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A pseudo-colony PCR method for rapid identification of transgenic sugarcane lines at *in vitro* stage

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Abstract: *Obtaining transgenic sugarcane lines in vitro is a laborious, expensive, and time-consuming process. To minimize these disadvantages, the presence of transgenes must be checked as soon as possible. Here, we describe a method for direct PCR amplification of DNA from sugarcane plantlets, using smaller quantities of vegetal material, low-cost lab reagents, and rapid processing. By using this methodology, we were able to identify 100% of the transgenic lines generated. The outlined protocol enabled us to perform an earlier checking of the transgene insertions on genomes of transformed lines reducing costs and labor at in vitro stages.*

Keywords: *Sugarcane, pseudo-colony PCR, transgene insertions, in vitro culture*

INTRODUCTION

Genetic transformation is a powerful tool to incorporate economic valuable traits in sugarcane, such as disease and insect resistance, improved tolerance to cold, salt, and drought, herbicide resistance, and accumulation of sugar and biomass (El Sheikha 2018, Budeguer et al. 2021, Verma et al. 2022). It is also a very arduous process, which consists of intensive and sophisticated tissue culture and plant regeneration procedures that must be optimized for each new sugarcane genotype to be transformed (Altpeter and Oraby 2010).

Hundreds of potential transgenic sugarcane lines are obtained when the transformation, selection, and regeneration procedures for a particular sugarcane variety are optimized. However, keeping these vegetal materials becomes a laborious, expensive, and time-consuming process. In this context, a quick check of transgenic lines using PCR is needed.

The transgene presence in transformed plants is checked by classical PCR using specific primers and total genomic DNA as a template (Noguera et al. 2015, Enrique et al. 2021). Aljanabi´s method is one of the most popular high-quality DNA extraction protocols (Aljanabi et al. 1999). However, in high-throughput screenings, this protocol requires significant quantities of leaf tissues, nitrogen, solvents, and labor time. Interestingly, Bellstedt et al. (2010) described a method, based on La Notte´s (1997) studies, that involves direct PCR amplification from material macerated in one buffer, followed by dilution and incubation in a second buffer. The effectiveness of this protocol was demonstrated for DNA amplification across a broad range of vascular plants, such as *Citrus limon*,

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Vitis vinifera, *Laurus nobilis,* and *Coffea arabica L.*, among others. Similar protocols have been published by Rogers and Parkes (1999), Sharma et al. (2012), and Ben-Amar and Mliki (2021) without DNA extraction and direct PCR amplification. However, none of them have processed sugarcane tissues. This work aimed to adapt a rapid and inexpensive alternative method to DNA isolation for direct polymerase chain reaction (PCR) for sugarcane plantlets, as colony PCR is for bacteria and other microorganisms.

MATERIAL AND METHODS

Plant and reference materials

The transgenic sugarcane lines were generated by transformation of the elite variety TUC 03-12 with a gene construct (pAST) containing the gene *npt*II for kanamycin selection, as previously described by Enrique et al. (2021). The non-transformed (NT) elite variety TUC 03-12 was used as genetic control material.

Rapid DNA isolation

Five mg of fresh tissues from putative transgenic lines and NT plantlets (*in vitro* and rusticated vegetal materials) were macerated in a 1.5 mL Eppendorf® tube with 0.7 mL grinding buffer 1 (15 mM Na_2CO_3; 35 mM $NaHCO_3$; 2% (m/v) PVP 40; 0.2% (m/v) BSA; 0.05% (v/v) Tween 20; 1% (m/v) sodium metabisulphite, pH 9.6) using a plastic pestle and 30 mg of sterilized sand to facilitate the disaggregation of tissues. Samples were centrifuged for 30 s at low speed (7000 rpm) using an Eppendorf® AG 5418 centrifuge (USA). Five µL of the clear supernatant were added in a 1.5 mL Eppendorf® tube containing 25 µL GES buffer 2 (0.1 M Glycine-NaOH, pH 9.0; 50 mM NaCl; 1 mM EDTA, pH 8.0; 0.5% (v/v) Triton X-100), incubated at 95 °C for 10 minutes, and immediately conserved on ice for 5 minutes. These samples could be used for PCR amplification or stored at -20 °C.

Conventional DNA extraction

Total genomic DNA was extracted according to the CTAB method described by Noguera et al. (2015) using 200 mg of ground young frozen leaves of the putative transgenic lines and the NT plantlets (*in vitro* and rusticated vegetal materials).

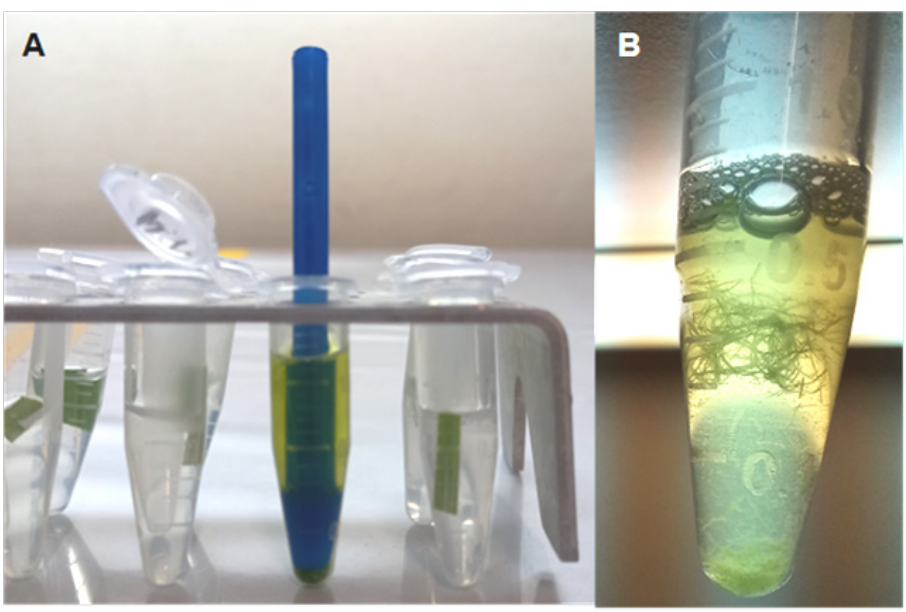

Figure 1. Processing of samples. A) A piece of sugarcane leaf (5 mg) is disaggregated in buffer 1 using a plastic pestle. B) Sugarcane leaf after the disaggregation process.

Detection of transgenic plants by PCR

One µL of the resulting solution was used as a DNA template directly in the PCR reaction. A fragment of 200 bp of the *npt*II gene was amplified by PCR using forward primer NPT2-F (5´-CTGCTCTGATGCCGCCGTGTTC-3´) and reverse primer NPT2-R (5´-CTTCGCCCAATAGCAGCCAGTCC-3´) to detect genetically transformed lines. PCR amplification of the *npt*II gene fragment was performed during 30 cycles according to the following conditions: denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s, and extension at 72 °C for 60 s. Besides, a fragment of 187 bp of *actin* gene was amplified by PCR using forward primer ACT3-F (5´-GATGGTGAGGACATCCAGC-3´) and reverse primer ACT2-R (5´-CCTCTCTTGGACTGTGCCTC-3´) and used as amplification control of tested samples. PCR amplification of the *actin* gene fragment was performed during 30 cycles according to the following conditions: denaturation at 95 °C for 45 s, annealing at 57 °C for 45 s, and extension at 72 °C for 60 s. Amplification products were analyzed in 1.5% (w/v) agarose gels stained with GelRed (Biotium, USA).

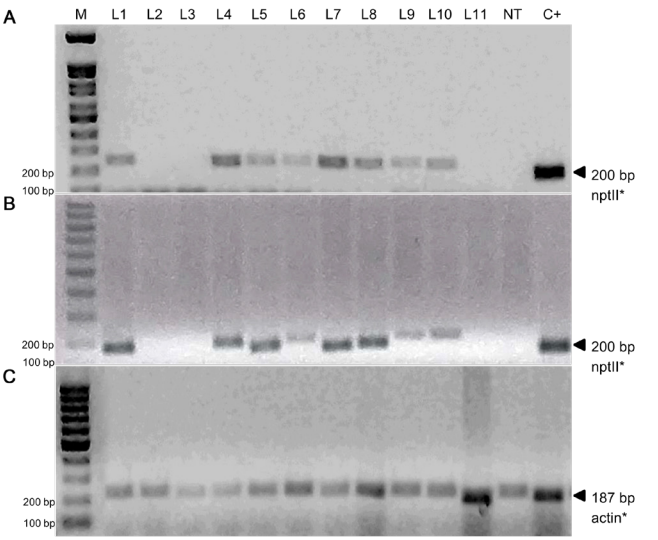

Figure 2. Comparison of two DNA isolation methods by PCR. A) Agarose gel showing the presence of a 200 bp fragment of the *nptII* gene in the DNA of transgenic lines (L1-L11), isolated by rapid protocol. B) Agarose gel showing the presence of the 200 bp transgene fragment in DNA of same transgenic lines, isolated by conventional method. C) Agarose gel showing the amplification of a 187 bp actin fragment in the assessed DNAs, isolated by rapid protocol. M, molecular marker; L1-L11, transformed lines; NT, non-transgenic parental line; C+, positive control of PCR reactions (dilution 10^{-3} of pAST for amplification of *nptII* fragment and DNA of TUC 03-12 (conventional method) for amplification of an actin fragment).

RESULTS AND DISCUSSION

We obtained more than 200 transformed sugarcane lines of TUC 03-12 parental variety after bombarding with a gene construct containing the *nptII* gene as a selection marker (data not shown). Subsequently, we isolated DNA using both rapid and conventional (Noguera et al. 2015) protocols to check the presence of the *nptII* transgene on genomes of transformed lines.

To isolate DNA for direct PCR amplification (rapid protocol), samples (5 mg) from *in vitro* and rusticated transformed sugarcane plantlets were macerated in buffer 1 using a plastic pestle, as shown in Figure 1A. Tissues were completely disaggregated after 60 seconds of processing (Figure 1B). Tissues from rusticated transgenic plantlets underwent complete disaggregation; however, greater mechanical force was required during the grinding process compared to the *in vitro* material. The sterilized sand facilitated the disaggregation of tissues containing a higher content of fibers. Furthermore, the composition of the grinding buffer (buffer 1) results in a good release of DNA from the plant tissue and protects it against oxidative and enzymatic damage, whereas the second buffer (GES) leads to the release of PCR inhibitors from DNA during the amplification reaction.

The presence of *the nptII* gene was checked by using conventional PCR. Amplification reactions confirmed 117 transgenic lines out of 208 tested transformed lines (Figure 2A). PCR amplification of isolated DNA from samples processed with the conventional protocol gave the same results (Figure 2B), demonstrating the reliability of the rapid protocol. It is important to notice that the band intensities were different between samples. This phenomenon could be caused by dissimilar quantities of DNA in samples and/or in the number of *npt*II insertions on genomes of the transgenic lines (Koressaar et al. 2009).

CONCLUSION

We optimized a pseudo-colony PCR protocol for sugarcane tissues using smaller quantities of vegetal material, low-cost lab reagents, and rapid processing. It is particularly suitable for processing tissues rich in polyphenols and

polysaccharides, such as sugarcane leaves. The protocol enabled an early reliable checking of the transgene insertions on genomes of transformed lines, hence reducing costs and labor at *in vitro* stages. The absence of a conventional DNA isolation step before PCR amplification and its high processivity makes it a cost-effective choice for projects requiring analysis of large sample numbers, such as transgenesis, genome editing, and barcoding.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current research are available from the corresponding author upon reasonable request.

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