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Development of a Real-Time PCR Method for the Differential Detection and Quantification of Four Solanaceae in GMO Analysis: Potato (*Solanum Tuberosum*), Tomato (*Solanum Lycopersicum*), Eggplant (*Solanum Melongena*), and Pepper (*Capsicum Annuum*)

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The labeling of products containing genetically modified organisms (GMO) is linked to their quantification since a threshold for the presence of fortuitous GMOs in food has been established. This threshold is calculated from a combination of two absolute quantification values: one for the specific GMO target and the second for an endogenous reference gene specific to the taxon. Thus, the development of reliable methods to quantify GMOs using endogenous reference genes in complex matrixes such as food and feed is needed. Plant identification can be difficult in the case of closely related taxa, which moreover are subject to introgression events. Based on the homology of β-fructosidase sequences obtained from public databases, two couples of consensus primers were designed for the detection, quantification, and differentiation of four Solanaceae: potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), and eggplant (*Solanum melongena*). Sequence variability was studied first using lines and cultivars (intraspecies sequence variability), then using taxa involved in gene introgressions, and finally, using taxonomically close taxa (interspecies sequence variability). This study allowed us to design four highly specific TaqMan-MGB probes. A duplex real time PCR assay was developed for simultaneous quantification of tomato and potato. For eggplant and pepper, only simplex real time PCR tests were developed. The results demonstrated the high specificity and sensitivity of the assays. We therefore conclude that β-fructosidase can be used as an endogenous reference gene for GMO analysis.

KEYWORDS: Real-time PCR; TaqMan; duplex; tomato; potato; pepper; eggplant; GMO; quantification; β-fructosidase; Solanaceae; introgression

INTRODUCTION

To be able to comply with the European regulation concerning the labeling and the traceability of GMO products and derived ingredients and to offer freedom of choice to consumers (*1*), the development of reliable, sensitive and accurate methods for GMO detection and quantification in food, feed and raw materials are required (*2*). For this purpose, three different categories of tests are described depending on the level of specificity needed: screening (*3*), construct-specific tests, and identification tests (*4*). Many of them have been reviewed and validated through interlaboratory studies via the Community Reference Laboratory (CRL) that was established in the context of Regulation No. (EC) 1829/2003 on GM Food and Feed (gmo-crl.jrc.it/) (*5*).

These tests for the detection and the quantification of GMOs are generally DNA-based methods. Real-time polymerase chain reaction (RT-PCR) with its different chemistries (TaqMan, Sybr Green, . . .) is the most popular technique because of the facility in performing and interpreting the assay (*6, 7*). It also allows the detection and quantification of small amounts of transgenes

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in complex matrixes such as processed food and feed samples (*8*). The percentage of GMO is calculated from a combination of two absolute quantification values: one for the specific GMO target and the second for the taxon endogenous gene. A plant endogenous reference gene must be nuclear, present in low copy number, and taxon specific and exhibit high homogeneity among cultivars (*9*).

In a recent paper (*12*), we discussed the bottlenecks faced when designing reference gene assays. Among the cited bottlenecks was the difficulty in differentiating closely related taxa and of detecting introgression events. An example of closely related taxa that require differentiation and additionally are subject to introgression events is the case of the Solanaceae. Among these are the taxonomically related (*10, 11*) taxa potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), and sweet pepper (*Capsicum annuum*). Genomic sequences have shown that the tomato and potato genomes are highly conserved (*10, 11*). This is being confirmed by international sequencing projects of many taxa of the Solanaceae family, which attempt to understand the basis of diversity and adaptation (http://www.sgn.cornell.edu/). One source of diversity is probably the occurrence of gene introgressions, whether they are naturally occurring or human directed (*16–18*).

It is known that species of *Solanum* and *Capsicum* participate in the introgression of desired traits, either with wild or indigenous (locally cultivated) taxa.

Examples include the interspecific cross between *S. lycopersicum* and the wild related taxa, *L. hirsutum* and *L. pennellii,*, to provide resistance to major insect pests identified *19–21*). Another case is the introgression of genes giving rise to resistance to the most serious eggplant diseases (bacterial and fungal wilts, and nematodes in particular) identified in *S. sisymbrifolium* Lam. and *S. torvum* (*22, 23*) (eggplant). Interspecific crossing was also described in potato (*24*) and in pepper (*18*). These introgressions occur when compatible lines are found nearby and are able to cross. Because of their sequence conservation and the possibility of introgression events, the Solanaceae present a particular challenge when developing a new endogenous reference gene assay. Most endogenous reference gene PCR assays developed to date have not examined related wild taxa that are thought to be involved in gene introgressions (based on phylogenetic studies), except for maize, whose putative ancestor, "Teosinte" has been studied (*25*). For example, in the case of the Solanaceae family, many reference gene detection systems have been reported (e.g., UGPase (uridine diphosphate (UDP)–glucose pyrophosphorylase) (*26*), PCI (metallo-carboxypeptidase inhibitor) for potato (*27*), and LAT52 (*28*) for tomato) without taking into consideration that these plants are subject to introgression and without determining inter- and intraspecies sequence variability. Moreover, no endogenous reference genes have been reported for eggplant and pepper, two taxa that will be subjected to genetic modifications in the near future (*29*).

Due to the high number of taxa to be detected and the large number of samples to be analyzed, cost and time effectiveness are major concerns in routine analysis. The use of multiplexed systems partially addresses this concern. Multiplexed conventional PCR systems that are able to detect up to nine GMO targets in a single reaction assay have been described (*30, 31*); however, no such system has been reported for the simultaneous detection and quantification of tomato and potato. The only duplex RT-PCR method that has been reported for the quanti-

Table 1. Potato and Tomato Lines and Cultivars Used in This Study and Covering the Geographic and Genetic Diversity of the Taxa

					Tomato				
S. lycopersicum	*S. lycopersicum*	*S. cerasiforme*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. pimpinellifolium*	*S. habrochaites*
acc. 38	acc. 46	acc. 78	var. *marmande*	var. *saint pierre*	var. *cigalou*	var. *corbarino*	var. *nagcarlan*	acc. 65	
S. lycopersicum	*S. lycopersicum*	*S. cerasiforme*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. pimpinellifolium*	*S. parviflorum*
acc. 39	acc. 47	acc. 79	var. *mécline*	var. *daniéla*	var. *cerise jaune*	var. *flora dade*	var. *plantense*	acc.66	
S. lycopersicum	*S. lycopersicum*	*S. cerasiforme*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. pimpinellifolium*	*S. chilense*
acc. 40	acc. 50	acc. 80	var. *momor*	var. *marmande RV*	var. *poivron des andes*	var. *valparaiso*	var. *rio grande*	acc. 68	
S. lycopersicum	*S. lycopersicum*	*S. cerasiforme*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. pimpinellifolium*	
acc. 41	acc. 51	acc. 81	var. *naine pormor*	var. *white beauty*	var. *grosse lisse*	var. *pingaun 7*	var. *sekaïchi*	acc. 67	
S. lycopersicum	*S. lycopersicum*	*S. cerasiforme*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. pimpinellifolium*	
acc. 42	acc. 53	acc. 82	var. *noire de crimée*	var. *sweetie*	var. *microtom*	var. *Plovdivska Konserva*	var. *Montfavet*	acc. 69	
S. lycopersicum	*S. cerasiforme*	*S. cerasiforme*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. pimpinellifolium*	*S. hirsutum*	
acc. 43	acc. 48	acc. 83	var. *Ohmiya Sunchong*	var. *est*	var. *yellow pear*	var. *ailsa craig*	acc. 62		
S. lycopersicum	*S. cerasiforme*	*S. cerasiforme*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. pimpinellifolium*	*S. Pennellii*	
acc. 45	acc. 52	acc. 49	var. *purple kalabash*	var. *orange queen*	var. *grosse de gros*	var. *kaki*	acc. 63		
S. lycopersicum	*S. cerasiforme*	*S. cerasiforme*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. lycopersicum*	*S. pimpinellifolium*	*S. Peruvianum*	
acc. 44	acc. 85	acc. 84	var. *san marzano*	var. *yellow stuffer*	var. *poivron jaune*	var. *caraïbo*	acc. 64		
					Potato				
S. tuberosum	*S. tuberosum*	*S. tuberosum*	*S. tuberosum*	*S. tuberosum*	*S. tuberosum*	*S. tuberosum*	*S. tuberosum*	*S. tuberosum*	*S. tuberosum*
var. *andigena*	var. *spegazziinii*	var. *operle*	var. *ratte*	var. *charlotte*	var. *rosabel*	var. *désirée*	var. *sirtema*	var. *safrane*	var. *russet barbank*
S. tuberosum	*S. tuberosum*	*S. tuberosum*	*S. tuberosum*	*S. tuberosum*	*S. tuberosum*	*S. tuberosum*	*S. phureja*	*S. acaule*	*S. demissum*
var. *kaptah vandel*	var. *rosa*	var. *vitelotte*	var. *BF15*	var. *chac 885 301*	var. *roseval*	var. *amandine*			
S. stoloniferum	*S. spegazzinii*	*S. verenei*							

Table 2. Eggplant and Pepper Lines and Cultivars Used in This Study and Covering the Geographic and Genetic Diversity

				Eggplant					
S. aethiopicum *Kumba* Group 25	*S. aethiopicum* *Kumba* Group 26	*S. aethiopicum* *Kumba* Group 27	*S. aethiopicum* *Kumba* Group 28	*S. aethiopicum* *Kumba* Group 29	*S. aethiopicum* *Kumba* Group 30	*S. aethiopicum* *Kumba* Group 31	*S. aethiopicum* *Kumba* Group 32	*S. macrocarpon* 57	*S. macrocarpon* 58
S. macrocarpon 59	*S. macrocarpon* 60	*S. macrocarpon* 61	*S. macrocarpon* 62	*S. macrocarpon* 63	*S. macrocarpon* 64	*S. melongena* cv. 73	*S. melongena* cv. 74	*S. melongena* cv. 75	*S. melongena* cv. 76
S. melongena cv. 77	*S. melongena* cv. 78	*S. melongena* cv. 79	*S. melongena* cv. 80	*S. melongena* cv. 81	*S. melongena* cv. 82	*S. melongena* cv. 83	*S. melongena* cv. 84	*S. melongena* cv. 85	*S. melongena* cv. 86
S. melongena cv. 87	*S. melongena* cv. 88	*S. melongena* Viollette de Barbentane	*S. melongena* parent pop de carto	*S. melongena* parent pop de carto R ralsto	*S. melongena* group G	*S. melongena* Liu Yé Qué	*S. melongena* plante aux œufs (groupe G/H)	*S. melongena* group F	*S. macrocarpon* Réunionita
S. macrocarpon Grosse violette de Gagnoa	*S. macrocarpon* Djackattous	*S. macrocarpon* Birm/S.0133	*S. macrocarpon* RNL037	*S. macrocarpon*	*S. macrocarpon*	*S. macrocarpon* parent pop carto	*S. incanum* gr. D	*S. incanum* gr. C	*S. linnaeanum*
S. hastifolium	*S. cyaneo-purpureum*	*S. torvum*	*S. viarum*	*S. cinereum*	*S. sisymbriifolium*	*S. scabrum*	*S. pyracanthos*	*S. quitoense*	
				Pepper					
C. annuum acc. 018	*C. annuum* acc. 019	*C. annuum* acc. 020	*C. annuum* acc. 021	*C. annuum* acc. 022	*C. annuum* acc. 023	*C. annuum* acc. 024	*C. annuum* acc. 025	*C. baccatum* acc. 061	*C. baccatum* acc. 062
C. annuum Florida VR2	*C. annuum* Perennial	*C. annuum* Avelar	*C. annuum* Serrano Vera Cruz	*C. annuum* Chay Angolano	*C. annuum* SC81	*C. annuum* Maroc1	*C. annuum* PI322719	*C. annuum* PI195301	*C. annuum* PI201234
C. annuum LP1	*C. annuum* Flambeau	*C. annuum* CM334	*C. annuum* PI187331	*C. annuum* Cuba3	*C. annuum* Bousso1	*C. annuum* Doux Long Landes	*C. annuum* Lamu	*C. annuum* H3	*C. annuum* Turrialba (C81)
C. annuum Managua CB10	*C. annuum* Antigua (390)	*C. annuum* Chihuahua	*C. annuum* El Tajin	*C. annuum* Panama City	*C. annuum* Pitalito	*C. baccatum* acc. 063	*C. baccatum* acc. 064	*C. baccatum* acc. 065	*C. baccatum* acc. 067
C. baccatum acc. 068	*C. baccatum* acc. 069	*C. baccatum* PI 238061	*C. baccatum* PI 260533	*C. baccatum* PI 439367	*C. chacoense* acc. 077	*C. chacoense* acc. 079	*C. chacoense* acc. 080	*C. chacoense* acc. 082	*C. chacoense* acc. 083
C. chacoense acc. 085	*C. chacoense* PI 260426	*C. chacoense* PI 260437	*C. chinense* acc. 088	*C. chinense* acc. 090	*C. chinense* acc. 092	*C. chinense* acc. 093	*C. chinense* acc. 094	*C. chinense* acc. 095	*C. chinense* acc. 096
C. chinense PI260438	*C. chinense* Hmong Rouge	*C. cardenasii* acc. 72	*C. cardenasii* acc. 073	*C. cardenasii* acc. 074	*C. cardenasii* ULLU PICA	*C. cardenasii* LA PAZ	*C. cardenasii* QUILACOLLO	*C. pubescens* ROCOTO	*C. pubescens* 54P
C. eximium EXI2 (Pic8 = PM642)	*C. eximium* EXI3 (PRA1 = PM592)	*C. galapogense* 11918 pl 4	*C. microcarpum* SA 326	*C. praetermissum* EXI x PRA 20-201	*C. annuum* Yolo Y				

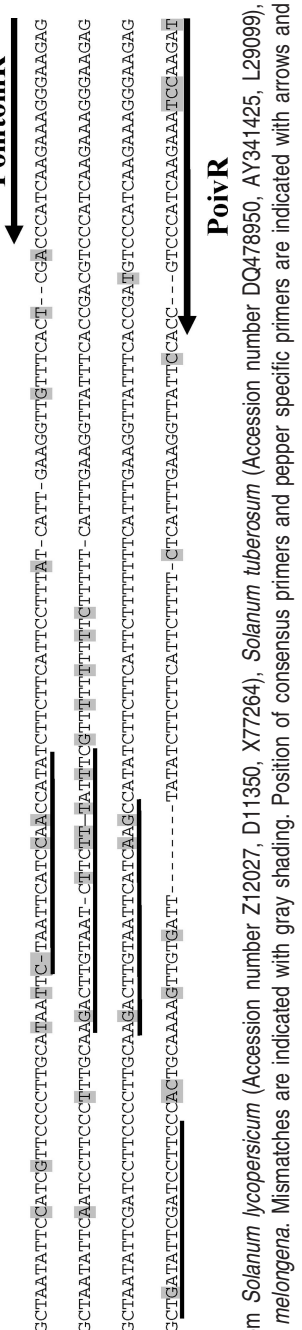

Figure 1. Sequence alignment of exon 7 of β-fructosidase from *Solanum lycopersicum* (Accession number Z12027, D11350, X77264), *Solanum tuberosum* (Accession number DQ478950, AY341425, L29099), *Capsicum annuum* (Accession number U87849), and *Solanum melongena*. Mismatches are indicated with gray shading. Position of consensus primers and pepper specific primers are indicated with arrows and probes are underlined.

fication of endogenous reference genes differentiates wheat and barley taxa (*32*)].

In the present work, we first describe an in silico analysis required before designing a specific endogenous reference gene assay, based on the selection of the gene and the fragment to be studied, here exon 7 of β-fructosidase. A second step consisted in the sequencing of the target to examine the intra- and interspecies sequence variability using lines, cultivars, and related wild taxa and also using taxa involved in the introgression of genes. The conserved region of our gene of interest was used for the design of four simplex real-time PCR reactions for the differentiation of potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), and pepper (*Capsicum annuum*). In addition, a duplex RT-PCR assay was designed to simultaneously detect and quantify potato and tomato using one pair of consensus primers and two specific probes. A representative collection of the genetic and the geographic diversity of the Solanaceae was established for the experimental study of the specificity. It included not only tomato, potato, pepper, and eggplant lines and cultivars, but also related wild plants.

MATERIAL AND METHODS

Solanaceae Material Used for Specificity Study. Fifty domesticated lines of tomato (*S. lycopercicum*), 17 lines of potato (*S. tuberosum*), 17 lines of eggplant (*S. melongena*), and 35 lines of pepper (*C. annuum*) constituted the collection of cultivated Solanaceae. Six potato wild lines (1 *S. phureja*, 1 *S. acaule*, 1 *S. demissum*, 1 *S. stoloniferum*, 1 *S. spegazzinii* and 1 *S. verenei*), 23 tomato wild lines (10 *S. cerasiforme*, 8 *S. pimpinellifolium*, 1 *S. hirsutum*, 1 *S. habrochaites*, 1 *S. pennellii*, 1 *S. parvoflorum*, and 1 *S. chilense*), 27 pepper wild lines (8 *C. baccatum*, 3 *C. cardenasii*, 6 *C. chacoense*, 7 *C. chinense*, 1 *C. galapogense*, 1 *C. microcarpum*, 1 *C. praetermissum*), and 27 wild eggplant (10 Mock tomato: 3 *S. aethiopicum* gr. *kumba*, 1 *S. aethiopicum* gr. *aculeatum*, 3 *S. aethiopicum* gr. *shum*, and 3 *S. aethiopicum* gr. *gilo*), 5 *S. macrocarpon*, 2 *S. incanum*, 1 *S. linnaeanum*, 1 *S. hastifolium*, 1 *S. cyaneo-purpureum*, 1 *S. torvum*, 1 *S. viarum*, 1 *S. cinereum*, 1 *S. sisymbriifoilium*, 1 *S. scabrum*, 1 *S. pyracanthos*, and 1 *S. quitoense*) were used for the specificity assessment.

Also, three GM tomato events, NCIMB 40015, NCIMB 40134, and Tg7RF, were used. The Solanaceae samples used in this study are listed in **Tables 1** and **2**. Other taxa were used for the experimental assessment of the specificity and are listed in Table 1 of the Supporting Information.

All the samples were provided as DNA or leaves from different INRA groups (Laboratoire de Génétique et d'Amélioration des Fruits et Légumes, INRA; Domaine St. Maurice, Avignon; Amélioration des Plantes et Biotechnologies Végétales, INRA; Agrocampus Rheu and Laboratoire d'Amélioration et de Santé des Plantes, INRA; Clermont Ferrand).

Isolation and Quantification of Plant Genomic DNA. The isolation of potato and tomato plant DNA was carried out by the cetyltrimethylammonium bromide (CTAB) protocol described in EN ISO 21571 (*33*). A rapid method (*34*) was used for the extraction of pepper and eggplant DNA. All the DNA samples were quantified using the Nanodrop (ND-1000 spectrophotometer, Chatsworth, CA) quantification method (*12*). The quality of the DNA and the PCR reactions was assessed using universal primers (*35*) and electrophoresis (*12*).

Primer Design and DNA Sequencing. All the primers used in this paper were designed using the alignment (**Figure 1**) of exon 7 of β-fructosidase (Accession number: Z12027, U87849, and AY341425) and are presented in **Table 3**. One pair of consensus primers was used for the amplification of tomato, potato, and eggplant DNA. The pepper test was designed using the same forward primer but with a different reverse primer. Primers for the extension reaction were determined using OLIGO v. 6.0 and were purchased from MWG-Biotech AG (Ebensburg, Germany). PCR products were purified using P100 (BIORAD Bio-Gel R P-100 Gel Fine 45–90 μm). The sequencing reaction was performed in a 10 μL reaction containing 10 nmol/L one primer

Table 3. Primers and Probes Used in This Study

primers	name	sequence 5′–3′	amplicon size	ref
Tomcpr	probe	FAM-ACTTGTAATCTTCTTTATTTCGT- MGB	143 bp (tomato)	this study
Pomcpr	probe	VIC-ACTTGTAATTCATCAAGCCAT -MGB	146 bp (potato)	
Poivcpr	probe	VIC-GATATTCGATCCTTCCCA-MGB	134 bp (pepper)	
Aubpr	probe	FAM-TAATTCATCCAACCATATCT-MGB	141 bp (eggplant)	
PomtomF	sense	CTGCCTCCGTCAAGATTTGGTCACT		
PomtomR	antisense	CTCTTCCCTTTCTTGATGG		
PoivR	antisence	ATCTTGGATTTCTTGATGGGACGGT		
Lat1	sense	AGACCACGAGAACGATATTTGC		(*28*)
Lat2	antisense	TTCTTGCCTTTTCATATCCAGACA	92 bp	
Lp	probe	HEX-CTCTTTGCAGTCCTCCCTTGGGCT-TAMRA		
PATF	sense	TGACAATTCATTCTACTCCACGAAA		(*27*)
PATR	antisense	TGTTACAAATTGGATCTGCGTGTT	104 bp	
PATP	probe	JOE-TGTTCTACCAACGGTGACGAAACTTTTTCA-TAMRA		

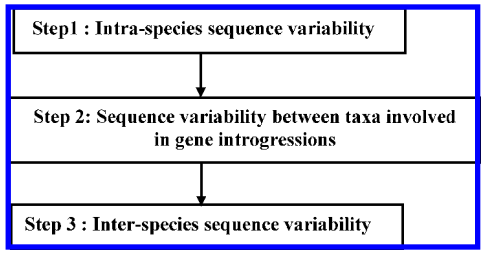

Figure 2. Description of three experimental steps required before designing an endogenous reference gene assay. These steps were carried out through the sequencing of the targeted sequence. Step 1: sequence variability is checked using a collection of cultivars and lines of the taxa. Step 2: sequence variability is checked using related wild taxa involved in gene introgressions. Step 3: sequence variability is checked using taxonomically close taxa. The variability observed using the three cited steps allows the design of specific endogenous reference gene assays.

Table 4. Description of the Variability Observed in Exon 7 of β-Fructosidase Using the Four Solananceae Taxa Studied

taxa	no. of SNPs	no. of indels
Reference Sequence: *Solanum lycopersicum* (Accession number Z12027)		
S. habrochaites	3	2
S. hirsutum	3	2
Reference Sequence: *Solanum tuberosum* (Accession number DQ478950)		
S. demissum	2	–
S. tuberosum var. rosabel	1	–
S. stoloniferum	5	–
S. spegazzinni	4	–
S. acaule	4	–
Reference Sequence:*Solanum melongena* var. Viollette de Barbentane		
S. macrocarpon acc. 60	3	–
S. quitoense	8	2
Reference Sequence: *Capsicum annuum* (Accession number U87849)		
C. chacoense	–	1
C. annuum var. El-Tajin	2	–
C. annuum var. LP1	2	–

(forward or reverse), 1 μL of BigDye^{TM} Terminator Cycle Sequencing reaction mixture (Applied Biosystems), 2 μL of the purified PCR product, 1 μL of buffer Big Dye, and 5 μL of H_2O. Reaction products were purified using G50 gel filtration (Sephadex TM G-50 superfine (Amersham Biosciences AB) and loaded onto ABI3730XL 96 capillary sequencers. All the sequences obtained were aligned and consensus regions were defined for the design of specific TaqMan probes targeting the revealed polymorphism. The sequence alignments and detection of polymorphism were performed using the software GENALYS, available at http://software.cng.fr.

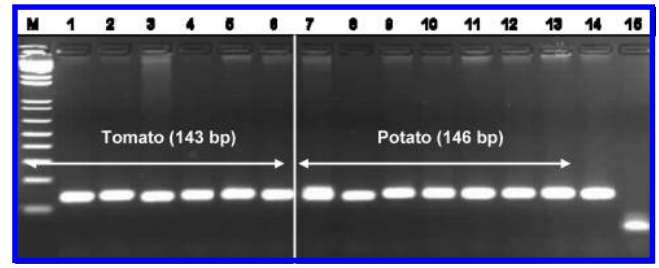

Figure 3. Amplification of DNA from 15 different tomato and potato related wild taxa. Lanes 1–16 correspond to *S. lycopersicum*, *S. habrochaites*, *S. hirsutum*, *S. pimpinellifolium*, *S. chilense*, *S. parviflorum*, *S. chimilewski*, *S. tuberosum*, *S. quitoense*, *S. phureja*, *S. acaule*, *S. demissum*, *S. stoloniferum*, *S. spegazzinii*, *S. verenei* and NTC (no template control). Lane M corresponds to the 1kb marker. 2% agarose gel was used.

Conventional PCR Conditions. Qualitative PCR was run on MJ Research thermocyclers (MJ Research, Waltham, MA). PCR amplifications were performed in a final volume of 25 μL. Each reaction mixture had 1X PCR buffer, 0.2 mM dNTP, 0.4 μM each primer, 25 ng of each DNA sample, and 1 unit of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA). The amplicons were resolved by electrophoresis on 2% agarose gels (Invitrogen, Carlsbad, CA). Gels were scanned with Image Master VDS (Amersham Bioscience, Amersham, UK).

Real-Time PCR Assays. To assess the amplification specificity, Sybr Green reactions containing 2.4 μL of Power SYBR I Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 1 μM of each of the specific primers were performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems). To design specific Solanaceae probes (**Table 3**), a Minor Groove Binder (MGB) TaqMan probe was used in order to discriminate between single mismatches (*36*). The design of these probes for the TaqMan assay was carried out using Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA). All probes were supplied by Applied Biosystems (Foster City, CA). The real time PCR reactions were run on an ABI PRISM 7900HT sequence detection system (Applied Biosystems). The quantitative reaction was performed in 5 μL final assay volume and contained 1 μM of each of the specific primers and 200 nM TaqMan-MGB probes added to 2.36 μL of master mix (TaqMan Universal PCR Master Mix No Amperase UNG (2x) and finally 1 μL of the plant DNA at 5 ng/μL. In addition, no primer dimers were detected with all the Solanaceae samples checked by real-time PCR using Sybr Green chemistry (data not shown).

RESULTS AND DISCUSSION

Selection of the Gene Sequence Used for Differentiation of the Four Targeted Solanaceae. Generally, the endogenous reference gene to be assayed has four requirements. It must be

Table 5. Specificity of the Primer Pairs and Probes Using Different Taxa of the Four Solanaceae Targeted[a]

taxa	β-fructosidase consensus primers	β-fructosidase pepper primers and probe	β-fructosidase tomato probe	β-fructosidase potato probe	universal primers
Arabidopsis thaliana	−	−	−	−	+
Beta vulgaris	−	−	−	−	+
Brassica napus	−	−	−	−	+
Brassica oleracea	−	−	−	−	+
Brassica nigra	−	−	−	−	+
Brassica juncea	−	−	−	−	+
Brassica rapa	−	−	−	−	+
Nicotiana tabacum	−	−	−	−	+
Triticum durum	−	−	−	−	+
Triticum aestivum	−	−	−	−	+
Zea diploperennis	−	−	−	−	+
Medicago truncatula	−	−	−	−	+
Oryza sativa	−	−	−	−	+
Phaseolus aureus	−	−	−	−	+
Pisum sativum	−	−	−	−	+
Secale cereale	−	−	−	−	+
Glycine max	−	−	−	−	+
Gossypium Barbadense	−	−	−	−	+
Gossypium hirsutum	−	−	−	−	+
Hordeum vulgare	−	−	−	−	+
Linum usitatissimum	−	−	−	−	+

[a] As shown in the table, no cross reaction was observed with any of the taxa tested other than the targets. Universal plant primers confirm the high quality of the DNA (ability for PCR amplification) and the absence of false negatives. (−) means no amplification observed in the electrophoresis analysis. (+) positive amplification observed in the electrophoresis analysis. (+/–) For *Capsicum chacoense*, it is the fragment amplified with the specific primers PomtomF/PoivR and not detected with the Poivpr probe because of the deletion. (tom); (po); (pe); (egg): tomato, potato, eggplant, and pepper involved respectively in the introgression of genes with the wild taxa.

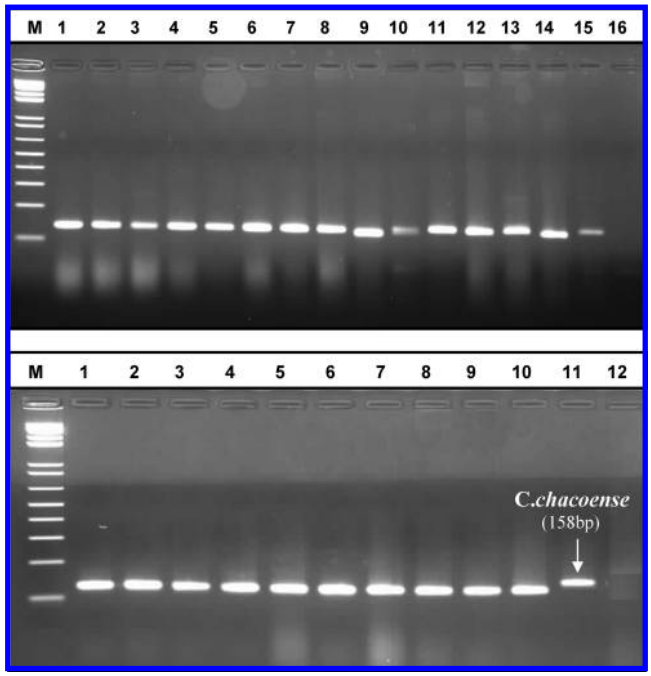

Figure 4. (**A**, top) PCR amplification from 15 eggplant related wild taxa. Lanes 1–16 correspond to *S. melongena* var. Violette de Barbentane, *S. aethiopicum* gr. kumba, *S. macrocarpon*, *S. incanum*, *S. linnaeanum*, *S. hastifolium*, *S. cyaneo-purpureum*, *S. torvum*, *S. viarum*, *S. cinereum*, *S. marginatum*, *S. sisymbriifoilium*, *Solanum scabrum*, *S. mammosum*, and *S. pyracanthos*, and no template control (NTC). Line M: 1kb marker. (**B**, bottom) PCR amplification product profiles of wild pepper taxa. Lanes 1–12 correspond to *C. annuum* acc. 018, *C. annuum* Florida VR2, *C. baccatum*, *C. cardenasii*, *C. chinense*, *C. galapogense*, *C. microcarpum*, *C. praetermissum*, *C. eximium*, *C. pubescens*, *C. chacoense* and no template control (NTC). Line M: 1kb marker. 2% agarose gel was used.

taxon specific and nuclear and present in low copy number, and it must exhibit high intraspecies homogeneity. Using tomato genomic sequences available through the international Solanaceae Genome Project (http://www.sgn.cornell.edu) and by using different plant DNA databases such as NCBI or the Adaptive Evolution Database (TAED) (http.//www.bioinfo.no/tools/TAED) (*37, 38*), we searched for reference gene sequences that belong to low copy number gene families. Among the sequences studied, we chose the sequence encoding the potato and tomato β-fructosidase (also called acid invertase or saccharase). This gene is present in all plants and is known to catalyze the hydrolysis of the disaccharide sucrose to the hexose sugars glucose and fructose (*39*). The full length cDNA sequences of potato and tomato shared an average overall homology of approximately 95%. On the basis of sequence differences and identified regions of homology, we designed a pair of consensus primers PomF/PomR for potato and tomato and PomF/PoivR for pepper. Eggplant cDNA was not available in the databases and was obtained later by sequencing.

In Silico Specificity. Once the primers were designed, we assessed the theoretical specificity of the primers and the probes by searching for similar sequences in the database through a BLAST search on the (NCBI) Web site. Potato, tomato, and pepper consensus primers were highly specific for the target taxa. We checked the location of our PCR target on the complete DNA sequence of β-fructosidase using Multialign software (*40*). As is shown in the alignment (**Figure 1**), exon 7 of the target gene was highly conserved among taxa of the Solanaceae family. Only the sequence corresponding to the wild tomato taxon *Solanum pennellii* is different (NCBI Accession number AJ272307) and was not detected with our consensus primers (data not shown). This sequence variability demonstrates that sometimes wild taxa are not detected and have to be tested for primer validation.

Sequencing of the Selected Regions. The consensus primers PomtomF/PomtomR were used for sequencing exon 7 of tomato, potato and eggplant and PomtomF/PoivR for pepper. Three sequencing steps were established that were shown to reveal all the polymorphism: first, between lines and cultivars (in-

Table 6. Study of the Specificity of Primers and Probes among Solanaceae Taxa and Their Involvement in Introgression Events[a]

taxa	possible introgression occurrence with *Solanum* and *Capsicum* genus	β-fructosidase consensus primers	β-fructosidase pepper primers and probe	β-fructosidase tomato probe	β-fructosidase potato probe	universal primers
Solanum lycopersicum	lyc	+	−	+	−	+
Solanum habrochaites	lyc	+	−	+	−	+
Solanum hirsutum	lyc	+	−	+	−	+
Solanum pimpinellifolium	lyc	+	−	+	−	+
Solanum pennellii	lyc	−	−	−	−	+
Solanum parviflorum	lyc	+	−	+	−	+
Solanum chilense	lyc	+	−	+	−	+
Solanum tuberosum	tub	+	−	−	+	+
Solanum quitoense	−	+	−	−	−	+
Solanum phureja	tub	+	−	−	+	+
Solanum acaule	tub	+	−	−	+	+
Solanum demissum	tub	+	−	−	+	+
Solanum stoloniferum	tub	+	−	−	+	+
Solanum spegazzinii	tub	+	−	−	+	+
Solanum verenei	tub	+	−	−	+	+
Capsicum annuum	ann	−	+	−	−	+
Capsicum baccatum	ann	−	+	−	−	+
Capsicum cardenasii	ann	−	+	−	−	+
Capsicum chacoense	ann	−	×	−	−	+
Capsicum chinense	ann	−	+	−	−	+
Capsicum microcarpum	ann	−	+	−	−	+
Capsicum praetermissum	ann	−	+	−	−	+
Capsicum galapogense	ann	−	+	−	−	+
Solanum melongena	mel	+	−	−	−	+
Solanum macrocarpon	mel	+	−	−	−	+
Solanum aethiopicum	mel	+	−	−	−	+
Solanum incanum	mel	+	−	−	−	+
Solanum linnaeanum	mel	+	−	−	−	+
Solanum hastifolium	mel	+	−	−	−	+
Solanum cyaneo-purpureum	mel	+	−	−	−	+
Solanum torvum	mel	+	−	−	−	+
Solanum viarum	mel	+	−	−	−	+
Solanum cinereum	mel	+	−	−	−	+
Solanum sisymbriifolium	mel	+	−	−	−	+
Solanum scabrum	mel	+	−	−	−	+
Solanum pyracanthos	mel	+	−	−	−	+

[a] Consensus primers detect all the taxa tested and belonging to the genus Solanum while the pepper specific primers detect only the pepper lines and cultivars including related wild taxa involved in genes introgressions. (+) means presence of amplification products with the expected size. (−) means no amplification observed in the electrophoresis analysis. (×) Detection only with primers and no quantification with pepper probe.

traspecies sequence variability); second, between related wild taxa involved in the introgression of genes; and finally, between taxonomically close taxa, such as tomato, potato, eggplant, and pepper (interspecies sequence variability). The steps are described in **Figure 2**.

Sequences obtained as well as the multiple alignments are described in Figures 1–4 of the Supporting Information. Few single nucleotide polymorphisms (SNPs) were present in the conserved target sequence except for related wild taxa (**Table 4**). These SNPs can be subsequently used for the design of allele specific molecular markers for genotyping in further studies.

No polymorphism was detected in our region of interest (exon 7) except in *C. chacoense* for pepper and in *S. quitoense* for eggplant. In fact, *S. quitoense* belongs to the genus Solanum, subgenus "Leptostemonum" (*41*) and is taxonomically close to *S. melongena* (*42, 43*). No artificial hybrids are reported with taxa from the subgenus "Potatoes". It is therefore unlikely that *S. quitoense* is involved in the introgression of genes and thus its sequence was not taken into account for the design of the quantitative Solanaceae reference system.

Design of TaqMan MGB Probes. Based on the sequence alignment of exon 7 of β-fructosidase, four specific probes were designed: Tomcpr, Pomcpr, Aubcpr, and Poivcpr in tomato, potato, eggplant, and pepper, respectively. The differentiation was facilitated through the use of MGB probes which are able to discriminate one single nucleotide polymorphism (SNP) in a given position (*36*). The use of these probes avoided problems of cross hybridization and the detection of false positives.

Development of Reference Gene-Specific Qualitative and Quantitative Real Time PCR Assays for Four Solanaceae. *Initial Assessment of the Experimental Specificity.* The consensus primers targeting the gene β-fructosidase gave rise to a PCR product of 143 bp for tomato, 146 bp for potato, and 141 for eggplant. Moreover, DNA from related wild tomato and potato taxa was also amplified with these primers (**Figure 3**) except for one taxa corresponding to a wild tomato (*Solanum pinnellii*). For the assessment of the specificity, we also used other plant DNA samples that are suspected to be frequently found in food to test their cross hybridization with our target (**Table 5**). The conventional PCR demonstrated that the consensus primers amplified not only tomato and potato cultivars but also other Solanaceae taxa such as eggplant (*Solanum melongena*) or Ethiopian nightshade (*Solanum aethiopicum*) (**Figure 4A**). DNA from pepper lines and cultivars was not amplified due to differences in the sequence of the reverse primer (**Figure 1**). Consequently, a new pepper-specific reverse primer was designed. Using the new couple of primers, we amplified only the pepper DNA, including domesticated and wild lines. The PCR product size was 134 bp for all the pepper lines with the same band intensity except for the taxa *Solanum chacoense*

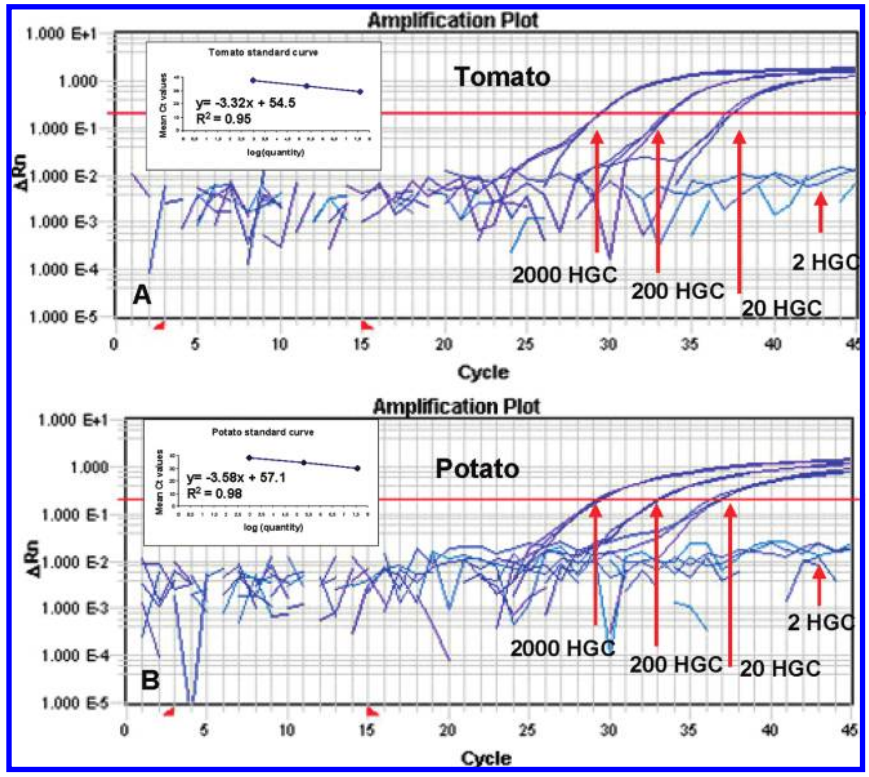

Figure 5. Amplification plots and standard curves for the duplex potato and tomato real time PCR assays. (**A**) Amplification plots generated by serial dilution of tomato and potato genomic DNA ranging from 2000 HGC to 2 HGC with the PomtomF and PomtomR primer pair and Tomcpr and Pomcpr probes. Assays were performed in triplicate. (**B**) Calibration curves generated from the amplification data given in (**A**). No amplification observed (Ct = 45) with the concentration 2 HGC confirming that the absolute LOD was greater than 2 HGC.

Table 7. Comparison of the Ct Values Using β-Fructosidase in Four Solanceae (Tomato, Potato, Eggplant, and Pepper)[a]

simplex study							duplex study						
HGC number	mean Ct	SD^r	SD^R	% RSD_r	% RSD_R	signal rate	HGC number	mean Ct	SD^r	SD^R	% RSD_r	% RSD_R	signal rate
		eggplant β-fructosidase							potato β-fructosidase				
2000	31.75	0.1	0.09	2.58	2.32	3/3	2000	29.611	0.13	0.06	3.63	1.67	3/3
200	34.64	0.31	0.12	8.01	3.10	3/3	200	33.181	0.2	0.11	5.58	3.07	3/3
20 (LODa)	37.52	0.45	0.21	11.62	5.45	3/3	20	36.332	0.16	0.08	4.46	2.23	3/3
2	–	–	–	–	–	0/3	2	40.0	–	–	–	–	0/3
		pepper β-fructosidase							tomato β-fructosidase				
2000	31.7	0.2	0.03	5.86	0.86	3/3	2000	28.475	0.11	0.04	3.31	1.20	3/3
200	34.9	0.19	0.06	5.57	1.72	3/3	200	32.331	0.10	0.09	3.01	2.71	3/3
20 (LODa)	37.4	0.31	0.18	9.09	5.18	3/3	20	35.146	0.19	0.1	5.72	3.01	3/3
2	–	–	–	–	–	0/3	2	40.0	–	–	–	–	0/3

[a] The presented amplification data was also used to determine the absolute LOD for both targets. Duplex values are described for potato and tomato and simplex values for pepper and eggplant. SD^r: repeatability standard deviation. SD^R: reproducibility standard deviation. RSD_r: relative standard deviation of repeatability. RSD_R: relative standard deviation of the reproducibility.

which gave rise to a fragment of 158 bp (**Figure 4B**). Indeed, this taxon presented an insertion of 24 nucleotides flanking the sequence. Thus, it is detectable only with the primers in qualitative PCR (**Figure 4B**).

Second Assessment of the Experimental Specificity. All the probes were tested on the whole collection of Solanaceae DNA. The real-time PCR system using the MGB probe developed for tomato detected only tomato in simplex and in the duplex mixture with potato, including three transgenic tomatoes (events NCIMB40015, NCIMB40134, and Tg7TF). It did not detect potato, eggplant, and pepper. Likewise, the potato and eggplant MGB probes detected all the potato and eggplant cultivars and lines, respectively, and did not detect any of the tomato or pepper DNA. The MGB probe for pepper was also specific and detected all the expected DNAs, except *Capsicum chacoense* because of the insertion. The specificity of primers and probes is summarized in **Table 6**. The described assay showed high specificity on a large collection of plants including cultivars and related wild taxa. Based on these results, we can expect that this assay will be applicable even following introgression of this gene from wild related taxa.

Intraspecies Conservation and Copy Number of β-Fructosidase. The intraspecies conservation of gene copy number is necessary for the reliability of the method. To test this, real-time PCR reactions were performed in triplicate using 5 ng DNA templates from the entire collection of potato, tomato, eggplant, and pepper. The mean cycle threshold (Ct) was calculated. To validate such an assay, a large and representative number of cultivars is required. 75 cultivars and lines of tomato, 23 of potato, 59 of eggplant, and 76 of pepper were used. Using the same primer pair for amplification of β-fructosidase, stable similar average Ct values were obtained for potato (27.76 $\pm$ 0.53) and for tomato (26.99 $\pm$ 0.38) in the duplex study. Values were slightly higher but again similar between pepper (28.7 $\pm$

0.32) and eggplant (28.02 ± 0.29) in the simplex assays. Similar rates of amplification were obtained for the different DNA templates, and no significant Ct variability was observed between the different cultivars and lines. This demonstrates the absence of variability in gene copy number and the stability of amplification efficiency among the taxa. Therefore, we can conclude that the primers and the probes used are suitable for the differentiation of tomato, potato, pepper, and eggplant taxa and that this gene can be used as an endogenous reference gene in GMO quantification.

β-Fructosidase, being a well-characterized gene, has previously been demonstrated to be low copy number in many taxa of the Solanaceae family (*44–46*). We confirmed these data using the ΔCt method. We compared the Ct values for potato and tomato to two previously described single copy genes, *LAT52* for tomato and *pci* for potato (*27, 28*), and found only minor differences in the ΔCt values, ranging from 0.6 to 1.05 for potato and from 3.3 to 3.59 for tomato (data not shown). This strongly suggests that β-fructosidase is present in low copy number in these two taxa.

Assessment of the Sensitivity. The absolute limit of detection (LOD) of our test was calculated as follows: two dilution series ranging from 2000 Haploid Genome Copies (HGC) to 0.02 HGC were prepared from potato (var. *Operle*) and tomato (var. *Nagcarlan*) individually. Equal volumes of each dilution were mixed and then were assayed in duplex. Dilution series were assayed in simplex for the quantification of our target in eggplant (cultivar RNL037) and pepper (cultivar Flambeau). Copy numbers of the respective target gene (β-fructosidase) in genomic DNA were derived from the haploid genomic weight of 0.49 pg/copy for tomato, 0.9 pg/copy for potato, 1.19 pg/copy for eggplant, and 3.27 pg/copy for pepper (*47*). These values are only estimations (*48*) and can be one of the critical points affecting the evaluation of the sensitivity of the tests. As expected, the ability to detect the taxa targeted decreased with decreasing copy numbers. **Figure 5** shows the amplification plots and the standard curves of potato and tomato and **Table 7** shows the amplification data (Ct values). For tomato and potato, the absolute LOD was estimated to be less than 20 HGC and simplex tests with pepper (var. *Chay angolano*) and eggplant (var. *parent pop de carto*) showed a LODa of 20 and 200 HGC, respectively. Using tomato and potato DNA templates, no fluorescence signal was observed with a DNA concentration of 2 HGC. Based on the slopes of the standard curves, the amplification efficiencies of the standards ranged from 92% to 99%, as calculated with the formula: $E = 10^{(-1/\text{slope})} - 1$. The linear correlation coefficient (R^2) of β-fructosidase ranged from 0.95 to 0.98 for potato, tomato, pepper, and eggplant. In addition, all the amplification plots gave a high ΔRn value (approximately 1 for all the taxa). The high efficiency and correlation coefficients obtained using this assay suggest strongly that it is well adapted to be used as a reference gene system for GMO quantification.

To further assess the reliability of these assays, the repeatability and the reproducibility of the assay were evaluated using the mixture of dilution series described above of tomato and potato DNA and the individual dilutions for eggplant and pepper. Each serial dilution was assayed in triplicate and repeated in three separate experiments. The repeatability standard deviation (SD^r) and reproducibility standard deviation (SD^R) were calculated. The SD^r values of tomato and potato ranged from 0.11 to 0.19 and 0.13 to 0.2, respectively, whereas it ranged from 0.1 to 0.45 for eggplant and from 0.2 to 0.31 for pepper (**Table 7**). The SD^R values ranged from 0.04 to 0.1 for tomato, from 0.06 to 0.11 for potato, from 0.09 to 0.21 for eggplant, and from 0.03 to 0.18 for pepper. These values demonstrate that the results are reproducible and repeatable and thus reliable in routine analysis.

In addition, the absolute limit of quantification (LOQa) was determined as previously described in the performance criteria requirements (engl.jrc.it/). For this we used the formula $RSD = [\ln(10)/b \times SD\ (Ct)$ (b, the slope of the standard curve; and SD, the standard deviation determined above) (*49*) to calculate the relative standard deviation of the repeatability (RSD_r) and of the reproducibility (RSD_R) and then deduced that the LOQa was 200 HGC for all the assays (**Table 7**).

The increasing number of GM plants and the lack of validated reference genes used for their quantification (http://gmo-crl.jrc.it) necessitate the development reliable assays for the quantification of GMOs as well endogenous reference genes. This presents an even greater challenge when the taxa to be assayed are closely related, such as is the case of the Solanaceae.

In this work, we describe the development of three assays for the detection of an endogenous reference gene β-fructosidase in four Solanaceae taxa: tomato, potato, eggplant, and pepper. Our approach relies on the use of a large collection of plant samples, including related wild taxa, for the development and validation of the assay. This collection, the first such of its kind for these taxa, includes a wide genetic diversity in the cultivated as well as the wild related taxa of Solanaceae. This assay thus contrasts others previously developed (*26, 27*) which did not take into account this diversity.

The assays include two simplex tests for the quantification of eggplant and pepper and one duplex real-time PCR method for the simultaneous detection and quantification of tomato and potato. The characterization of the β-fructosidase fragment was performed and carried out following sequencing to identify intra- and interspecies variability. Despite strong homology of the cDNA sequences of tomato and potato β-fructosidase, we were able to develop a TaqMan assay that differentiates the two taxa. This duplex assay offers the additional advantage of being more cost and time efficient. All our results suggest that the assays described here should be applicable to the quantification of the β-fructosidase in GM tomato and potato in complex samples such as food in combination with GMO event specific assays.

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Supporting Information Available: Cultivars of taxa used for the first assessment of the specificity (Table 1); illustration of sequence alignment of the region targeted in the Solananceae taxa (Figure 1 for tomato, Figure 2 for potato, Figure 3 for eggplant, and Figure 4 for pepper). These information are available free of charge via the Internet at http://pubs.acs.org.

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