Article

Methodologies for the analysis of expression and quantification of proteins conferred in tissues of GM crops

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Abstract

The Cry toxins of *Bacillus thuringiensis* (Bt) are used as bioinsecticides in agriculture. Reliable methods are needed to analyze the characteristics that are conferred to genetically modified (GM) plants during their development, before commercialization. The objective of the work was to standardize methodologies for the analysis of the expression of genes and proteins conferred to GM cotton during its different phenological stages in the field. As the main practical application of the study, the standardized methodologies can be used in the characterization of GM crops that are developed and for which it is necessary to carry out their risk analysis. To do this, samples of plant tissue were processed in different phenological stages obtained from GM cotton commercial farms in the Yaqui Valley. In the different tissues, gene and protein expression was quantified by RTq-PCR and Elisa analysis, respectively. The results obtained show variation in the expression of the genes conferred throughout the development of the same variety and between the different sites where the crops were located. The highest levels of expression were identified, as expected, in the early stages of the culture (average values of 8.5 μg g^{-1} for Cry1Ac and 63.1 μg g^{-1} for Cry2Ab) compared with that observed in late or mature stages (mean values 0.05 and 0.3 μg g^{-1} for Cry1Ac and Cry2Ab, respectively). Therefore, it is concluded that the RTq-PCR and ELISA techniques are suitable to evaluate the spatial and temporal expression of genes that are conferred to GM plants, information required to characterize the exposure to the hazard and to perform the risk analysis.

Keywords: gene expression analysis, GM crops, protein expression analysis.

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Introduction

The latest report of the international service for the acquisition of agri-biotech applications (ISAA) of 2017 reports 498 events, in 29 genetically modified (GM) crops that have been developed and approved for different commercial purposes around the world. The characteristics that confer the events with greater time in the world market, including the Mexican one, are the one of the tolerances to herbicides and the resistance to insect's lepidoptera pests.

Cry proteins are the most used for the management and control of insect pests. Transgenic plants have been used to reduce the losses caused by the attack of these insects. Cotton varieties resistant to insect pests, which have been used in Mexico for commercial purposes, incorporate the Cry1Ac and Cry2Ab genes (Bayer, 2012), which give the plant the expression of specific insecticidal proteins to control the main insect species. Lepidoptera crop plague. *Bacillus thuringiensis* (Bt) is a pathogenic bacterium for some insects, since it forms toxic proteins known as Cry, Vip and Cyt. At least four different types of Cry proteins are reported, according to their structure (Bravo *et al*., 2011).

There are different methods that allow to detect in a qualitative and quantitative way the presence of genes and transgenic proteins in the different GM crops. These methods may be based on the detection of conferred deoxyribonucleic acid (DNA) or the immunological detection of the protein they encode. The method that is being used most frequently in the detection of GM organisms (GMOs) is the polymerase chain reaction in real time (RTq-PCR), which allows the quantification of the introduced DNA allowing to estimate its presence in derived products even in complex mixtures (Pla *et al*., 2006; Elenis *et al*., 2008).

Immunological detection methods are basically developed in two ways: the lateral flow strips or test strips and the enzyme-linked immunodetection assay (Elisa). Through the use of the test strips, the absence or presence of a specific protein can be determined, which is done by a qualitative analysis, which can be semi-quantitative, including statistical considerations in the sampling. The Elisa test is based on the use of specific antibodies to detect the protein of interest and in turn exclude proteins that are found in the product being analyzed; it is a very sensitive, versatile and quantitative method (Corona *et al*., 2006).

The quantification in time and space of the proteins that are conferred to a GM crop is part of its characterization to generate information required in the different analyzes prior to commercialization. For example, the levels of expression of the protein conferred in the different parts of the plants, is used as base information of the environmental risk analysis and the data of the concentration of the protein conferred to the GM in the harvested grain is used in the analysis of dietary exposure.

The objective of the present investigation was to standardize methodologies for the analysis of the expression of genes and proteins conferred to GM cotton during its different phenological stages in the field. As the main practical application of the study, the standardized methodologies can be used in the characterization of GM crops that are developed and for which it is necessary to carry out their risk analysis.

Materials and methods

Study zone

The samplings were carried out in the Yaqui Valley, located in the south of the state of Sonora, in four different agricultural plots and where two varieties of genetically modified cotton were planted, during the 2014 cycle (Table 1). A variety that does not express the Cry1Ac and Cry2Ab proteins was also planted in each agricultural plot, as a refuge.

Table 1. Georeferences of the plots sampled.

Property	Geographical coordinates			
Guamuchil	N	27°26’28.4”	W	109°59’36.0”
5 de febrero	N	27°22’31.9”	W	109°56’06.9”
Field 9	N	27°22’13.0”	W	109°54’35.1”
Field 1	N	27°22’10.5”	W	109°54’22.3”

Take and sample preparation

Samples of leaves were taken in six dates, in the first four dates samples of square were also taken and acorns were collected in the last two dates. The sample of vegetal tissue was taken at the height of the third node from top to bottom. For immunological detection, a total of 120 samples were taken, corresponding to 60 leaves, 40 squares and 20 acorns, of these 24 (12 of leaves, eight of squares and four of leaves) were from the refuge. For gene detection only samples were taken on three dates, taking five samples each time, from each field, which were processed independently.

For the case of the leaf, it was taken into account that it was completely unfolded and integrated, so that the square and acorn were careful not to have any alteration at the time of sampling. 30 mg of leaf tissue, 80 mg of square tissue and 9 mg of acorn tissue were taken, all fresh. For the immunological detection tests, the tissues were processed fresh with their respective extraction buffer and the maceration proceeded. For gene detection the tissues were placed in contact with liquid nitrogen and subsequently crushed with the help of a sterile micropystile.

Immunological detection

Side flow strips

Before proceeding with protein and gene quantification, the presence of transgenic proteins was evaluated by the use of lateral flow strips, following the methodology suggested by the manufacturer (EnviroLogix, Portland, ME, USA). For this, a 1.5 mL tube was made of a sample composed of five portions of the different tissues of cotton, 0.5 mL of extraction buffer for leaf analysis and 1.5 mL for square and acorn were added. The sample was homogenized with the help of a micropystile. Subsequently, a strip was introduced into the tube and left for 10 min to observe and interpret the results. The samples that were positive were analyzed by the Elisa test and real time PCR.

Immunosorbent assay linked to enzymes (Elisa)

A 'sandwich' test was performed for enzyme-linked immunosorbent assay (Elisa) following the protocol of the QualiPlate AP 051 Kit of EnviroLogix. For the calibration curves, five standards were used in concentration of 3.13, 6.25, 12.5, 25 and 50 ng mL^{-1} of protein Cry1Ac and Cry2Ab. The contents of the wells were mixed and then placed in incubation at room temperature for one hour. After incubation the contents of the wells were shaken, the wells were completely flooded with the washing buffer, then emptied. This washing step was repeated three times.

100 µL of *p*-nitrophenyl phosphate (substrate S1) was added to each well, mixed and incubated at room temperature for 30 min. It was read on the SpectrOstar Nano spectrum (BMG Lab Tech, Ortenberg, Germany) at 405 nm for the Cry2Ab protein. Four washes were made to subsequently add 100 µL of 3,3', 5 5'-tetramethylbenzidine (substrate S2) to each well, incubated at room temperature for 30 min and read at 650 nm for Cry1Ac. The interpretation of the results was made based on the absorbances of each of the samples analyzed (Table 2).

Table 2. Concentrations and absorbance measurements for the Cry1Ac and Cry2Ab proteins.

Concentration (ng mL^{-1})	Absorbance Cry1Ac	Absorbance Cry2Ab
0	-0.005	0.0045
3.13	0.269	0.0525
6.25	0.522	0.1825
12.5	1.139	0.3685
25	1.975	0.7025

Gene detection

Extraction of RNA from plant tissues

The plant tissue to be analyzed was placed in a 1.5 ml tube and 500 µL of PureLink® Plant RNA Reagent (Life Technologies™, Carlsbad, California, USA) was added and subsequently homogenized. The samples were incubated for 5 min at room temperature keeping the tube horizontally for maximum extraction. They were centrifuged at 14 000 RPM for two min at 4 °C and the supernatant was transferred to a new sterile tube. 100 µL of 5 M NaCl was added to clarify. 300 µL of chloroform was added and mixed vigorously.

It was centrifuged at 14 000 RPM for 10 min at 4 °C and the supernatant was transferred to a new sterile tube. The RNA was precipitated with an equal volume of 100% cold isopropanol to the aqueous phase which was separated and mixed by inversion. It was incubated for 10 min at room temperature. It was incubated at room temperature for 10 min and subsequently centrifuged at 14 000 RPM for 10 min at 4 °C. The supernatant of the tube was decanted leaving the pellet of RNA, for the washing 500 µL of 75% ethanol was added, it was stirred and centrifuged at 14 000 RPM for five minutes at 4 °C, afterwards the RNA pellet was dried by approximately 5-10 min and resuspension was carried out with 50 µL of DEPC water mixing with the micropipette. It was left to resuspend at 4 °C and then proceeded to determine the concentration and purity of the extraction.

Synthesis of the cDNA by reverse transcription

The retrotranscription was performed using the GoTaq® Probe 2-Step RT-qPCR System kit (Promega, Madison, Wisconsin, USA) and was carried out in 0.2 mL PCR tubes containing the following mixture for each reaction: 2 µL RNA (200 ng), 1 µL of oligo (dT) 15 primers, 1 µL of random primers and 3 µL of free water of RNAse/DNase to complete a volume of 7 µl. RNA and primers were denatured in a heating block at 70 °C for 5 min, placed immediately on ice for 5 min and then centrifuged for 10 s.

For the alignment and synthesis of cDNA the following components were added: 4.9 µL of free RNAse/DNAse water, 4 µL of GoScript 5X reaction buffer, 1.6 µL of MgCl2, 1 µL of nucleotides, 0.5 µL of recombinant RNasin as inhibitor, 1 µL of GoScript reverse transcriptase, to complete a volume of 13 µL and add it to the 7 µL of the denaturation step. For the alignment they were placed at 25 °C for 5 min, for elongation at 42 °C for 45 min and to inactivate the reverse transcriptase at 70 °C for 15 min. It is proceeded to RT-PCR or were stored at -20 °C.

Real-time polymerase chain reaction (RT-PCR)

Real-time PCR amplification was carried out in 0.2 mL PCR tubes containing the following mixture for each reaction for the samples: 2 µL of RNA (200 ng), 7 µL of RNAse/DNase-free water, 10 µL of GoTaq® Probe qPCR Master Mix (Promega, Madison, Wisconsin, USA) and 1 µL of the primer/probe mix (300 nM), finally obtaining a volume of 20 µL for each reaction.

To perform the calibration curves, the mixture for each reaction was as follows: 5 µL of each point on the curve, 4 µL of RNAse/DNAse-free water, 10 µL of GoTaq® Probe qPCR Master Mix and 1 µL of the first mix/probe (300 nM), to obtain a final volume of 20 µL for each reaction. All samples were analyzed in the 7500 Fast Real-Time PCR system (Life Technologies™, Carlsbad, California, US) under the conditions shown in Table 3. The primers used for the amplification in each of the reactions are shown in Table 3. For the quantification, a calibration curve was made by serial dilutions from an initial concentration of 200 000 copies per microliter (Table 4 y 5).

Table 3. Running conditions in the thermal cycler in real time.

Step	Cycles	Temperature	Time (min)
GoTaq® Polymerase Activation	1	95 °C	02:00
Denaturation	40	95 °C	00:15
Alignment	40	60 °C	01:00

Table 4. Sequence of primers used.

Primers	Sequence	Amplicon
Cry1Ac	(F) GACTCTAACCTCTTGGGACACT	134 pb
	(R) AGAACGGTGACTTCAACAATGG	
Cry2Ab	(F) AATTCTAACTACTTCCCCGACTACTTC	121 pb
	(R) ACGGAGAGGCGATGTTCCTG	

Table 5. Points of the calibration curve for gene quantification.

Point	No. of copies (μL)
1	200 000
2	20 000
3	2 000
4	200
5	20
6	2
7	0.2

Analysis of results

The results obtained in protein detection were analyzed by means of an analysis of variance (Anova), based on a linear model of fixed effects for $p< 0.05$, from the means obtained in the analyzes. The dates were taken as a source of variation (six levels), without interaction with the varieties or the sites, elements that were only taken to increase the size of the sample. For the conformation of the calibration curves of the Cry1Ac and Cry2Ab proteins, a simple linear regression analysis was performed, presenting the regression equation and the coefficient of determination. The results obtained in gene detection were analyzed by means of the evaluation of medians in the Kruskal-Wallis test with $p< 0.05$. The statistical package Statgraphics Centurion XV (StatPoint Technologies, Inc.) was used.

Results and discussion

Immunological detection

Side flow strips

Prior to the analyzes by ELISA and RT-qPCR, analyzes were performed by immunochromatography, with 96 of the 120 samples collected and presumably expressing the Cry1Ac and Cry2Ab proteins. Dohare and Tank (2014) detected the presence of the same proteins, which were sought in this study, by analyzing a total of three varieties of genetically modified cotton from the Bollgard II® line. While Larbouga *et al*. (2013) used the same tool for a similar study five varieties of cotton from the same line.

This technology allows the immobilized and specific antibodies for each protein to be incorporated into a strip of nitrocellulose coupled to a molecule that will react in the presence of the proteins analyzed (Lipton *et al*., 2000; Wang *et al*., 2007). That is why this tool is widely used today due to the speed and ease of obtaining results, either in the field or in the laboratory.

Quality parameters in Elisa

To verify the linearity and quantification of the transgenic proteins, calibration curves were made with five known concentrations at different levels. For the Cry1Ac protein R^2 was obtained higher than 0.99, the same for the Cry2Ab protein. The values of R^2 recommended to ensure the linearity of the method is ≥0.99, agreeing with the results obtained by Shan *et al.*

(2007), in the construction of curves for the quantification of the Cry1Ac protein in different cotton tissues. In the Figure 1 shows the linearities of the calibration curve system for the Cry1Ac and Cry2Ab proteins. The accuracy and precision of the method was evaluated by performing all the analyzes in duplicate of each of the quantified samples. Karuri *et al*. (2013) obtained coefficients of variation of less than 15%, similar to those obtained in this study, which reflects the quality of the results.

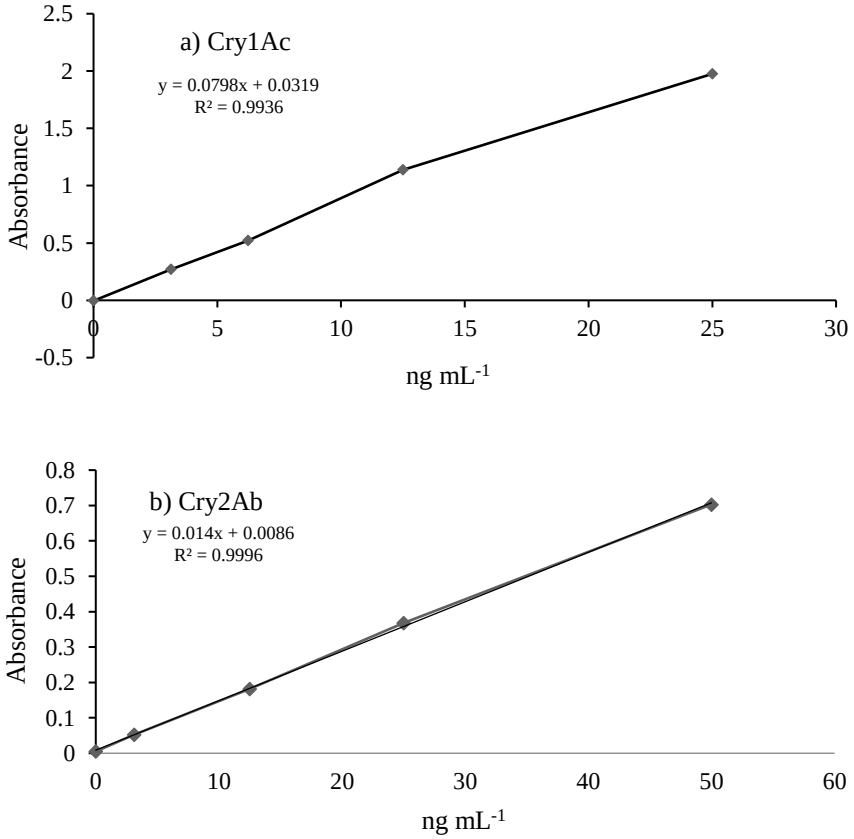


Figure 1. Calibration curves for the Elisa test of the proteins, a) Cry1Ac and b) Cry2Ab.

Quantification of Cry proteins through Elisa

The results of the analysis of the quantification of protein expression for Cry1Ac and Cry2Ab in the four farms analyzed, in six moments and two different cultivars, showed an expression rate which decreased over time, with highly significant differences ($p< 0.0025$ for Cry1Ac and $p< 0.0045$ for Cry2Ab), reaching levels to which it could not be detected, this for the square and acorn cases. The minimum level of Cry1Ac protein quantified in leaf (Table 5) was 0.3 µg g^{-1}, while in tables and acorns (Table 6 and 7) the trend occurred to zero, as it was undetectable in the field on February 5. The maximum levels of expression occurred in leaves, with up to 8.5 µg g^{-1} being found in early stages of cultivation, while in tables and acorns it was 2.5 µg g^{-1}. Greenplate *et al.* in 2003, in a similar study with the cotton variety MON-15985, they were able to determine the Cry1Ac protein; through the Elisa test, obtaining average values of 4.8 µg g^{-1} in leaf tissue, results very similar to those obtained in this study.

Table 6. Concentration of protein in leaf (µg g^{-1} in dry weight).

Site \ Date	21/05/14	04/06/14	19/06/14	14/07/14	21/07/14	29/07/14
	Leaf	Leaf	Leaf	Leaf	Leaf	Leaf
			Cry1Ac			
Field 1	7.88	7.2	3.7	2.36	2.11	0.91
Guamuchil	8.5	4.75	1	3.65	1.59	1.51
5 de febrero	5	4.08	1.74	1.63	0.33	0.91
Field 9	ND	5.14	1.91	1.42	1.57	1.5
			Cry2Ab			
Field 1	63.13	66.67	63.44	13.91	35.49	17.57
Guamuchil	64.32	66.71	69.11	21.22	31.74	15.11
5 de febrero	62.88	74.09	63.2	9.68	33.92	14.47
Field 9	61.46	72.26	64.04	8.51	34.90	18.09

ND= not detected.

Table 7. Protein concentration in square and acorn (µg g^{-1} in dry weight).

Site \ Date	21/05/14	04/06/14	19/06/14	14/07/14	21/07/14	29/07/14
	Square	Square	Square	Square	Acorn	Acorn
			Cry1Ac			
Field 1	1.87	2.2	1.16	0.18	0.18	0.16
Guamuchil	1.52	1.08	0.65	0.38	0.06	0.06
5 de febrero	1.03	2.08	0.73	0.08	ND	ND
Field 9	1.71	2.47	0.56	0.04	0.35	0.08
			Cry2Ab			
Field 1	70.75	81.73	59.02	1.07	24.35	18.51
Guamuchil	52.02	58.19	64.38	2.35	19.21	17.52
5 de febrero	59.68	92.64	80.92	0.64	12.34	16.7
Field 9	73.6	88.73	58.1	0.36	17.11	19.07

ND= not detected.

In the analysis of the expression of proteins conferred to GM crops, it is important to bear in mind their direct relationship with the growth and development of the plant itself and that both are influenced by environmental factors. The temperature, light, salinity, water availability, nutritional status and environmental interactions (with biotic agents of their environment) are identified as the main environmental factors that affect the growing conditions and therefore the development of the plants and their composition Sivasupramaniam *et al.* (2008); (Jamal, 2009); Knight *et al*. (2013).

According to Kranthi *et al*. (2005), the Cry1Ac protein tends to vary between the different hybrids and the different tissues of the plant, finding the highest levels in terminal leaves, followed by square, acorns and finally the flowers. This variation was clearly observed throughout the

samplings and analyzes carried out. A very similar behavior was observed and quantified with the Cry2Ab protein in all tissues analyzed. The highest levels were quantified in leaves with 74 μg g^{-1} in the vegetative stage, while in later stages lower levels of up to 8.5 μg g^{-1} were obtained, without reaching undetectable levels. In table and acorn, similar levels were obtained to leaf levels in early stages, quantifying up to 92.6 μg g^{-1}, while in late stages levels of up to 0.3 μg g^{-1} were obtained, Knight *et al*. (2013).

Gene detection

Extraction and determination of the concentration and purity of RNA from plant tissues

The absorbance at 260 nm and the A260/280 ratio gives us an indication of the amount of RNA and the degree of contamination of organic and inorganic impurities. RNA extracts were obtained from leaf, square and acorn tissues with A260/A280 ratios between 1.7-2, which indicates a high degree of purity of isolated RNA, according to Kang (2012). The extraction yields from leaf were superior to 1 000 ng μL^{-1}, while for square and acorn they were between 50 and 340 ng μL^{-1}.

Quality parameters in gene detection

To verify the linearity and gene quantification of the transgenic proteins, calibration curves were made with six concentrations, in number of copies, known at different levels. For the protein Cry1Ac R^2 was obtained higher than 0.99 in most cases, the same for the Cry2Ab protein. The values of R^2 recommended to ensure the linearity of the method is ≥0.99. In the Figure 2 shows the linearities of the calibration curve system for the gene quantification of the Cry1Ac and Cry2Ab proteins.

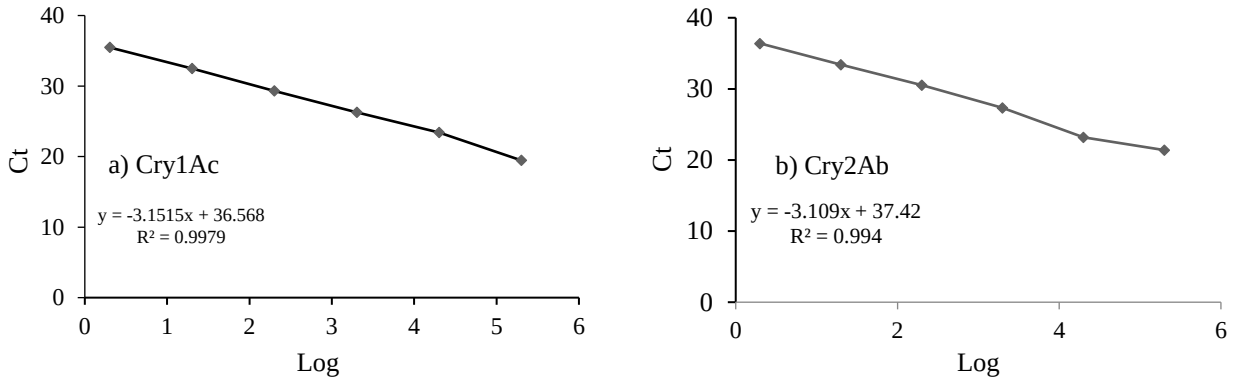


Figure 2. Calibration curve for the Cry1Ac (A) and Cry2Ab (B) proteins in RT-Qpc.

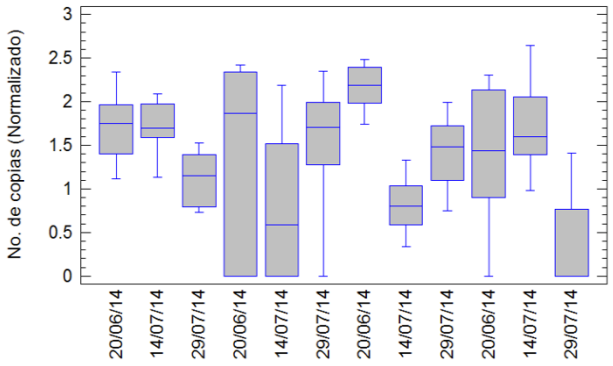
The efficiency of the method (E) was measured, which is the adequate duplication of the sequence of interest in each cycle. The efficiencies obtained were 107.63% for Cry1Ac and 109.72% for the Cry2Ab protein. An adequate reaction yields efficiency with values of 90-110%. For Kavanagh *et al*. (2011) the slope should be another quality parameter evaluated in the polymerase chain reaction tests, those obtained in this study were -3.1515 for Cry1Ac and -3.109 for Cry2Ab, which fall within those recommended by bibliography that they should oscillate between -3.58 and -3.1.

The results obtained in the validation meet the criteria established in the minimum information guide for the publication of quantitative real-time PCR experiments (MIQE), published by Bustin *et al*. (2009) and used by Dinon *et al*. (2011), for the development and validation of transgenic protein detection methods by real-time PCR.

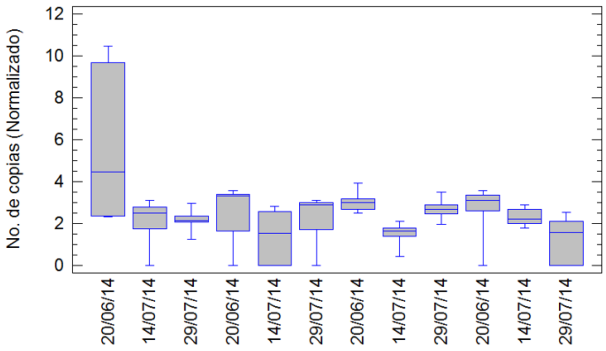
Real-time polymerase chain reaction (RT-qPCR)

Once the complementary DNA was synthesized, we proceeded to quantify the genes coding for the Cry1Ac and Cry2Ab proteins expressed in the aforementioned tissues. Due to the dispersion obtained from the data, we proceeded to perform the Kruskal-Wallis test analyzing the median of the data to conclude whether or not there was a statistically significant difference. These results showed that there is no significant difference, with respect to time, in the expression of the protein Cry1Ac (p= 0.84) and Cry2Ab (p= 0.446) in leaves; if there is a difference in tables and acorns for both proteins (p= 0.0004 for Cry1Ac, p= 0.001 for Cry2Ab).

It was observed in each of the fields that, as the analysis was carried out at later stages, the quantification of the gene was lower, in some cases tending to zero (Figures 3 and 4), but there was always expression. In a similar study conducted by Adamczyk and Meredith in 2004, gene expression of the Cry1Ac protein tends to vary between different varieties and parts of the plant throughout the season. Adamczyk *et al*. (2009), correlated the amounts of the Cry1Ac protein with the mRNA transcripts, finding differences in the varieties studied.



a) expression of the Cry1Ac protein in leaf.

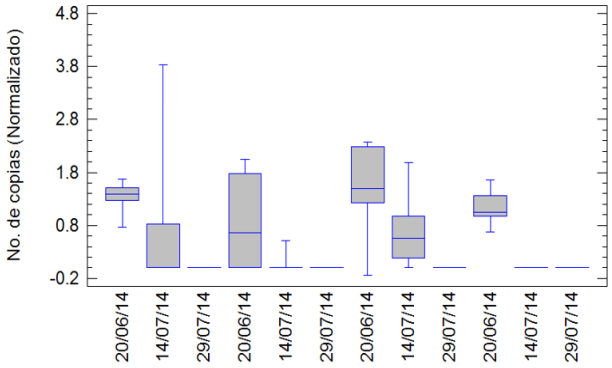


b) expression of the Cry2Ab protein in leaf.

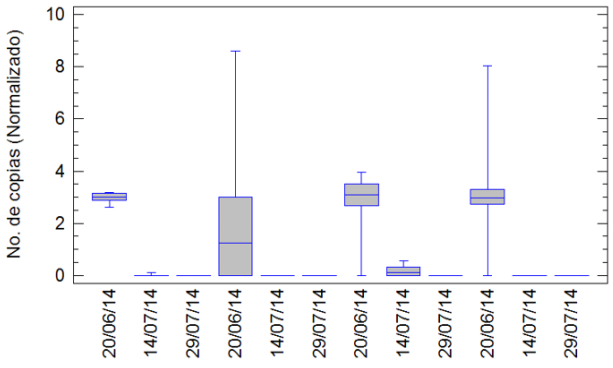
Figure 3. Quantification of the genes of the Cry1Ac and Cry2Ab proteins in GM cotton leaf tissues.

The order of the dates for the properties from left to right is: Field 1, 5 de febrero, Guamuchil and Field 9.

The date corresponding to the acorn analysis is that of 07/29/2014, the rest corresponds to tables. The order of the dates for the properties from left to right is: Field 1, 5 de febrero, Guamuchil and Field 9.



a) expression of the Cry1Ac protein in square and acorn.



b) expression of the Cry2Ab protein in square and acorn.

Figure 4. Quantification of the genes of the Cry1Ac and Cry2Ab proteins in GM cotton square and acorn tissues.

Olsen *et al*. (2005); Xia *et al*. (2005), concluded that the mechanism is to some extent unknown. Adamczyk and Gore (2004); Kranthi *et al*. (2005); Olsen *et al*. (2005); Wan *et al*. (2005), concluded that the expression of the Cry1Ac gene may decrease the effectiveness of the technology in crops due to the levels of expression observed in late stages, recommending constant monitoring to establish the baselines of each insect problem and avoid with it the development of resistance in white insects.

For the case of the Cry2Ab protein in leaf, acorns and squares the results show that there is variation and difference of expression in the tissue analyzed between the different dates and fields studied. Bakhsh *et al*. (2012) observed that the expression of the Cry2Ab gene undergoes variations among the varieties studied, as well as in the different tissues that make it up. Monsanto (2011) indicated that the variations of gene expressions depend on the periods of production, physiology of each plant, genetic variability of each plant, environmental conditions and agricultural practices; in addition, there may be variations between plants of the same batch and that cellular conformation may compromise the expression of proteins added to the plant genome.

Wessel *et al*. (2001) suggested that the activity of the promoter region of the transgene can be characterized only by the distribution of the different levels of expression in the plant, at each level (or tissue) a different behavior occurs. The temporal and spatial expression characteristic of a transgene in different independent transgenic lines must be intrinsic to the activity of the promoter. Therefore, the promoter who is the one who directs the expression of the transgene has different behaviors in each cell type in the plant, defining the spatial regulation of the expression (Head and Greenplate, 2012).

Conclusions

It was possible to standardize molecular methodologies for the analysis of gene expression and quantification of proteins conferred in the two varieties of GM cotton cultivated in the Yaqui Valley.

The Elisa analysis allowed to determine the concentration of each of the proteins throughout the phenological development of the variety studied and in each site.

The observed expression tendencies, levels and stage of crop development, ratify the information that has been published about the GM cotton plants used in the study. As expected, maximum expression values were observed in the initial phases of the culture.

The RTq-PCR and ELISA techniques are suitable to evaluate the spatial and temporal expression of genes that are conferred to GM plants. The use of these techniques will generate information necessary for risk analysis, in particular to characterize exposure, to new GM crops that are intended to release the environment and then enter the food chain.

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