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Detection of genetically modified maize in processed products, dry grains, and corn ears intended for fresh consumption in South Brazil

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ABSTRACT. Conventional and genetically modified (GM) maize cultivars have been widely planted in Brazil to produce grains for processed food, feed, or to be consumed fresh as corn ears. This study used real-time PCR to detect GM maize in processed products and fresh commercial corn ears produced in the last two years in South Brazil. Eighteen conventional and GM maize cultivars were obtained from seed production companies and 50 commercial samples (including canned corn, corn flour, dry grains, and fresh corn ears) were purchased in small local stores and supermarkets. All samples were analyzed by real time TaqMan PCR to detect one constitutive maize gene (*hmg*) and three genetic regions present in GM plants (p-35S promoter, major gene *cry* 1A.105, and t-Nos terminator). Each commercial sample was classified as conventional or GM based on the PCR results. PCR targeting the

hmg gene generated positive results from all DNA samples, which were further tested with the GM targets. These targets were not detected in the five conventional maize cultivars, but were detected in the GM seeds hosting these fragments. Analysis of processed foods identified four cultivars as conventional and six as GM, which were mostly correctly labeled. Seven (53.8%) dry grain samples were classified as conventional, while six (46.2%) were classified as GM. Three (11.1%) corn ear samples were identified as conventional, and the remaining 24 (88.9%) were GM maize. These results demonstrate the high frequency of GM maize in processed products, including fresh corn ears intended for consumption in South Brazil.

Key words: GM crops; Real-time PCR; Transgenic maize

INTRODUCTION

Maize (*Zea mays*), or corn, is one of the most widely consumed grains by humans and livestock throughout the world. As a component of the human diet, it can be consumed fresh as corn ears, used as a staple food for processed meals (polenta, tortillas, burritos), or as a snack food, such as popcorn and corn chips. In general, variation in the moisture content accounts for the different properties of these foods; while corn ears have more than 70% water content, dry grains (used in processed foods) have only 15 to 20% (Silva et al., 2010).

Due to its importance in human and animal diets, maize is a leading crop plant in Brazil, with a plantation area of 15.8 million hectares and a total production of 85.5 million tons in the 2014/2015 crop. It is the second most cultivated grain, accounting for 40% of the total crop production in the country (CONAB, 2015). Data from maize producing companies reveal that 478 cultivars were available to farmers during the last crop in Brazil, including 292 genetically modified (GM or transgenic) and 186 conventional cultivars (Cruz, 2014; Cruz, 2015). In addition, there were 320 different genetic materials, 186 of which were marketed as GM and conventional, and 134 commercialized only as GM crops (lacking the conventional option). All of these cultivars can be used to produce dry grains, silage for livestock animals, and corn ears. In the last crop, only 17 cultivars were recommended for the production of corn ears, while 474 cultivars were indicated for dry grain production and whole plant silage. Four other cultivars were recommended for the production of popcorn and corn starch (Cruz, 2015).

GM corn crops express insecticidal genes (*cry* or *vip*), derived from the soil bacterium *Bacillus thuringiensis* (Bt), in order to control the major Lepidoptera species. Currently commercialized GM biotech crops have different events to achieve this effective control, such as Herculex I (TC1507), YieldGard (MON810), Agrisure TL (Bt11), TL Viptera (MIR162), and VT PRO (MON890314). Plant leaves produce different Cry proteins according to the inserted event, such as Cry 1F, Cry 1Ab, Cry 1A.105, Cry 2Ab2, Cry 3Bb1, Cry 34Ab1, and Cry 35Ab2. More recently, GM seeds with insecticidal traits stacked with tolerance to the herbicides glyphosate (Roundup Ready NK603 and TG GA21) or glufosinate-ammonium (Liberty Link Technology) have also been commercialized in Brazilian producing regions (Cruz, 2015). All of these GM events carry at least one promoter and one terminator region, in addition to the main gene (*cry*, *vip*, *epsps*, etc.). The promoter region p-35S (from the cauliflower mosaic virus) and the terminator t-Nos (from the nopaline synthase gene) are the

most commonly used in GM maize seeds (Wolf et al., 2000; Dinon et al., 2011).

The use of GM crops has many social, environmental, and economic advantages. GM crops reduce the use of chemical insecticides; provide benefits to human health, the environment, and biodiversity; and lead to higher productivity increases income for farmers (Romeis et al., 2006; Hutchison et al., 2010; Tabashnik, 2010; Ronald, 2011). These advantages explain the intensive use of biotech crops since their first commercial release in 1996. The total cumulative area of transgenic crops exceeds 1.8 billion hectares with a 3 to 4% consistent increase per year. As a consequence, GM maize products have been increasingly marketed in the last few years (James, 2014).

The production of food and feed from GM plants is subject to specific regulation. Products for human or animal use containing more than 1% GM organisms have to be labeled to inform consumers (Brazilian Government - Law No. 4680, April 25, 2003; Marinho et al., 2014). The aim of the present study was to detect GM maize in industrially processed products, dry grains, and corn ears commercialized for fresh consumption in the Rio Grande do Sul State, south of Brazil. All samples were tested for the presence of two genetic regions usually present in GM crops (the p-35S promoter and the t-NOS terminator) and one main gene (*cry* 1A.105) present in the most commercialized GM corn crops grown in this region (VT PRO).

MATERIAL AND METHODS

Samples

Seeds of 18 maize cultivars were kindly provided by cooperatives, seed companies, and agricultural markets (Table 1). The cultivars were obtained from the commercial companies Agroceres, Biomatrix, Dow Agroscience, Dekalb, Santa Helena Seeds, and Syngenta (Celeron, Formula, and Status brands). These cultivars were approved to be planted in the last Brazilian crop by the National Biosafety Technical Commission. In addition, 50 commercial samples were purchased from small local stores and supermarkets. The products included canned corn (N = 4), corn flour (N = 6), dry grains currently used in feed (N = 13), and fresh corn ears (N = 27). All these samples were produced between 2014 and 2015. The corn ears were marketed in 27 different commercial plastic bags, each containing three or four corn ears, and were produced by 17 different companies located in 12 cities from Rio Grande do Sul State, Brazil (Figure 1).

DNA extraction

All samples were processed prior to DNA extraction. Grains were first macerated and 0.1 to 0.2 mg was used for the analysis of DNA. DNA was extracted by an adapted silica method (Boom et al., 1990) using commercial reagents (Simbios Biotecnologia, Cachoeirinha, RS, Brazil). Briefly, 1350 µL lysis solution (5 M guanidine tiocianate, 0.1 M Tris-HCl, pH 6.4) was added to each sample, which were then incubated at 60°C for 10 min. After centrifugation (10,000 *g*, 3 min), the supernatant was transferred to a tube containing 20 µL silica suspension. This mixture was stirred and centrifuged (10,000 *g*, 3 min), and the pellet was washed twice with 1000 µL wash solution A (5 M guanidine tiocianate, 0.1 M Tris-HCl, pH 6.4), twice with wash solution B (75% ethanol), and once with wash solution C (100% ethanol). The silica was dried and the DNA was separated using 50 µL eluting solution (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Table 1. Maize seed cultivars used in the present study.

Type	Cultivars	*hmg*	p-35S	cry 1A.105	t-Nos
Transgenic	BM 915 PRO	+	+	+	+
	SHS 7920 PRO	+	+	+	+
	BM 3063 PRO2	+	+	+	+
	BM 3066 PRO2	+	+	+	+
	SHS 7915 PRO	+	+	+	+
	SHS 7990 PRO2	+	+	+	+
	DKB 240 PRO	+	+	+	+
	2B647 PW	+	+	+	+
	AG 5011	+	+	-	-
	CELERON TL	+	+	-	+
	FORMULA TL	+	+	-	+
	STATUS VIP	+	-	-	+
	STATUS VIP3	+	+	-	+
Conventional	AG 8025	+	-	-	-
	BM 911	+	-	-	-
	FORMULA	+	-	-	-
	CELERON	+	-	-	-
	STATUS	+	-	-	-

hmg - endogenous corn gene; p-35S - promoter region; cry 1A.105 - main maize gene for the MON89034 event; t-Nos - terminator region. Biomatrix (BM), Santa Helena Seeds (SHS), Dekalb (DKB), Dow Agroscience (2B), Agroceres (AG), and Syngenta (Celeron, Formula, and Status brands).

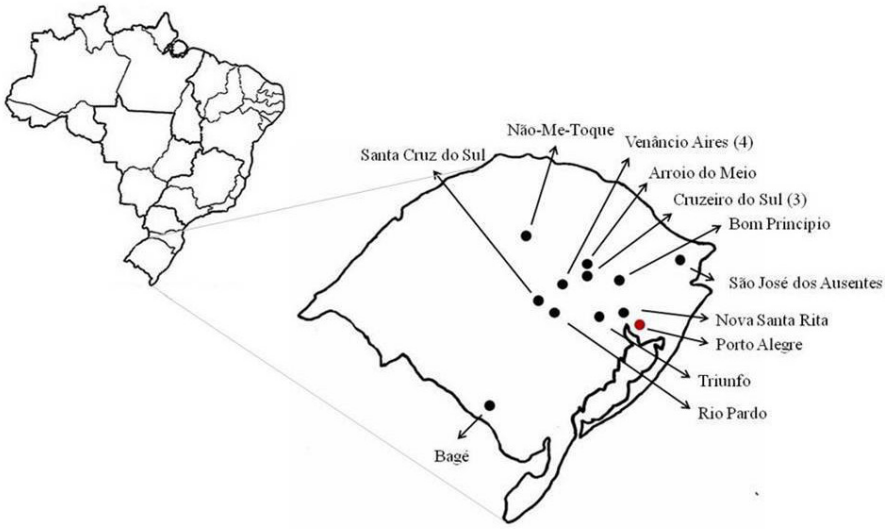

Figure 1. Cities of the corn ears producers in Rio Grande do Sul. The state capital, Porto Alegre, is highlighted in red.

Primers and probes

Primers and probes targeting high mobility group (*hmg,* a constitutive maize gene), p-35S, the *cry* 1A.105 gene, and t-Nos were selected based on the results of previous studies (Table 2). All primers and probes were evaluated using the Primer Express software (Applied Biosystems, Norwalk, CT, USA). Oligonucleotides were purchased from Applied Biosystems or Integrated DNA Technologies (Coralville, IA, USA).

Table 2. Primers and TaqMan probes used in the real time PCR.

Primers	Sequence (orientation 5'-3')	Amplicon size (bp)	Reference
p-35S - F	GCC TCT GCC GAC AGT GGT	82	Waiblinger et al., 2008 Huber et al., 2013
p-35S - R	AAG ACG TGG TTG GAA CGT CTT C		
p-35S - P	FAM-CAA AGA TGG ACC CCC ACC CAC G- ZEN-IOWA BLACK FQ		
t-Nos - F	CAT GTA ATG CAT GAC GTT ATT TAT G	84	Reiting et al., 2007 Huber et al., 2013
t-Nos - R	TTG TTT TCT ATC GCG TAT TAA ATG T		
t-Nos - P	VIC-ATG GGT TTT TAT GAT TAG AGT CCC GCA A- ZEN-IOWA BLACK FQ		
cry1A.105 - F	TCAGAGGTCCAGGGTTTACAGG	113	Dinon et al., 2011
cry1A105 - R	GTAGTAGAGGCATAGCGGGATTCTTG		
cry1A105 - P	FAM-AGACATTCTTCGTCGCACAAGTGGAGGACC-ZEN –IOWA BLACK FQ		
ZM1- F(*hmg*)	TTGGACTAGAAATCTCGTGCTGA	79	Corbisier et al., 2010
ZM1- R(*hmg*)	GCTACATAGGGAGCCTTGTCCT		
ZM1- P(*hmg*)	VIC-CAATCCACACAAACGCACGCGTA- IOWA BLACK FQ		

F: Forward primer; R: reverse primer; FAM and VIC TaqMan reporter dye labels; MGB and ZEN-IOWA BLACK FQTaqMan quencher dye labels.

Real time-PCR

Real-time TaqMan PCR assays were performed using StepOne Plus® (Applied Biosystems). First, PCR with the *hmg* primers/probe set was carried out to evaluate the quality of all extracted DNA. Next, three separate PCRs were run to detect p-35S, t-Nos, and *cry*1A-105. All reactions were carried out in a total volume of 30 µL with 2 µL DNA template, 1.5 U Taq DNA polymerase and the respective enzyme reaction buffer (Ludwig Biotecnologia, Porto Alegre, RS, Brazil), 1.5 mM $MgCl_2$, 0.06 mM each dNTP, 0.25 µM each primer, and 0.125 µM probe(s). The following cycling parameters were used: one cycle of 95°C for 3 min, 40 cycles of 95°C for 15 s, and 60°C for 60 s. Cycle threshold was defined for each sample and compared to that of the positive and negative controls.

RESULTS

TaqMan PCR conditions

All probes and primers used in this study were first tested using two GM crop cultivars hosting the VT PRO event (BM 915 PRO and SHS 7920 PRO). Both generated positive results for the four targets: the constitutive maize gene *hmg*, the promoter region p-35S, the main Bt gene *cry* 1A.105, and the terminator region t-Nos. The TaqMan PCR assays proved to be effective at detecting all four different targets for these two cultivars (Table 1).

Detection of GM maize events in commercial cultivars

The remaining 16 maize seeds, including five conventional and 11 GM crops, were analyzed using the four PCR assays described (Table 1). Briefly, all samples were found to be positive for the endogenous maize gene *hmg*. Conventional maize seeds (AG 8025, BM 911, Celeron, Formula, and Status) presented negative results for the other three targets. Of the 11 GM crops, 10 amplified for the p-35S promoter and the t-Nos terminator gene fragments, while six were also positive for the *cry*1A.105 gene. These remaining six maize cultivars were all positive for *cry*1A.105, p-35S, and t-Nos. As defined by the producers, all of those cultivars host the GM event PRO (MON89034). The cultivars AG 5011 and Status VIP presented

positive results only for p-35S and t-Nos, respectively. The remaining three samples (Celeron TL, Formula TL, and Status VIP3) presented positive results for both the p-35S and t-Nos regions (Table 1).

Detection of GM maize events in processed foods and dry grains

All 23 industrially processed products (six corn flours, four canned corns, and 13 dry grains for animal feed) tested positive for the *hmg* gene (Table 3). In 11 samples, none of the maize GM targets was detected, and they were classified as conventional (non-GM). Of the other 12 samples, at least one of the GM targets was detected, and they were defined as GM foods. Seven samples were positive for the three GM targets, two for the p-35S and t-Nos regions, two for p-35S, and one for t-Nos (Table 3). Among the 12 transgenic samples, only the five corn flours had a GM label on their packaging.

Table 3. Description of the commercial samples: industrially processed foods (corn flours and canned corn), dry grains, and corn ears.

	N	*hmg*	p-35S	cry 1A.105	t-Nos	Classification
Corn flours	1	+	-	-	-	Conventional
	5	+	+	+	+	GM
Dry grains	2	+	+	-	+	GM
	2	+	+	+	+	GM
	1	+	+	-	-	GM
	7	+	-	-	-	Conventional
	1	+	-	-	+	GM
Canned corn	3	+	-	-	-	Conventional
	1	+	+	-	-	GM
Corn ears	19	+	+	+	+	GM
	3	+	-	-	-	Conventional
	1	+	+	-	-	GM
	1	+	-	-	+	GM
	3	+	+	-	+	GM

hmg - endogenous corn gene; p-35s - promoter region; cry 1A.105 - main maize gene for the MON89034 event; t-Nos - terminator region.

Detection of GM maize events in corn ears

Real-time PCR assays were used to detect the presence of transgenic maize in 27 commercial samples of corn ears. Grains of all samples tested positive for the endogenous *hmg* gene. Analysis of the three specific GM targets revealed that only three samples were negative, and these were classified as conventional. The remaining 24 (88.9%) corn ear samples were positive for at least one DNA region of the GM crops (Table 3). Interestingly, 19 samples were positive for the three targets, meaning that they all carry the transgenic event VT PRO (MON89034).

DISCUSSION

GM maize food and feed have been marketed worldwide. In Brazil, all food products intended for human and animal consumption containing over 1% GM crops is required to be properly labeled (Brasil, 2003). Consequently, immunological and molecular biology

techniques have been developed to detect the presence of specific GM DNA fragments in food and feed products. Immunological assays (such as enzyme linked immunosorbent assay and immunochromatography) are fast and user-friendly and require relatively low investment in terms of equipment and personnel. The immunochromatographic strip test is the most-disseminated assay used in the field, and is even used by farmers to separate GM from non-GM crops. However, this assay detects specific transgenic proteins (Bt crystal protein in maize or EPSPS in soybean), which are often not readily available in some plants, seeds, and consequently, food products (Nascimento et al., 2012; Cantelmo et al., 2013).

Molecular biology assays (mainly PCR) are specific, sensitive, and have been widely used to detect the presence of GM crops. Although requiring special reagents and equipment, PCR can detect the presence of specific exogenous genes inserted into the plant genome (including the seeds), and represents the most widely used method to detect GM food and feed (Dinon et al., 2011; Branquinho et al., 2013; Cantelmo et al., 2013). In the present study, we used real time PCR to detect three GM-specific fragments: the promoter region p-35S derived from the cauliflower mosaic virus (Waiblinger et al., 2008; Huber et al., 2013), the terminator region, t-Nos, derived from the nopaline synthase gene of *Agrobacterium tumefaciens* (Reiting et al., 2007; Huber et al., 2013), and the main *cry1A.105* gene from Bt (Dinon et al., 2011). These PCR assays were successfully implemented, in addition to an assay targeting the constitutive *hmg* gene, which was used as an endogenous control (Corbisier et al., 2010). Two GM crop cultivars carrying the VT PRO event (BM 915 PRO and SHS 7920 PRO) presented positive results for all three targets. Furthermore, the other conventional and GM maize cultivars presented results that were expected based on register information, as demonstrated in previous studies (Reiting et al., 2007; Waiblinger et al., 2008; Dinon et al., 2011; Huber et al., 2013).

All of the food samples analyzed were positive for the endogenous *hmg* gene, demonstrating the absence of inhibition in the PCR and the presence of sufficient genetic material in these highly processed foods. Previous studies have demonstrated that it is not possible to detect oils and refined starches derived from GM crops because of the excessive heat, low pH, and enzymes used in the processing of corn flour products (Conceição et al., 2006), The five flour samples that had a GM label were positive for all of the tested targets, showing the presence of grains with the VT PRO event. The only non-labeled flour was negative for the three GM targets. A study was previously performed to verify compliance with Brazilian laws on the reporting of GM crops in food (Branquinho et al., 2013). Those authors reported the occurrence of several food products with GM organisms that were not properly labeled between 2011 and 2012. In the following year, the same products were properly labeled in accordance with Brazilian law. Another study conducted in Turkey revealed the presence of GM maize events in the feed composition used for livestock production (Meriç et al., 2014).

Conversely, the majority of corn ears (24 of 27, 88.9%), collected in the market and produced by small companies in different regions of Rio Grande do Sul State, were positive for at least one GM target (p-35S, t-Nos, and/or the *cry* 1A.105 gene). Those results demonstrate the intensive use of GM cultivars to produce commercial corn ears for human consumption. Furthermore, 19 samples were positive for the four targets, demonstrating that they are probably cultivars containing the GM VT PRO (MON89034) event. This may be due to the majority of the GM maize cultivars commercialized in the current season (55%) carrying the VT PRO event (Cruz, 2014; Cruz, 2015).

Crops used for corn ear production have been developed to meet characteristics sought

by consumers, which include intense yellow grains, fruity and sugary taste, soft texture, and being free of spike damage. Some of these are still planted to produce corn ears and/or sweet corn (Lopes et al., 2014). In the last crop season (2014/15), 17 maize cultivars were suitable for the production of corn ears, and only one of those hosts the MON89034 event (VT PRO). This specific cultivar has not been widely distributed and commercialized to the small farmers that plant and sell corn ears to the commercial companies (Cruz, 2014). It is likely that several of the 292 GM cultivars used for other purposes (dry grain, silage) have been widely planted in the field. Maize in Brazil is produced widely by small farmers, who do not usually have the opportunity to select cultivars and purchase the seeds from local markets with restricted commercial options. Therefore, seeds are used for the production of dry grains and silage because they are commonly available in the market (Cruz, 2015).

Non-transgenic corn crops can be pollinated by transgenic plants grown in neighboring locations (Devos et al., 2009). Good management practice of transgenic crops (use of natural barriers, cultivation of conventional plants in the borders, postponing or advancing sowing to the flowering period) could help to avoid the contamination of non-transgenic crops (Palaudelmàs et al., 2012). Of note, producers have responsibility, especially in organic production, for the accuracy of the information provided about the crop (CIB, 2011). According to Brazilian law, the use of transgenic grains is also forbidden in organic products; therefore, producers should avoid any possible contamination with GM maize (MAPA, 2015; Santos, 2015). Currently, 46% of maize produced comes from family farms in Brazil, many of which are focused on organic corn production (MDA, 2015).

The present study has two limitations. First, not all of the 21 transgenic maize events released for sale in Brazil were tested (although the GM events tested here represent more than 95% of the GM seeds produced in the last crop). Second, the number of analyzed samples used to represent the southern region of Brazil or the whole country is low.

In conclusion, GM maize was detected in industrially processed commercial products and fresh corn ears in Rio Grande do Sul State, south of Brazil. There was a high frequency of GM maize in processed products, dry grains, and corn ears intended for fresh consumption in South Brazil. Most food was found to carry the *cry* 1A.105 gene, demonstrating the intensive use of GM maize cultivars with the VT PRO (MON89034) event.

Conflicts of interest

The authors declare no conflict of interest.

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