Plant Mol Biol (2014) 84:443–454
DOI 10.1007/s11103-013-0140-2

An improved chemically inducible gene switch that functions in the monocotyledonous plant sugar cane

Mark Kinkema · R. Jason Geijskes · Kylie Shand · Heather D. Coleman · Paulo C. De Lucca · Anthony Palupe · Mark D. Harrison · Ian Jepson · James L. Dale · Manuel B. Sainz

Received: 10 March 2013 / Accepted: 7 October 2013 / Published online: 20 October 2013

Abstract Chemically inducible gene switches can provide precise control over gene expression, enabling more specific analyses of gene function and expanding the plant biotechnology toolkit beyond traditional constitutive expression systems. The *alc* gene expression system is one of the most promising chemically inducible gene switches in plants because of its potential in both fundamental research and commercial biotechnology applications. However, there are no published reports demonstrating that this versatile gene switch is functional in transgenic monocotyledonous plants, which include some of the most important agricultural crops. We found that the original *alc* gene switch was ineffective in the monocotyledonous plant sugar cane, and describe a modified *alc* system that is functional in this globally significant crop. A promoter consisting of tandem copies of the ethanol receptor inverted repeat binding site, in combination with a minimal promoter sequence, was sufficient to give enhanced sensitivity and significantly higher levels of ethanol inducible gene expression. A longer CaMV 35S minimal promoter than was used in the original *alc* gene switch also substantially improved ethanol inducibility. Treating the roots with ethanol effectively induced the modified *alc* system in sugar cane leaves and stem, while an aerial spray was relatively ineffective. The extension of this chemically inducible gene expression system to sugar cane opens the door to new opportunities for basic research and crop biotechnology.

Keywords *alc* gene switch · Sugar cane · *Saccharum* · Ethanol inducible · Gene expression · Monocot

Accession numbers: scoalcR (KC189903), scoGUS (KC189904), var4 (KC189905), var5 (KC189906), var8 (KC189907).

Electronic supplementary material The online version of this article (doi:10.1007/s11103-013-0140-2) contains supplementary material, which is available to authorized users.

M. Kinkema · R. J. Geijskes · K. Shand · H. D. Coleman · P. C. De Lucca · A. Palupe · M. D. Harrison · J. L. Dale · M. B. Sainz
Syngenta Centre for Sugar Cane Biofuels Development, Centre for Tropical Crops and Biocommodities, Queensland University of Technology, Brisbane, QLD 4001, Australia

Present Address:
M. Kinkema (✉)
AgBiTech, 8 Rocla Court, Glenvale, QLD 4350, Australia
e-mail: Mark.Kinkema@qut.edu.au

Present Address:
R. J. Geijskes · P. C. De Lucca
Syngenta Proteção de Cultivos Ltda., Rodovia BR 452 km 142, Uberlândia, MG 38407-049, Brazil

Present Address:
H. D. Coleman
Department of Biology, Syracuse University, 107 College Place, Syracuse, NY 13244, USA

Present Address:
A. Palupe · J. L. Dale
Centre for Tropical Crops and Biocommodities, Queensland University of Technology, Brisbane, QLD 4001, Australia

I. Jepson
Syngenta Biotechnology Inc., Research Triangle Park, NC 27709, USA

Present Address:
M. B. Sainz
Syngenta Proteção de Cultivos Ltda., Av Maurílio Biagi 800 9º andar, Ribeirão Prêto, SP 14020-750, Brazil

Introduction

Constitutive expression of transgenes has been the traditional approach used to dissect the function of genes in developmental and physiological processes in plants. The value of this approach, however, is limited when applied to genes that are detrimental to plant development or have multiple functions that may be epistatic (Laufs et al. 2003). More sophisticated technologies that enable precise control over the spatial and temporal expression of genes can overcome these technical challenges. Biotechnology is also utilizing more specific expression tools for crop improvement. For example, while the first commercial, genetically modified crops used constitutive promoters to control the expression of herbicide tolerance (Shah et al. 1986) and insect resistance (Perlak et al. 1991) genes, we are now seeing the emergence of commercial, genetically modified crops with improved output traits such as golden rice (Paine et al. 2005), high-lysine corn (Houmard et al. 2007), and Enogen® corn that were created using tissue-specific promoters to drive transgene expression.

Chemically inducible gene switches enable both temporal and spatial regulation of gene expression, and can be used to more precisely characterize gene function in plants (Caddick et al. 1998; Deveaux et al. 2003; Laufs et al. 2003; Maizel and Weigel 2004). These gene switches also hold great promise for crop improvement that cannot be achieved using constitutive expression of transgenes. For example, they can be used to create traits such as synchronized flowering and fruit ripening, modified plant architecture and improved yield (Ait-ali et al. 2003), conditional male sterility in hybrid seed production (Unger et al. 2002), and antibiotic marker-free plants (Hare and Chua 2002). Furthermore, chemically inducible gene switches have the potential to restrict the production of industrial proteins or pharmaceuticals that may otherwise be deleterious to plant development until maximum biomass has been generated, thereby maximizing recombinant protein yields (i.e. plant biofactories).

A number of chemically inducible gene expression systems have been developed for plants. These systems are induced by chemicals such as the antibiotic tetracycline, copper, steroids (dexamethasone and estrogen), insecticides, and ethanol (Padidam 2003; Moore et al. 2006). While all of these different systems have been successfully used in different dicotyledonous plants, only a few have been shown to be functional in monocotyledonous plants. The dexamethasone inducible GVG (Ouwerkerk et al. 2001) and estrogen inducible XVE (Okuzaki et al. 2011) systems are capable of driving ligand-dependent transgene expression in rice, although the latter has only been evaluated in young seedlings. While these systems show promise for regulating gene expression in the laboratory, the major disadvantage of these two systems is that the inducing chemicals are steroid hormones, which would preclude their use in the field. The insecticide inducible VGE system, however, utilizes the commercially available, registered, non-steroidal ecdysone agonists tebufenozide and methoxyfenozide (Martinez et al. 1999; Padidam 2003), and has been used successfully in maize to conditionally restore fertility to the maize *ms45* male-sterile mutant (Unger et al. 2002).

The *alc* gene switch is considered to be one of the best chemically inducible gene expression systems for use in plants (Padidam 2003; Moore et al. 2006) because of its utility in both the laboratory and field (Li et al. 2005). This system consists of the AlcR transcription factor and one of its target promoters, *alcA*, which are components of the ethanol utilization regulon of the filamentous fungus *Aspergillus nidulans* (Felenbok 1991). AlcR binds to specific sites in the *alcA* promoter and, in the presence of an inducer (i.e. ethanol or acetaldehyde), initiates transcription of the downstream gene. In plants, the *alc* gene switch can temporally regulate gene expression throughout the plant or in a spatially-restricted manner by putting *alcR* under the control of either a constitutive or tissue-specific promoter, respectively (Caddick et al. 1998; Deveaux et al. 2003; Maizel and Weigel 2004). This chemically inducible system displays little or no apparent basal expression in plants in the absence of the inducer, and enables rapidly inducible and reversible gene expression following ethanol treatment (Caddick et al. 1998; Salter et al. 1998; Roslan et al. 2001). The inducer, ethanol, is inexpensive, biodegradable, readily available, and non-phytotoxic at the levels necessary to induce gene expression. While the *alc* gene switch has been shown to function in a variety of dicotyledonous plants including tobacco (Caddick et al. 1998), Arabidopsis (Roslan et al. 2001), poplar (Filichkin et al. 2006), potato (Sweetman et al. 2002), oilseed rape (Sweetman et al. 2002), and tomato (Garoosi et al. 2005), it has not been reported to be functional in monocotyledonous plants.

Sugar cane is the world's largest crop by tonnage (1.7 billion metric tons in 2010), and is grown on over 24 million hectares in around 120 countries (FAOSTAT 2008). It is a valuable industrial crop used for the production of approximately 75 % of the world's sugar and a significant portion of the world's biofuel (FAOSTAT 2008). Sugar cane is also the most productive biomass crop, yielding an annual dry biomass of approximately 39 t/ha (bagasse and trash), which is significantly higher than that of other biomass crops such as *Miscanthus* (29.6 t/ha), switchgrass (10.4 t/ha), and maize (17.6 t/ha grain plus stover) (Heaton et al. 2008). The difficulties and limitations associated with the classical breeding in this highly polyploid crop, and the broad potential of genetic engineering, make biotechnology an attractive approach for the improvement of sugar cane

(Lakshmanan et al. 2005). Given its high biomass yield, the cellulose present in sugar cane is an enormous and renewable source of fermentable sugars for biofuel production. The creation of transgenic plants capable of expressing the cellulase and hemicellulase enzymes needed to convert the biomass into fermentable sugars will greatly improve the economic sustainability of cellulosic ethanol production (Sainz 2009; Harrison et al. 2011). A functional *alc* gene switch could be valuable for this application because the constitutive expression of either individual or combinations of cell wall hydrolysing enzymes may be detrimental to plant development. Furthermore, dissecting the function of genes involved in complex, developmentally-regulated processes in sugar cane such as sucrose biosynthesis, transport, and metabolism would be greatly facilitated by a system that enables precise spatial and temporal control over transgene expression.

Here, we report the development of an improved *alc* gene switch that functions effectively in a broad-acre, commercially-grown monocotyledonous plant, namely sugar cane. Transgenic sugar cane possessing this improved *alc* gene expression system displayed stable, ethanol inducible gene expression. The improved *alc* gene switch was effectively induced in large, glasshouse-grown sugar cane plants through the application of ethanol to the roots. The improvement of this chemically inducible gene expression system may enhance its application in basic plant biology research and the genetic improvement of crops.

Materials and methods

Vector construction

For the non-optimized vectors, a *Zm-Ubi1-GS* (GUS syntron) construct was generated that contained the *Zm-Ubi1* promoter, the *GUS* reporter gene containing a synthetic intron (*GS*), and the nopaline synthase (*nos*) terminator. The *GS* sequence consisted of a 232 bp 1st exon, 84 bp synthetic intron, and 1,580 bp 2nd exon. For the non-optimized *alc* gene switch, a *Zm-Ubi1-alcR alcA-GS* construct was generated that contained the *alcR* coding sequence under the control of *Zm-Ubi1*, and *GS* under the control of the original *alcA* promoter.

Sugar cane optimized *alc* switch constructs (Fig. 1a) were generated by incorporating the following modifications: (1) the *alcR* and *uidA* (*GUS*) coding sequences were optimized for expression in sugar cane (*scoalcR* and *scoGUS*, respectively) by Geneart AG through the modification of the *alcR* and *GUS* coding sequences without altering the encoded protein sequences. The sequences were modified to increase the overall G+C content, improve the codon adaptation index (how well the codons match the codon usage preference of sugar cane), and eliminate potential cryptic splice sites, polyadenylation consensus sequences, and potential ribosome binding sites. (2) the TMV omega translational enhancer sequence (5′-gtattttttacaacaattaccaacaacaacaaacaacaaacaacattacaattactatttacaattaca-3′) followed by an alternative Kozak sequence (gcggccgcc) were placed immediately upstream of the *scoalcR* and *scoGUS* coding sequences; the nine different *alcA* promoter variants, in addition to the original *alc* switch, were synthesized by Geneart and cloned directly upstream of the *TMV omega-scoGUS*. The *Zm-Ubi1-scoGUS* consisted of the *Zm-Ubi1* promoter followed by TMV omega and the alternative Kozak sequence upstream of *scoGUS*.

Plant transformation, growth, and maintenance

Field-grown sugar cane material used for callus production was kindly provided by the Bureau of Sugar Experiment Stations (BSES Ltd.). Transgenic plants were regenerated from embryogenic sugar cane callus that had been transformed either by microprojectile bombardment (MPB) (non-optimized vectors; cultivar Q117) or *Agrobacterium*-mediated transformation (sugar cane optimized vectors; cultivar KQ228). Transformation using MPB and *Agrobacterium*-mediated transformation was carried out as described previously (De Lucca et al. 2010; Harrison et al. 2011).

Plants were initially moved from tissue culture and placed in seedling trays containing soil and incubated in a growth chamber. After transfer to soil, the plants were analyzed for the presence of the transgene and copy number (1, 2, >2) using TaqMan real-time PCR analysis (Ingham et al. 2001) of all the relevant transgenes (i.e. *nptII*, *GUS*, *scoGUS*, *alcR*, *scoalcR*). Samples from a transgenic sugar cane plant possessing a single copy of the *nptII* gene (as determined by Southern blot analysis) were included as a control in the TaqMan assay. At approximately 8 weeks post transfer to soil, the T0 plants were moved to 4.5 l pots and maintained in a glasshouse at 28 °C with only natural lighting. At approximately 7 months post transfer to soil, T0 plants were transferred into 14 l pots until maturity (approximately 12 months of age). Setts containing a single bud were obtained from stems of mature T0 and T0V1 (vegetatively-propagated plants from setts of T0 plants) plants, germinated in soil, and maintained in 14 l pots in the glasshouse.

Ethanol treatment

Transgenic plants possessing the original non-optimized *alc* gene switch (generated using MPB) were characterized at approximately 15 weeks post transfer to soil. Ethanol treatment was carried out using 10 % (v/v) ethanol and

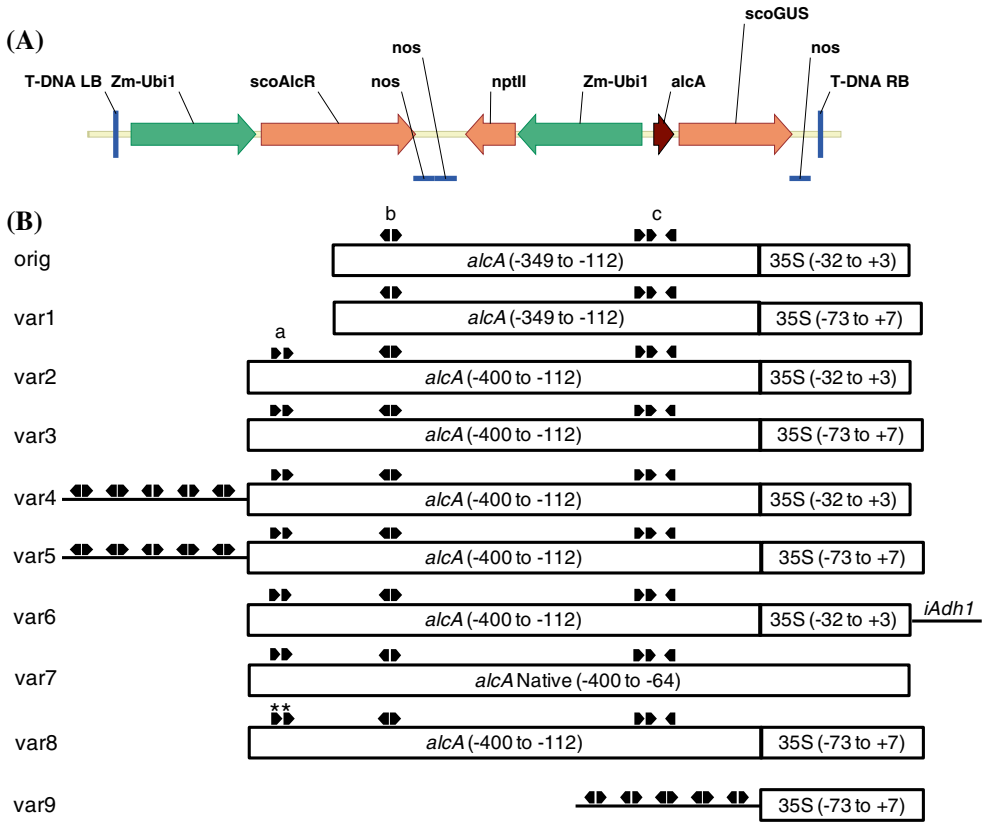

Fig. 1 Schematic representations of the *alcA* constructs. **a** Overview of the *alc* gene switch construct design. **b** Schematic representations of the original (orig) *alcA* promoter developed for plants and *alcA* promoter variants (var) that were tested for ethanol inducibility in sugar cane. The AlcR binding sites (*a*, *b*, *c*) within the *alcA* promoter consist of either two direct repeats (binding site "a"), two inverted repeats (binding site "b"), or three half sites consisting of both direct and inverted repeats (binding site "c"). **Indicates the AlcR "a" binding sites that were mutated to more closely resemble the consensus binding site sequence. *iAdh1*, intron from the maize *Adh1* gene. Numbering for the *alcA* promoter indicates nucleotide positions relative to the *alcA* translational start site. Numbering for the CaMV 35S minimal promoter indicates nucleotide positions relative to the CaMV 35S transcriptional start site

subsequently with 2 % (v/v) ethanol (700 ml per 4.5 l pot root drench and an aerial spray until runoff). After treatment, the plants were enclosed using plastic sheeting to maximize their exposure to the ethanol vapor. Leaf samples were taken just prior to ethanol treatment and from the same leaf at three and 6 days post treatment (d.p.t.) for histochemical analysis as described below.

Transgenic plants possessing the sugar cane optimized constructs (generated using *Agrobacterium*-mediated transformation) were initially characterized at 4–7 weeks post transfer to soil using either a 2 % (v/v) ethanol treatment (daily 2 % (v/v) ethanol root drench and aerial spray for four consecutive days) or with 1 % (v/v) ethanol using a single root drench (800 ml per seedling tray) and aerial spray until runoff. Leaf samples were taken just prior to ethanol treatment and at five d.p.t. from the same leaf. For analysis of ethanol inducible expression in older plants, all the single copy plants for each construct were selected and transferred to the glasshouse. Approximately 6-month-old T0 plants were treated with 5 % (v/v) ethanol using a single root drench (700 ml per 4.5 l pot; approximately 15.5 ml of 5 % ethanol per 100 ml of soil) and aerial spray. Leaf samples were taken just prior to ethanol treatment and at two, four, and seven d.p.t. from the same leaf. Analysis of ethanol inducible expression in T0V1 and T0V2 plants utilized a 5 % (v/v) ethanol induction consisting of either a single root drench (2 l per 14 l pot) and aerial spray until runoff, root drench alone, or aerial spray alone. Ethanol toxicity experiments were carried out on approximately 6-month-old wild type (cultivar KQ228) rationed (i.e. regrown after harvesting) plants and 4-week-old wild type (cultivar KQ228) sett plants using a soil root drench.

Analysis of GUS accumulation

Leaf samples equal to approximately five standard hole punches were taken from the first fully unfurled leaf. For histochemical analysis, leaf samples were vacuum infiltrated (30 min) with 50 mM Na_3PO_4, 0.01 M EDTA, 500 nM ferrocyanide, 500 nM ferricyanide, 0.1 % (v/v) Triton

X-100 and 1 mM 5-Bromo-4-chloro-3-indolyl-beta-D-glucuronic Acid (X-Gluc) (Phytotechnology laboratories), and incubated at 37 °C for 48 h. Following staining, chlorophyll was removed from the leaf samples by repeated washing in 100 % ethanol. Transverse stem sections were incubated in the staining buffer (without vacuum infiltration) for 24 h and subsequently stored in 100 % ethanol.

GUS accumulation was quantitatively analyzed by ELISA as previously described (Harrison et al. 2011). A sample from the first fully unfurled leaf equal to approximately four standard hole punches was taken and placed into one well of a 96-well sample block on ice. Each plant was sampled in duplicate from the same leaf. Samples were subsequently frozen at −80 °C and freeze-dried prior to analysis.

RNA analysis

Samples for RNA analysis were taken from the first fully unfurled leaf and a young stem section (internode 4) of approximately 6-month-old plants. RNA was isolated using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions, and 1 μg of RNA was used to make cDNA using the M-MLV reverse transcriptase system (Promega) according to the manufacturer's instructions. Primers specific to the sugarcane *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) gene (Forward: 5′-cacggccactggaagca-3′; Reverse: 5′-tcctcagggttcctgatgcc-3′) were used in a PCR to verify that the cDNA synthesis was successful and that there was no genomic DNA contamination in the reverse transcriptase (RT) negative samples. Primers specific to the *scoGUS* gene (Forward: 5′-cgtgcgtgaacgtgatgttct-3′; Reverse: 5′-agcgcttttgcagcaggaa-3′) were used in a PCR (95 °C for 3 min, and 25 or 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min), and the PCR products were subsequently visualized using SYBR safe DNA gel stain (Invitrogen).

Results

Functional analysis of the original *alc* gene switch in sugar cane

There are no reports demonstrating that the original *alc* gene switch is functional in monocotyledonous plants. The functionality of the *alc* gene switch was evaluated in sugar cane using the AlcR transcription factor constitutively expressed from the *Zm-Ubi1* promoter, and the original ethanol inducible *alcA* promoter developed for plants (Caddick et al. 1998) driving expression of the *beta-glucuronidase* (*GUS*) reporter gene (Jefferson et al. 1987). No GUS accumulation was detected by histochemical staining in any of the 21 confirmed transgenic *alc* gene switch plants (generated using MPB) following induction with either 2 % (v/v) or 10 % (v/v) ethanol (data not shown). The lack of any detectable ethanol inducible expression in transgenic sugar cane may be due to different factors such as low expression of the ethanol receptor AlcR and/or low sensitivity of the *alcA* promoter.

Development of a functional *alc* gene switch in sugar cane

To develop a functional *alc* gene switch in sugar cane, we designed and tested nine different *alcA* promoter variants (Fig. 1b; Supplemental Table S1). Modifications to the original *alcA* promoter engineered for plants included (1) a longer CaMV 35S minimal promoter element because this was shown to substantially increase chemically inducible gene expression in tobacco (Martinez et al. 1999); (2) incorporation of the entire 5′ region of the *A. nidulans alcA* promoter which possesses an additional AlcR binding site shown to be important for ethanol inducibility (Panozzo et al. 1997); (3) incorporation of five tandem copies of the AlcR inverted repeat binding site; (4) incorporation of a maize *Adh1* intron which has been shown to increase gene expression in plants (Callis et al. 1987); (5) mutation of the weak AlcR binding site "a" to better match the consensus binding site sequence (Nikolaev et al. 1999) for improved transcription factor binding and; (6) use of the native *A. nidulans alcA* promoter sequence instead of the heterologous CaMV 35S minimal promoter. For improved expression of *alcR*, all of the constructs utilized a sugar cane optimized *alcR* gene (*scoalcR*; driven by *Zm-Ubi1*; Fig. 1a). In addition, a sugar cane optimized *GUS* gene (*scoGUS*; driven by the different *alcA* promoters) was also used for improved expression (Fig. 1a). *Agrobacterium*-mediated transformation was used to generate a total of 448 independent transgenic events, with between 25 and 66 confirmed transgenic sugar cane plants for each construct (Table 1).

Ethanol inducible expression was initially investigated using GUS histochemical staining of leaves from 4 to 7 week old plants. To do this, the transgenic plants were divided into two groups. One group was evaluated for ethanol inducible expression in response to a repeated (daily) 2 % (v/v) ethanol root drench and aerial spray for four consecutive days. The other group was evaluated for ethanol inducible expression in response to a milder ethanol induction consisting of a single 1 % (v/v) ethanol root drench and aerial spray. GUS histochemical staining was assessed in both groups after 5 days. Following the daily 2 % ethanol treatment, GUS accumulation was detected in a considerably higher proportion of transgenic plants containing some of the *alcA* promoter variants (up to 80 % for var4) than was observed for the original *alcA* promoter (17 %) (Table 1; Supplemental Table S2). The milder ethanol

Table 1 GUS histochemical staining in the transgenic sugar cane plants at 4–7 weeks post transfer to soil

Construct	GUS histochemical staining		
	Total number of transgenic plants	Induced by 2 % ethanol[a]	Induced by 1 % ethanol[a]
orig	25	2/12 (17 %)	0/13 (0 %)
var1	44	6/30 (20 %)	0/14 (0 %)
var2	31	1/17 (6 %)	0/14 (0 %)
var3	66	8/16 (50 %)	2/50 (4 %)
var4	44	8/10 (80 %)	20/34 (59 %)
var5	57	12/27 (44 %)	19/30 (63 %)
var6	44	0/6 (0 %)	0/38 (0 %)
var7	33	10/18 (56 %)	1/15 (7 %)
var8	40	4/20 (20 %)	0/20 (0 %)
var9	64	12/32 (38 %)	13/32 (41 %)

Histochemical staining was assessed at 5 days post treatment with either a daily 2 % root drench and aerial spray or a single 1 % root drench and aerial spray

[a] Plants were considered induced if, following ethanol treatment, the leaf samples clearly possessed stronger GUS staining than the uninduced samples based on visual examination of the samples following removal of the chlorophyll

treatment further differentiated the ethanol sensitivity of the different promoters, with only those plants containing var4 (59 %), var5 (63 %), and var9 (41 %) having a high proportion of ethanol inducible expressing plants (Table 1; Supplemental Table S2). Var3 and var7 were the only other promoters to yield detectable expression in response to the milder ethanol treatment, with just 4 and 7 % of the plants showing visible GUS staining, respectively (Table 1). The var4, var5, and var9 promoters all incorporate five tandem copies of the AlcR inverted repeat binding site (Fig. 1b), indicating that this modification substantially improved the performance of the *alc* gene switch in sugar cane.

Characterization of transgenic plants containing only a single copy of the expression construct is essential for an accurate comparison of different promoters. Therefore, single copy plants for each of the constructs were selected following copy number assessment (Supplemental Table S2). These single copy plants were assessed for ethanol inducible *GUS* expression at 6 months (1.5–2 m tall) post-transfer to soil using a 5 % root drench and aerial spray. No GUS activity was detected in the plants prior to ethanol treatment (data not shown). Following treatment, significantly higher ethanol inducible expression was detected in the leaves of sugar cane plants with the novel promoter variants (var4, var5 and var9) incorporating five tandem copies of the AlcR inverted repeat binding site (Fig. 2a). The pentamer alone (var9), without any additional *alcA* promoter sequence, was sufficient for enhanced ethanol inducible expression (Fig. 2a). Ethanol inducible expression was also assessed in the stem of transgenic sugar cane possessing the original *alc* gene switch promoter and the var5 and var9 promoters using GUS histochemical staining. No GUS accumulation was detected in any of the plants prior to ethanol treatment (Fig. 2b). Following ethanol induction, GUS accumulation was detected in the stem of transgenic sugar cane possessing the improved var5 and var9 promoters, but not the original *alc* gene switch promoter (Fig. 2b).

In addition to the tandem copies of the AlcR inverted repeat binding site, the choice of minimal promoter also showed a substantial effect on ethanol inducible expression. Promoter variants containing the longer CaMV 35S minimal promoter (−73 to +7) had consistently higher levels of ethanol inducible expression than otherwise identical variants containing a shorter CaMV 35S minimal promoter (Fig. 2a; compare orig to var1, var2 to var3, and var4 to var5). Maximum levels of GUS accumulation from transgenic events possessing the strongest ethanol inducible promoter variant (var5) were in the range obtained with the constitutive *Zm-Ubi1* promoter (53–193 ng GUS/mg protein; Fig. 3).

The ideal chemically inducible gene switch should have no expression in the absence of inducer in addition to providing high levels of inducible expression. Using a quantitative GUS ELISA, no GUS accumulation was detected in the leaves of 24 of 27 6-month-old transgenic sugar cane plants containing the improved var4, var5, and var9 promoters prior to ethanol treatment (with a lower detection limit of approximately 1.8 ng GUS ml^{-1}), and only low levels (1.1 ± 0.63 ng GUS mg^{-1} protein) were detected in the remaining three plants. Analysis of promoter activity at the transcriptional level, however, revealed that a low level of GUS transcript was present in both the leaves and stems of uninduced plants (Fig. 4).

Induction of the *alc* gene switch in response to root drench vs aerial spray

Ethanol induction of the 6-month-old, single copy transgenic sugar cane described above was achieved using a combination of an aerial spray and root drench. Therefore, we examined the relative effectiveness of using either an aerial spray or root drench alone to induce the improved *alc* gene switch in sugar cane. This experiment was carried out using clones from stem cuttings (setts) of a single-copy, mature var9 plant. Strong GUS staining was detected in the leaves, and young and mature stem sections of all three clones that were induced using a 5 % ethanol root drench alone (representative images shown in Fig. 5). In contrast, GUS staining was either absent or weak in both the leaves and stems of plants treated with a 5 % ethanol aerial spray alone (Fig. 5). Plants treated with a 5 % ethanol root drench remained healthy, with little or no detectable effects other than occasional yellowing of the oldest leaves.

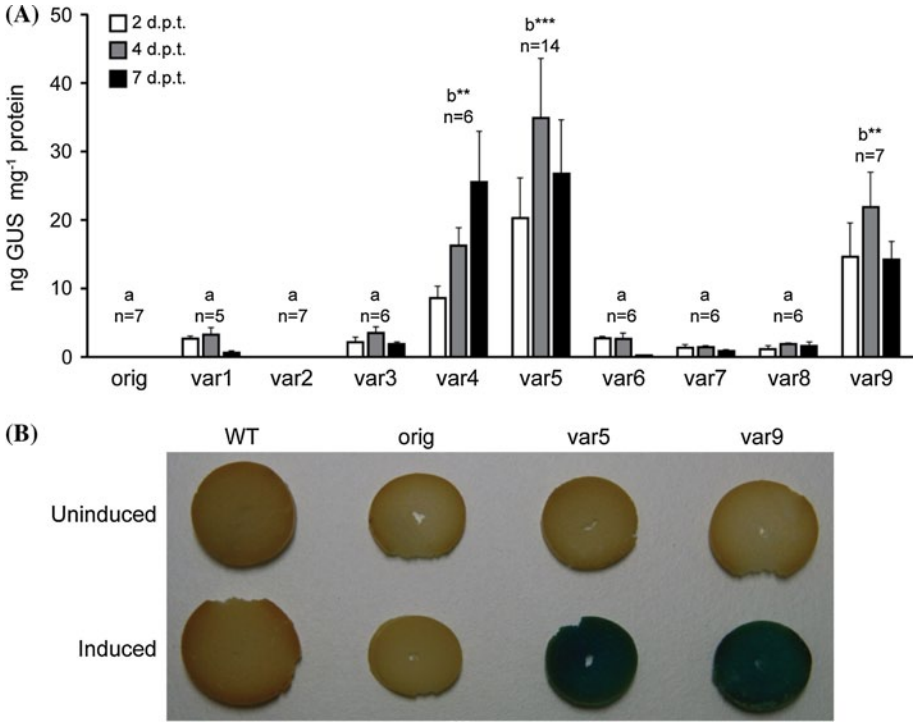

Fig. 2 Ethanol inducible expression in transgenic plants containing the different *alcA* promoter variants. **a** GUS accumulation in leaf of 6-month-old sugar cane at different days post treatment (d.p.t.) with a 5 % ethanol root drench and aerial spray. Data are mean ± SE. n = number of independent, single copy transgenic plants analyzed for each construct. Data with different letters are significantly different. $^{**}P < 0.01$; $^{***}P < 0.001$. Statistical analyses were carried out using a one-way ANOVA with Tukey post test. **b** Representative GUS staining in uninduced and induced (4 d.p.t.) transverse stem sections (internode 8) taken from wild type (WT) plants, and independent transgenic vegetative subclones (T0V1) containing the original (orig) *alcA* promoter, variant5 (var5), or variant9 (var9). Ethanol induction was carried out using a 5 % ethanol root drench and aerial spray

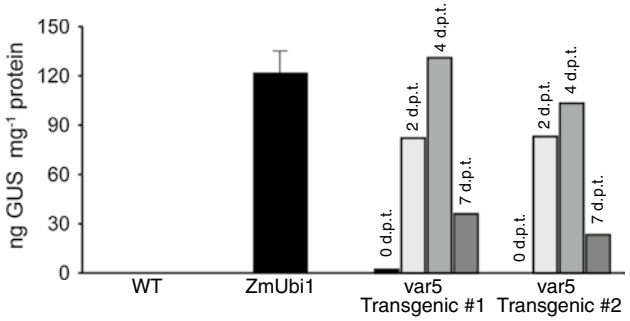

Fig. 3 Comparison of constitutive and ethanol inducible expression in transgenic sugar cane. GUS accumulation in leaf of 6-month-old wild type sugar cane and transgenic plants containing a single copy of the *Zm-Ubi1-scoGUS* or var5-*scoGUS* construct. Mean GUS accumulation is shown for untreated wild type and *Zm-Ubi1-scoGUS* (n = 11) plants and two independent var5-*scoGUS* plants prior to induction and at various days post treatment (d.p.t.) with a 5 % ethanol root drench and aerial spray

To assess the relative toxicity of ethanol on sugar cane at different stages of development, we treated both young (4-weeks-old) sugar cane plants and older (approximately 6-months-old) plants with different concentrations of an ethanol root drench. Young sugar cane plants were relatively tolerant to ethanol treatment, however some growth retardation and leaf senescence was visible in plants treated with 10 % ethanol, and some plants treated with 7.5 % ethanol showed a relatively low occurrence of leaf senescence (Fig. 6a and Supplemental Fig. S1). Young sugar cane plants treated with either 2.5 or 5 % ethanol did not display any detectable phenotype, and appeared similar to the controls treated with water alone (Fig. 6a and Supplemental Fig. S1). Similarly, more mature plants treated with a 5 % ethanol root drench did not display any detectable phenotype and appeared similar to the controls (Fig. 6b). In contrast, a 10 % ethanol root drench was highly toxic to older plants and caused severe leaf senescence throughout the plant, including the upper growing region (Fig. 6b). In older plants treated with a 7.5 % ethanol root drench, leaves of the upper growing region remained healthy while older leaves senesced (Fig. 6b).

Stability of ethanol inducible gene expression in sugar cane

Transgene expression stability between generations is an important characteristic for any plant expression system.

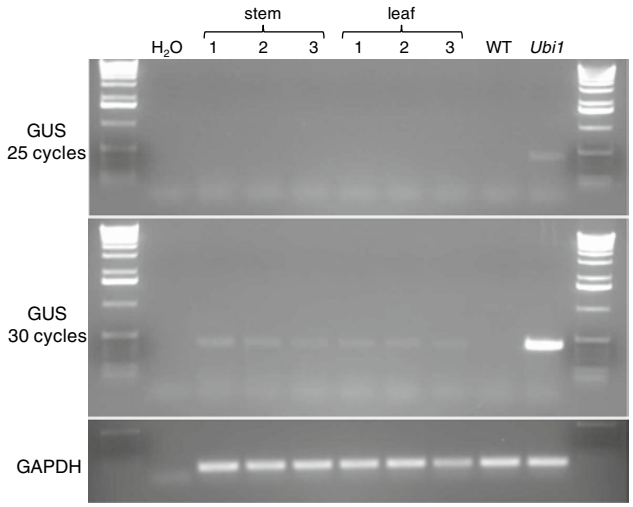

Fig. 4 RT-PCR analysis of the basal expression levels from the *alc* gene switch in transgenic sugar cane. RNA was isolated from the stem and first fully unfurled leaf of three independent, 6-month-old vegetative subclones (T0V2) from a representative, single copy var9 *alc* gene switch event. RNA from the first fully unfurled leaf of a wild type (WT) and constitutive *Zm-Ubi1-scoGUS* (*Ubi1*) plant were used as negative and positive controls, respectively. A PCR containing water alone (H_2O) also served as a negative control. Primers specific for the *scoGUS* gene were used in a PCR, and the resulting products were visualized after either 25 or 30 cycles. Primers specific for *GAPDH* were used as a control to verify the success of cDNA synthesis

Sugar cane is a vegetatively propagated crop that is typically replanted every 3–4 years. To assess the stability of the improved *alc* gene switch in sugar cane, setts from the 12 month old primary transgenic plants (T0) were planted and the resulting vegetatively propagated plants (T0V1) were assessed for ethanol inducible gene expression using a 5 % ethanol root drench and aerial spray. As shown in Fig. 7, ethanol inducible expression was maintained in the vegetatively propagated plants obtained from the independent, primary transgenic plants.

Discussion

The *alc* gene switch has proven to be a valuable tool for analyzing gene function, and for plant biotechnology applications (Deveaux et al. 2003; Maizel and Weigel 2004; Werner et al. 2011), but to date this system has been limited to dicotyledonous plants. The improved *alc* gene switch described here will enable this valuable gene expression technology to be utilized in the globally-important monocotyledonous crop sugar cane. A single ethanol treatment was sufficient to provide ethanol inducible gene expression in large (approximately 1.5–2 m tall), single copy, sugar cane plants grown in tall (5–6.5 m) glasshouses. To our knowledge, these are the largest plants to be described for use with the *alc* gene switch. For most transgenic plants it was not possible to determine the fold induction of GUS protein accumulation in response to ethanol treatment because no GUS accumulation was detected prior to induction. However, in one transgenic plant that possessed a detectable level of GUS protein prior to induction, ethanol treatment increased GUS protein accumulation by approximately 100-fold (Fig. 3, var 5 transgenic #1). The low level of leaky expression may make this system suitable for the inducible expression of a variety of different proteins, however it is currently not known how it will perform when regulating the expression of a protein that is highly toxic to plant cells. Ethanol induction was achieved without maintaining the plants in a tightly confined environment that has typically been used in other plant systems to maintain a high and continuous ethanol vapor for optimal induction (Roslan et al. 2001; Sweetman et al. 2002; Filichkin et al. 2006). It is still possible, however, that ethanol vapor from the root drench and aerial spray contributed to the ethanol inducible expression in the glasshouse experiments. The application of ethanol to the roots effectively induced the *alc* gene switch in sugar cane, while an aerial spray was relatively ineffective. These results are consistent with a previous study showing that a root drench gave approximately fivefold higher expression than an aerial spray in tobacco leaves (Sweetman et al. 2002). We found that root treatment induced the *alc* gene switch in both mature and young stem, as well as the upper leaves of the sugar cane, suggesting that the ethanol was being transported throughout the plant in the xylem. Plants treated with a single 5 % root drench (with or without an aerial spray) remained healthy, with the exception of some yellowing of the oldest leaves. The effect of repeated ethanol root drenches on sugarcane growth and development are currently not known. While the aerial spray utilized in these studies did not efficiently induce the *alc* gene switch, it is possible that improved induction may be achieved by ensuring that the stomata are fully open at the time of application, and/or by using an ethanol formulation that includes a surfactant.

While the original *alc* gene switch is functional in a range of dicotyledonous plants, there are no published reports demonstrating its functionality in monocotyledonous plants. Our results support the hypothesis that the original *alc* gene switch developed for plants functions poorly in monocotyledonous plants, in particular sugar cane. Using standard expression constructs that were not modified for optimal expression in sugar cane, no ethanol inducible expression was detected using the original *alc* gene switch that has been shown to function in a variety of dicotyledonous plants (Caddick et al. 1998; Roslan et al. 2001; Sweetman et al. 2002; Garoosi et al. 2005; Filichkin et al. 2006). Using gene optimized *alcR* (*scoalcR*) and *GUS*

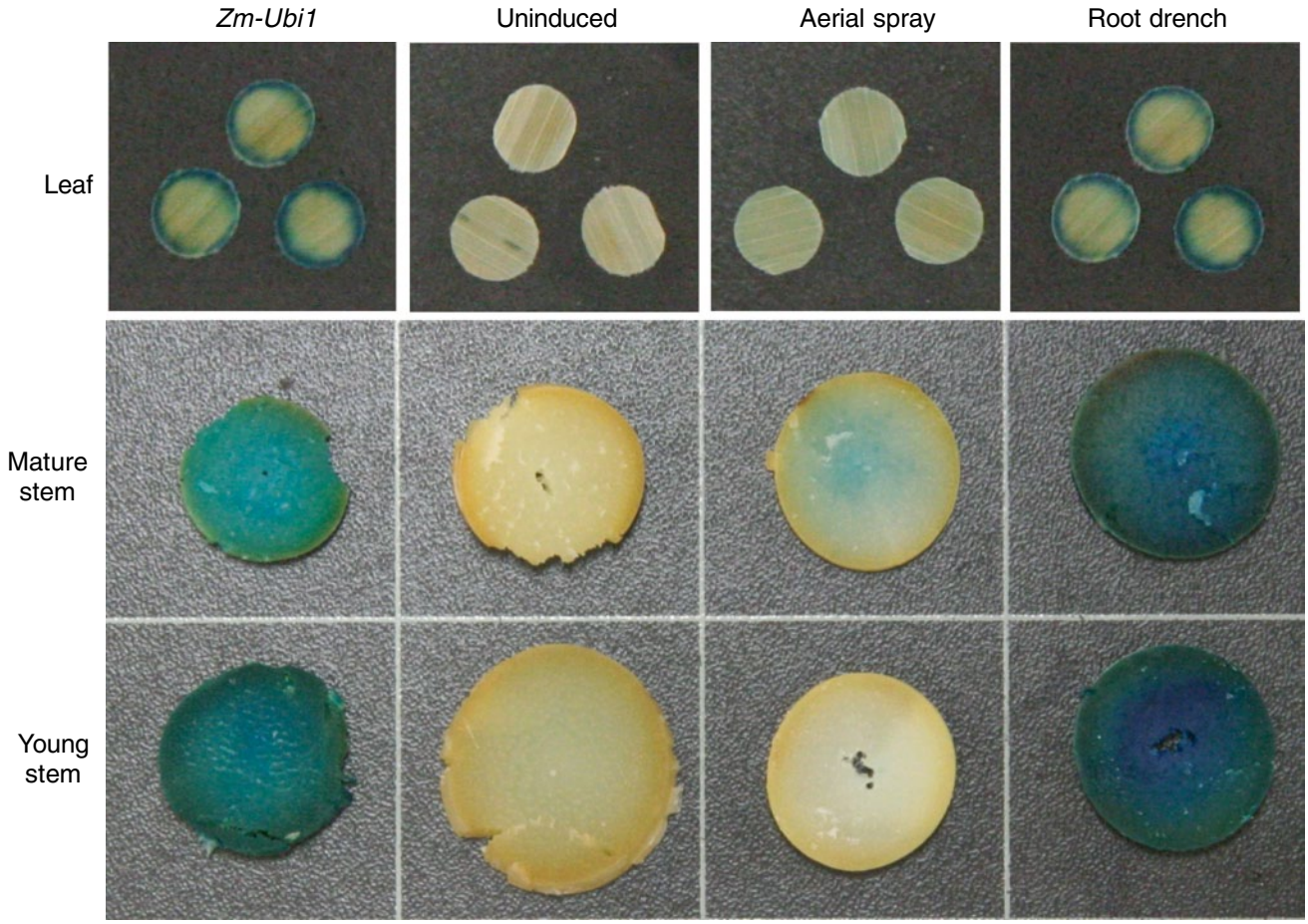

Fig. 5 Comparison of ethanol inducible expression in response to aerial spray or root drench. GUS staining in leaf and transverse stem sections (Young, internode 4; Mature, internode 13) of 6-month-old constitutive *Zm-Ubi1-scoGUS* (*Zm-Ubi1*), and T0V2 vegetative subclones from a representative var9 *alc* gene switch event. The *alc* switch subclones were analyzed prior to induction (uninduced), and after induction (4 d.p.t.) using either an aerial spray or root drench. GUS staining was similar for the three independent clones analyzed for each treatment, and representative images are shown

(*scoGUS*) coding sequences for improved expression in sugar cane, only 2 of 12 transgenic plantlets (17 %) showed detectable ethanol inducible expression from the original *alcA* promoter, and both of these plantlets possessed multiple copies of the expression construct (Supplemental Table S2). This ethanol inducible expression was only detected in plantlets in response to a relatively strong ethanol induction, and was undetectable in the 13 plantlets exposed to a milder ethanol treatment. Increased expression of the *scoalcR* and *scoGUS* sequences relative to the non-optimized sequences was expected to enhance the performance of the gene switch. Expression from a *Zm-Ubi1-scoGUS* construct has been shown to be approximately 32–50 fold higher than the expression from a standard *Zm-Ubi1-GUS* (Kinkema, unpublished data). At present it is not clear why the functionality of the original *alc* gene switch differs so dramatically between dicotyledonous and monocotyledonous plants.

Six different modifications to the *alcA* promoter were evaluated in order to develop a functional *alc* gene switch for sugar cane. Interestingly, the only modification that significantly improved the *alc* gene switch in sugar cane was the incorporation of five tandem copies of the AlcR inverted repeat binding site. This was surprising because some of the other modifications have been shown to substantially improve chemically inducible gene expression in either *A. nidulans* or transgenic plants. For example, deletion of AlcR binding site "a" within the *alcA* promoter decreased ethanol inducible expression by approximately 70 % in *A. nidulans* (Panozzo et al. 1997), but the addition (or deletion) of this binding site to the *alc* promoter did not have any significant effect on the *alc* gene switch in sugar cane (Fig. 2a; compare orig to var2 and var1 to var3). However, we cannot rule out the possibility that the AlcR binding site "a" may have a modest effect on the *alc* gene switch in sugar cane because a higher proportion of

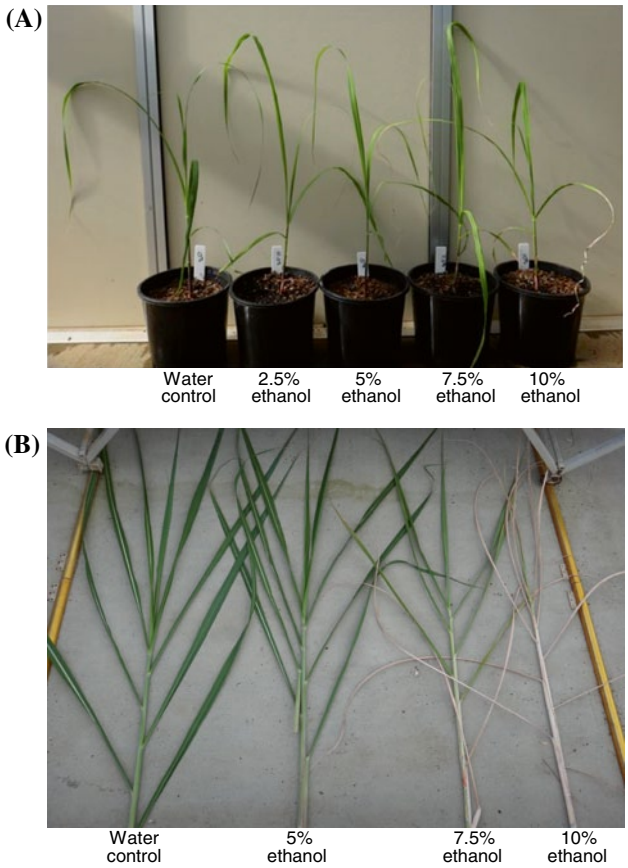

Fig. 6 Ethanol toxicity in sugar cane. Plants were treated using a single ethanol root drench at the indicated concentrations. Photographs were taken 10 days after treatment. **a** 4-week-old sugar cane plants treated with different concentrations of ethanol. Photograph shows a selected plant for each of the ethanol treatments for comparison. See Supplemental Fig. S1 for images of all plants for each treatment. **b** Photographs for the approximately 6-month-old plants, showing the upper, approximately one-third of the sugar cane plants

plants containing a construct possessing this binding site (var3) showed detectable ethanol inducible expression relative to the comparable construct lacking this site (var1; Table 1). In tobacco, a longer CaMV 35S minimal promoter increased insecticide inducible gene expression up to 20-fold (Martinez et al. 1999), while a similar modification had a more modest effect on the *alc* gene switch in sugar cane (Fig. 2a; compare orig to var1, var2 to var3, and var4 to var5). It was not obvious that tandem copies of the AlcR binding site would improve the functionality of the *alc* gene switch because a previous study found that four tandem copies of the AlcR direct repeat binding site did not enhance ethanol inducible expression over that of the original *alc* gene switch (Jepson et al. 2001). Furthermore, the *alcA* promoter sequence already contains three separate AlcR binding site regions (Fig. 1). The discovery that tandem copies of the AlcR inverted repeat binding site appear to be unique in their ability to significantly improve the *alc* gene switch may be due to the fact that two molecules of AlcR can simultaneously bind to an inverted repeat while only one molecule binds the direct repeat (Lenouvel et al. 1997; Panozzo et al. 1997).

Studies in *A. nidulans* have suggested that the position of the AlcR binding site (240 bp upstream of the transcriptional start site (TSS)) is critical for ethanol inducible expression from the *alcA* promoter (Panozzo et al. 1997). However, the strong ethanol inducibility of var9 (in which all of the AlcR binding sites are within 186 bp of the TSS) suggests that the exact location of these binding sites are not critical for ethanol inducible expression in plants, although it is possible that even higher ethanol inducible expression could be achieved by optimizing the position of these AlcR binding sites.

In conclusion, we have developed a functional *alc* gene switch for sugar cane that exhibits increased ethanol sensitivity and inducibility. Five tandem copies of the AlcR inverted repeat binding site alone (without any additional *alcA* sequence) were sufficient to significantly improve the functionality of the *alc* gene switch. The functionality of the improved *alc* gene switch in sugar cane, a plant that is relatively recalcitrant to genetic engineering (Lakshmanan

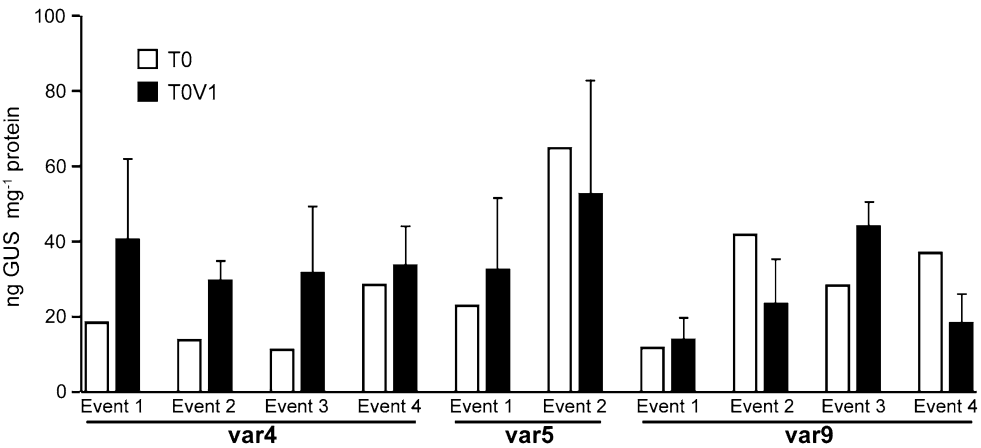

Fig. 7 Ethanol inducible expression in vegetatively propagated transgenic sugar cane plants. GUS accumulation in leaves of 6-month-old T0 and 4-month-old T0V1 transgenic sugar cane at 4 days post treatment with a 5 % ethanol root drench and aerial spray. The T0 and T0V1 plants were at approximately the same developmental stage based on plant height. Data for the T0V1 are mean ± SD from 2 to 3 independent plants

et al. 2005), makes it possible that the successful application of this switch will also extend to other monocotyledonous plants. Furthermore, the enhanced ethanol sensitivity and inducibility achieved with this system may also provide benefits over the current *alc* gene switch used in dicotyledonous plants. However, future work is required to assess the utility of this gene switch in other plants. In addition, the speed of induction and stability of expression in multiple generations (beyond the T0V1 generation) remain to be determined. Finally, while the volume of ethanol used to induce the *alc* gene switch in sugar cane (15.5 ml per 100 ml of soil) is in the range used by other researchers for activation of the *alc* gene switch in other plants (5–16 ml per 100 ml soil; Roslan et al. 2001; Sweetman et al. 2002; Garoosi et al. 2005; Filichkin et al. 2006), experiments in the field will be essential to assess the utility of this chemically inducible system for commercial biotechnology applications.

Acknowledgments We thank Michele Yarnall and Rachel Whinna (Syngenta Biotechnology Inc.) for carrying out all of the GUS qELISA analyses, Jamie Huang and Wenling Wang (Syngenta Biotechnology Inc.) for TaqMan analysis, Shujie Dong (Syngenta Biotechnology Inc.) for help with sample deliveries, and Kerry Caffall (Syngenta Biotechnology Inc.) for critical review of the manuscript. The authors also thank the staff at the Queensland Crop Development Facility (Department of Employment, Economic Development and Innovation, Queensland State Government) for their assistance with the growth of transgenic sugar cane. The Syngenta Centre for Sugarcane Biofuels Development is supported by Syngenta, the Queensland University of Technology, Farmacule Bioindustries, and by a grant from the National and International Research Alliances Program of the Queensland State Government. Dr. Mark Harrison is the recipient of a Smart State Fellowship from the Queensland State Government. The Queensland Crop Development Facility is funded by a grant from the Smart State Research Facilities Fund Scheme of the Queensland State Government. The work described in this manuscript is the subject of a patent application by Dr. Mark Kinkema and Dr. Manuel Sainz.

References

Ait-ali T, Rands C, Harberd NP (2003) Flexible control of plant architecture and yield via switchable expression of *Arabidopsis gai*. Plant Biotechnol J 1:337–343

Caddick MX, Greenland AJ, Jepson I, Krause K-P, Qu N, Riddell KV, Salter MG, Schuch W, Sonnewald U, Tomsett AB (1998) An ethanol inducible gene switch for plants used to manipulate carbon metabolism. Nat Biotechnol 16:177–180

Callis J, Fromm M, Walbot V (1987) Introns increase gene expression in cultured maize cells. Genes Dev 1:1183–1200

De Lucca PC, Dong S, Geijskes RJC, Dunder EM, Sainz MB (2010) Methods for Agrobacterium-mediated transformation of sugar cane. Patent application WO 2010/151634A1

Deveaux Y, Peaucelle A, Roberts GR, Coen E, Simon R, Mizukami Y, Traas J, Murray JAH, Doonan JH, Laufs P (2003) The ethanol switch: a tool for tissue-specific gene induction during plant development. Plant J 36:918–930

FAOSTAT (2008) http://faostat.fao.org

Felenbok B (1991) The ethanol utilization regulon of *Aspergillus nidulans*: the *alc*A-*alc*R system as a tool for the expression of recombinant proteins. J Biotechnol 17:11–18

Filichkin SA, Meilan R, Busov VB, Ma C, Brunner AM, Strauss SH (2006) Alcohol-inducible gene expression in transgenic *Populus*. Plant Cell Rep 25:660–667

Garoosi GA, Salter MG, Caddick MX, Tomsett AB (2005) Characterization of the ethanol-inducible *alc* gene expression system in tomato. J Exp Bot 56:1635–1642

Hare PD, Chua NH (2002) Excision of selectable marker genes from transgenic plants. Nat Biotechnol 20:575–580

Harrison MD, Geijskes J, Coleman HD, Shand K, Kinkema M, Palupe A, Hassall R, Sainz M, Lloyd R, Miles S, Dale JL (2011) Accumulation of recombinant cellobiohydrolase and endoglucanase in the leaves of mature transgenic sugar cane. Plant Biotechnol J 9:884–896

Heaton EA, Dohleman FG, Long SP (2008) Meeting US Biofuel goals with less land: the potential of Miscanthus. Glob Change Biol 14:2000–2014

Houmard NM, Mainville JL, Bonin CP, Huang S, Luethy MH, Malvar TM (2007) High-lysine corn generated by endosperm-specific suppression of lysine catabolism using RNAi. Plant Biotechnol J 5:605–614

Ingham DJ, Beer S, Money S, Hansen G (2001) Quantitative real-time PCR assay for determining transgene copy number in transformed plants. Biotechniques 31:132–134

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Jepson I, Greenland AJ, Caddick MX, Tomsett AB (2001) Expression system. Patent WO 01/09357

Lakshmanan P, Geijskes RJ, Aitken KS, Grof CPL, Bonnett GD, Smith GR (2005) Sugarcane biotechnology: the challenges and opportunities. In vitro Cell Dev Biol Plant 41:345–363

Laufs P, Coen E, Kronenberger J, Trass J, Doonan J (2003) Separable roles of *UFO* during floral development revealed by conditional restoration of gene function. Development 130:785–796

Lenouvel F, Nikolaev I, Felenbok B (1997) *In vitro* recognition of specific DNA targets by AlcR, a zinc binuclear cluster activator different from the other proteins of this class. J Biol Chem 272:15521–15526

Li R, Jia X, Mao X (2005) Ethanol-inducible gene expression system and its applications in plant functional genomics. Plant Sci 169:463–469

Maizel A, Weigel D (2004) Temporally and spatially controlled induction of gene expression in *Arabidopsis thaliana*. Plant J 38:164–171

Martinez A, Sparks C, Hart CA, Thompson J, Jepson I (1999) Ecdysone agonist inducible transcription in transgenic tobacco plants. Plant J 19:97–106

Moore I, Samalova M, Kurup S (2006) Transactivated and chemically inducible gene expression in plants. Plant J 45:651–683

Nikolaev I, Lenouvel F, Felenbok B (1999) Unique DNA binding specificity of the binuclear zinc AlcR activator of the ethanol utilization pathway in *Aspergillus nidulans*. J Biol Chem 274:9795–9802

Okuzaki A, K-i Konagaya, Nanasato Y, Tsuda M, Tabei Y (2011) Estogen-inducible GFP expression patterns in rice (*Oryza sativa* L.). Plant Cell Rep 30:529–538

Ouwerkerk PBF, de Kam RJ, Hoge HC, Meijer AH (2001) Glucocorticoid-inducible gene expression in rice. Planta 213:370–378

Padidam M (2003) Chemically regulated gene expression in plants. Curr Opin Plant Biol 6:169–177

Paine JA, Shipton CA, Chaggar S, Howells RM, Kennedy MJ, Vernon G, Wright SY, Hinchliffe E, Adams JL, Silverstone AL, Drake R (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nat Biotechnol 23:482–487

Panozzo C, Capuano V, Fillinger S, Felenbok B (1997) The zinc binuclear cluster activator AlcR is able to bind to single sites but requires multiple repeated sites for synergistic activation of the *alcA* gene in *Aspergillus nidulans*. J Biol Chem 272:22859–22865

Perlak FJ, Fuchs RL, Dean DA, McPherson SL, Fischhoff DA (1991) Modification of the coding sequence enhances plant expression of insect control protein genes. Proc Natl Acad Sci USA 88:3324–3328

Roslan HA, Salter MG, Wood CD, White MR, Croft KP, Robson F, Coupland G, Doonan J, Laufs P, Tomsett AB, Caddick MX (2001) Characterization of the ethanol-inducible *alc* gene expression system in *Arabidopsis thaliana*. Plant J 28:225–235

Sainz M (2009) Commerical cellulosic ethanol: the role of plant-expressed enzymes. In vitro Cell Dev Biol Plant 45:314–329

Salter MG, Paine JA, Riddell KV, Jepson I, Greenland AJ, Caddick MX, Tomsett AB (1998) Characterization of the ethanol-inducible *alc* gene expression system for transgenic plants. Plant J 16:127–132

Shah DM, Horsch RB, Klee HJ, Kishore GM, Winter JA, Tumer NE, Hironaka CM, Sanders PR, Gasser CS, Aykent S, Siegel NR, Rogers SG, Fraley RT (1986) Engineering herbicide tolerance in transgenic plants. Science 233:478–481

Sweetman JP, Chu C, Qu N, Greenland AJ, Sonnewald U, Jepson I (2002) Ethanol vapor is an efficient inducer of the *alc* gene expression system in model and crop plant species. Plant Physiol 129:943–948

Unger E, Cigan AM, Trimnell M, Xu RJ, Kendall T, Roth B, Albertsen M (2002) A chimeric ecdysone receptor facilitates methoxyfenozide-dependent restoration of male fertility in *ms45* maize. Transgenic Res 11:455–465

Werner S, Breus O, Symonenko Y, Marillonnet S, Gleba Y (2011) High-level recombinant protein expression in transgenic plants by using a double-inducible viral vector. Proc Natl Acad Sci USA 108:14061–14066