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Genetic characterization and field evaluation to recover parental phenotype in transgenic sugarcane: a step toward commercial release

Aldo Noguera · Ramón Enrique · María Francisca Perera · Santiago Ostengo · Josefina Racedo · Diego Costilla · Silvia Zossi · María Inés Cuenya · María Paula Filippone · Björn Welin · Atilio Pedro Castagnaro

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Abstract Sugarcane commercial variety RA 87-3 was transformed with a genetic construct harboring the *epsps* gene from Agrobacterium strain CP4 conferring tolerance to glyphosate and *nptII* gene for kanamycin selection. Transformed lines were multiplied in greenhouse, and herbicide tolerance was evaluated using different concentrations (3, 4, 8 and 16 l/ha) of glyphosate (Helm 48 % p/v). All herbicide-tolerant (HT) lines were field tested to confirm glyphosate tolerance and perform preliminary evaluations of phenotypic resemblance to parental cultivar. All transformed lines maintained herbicide tolerance, but many showed phenotypic changes and/or growth aberrations. Ten HT lines, showing close growth resemblance to RA 87-3, were analyzed using nine compulsory morphologic markers proposed by the International Union for the Protection of New Varieties of Plants (UPOV) and 339 molecular markers. Out of the ten HT lines tested, six showed minor morphologic and genetic variations and were selected for field testing over two vegetative crop cycles (plant cane and first ratoon) at two production areas in Argentina. The six field-tested HT lines were found to be almost indistinguishable when comparing agronomic and industrial characteristics and chemical composition. Stable heritance of the CP4 *epsps* gene and glyphosate tolerance throughout different clonal generations were confirmed by RT-qPCR and Southern blot. Taking into account all results, two out of the six lines tested were selected for a possible commercial release. Our study confirms the utility of genetic transformation as a complementary tool to classical breeding procedures and highlights the usefulness of UPOV traits together with molecular markers for early selections of transgenic events that closely resemble their parental genotype.

Keywords Saccharum hybrid · Glyphosate · CP4 *epsps* gene · Herbicide tolerance

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A. Noguera · R. Enrique · M. F. Perera · S. Ostengo · J. Racedo · D. Costilla · S. Zossi · M. I. Cuenya · M. P. Filippone · B. Welin (✉) · A. P. Castagnaro (✉)
Instituto de Tecnología Agroindustrial del Noroeste Argentino (ITANOA), Estación Experimental Agroindustrial Obispo Colombres (EEAOC) - Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. William Cross 3150, C.P. T4101XAC, Las Talitas, Tucumán, República Argentina
e-mail: bwelin@eeaoc.org.ar

A. P. Castagnaro
e-mail: atilio@eeaoc.org.ar

Introduction

Sugarcane (*Saccharum* hybrid) is the world's leading sugar producing crop, accounting for more than 70 %

of the world sugar supply (Dillon et al. 2007). Additionally, there is an increasing global interest in expanding and improving sugarcane production since this crop is an important resource of biomass for energy production and a promising feedstock for different types of value-added chemicals and polymers. Due to the extremely complex genome of sugarcane (Grivet et al. 2001), conventional breeding is slow and laborious, and in many cases, agronomic and/or industrial traits of interest are not found in the cultivated germplasm or in closely related species, preventing the introgression through sexual crossing. Consequently, genetic transformation is an invaluable tool in order to overcome these limitations contributing directly to the introduction of novel and improved traits, which can generate increased yields, lower management and manufacturing costs and lead to a more sustainable crop production.

Several genes have been introduced successfully by genetic engineering in sugarcane, including genes of commercial interest such as the bar gene conferring glufosinate ammonium tolerance (Enríquez-Obregón et al. 1998; Gallo-Meagher and Irvine 1996; Falco et al. 2000; Leibbrandt and Snyman 2001), gene *cry*1A conferring insect resistance ((Rapulana and Bouwer 2013) and isomaltulose synthase gene for production of the disaccharide isomaltulose (Basnayake et al. 2012). However, until now only one official commercial release of a transgenic sugarcane has been reported, a variety with improved drought tolerance released in Indonesia in 2013 (http://www.isaaa.org/resources/publications/briefs/46/).

In order to be able to deregulate a transgenic crop variety in Argentina, extensive field testing is essential to properly evaluate potential impact on agricultural environments and food safety (http://www.minagri.gov.ar/site/agregado_de_valor/biotecnologia/20-CONABIA/index.php). In addition, molecular and biochemical studies are required to ensure stable expression of the introduced gene(s) in a plant that retains its genetic integrity, chemical composition and agronomic characteristics. The two last items are especially important in crops like sugarcane, where it is a well-known fact that genetic transformation and in vitro regeneration processes can cause important epigenetic and genetic changes through somaclonal variation and as a consequence a very heterogeneous offspring with many different phenotypic and growth aberrations (Gallo-Meagher and Irvine 1996; Arencibia et al. 1999, 2000; Sala et al. 1999; Gilbert et al. 2009). It is therefore of uttermost importance to be able to regenerate transformed lines with stable inheritance of an introduced trait(s) with comparable agronomic and industrial performance to parental elite cultivars for a possible commercial release. Another important factor adding to this concept is the impossibility to backcross a genetically introgressed trait(s) to an elite variety in this cultivar due to its complicated genetics and asymmetric inheritance, which impede recovery of the desired elite phenotype. This fact implicates that for every commercial sugarcane variety, a new transformation event will have to take place in order to produce a progeny comparable to the parental elite line.

The development of glyphosate-tolerant crops remains one of the most important commercial applications of plant genetic engineering (Green 2009b), and at the moment, there are seven glyphosate-tolerant crops commercially released in the world including alfalfa, rape, cabbage, cotton, maize, soybean and sugar beet (Green 2009a). In 2011, molecular genetically modified (GM) crops were planted on more than 148.5 million hectares, with GM herbicide-tolerant (HT) crops accounting for 63 % of the total GM crop area. The same year, 21 million ha of HT GM crops were planted in Argentina, including more than 99 % of all soybean production (Brookes and Barfoot 2012).

Studies conducted in sugarcane have estimated yield loss due to weeds as high as 70 % depending on the severity of infestation (Gupta 1960; Ibrahim 1984). In Argentina, more than 300 weedy species affect sugarcane production and yield loss has been estimated as high as 50 % in some production areas (Olea et al. 2009). High costs of weed control and environmental concerns have prompted the need for cheaper, more effective and more environmental friendly alternatives, such as the use of broad-spectrum biodegradable herbicides such as glyphosate and glufosinate ammonium.

In this study, we demonstrate that transformation of sugarcane by the microprojectile bombardment method allows recovering genetically transformed lines, from a relatively small number of transformation events, showing highly similar genetic, phenotypic, agronomic and industrial properties compared with the parental elite variety used for transformation, a prerequisite for a possible commercial release of transgenic sugarcane. In addition, we demonstrate the

usefulness of the combination of molecular and morphological markers as valuable tools to identify genetically transformed lines early in the transformation process, with comparable agronomic and industrial characteristics to their recipient genotype.

Materials and methods

Plant material and tissue culture conditions

Embryogenic calli of RA 87-3 parental elite variety for DNA bombardment were prepared as described previously (Bower and Birch 1992).

Construct and gene transfer

Plasmid pEA1 contains the synthetic CP4 *epsps* and nptII genes. Both genes were fused to strong constitutive plant monocot promoters, the ubiquitin promoter from maize for the *nptII* gene and the rice actin promoter for the synthetic CP4 *epsps* gene. The CP4 *epsps* gene is fused to chloroplast transit peptide (cTP4) coding sequence from Petunia hybrida. The entire genetic construct was provided by Biosidus AG S.A.

For particle bombardment of plant calli, 3.2 µl of purified pEA1 plasmid was co-precipitated onto tungsten particles (M10, with a 0.8 µm average diameter). Sugarcane calli in Petri dishes were placed at a distance of 12 cm from the microcarrier containing the DNA covered tungsten particles. Bombardment was performed using a microparticle accelerator developed at Copersucar Technology Center according to the model described by (Finer et al. 1992), under vacuum using a helium pressure of 1200 psi. Optimization of bombardment conditions was established using the plasmid pFN1 containing the β-glucuronidase reporter gene (gusA) (Vellicce et al. 2011).

Selection, regeneration and micropropagation conditions

Selection of transformed plant was carried out using a culture medium containing 45 mg l^{-1} geneticin according to Bower et al. (1996) with modifications. Selected shoots were regenerated on medium supplemented with 0.7 mg l^{-1} BAP without 2,4-D under a 16-h light regime. Regenerated shoots were thereafter micropropagated and rooted according to a previously optimized protocol for sugarcane variety RA87-3 (Noguera et al. 2010) and acclimated under special greenhouse conditions (2500 lux and 80–100 % humidity) to ex vitro growth conditions.

Herbicide tolerance assays under greenhouse conditions

In order to evaluate transformed lines for glyphosate tolerance, plants were sprayed with a 3 l/ha glyphosate solution from a commercial formulation (Helm 48 % p/v, Helm Argentina) and surviving plants were evaluated at 1, 2 and 4 weeks after herbicide application.

Detection of transgenic plants by PCR

Genomic DNA was extracted from 200 mg of freeze-dried leaf material of 88 geneticin-resistant lines and non-transformed control plants, using the method described by Aljanabi et al. (1999). A fragment of 561 pb of epsps gene was amplified by PCR using forward primer EPA-F (5′-GCTCGATTTCGGCAATGC-3′) and reverse primer EPA-R (5′- GTTTGATGACTTC GATGTCGG-3′) to detect genetically transformed lines. PCR amplification of the CP4 *epsps* gene was performed during 30 cycles according to the following conditions: denaturation at 95 °C for 30 s, annealing at 64 °C for 30 s and extension at 72 °C for 60 s.

Chlorophyll content determination

Samples from greenhouse transgenic lines (G) and non-transformed plants treated with 3 to 8 l/ha glyphosate were collected after 0, 1, 2 and 4 weeks. Total chlorophyll content was extracted from a sugarcane disk leaf of approximately 15 mg with 500 µl of 80 % acetone solution. Samples were incubated for 1 h at 4 °C and diluted tenfold before spectrophotometric quantification. Total chlorophyll (a and b) content was measured with an Agilent 8453 UV–visible spectrophotometer (USA) following the method described by Lichtenthaler (1987).

Reverse transcription quantitative real-time PCR

Young leaves from transformed HT lines 28 and 37 and non-transformed parental variety RA87-3 were

collected in the greenhouse (G) and three consecutive crop ages (plant cane, P; first ratoon, R1; and second ratoon, R2) and used for total RNA extraction. Total RNA was isolated according to the manufacturer's instructions (SV Total RNA Isolation Kit; Promega, USA). First-strand cDNA synthesis was performed with the SuperScriptII reverse transcriptase (Invitrogen) with 1-μg DNase-treated total RNA and oligo-dT15 as primers. Synthesized cDNA was used for reverse transcription quantitative real-time PCR (RT-qPCR). The reactions were carried out in the presence of the double-stranded DNA-specific dye SYBR green (Biodynamics, Argentina) and monitored in real time with the Mini Opticon Real-Time PCR System (Bio-Rad, USA). The following oligonucleotide primers were used in the study: forward primer EPS4-F (5′-CGCGCATCACCGGCCTTCTGG-3′) and reverse primer EPS4-R (5′-CCGCCATTGCCGACGCCATC-3′). A sugarcane actin amplicon was used as reference gene and was amplified using forward primer ACT2-F (5′-AATGGTCAAGGCTGGTTTTG-3′) and reverse primer ACT2-R (5′-CCTCTCTTGGACTGTGCCTC-3′). All of these amplicons have a product size approximately of 125 bp. The reaction contained 1× SYBR Green PCR Master Mix (Mezcla Real®, Biodynamics, Argentina), 0.4 μM of the forward and reverse primers and 1 μl of cDNA (1:5 dilution) in a total volume of 15 μl. No-template controls were also included as negative controls. PCR amplification was performed for 39 cycles according to the following conditions: denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 68 °C for 40 s. Non-transgenic leaves from RA87-3 served as reference sample, and the results were normalized against the actin gene amplification. The relative expression of transcripts was calculated using the threshold cycle values (C_t) obtained from each sample as follows: relative expression $= 2^{-\Delta\Delta}$, being $\Delta C_t = C_t$ sample $- C_t$ actin and $\Delta\Delta C_t = \Delta C_t$ sample $- \Delta C_t$ ref sample (Livak and Schmittgen 2001). Average values were calculated from triplicate samples.

Glyphosate tolerance and phenotypic growth characteristics in the field

Sugarcane plants (P) (3 months of age) were sprayed with a 3 l/ha glyphosate solution from a commercial formulation (Helm 48 % p/v, Helm Argentina), and surviving plants were evaluated at 15 and 30 days after herbicide application. Ten sugarcane plants (10 months of age) from each of the transgenic line (6, 15, 18, 22, 23, 27, 28, 29, 36 and 37) were evaluated for qualitative and quantitative characters following the test guidelines proposed by UPOV (2005), which are applied to all vegetative propagated varieties of *Saccharum* L. Out of the characters proposed by UPOV, the nine compulsory ones used to identify sugarcane varieties were used in the phenotypic evaluation in this study.

Molecular marker analysis

Total DNA was extracted by the previously described CTAB method (Aljanabi et al. 1999) from ground frozen tissue of the following glyphosate-tolerant lines (6, 15, 18, 22, 23, 27, 28, 36 and 37) and of conventional propagated and micropropagated parental variety RA87-3, used as controls. Three other sugarcane varieties from the EEAOC germplasm collection LCP85-384, TUC97-7 and TUC95-24 were included to validate the usefulness of the molecular technique. Nine combinations of TRAP primers were used to characterize the genotypes. The fixed primers (Alwala et al. 2006) were designed from four genes associated with sucrose metabolism, and the arbitrary reverse primers were obtained from Li and Quiros (2001). The amplification reaction mix, optimized in our laboratory, contained: 1× buffer Taq DNA polymerase, 2.5 mM $MgCl_2$, 0.1 U Taq DNA polymerase, 0.16 μM of both primers (Invitrogen, Life Technologies), 88 μM of each dATP, dTTP and dGTP, 72 μM dCTP, 1.4 μM Cy5.5-dCTP (GE Healthcare Life Sciences) and 100 ng DNA. Amplification parameters were as follows: one cycle at 94 °C for 4 min; 35 cycles at 94 °C for 45 s, 45 °C for 45 s and 72 °C for 1 min; and one cycle at 72 °C for 7 min. Amplification products were separated by electrophoresis on denaturing polyacrylamide gels in a 4300 DNA Analyzer (LI-COR). Gels were analyzed by using the SagaMX AFLP® Software (LI-COR), and all amplified bands were included in the analysis and scored in a dominant manner.

Molecular markers and also morphological traits were transformed into either a 0 or a 1 binomial matrix. Similarity by using molecular, morphological or both kinds of markers, a Jaccard (Sj) coefficient was calculated (Sneath and Sokal 1973a). Cluster analyses were carried out using McQuitty similarity analysis

(UPGMA) (Sneath and Sokal 1973b). The FIND module was used to identify all trees, and they were compiled by the CONSEN module to test the robustness of the tree topology. All calculations were carried out by using InfoStat software (Di Rienzo et al. 2009).

Southern analysis

A 428-bp *epsps* gene fragment and a 598-bp *nptII* gene fragment were PCR amplified from pEA1 and used as DNA probes to hybridize with genomic DNA extracted from freeze-dried leaf material of glyphosate-tolerant plants and non-transformed plants in two crop ages (P and R1). DNA (approximately 20 µg per sample) and plasmid pEA1 (10 pg) digested with the restriction enzymes MfeI or Bpu10I were separated by electrophoresis on a 0.8 % agarose gel and transferred to a nylon membrane (Hybond+, Amersham). Filters were hybridized with the *epsps* and *nptII* gene fragments priorly labeled with ^{32}P-dCTP using the kit (Prime-a-Gene, Promega). Unincorporated nucleotides were removed by passing the reaction solution through a G-25 microcolumn (GE). After overnight hybridization at 65 °C, membranes were washed three times with different concentrations of a saline sodium citrate (SSC) buffer (2×; 1× and 0.5×) with 0.1 % SDS and exposed to a phosphor screen (GE) and scanned after 24 h of exposure using a PhosphorImager Storm 845 (GE).

Comparison of agronomic traits and industrial properties among six herbicide-tolerant lines and their parental variety RA87-3

Agronomic traits and industrial properties were evaluated at two crop ages (P and R1) for six HT transgenic lines and in vitro and field-propagated non-transformed parental variety RA 87-3 at two independent production areas in northwest Argentina (provinces of Tucumán and Salta). Trials in each environment were planted with a randomized complete block design (RCBD) with three replicates. Plots of three seven-meter-long rows were used as the experimental unit, and cane yield per plot (kg cane/plot) was evaluated. Ten stems from each transgenic line and RA87-3 plants were evaluated in triplicates (plots) in both field trials to evaluate agronomic traits (weight, height and diameter of stalks). The same samples were used to evaluate protein, sugar and fiber content. Total proteins were determined by the Kjeldahl method, according to AOAC (2009), using a distiller Buchi B-324. ADF (acid detergent fiber) and NDF (neutral detergent fiber) were determined using a fiber analyzer ANKOM 2000, according to the methodology ANKOM (2006a, b). Sucrose was determined by high-performance liquid chromatographic (HPLC) using a pump and autosampler Alliance. The column used was Sugar-Pak (Waters), using as mobile phase 0.05 M calcium EDTA solution at a flow rate of 0.5 ml/min and refractive index detector (Waters). For each variable, data were statistically analyzed by ANOVA and Fisher's LSD test (significance level 5 %).

Results

Transformation and selection of glyphosate-tolerant transgenic lines

One thousand seven hundred twenty eight (1728) calli from commercial sugarcane variety RA 87-3 were bombarded with plasmid pEA1 containing the synthetic CP4 *epsps* gene, conferring glyphosate tolerance, and the *npt*II gene from *E. coli* for kanamycin/geneticin selection of transformed cells. Eighty eight plantlets were selected primarily as geneticin resistant, but only 43 lines survived the complete in vitro regeneration process. Out of these plants, 36 were tested as PCR positive (2.1 % transformation efficiency) for the CP4 *epsps* gene (data not shown).

In order to determine tolerance against glyphosate, all surviving transformed plants, including the seven PCR-negative ones, were propagated and evaluated under greenhouse (G) conditions. Out of the 43 plant lines tested, 17 were found to be tolerant to glyphosate application (>5 fold the recommended field concentration of 3 l/ha), while five lines showed intermediate tolerance and 21 lines did not show any measurable tolerance to the herbicide at the concentrations tested.

Field testing of glyphosate tolerance and phenotypic growth characteristics

The 17 highly glyphosate-tolerant lines, as deduced from greenhouse experiments, were planted in a small-scale field trial in order to confirm herbicide tolerance

and to preliminary evaluate field growth and plant phenotype as severe growth aberrations (e.g., stunting, excessive sprouting, atypical buds) had been observed during multiplication of several of the transformed lines in the greenhouse. All lines tested maintained their tolerance to glyphosate, but several of them showed a clear growth difference when compared to the recipient genotype, similar to our observation during greenhouse growth. Ten transgenic lines, selected for good phenotypic resemblance to the parental variety, were further analyzed by using nine morphological markers proposed by International Union for the Protection of New Varieties of Plants (UPOV) that are compulsory for sugarcane variety registration. None of the ten tested lines showed a 100 % identity to parental line RA 87-3 when analyzing the UPOV markers, but several lines only differed in minor phenotypic characteristics such as coloration of plant tissues (Table 1). Four of the HT lines tested (6, 23, 29 and 36) differed markedly with more than 50 % of the markers differing from the parental variety and were therefore discarded from further studies. The remaining six lines (15, 18, 22, 27, 28 and 37) showing identity in at least five of the nine markers were selected for further analysis and possible candidates for a commercial release. The two best lines as deduced from this study were lines 28 and 37 with six and seven identical matches to the UPOV markers, respectively.

CP4 *epsps* gene expression levels and its relationship to chlorophyll content variation in transgenic lines treated with different concentrations of glyphosate

Reverse transcription quantitative real-time PCR (RT-qPCR) was used to assess expression levels of the CP4 *epsps* gene in the six glyphosate-tolerant lines (15, 18, 22, 27, 28 and 37) and to evaluate a possible relationship between expression level and herbicide tolerance. Leaves of plants grown in the greenhouse (G) were used in this analysis. Levels of relative gene expression were found to be increased 15- to 25-fold for the six lines examined, as compared to non-transformed control plants. The highest relative CP4 *epsps* expression levels were found for lines 18, 28 and 37 (Fig. 1a).

In order to quantify and visualize tolerance to glyphosate application, leaf samples (G) from the six transformed lines and non-transformed parental variety plants (RA 87-3) were taken at different time points after herbicide treatment using different glyphosate concentrations (0, 3, 4, 8 l/ha). Chlorophyll content related to tolerance ability of the lines was similar at all glyphosate concentrations at 0, 1, 2 and 4 weeks after application. While this content was similar to that from the non-treated non-transformed plants, dramatic differences were observed with respect to the herbicide treated non-transformed plant (Fig. 1b–e). Treated non-transformed plants lost almost all chlorophyll content already 1 week after herbicide treatment, while tolerant transformed lines retained chlorophyll content in a similar fashion to non-treated RA 87-three plants (supporting information) throughout the 4 weeks of testing. The HT line with the lowest expression level (22) and the one with the highest level (37) showed a similar HT, suggesting that there was no direct relation between HT and expression levels of CP4 *epsps* gene at the concentrations of glyphosate applied (data not shown).

Genotypic evaluation of transformed lines using molecular markers

To establish whether the similar morphology between the HT lines and their parental variety was also true at the genetic level, nine independent target region amplification polymorphism (TRAP) markers were used to characterize the aforementioned ten transgenic lines evaluated by morphological markers. In addition to the HT lines, three sugarcane varieties, selected from a previous study where the usefulness of different marker techniques to identify and estimate genetic diversity was assessed (Perera et al. 2012), were included in the study: where two genotypes present a high similarity degree (TUC95-24 and TUC97-7) to RA87-3 as well as a more distant genotype (LCP85-384). A total of 339 unambiguous bands were scored, out of which 212 (62 %) were polymorphic, with an average of 37 bands per primer combination (data not shown). In the dendrogram obtained (data not shown), no genetic difference at all could be detected for five (15, 18, 22, 28 and 37) of the six lines showing high phenotypic resemblance to the parental genotype RA87-3. The remaining line, 27, presented two different bands out of the 277 that characterized the variety (99 % similarity), while the four transformed lines that exhibited a distinct growth

Table 1 Characterization of ten transgenic lines and parental variety RA 87-3 using the morphological parameters of UPOV

Characteristics[a]	15	18	22	27	28	37	6	29	36	23	RA 87-3*	RA 87-3
Leaf sheath adherence	**Weak**	**Weak**	Medium	Medium	Medium	**Weak**	Medium	**Weak**	Medium	Medium	**Weak**	**Weak**
Stem diameter	Thin	Thin	**Thick**	**Thick**	**Thick**	**Thick**	Medium	Thin	Medium	Thin	**Thick**	**Thick**
Stem shape	**cyl.**	**cyl.**	**cyl.**	**cyl.**	**cyl.**	**cyl.**	**cyl.**	Conoidal	**cyl.**	**cyl.**	**cyl.**	**cyl.**
Color where exposed to sun	184A	184A	185A	71A	184A	**79A**	59A	199A	59B	183A	79C	**79A**
Color where not exposed to sun	166A	153D	151A	151A	**183A**	152D	59A	187A	54B	186B	151A	**183A**
Zigzag alignment expression	**Absent**	**Absent**	**Absent**	**Absent**	**Absent**	**Absent**	**Absent**	**Absent**	**Absent**	**Absent**	**Absent**	**Absent**
Bud shape	**Ovate**	**Ovate**	**Ovate**	**Ovate**	**Ovate**	**Ovate**	**Ovate**	**Ovate**	**Ovate**	Triangular-pointed	**Ovate**	**Ovate**
Necklace color leaf sheath	199A	200B	146B	146B	**200A**	146B	152A	146C	144B	144A	**200A**	**200A**
Width at the longitudinal midpoint leaf	**Narrow**	**Narrow**	**Narrow**	**Narrow**	**Narrow**	**Narrow**	**Narrow**	**Narrow**	**Narrow**	**Narrow**	**Narrow**	**Narrow**
Number of coincidences between the line and the parental variety	5	5	5	5	6	7	4	4	4	3		

Alphanumerical codes stipulated in the UPOV classification system (e.g., 184A)

Measures were taken in plant cane (P) of 10-month-old plants

RA87-3*, in vitro propagated

RA87-3, conventionally propagated

In bold, coincidences with respect to controls

cyl. cylindrical

[a] Nine compulsory characters to identify sugarcane varieties according UPOV

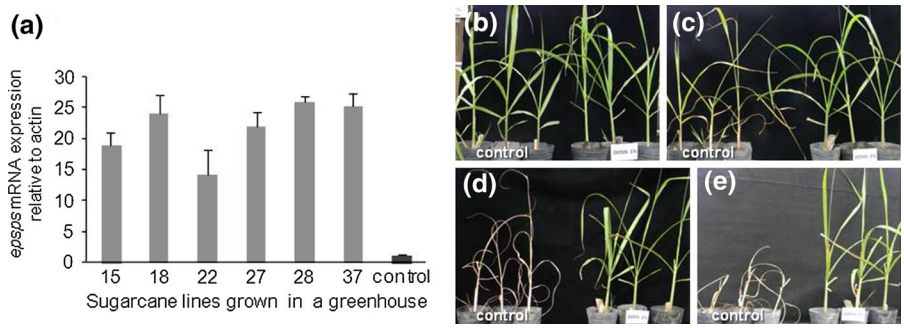

Fig. 1 Quantitation of *epsps* gene expression levels by RT-qPCR and evaluation of glyphosate tolerance in transgenic lines. **a** Reverse transcription quantitative real-time PCR analysis of transgenic lines. Average *n*-fold changes in *epsps* gene expression in six transgenic lines compared to non-transformed RA87-3 plant are calculated from triplicate samples. **b–e** Phenotypic changes of transformed line (37) plants at 0, 1, 2 and 4 weeks, respectively, after herbicide treatment (3 l/ha glyphosate). Non-transformed RA87-3 plants are used as control. Samples from plants grown at greenhouse (G) are used in both studies

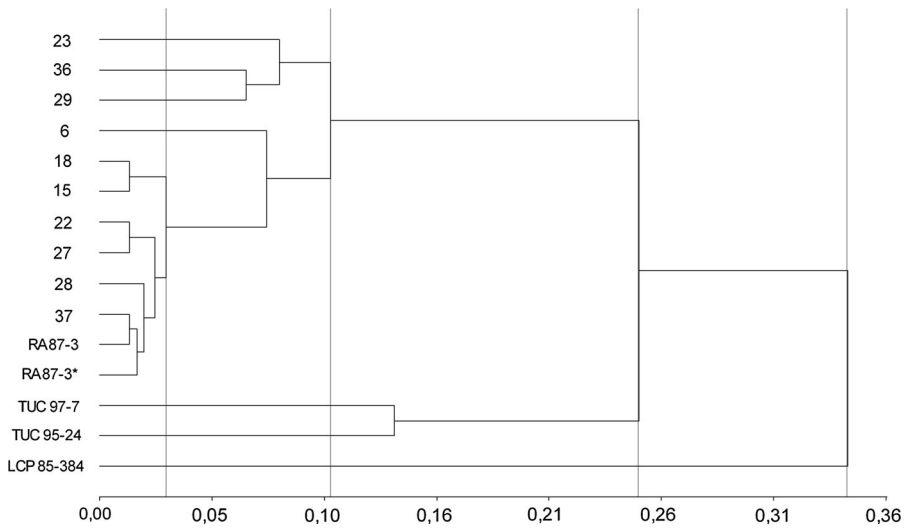

Fig. 2 Molecular and morphological evaluation of transgenic lines. Dendrogram of the four sugarcane genotypes and the RA87-3 transgenic lines is based on the analysis of 339 alleles from nine TRAP primer combinations and 60 alleles produced from the nine compulsory morphological traits proposed by UPOV, by using Jaccard coefficient and UPGMA clustering method with InfoStat, presented as distance (1-S, S: similarity). *Lines* represented similarity of 0.97, 0.89, 0.75 and 0.65

phenotype as shown by phenotypic markers compared with parental line RA87-3 showed a higher degree of polymorphism (92–95 % similarity). However, all ten HT lines tested showed higher similarity to RA87-3 (>92 %) compared with 78 % found for varieties genetically close and 68 % for the genetically distant variety of the EEAOC germplasm collection.

As molecular markers can be combined with morphological traits in order to better characterize genotypes, reveal genetic relationships and genetic diversity (Perera et al. 2012), a dendrogram was constructed by combining both methods (Fig. 2). The morphological characterization of TUC95-24, TUC97-7 and LCP85-384 was obtained from previous studies. The topology of the dendrogram was similar to that obtained with molecular markers; transgenic lines shared more than 89 % similarity with the parental line, while the varieties genetically close shared 75 % and the genetically distant variety shared 65 % similarity. Since morphological traits revealed more polymorphism, their inclusion allowed us to differentiate between all HT lines tested. When combining morphological and molecular markers, HT lines 28 and 37 were found to be most similar to

the parental variety RA87-3 (more than 98 % similarity) (Fig. 2).

Transgene copy number, genetic stability and CP4 *epsps* expression

In order to study number of CP4 *epsps* transgene insertions in the plant genome, Southern blot analysis was performed using restriction enzymes *Mfe*I and *Bpu*10I, which both cut once in the pEA1 plasmid. All HT transformants tested in two crop ages, plant crop (P) and first ratoon (R1) contain multiple inserts (>5). Evidence for the presence of at least seven integration events was obtained for the four transgenic lines (15, 18, 28 and 37) when using the CP4 *epsps* gene as probe (Fig. 3a, b) and another independent probe corresponding to the *nptII* gene (data not shown).

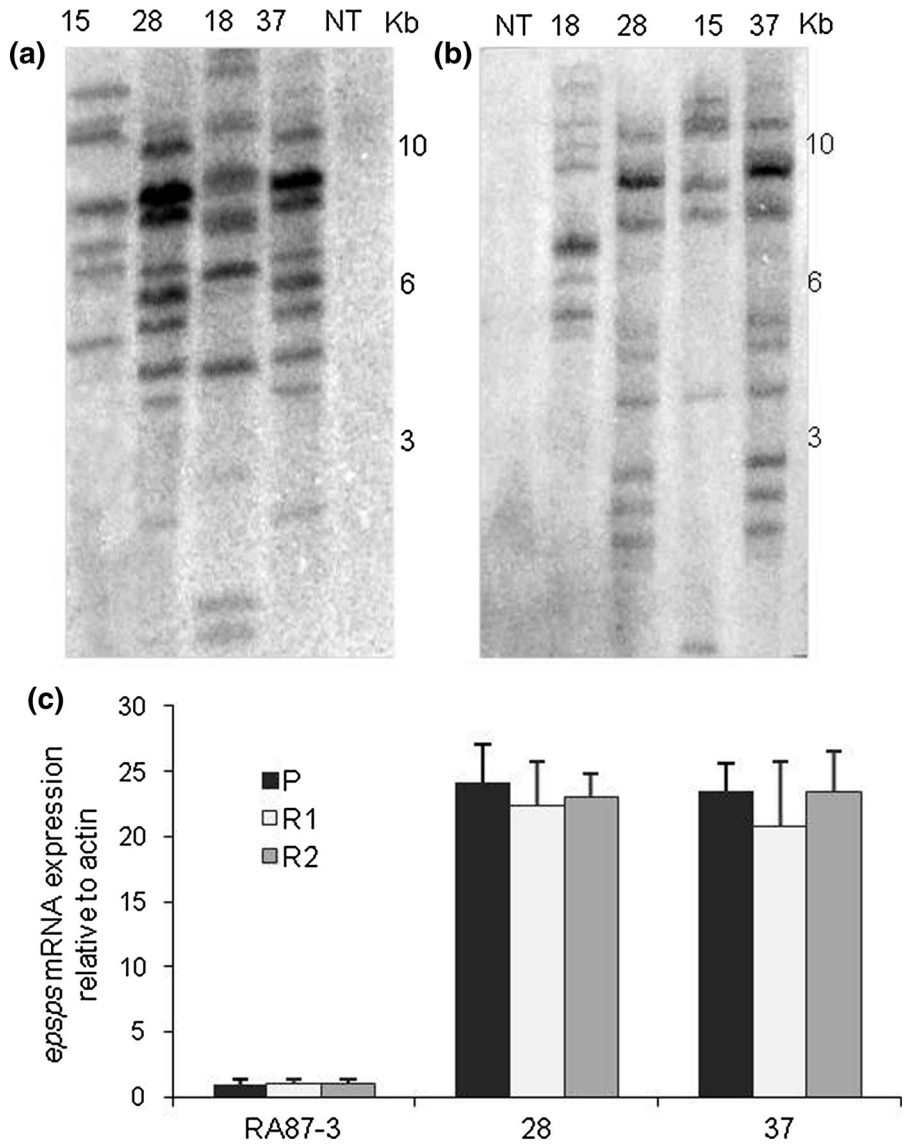

Fig. 3 Molecular characterization of transgenic lines. **a**, **b** Analysis of number copies in transgenic lines in plant cane (P) by means of Southern blot using Bpu10I and MfeI enzymes, respectively, with epsps probe. (NT) Non-transformed RA 87-3 variety is used as control. **c** Quantitation of stability of *epsps* gene expression in three propagation generations (P, R1 and R2) by RT-qPCR. Average *n*-fold changes in *epsps* gene expression in two transgenic lines (28, 37) compared to non-transformed RA87-3 plant are calculated from triplicate samples. *P* plant cane, *R1* ratoon 1, *R2* ratoon 2

One of the most important data when analyzing a transgenic variety for a possible commercial release is the stable heritance of the introduced DNA harboring the genes responsible for the characteristic(s) of interest. In addition, when working with a vegetatively propagated plurianual crop, like sugarcane, it is important to show stable expression of the transgene(s) in multiple crop cycles (P, R1 and R2). All six transgenic HT lines, which were selected for further studies and a possible commercial release, have been propagated for at least four generations and three of these generations have been grown during three consecutive years (crop ages) in the field maintaining their glyphosate tolerance at the same level as originally tested in the first generation under greenhouse conditions (G). To confirm stable transcription of the CP4 *epsps* gene throughout the different clonal generations, RT-qPCR analysis was performed. The CP4 *epsps* gene is transcribed at similar levels (20–25-fold) for the two transgenic lines (28 and 37) tested in the third generation analyzed during three consecutive crop ages (P, R1 and R2) (Fig. 3c). The relative expressing levels of CP4 *epsps* are in agreement with those previously shown in Fig. 1a for plant cane of the first-generation greenhouse grown plants. These results confirm the stable heritance of DNA observed in Southern blot assays in both crop ages, P (Fig. 3a, b) and R1 (data not shown).

Agronomic performance of the six herbicide-tolerant lines

Field trials at the two most important sugarcane production areas in Argentina, the provinces of Tucumán and Salta, were performed over two crop ages (P and R1). Agronomical parameters, statistically analyzed by ANOVA, included stalk height, stalk weight, stalk diameter and yield per plot. Two independent RA87-3 controls were employed in the trial, in vitro and conventionally field propagated since previous reports have shown that in some cases, in vitro propagated sugarcane shows growth differences as compared to field-propagated plants (Arencibia et al. 1999, 2000; Sala et al. 1999) (Table 2). These analyses showed statistically significant differences of some agronomical parameters studied at the two test sites for some of the lines tested (i.e., lines 15, 18 and 22). However, the two genetically most similar, as compared to parental variety RA 87-3, transformed HT lines (28 and 37) did not show any statistically significant difference as compared to in vitro or field-propagated parental variety RA87-3 (Table 2).

Chemical composition of the six evaluated transgenic lines

As important as the aforementioned agronomical parameters are the industrial parameters of sugarcane production (i.e., sucrose and fiber content), which were determined together with total protein content. Samples for chemical analysis were taken prior to harvest from 8- to 11-month-old plants, at two crop ages (P and R1) at both field trial sites (Tucumán and Salta). Transgenic HT lines did not show any statistically significant difference in the parameters measured when compared with their recipient variety, except transformed line 18 that exhibited a difference for total protein content in the first year of growth (P) at one field site (Salta) (Table 3).

It is therefore safe to conclude from these studies that all six HT lines are very similar agronomically and chemically to their parental variety and could therefore be considered as good candidates for a commercial release.

Discussion

This is the first complete study presented on genetically transformed glyphosate-tolerant sugarcane, including field studies on agronomical and industrial performance. Our results show the capability to produce a genetically stable and closely related progeny of an elite parental variety, a prerequisite for future commercial release. Initially, an efficient biolistic-mediated transformation protocol for the local elite sugarcane cultivar RA87-3 was developed with a callus transformation efficiency of 2.1 %, which is slightly higher than that previously reported in sugarcane (1.6 %) by (Vickers et al. 2005). It is interesting to notice that all transformed HT lines examined contained multiple transgene inserts, something that has been previously reported in sugarcane when using circular DNA for transformation (Franks and Birch 1991). A high number of inserts could be correlated with a higher protein production as has previously been reported (Altpeter et al. 2005) and correspond to a higher glyphosate tolerance. As this

Table 2 Agronomic traits of six glyphosate-tolerant transgenic lines and two differently multiplied control plants (in vitro and field propagated) of parental variety RA 87-3 evaluated at the two major sugarcane production regions in Argentina during two consecutive growth seasons: plant cane (P) and first ratoon (R1)

Lines	Stalk height (cm)[a]		Stalk weight (Kg)[a]		Stalk diameter (cm)[a]		Yield per plot (Kg)	
	P	R1	P	R1	P	R1	P	R1
Tucumán								
15	148.3a	193.3bcd	0.56c	0.68b	2.14d	2.03b	195.7a	227.0a
18	151.7a	180.0d	0.58bc	0.69b	2.31bcd	1.93b	162.7a	236.3a
22	165.0a	190.0cd	0.81a	0.93a	2.62a	2.36a	196.7a	235.0a
27	160.0a	206.7abc	0.74ab	1.00a	2.51abc	2.37a	186.7a	239.7a
28	156.7a	210.0ab	0.68abc	0.95a	2.29cd	2.36a	167.7a	231.7a
37	158.3a	200.0abc	0.76a	0.92a	2.39abc	2.31a	202.3a	218.7a
RA87-3*	161.7a	213.3a	0.76a	0.94a	2.54ab	2.48a	185.7a	247.7a
RA87-3	161.7a	216.7a	0.79a	1.03a	2.41abc	2.42a	178.0a	243.3a
Salta								
15	195.0a	170.0c	0.59b	0.54b	1.97c	1.77b	196.2ab	157.3c
18	223.3a	190.0bc	0.73ab	0.63b	2.10bc	2.19a	248.7a	183.3bc
22	220.0a	206.7ab	0.87a	0.83a	2.41a	2.41a	206.7ab	202.7ab
27	228.3a	223.3a	0.88a	0.89a	2.34a	2.35a	195.2b	198.7ab
28	216.7a	216.7a	0.78a	0.94a	2.26a	2.39a	206.5ab	200.3ab
37	233.3a	220.0a	0.88a	0.98a	2.27ab	2.39a	210.7ab	219.7a
RA87-3*	223.3a	216.7a	0.81a	0.85a	2.23ab	2.38a	220.8ab	217.7ab
RA87-3	236.7a	230.0a	0.86a	0.87a	2.28ab	2.43a	232.0ab	227.3a

RA87-3*, in vitro propagated

Statistical analysis performed by ANOVA and Fisher's LSD test

Means followed by the same letter within a column for each region are not significantly different ($p > 0.05$)

Plot size: three rows of seven meter long

[a] Data obtained from a sample of ten stalks ($n = 10$) per replicate

study only analyzed genetically transformed lines showing high HT in Southern blot analysis, there could be a correlation between HT and multiple transgene inserts (Fig. 3a, b). However, this is not very likely since a recent study in sugarcane showed no correlation of higher gene expression levels with higher numbers of transgene insertions (Jackson et al. 2013).

Our results show that the use of TRAP molecular markers is a rapid and recommendable first approach to select for promising transgenic lines resembling their parental genotype. Even more, it seems a more adequate approach to detect genetic changes caused during entire transformation process than other molecular markers techniques such as RAPD (Taylor et al. 1995) and AFLP (Joyce et al. 2014) previously evaluated in sugarcane that failed to detect consistent genome differences between clones with visually distinct phenotype variation. In contrast to these reports, this work clearly demonstrate a high degree of reproducible polymorphism using TRAP markers among transformed lines that also exhibited a distinct growth phenotype from transformed parental variety. This result is somewhat surprising considering the nature of TRAP markers which are oriented at specific loci and not distributed throughout the genome. The reason behind the high polymorphism found needs further studies to be completely elucidated, but it is evident that a high frequency of genetic changes has occurred within the genome.

The usefulness of the UPOV's morphological markers combined with molecular markers in sugarcane research has been shown previously and can be used to assist sugarcane breeders in estimating genetic diversity, electing parents for crossings, identifying superior lines and to protect intellectual property

Table 3 Chemical composition traits of six glyphosate-tolerant transgenic lines and two differently multiplied control plants (in vitro and field propagated) of parental variety RA 87-3 evaluated at the two major sugarcane production regions in Argentina during two consecutive growth seasons: plant cane (P) and first ratoon (R1)

Lines	Protein (g/100 g)[a]		ADF (g/100 g)[a]		NDF (g/100 g)[a]		Sucrose (g/100 g)[a]	
	P	R1	P	R1	P	R1	P	R1
Tucumán								
15	2.14a	1.46a	16.41a	20.04a	31.69a	32.29a	18.26a	21.12a
18	2.22a	1.19a	17.74a	20.14a	33.01a	31.78a	18.22a	21.12a
22	2.09a	1.61a	18.56a	21.72a	33.22a	33.19a	18.59a	20.72a
27	2.31a	1.38a	20.18a	19.89a	36.30a	31.83a	18.48a	20.52a
28	2.30a	1.63a	18.63a	21.26a	35.07a	32.75a	19.03a	20.87a
37	2.41a	1.42a	19.03a	22.25a	35.33a	34.37a	18.80a	21.67a
RA87-3*	2.37a	1.42a	18.41a	20.86a	34.92a	32.53a	18.51a	21.09a
RA87-3	2.12a	1.48a	18.18a	21.21a	35.08a	31.61a	18.77a	21.51a
Salta								
15	1.25bc	1.96a	14.54a	21.57a	28.04a	35.81a	19.81a	19.70a
18	1.09c	1.38a	13.62a	20.94a	25.87a	35.84a	21.05a	19.14a
22	1.33b	1.52a	16.37a	20.07a	28.91a	33.83a	20.21a	20.80a
27	1.40ab	1.56a	15.34a	21.51a	27.07a	35.90a	20.73a	20.28a
28	1.36ab	1.67a	14.58a	21.89a	26.07a	36.25a	20.95a	20.62a
37	1.26bc	1.54a	13.50a	21.62a	26.83a	35.68a	20.89a	20.25a
RA87-3*	1.55a	1.58a	15.11a	18.64a	30.22a	31.74a	20.29a	19.66a
RA87-3	1.39ab	1.77a	15.43a	19.77a	27.90a	34.16a	20.47a	20.41a

RA87-3*, in vitro propagated

Statistical analysis performed by ANOVA and Fisher's LSD test

Means followed by the same letter within a column for each region are not significantly different ($p > 0.05$)

Plot size: three rows of seven meter long

[a] Data obtained from a sample of ten stalks ($n = 10$) per replicate

rights (Perera et al. 2012). The present work shows that the combination of TRAP and morphological markers is a commendable approach in order to evaluate, characterize and select genetically transformed lines with highly similar phenotypes and growth behavior as compared to the recipient genotype. It must be highlighted that this is the first time that this kind of integral characterization, including UPOV's traits, is conducted to evaluate a transgenic sugarcane variety.

Many reports state that in vitro micropropagation of sugarcane can cause less vigorous and productive plants (Gilbert et al. 2009; Vickers et al. 2005; Basnayake et al. 2012; Hoy et al. 2003). In a recent field study in Australia, where seven different in vitro cultured-derived transgenic varieties were compared with field-propagated cane, it was found that in vitro propagated plants were shorter and had thinner stalks than those field propagated during the first growth season. However, already in the second year of field-growth yields and growth, phenotypes were indistinguishable between in vitro and field-propagated plants. The experience from our in vitro propagation program at the EEAOC confirms this statement since first year plantation of in vitro propagated plantlets can in some cases give rise to shorter and thinner stalks; however, during subsequent field propagation, such differences disappear (data not shown). For the aforementioned reason, all transgenic lines were micropropagated under an optimized scheme minimizing somaclonal variation and were after that field propagated for at least 2 years before evaluating agronomic and industrial traits. However, to exclude that any possible differences observed in field tests had originated from in vitro propagation effects, two different control parental varieties (in vitro propagated and field

propagated) were employed in all field tests. All our results from our field tests corroborate that there are no effect what so ever originating from the in vitro propagating procedure as no difference in the parameters measured could be detected between in vitro propagated and field-propagated plants of the parental variety RA 87-3.

Results from our field tests showed only minor differences among the six transgenic lines and the parental variety RA87-3 when analyzing different agronomical traits and chemical composition (Tables 2, 3). All results obtained in this study for sucrose, protein, acid detergent fiber and neutral detergent fiber are within the range of values reported by the Organisation for Economic Co-operation and Development (OECD) (http://www.oecd.org/science/biotrack/48962816.pdf). Similar results have been obtained in other field studies of transgenic sugarcane where it was shown that it is indeed possible to completely recover a parental phenotype with comparable agronomical and industrial traits (Leibbrandt and Snyman 2003; Gilbert et al. 2005). These findings taken together with our study show the potential usefulness of genetic engineering technology in sugarcane breeding. However, as has been demonstrated in this study and several others, thorough genetic and field evaluation studies are necessary in order to find promising transformed lines comparable in all aspects to their parental genotype.

Taking into account all information obtained in this work (phenotypic, morphological, genetic, agronomical and chemical evaluation), we were able to select two HT lines that were almost identical to their parental variety. It was therefore no great surprise when Southern blot analyses showed that these lines (28 and 37) had most likely originated from the same transformation event (Fig. 3a, b). The fact that these two lines were selected without prior knowledge that they had probably originated from the same transformation event is a good indication that our studies are reproducible and reliable. Furthermore, our results suggest that at least 30–40 independent transformation events are necessary to encounter transformed lines with similar growth and production characteristics to a parental variety when using a callus transformation protocol in sugarcane. These numbers are based on the fact that we found at the most 2–3 outstanding candidates for commercial release out of an initial number of 36 CP4 *epsps* PCR-positive lines.

In many countries, there are major concerns related to public reception of new GM crops. Argentina is probably one of the most amenable countries in this aspect, where the adoption of GM technology has been more successful and rapid than in any other part of the world, including USA (James 2013). This makes Argentina an excellent candidate to commercially release one of the first transgenic sugarcane varieties in the world. Another positive aspect of developing GM sugarcane is the fact that sugar produced from glyphosate or glufosinate-tolerant GM sugar beet has already been approved for commercialization in some countries of the European Union, Australia, Mexico, Canada, USA and Japan (CERA 2008). The fact that over 500.000 ha of glyphosate-tolerant sugar beets are already grown in the USA (USDA) is an important helping factor as global sugar industry has accepted GM technology for sugar production.

In conclusion, our results demonstrate the potential of genetic transformation as an effective and complementary tool to classical breeding approaches in order to introduce new and desirable traits in the complex sugarcane genome. In addition, our results from field testing show that it is possible to recover a transgenic line with all characteristics of the recipient genotype which is necessary to enable a commercial release, and by using TRAP markers, it is possible to select for good genetic candidates at a very early stage of the transformation process. These encouraging results will hopefully help in advancing toward the release of GM sugarcane varieties in Argentina and other countries with many different traits that will enable a more efficient and sustainable production worldwide of this important crop.

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