Anal Bioanal Chem (2008) 392:395–404
DOI 10.1007/s00216-008-2265-8

ORIGINAL PAPER

Real-time and conventional PCR detection of Liberty Link® rice varieties and transgenic soy in rice sampled in the Mexican and American retail markets

Maricarmen Quirasco · Bernd Schoel · Pradheep Chhalliyil · John Fagan · Amanda Gálvez

Received: 5 April 2008 / Revised: 22 June 2008 / Accepted: 25 June 2008 / Published online: 31 July 2008

Abstract Samples of rice from Mexican and USA retail stores were analyzed for the presence of transgenic (GM) events using real-time PCR. In screening for the *CaMV35S* promoter sequence (*35SP*), positive results were found in 49 and 35% of the Mexican and American samples, respectively. In further investigations in Mexican samples, 43% were positive for *P35S::bar*, with two above the quantifiable limit; these were 0.07% and 0.05% GMO. Fourteen out of the sixteen positive samples were labeled as imported from the USA. In testing samples bought in American retail shops, 24% showed positive results, all below the quantifiable range. It could be deduced that *P35S::bar* positive samples were Liberty Link® (LL) rice. In distinguishing between LL601 and LL62, end-point PCR was used, corroborating the *P35S::bar* amplicon length difference of these events. LL62 was found in one rice sample purchased in Mexico and two in the USA samples. Its presence was verified with the 35S terminator sequence. All other LL positive samples contained LL601. None of the samples analyzed showed the presence of Bt63 rice. The LL rice varieties found have been identified as not being commercially cultivated, and so their presence requires further investigation. *35SP* was also present in samples which did not have any LL rice. Maize sequences could not be detected in any of the samples; however, soybean DNA was found in Mexican and USA rice samples. The Roundup Ready® trait was detected in trace amounts in 16 and 6% of the rice samples bought in Mexico and the USA, respectively. Real-time PCR was shown to be the method of choice for the sensitive and rapid screening of commodities and retail samples for the detection of GM and other contamination.

Keywords GM rice · Quantitative PCR · LLRICE601 · LLRICE62 · *P35S::bar* · Adventitious presence

The three authors Maricarmen Quirasco, Bernd Schoel and Pradheep Chhalliyil contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00216-008-2265-8) contains supplementary material, which is available to authorized users.

M. Quirasco · A. Gálvez (✉)
Departamento de Alimentos y Biotecnología,
Facultad de Química,
Universidad Nacional Autónoma de México,
04510 México, D. F., México
e-mail: galvez@servidor.unam.mx

B. Schoel · P. Chhalliyil · J. Fagan
Genetic ID NA, Inc.,
P. O. Box 1810, Fairfield, IA 52556, USA

Introduction

Rice world production amounts to 417.64 million metric tons (MT) [1]. Between 2005 and 2007 the USA provided about 11% of the global rice trade [2]. Over 70% of all long-grain rice is grown and commercialized in that country [3]. In contrast, Mexican production has oscillated over the past decade, with an abrupt decrease in the past five years: in 1985 a top figure of 808 thousand MT was obtained, which subsequently sharply decreased, reaching only 287 thousand MT in 1993 [4]. After a new peak in 1997, with 469.5 thousand MT produced, by 2006 only 244.47 thousand MT were harvested [5]. The Mexican market was driven to import rice mainly from the USA, since both countries belong to the North American Free Trade Agreement (NAFTA). In 2006 rice imports totaled 785.8 thousand MT according to the USA Rice Federation [3], or 808.5 thousand MT according to the Department of

Commerce, US Census Bureau [6], with a value of approximately 204 M USD. Thus, Mexico became by far the biggest importer in the continent, and the USA the largest market for long-grain and other rice varieties [7]. As one of the major suppliers of rice, the USA exported approximately 20,000 MT of long-grain, semi-milled and wholly milled rice to the EU on average per month.

In the current global commodity market, the main transgenic crops are soybean and corn, followed by canola and cotton. Transgenic rice is not a commercial commodity, although several genetically modified (GM) varieties have been developed to confer herbicide tolerance or insect resistance. Three experimental GM rice varieties were registered in biotech databases such as AgBios [8]: Liberty Link® (Bayer CropScience) varieties LLRICE601, LLRICE06 and LLRICE62, although LLRICE601 is not registered in the Cartagena Protocol Biosafety Clearing-House [9]. All three were engineered to contain a stably integrated *bar* gene which encodes for phosphinotricin-N-acetyltransferase (PAT). This enzyme catalyzes the conversion of L-phosphinotricin, the active ingredient glufosinate ammonium, to an inactive form, therefore conferring resistance to the herbicide. The donor organism of the *bar* gene is *Streptomyces hygroscopicus*, strain HP632 [10], which was introduced through direct gene transfer of plasmid DNA. The LLRICE06 event contains several complete and partial copies of the *bar* gene. The LLRICE62 presents one complete copy of the gene [11]. GM varieties LLRICE62 and LLRICE06 were approved for release into the environment in the USA in 1999. They were later approved for food and/or feed in the USA and Canada in 2000 and 2006, respectively. The latest variety to be authorized, as recorded in the AgBios database, was LLRICE601. This variety was field-tested between 1998 and 2001 [12] and approved for release into the environment in US territory in November 2006 after its detection in European imports. Regarding food safety for human consumption, the FDA published a statement which affirms that the PAT protein is considered safe according to the authorization of the LL06 and LL62 varieties. Due to the fact that the company did not plan on commercializing the LL601 variety, no petition for authorization was submitted for either experimental or commercial release in 1998–2001. Based on the available data and information about the safety of the PAT protein, molecular characterization, and the nutritional composition of grain from LLRICE601 provided by Bayer CropScience, the FDA concluded in August 2006 that the presence of this bioengineered rice variety in the food and feed supply poses no food or feed safety concerns [13]. Nevertheless, the normal track for the food safety approval of LL601 is still pending.

Conventional products contaminated unintentionally are not subject, under the European Directive, to traceability or labeling requirements if they contain authorized GM organisms (GMO) below a 0.9% threshold level, provided that the presence of GM material is adventitious or technically unavoidable during harvesting, storage, transport, or processing [14]. However, unauthorized GM material is tolerated to a maximum of 0.5% in conventional food and feed, provided its presence is adventitious or technically unavoidable, for a limited number of events which have benefited from a favorable risk evaluation [15]. Of the GMOs authorized in accordance with Directive 2001/18/EC of the European Parliament and of the Council on the deliberate release into the environment of GMOs, 22 maize, rape, soybean, cotton and sugar beet varieties were authorized for use in food/feedstuffs. No GM rice variety has been listed as authorized or risk-evaluated in Europe [16]. However, in August 2006, Switzerland and the EU found the presence of LLRICE601. Since this date, a non-GM certificate has been required, and rice exports to the EU have dropped sharply.

In Mexico, no applications for the authorization of LLRICE601 had been submitted. By March 2007 one authorization had been granted for LLRICE62 for food and/or feed [17]. Considering that Mexico imports 90% of its rice from the USA, this fact made it necessary to investigate the status of GM rice and to screen samples from Mexican retail stores. This study presents data for GM rice and other species in samples from Mexican and, for comparison, North American retail stores.

Materials and methods

Sample materials The samples comprising long, medium, whole brown, and precooked rice grains, two rice flours, and a rice cereal were purchased between September and October 2006 at various retail stores in Mexico (Monterrey and Mexico City) and the USA. Countries of origin, as indicated on the package, were Mexico, USA, Thailand, India and Italy. The average amount of rice grain was 399 g for the Mexican and 547 g for USA rice grain. The minimum amount of a sample was 200 g, equivalent to 11,000 grains (180 g per 10,000 grains).

Genomic DNA extraction Each sample was ground and homogenized in an electric blender. DNA extraction was done in duplicate per sample using the Fast ID Genomic DNA Extraction Kit (Genetic ID NA, Inc., Fairfield, IA, USA). Three mL of Genomic Lyse buffer and 30 μL of proteinase K (20 mg/mL) were added to 2 g samples. They were vigorously mixed and incubated at 65 °C for 30 min. Then 3 mL of chloroform were added, vortexed and centrifuged at 3,000 rpm for 15 min. Subsequently 1 mL of supernatant was mixed with an equal volume of

Genomic Bind buffer and applied to the DNA Binding Columns using a vacuum manifold. Columns were washed once with 1 mL of Genomic Bind buffer and three times with 75% ethanol. Finally, DNA was eluted with 200 μL of 1X TE buffer.

Determination of yield and quality of nucleic acid extracts The quantity and quality of DNA extracts were assessed by UV spectroscopy (Genespec-I spectrophotometer, Hitachi Genetic Systems, MiraiBio, San Francisco, CA, USA). Samples were then diluted to 20 ng/μL DNA for GMO analysis.

Primers and reference Primer/probe sets for the detection of commodities were used that target endogenous housekeeping genes: phospholipase D (*PLD*) for rice, lectin for soy, and invertase for corn. *35SP* and *35ST*, which target *35SCaMV* promoter and terminator, were used for GMO screening. *P35S::bar* was used to detect the Liberty Link® trait in rice, which includes LL601 and LL62 (Bayer CropScience, Monheim am Rhein, Germany). Other primer/probe sets for GM varieties that were used were Bt63 for rice and RR for detecting the Roundup Ready® resistance trait [18, 19]. All oligonucleotide primers and the FAM-labeled *PLD*, lectin, *35SP*, and LL601 TaqMan® probes were synthesized at Integrated DNA Technologies, Coralville, IA, USA, and are listed in Table S1 of the "Electronic supplementary material"). The FAM-labeled MGP TaqMan® probe for *P35S::bar* was synthesized by Applied Biosystems (ABI, Foster City, CA, USA). LL601 reference was traceable to a LLRICE601 DNA Bayer CropScience verified reference material. LL62 was obtained from AOCS (Urbana, IL, USA); Roundup Ready® Soy, Corn and Bt63 references were from Genetic ID NA, Inc. (Fairfield, IA, USA).

Real-time qPCR Real-time qPCR amplification was performed on an ABI 7500 Fast Real-Time PCR System. The 25 μL reaction volume contained 12.5 μL of TaqMan® Fast Universal PCR Master Mix (ABI) and a total of 200 ng of DNA per reaction. For *PLD*, the final concentration was 0.2 μM each for forward and reverse primer and the probe, and for *P35S::bar* or LL601 it was 0.4 μM each for forward and reverse primer, and 0.2 μM for the probe [20]. For lectin and *35SP* 0.5 μM for forward and reverse primer and 0.125 μM for the probe were used. Two reactions for each target gene were performed per sample duplicate. Cycling conditions were 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C and 1 min at 60 °C [21]. Non-template controls were included in each experiment.

Data analysis of qPCR To assess the GMO content using *35SP*, the threshold cycle number values (Ct values) of each reaction were converted using Ct/log concentration standard curves into units appropriate for each target gene. Standard curves were accepted only when the slopes indicated 100% PCR efficiencies within acceptable tolerances (±10%) ranging from −3.1 to −3.6, demonstrating no inhibition over the range of the standard curve. In order to obtain the GMO concentration per sample, the average of normalized duplicate values were used, where the value of the GM target gene was divided by the value of the endogenous reference gene. Thus, quantities are expressed as percentage (% GMO) as follows:

$$\left(\frac{\text{Number of transgenic genomes}}{\text{Number of non}-\text{transgenic rice genomes}}\right)\times 100.$$

For *P35S::bar* and LL601, the ΔΔCt method was used [22]. Values were rounded to two decimals.

Conventional endpoint PCR Qualitative endpoint PCR was performed on a GeneAmp 9700 PCR System thermocycler (ABI). The 50 μL reaction volume contained 0.025 U/μL Qiagen HotStarTaq DNA Polymerase (Qiagen, Venlo, The Netherlands), a total of 200 ng of DNA per reaction, 2.7 mM $MgCl_2$, 0.1 mM dXTP, and 1 μM each for forward and reverse primer. One reaction for each target gene was performed per sample duplicate. Positive and negative non-template controls were run in duplicate. Cycling conditions were 15 min at 95 °C, 40 cycles of 20 s at 94 °C and 55 min at 55 °C, followed by 10 min at 72 °C.

Electrophoresis and data analysis of qualitative PCR Amplified PCR products were run on a horizontal 2% NuSieve® 3:1 agarose gel (Lonza, Rockland, ME, USA) containing 10 μL GelStar (Lonza) per 350 mL gel. Twenty microliters of loading buffer were added to the PCR reaction product (15% w/v Ficoll 400, 0.025% Bromophenol Blue, Sigma-Aldrich, St. Louis, MO, USA or Baker Chemicals, Phillipsburg, NJ, USA), and then 20 μL were loaded per slot. After size separation digital images were taken using the Gel Doc imaging system (BioRad Laboratories, Hercules, CA, USA). Sample signal intensities were compared to references. A molecular 50–2,000 bp DNA ladder was used for size comparison (Sigma-Aldrich)

Results and discussion

The scope of this work was to screen rice in the Mexican market after unauthorized varieties were found in Europe. Samples were obtained from retail supermarkets and thus bagged and handled through the channels of wholesale commodities distribution. Besides national Mexican production, samples included grains imported mainly from the

USA. Rice purchased at USA supermarkets was also tested. Samples purchased in both countries included precooked grains as well as natural whole-grain brown rice, medium-grain and long-grain rice varieties. As indicated by package labels, besides the USA and Mexico, other countries of origin were India, Thailand and Italy.

Real-time qPCR was chosen in order to screen for the presence of GM material. Following the *Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing* from the European Network of GMO Laboratories, to define an LOD the presence of the transgenic material should be detected at least 95% of the time, and so false negative results can only be found in ≤5% of the cases. In this work, an LOD of 0.005% was achieved for the amount of sample size processed (approximately 400–550 g). This was verified experimentally for *P35S::bar* and LL601, where 24 out of 24 PCR reactions were found to give positive results at concentrations of both 0.005% and 0.01% GMO. The LOD can be deduced by considering the copy numbers of the target gene. The C value for *Oryza sativa* is 0.439 [24], therefore the number of genome copies in a solution containing 200 ng of DNA is 4.56×10^5. This results in 23 copies at 0.005% with 200 ng DNA per reaction. It has been reported that about ten copies are needed for reliable detection [25, 26]. The Community Reference Laboratory from the European Union recommends that the LOD should be 1/20th and the LOQ less than 1/10th of the value of the target concentration [27]. In the case of an adventitious presence in Europe, labeling is required for a GMO concentration equal or higher to 0.9% [14]. Therefore, for trade purposes with that region, the suggested LOD value would be ~0.045% GMO and LOQ ~0.09% GMO. Thus, the limits found experimentally in this work are below these suggested values. This is important considering that the target GM material should not be present.

Screening for *CaMV35S* promoter

The first screening was performed using primers and probe for *CaMV35S* promoter, which allowed the detection of transgenic positive samples. No cross-contamination with cauliflower mosaic virus was found, as demonstrated by-end point PCR with primers specific for CaMV (data not shown). The frequency of positive samples was higher in those purchased in Mexico (Table 1) than in samples from US supermarkets (Table 2). Samples purchased in either country but produced in Asia or Europe were GM-negative.

Of the samples purchased in Mexico, 18 out of 37 were positive, and among those positive samples, four presented values that were equal to or higher than the LOQ (≥0.05% GMO). Of the 35S promoter (*35SP*)-positive samples, 15 were American imports, whereas three were declared as being of Mexican origin. Twelve out of the 34 samples purchased in the USA were detected positive for the *CaMV35S* promoter. None of the samples from the USA were above the LOQ. The positive samples from this screening were considered for more detailed analysis.

Table 1 Real-time PCR results for the detection of 35S Promoter in rice sampled in Mexico

Sample ID	Grown/produced in [a]	*35SP* real-time PCR [b]
MX 1	Mexico	N
MX 2	USA	D
MX 3	Mexico	D
MX 4	Mexico	N
MX 5	Mexico	N
MX 6	Mexico	D
MX 7	USA	D
MX 8	USA	D
MX 9	USA	N
MX 10	USA	D
MX 11	Mexico	N
MX 12	Mexico	N
MX 13	USA	D
MX 14	USA	0.05%
MX 15	USA	D
MX 16	Mexico	N
MX 17	Mexico	N
MX 18	USA	D
MX 19	USA	D
MX 20	Mexico	N
MX 21	USA	N
MX 22	USA	N
MX 23	USA	0.16%
MX 24	USA	N
MX 25	USA	N
MX 26	Mexico	N
MX 27	USA	0.08%
MX 28	USA	N
MX 29	USA	N
MX 30	USA	N
MX 31	Mexico	N
MX 32	Italy	N
MX 33	USA	D
MX 34	USA	D
MX 35	USA	D
MX 36	Mexico	D
MX 37	USA	0.06%

[a] Grown or produced as labeled on package.
[b] Target gene analyzed with reference to LL62 rice: *N*, not detected: absence of signal or average less than 0.005% GMO; *D*, detected: average GMO value 0.005–0.045%

Screening for GM rice varieties

The *35SP*-positive samples were then analyzed for the presence of Liberty Link® and Bt63 rice. For Liberty Link® rice, both LLRICE601 and LLRICE62, the *P35S::bar* was used, whereas a specific target gene was utilized for LL601,

Table 2 Real-time PCR results for the detection of 35S Promoter in rice sampled in the USA

Sample ID	Grown/produced in [a]	*35SP* real-time PCR [b]
USA 1	USA	N
USA 2	USA	D
USA 3	USA	N
USA 4	USA	D
USA 5	USA	N
USA 6	USA	D
USA 7	USA	D
USA 8	USA	N
USA 9	USA	N
USA 10	USA	N
USA 11	USA	N
USA 12	USA	N
USA 13	Thailand	N
USA 14	Thailand	N
USA 15	Thailand	N
USA 16	USA	N
USA 17	USA	N
USA 18	USA	D
USA 19	USA	N
USA 20	USA	N
USA 21	Thailand	N
USA 22	USA	N
USA 23	India	N
USA 24	USA	N
USA 25	USA	D
USA 26	USA	N
USA 27	USA	N
USA 28	USA	N
USA 29	USA	D
USA 30	USA	D
USA 31	USA	D
USA 32	USA	D
USA 33	USA	D
USA 34	USA	D

[a] Grown or produced as labeled on package.
[b] Target gene analyzed with reference to LL62 rice: *N*, not detected: absence of signal or average less than 0.005% GMO; *D*, detected: average GMO value 0.005–0.045%

according to Bayer CropScience methods [20, 22]. From Mexican samples, 16 out of 37 were positive for LL601, targeting either *P35S::bar* or the event-specific construct (Table 3). Samples MX27 and MX34 analyzed with *P35S::bar* were Liberty Link® rice-positive just at or above the LOQ, with levels of 0.07% and 0.05% GMO. Quantitative values for the target genes *35SP*, *P35S::bar* and the event-specific LL601 were 0.08% (±0.0182), 0.07% (±0.0245) and 0.06% (±0.0064) for MX27 and 0.04% (±0.0216), 0.05% (±0.0021) and 0.04% (±0.0085) for MX34 (standard deviation, SD, in brackets). LL601 was the only GM event found in these two samples.

The relative standard deviations RSD_r obtained by dividing the standard deviation by the average value were 0.23, 0.35, and 0.10 for MX 27 and 0.54, 0.04, and 0.21 for MX34 for 35S promoter, *P35S::bar* and LL601 target genes respectively (data for calculations in Table 3). With only one exception, these RSD_r values were lower than the one reported for a GMO content as low as 0.1% GMO (RSD_r of 0.40) [28]. The European regulation as implemented by the Community Reference Laboratory for GM Food and Feed (CRL) accepts an RSD_r of up to 0.50 for concentrations at and below 0.2% GMO with an intermediate level of reproducibility [27]. Of additional interest is the fact that both of the samples MX27 and MX34 were precooked grains, implying that they had several heat treatments with a concomitant starch gelatinization process. This might interfere with the efficiency of DNA extraction, increasing the possibility of erroneous results. However, the DNA extraction method used rendered good amplification yields in qPCR.

In accordance with generally accepted international practice, it is recommended that quantitative results should be expressed together with the expanded uncertainty (*U*), using a coverage factor *k* of two. This gives a coverage probability of about 95% assuming a normal distribution, and means that there is an ~95% confidence level that the value measured is within the limits of ±*U* of the average value. The expanded uncertainty *U* is calculated as $U = k \times SD$ [29, 30]. For example, for the *P35S::bar* results, *U* is 0.049% for MX27, which means that 95% of GMO measurements can be expected in the range of 0.021% to 0.119% GMO, which is above the LOQ. Likewise, *U* is 0.004% for MX34, which means that 95% of data can be expected in the range of 0.046% to 0.054% GMO at the LOQ. Thus, the results obtained can be considered to be a fair representation of the GMO content in each sample. This is reflected in the values for all three target genes *35SP*, *P35S::bar* and LL601.

From the 34 samples purchased in the USA, nine showed positive for *P35S::bar*, and in six out of the 34, LLRICE601 was detected above 0.005% GMO in qPCR. None of the samples were within the quantifiable range. This is in accordance with results for *35SP* and *P35S::bar* as well as with end-point PCR, except for the samples labeled USA4 and USA33, where amplification signals for LL601 were detected but concentrations were below 0.005% GMO (Table 4).

LL601 and LL62 can be discriminated by the disparity in length of their resulting end-point PCR amplicons for *P35S::bar*. The amplicon lengths were ~150 bp and ~110 bp for LL601 and LL62, respectively [31]. Thus, it was possible to detect trace amounts of LL62 (≥0.005% and <0.045%) in one sample purchased in Mexico, MX37 (Fig. 1a, Table 3) and in two samples purchased in American supermarkets, USA31 and USA34, (Fig. 1a, Table 4). The presence of LL62 was verified by testing for the 35S terminator (*35ST*) sequence that is present in LL62 but not LL601 (Fig. 1b) [32].

Table 3 Real-time qPCR and conventional PCR results for various GM-specific and species-specific target genes in rice sampled in Mexico

Sample ID	Grown/ produced in [a]	*35SP* qPCR	*P35S::bar* qPCR	LL601 qPCR	*P35S::bar* PCR [b]	*35ST* end-point PCR	Bt63 end-point PCR	Corn species qPCR	Soy species qPCR [c]	Roundup Ready qPCR
MX 2	USA	D	D	D	LL601	N	N	N	N	N
MX 3	Mexico	D	D	D	LL601	N	N	N	N	N
MX 6	Mexico	D	D	D	LL601	N	N	N	D	N
MX 7	USA	D	D	D	LL601	N	N	N	N	N
MX 8	USA	D	D	D	LL601	N	N	N	D	D
MX 10	USA	D	D	D	LL601	N	N	N	D	D
MX 13	USA	D	D	D	LL601	N	N	N	D	N
MX 14	USA	0.05% (0.0086)	D	D	LL601	N	N	N	0.06% (0.0276)	D
MX 15	USA	D	D	D	LL601	N	N	N	N	N
MX 18	USA	D	D	D	LL601	N	N	N	D	N
MX 19	USA	D	D	D	LL601	N	N	N	N	N
MX 22	USA	N	N	N	—	—	—	—	D	N
MX 23	USA	0.16% (0.0085)	D	D	LL601	N	N	N	0.42% (0.0007)	D
MX 24	USA	n	N	N	—	—	—	N	0.09% (0.0170)	N
MX 27	USA	0.08% (0.0182)	0.07% (0.0245)	0.06% (0.0064)	LL601	N	N	N	D	N
MX 28	USA	N	N	N	—	—	—	—	D	N
MX 31	Mexico	N	N	N	—	N	N	N	D	N
MX 33	USA	D	D	D	LL601	N	N	N	N	N
MX 34	USA	0.04% (0.0216)	0.05% (0.0021)	0.04% (0.0085)	LL601	N	N	N	N	N
MX 35	USA	D	D	D	LL601	N	N	N	D	N
MX 36	Mexico	D	N	N	N	N	N	N	D	D
MX 37	USA	0.06% (0.0146)	N	N	LL62	P	N	N	0.11% (0.0035)	D

[a] Grown or produced as labeled on package.
[b] The presence of LL601 or LL62 is indicated as LL601 or LL62, with the difference confirmed by amplicon length (end-point PCR).
[c] Soy-specific target gene values are in relation to a soy reference of 200 ng sample DNA per reaction.
—, Not done; *N*, not detected: absence of signal or average less than 0.005% GMO; *D*, detected: average GMO value between 0.005% and less than 0.045%; *P*, positively detected for conventional PCR; value (SD) for results at/above the limit of quantification of 0.05%.
References were LL62 rice for 35S Promoter, 35S Terminator; LL601 rice for 35S::bar and LL601 target gene; LL62 and LL601 for 35S::bar end point PCR; Bt63 for Bt63 target gene; corn for corn species target gene; Roundup Ready soy for soy species and Roundup Ready target genes.

Detection of LL601 is of interest due to its international regulatory status. LL62 and LL06 are the only Liberty Link® deregulated varieties which have gone through safety evaluations and have been considered safe for use in food and the environment, but none of these lines were ever commercialized [9, 12]. The regulated line "Cocodrie LLRICE601" was field-tested between 1999 and 2001 under a Bayer CropScience contract. Authorization for LLRICE601 was not submitted until late 2006 in the USA; it was granted approval for environmental release given an extension of the nonregulated status issued for the rice lines LLRICE06 and LLRICE62 [32, 36]. No GM rice authorization has been issued in Mexico, except for LLRICE62 in 2007. Given that none of these three varieties were cultivated at a commercial level, they are not expected to be present in samples of conventional rice in the market.

Furthermore, in a statement dated March 5, 2007, APHIS indicated the presence of trace levels of a genetic material not yet approved for commercialization in a long-grain rice seed known as Clearfield 131 (CL131) [33]. Results regarding GM presence in CL131 were confirmed and further distribution or planting of this seed has been stopped. CL131 rice seed saved from prior crop years cannot be further distributed or planted. The USDA investigation in 2007 indicated the possibility that a variety containing LLRICE62 and another LLRICE variety now identified as LLRICE604 may have mixed with CL131 [33]. Experimental unconfined release into the environment of GM crop varieties in countries that are seed producers and exporters of commodity crops created the potential for gene flow.

Recently, Akiyama et al. detected the presence of Bt rice in vermicelli products [18] by identifying a construct similar

Table 4 Real-time qPCR and conventional PCR results for various GM-specific and species-specific target genes of rice sampled in the USA

Sample ID	Grown/ produced in [a]	*35SP* qPCR	*P35S::bar* qPCR	LL601 qPCR	*P35S::bar* PCR [b]	*35ST* end-point PCR	Bt63 end-point PCR	Corn species qPCR	Soy species qPCR [c]	Roundup Ready qPCR
USA 2	USA	D	D	D	LL601	N	N	N	N	N
USA 4	USA	D	D	N	LL601	N	N	N	D	D
USA 6	USA	D	D	D	LL601	N	N	N	N	N
USA 7	USA	D	D	D	LL601	N	N	N	N	N
USA 9	USA	N	N	N	—	—	—	—	D	N
USA 18	USA	D	D	D	LL601	N	N	N	N	N
USA 24	USA	N	N	N	—	—	—	—	0.23% (0.0566)	N
USA 25	USA	D	D	D	LL601	N	N	N	D	N
USA 30	USA	D	D	D	LL601	N	N	N	N	N
USA 31	USA	D	D	N	LL62	P	N	N	D	N
USA 32	USA	D	N	N	N	N	N	N	D	D
USA 33	USA	D	D	N	LL601	N	N	N	N	N
USA 34	USA	D	N	N	LL62	P	N	N	N	N

[a] Grown or produced as labeled on package.
[b] The presence of LL601 or LL62 is indicated as LL601 or LL62, with the difference confirmed by amplicon length (end-point PCR).
[c] Soy-specific target gene values are in relation to a soy reference of 200 ng sample DNA per reaction.
—, Not done; *N*, not detected: absence of signal or average less than 0.005% GMO; *D*, detected: average GMO value between 0.005% and less than 0.045%; *P*, positively detected for conventional PCR; value (SD) for results at/above the limit of quantification of 0.05%.
References were LL62 rice for 35S Promoter, 35S Terminator; LL601 rice for 35S::bar and LL601 target gene; LL62 and LL601 for 35S::bar end-point PCR; Bt63 for Bt63 target gene; corn for corn species target gene; Roundup Ready soy for soy species and Roundup Ready target genes.

to the Shanyou 63 (Bt63) rice line, an insect-resistant variety developed and field-tested in China in 1999 [34]. This variety has not been authorized for environmental release or food use in the European Union or other countries, including Mexico [18, 35]. In this work, all samples were screened for Bt63 rice targeting *Bacillus thuringiensis* (Bt) constructs, such as the junction between the gene *cry1Ab/cry1Ac* coding sequence and the nopaline synthase terminator (*nos*) [18]. None of the samples yielded a positive result for Bt63 (Tables 3 and 4, and Fig. 1c).

Detection of other species and GM material

Several of the samples tested in this work had significantly higher *35SP* target gene values than *P35S::bar* or LL601 target gene values, even considering that LLRICE601 has two copies of the *CaMV35S* promoter [36]. In particular, sample MX37 showed that *35SP* was quantified (0.06%± 0.0146), but no LLRICE601 was found. MX14 showed a higher amount of *35SP* (0.05%±0.0086) than LL601 (<0.045% GMO) (Table 3). The *CaMV35S* promoter can be found in other commercially approved GM species and commodities such as soybean and corn. Therefore, all samples were tested for the presence of maize or soybean by targeting species-specific sequences. Maize DNA was not detected in any of the samples (Tables 3 and 4). Out of the 37 rice samples purchased in Mexico, 15 showed positive for soybean, with amounts between 0.06 and 0.42% of total DNA. Twelve of the 15 samples were imports from the USA. Nine of the 15 were non-GM, whereas in six the Roundup Ready® (RR) trait was detected. The highest soybean contents found were 0.42% (±0.0007) and 0.11% (±0.0035) for MX23 and MX37, respectively. Of all of the samples tested from both countries, MX23 had the highest soybean content. Out of the 34 rice samples from the USA (Table 4), six showed positive for soybean but only two were RR™ positive in trace amounts. In Fig. 2, the frequencies of the GM material present in samples are shown.

The presence of soy is not explained by soybean grains commingled with rice grains. This would be visually noticeable in rice packages. However, through the commodity chain from the previous processing of soybean, powdered residues appear to have remained in grain storage, handling facilities and equipment, as detected in our assays. The rice production chain, similar to any other commodity chain, is a complicated multistage process from plantlets in nurseries to rice paddy, which in turn is husked to produce brown rice. Mills eliminate germ and other layers in order to render white grains that are sieved and bagged. This rice is transported and distributed along the commercial chain, sold to intermediaries and exported. In the importing country, rice is then bagged into smaller portions for retail. During this lengthy process there is a strong possibility that transporting and processing equipment that is not cleaned between loads will be used. The

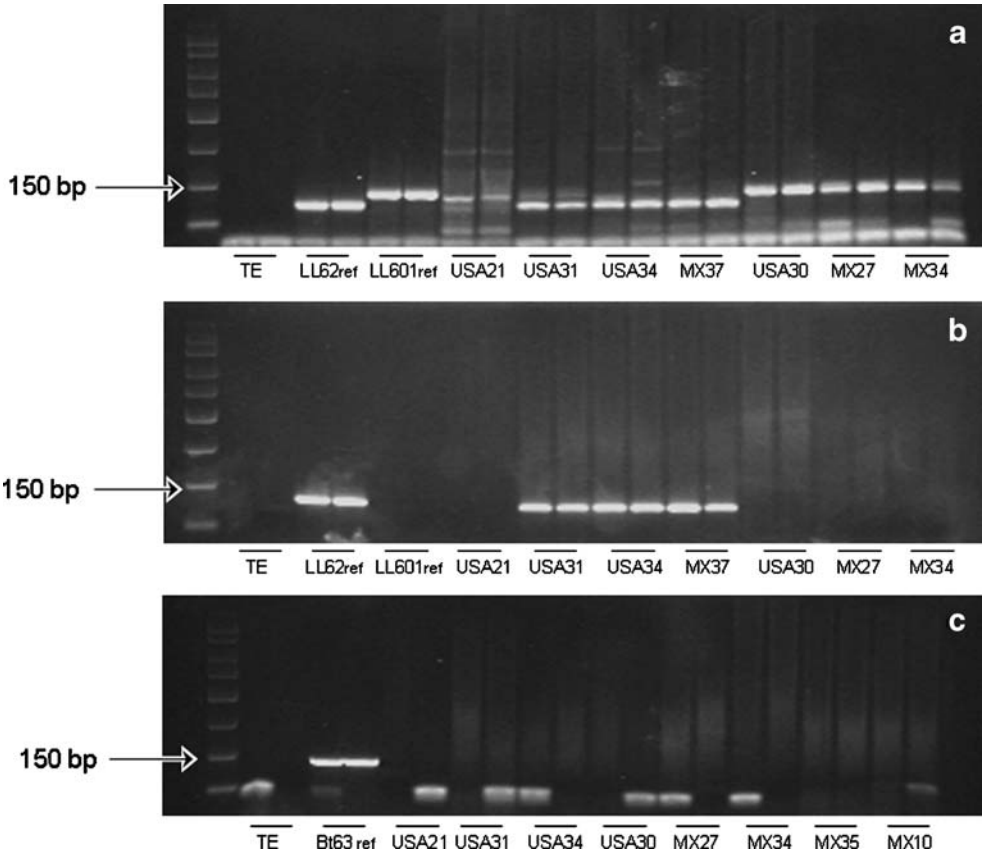

Fig. 1a–c Conventional end-point PCR gel electrophoresis results for 35S::bar (**a**), 35ST (**b**), and Bt63 (**c**)-specific primer sets. PCR reactions for the (**a**) and (**b**) lanes, *from left to right*, were loaded with 1X TE, LL62 reference, LL601 reference, samples USA21, USA31, USA34, MX37, USA30, MX27, and MX34. PCR reactions for lanes in (**c**), *from left to right*, were loaded with 1X TE, Bt63 reference, USA21, USA31, USA34, USA30, MX27, MX34, MX35, and MX10. The double-stranded DNA ladder indicates the following fragment sizes: 50, 150, 300, 500, 750, 1000, 1500, 2000 base pairs

chance of finding material from other rice varieties as well as other commodities such as canola, maize or soybeans is high. Ninety-four percent of soybean trade in the USA is GM [37], but the amount of GM soybean found in this study is not high enough to trigger the labeling system in the EU. This is an example of an adventitious presence that in turn is defined as the unintended, technically unavoidable presence of genetically engineered material in an agri-food commodity [38]. This can occur throughout the commodity chain, from seed production to blending of ingredients for processed foods or feeds. The amount of soy (a potential allergen) quantified was, e.g., 0.42% GMO for a Mexican sample, corresponding to 45 mg of soy protein per portion of cooked rice, or ten times less the amount required to trigger an allergic reaction [39].

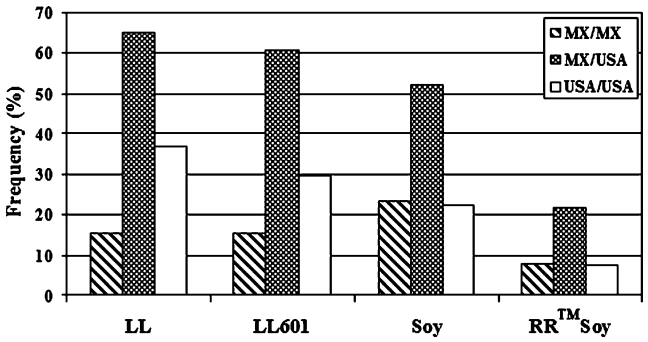

Fig. 2 Frequencies of the presence of GM rice: Liberty Link (LL) or LLRICE 601 (LL601), soy and GM soy; Roundup Ready (RR™), in samples collected in Mexico or USA. Samples were collected/grown in: Mexico/Mexico (MX/MX), Mexico/USA (MX/USA), and USA/USA, respectively

Conclusions

Rice samples were analyzed for the presence of GMO and other unrelated species using real-time PCR. LL601 was found in 43% of Mexican and in 27% of US retail rice samples. The presence of LL601 in commercial rice cannot be considered an adventitious presence, because it is not an approved transformation event, it was not commercialized, and therefore it is not possible that the source of this presence could be due to a technically unavoidable admixture of grains. Interestingly, LL62 or an unknown Liberty Link® rice of similar construct was detected at trace levels in one Mexican and two USA retail rice samples. This is the first time that such an event has been detected and reported. This indicates the contamination of rice with a noncommercialized rice event other than LL601. Soy, both non-GM and RR™ soy, but not maize, was detected in 41% of Mexican and 18% of USA retail rice samples. This is again the first report of soy contamination in rice

samples, and it is of interest for the tracing of potential allergens. Using the general strategy presented in this work, it was possible to discriminate among different probable combinations of sources of GM and non-GM sequences in the commodity chain. Real-time PCR together with an efficient DNA extraction proved to be a powerful tool for growers and exporters, as well as importers, retailers and customs laboratories from both trade partners, for the screening of rice commodities in order to fulfill national and international regulations in trade. Other methodologies such as multiplex PCR would save costs and allow high throughput. Careful validation is necessary to ensure the sensitivity required for routine analysis. Considering the quantity and the variety of genetically modified organisms in the commodity grains that Mexico trades with the USA on a daily basis, new molecular approaches, such as oligonucleotide microarrays, could be considered for screening purposes and require further development.

References

1. United States Department of Agriculture, Foreign Agricultural Service (2008) World rice production, consumption, and stocks. Available at: http://www.fas.usda.gov/psdonline/, last accessed 8 July 2008
2. United States Department of Agriculture, Foreign Agricultural Service (2008) Circular Series FG 02–08. Grain: world markets and trade. Available at: http://www.fas.usda.gov/grain/circular/2008/02-08/grainfull02-08.pdf, last accessed 8 July 2008
3. Childs N (2007) Electronic outlook report from the Economic Research Service: Rice situation and outlook yearbook (RCS-2007). United States Department of Agriculture, Washington, DC
4. Mexican Congress (2001) Meeting minutes: Importación de Arroz. http://www.diputados.gob.mx/buenfil/20sep01.htm, last accessed 8 July 2008
5. SAGARPA, Subsecretaría de Agricultura (2007) Mexican Ministry of Agriculture. http://www.siap.sagarpa.gob.mx, last accessed 8 July 2008
6. United States Department of Agriculture, Foreign Agricultural Service (2007) USA trade exports: FATUS commodity aggregations. http://www.fas.usda.gov/ustrade/USTExFatus.asp?QI=, last accessed 8 July 2008
7. United States Department of Agriculture, Foreign Agricultural Service (2007) Outstanding export sales and exports by country, region and marketing year: long grain rice. http://www.fas.usda.gov/export-sales/rice.htm, last accessed 8 July 2008
8. AGBIOS (2008) GMO crop database. AGBIOS, Merrickville, Canada. http://www.agbios.com/dbase.php, last accessed 8 July 2008
9. Convention on Biological Diversity (2008) Biosafety clearing-house (BCH): Cartagena Protocol on Biosafety. Convention on Biological Diversity. Montreal, Canada. http://bch.cbd.int/database/organisms/uniqueidentifiers/, last accessed 8 July 2008
10. Thompson CJ, Movva NR, Tizard R, Crameri R, Davies JE, Lauwereys M, Botterman J (1987) EMBO J 6:2519–2523
11. Van Wert S (1998) Petition for the determination of nonregulated status: LibertyLink rice transformation events LLRICE06 and LLRICE601 (AgrEvo submission to APHIS, USDA). http://www.agbios.com/docroot/decdocs/05-242-038.pdf, last accessed 8 July 2008
12. United States Department of Agriculture (2006) Release No. 0307.06. Statement by Agriculture Secretary Mike Johanns regarding genetically engineered rice. Available at: http://www.usda.gov/wps/portal/, last accessed 8 July 2008
13. United States Food and Drug Administration, CFSAN/Office of Food Additive Safety (2006) US Food and Drug Administration's statement on report of bioengineered rice in the food supply. http://www.cfsan.fda.gov/~lrd/biorice.html, last accessed 8 July 2008
14. European Commission, Directorate on General, Health and Consumer Protection (2007) Legal notice: biotechnology exemption. http://ec.europa.eu/food/food/biotechnology/exemption/index_en.print.htm, last accessed 8 July 2008
15. European Commission (2007) List of the genetically modified material which has benefited from a favourable risk evaluation within the meaning of Article 47 of Regulation (EC) No 1829/2003, in accordance with Article 18 of Regulation (EC) No 641/2004. http://ec.europa.eu/food/food/biotechnology/gmfood/events_en.pdf, last accessed 8 July 2008
16. European Commission, Directorate on General, Health and Consumer Protection (2007) Community register of genetically modified food and feed. http://ec.europa.eu/food/dyna/gm_register/index_en.cfm, last accessed 8 July 2008
17. SALUD, Comisión Federal para la Protección contra Riesgos Sanitarios (2008) Inocuidad de productos biotecnológicos: nueva tabla_internet2.pdf. http://www.cofepris.gob.mx/inf/pdf/biotecnologicos.zip, last accessed 8 July 2008
18. Akiyama H, Sasaki N, Sakata K, Ohmori K, Toyota A, Kikuchi Y, Watanabe T, Furui S, Kitta K, Maitani T (2007) J Agric Food Chem 55:5942–5947
19. Brodmann PD, Ilg ED, Berthoud H, Herrmann A (2002) J AOAC Int 85:646–653
20. Bayer CropScience (2006) Grain testing method for detection of rice GM events containing *P35S::bar* sequences using RT-PCR protocols PGS0494 and PGS0476. http://gmo-crl.jrc.it/doc/P35S-bar%20sqRT-PCR%20-%20PGS0494-476%20310806.pdf, last accessed 8 July 2008
21. International Organization for Standardization (2003) International Standard (ISO/DIS) 21570 methods of analysis for the detection of genetically modified organisms and derived products: Quantitative nucleic acid based methods. ISO, Geneva, Switzerland
22. Bayer Cropscience (2006) Grain testing method for detection of rice GM event LLRICE601 using RT-PCR protocols PGS0505 and PGS0476. http://gmo-crl.jrc.it/doc/LLRICE601%20sqRT-PCR%20-%20PGS0505-0476%20300806.pdf, last accessed 8 July 2008
23. Hird H, Powell J, Johnson ML, Oehlschlager S (2003) J AOAC Int 86:66–71
24. Munich Information Center for Protein Sequence (2008) The *Oryza sativa* database (MOsDB). Munich Information Center for Protein Sequence, Munich, Germany. http://mips.gsf.de/proj/plant/jsf/rice/index.jsp, last accessed 8 July 2008
25. Huebner P, Waiblinger HU, Pietsch K, Brodmann P (2001) J AOAC Int 84:1855–1864
26. Kay S, Van den Eede G (2001) Nature Biotech 19:405
27. European Network of GMO Laboratories (2005) Definition of minimum performance requirements for analytical methods of GMO testing. http://gmo-crl.jrc.it/doc/Method%20requirements.pdf, last accessed 8 July 2008
28. Feinberg M, Fernandez S, Cassard S, Charles-Delobel C, Bertheau Y (2005) J AOAC Int 8:558–573
29. United Kingdom Accreditation Service (2007) The expression of uncertainty and confidence in measurement (M3003), 2nd edn. United Kingdom Accreditation Service, Feltham, UK. http://www.ukas.com/Library/downloads/publications/M3003.pdf, last accessed 8 July 2008
30. Ellison SLR, Rosslein M, Williams A (2000) EURACHEM/CITAC guide number 4: Quantifying uncertainty in analytical measurement, 2nd edn. LGC, Teddington, UK. http://www.eurachem.org/guides/QUAM2000-1.pdf, last accessed 8 July 2008

31. European Commission, Directorate General, Joint Research Centre, Institute for Health and Consumer Protection (2007) Report on the verification of a construct-specific detection method for identification of rice GM-events containing P35S::bar using a real-time PCR assay. http://gmo-crl.jrc.it/doc/Verification%20Report%2035S-BAR%20CRL.pdf, last accessed 8 July 2008
32. AGBIOS (2008) GMO crop database: search results for LLRICE601. AGBIOS, Merrickville, Canada. Available at: http://www.agbios.com/dbase.php, last accessed 8 July 2008
33. United States Department of Agriculture Statement (2007) Report of Liberty Link rice incidents. http://www.aphis.usda.gov/newsroom/content/2007/10/content/printable/RiceReport10-2007.pdf, last accessed 8 July 2008
34. Tu J, Zhang G, Datta K, Xu C, He Y, Sangh Q, Singh Khush G, Kumar Datta S (2000) Nature Biotech 18:1101–1104
35. Mäde D, Degner C, Grohman L (2006) Eur Food Res Technol 224:271–278
36. United States Department of Agriculture, Animal and Plant Health Inspection Service (2006) Finding of no significant impact: Extension of the nonregulated status to rice line LLRICE601; Response to comments APHIS No. 06-234-01p. http://www.agbios.com/docroot/decdocs/06-332-005.pdf, last accessed 8 July 2008
37. GMO Compass (2008) Genetically modified plants: global cultivation area: soybean. http://www.gmo-compass.org/eng/agri_biotechnology/gmo_planting/342.genetically_modified_soybean_global_area_under_cultivation.html, last accessed 8 July 2008
38. Agriculture and Agri-Food Canada, Canadian Food Inspection Agency, Canadian Grain Commission (2004) Industry and government perspectives on adventitious presence of products of genetic engineering in seeds, grains, oilseeds and special crops. http://www.agr.gc.ca/itpd-dpci/topics/paper_e.pdf, last accessed 8 July 2008
39. Cordle CT (2003) Suppl J Nutr 1213S–1219S