 16(4):366–371
DOI 10.1007/s12355-013-0294-x

RESEARCH ARTICLE

Advances in *Agrobacterium*-Mediated Sugarcane Transformation and Stable Transgene Expression

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Received: 18 October 2013 / Accepted: 6 December 2013 / Published online: 4 January 2014
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Abstract The sugarcane plant, with complex genetic characteristics and a lengthy breeding process, is challenging for genetic improvement through traditional breeding. Genetic engineering offers an opportunity for trait-specific sugarcane variety improvement. Transformation and transgene expression are key technologies to enable the application of biotechnology to sugarcane improvement, and technical challenges have been reported in both of these areas. An efficient *Agrobacterium*-mediated transformation system for multiple diverse sugarcane varieties has been developed to an industrial scale. The transgenic events generated have demonstrated high level transgene expression over multiple seasons in the field. Syngenta is now applying this technology on a larger scale in sugarcane improvement research.

Paper presented at the XXVIII Congress of the International Society of Sugar Cane Technologists, Sao Paulo, Brazil, 24–27 June 2013 and published here with the agreement of the Society.

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Keywords Sugarcane · *Agrobacterium* · Transformation · Desiccation · Expression

Abbreviations

CFP	Cyano fluorescent protein (AmCyan)
GFP	Green fluorescent protein
TSP	Total soluble protein
V1	The first vegetative propagation
V1R1	The first ratoon of first vegetative propagation

Introduction

The genetic modification of sugarcane (*Saccharum* spp. interspecific hybrids) has advanced significantly since the first successful reports in the early 1990s (Bower and Birch 1992). An increasing number of sugarcane industries around the world have devoted significant resources towards developing transformation platforms to explore the potential of genetic modification for sugarcane improvement. As a result, the production of transgenic sugarcane plants has been described in a number of cultivars utilising a range of explants and gene delivery methods (Lakshmanan et al. 2005). *Agrobacterium*-mediated transformation has advantages over other gene delivery technologies.

The potential to generate high quality integration events with low copy and stable transgene expression, which are preferred by regulatory agencies, is a significant driver to develop *Agrobacterium*-mediated transformation methods. However, when we started this program at Syngenta, there were only a handful of labs in the world that had demonstrated successful sugarcane transformation via *Agrobacterium* (Zhangsun et al. 2007; Arencibia et al. 1998; Elliott et al. 1998; Enriquez-Obregon et al. 1998), and available systems were not efficient enough to support large scale

commercial event production and were largely variety-dependent. Moreover, a number of publications had reported transgene silencing (Hansom et al. 1999; Wei et al. 2003; Yang et al. 2003; Mudge et al. 2009), indicating that this could be a major potential issue in the development of commercial transgenic sugarcane products.

The key objectives for this project were: (1) to develop a consistently efficient commercial sugarcane *Agrobacterium*-mediated transformation platform across different sugarcane varieties and (2) to achieve predictable and stable transgene expression through vegetative propagation and over multiple sugarcane growing seasons under field conditions.

Materials and Methods

Plant Material

Plant materials were obtained from either field- or greenhouse-grown sugarcane plants. Varieties Q117 and KQ228 were field-grown (kindly provided by BSES Limited, Meringa Sugarcane Research Station, Gordonvale, QLD, Australia) while varieties L97-128 (kindly provided by Dr Kenneth Gravois, Louisiana State University), CP84-1198, CP89-2143 (kindly provided by the Canal Point USDA Sugarcane Breeding Station), and SP70-1143 (kindly provided by Sugarcane World Collection in Coral Gables, FL, USA) were grown at the Syngenta Greenhouse complex, Research Triangle Park, NC, USA. Sugarcane tops and tillers containing the immature leaf whorl used as source material for embryogenic cultures were collected and initiated into tissue culture within 36 h of harvest.

Transverse sections of immature sugarcane leaf whorls were prepared essentially as described by Bower and Birch (1992). Transverse sections (approximately 25–30) of immature leaf whorl between 1 and 2 mm in thickness were taken from just above the meristem and placed onto callus induction media. Transverse sections from Q117 and KQ228 were initiated on EM3 medium (Murashige and Skoog (MS)salts and vitamins, 500 mg/L casein hydrolysate, 100 mL/L young coconut water, 20 g/L sucrose, 3 mg/L 2,4-D and 8 g/L Agar) while L97-128, CP84-1198, CP89-2143, and SP70-1143 were initiated on SC-D2 medium (MS salts, B5 vitamins, 30 g/L sucrose, 2 mg/L 2,4-D and 7 g/L Phytoblend) (Murashige and Skoog 1962). Cultures were maintained in the dark at 27 ± 1 °C and subcultured onto fresh media every 12–14 days for 28–42 days. For Q117 and KQ228, younger target tissue was used that included the original leaf roll material attached to the embryogenic cultures. For all other varieties, embryogenic calli were selected as target tissue for transformation providing good regeneration and consistent transformation. Calli were selected based on morphological characteristics such as compact structure and colour and were fragmented in small clusters (2–4 mm) prior to transformation. This was similar for all six sugarcane cultivars tested.

Preparation of *Agrobacterium*

Agrobacterium cultures harbouring a vector comprising a range of visual or selectable marker genes, such as *AmCyan1* (licensed from Clontech Laboratory, Inc.), *nptII* or *pmi*, were streaked out on Luria-Bertani LB medium containing appropriate antibiotics and grown at 28 °C for 3 days. Prior to transformation, a single colony was selected and streaked onto a fresh LB plate and grown for 1–2 days at 28 °C.

Agrobacterium was prepared for inoculation by initiating a fresh *Agrobacterium* culture from a plate into a 30 mL starter culture of liquid LB medium and grown for 4–5 h at 28 °C in a shaker at 200 revolutions per minute (r/min). The starter culture was transferred to a 500 mL Erlenmeyer flask with 100–150 mL of fresh LB medium. The culture was grown for 12–14 h at 28 °C at 150 r/min to an optical density (OD_{600}) of 0.2–1.0 at 600 nm. The *Agrobacterium* culture was then centrifuged for 20 min at 2,000 r/min at 25 °C. The pellet obtained was resuspended in 150 mL L ½ strength MS medium (without sucrose) supplemented with 400 µM acetosyringone. This culture was then maintained at 28 °C at 50 RPM for 4 h prior to infection. The OD_{600} was adjusted to a desired level before infection of the plant material to be transformed.

Infection and Co-cultivation

Embryogenic callus target tissue was weighed to ensure all experiments could be compared. For example 10 g of Q117 embryogenic callus was used per treatment and was placed into a 200 mL culture vessel. Embryogenic callus tissue was heat shocked at 45 °C for 5 min by adding 50 mL of pre-warmed ½ strength MS (without sucrose) medium and then maintained in a water bath at 45 °C. MS medium was then drained from the callus, and 25–30 mL of the *Agrobacterium* inoculation suspension was added to each vessel and mixed gently. The callus/*Agrobacterium* mixture was vacuum-infiltrated by placing it into a vacuum chamber for 10 min at −27.5 mmHg of vacuum. The callus/*Agrobacterium* mixture was then rested for 5–10 min in the dark.

The *Agrobacterium* suspension was then drained from the callus, and the remaining callus culture was blotted dry (Whatman Grade 1 paper) to remove excess *Agrobacterium* suspension.The callus was then transferred for co-cultivation to 90 × 25-mm Petri dishes containing no co-culture medium or dry filter papers and sealed with NESCOFILM® or MICROPORE™ tape (3 M; Minneapolis, MN, USA).

Controls were transferred to semi-solid co-cultivation media. The dishes were incubated in the dark at 22 °C for 2–3 days.

Post-transformation

After co-cultivation, the callus material was transferred to MS 1 medium (MS salts, MS vitamins, 20 g/L sucrose, 8 g/L agar, 50 g/L coconut water and containing 200 mg/L of Timentin) and kept in the dark at 25 °C for 4–7 days. The first selection step was made in MS 2 medium (MS salts, MS vitamins, 20 g/L sucrose, 8 g/L agar, 50 g/L coconut water, 50 mg/L geneticin (for NPTII) or 5 g/L Sucrose/8 g/L mannose (for PMI) and containing 200 mg/L of Timentin) for 14–15 days in the dark at 27 ± 1 °C.

Regeneration and Rooting

Regeneration was conducted on MS 3 medium [MS salts and vitamins, 20 g/L sucrose, 8 g/L agar, 1 mg/L benzylaminopurine (BAP), 50 mg/L geneticin(for NPTII) or 30 g/L sucrose/5 g/L mannose (for PMI) and containing 200 mg/L of Timentin] 27 ± 1 °C in 16 h light. Gradual increases in light intensity were required. For the first week, the culture was left at low light intensity and, for the next 3 weeks, the culture was grown at moderate light intensity.

Shoot formation was seen between 2 and 4 weeks. When the first leaves appeared, the shoots were transferred to MS 4 medium (MS salts, MS vitamins, 20 g/L sucrose, 8 g/L agar, 50 mg/L geneticin (for NPTII) or 30 g/L sucrose/5 g/L mannose (for PMI) and containing 200 mg/L of Timentin) until the plants grew to 4–5 cm in height. They were then sampled for plant analysis and transferred to soil.

Event Confirmation

The confirmation of events containing a transgene insertion was conducted routinely via a TaqMan quantitative real time PCR assay. Initial events generated in Q117 and L97-128 were validated via Southern analysis and a confirmed single copy event was then used as a copy number control in TaqMan assay deployment.

Field Growth of Transgenic Events

Events positive for transgene integration and expression (data not shown) were grown for approximately 6 months in the greenhouse before being harvested. Samples were collected for expression analysis and one-eyed setts were sent to Syngenta's Vero Beach Research Center in Florida for the field trialing of five clonal plants per transgenic event. Setts were germinated in pots and then transferred to the field 3 months after germination.

Plant Analysis for Transgene Expression

Top dewlap leaf samples from events in the field were taken at 3, 6, 9 and 12 months in both the plant cane and 1 subsequent ratoon generation. Samples were analysed by quantitative ELISA assay for the fluorescent marker AmCyanprotein.

Results

Agrobacterium-Mediated Transformation of Sugarcane

Sugarcane can be efficiently transformed by subjecting the *Agrobacterium*-inoculated plant tissue to extreme desiccation during the co-cultivation step, in which plant tissue scan lose weigh to veran extended period of time (data not shown; DeLucca 2010).By culturing the *Agrobacterium*-inoculated plant tissue in the desiccating environment during the co-cultivation step, transformation efficiency based on either transient expression or stable event callus line production was increased significantly (Table 1). Figure 1 illustrates the efficient transformation process that has been developed. High level expression of transient and stable CFP/GFP gene expression was observed 5 days after infection (Fig. 1a, d), and 3 weeks after infection (Fig. 1b, e), respectively.

As shown in Table 2, utility of this method employing desiccation has been demonstrated on a numberof commercial and 'model' varieties. High level transformation efficiency has been obtained with both antibiotic and non-antibiotic selection systems (applying selection agents geneticin and mannose, respectively).

The most comprehensively studied of these are Q117 and L97-128 (Table 2) with which we were able to develop the most productive transformation processes.

For these two varieties, we are able to reach transformation efficiency of up to 20 events/g target tissue.

Subsequent work to apply this method to additional varieties has demonstrated utility in more than eight varieties, although the level of transformation efficiency is variable. Additional sugarcane varieties successfully transformed via *Agrobacterium* include but not limited to CP72-1210, Q208, KQ228, SP70-1143, CP89-2143, L99-226, L99-233, and CP84-1198.

Transgene Expression in Sugarcane Growing in the Field

Transgene expression was monitored in the *Agrobacterium*-mediated transgenic events produced in three different sugarcane varieties using the transformation protocol described. The ELISA data (Table 3) indicate that

Table 1 Summary of transgenic event generation efficiency following various co-cultivation conditions (*Agrobacterium* strain EHA101was used for all conditions)

Cultivar	Strain and construct	Co-cultivation treatment[c]	Transient expression[b]	Stable callus lines[e]
CP84-1198	EHA101 (pNOV2145)	Co-culture medium[a]	*	N/A[d]
		Filter	***	N/A
		No medium	***	N/A
CP72-1210	EHA101 (pNOV2145)	Co-culture medium[a]	*	N/A
		Filter	***	N/A
		No medium	***	N/A
L99-226	EHA101 (pNOV2145)	Co-culture medium[a]	*	N/A
		Filter	***	N/A
		No medium	***	N/A
L97-128	EHA101 (pNOV2145)	Co-culture medium[a]	*	N/A
		Filter	***	N/A
CP84-1198	EHA101 (pNOV2145)	Co-culture medium[a]	*	1
		Filter	***	3
CP84-1198	EHA101 (13601)	Co-culture medium[a]	**	11
		Filter	*****	31
L97-128	EHA101 (pNOV2145)	Co-culture medium[a]	*	1
		Filter	*****	8

[a] Co-culture medium was used during co-cultivation stage; 'Filter' represents only wet filter paper used during co-cultivation; 'No medium' represents explants that were co-cultivated on an empty plate without any medium or filter paper

[b] More * indicates higher transient expression

[c] Equal amount of explants were used for the different treatments

[d] N/A = data not available or not collected

[e] Embryogenic callus with multicelled CFP expressing stable transformation events or 'sectors' at 3 weeks after infection

AmCyan/CFP transgene expression was stable in V1 (The first vegetative propagation) plants through two growing seasons. Transgene expression in these five transgenic events in three different varieties is stable through multiple growing seasons in the field.

Discussion

We have developed an efficient *Agrobacterium*-mediated sugarcane transformation method that can now be applied on an industrial scale to sugarcane improvement research. The key to this high efficiency *Agrobacterium*-mediated transformation platform is the application of desiccation during the co-cultivation stage. Desiccation before, during and after co-cultivation has been previously reported to improve transformation efficiency in wheat and sugarcane (Arencibia et al. 1998; Zhangsun et al. 2007; Cheng et al. 2003).

In our work, desiccation greatly enhanced gene delivery/transient expression efficiency and also improved the survival and growth of the embryogenic target tissue in subsequent culture, when compared with standard transformation methods. In comparison to methods used for varieties such as Q117 (Elliott et al. 1998; Joyce et al. 2010), where semi-solid media was used for co-cultivation, we observed almost no browning or necrosis in the target tissue at 1 week after co-cultivation. With semi-solid media co-cultivation, we observed much more browning and necrosis and we rarely observed the development of multicelled CFP expressing stable transformation events or 'sectors'.

When the co-cultivation step is carried out in the absence of media (beyond the residual inoculation media in which the bacterial cells are suspended), the material showed minimal necrosis, almost indistinguishable from control cultures not exposed to the bacteria, and most of the transformed cells are viable, as evidenced by the development of many multicelled CFP expressing stable transformation events or 'sectors'.

The production of multicelled CFP expressing stable transformation events or 'sectors' correlated well with subsequent regeneration of whole plant transgenic events. This transformation technology is now functioning at an efficiency that can be applied on an industrial scale. In varieties such as Q117 and L97-128, transgenic events can be efficiently produced at a transformation efficiency of 20 events per gram of fresh target tissue. These events, when

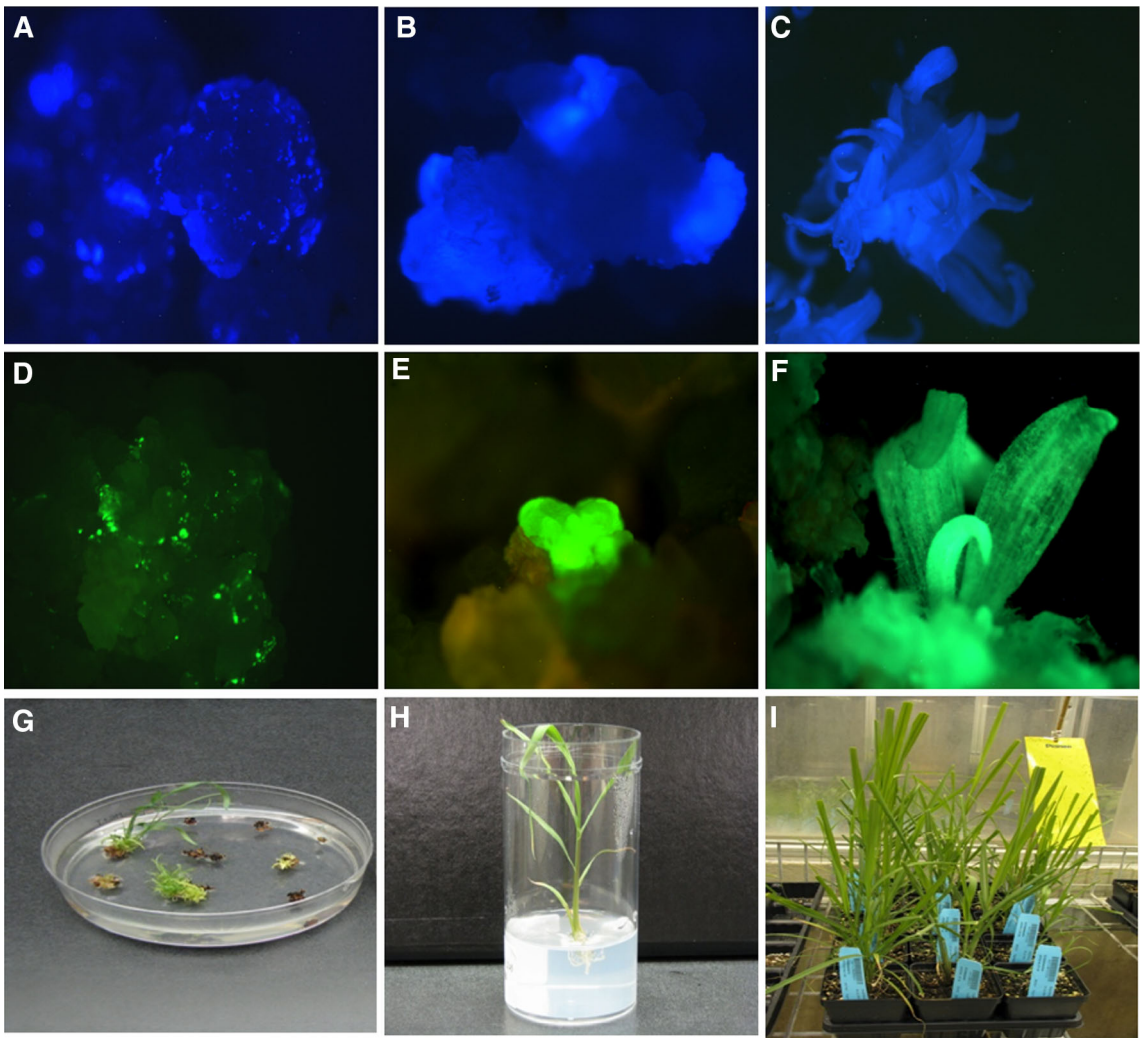

Fig. 1 Efficient sugarcane transformation system development. **a** Embryogenic callus piece exhibiting transient CFP expression indicating successful gene delivery (5 days after infection). **b** Embryogenic callus piece with three separate multicelled CFP expressing stable transformation events or 'sectors' at 3 weeks after infection. **c** Transgenic CFP expressing shoot. **d** Embryogenic callus piece exhibiting transient GFP expression indicating successful gene delivery to some cells. **e** Embryogenic callus piece with a multicelled GFP expressing stable transformation event or 'sectors' at 3 weeks after infection. **f** transgenic GFP expressing shoot. **g** Shoots on selection media. **h** Plantlet on rooting media. **i** Plantlets in greenhouse

Table 2 Estimated sugarcane transformation efficiency in 'model' sugarcane varieties

L97-128	PMI/mannose	15
L97-128	NPTII/geneticin	20
Q117	NPTII/geneticin	5[a]

Events/g target tissue is the total number of independent transgenic events produced per gram of inoculated embryogenic callus. PMI is the phosphomannoseisomerase selectable marker gene using mannose as a selection agent. NPTII is the neomycin phosphotransferase II selectable marker gene using geneticin as a selection agent

[a] Target tissue included original leaf roll material along with embryogenic callus

screened by TaqManquantitative PCR, can be quickly assessed for transgene copy number. Events with low (1 or 2) transgene copy number were transferred to the greenhouse for further analysis.

Confirmation of transgene expression was measured via qRT-PCR (data not shown) and quantitative ELISA (qELISA) showing the expected range of expression from undetectable to high expression, subject to variations due to intactness of integration events and transgene insertion site. Transgene expression stability was monitored through vegetative propagation and ratooning in the field. Expression was consistent across generations when a single event

Table 3 Long-term CFP transgene expression based on the analysis of 3 independent transformation events in both variety 1 and 2 and 2 independent events in variety 3

Generation	2010		2011	
	V1 (Field)		V1R1 (Field)	
CFP (ng/mg TSP)	Mean	SD	Mean	SD
Variety 1	455	111	225	83
Variety 2	522	218	426	95
Variety 3	467	12	738	72

V1 the first vegetative propagation from stalk material of T0 plants. V1R1 the first ratoon of first vegetative propagation

was tested in the plant cane and ratoon generations. Further expression analysis of these events is in progress.

Conclusion

The *Agrobacterium*-mediated transformation method for sugarcane reported here has now been validated in over eight sugarcane varieties by more than six researchers at two separate laboratories, providing the most robust sugarcane transformation system reported to date. The events generated showed a high proportion of single copy, commercially relevant insertions based on copy number, insert integrity and lack of vector backbone sequences.

Further field analysis of these events has demonstrated transgene stability across clones from a single event and across multiple generations. This stability of transgene expression across sugarcane varieties and through multiple growing seasons demonstrates the broad utility of our transformation technology in the application of biotechnology for sugarcane variety improvement.

This technology is currently being applied to support multiple sugarcane biotechnology crop improvement projects to bring yield-enhanced products to the market.

Acknowledgments Work conducted on Q117, Q208 and KQ228 was conducted at the Syngenta Centre for Sugarcane Biofuels Development, a collaboration between Syngenta, Queensland University of Technology and Farmacule Bioindustries. We thank BSES Limited for the supply of sugarcane materials used in transformation in Australia, and Louisiana State University and the USDA for providing the varieties used for experiments conducted in the US.

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