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Multiplex-PCR Detection of Accompanying GMO Transgenic Sequences

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***Abstract*—The goal of this study was the development and optimization of a multiplex-PCR protocol for the simultaneous detection of three accompanying transgenic sequences. The DNA was extracted and the target sequences were separately detected by PCR. Multiplex PCR was performed with DNA from maize, cotton and soybean for the detection of transgenic sequences, allowing the amplification of nos (125 bp), ntpII (271 bp) and luc (450 bp) at 62º C. Optimizing the multiplex PCR technique for the amplification of the accompanying transgenic sequences could become a very valuable tool for detecting genetically modified organisms (GMO).**

***Keywords t*— nos, luc, ntpII**

I. INTRODUCTION

Polymerase Chain Reaction (PCR) based techniques have been preferred for the detection of GMOs mainly due to the specificity and sensitivity but also because they are fast, reproducible and can be automated (Tamarin, 1996). Multiplex PCR is a special kind of PCR that simultaneously amplifies two or more target sites during the same enzymatic reaction, instead of the synthesis of multiple loci sequences by individual reactions, which represents both advantages in terms of time and cost. There are reports on the application of multiplex PCR for the detection of transgenic events on corn, soybean and canola, where the reaction conditions have been standardized for the different plants and transgenic events (James et al., 2003). Multiplex PCR systems have also been developed for the identification of five genetically modified corn lineages using specific primers for the transgenic events (Matzuoka, et al., 2001). Research studies have lead to the optimization of multiplex PCR protocols targeting samples from genetically modified soybean and corn, amplifying different transgenic events (Permingeat et al., 2002; Germini et al., 2004). All the reported studies have used the sequences of the transgenic events to optimize the multiplex PCR systems. However, various gene sequences are incorporated during the creation of transgenic organisms such as the promoter, terminators, markers and reporter genes; these sequences are always adjacent to the transgene, and for this reason they are named accompanying sequences (Germini et al., 2004). The promoter regulates the inserted gene, enabling its expression and the quantity in which it is being expressed in the transformed organism. On the contrary, the terminator stops the expression of the gene. It is common to add a marker gene with the purpose of identifying the plant cells or tissues that have successfully integrated into the transgene. The reporter genes encode an activity that can be easily measured (James et al., 2003). The objective of the present study was to design a multiplex-PCR protocol for the identification of transgenic residues in corn, soybean and cotton plants based on the PCR amplification of the sequences that accompany the transgene event.

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II. MATERIALS AND METHODS

A. Plant material

Seventy two grain samples were randomly taken from the same number of import batches during 2005, 29 corresponding to corn, 22 to soybean and 21 to cotton. These imports came from the United States of America. The seeds were planted in polystyrene vessels with sterile soil and were provided with distilled water every two days. Plants were exposed to the sunlight for three weeks, growing in this from 10-15 cm; and leaf tissue samples were taken at this height. Seeds were disinfected with a 3% chlorine solution, wrapped in paper, wetted and put within an incubator at 25º C, under these conditions the plantlets appeared within six days.

B. DNA isolation and PCR primers

DNA isolation was performed by means of the Graham et al., (1995) method.

The most frequently used accompanying transgenic sequences and the primers designed for their detection were searched on the literature (http://www.agbios.com/dbase.php) (Table 1). The sequences used in this research were the promoters ubi and 35S, the terminators ocs y nos, the marker genes ntpII and bar and the reporters gus and luc. The primer were tested to fill of the requisites for good primers as defined by Sharrocks, 1994 using the software Oligo analyzer 3.1 (http://www.idtdna.com/analyzer/Applications/

OligoAnalyzer/). The software FastPCR was employed to analyze the primers sequences and to determine whether the designed oligos were suited for multiplex PCR.

C. Multiplex-PCR Optimization

After determining the DNA integrity, the multiplex PCR was performed employing a PCR Thermocycler model Px2 (Thermo Electron Corporation, Milford, MA. USA). The PCR reaction volume was 30 µL for a double PCR and 34 µL for triple PCR; containing 14.8 µL deionized sterile distilled water, 2.5 µL of 10X PCR buffer (Invitrogen, Carlsbad Ca. USA) 2.0 µL of 25 mM MgCl2 (Invitrogen, Carlsbad Ca. USA) (MgCl2 concentrations were adjusted by adding 0.41,

0.83, 1.25 and 1.6 mM for the double reaction; 0.37, 0.73, 1.10 and 1.47 mM for the triple reaction), 2.5 µL dNTP (Invitrogen, Carlsbad Ca. USA) (0.83 mM for the double reaction and 0.73 mM for the triple one), 2.0 of 10 pM forward primer, and 2.0 µL of 10 pM reverse primer (Table 1). After thoroughly mixing the reagents, 0.5 µL of 5 U/µL Taq DNA Polymerase (Bioline, London UK) and 2.0 µL of template DNA were added (Viñas, 2004). The optimum annealing temperature for the polymerase chain reaction was determined by running sets of reactions from 57º to 64º C, with 62º C yielding the best results (Table 1). Denaturing was done at 94º C and polymerization at 72º C in all the cases, with 1 minute per PCR cycle step and 35 cycles for the overall reaction. A 2% agarose (Invitrogen, Carlsbad Ca. USA) gel electrophoresis was ran to visualize the amplified PCR products, with a 100 bp DNA ladder (Invitrogen Carlsbad Ca. USA) used to assess the products base pair lengths.

III. Results and Discussion

A. *Multiplex PCR for two sequences*

The multiplex PCR reaction for two gene sequences was carried out using the DNA template employed for the single PCR reactions, following the protocol described by Viñas (2004), with the magnesium chloride concentration optimized (Zangenberg et al., 1999) by running reactions with 0.41, 0.83,1.25 and 1.65 mM MgCl2; with the last concentration yielding the best results. Permingeat et al. (2002) obtained good results with 1.5, 2.0 and 3.0 mM of MgCl2. Similarly, James et al. (2003) and Germini et al. (2004) used the magnesium chloride at 1.5 mM, which are very close to the concentration used in this research. Once the magnesium chloride concentration was optimized, and with 62º C as the annealing temperature, distinctive bands with 271 pb and 450 bp sizes were obtained, corresponding to the marker gene ntpII and the reporter luc (Fig. 1).

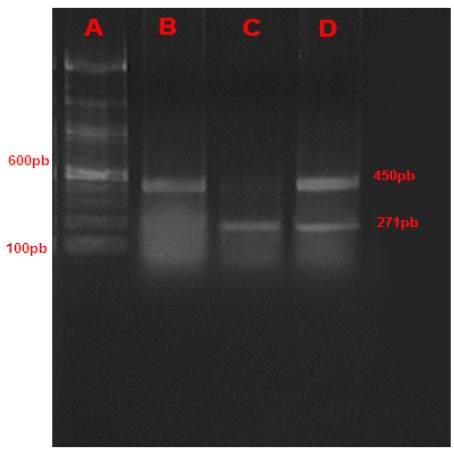

Figure 1. Multiplex PCR for two soybean sequences. A. 100 bp marker ladder (Invitrogen). B. luc sequence. C. ntpII sequence. D. Both sequences

B. *Multiplex PCR for three sequences*

The reaction was set the same as the double PCR, adjusting the annealing temperature to a value that enabled the simultaneous amplification with the three sets of primers. PCR reactions were run at 64º, 62º and 57º C, the corresponding annealing temperatures for ntpII, luc and nos. When these primers are used together it was determined that the best results are obtained with an annealing temperature of 62º C. Optimization of the magnesium chloride concentration was determined by running sets of reactions using 0.37, 0.73, 1.10 and 1.47 mM MgCl2, the latter one conducing to the best results. The optimum magnesium chloride concentration found is similar to the concentration used by other researchers in multiplex PCR protocols (Permingeat et al., 2002; James et al., 2003; Germini et al., 2004). With the described conditions, distinctive bands of the expected base pair sizes were obtained, with a 450 bp band corresponding to the reporter gene luc, a 271 bp band for the marker gene ntpII and a 125 bp for the terminator gene nos (Fig. 2).

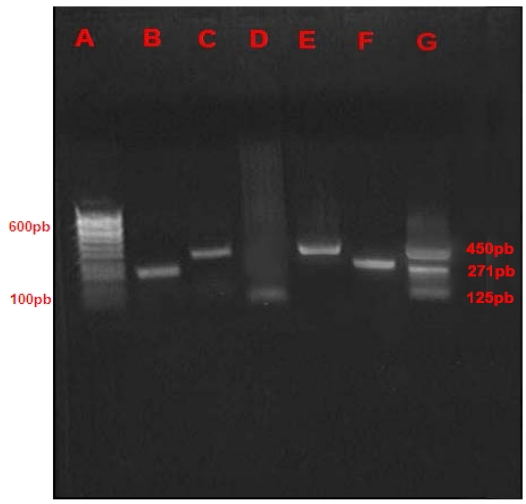

Figure 2. Multiplex PCR (three sequences). A, 100 bp marker ladder (Invitrogen). B and F, ntpII sequences (271 bp). C and E, luc sequences (450 bp). D, nos sequences (125 bp). G, the three sequences

Most research aimed at detecting transgenic plants by PCR has employed primers against the transgenic events (Permingeat et al., 2002; James et al., 2003; Germini et al., 2004). By contrast, here we identified the most common gene sequences accompanying the transgenic events, which is a useful contribution that reduces the possibilities of losing a positive sample. To date, 108 different events have been reported for transgenic crops (www.agbios.com). The probabilities of detecting a positive sample based on one transgenic event at a time is equal to 1/108 transgenic events, meaning a probability of 0.9%, while the detection based on the accompanying sequences significantly increase the probabilities of identifying a transgenic sample because they are present together with many events. For instance, the nos terminator gene, used in this study, occurs in 77% of the transgenic events, therefore the probability of identifying a true transgenic sample would be 77%, and this chance will increase when simultaneously employing several accompanying sequences.

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Table 1. Gene or gene sequence, primers sequence, PCR product size, and annealing temperature, used for the identification transgenic sequences in corn, soybean and cotton samples.

Gene or gene sequence	Primer sequence	Size bp	Annealing Temp. ºC
ubi	F-5'-gctaacttgccagtgtttctctttgg-3' R-5'-ggctggcattatctactcgaaacaag-3'	220	55
35 s	F-5'-gctcctacaaatgccatca-3' R-5'-actgcgtgttagggtgatag-3'	238	60
bar	F-5'-gcacagggcttcaagagcgtggtc-3' R-5'-gggcggtaccggcaggctgaa-3'	177	55
ntpII	F-5'-gaggctattcggctatgact-3' R-5'-aaggtgagatgacaggagat-3'	271	64
gus	F-5'-ggtgggaaagcgcgttacaag-3' R-5'-accgccttcgttgcgcatttg-3'	150	55
luc	F-5'-cgccaaaaacataaagaaaggc-3' R-5'-tgtccctatcgaaggactctgg-3'	450	64
ocs	F-5'-ctcgagctgctttaatgagatatgcg-3' R-5'-tctagactgctgagcctcgacatgttg-3'	120	55
nos	F-5'-gaatcctgttgccggtcttg-3' R-5'-gcgggactctaatcataaaaacc-3'	125	57