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Development of a further transgenic sugarcane cultivar resistant to glyphosate herbicide

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ABSTRACT

A previous development of transgenic glyphosate-resistant sugarcane suitable for commercial release was carried out through genetic transformation of cultivar RA 87-3. During the time elapsed to develop this technology, new commercial cultivars produced by the local breeding program have been adopted by farmers. The complex genetics of modern sugarcane cultivars, which are interspecific hybrids, highly polyploid and frequently aneuploid, make the introgression by backcrossing of the transgene into other varieties extremely difficult. Direct transformation of new commercial cultivars or promising clones at final stages of a breeding scheme could greatly improve the development and adoption of transgenic sugarcane by both farmers and millers. The aim of this study was to obtain glyphosate-resistant transgenic events of the two recently released cultivars, TUC 95-10 and TUC 03-12, through the introduction via microprojectile bombardment of plasmids harbouring the *epsps* and *nptII* genes. A total of 23 and 8 independent bombardments experiments were carried out on TUC 95-10 and TUC 03-12, respectively. The stable transformation and integration of both *epsps* and *nptII* genes were determined by using PCR with specific primers. Transgenic events were evaluated for glyphosate tolerance under *ex vitro* conditions and for genetic similarity with donor plant by using target region amplification polymorphism (TRAP) molecular markers. Although plantlets of both varieties regenerated from calli, TUC 95-10 showed a low tissue culture-response since only two events were obtained, whilst a total of 22 plantlets regenerated for TUC 03-12. One line of TUC 95-10 and three lines of TUC 03-12 were PCR-positive for both genes and different levels of herbicide-tolerance were observed. Genetic analyses of 213 TRAP *loci* from transgenic lines derived from TUC 03-12 indicate that two events show 99.30% of similarity with the non-transformed TUC 03-12 control and the third had 98.7% similarity. These are encouraging results as, based on our previous experience, the molecular-marker data suggest that the events are practically identical to their parental cultivar, and are suitable for future comparative field testing.

Key words: Genetic transformation, biolistic, genetically modified organism, sugarcane, glyphosate.

RESUMEN

DESARROLLO DE UN NUEVO CULTIVAR TRANSGÉNICO DE CAÑA DE AZÚCAR RESISTENTE AL HERBICIDA GLIFOSATO

El previo desarrollo de un evento transgénico de caña de azúcar resistente al glifosato aceptada para su liberación comercial fue llevado a cabo a partir de la transformación genética de la variedad RA 87-3. Durante el tiempo transcurrido para desarrollar esta tecnología, nuevas variedades comerciales liberadas por el programa de mejoramiento local están siendo adoptadas por los agricultores. La complejidad genómica de las variedades modernas de caña de azúcar, caracterizadas por ser híbridos interespecíficos, con un alto grado de poliploidía y frecuente aneuploidía, hace prácticamente imposible la introgresión del transgén por retrocruzamientos en otras variedades. La transformación directa de nuevas variedades comerciales o clones promisorios que se encuentren en etapas finales de un esquema de mejoramiento, podrían mejorar enormemente el desarrollo y la adopción de la caña de azúcar transgénica tanto por los agricultores como por los industriales. El objetivo de este estudio fue obtener eventos transgénicos resistentes al glifosato de las variedades TUC 95-10 y TUC 03-12 a partir del bombardeo de microproyectiles recubiertos por plásmidos que contengan los genes *epsps* y *nptII*. Se realizaron experimentos independientes con un total de 23 y 8 bombardeos de las variedades TUC 95-10 y TUC 03-12, respectivamente. La transformación e integración estable de los genes *epsps* y *nptII* se determinaron mediante el uso de la técnica de PCR con cebadores específicos. Los eventos transgénicos fueron evaluados en condiciones *ex vitro* para determinar la tolerancia al glifosato, mientras que también se analizó su similitud genética con la variedad que les dio origen mediante marcadores moleculares TRAP. Aunque se obtuvieron plántulas de ambas variedades, la variedad TUC 95-10 mostró una menor respuesta al cultivo de tejido, ya que solo se obtuvieron dos eventos, mientras que para la variedad TUC 03-12 se regeneraron un total de 22 plántulas. Un evento regenerado a partir de TUC 95-10 y tres eventos derivados de TUC 03-12 fueron PCR positivos para ambos genes, observándose diferentes niveles de tolerancia al herbicida. El análisis genético de 213 *loci* TRAP en las 3 líneas transgénicas derivadas de TUC 03-12 indican que dos eventos comparten un 99,30% de similitud con el control no transformado, mientras que el evento restante presenta un 98,7%. Estos resultados son alentadores, ya que basados en conocimientos previos, los marcadores moleculares indicarían que los eventos obtenidos son prácticamente idénticos a su variedad donante, con lo cual serían adecuados para conducir ensayos comparativos adicionales a campo.

Palabras clave: Transformación genética, biobalística, organismo genéticamente modificado, caña de azúcar, glifosato.

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INTRODUCTION

Modern sugarcane cultivars are interspecific hybrids obtained through human intervention. They are highly polyploid and often aneuploid, with chromosome numbers ranging from 100 to 130 (Daniels and Roach 1987). Due to this genetic complexity, conventional breeding is slow and laborious. On average, one commercial variety can be obtained for every 250,000 seedlings evaluated in the first stage of the breeding program (Cheavegatti-Gianotto *et al*. 2011), taking no less than 10 years for the selection of superior genotypes.

Even though biotechnological methods offer a valuable tool for breeding programmes in order to transfer one or few characteristics of interest into a given variety through genetic transformation, several years of field trial evaluation post-transformation are also required (see Noguera *et al*. 2015). This is necessary in order to select genetically and phenotypically stable and agronomically acceptable material for commercial release (Gilbert *et al*. 2005). However, during the time taken to produce a genetically-modified line, new higher productive cultivars can be released by the breeding program, rendering obsolete the genetic background of the transgenic event. Moreover, introgression of the event into other lines by using backcrossing is largely precluded due to the high heterozygosity and chromosomal rearrangements that occur during meiosis.

As Cristofoletti *et al*. (2018) recently mentioned, a transformation process applied to promising clones at pre-commercial stages of a breeding pipeline could greatly improve the development of commercial transgenic sugarcane. Additionally, revision of the deregulatory system should ideally accompany this process, through the creation of a reduced and simplified process when the same, or essentially the same, genetic construct that has been previously deregulated is transferred into another cultivar of the same crop. In Argentina, this situation has already been raised by the National Advisory Commission on Agricultural Biotechnology (Comisión Nacional Asesora de Biotecnología Agropecuaria, CONABIA). From this, a resolution (Nº 318/2013- Ministry of Agriculture, Livestock and Fisheries of Argentina) would allow an abbreviated deregulation process for the use of transgenic technology in asexually propagated crops such as sugarcane.

The aim of our study was to obtain glyphosate-resistant transgenic events of TUC 95-10 and TUC 03-12, through the microprojectile bombardment of plasmids harbouring the *epsps* and *nptII* genes. These commercial sugarcane cultivars were released by the local breeding programme in 2011 (TUC 95-10) and 2015 (TUC 03-12) and are being adopted by farmers due to their increased yields. The genetic construct is the same previously used for obtaining transgenic sugarcane (derived from variety RA 87-3; Noguera *et al*. 2019) resistant to glyphosate and suitable for commercial release according to CONABIA. An abbreviated deregulation process is expected in order to make the new technology available to the production sector, which would improve productivity and contribute to the sustainability of the crop.

MATERIALS AND METHODS

Plant material, genetic construct and gene transfer

Embryogenic calli of TUC 95-10 and TUC 03-12 were prepared as described previously (Bower and Birch 1992).

Plasmid pEA1, containing the synthetic CP4 *epsps* and *nptII* genes, was used for genetic transformation. Both genes are controlled by 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 the chloroplast transit peptide (cTP4) coding sequence from Petunia hybrida. The entire genetic construct was provided by Biosidus AG S.A.

For particle bombardment of calli, purified pEA1 plasmid was precipitated onto tungsten particles (M10, 0.8 µm average diameter), following a previously described protocol (Brasileiro and Carneiro 1998). Sugarcane calli in Petri dishes were placed at a distance of 12 cm from the microcarrier. Bombardment was performed using a microparticle accelerator developed at the Copersucar Technology Center (now the Centro de Tecnologia Canavieira) in Piracicaba, Brazil, according to the model described by Finer *et al*. (1992), under vacuum using a helium pressure of 7584 kPa.

Selection, regeneration and micropropagation conditions

Selection of transformed plants was carried out in the dark using a culture medium containing 45 mg/L geneticin according to Bower *et al*. (1996) with modifications. Selected shoots were regenerated on medium supplemented with 0.7 mg/L BAP without 2,4-D under a 16-h photoperiod. Regenerated shoots were then micropropagated and rooted according to a previously optimized protocol for sugarcane variety RA 87-3 (Noguera *et al*. 2010) and acclimated to ex vitro growth under special conditions (2500 lux and 80–100% humidity). This micropropagation step is necessary to generate material for further testing.

Detecting transgenic events by PCR

Genomic DNA was extracted from 200 mg of leaf material from geneticin-resistant lines and non-transformed control plants by using the method described by Aljanabi *et al*. (1999). DNA concentration and quality were determined

by measuring the OD_{260} in a spectrophotometer and by agarose gel electrophoresis (1%).

The presence of CP4 *epsps* was detected by PCR using GLA-F (5´-GCAAATCCTCTGGCCTTTCC-3´) and GLA-R (5´-GCACGTTGAGGATGGTGAC-3´), whilst the presence of *nptII* was detected by using: NPTII-F (5'-ACTTGTCGACATGATTGAACAAGATGGATTG-3') and NPTII-R2 (5'-TTATAAGCTTGAAGAACTCGTCAAGAAG3-'). PCR conditions were 30 cycles under following conditions: denaturation at 95°C for 30 s; annealing at 70°C (primers GLA) or 69°C (primers NPTII) for 30 s; and extension at 72°C for 60 s. Products obtained were analyzed by agarose gel electrophoresis (1%).

Molecular-marker analysis of transgenic lines

Transgenic events and their parental genotype were analyzed by molecular markers in order to establish genetic similarity, following the protocol described by Noguera et al. (2015). Sugarcane DNA samples were characterized with seven combinations of TRAP primers, involving fixed primers associated with sucrose metabolism genes (SuPS1, DirH, Sut4 and Sut) and the arbitrary reverse primers (Arbi1, Arbi2 and Arbi3) as described by Li and Quiros (2001). The amplification reaction mixture, optimized in our laboratory, contained 1x buffer Taq DNA polymerase, 2.5 mM $MgCl_2$, 0.1 U Taq DNA polymerase, 0.16 µM of both primers (Invitrogen, Life Technologies), 0.088 mM of each dATP, dTTP and dGTP, 0.072 mM dCTP, 0.8 µM Cy5.5-dCTP (GE Healthcare Life Sciences) and 100 ng DNA. PCR conditions were: one cycle at 94°C for 4 min; 5 cycles at 94°C for 45 s; 35°C for 45 s and 72°C for 1 min; 35 cycles at 94°C for 45 s, 50°C for 45 s and 72°C for 1 min; and one final 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), scoring all amplified bands in a dominant manner. A binomial matrix (0 and 1) was used to estimate a distance matrix (1-S) where similarity (S) among genotypes was calculated by using the Jaccard coefficient (Sneath and Sokal 1973). Cluster analyses were carried out with UPGMA method using InfoStat software (Di Rienzo *et al*. 2009).

Herbicide-tolerance assays under ex vitro conditions

Plants transformed were evaluated in order to determine their glyphosate tolerance. Experiments were carried out in triplicate, and two different concentrations of glyphosate from a commercial formulation (YPF Glifosato concentrado HD 62% p/v, Tecnomyl S.A., Argentina) were tested. A solution containing 7.2 or 11.5 g/L of herbicide (N-phosphonomethyl glycine) was applied with a one-nozzle (Teejet 8002VS), handheld boom attached to a CO_2-pressurized backpack sprayer (RYD SPRAYERS). Plants were evaluated at 1, 2 and 3 weeks after herbicide application. The event TUC87-3RG glyphosate-resistant (Noguera *et al*. 2015, 2019), approved by CONABIA, was used as resistant control, whilst conventionally-propagated parental cultivars (TUC 95-10 or TUC 03-12) were included as susceptible controls. Treated plants were also compared with controls without applied herbicide.

RESULTS

Transformation and selection of transgenic lines

A total of 423 plates with calli of TUC 95-10 were bombarded with plasmid pEA1 in 23 independent transformation experiments. From these, 23 plantlets were selected primarily as geneticin resistant, but only two lines survived the complete *in vitro* regeneration process. One event derived from TUC 95-10, called TUC95-10 L20, showed PCR products for NPTII and GLA primers pairs.

A total of 135 plates with calli of TUC 03-12 were bombarded with plasmid pEA1 in 8 independent transformation experiments. From these, 31 plantlets were selected primarily as geneticin resistant, yielding 22 lines that survived the complete process of propagation *in vitro*. Of these plants, three events (L1, L3 and L5) were tested as PCR positive for the CP4 epsps and NPTII genes.

Glyphosate resistance

To determine tolerance against glyphosate, TUC 95-10 L20, three transformed lines (L1, L3 and L5) from TUC 03-12, as well as susceptible and resistant controls, were evaluated under *ex vitro* conditions (Figure 1). Similar results were observed for both glyphosate concentrations tested (7.2 or 11.5 g/L of herbicide (N-phosphonomethyl glycine)). TUC 95-10 L20 was herbicide resistant, since no damage was observed after 3 weeks. Concerning events derived from TUC 03-12, L3 plants were susceptible to glyphosate application, showing more damage than the susceptible control, even only 1 week post-application. L1 showed intermediate tolerance, as during the first week no symptoms were visible, with intermediate damage appearing 2 and 3 weeks after herbicide application. In contrast, L5 plants were glyphosate resistant at both concentrations, maintaining growth similar to the L5 control (without herbicide application) to the end of the 3-week assay.

Genotypic evaluation of transgenic events using TRAP molecular markers

This study was conducted by using duplicated DNA samples from TUC 03-12 and transgenic lines derived from that cultivar. Even though TUC 95-10 L20 was glyphosate-resistant, it was not included in molecular analysis because of the lack of phenotypic resemblance to their parental genotype. In order to evaluate the accuracy of the

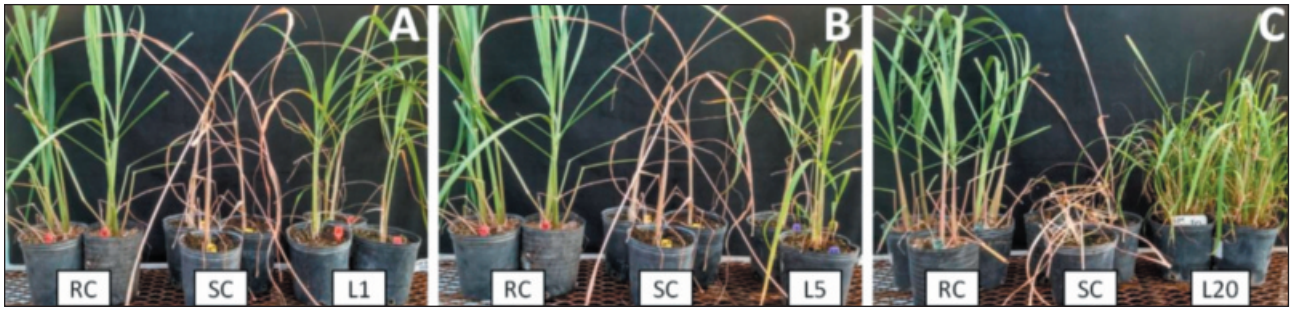

Figure 1. Glyphosate-resistance of transgenic sugarcane expressing synthetic CP4 *epsps* gene, evaluated two weeks after herbicide treatment (glyphosate 7.2 g/L). RC, Resistant Control (glyphosate- resistant TUC87-3RG); SC, Susceptible Control (A y B, TUC 03-12 and C, TUC 95-10); transgenic lines L1, L5 and L20.

technique through assuring that a significant number of polymorphic bands were analyzed, TUC 95-10 was included as a control.

Seven TRAP markers generated a total of 213 *loci*. A dendrogram illustrating the distance (1-S) between genotypes (Figure 2) showed that L3 and L5 are very close to their parental genotype TUC 03-12, with more than 99% similarity. L1 showed a lower similarity with TUC 03-12, although it was also high at 98.7%.

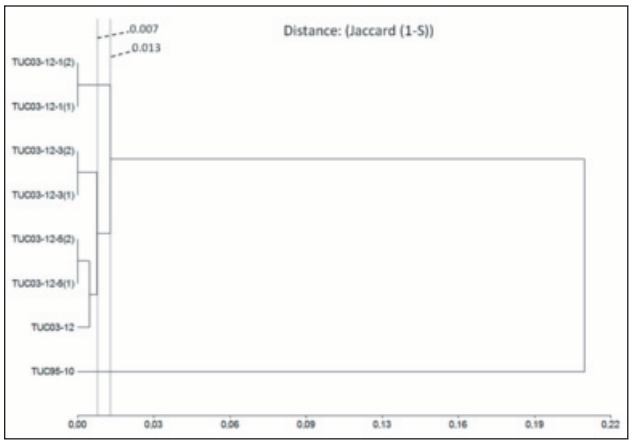

Figure 2. Dendrogram illustrating genetic distances based on Jaccard coefficient among transgenic sugarcane genotypes L1 (TUC 03-12-1), L3 (TUC 03-12-3) and L5 (TUC 03-12-5), and their donor variety TUC 03-12; genotype TUC 95-10 was included as a control, in order to evaluate the power to differentiate between lines. Analysis was performed from distances obtained from 213 *loci* generated with seven TRAP markers by using UPGMA clustering method with InfoStat.

DISCUSSION

Genetic transformation is a powerful tool for sugarcane breeding, since the genomic complexity of the crop makes one-gene transfer through conventional improvement via sexual crossing extremely difficult. Despite its numerous advantages, only few literature reports are available about transgenic sugarcane with commercial purposes. Moreover, despite the economic importance of the crop globally, no country around the world currently commercially grows transgenic sugarcane. However, is important to highlight that Brazil recently approved CTC20bt, a transgenic sugarcane that is insect-resistant, which is in seedling propagation process, in order to gradually increase the planted area and to achieve international approvals for commercialization the sugar produced from the genetically modified sugarcane.

A transformation protocol requires both a tissue-culture procedure that allows recovery of as many regenerated plants as possible together with a highly-efficient transformation method. Several tissue-culture stages are required for gene transfer, including selection and plant regeneration that are the main determinants of genetic transformation efficiency via particle bombardment in sugarcane (Singh *et al*. 2013). It is well known that both tissue culture-response and transformation rates are genotype-dependent, among many other factors. Our results agree with this, since calli from TUC 95-10 was bombarded three-times more than TUC 03-12, and only two plantlets from the former survived the entire *in vitro* process compared with 22 plantlets from latter. It is important to mention that calli of untransformed controls, as well as controls subjected to microprojectile bombardment without DNA, yielded numerous plantlets, and biolistic efficiency was previously optimized in our laboratory by using reporter genes indicating a high number of stable transformed cells, 10 days after transformation. Hence, it is possible to consider that mechanisms could exist whereby transformed cells are able to silence or expel transgenes (Iyer *et al*. 2000).

The presence of the CP4 *epsps* and *nptII* genes, as determined by using specific PCR, allowed the identification of one transgenic line derived from TUC 95-10. The event TUC95-10 L20 showed high herbicide tolerance, although dramatic phenotypic differences were noticeable with respect to their parental genotype. Due to this, the line was not subjected to TRAP molecular analysis and was discarded, in line with the aim of obtaining genotypes suitable to the commercial release.

Three transgenic events obtained from TUC 03-12 showed different tolerance levels to glyphosate, including

TUC 03-12 L5 which had the highest resistance. This event displayed more than 99% similarity to its parental genotype, highlighting the potential to be considered for further field testing and molecular studies conducive to the deregulation process.

CONCLUSIONS

A transgenic event tolerant to herbicide glyphosate derived from TUC 03-12 was obtained through the biolistic transformation method. The high similarity observed (more than 99%) with their parental genotype is an encouraging result, since based on our previous knowledge, molecular markers would indicate that TUC 03-12 L5 is suitable for future comparative field testing.

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