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STUDY OF GENE FLOW FROM GM COTTON (*Gossypium hirsutum*) VARIETIES IN "EL ESPINAL" (TOLIMA, COLOMBIA)

Estudio del flujo de genes desde variedades GM de algodón (*Gossypium hirsutum*) en El Espinal (Tolima, Colombia)

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ABSTRACT

In 2009, 4088 hectares of genetically modified (GM) cotton were planted in Tolima (Colombia), however there is some uncertainty about containment measures needed to prevent the flow of pollen and seed from regulated GM fields into adjacent fields. In this study, the gene flow from GM cotton varieties to conventional or feral cotton plants via seed and pollen was evaluated. ImmunostripTM, PCR and ELISA assays were used to detect gene flow. Fifty six refuges, 27 fields with conventional cotton and four feral individuals of the enterprise "Remolinos Inc." located in El Espinal (Tolima) were analyzed in the first half of 2010. The results indicated seed mediated gene flow in 45 refuges (80.4 %) and 26 fields with conventional cotton (96 %), besides pollen mediated gene flow in one field with conventional cotton and nine refuges. All fields cultivated with conventional cotton showed gene flow from GM cotton. Two refuges and two feral individuals did not reveal gene flow from GM cotton.

Keywords: biosafety, *cry1Ac*, GM crops, transgenic cotton.

RESUMEN

En el 2009 se plantaron 4088 hectáreas de algodón genéticamente modificado en el departamento de Tolima (Colombia), sin embargo, hay ciertas incertidumbres acerca de las medidas de contención necesarias para impedir el movimiento de polen y semillas desde los campos GM regulados, hacia los campos adyacentes de cultivos convencionales. En este estudio se evaluó el flujo de genes mediado por polen y semillas desde variedades GM hacia variedades convencionales o individuos ferales, en el cultivo del algodón. Para detectar el flujo de genes se utilizaron ImmunostripTM, PCR y ELISA. Cincuenta y seis refugios, 27 campos con algodón convencional y cuatro individuos ferales de la empresa "Remolinos S.A." localizada en El Espinal (Tolima) fueron analizados en el primer semestre de 2010. Los resultados indicaron presencia de plantas GM en 45 refugios (80,4 %) y 26 campos de algodón convencional (96 %), además de un flujo génico mediado por polen en un campo de algodón convencional y nueve refugios. En todos los campos cultivados con algodón convencional se evidenció flujo de genes desde algodón GM. Solo en dos refugios y en dos individuos ferales no se evidenció flujo de genes desde algodón GM.

Palabras clave: algodón transgénico, bioseguridad, *cry1Ac*, cultivos GM.

INTRODUCTION

Transgene flow occurs via pollen or seeds (Heuberger *et al.*, 2010; Mallory-Smith and Zapiola, 2008; Kim *et al.*, 2008). Cotton ranks third among genetically modified crops (James, 2009) and is a self-pollinating crop with low outcrossing rates. Probably for this reason there have been few studies of pollen and seed mediated gene flow (Heuberger *et al.*, 2010). Seed mediated gene flow has received less attention than pollen mediated gene flow (Heuberger *et al.*, 2010; Beckie and Hall, 2008). Some studies have reported gene flow from plots cultivated with genetically modified cotton to conventional cotton via pollen (Llewellyn and Fitt, 1996; Freire, 2002; Glover, 2002; Shirong, 2002; Messeguer, 2003; Van Deynze *et al.*, 2005; Zhang *et al.*, 2005; Llewellyn *et al.*, 2007; Mallory-Smith and Zapiola, 2008; Heuberger *et al.*, 2010;) and/or via seeds (Mallory-Smith and Zapiola, 2008; Heuberger *et al.*, 2010). The presence of pollinating insects (bees, bumblebees, wasps, flies, and butterflies) has been identified as the main cause (Tian *et al.*, 2004; Zhang *et al.*, 2005).Other factors such as the number of pollinators, increased or reduced insecticide applications (Van Deynze *et al.*, 2005, Zhang *et al.*, 2005), wind, crop location, environmental conditions, climate (Hokanson *et al.*, 1997; Amand *et al.*, 2000; Elliott *et al.*, 2004; Tian *et al.*, 2004; Van Deynze *et al.*, 2005; Zhang *et al.*, 2005; Llewellyn *et al.*, 2007), reduction of forest and close presence of fruit crops (Llewellyn *et al.*, 2007) might influence the cross-pollination rate between cotton genotypes.

Lack of control and precautions to prevent seed or pollen mediated transgene flow could, through introgression with compatible secondary recipients, lead to new and more resistant weeds (Messeguer, 2003), loss of biodiversity (Amand *et al.*, 2000; Messeguer, 2003), food safety risks (Messeguer, 2003), potential effects on pest resistance. Since the purpose of refuge areas is disturbed and could affect major pests (Chilcutt and Tabashnik, 2004), production of hybrid and new genotypes may impinge on the long term stability of natural ecosystems (Rognli *et al.*, 2000; Gueritaine *et al.*, 2002; Ilardi and Barba, 2002; Rieger *et al.*, 2002; Beckie *et al.*, 2003; Burke and Rieseberg, 2003; Zhang *et al.*, 2005).

Pollen flow from GM crops to neighboring non-GM crops might even cause a legal problem because the spread of transgenes can be considered as damage to the property of farmers cultivating non-GM crops (Kim *et al.*, 2008). It is also considered a restriction for the exportation of cotton seeds into countries where the biotech traits may not be approved or deregulated (Van Deynze *et al.*, 2005). It could also threaten the intellectual property rights of biotechnology companies, markets for non-GM products and resistance management strategies for insect and weeds (Heuberger *et al.*, 2010; Mallory-Smith and Zapiola, 2008; Mellon and Rissler, 2004; Smyth *et al.*, 2002).

To limit such risks, Colombia established the law 740 of 2002 ratifying the "Cartagena Protocol on Biosafety", which aims to ensure an adequate level of protection in the field for the transfer, handling and safe use of GMOs that might have adverse effects on the conservation and sustainable use of biodiversity. However, in Colombia only three wild species (*G. arboreum*, *G. herbaceum* and *G. barbadense*) were encountered (Garrido, 2007) which can be crossed, and for which some hybridization has been applied to cotton breeding (Fryxell *et al.*, 1992, Zhang *et al.*, 2005). Mendoza and Aramendiz (1985) reported 5 % of natural crossing in hybridization studies of cotton in Colombia, Díaz *et al.*, (2002) reported 1,25 % of natural hybridization.

In 2009, the national authority on the subject, the Instituto Colombiano Agropecuario, ICA, issued its resolution 682, which establishes the management and biosafety plan for the cultivation of GM cotton in the country. It establishes that each GM cotton field planted with an insect resistant cotton variety must have an associated refuge representing 4 % or 20 % of the total area, in which a different (non-modified or herbicide resistant) variety must be planted.

Insect resistant and/or herbicide tolerant varieties of GM cotton have been planted in Colombia since 2002. The cultivated area grew from 2000 ha to 37657 hectares from 2002 to 2010 (Agrobio, 2011). However, studies on gene flow from GM cotton to non-GM counterparts and/or wildlife in Colombia have not been reported. In this study, seed and pollen mediated gene flow from transgenic cotton varieties carrying the *cry1Ac* gene and/or the 35S Figwort mosaic virus promoter to conventional or feral cotton individuals was tested at "Remolino Inc." (El Espinal, Tolima), to evaluate the current prevention measures for the movement of pollen and seeds from regulated GM fields into adjacent cotton fields.

MATERIALS AND METHODS

Field Sampling

Sampling was performed in the first half of 2010, in "El Espinal" (Tolima, Colombia), located at 04°09"N and 74° 53"W, 323 meters above sea level and with a temperature ranging between 27 °C and 40 °C (Unibague, 2007).

Samples were taken at "Remolino Inc." which is an association of cotton farmers located in the town of El Espinal (Tolima - Colombia). According to the farmer's register provided by the association, there were fields planted with conventional as well as with GM cotton. A total of 390 fields planted with GM cotton had associated refuge areas. Of these refuges, 56 (14.3 %) were sampled. On the other hand, 27 out of 42 (62 %) fields planted with conventional varieties were sampled. The distance between these conventional cotton fields and GM cotton fields varied from 4 m to 2570 m. Finally, four conventional cotton feral individuals were found in the area and thus included in the analysis.

At the time of sampling, the transgenic varieties grown in the area were: B2F 141 DP, DP 455 BG/RR, FM 9063 B2F, FM 9162 B2F, FM 9171 B2F, FM 9180 B2F and Nuopal RR. All

these lines carry the *cry1Ac* transgene, which confers resistance to lepidopterans, and the Figwort mosaic virus promoter. Most refuge areas associated to transgenic plots were planted with Delta Opal RR variety and three refuges were cultivated with the DP90 National variety. The conventional varieties planted were: DP90 National, Corpoica M123, Corpoica M129 and Corpoica M137.

The sampling method proposed by Ortiz *et al.*, (2005) was used as a model. Thus, nine randomly located sample points following a "W" shape were marked in each conventional field and refuge. Four leaves and five bolls from the same plant were taken per point. Leaf samples were stored in resealable plastic bags and bolls were stored in paper bags. Field number, collection date, farm owner, geo-reference, number of acres, crop type, planting date, germination date and collector was recorded in each sampling site following recommendations by Maldonado *et al.*, (2007). The distance to the nearest GM crop was calculated. In the laboratory, the leaves were stored at -20 °C and seeds were kept at room temperature.

Detection of Genes and Transgenic Proteins in Collected Material

The sample analysis procedures were conducted in the Molecular Biology laboratory located in the Biology Department of "Universidad Nacional de Colombia" (Bogotá, Colombia).

Parental female analysis. On leaves collected from each plant, an ImmunostripTM assay was applied (Catalog No. STX 06200 Agdia) to detect the *cry1Ac* protein. Eppendorf tubes (1.5 ml) and previously sterilized blunt micropipette tips (100-1000 µL) were used. The procedure was performed according to Immunostrip™ instructions.

Fields that had at least one positive sample for protein *cry1Ac* were excluded from leaf DNA extraction and PCR. Similarly, fields that had at least one positive sample for the presence of transgene *cry1Ac* by PCR were excluded from progeny analysis because it implied that the sampled area was contaminated, and this contamination increased the likelihood of finding hybrids.

The protocol described by Phillips *et al.*, (2003) combined with the cleaning protocol proposed by Falcón and Valera (2007) was used for DNA extraction. DNA quantification was performed using a Thermo Scientific Nanodrop 2000.

PCR was performed for each of the samples, using the primers reported by Randhawa *et al.* (2010), to amplify the *cry1Ac* transgene (F: GACCGCTTACAAGGAGGGATACG, and R: ACGGAGGCATAGTCAGCAGGACC). Primers in this study were designed using Primer 3 online software to amplify the Figwort mosaic virus promoter sequence (F: GTCCAAAG CCTCAACAAGGT, R: TCTTTTGTGGTCGTCACTGC). Primers reported by Lee *et al.* (2007) were used to amplify the endogenous cotton gene fsACP (F: CAAACAAGAGACC GTGGATAAGGTA and R: CAAGAGAATCAGCTCCAAGAT CAAG). The primers used were synthesized by Invitrogen. Amplification reactions were performed in a BioRad My Cycler® thermocycler, in a total volume of 25 µL containing 1x reaction buffer: 8.875 µL of nuclease-free water, 2.5 µL of 10X Taq buffer, 2.5 µL of dNTPs (2 mM of each dNTP), 2 µL of each primer (0.8 µM), 2 µL of $MgCl_2$ (2 mM) and 0.125 µL of Taq DNA polymerase (Fermentas kit from Qiagen). All PCR reactions were performed under the following conditions: initial denaturation, 95 °C, 3 min; 35 cycles comprising: denaturation, 95 °C, 30 seconds; annealing, 63 °C for fs ACP primers, 60.4 °C for Figwort and 64.4 °C for *cry1Ac*, 30 seconds; extension, 72 °C, 45 seconds; final extension, 72 °C, 5 min.

DNA extracted from seeds of NO/RR (Nuopal / Roundup ready) variety was used as PCR positive control, and as a negative control, DNA extracted from seeds of feral cotton individuals.

Leaf DNA extraction and PCR were performed using leaves from fields whose samples were negative with the Immunostrip™ assay, this was done in order to identify false negative results with this assay, and identify other events that have been released in the study area.

Positive results with both Immunostrip™ and PCR are interpreted as seed mediated gene flow. Therefore, progeny analysis to evaluate pollen mediated gene flow was carried out with seeds of fields in which all parental plants were negative with Immunostrip™ and PCR.

Progeny analysis. Seed DNA extraction of conventional varieties (DP90 National, Corpoica M123, M129, M137) and feral individuals was done to standardize the level of detection of the PCR kit. Briefly, the experimental protocol involved different sample sizes: 100, 200 and 300 conventional or feral seeds, each with one transgenic seed. Each sample was processed using DNeasy Plant Maxi kit from Qiagen.

To detect pollen mediated gene flow and hybridization in fields, the bolls selected were those in which all the parental plants were negative. The seeds were extracted removing the lint manually and were mixed with seeds from the same field. Three hundred seeds were randomly taken and, using a blender, a fine powder was obtained, from which two 0.5 g samples were taken for DNA extraction using DNeasy Plant Maxi kit from Qiagen

Finally, to detect the Bt *cry1Ac* toxin in positive PCR samples and in varieties DP90 National, Corpoica M123, M129 and M137, an ELISA assay was used (kit DAS ELISA from Agdia to detect proteins Cry1Ab/Ac: catalog number PSP06200). The assay was performed by triplicate for each sample. The reading was measured using an Elisa plate reader at 655 nm. We quantified the concentration of Bt *cry1Ac* toxin using the Pierce BCA protein assay kit (Thermo Scientific). Six dilutions were used to approximate the toxin concentration of the samples tested.

Genes or constructs of GM cotton that has been released in Colombia were used to design the primers and to select ELISA

and Immunostrip™ assays. Thereby, we evaluated the presence and translation of the *cry1Ac* transgene and the presence of the Figwort mosaic virus promoter. As a PCR positive control, the endogenous gene fsACP primers were used.

The eppendorf tubes and tips were autoclaved at 15 psi (121 °C) for 20 minutes; PCR tubes and micropipettes were sterilized with UV light for 40 minutes and blender jars were disinfected in 4.5 % sodium hypochlorite for 30 minutes.

RESULTS AND DISCUSSION

Analysis of Female Parentals

Cry 1Ac Protein detection. Immunostrip™ assays were used to detect the *cry1Ac* protein in maternal tissue from 27 conventional cotton fields. Nine samples were taken in each field. All samples were negative for the *cry1Ac* toxin in 11 (41 %) of the 27 fields (Fig. 1A), while the remaining 16 (59 %) fields had at least one positive sample for *cry1Ac* (Table 1). The distance between the fields with one or more positive samples and GM cotton fields varied in a wide range, from 4 m to more than 2 km. There are no clear regulations as to the minimum distance that must be observed between GM and conventional cotton fields. It is important to note that some fields were located at about 5 m from the road to Ibagué (Tolima).

In maternal tissue from 56 refuge areas analyzed, there were 20 (36 %) refuges that scored negative for the *cry1Ac* toxin

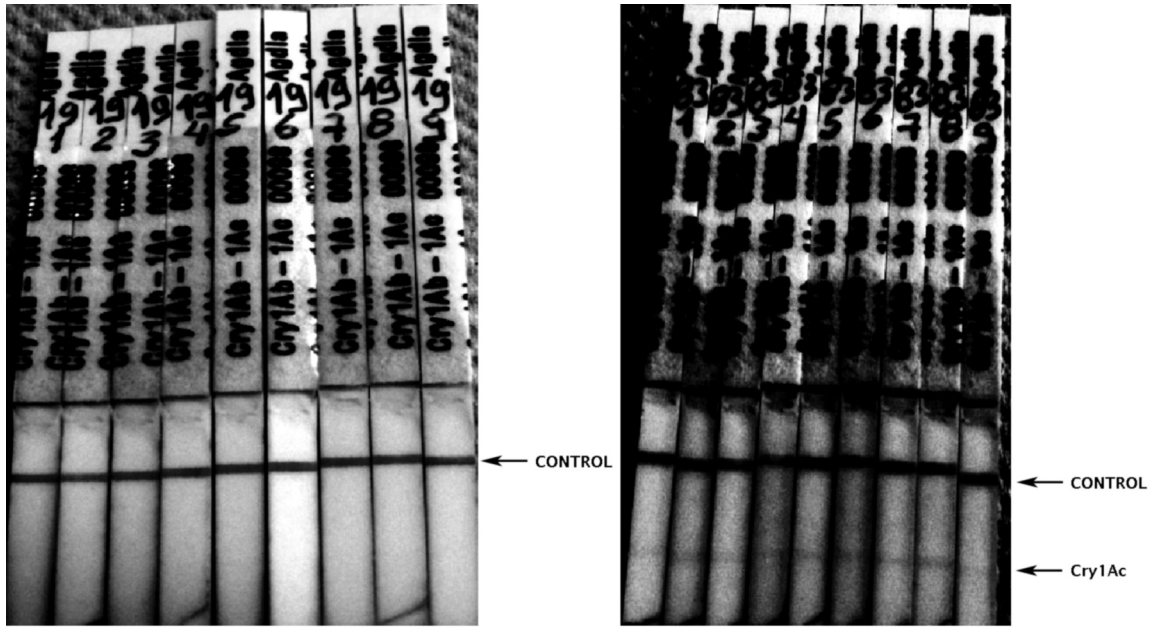

Figure 1. Immunostrip™ detection of cry1Ac in leaf tissue of maternal cotton plants. A. Conventional cotton field where all samples tested negative. B. Refuge where all samples tested positive.

Table 1. Detection of the *cry1Ac* protein in female cotton parentals using Immunostrip™.

Number of positive samples per field	Number of conventional fields	Number of refuges	Number of feral individuals	Total
0	11	20	4	35
1	8	11	0	19
2	4	9	0	13
3	1	3	0	4
4	2	2	0	4
5	0	4	0	4
6	0	2	0	2
7	1	1	0	2
8	0	0	0	0
9	0	4	0	4
Total negative	11	20	4	35
Total positive	16	36	0	52

using the ImmunostripTM assay, four (7 %) refuges in which all nine samples were positive (Fig. 1B) and 32 (57 %) refuges with least one positive sample for *cry1Ac* (Table 1). Samples of feral individuals were negative for *cry1Ac* toxin expression.

PCR Detection of the cry1Ac transgene. In those fields in which no presence of the *cry1Ac* toxin was detected by the ImmunostripTM assay, leaf DNA was extracted for PCR analysis. The DNA concentrations and 260/280 ratios ranged from 61.2 to 1218.7 ng/µl and 1.60 to 1.99, respectively. Despite the differences in concentration and 260/280 ratios, the DNA was suitable for PCR amplification, as confirmed by the use of the fsACP primers, which served as positive PCR controls (Fig. 2A) (Van Deynze *et al.*, 2005). Lee *et al.* (2007) used these primers to amplify the fsACP gene expressing cotton fiber specific protein (FsACP) in order to distinguish cotton from other crops such as corn, soybeans, rice and barley, and as controls in detection methods for Monsanto's cotton.

Using the primers to amplify the *cry1Ac* gene, PCR was performed with DNA extractions from samples of the 11 conventional cotton fields, 20 refuges and four feral individuals. An amplicon of 228 bp was obtained in at least one sample from 10 fields planted with conventional cotton

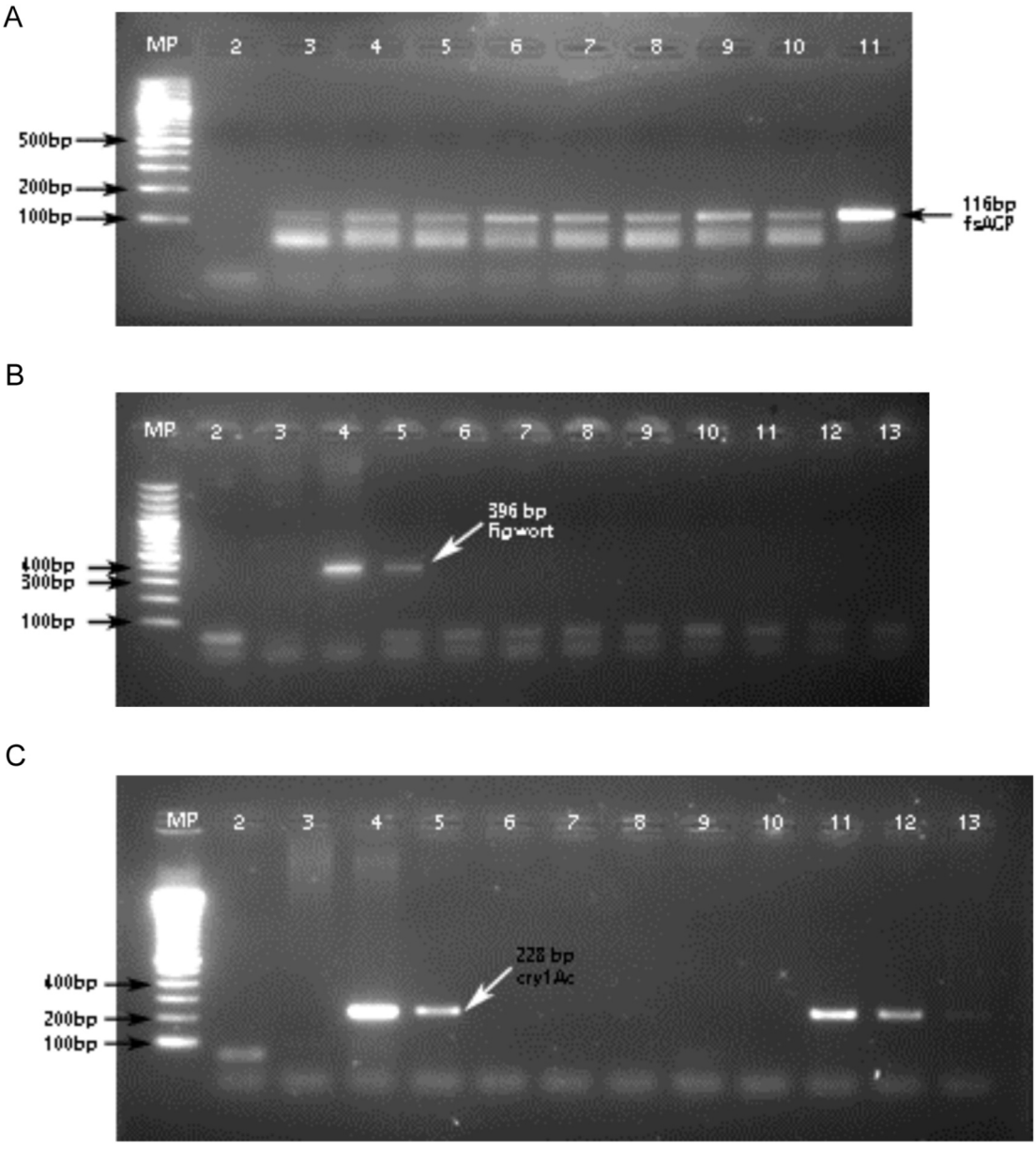

Figure 2. PCR detection of control and transgene sequences in leaf DNA from maternal cotton plants. A. fsACP, MP: 100bp molecular weight marker, lane 2: water, lanes 3-11: samples 1 to 9. B. 35S Figwort mosaic virus promoter, lane 2: water, lane 3: negative control: feral cotton, lane 4: positive control: Nuopal/RR, lanes 5-13: samples 1 to 9. C. *cry1Ac*, lanes as in B.

and in 9 refuges (Fig. 2C). All samples in the remaining 11 refuges were negative. All samples of one field planted with conventional cotton and the feral individuals were negative (Table 2). This amplicon size was also obtained by Randhawa *et al.* (2010) in PCR assays to detect MON531 and MON 15985 cotton.

amplified only when using the *cry1Ac* primers. This could be due to contamination of seeds coming from GM varieties carrying that transgene with a different promoter, like MON 531, which carries the CaMV35S promoter and has been cultivated in the area. This remains to be confirmed.

Table 2. PCR detection of the *cry1Ac* sequence in leaf DNA from female cotton parentals from fields where no *cry1Ac* was detected by Immunostrip™.

Number of positive samples	Number of conventional fields		Number of refuges		Number of feral individuals	
			Primers			
	Figwort	*cry1Ac*	Figwort	*cry1Ac*	Figwort	*cry1Ac*
0	7	1	0	11	4	4
1	3	4	1	2	0	0
2	1	1	0	3	0	0
3	0	0	0	2	0	0
4	0	1	0	1	0	0
5	0	3	0	0	0	0
6	0	0	0	1	0	0
7	0	0	0	0	0	0
8	0	1	0	0	0	0
9	0	0	0	0	0	0
Total negative	7	1	0	11	4	4
Total positive	4	10	1	9	0	0

PCR Detection of the 35S Figwort Mosaic Virus Promoter. The primers to amplify the Figwort mosaic virus promoter region were used on 112 extractions corresponding to the four feral individuals and the samples from 12 fields where the *cry1Ac* protein was not detected by the Immunostrip™ assay (11 conventional cotton fields and 1 refuge). The remaining 19 refuges that tested negative for the protein corresponded to Delta Opal RR cotton, which carries this promoter. In four of the conventional fields and in the refuge, at least one sample produced an amplicon of 396 bp, which is the size expected for the amplification of this promoter (Fig. 2B). No samples were positive in seven fields planted with conventional cotton, and the feral individuals were all negative (Table 2).

These results showed the presence of the transgenic sequences in 10 additional fields planted with conventional cotton and nine additional refuges. This could be due to false negatives obtained by the Immunostrip™ assay, or by plants carrying but not expressing the transgene.

Analyzing female parentals with Immunostrip™ and PCR, we found that there is gene flow via seeds into 26 out of 27 fields planted with conventional cotton (96 %) and into 45 out of 56 sampled refuges (80 %). In feral individuals no gene flow via seeds was detected (Tables 1 and 2).

Five samples tested positive both for the 35S figwort mosaic virus promoter and *cry1Ac* using PCR. However, six samples

Gene flow via seeds towards fields planted with conventional varieties located approximately 4-10 m away from fields planted with transgenic varieties could be due to transgenic seed falling inadvertently in these fields, or seeds that were disseminated or scattered by the wind. This pattern has also been shown by Messeguer (2003) and Heuberger *et al.*, (2010). Gene flow observed in conventional fields located further away from fields planted with transgenic cotton could be due to contamination of seeds (Van Deynze *et al.*, 2005) (mixture of seeds that can be sourced), inadvertent planting of seeds during harvest (Messeguer, 2003), or as a result of human errors committed during planting, harvesting or processing of the seed (Heuberger *et al.*, 2010).

However, it should be noted that factors such as dumping of seed in production areas or during transport and their use for feeding livestock, may also be involved and have been reported as potential causes for gene flow via seeds (Phillips *et al.*, 2003).

In refuges where not all samples were positive, but only some plants, gene flow was probably because there was mixing of seeds during planting, either due to contamination of the source (mixed seed source) or by mechanical mixing (Van Deynze *et al.*, 2005), especially when planting was done using the same machines or same equipment.

Heuberger *et al.*, (2008) and Heuberger *et al.*, (2010) found gene flow in experimental plots in Arizona planted with non-

Bt cotton and mixture of seed in bags that should contain only non-Bt cotton seeds. These results show that seed mixture and seed manipulation may be important sources of contamination of fields planted with non-GM cotton.

All of the samples tested in four of the refuges were positive. It is unlikely to find this result due to accidental seed mixing and could be indicating a different situation. It is possible that the information that was received about the fields is wrong or that farmers are not complying with legal rules on the establishment of refuges. The national authority on this subject, Instituto Colombiano Agropecuario, ICA (2009), prescribes the use of two different refuge schemes with a ratio of the area of the GM field/refuge of 80/20 or 96/4.

With regard to the feral individuals, no gene flow via seed was found, which could be due to a greater distance from the sources of pollen and seed from transgenic plants, given that these trees were found in the urban area of the town and not close to cotton fields. The sampled trees were located between the police station and the bus terminal of El Espinal, being trees of about three to four meters in height and five to seven years old, according to information supplied by local people and field technicians of "Remolino Inc.".

Progeny Analysis

PCR detection level. Previous to the assay to establish the detection level of the PCR kit, all DNA samples from seeds of conventional varieties DP90 National, Corpoica M123, M129 and M137 and feral individuals were amplified with fsACP primers indicating that DNA extracted is free of PCR inhibitors (Van Deynze *et al.*, 2005). When using *cry1Ac* primers, all samples were amplified, except samples of feral individuals. However, DNA samples of the variety M123 and feral individuals did not amplify with Figwort primers, while in the other varieties amplicons were obtained. These results show that seeds provided as conventional cotton are contaminated by blending or are product of hybridization between GM and non-GM cotton plants. Variety M123 amplified for *cry1Ac* but not for the Figwort promoter, which suggests that the contamination of these seeds was with the GM cotton MON 531, which is cultivated in the area and carries the *cry1Ac* gene and the CaMV35S promoter, not the Figwort promoter.

The Elisa test verified these results as it identified *cry1Ac* transgene translation in samples of seed corresponding to conventional varieties DP90 National, Corpoica M123, M129 and M137. The level of protein expression was less than 150 µg/ml.

The assay performed to test the detection level of the PCR kit showed that is possible to detect DNA from one transgenic seed in 100, 200 or 300 non-transgenic seeds. This is valid both for the *cry1Ac* and Figwort primers. For Ortiz and colleagues (2005), the maximum sample size with which it is possible to detect a transgenic seed is 300 seeds; therefore, for the present study 300 seeds were used as sample size for mixing, grinding and DNA extraction.

Pollen mediated gene flow. For 12 fields (one conventional and 11 refuges) for which leaf analysis was negative, seed DNA extraction was performed. The DNA concentration obtained ranged between 8.7 and 85.8 ng/µL and the 260/280 ratio was 1.6 - 1,9. Amplicons with primers fsACP were observed in all DNA samples.

Primers for the Figwort promoter were used to perform PCR on DNA extracted from samples from the only conventional cotton field that did not present any seed mediated gene flow and positive results were obtained, showing pollen mediated gene flow in this case (Fig. 3A). Samples of feral individuals were negative for the presence of Figwort promoter.

PCR was done using primers to amplify the *cry1Ac* gene on samples from the 11 refuges where all parental female plants tested negative for protein expression or PCR assays. Nine positive and two negative results were obtained; samples from the conventional field tested were positive and feral individuals were negative for the presence of *cry1Ac* gene (Fig. 3B).

Using the Elisa test we found that two refuges were negative for *cry1Ac* gene expression, which is in agreement with refuges in which no *cry1Ac* transgene amplification was found (data not shown). The nine remaining refuges and the conventional field analyzed with this assay were positive for *cry1Ac* toxin, in which the quantification of protein was approximately less that 200 µg/ml.

The samples tested that were positive for both presence and expression of *cry1Ac* transgene demonstrate that there is gene flow via pollen from genetically modified cotton to conventional cotton. Similar results were obtained by Freire (2002), Van Deynze *et al.* (2005), Zhang *et al.* (2005), Llewellyn *et al.* (2007) and Heuberger *et al.* (2010), who reported gene flow from GM cotton cultivated plots to conventional cotton. The flow of transgenes via cotton pollen has been reported in the United States, Australia, China, South Africa and Argentina (Van Deynze *et al.*, 2005).

Gene flow via pollen reported in this study should be analyzed taking into account that gene flow via seed is very high (80 % and 96 % of the fields analyzed in refuges and in fields planted with conventional varieties of cotton, respectively). Additionally, the amount of GM cotton crops that were in the area during sampling may lead to a higher occurrence of gene flow via pollen, being more likely to be present. Isolation distances are clearly established, and we found fields planted with conventional cotton surrounded by fields planted with GM cotton (separated about 4-10 m). The scheme 96/4 is preferentially used by the farmers of "Remolino Inc.", which allows concluding that the source of transgenes is higher due to that fact. Due to the high incidence of seed mediated gene flow, the analysis of pollen mediated gene flow was performed only in one conventional field and 12 refuges. However, this study shows evidence of the presence of hybrid cotton in the region. In addition, gene flow via pollen can be related with the presence of pollinating insects. This has been reported as the

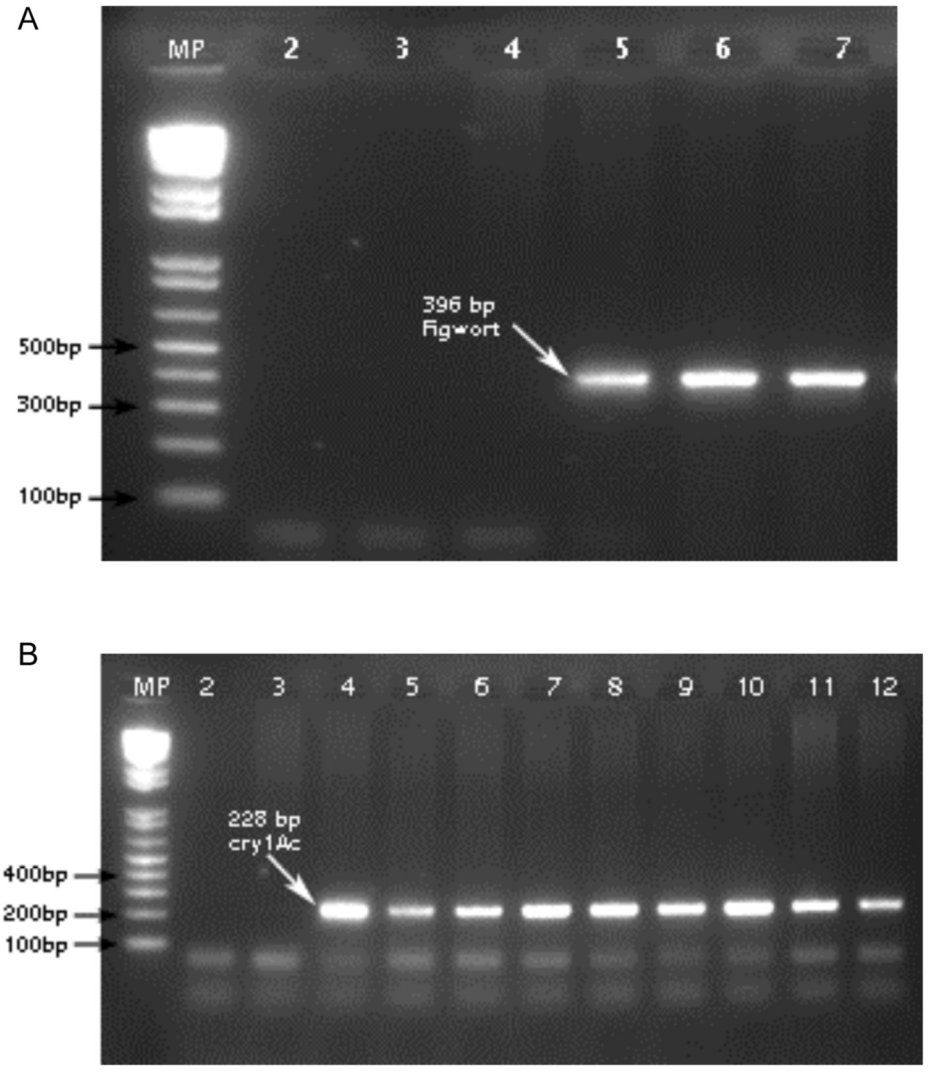

Figure 3. PCR detection of control and transgene sequences in DNA from sampled cotton seeds. A. 35S Figwort mosaic virus promoter, MP: 100bp molecular weight marker, lanes 2 and 3: water, lane 4: negative control: feral cotton, lane 5: positive control: Nuopal/RR, lanes 6 and 7: sample from conventional cotton field. B. *cry1Ac*, lane 2: water, lane 3: negative control: feral cotton, lane 4: positive control: Nuopal/RR, lanes 5, 6, 9-12: field samples from refuges, lanes 7, 8: samples from conventional cotton fields.

main reason for gene flow to occur via pollen and natural hybridization in cotton (Van Deynze *et al.*, 2005), either by the presence of bees (Llewellyn and Fitt, 1996; Van Deynze *et al.*, 2005; Zhang *et al.*, 2005; Llewellyn *et al.*, 2007), bumblebees and melisodes (Van Deynze *et al.*, 2005; Zhang *et al.*, 2005), or bumblebees (Llewellyn *et al.*, 2007; Zhang *et al.*, 2005), or other insects such as wasps, flies, and butterflies (Tian *et al.*, 2004; Zhang *et al.*, 2005).

Other secondary causes of pollen mediated gene flow could be the amount and behavior of pollinators due to the decrease in insecticide applications that affect pollinator activity and pollen dispersal (Van Deynze *et al.*, 2005; Zhang *et al.*, 2005, Carpenter and Gianessi, 2001; Betz *et al.*, 2000; Mendoza and Aramendiz, 1985), the location of the lots, environmental conditions, the climate where cotton is grown (Hokanson *et al.*, 1997; Amand *et al.*, 2000; Elliott *et al.*, 2004; Tian *et al.*, 2004; Van Deynze *et al.*, 2005; Zhang *et al.*, 2005; Llewellyn *et al.*, 2007;), the presence of nearby fruit crops (Llewellyn *et al.*, 2007) and wind (Van Deynze *et al.*, 2005). This study does not allow the differentiation of these causes of gene flow, especially due to the predominance of seed mediated gene flow.

With the results obtained in this study and taking into account that there are no regulations regarding isolation distances between GM cotton crops and conventional cotton, it is necessary to evaluate the idea of isolation distances between these fields. Especially when considering that the conventional field that was separated about 4 m from fields planted with transgenic cotton showed hybridization events. Studies in other countries have shown that gene flow via pollen

decreases exponentially with increasing distance from the pollen source (Zhang *et al.*, 2005; Llewellyn and Fitt, 1996; Messeguer, 2003), and is dependent on environmental conditions; gene flow has been detected at 20 and 25 m in Arizona, Arkansas, Mississippi and Northern California (Van Deynze *et al.*, 2005; Zhang *et al.*, 2005) and more than 1600 m in California (Van Deynze *et al.*, 2005; Kim *et al.*, 2008).

With regard to gene flow observed in refuge areas, several authors (Llewellyn and Fitt, 1996; Chilcutt and Tabashnik, 2004; Luna *et al.*, 2001; Morris *et al.*, 1994) demonstrate that many factors including refuge size, shape and distance from the Bt crop, pollen longevity and setting rate, similarity of Bt and non-Bt hybrids in maturation times and height of male and female flowers are the principal causes of this phenomenon.

The study of gene flow in El Espinal (Tolima-Colombia), was made in the first half of 2010. Our results indicate seed mediated gene flow in 45 refuges (80 % of total sampled) and 26 fields with conventional cotton (96 %), besides pollen mediated gene flow in one field with conventional cotton and nine refuges. Figwort promoter sequence was identified in 18.5 % of conventional cotton fields sampled and 33.3 % of refuges. Transgene *cry1Ac* expression was detected and/or verified in 100 % of cultivated conventional cotton fields and 96.4 % of refuge areas.This study shows that standards are not being met since Bt plants are present in refuges and conventional fields. Therefore it is necessary to evaluate strategies to prevent or reduce gene flow. These strategies should aim at regulating isolation distances between GM and non-GM cotton fields, as well as controlling the accidental or intentional mixing of seeds and possible leaks during transportation.

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