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Full Length Research Paper

Simultaneous detection of three transgenic events in crops using multiplex polymerase chain reaction (PCR)

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In spite of all advantages for using end-point polymerase chain reaction (PCR) this technique can only detect one gene DNA sequence. However multiplex PCR has been proposed for employing in the detection of two or more DNA sequences or genes. This implies saving time and money. The objective of this study was to adapt the multiplex PCR technique for detection of either two or three of the most common transgenic genes present in different plants. The DNA was isolated from maize, soybean and cotton plants. The optimization of the multiplex PCR was performed changing the concentration of $MgCl_2$, primer annealing temperature and primer design. With this optimization it was possible to amplify three genes (*cry* 1Ab, *epsps* and *als*) in same reaction of PCR. These results may lead to increased efficiency in detection of genetically modified organisms.

Key words: Multiplex polymerase chain reaction (PCR), *cry 1Ab, epsps, als.*

INTRODUCTION

The negative effects caused to plants by insects and environment has led to the development of new genomic strategies to create and characterize the genetic diversity in plants (Roessner et al., 2001; Stein et al., 2009). This diversity is achieved commonly by the insertion of a recombinant piece of DNA into the genome of the organism. This diversity is gotten commonly through the insertion of a recombinant piece of DNA into the organism's genome (Nesvold et al., 2005). Commonly, soybean, cotton and maize are genetic modified with different aims such as herbicide tolerance or insect protection (Phipps et al., 2003; Calsamiglia et al., 2007). In this case, the genes *bar*, *pat* and *cry*3A/35/Ab1 are used to confer tolerance and protect against both root-worm and herbicides that contain glufosinate-ammonium (Thompson et al., 1987; Toyama et al., 2003; Bae et al., 2008; Stein et al, 2009). These genes are some of the most common transgenic sequences used.

In spite of the great agricultural advantages of the transgenic crops, they are not accepted in some countries because of the suspicion of the consumer as result of the allergic reactions observed with some transgenic crops, and the lack of worldwide regulations to these crops. The negative side effects to the environment by the massive farming of transgenic crops, for example loss of genetic diversity and the creation of higher adapting weeds, as documented in rye grass (*Lolium perenne* L.) populat-ions, Quack grass *(Agropyron repens)*, birdsfoot trefoil *(Lotus corniculatus)* and species of *Cirsium* that are transgenic crops become weeds (Holt, 1993) and the migration of transgenic genes to their wild relatives and less

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likely the migration of transgenic genes to other unrelated organisms by horizontal transference (Goldburg, 1992). Also, the contamination of food with transgenic residues has persuaded different countries to restrict the importation of food made with transgenic plants or labeling the food or the ingredients as from transgenic crops (Sasson, 2001). Moreover, prior reports have mentioned that transgenic DNA could be transferred and accumulated in different animal products (Klotz et al., 1999, Reuter and Aulrich, 2003; Calsamiglia et al., 2007). Chowdhury et al. (2003) detected transgenic traits by either PCR or ELISA of c*ry*1ab in the gastrointestinal contents. However, Phipps et al. (2005) mentioned that the transgenes and its products have been not detected in the final product. These authors analyzed several samples of milk by both PCR and Enzyme-Linked Immuno-Sorbent Assay (ELISA) and they did not detect transgenes.

Then, it is necessary before releasing the transgenic, to evaluate the effects of the transgenic genes and the genetically modified organisms (GMO) on health and environment. But it has been noticed that release of transgenic either hostile intention or escapes from experiments without public notice. Despite the controversy that exist in Mexico there is little legislation about this topic. To help competent authorities and others responsible for food and environmental safety, it is urgent to provide suitable analytical tools for discovering Unknown GMOs (Nesvold et al., 2005).

The analytical Tools for detection of GMO´S are generally divided into two methodologies. One methodology is to detect the protein products and the other is based on detection of the genes that have been introduced into the plant. This methodology is commonly performed using PCR (Spinegelhalter et al., 2001). Most PCR protocols for GMO detection involve reactions for amplification of only one segment (Gachet et al., 1999), though the multiplex PCR has been developed for amplifying two or more sequences simultaneously in the same reaction (Edwards and Gibbs, 1995). To perform multiplex-PCR, it is necessary that mixed reagents and thermo-cycler program be adequate to allow detection of each sequence which do not inhibit detection of other sequences. In this case, some parameters such as magnesium and primers concentration, as well as type and amount of DNA polymerase must be adjusted experimentally (Méndez-Álvarez and Pérez-Roth, 2004). The objective of this work was the optimization of a multiplex PCR for the detection of three of the most common transgenic events in soybean, maize and cotton plants.

MATERIALS AND METHODS

Vegetal material

Seventy-two grain samples were analyzed, 29 were of maize, 22 of soybean, and 21 of cotton. The grains samples were taken randomly during the year 2005 at Nuevo Laredo, Tamaulipas, Mexico. These lots were obtained from grain imports from different Countries. At least 300 seeds of each sample were planted and seeded in a container with sterile soil. After that, they were watered every two days with sterile distilled water. These containers were placed under greenhouse conditions during three weeks; during this period the plants grew approximately 10 to 15 cm. At this point, tissue was collected from each single plant leaf for each sample. In some cases because of technical problems some samples were germinated in an incubator, placing the seed (previously disinfected with a chlorine solution to 3%) in paper towels watered with sterile distilled water, after that, they were placed in an incubator at 25℃. Seedlings were obtained 6 days later, only from these seedlings; leaf tissue was collected.

DNA isolation

The DNA isolation was performed using the method reported by Graham et al. (1995), which is explained briefly as follows: 0.1 g of vegetable material was frozen in liquid nitrogen. Samples were ground to fine powder, which was transferred to an Eppendorff tube and 1 ml of buffer CTAB (57.1 ml NaCl 5 M (SIGMA-ALDRICH St Louis MO, the USA), 10.2 ml Tris-HCl 2 M (SIGMA-ALDRICH St Louis MO, the USA) pH =8.0, 8.2 ml EDTA 0.5 M (SIGMA-ALDRICH St Louis MO, the USA), 4.1 g CTAB in 124.5 ml of distilled water) and 20 µl of bovine serum albumin 20% (SIGMA-ALDRICH St Louis MO, the USA) to 20% were added to the tube.

The tubes were vortexed and placed in water bath for 20 min at 55℃. Immediately, the tubes were centrifuged at 125 00 rpm during 10 min. Later the superior phase was transferred to a new tube and an equal volume to the chloroform: isoamilic alcohol (24: 1) (SIGMA-ALDRICH St Louis MO, the USA) mixture was added. The tubes were mixed by gentle inversions during 2 min. A new centrifugation was carried out to 12,500 rpm for 10 min. The liquid phase was transferred to a new tube. Later 50 µl of ammonium acetate 7.5 M (SIGMA-ALDRICH St Louis MO, the USA) and 800 µl of cold ethanol (SIGMA-ALDRICH St Louis MO, the USA) were added to 96%. After that, the tubes were mixed by gentle inversions and were placed in a freezer for about one hour to -20℃. After this time the tubes were removed from the freezer and were centrifuged to 12,500 rpm for about 5 min. The supernatant was discarded and the DNA paste was washed with alcohol to 70% twice, mixing by gentle inversions. The DNA pastes were suspended in 80 µl of NaOH 8 mM (SIGMA-ALDRICH St Louis MO, the USA). The microtubes were stored in a refrigerator at 4℃. In order to verify the integrity of the extracted DNA an agarose (1.5%) gel electrophoresis was performed.

Primer design

The primers for three transgenic events (*als*, *cry*1Ab and *epsps)* were selected from literature (Tables 1 and 2). Once primers were theoretically verified about high G-C frequency at their ends, in addition primers were checked for no homology with itself or with other primers, annealing temperature according to Wallace's equation (Wallace et al., 1979); primer length for high hybridization was verified to guarantee a good primer design (Sharrocks, 1994). Theoretical estimated parameters were verified using the software Oligo Analyzer version 3.0 (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/).

Optimization of the multiplex PCR technique

The software FastPCR ® was used to test the primers employed to perform single PCR reactions. This software was used to determine interactions among, and if these primers can be used in a multiplex PCR reaction. The single and double PCR reaction were carried out

Table 1. Target gene, primer sequence, band size and annealing temperature using for transgenic event detection.

Gene sequence	Primer sequence	Band size (bp)	Annealing temperature (ºC)	Reference
als	F-5'-gggttacgcacgcgccaccgg-3' R-5'-ggctgatcccagtcaggtatc-3'	397	64	(11)
cry 1Ab	F-5'-accatcaacagccgctacaacgacc-3' R-5'-tggggaacaggctcacgatgtccag-3'	184	62	(2)
epsps	F-5'-tggcgcccaaagcttgcatggc-3' R-5'-ccccaagttcctaaatcttcaagt-3'	356	62	(13)

Table 2. Primer combinations that should be avoided when using the Multiplex-PCR proposed in this study.

Event	Primers	Event	Primers
epsps	F-5'-tggcgcccaaagcttgcatggc-3' R-5'-ccccaagttcctaaatcttcaagt-3'	***cry* 3A**	F-5'-acatgcatgcattaactagaaagtaaagaagtag-3' R-5'-acatgcatgcaagcttacagagaaatacacgaggg-3'
epsps	F-5'-tggcgcccaaagcttgcatggc-3' R-5'-ccccaagttcctaaatcttcaagt-3'	***cry*11A**	F-5'-acatgcatgcagtcatgttagcacaagagga-3' R-5'-acatgcatgctttaggtctttaaaaattaga-3'
epsps	F-5'-tggcgcccaaagcttgcatggc-3' R-5'-ccccaagttcctaaatcttcaagt-3'	**ACCasa**	F-5'-taggactggtaccgtaaagcagagtaacacaaggtcag-3' R-5-taggactctcgagagtctttcggaacctcacaccataagg-3'

in an entire volume of 30 µl and in the case of the triple PCR reaction 34 µl were used as entire volume. The PCR cocktail was composed as follow; 2.5 µl of Buffer 10× (Invitrogen, Carlsbad Ca. It uses), 2 µl of $MgCl_2$ (Invitrogen, Carlsbad Ca). Eight different $MgCl_2$ concentrations were tested (0.41, 0.83, 1.25 and 1.6 mM for double PCR reactions and 3.37, 0.73, 1.10, and 1.47 mM for the triple PCR), 2.5 µl of dNTPs (10 pM)(Invitrogen, Carlsbad Ca). It uses (0.83 µl of dNTPs in the double PCR and 0.73 µl in the triple PCR reaction), 0.2 µl of Taq Platimun (Bioline, London UK) (5 U/ µl) and 14.8 µl of distilled water deionized sterile, 2 µl of the forward primer 10 pM and 2 µl of the reverse primer 10 pM, and finally 2 µl of sample of DNA (Vineyards, 2004). The PCR was performed under the following conditions, 35 cycles of 3 steps each one, a denaturalization step to 94℃ for a minute, an annea ling step to 62℃ for a minute, and an elongation step to 72℃ fo r a minute. During the amplification process, a thermocycler Thermal cycler model Px2 (Thermo Electron Corporation, Milford, MA.) was used. Amplified bands were visualized performing an agarose (1.5%) gel electrophoresis. Size of amplified band was determined using as reference the molecular markers 50 bp and 100 bp DNA molecular ladder (Invitrogen Carlsbad Ca. USA). Also the amplified bands were sequenced to corroborate the identity of the transgenic amplified events.

RESULTS

Determination of transgenic residues in imported corn, soybean and cotton samples

The samples were considered to be genetically modified if at least one of the genes *als*, *cry*1Ab and *epsps* were amplified by PCR. Hundred percent of the corn, soybean and cotton analyzed contained at least one transgene. This result suggests that the grains and plants imported to Mexico are only GMO. Moreover, in Mexico there is no control and the non-transgenic and transgenic grains and probably those plants are being all mixed up during harvesting, transporting and storing.

The combinations of two pairs of primers to carry out double PCR reactions were used to amplify both genes *cry*1Ab and *als*, the amplicons showed a size of 184 and 397 bp, respectively (Figure 1). Then $MgCl_2$ concentration was estimated and established within 1.6 mM. This $MgCl_2$ concentration is almost the double of the amount recommended for single PCR (Williams, 1989). Optimal concentration of $MgCl_2$ is very important for the PCR reaction, because it gives a specific amplification and reduction of the number amplified products (Henegariu et al., 1997; Ellsworth et al., 1993).

The reaction with three pairs of primers was performed in order to amplify the *cry*1ab, *epsps* and *als* genes. Previously, the optimal annealing temperature for triple PCR was determined by using temperature gradients. The results indicated that the optimal annealing temperature for the three pair of primers tested was 63.3°C. Also $MgCl_2$ concentration was optimized, in this case four different concentrations (0.37, 0.73, 1.10 and 1.47 mM) were tested. The optimal $MgCl_2$ concentration was 1.47 mM (Figure 2). Finally, of the 132 samples analyzed, 37

Figure 1. Multiplex PCR (two events) A, B and C are maize samples, D, E, F, G and H are soybean samples. M: Molecular Marker Invitrogen DNA 100 bp ladder. A, B and E amplification of transgenic events *cry* 1Ab (184 bp) and *als* (397 bp).

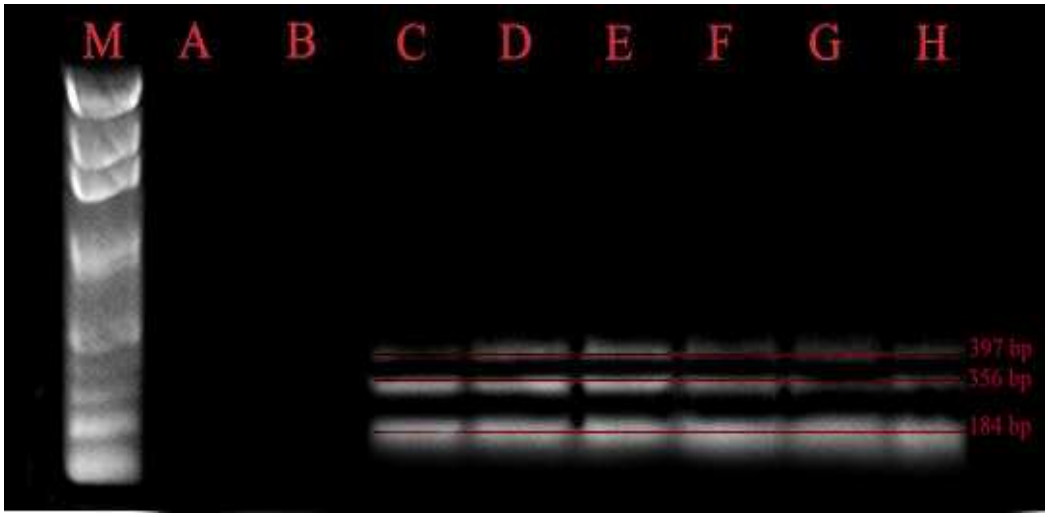

Figure 2. Multiplex PCR (three events) A, B, C and D are maize samples, E, F, G and H soybean sample. M Molecular Marker Invitrogen DNA 100 bp ladder. C, D, E, F and H amplification of transgenic events *cry* 1Ab (184 bp), epsps (356 bp) and *als* (397 bp).

have been modified with the *epsps* gene, 17 with the *als* gene and 27 with the *cry*1Ab/ac gene (http://www.agbios.com/dbase.php).

DISCUSSION

In this work we amplified three different transgenic gene sequences by PCR in plants from different imported grain (maize, soybean and cotton) lots to Mexico. Previously, Phipps et al. (2003) confirmed by PCR the presence of the *cry1ab* (203 bp) in soybean and corn, while, Chowdhury et al. (2003) identified fragments of *cry*1ab (110 and 437 bp, respectively) in the gastrointestinal contents of pigs. However, Nair et al. (2002) reported that no detected fragment of *cp*4 *epsps* gene neither Northern blot nor Real Time (RT)-PCR but they detected this gene by western blot.

Despite methods based on PCR technology implemented to GMO detection, difficulties still exist to analyze several targets simultaneously (Rudi et al., 2003; Palaudelmas et al., 2008). In the present study three different set of primers were used in order to search transgenes in the sample described above. Prior to this, some protocols have been reported to perform multiplex PCR for GMO detection. Moreover, Rudi et al. (2003) mentioned that the developments of multiplex PCR are generally limited by the complexity of the amplification reaction, and the possible primer dimer formation. Another controversy with multiplex PCR is the amount of the DNA used; in this work the DNA concentration employed was 100 ng and amplicons were obtained (Figure 1 and 2), but we were not able to quantify the products of PCR. Earlier, Permingeat et al. (2002) reported the amplification by PCR-multiplex of two different transgenes (*cry1Ab* and *pat)* from 4 different lines of genetically modified maize. Additionally, Rudi et al. (2003) developed a quantitative multiplex DNA array based PCR method, and used 10-50 pg of ADN as template. In accordance with prior reports, the probes such as TaqMan or Cyber Green could help to increase the specificity and let us quantify the product of PCR, however, that is an expensive technology and lot of countries need to develop cheap technologies. The multiplex PCR showed in this paper can help developed countries save more money.

Conclusion

The optimization of a multiplex PCR for the simultaneous detection of 3 of the most common transgenic events (*epsps*, *cry* 1Ab and *als*) in modified genetically crops was obtained, amplifying bands of 356, 184, and 397 bp from each gene respectively. The optimized parameters were the $MgCl_2$ concentration and annealing temperature; which obtained the clearest and neatest amplified band with an $MgCl_2$ concentration of 1.47 mM, and an annealing temperature of 63.3°C. This multip lex PCR procedure may be extended for simultaneous detection of more than three transgenic events only avoiding the primer combination established in the Table 2.

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REFERENCES

Bae TW, Vanjildorj E, Song SY, Nishiguchi S, Yang SS, Song IJ, Chandrasekhar T, Kang TW, Kim JI, Koh YJ, Park SY, Lee J, Lee YE, Ryu KH, Riu KZ, Song PS, Lee HY (2008). Environmental Risk Assessment of Genetically Engineered Herbicide-Tolerant Zoysia japonica. J. Environ. Qual., 37

Calsamiglia S, Hernandez B, Hartnell GF, Phipps R (2007). Effects of Corn Silage Derived from a Genetically Modified Variety Containing Two Transgenes on Feed Intake, Milk Production, and Composition, and the Absence of Detectable Transgenic Deoxyribonucleic Acid in Milk in Holstein Dairy Cows. J. Dairy Sci., 90: 4718–4723.

Chowdhury EH, Kuribara H, Hino A, Sultana P, Mikami O, Shimada N, Guruge KS, Saito M, Nakajima Y (2003). Detection of corn intrinsic and recombinant DNA fragments and Cry1Ab protein in the gastrointestinal contents of pigs fed genetically modified corn Bt11. J. Anim. Sci., 8: 2546 2551.

Edwards MG, Gibbs RA (1995). Multiplex PCR In Dieffenbach. Pages 157-171 in CW Dveksler GS eds. PCR Primer A Laboratory Manual. Cold Spring Harbor Laboratory Press: Plainview. New York.

Ellsworth DL, Rittenhouse KD, Honeycutt RL (1993). Artifactual variation in randomly amplified polymorphic ADN banding patterns. BioTechniques, 14(2): 214-217.

Gachet E, Martin GG, Vigneau F, Meyer G (1999). Detection of genetically modified organisms (GMOs) by PCR: a brief review of methodologies available. Trends on Food Science Technology 9:380-388.

Graham HC, Mayers P, Henry RJ (1995). A simplified method for the preparation of fungal genomic ADN for PCR an RAPD analysis. BioTechniques 16: 48-50.

Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH (1997). Multiplex PCR: Critical parameters and step-by-step protocol. BioTechniques 23(3): 504-511.

Holt JS, Powles SB, Holtum JAM (1993). Mechanisms and Agronomic Aspects of Herbicide Resistance. Annual Review Plant Physiology Plant Molecular Biology 44: 203-229.

Integrated DNA Technologies: http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/.

Klotz-Ingram C, Jans S, Fernanádez-Cornejo J, McBride W (1999). Farm level production effects related to the adoption of genetically modified cotton for pest management. AgbioForum. USA. 2-2: 73-84.

Méndez-ÁS, Pérez RE, La PCR (2004) múltiple en microbiología clínica. Enferm. Infec. Microbiol. Clin., 22: 182-92.

Nair RS, Fuchs RL, Schuette SA (2002). Roundup Ready 1 Soybeans Current Methods for Assessing Safety of Genetically Modified Crops as Exemplified by Data on. Toxicol. Pathol., 30 (117).

Nesvold H, Kristoffersen AB, Holst-Jensen A, Berdal KG (2005). Design of a DNA chip for detection of unknown genetically modified organisms (GMOs). Bioinfo., 21(9): 1917-1926.

Permingeat HR, Reggiardo MI, Vallejos RH (2002). Detection and quantification of transgenes in grains by multiplex and real-time PCR. J. Agric. Food Chem., 50: 4431-4436.

Phipps RE, Deaville ER, Maddison BC (2003). Detection of Transgenic and Endogenous Plant DNA in Rumen Fluid, Duodenal Digesta, Milk, Blood, and Feces of Lactating Dairy Cows. J. Dairy Sci., 86: 4070-4078.

Phipps RH, Jones AK, Tingey AP, Abeyasekera S (2005). Effect of Corn Silage from an Herbicide-Tolerant Genetically Modified Variety on Milk Production and Absence of Transgenic DNA in Milk. J. Dairy Sci., 88: 2870–2878.

Reuter T, Aulrich K (2003). Investigations on genetically modified maize (Bt-maize) in pig nutrition: Fate of feed ingested foreign DNA in pig bodies. Eur. Food Res. Technol., 216: 185-192.

Roessner U, Luedemann A, Brust D, Fiehn O, Linke T, Willmitzer L, Fernie R (2001). Metabolic Profiling Allows Comprehensive Phenotyping of Genetically or Environmentally Modified Plant Systems. Plant Cell, 13: 11–29.

Rudi K, Rud I, Holck A (2003) A novel multiplex quantitative DNA array based PCR (MQDA-PCR) for quantification of transgenic maize in food and feed. Nucl. Acids Res., 31, e62.

Sasson A (2001). Cultivos transgénicos: hechos y desafíos. CBGC, Monografía. La Habana. pp. 142-143.

Sharrocks AD (1994). The design of primers for PCR. Pages 5-11 in, H G Griffin AM Griffin, eds. PCR Technology, Current Innovations. CRC Press, London.

Stein HH, Rice DW, Smith BL, Hinds MA, Sauber TE, Pederson C, Wulf DM, Peters DN (2009). Evaluartion of corn grain with the genetically modified input trait DAS-59122-7 fed to growing-finishing pigs. J. Anim. Sci., 87: 1254-1260.

Thompson CJ, Movva NR, Tizard R, Crameri R, Davies JE, Lauwereys M, Bottermann J (1987). Characterization of the herbicide-tolerance gene bar from *Streptomyces hygroscopicus*. EMBO J., 6: 2519–2523.

Toyama K, Bae CH, Kang JG, Lim YP, Adachi T, Riu KZ, Song PS, Lee HY (2003). Production of herbicide-tolerant zoysiagrass by Agrobacterium-mediated transformation. Mol. Cells 16: 19–27.

Wallace RB, Shaffer J, Murphy RF, Bonner J, Hirose T, Itakura K (1979). Hybridization of synthetic oligodeoxyribonucleotides to X 174 ADN: the effect of single base pair mismatch. Nucleic Acids Research 6: 3543

Williams JF (1989). Optimization Strategies for the Polymerase Chain Reaction. BioTechniques 7(7): 762-769.