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Real-Time and Conventional Polymerase Chain Reaction Systems Based on the Metallo-Carboxypeptidase Inhibitor Gene for Specific Detection and Quantification of Potato and Tomato in Processed Food

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ABSTRACT

In this paper, a method for the specific detection and quantification of potato and tomato DNA in food samples with the use of conventional and real-time polymerase chain reaction (PCR) is described. This method is adequate for use in food quality routine assays involving highly processed samples for which very tiny amounts of DNA are expected. Detection was achieved by amplifying a region of the metallo-carboxypeptidase inhibitor gene from either the potato (*PCI*) or the tomato (*MCPI*) and by using specific primers complementary to the propeptide regions of these inhibitors, which were found to differ for the potato and tomato proproteins. Conventional and real-time PCR systems were based on the same potato- or tomato-specific primer pairs, and quantification was carried out with a TaqMan chemistry-based probe. The methods developed proved to be very specific and sensitive and highly reliable for the identification and quantification of DNA from both plant species. In addition, the construction of plasmids *pPAT* and *pTOM,* suitable for use as external calibration standards for the elaboration of comparative amplification profiles, is reported. Limits of detection and quantification with the use of these plasmid standards are given. Specificity and copy number conservation among different cultivars were analyzed, and the reliability of these systems was tested through their application to the analysis of commercial food samples including potato and/or tomato as components.

The identification of food species is becoming a very important issue concerning the assessment of food composition, which is necessary for the provision of proper consumer information. The increasing demand for transparency in the food industry by consumers has provoked a strong demand for appropriate detection methods that allow the identification of the different components of processed foods. In addition, European norms mandate the labeling of foods with detailed ingredients to inform and preserve the free choice of consumers *(1, 12).* Therefore, methods and techniques required to achieve these objectives are rapidly being developed.

The estimated areas used for potato and tomato cultivation worldwide in 2001 *(13)* were 19,300,000 and 3,700,000 ha, respectively, for a global production of 308,200,000 Mt of potatoes (mostly in cold countries such as Russia) and 100,300,000 Mt of tomatoes (mostly in warmer countries such as Italy). These two plant species are components of many food products (pizza, tomato sauces, vegetable soups) and are frequent food industry by-products (potato starch); methods for the detection of these ingredients have not been developed to date.

In recent years, the commercialization of genetically modified (GM) potatoes and tomatoes has been approved in different countries. Transgenic potatoes are the fifth most cultivated transgenic crop, representing a total worldwide area of about 100,000 ha *(19).* The European Union (EU) and Japan have established mandatory rules for the labeling of all GM organism (GMO) foods or food ingredients with a list of components exceeding 1% (EU *(9)*) and 5% (Japan *(3)*) threshold levels, and 1% for every GMO food additive introduced into the market (EU *(10)*). Therefore, quantification of the relative amounts of GMO ingredients is crucial in the analysis of food samples, with quantitative estimation requiring a species-specific endogenous control that provides an estimate of the total amount of the species in question in the sample.

Different techniques based on protein and DNA detection have been used for food identification and quantification. DNA-based methods have proved to be more reliable because of the stability of DNA under the conditions associated with the high temperatures, pressures, and chemical treatments used in the preparation of some food products. One of the techniques currently used, real-time quantitative polymerase chain reaction (PCR), is a powerful technology that is highly accurate, is relatively fast, is simple, and results in elevated sensitivity and specificity *(16).* The use of real-time quantitative PCR with TaqMan technology for specific detection and quantification of viruses

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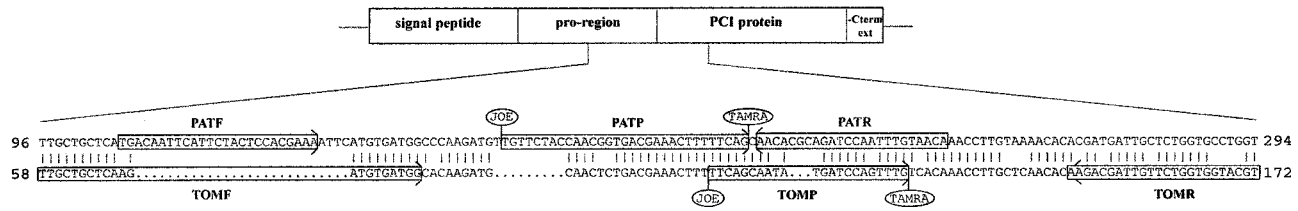

FIGURE 1. *Alignment of the potato* PCI *and the tomato* MCPI *nucleotide fragments harboring the designed PCR primers and probes. Primers and probes are boxed in arrows pointing in the 5′-to-3′ direction. The names of the primers and dual-labeled fluorescent probes are indicated. Gaps are included for best-fit alignment. Sequence numbering is given for accession numbers AF060551 and X59282, corresponding to the potato and the tomato cDNAs, respectively.*

(7), microorganisms *(24),* and plants such as maize, soybean, and rapeseed *(5, 18, 27–29)* has been reported.

To date, no reports have been published describing methods for the species-specific tomato and potato quantification. A PCR-based method for the identification of potato DNA was developed, but it was not optimized for quantification *(1).* A particular problem in dealing with these two plant species is their strong similitude at the DNA level. In this regard, nearly all restriction fragment length polymorphism probes that have been mapped for the tomato were found to cross-hybridize with potato DNA and were successfully used for potato mapping *(6).* This mapping allowed the determination, with a high degree of precision, of the breakpoints corresponding to five chromosomal inversions that differentiate the tomato and the potato genomes *(26).* Potato and tomato genes generally share high percentages of identity, especially in the protein-coding regions, where levels of homology can be >90%, with differences between the two species often corresponding to point mutations that tend to be silent or to result in conservative amino acid exchanges. A search of GenBank for specific gene sequences encoding open reading frames that would be relatively dissimilar in these two otherwise closely related species led to the identification of the wound-inducible metallo-carboxypeptidase inhibitor (*PCI*) gene *(30),* which, in *Solanum tuberosum,* is encoded by a small gene family *(23). PCI* encodes a small protease inhibitor that is active against proteases of the digestive tracts of insects and accumulates in response to mechanical wounding or insect injury, thus making up part of a plant's defense system against insect attack *(15).*

Nucleotide sequence alignment of the cDNAs for the tomato (*MCPI*) and potato (*PCI*) precursors showed an overall 84.9% homology between the two genes, with major differences being found in the region encoding the N-terminal proinhibitor domain *(30).* Particularly striking is the presence in this region of a 27-bp insertion (at positions 110 through 137) in potato cDNA which is absent from the tomato message. We designed primer pairs for this region (Fig. 1) for the development of PCR methods capable of discriminating between tomato and potato template DNAs. Here we describe a method, based on the TaqMan technology, that allows specific, sensitive, and accurate quantification of potato and tomato DNA in processed food samples. The specificity of the method is based on the design of a primer pair and a TaqMan probe complementary to the most differential regions of these genes. The reduced amplicon size, aside from providing a high level of reaction efficiency, allows the amplification of target sequences present in processed food samples that have been subjected to conditions that are expected to result in partial DNA degradation. Furthermore, we obtained cloned target constructs suitable for use as external calibration standards in quantitative assays and demonstrated their accuracy as quantification standards. We report on the application of this PCR-based method to potato- and tomato-derived processed food products.

MATERIALS AND METHODS

Plant materials. Leaves from 18 different potato lines (Ackensegen, Bamberger Hörnchen, Roseval, Jubel, Edzell Blue, Aura, Shetland black, Hermanns Blaue, Pink fir Apple, Naglerner Kipfler, Vitelotte, Sharon Blue, Reichskanzler, Piroschka, Highland Burgundy Red, Flava, Hindenburg, and Mamantambo) were kindly provided by GeneScan (Freiburg, Germany). Leaves from 14 tomato lines (*Lycopersicon hirsutum,* Corbarino, Orange Queen, Yellow Stuffer, Momor, Noire de Crimée, *Lycopersicon pimpinellifolium,* Valparaiso, *Lycopersicon peruvianum,* Marmande V.R., Naines Pomor, Médine, Flora Dade, and Ohmiya Suncherry) were provided by the Institut National de la Recherche Agronomique (INRA; Versailles, France). Leaves from *Lycopersicon esculentum* var. Ailsa Craig, *Solanum tuberosum* var. Désirée, *Arabidopsis thaliana* ecotype Columbia, *Brassica napus, Brassica oleracea, Helianthus annuus, Hordeum vulgare, Ipomoea batatas, Oryza sativa, Sorghum bicolor, Zea diploperennis,* and *Zea mays* cv. W64A were from plants cultivated in greenhouses at the Institut de Biologia Molecular de Barcelona–Consejo Superior de Investigaciones Científicas. DNA extracted from *Secale cereale, Triticum aestivum, Panicum miliaceum, Glycine max, Lens esculenta, Vicia faba, Phaseolus vulgaris,* and *Lupinus albus* was provided within the framework of the Qpcrgmofood European project (QLK1-1999-01301). Food samples were purchased at the local market.

Plant DNA isolation and quantification. Large-scale genomic DNA was isolated from 30 g of leaves according to Dellaporta et al. *(8)* and purified by cesium chloride density gradient centrifugation. Small-scale genomic DNA was isolated from 0.1 g of plant material by a hexadecyltrimethylammonium bromide (CTAB)–based protocol described previously *(22).* DNA concentrations were spectrophotometrically quantified with a GeneQuant RNA/DNA Calculator (Amersham Pharmacia). DNA concentrations were further assessed by agarose gel electrophoresis and ethidium bromide staining. UV-fluorescent emission was recorded and quantified with Quantity One software (Bio-Rad). All DNA solutions were tested for the possible presence of PCR inhibitors through real-time PCR reactions specific for the control plasmid

TABLE 1. *Oligonucleotides used in this study*

PCR system Name	Description	Sequence
Potato		
PATF	Forward primer	5′-TGA CAA TTC ATT CTA CTC CAC GAA A-3′
PATR	Reverse primer	5′-TGT TAC AAA TTG GAT CTG CGT GTT-3′
PATP	Forward probe	JOE-5′-TGT TCT ACC AAC GGT GAC GAA ACT TTT TCA G-3′-TAMRA
Tomato		
TOMF	Forward primer	5′-TTG CTG CTC AAG ATG TGA TGG-3′
TOMR	Reverse primer	5′-ACG TAC CAC CAG AAC AAT CGT CT-3′
TOMP	Forward probe	JOE-5′-TTC AGC AAT ATG ATC CAG TTT GTC ACA AAC CTT G-3′-TAMRA

pMON3 (18); reactions involved 100 copies of target DNA and were run in the presence of either 100 ng of each DNA solution or the same volume of water. Threshold cycles (the threshold cycle [C_T] is the PCR cycle at which the fluorescence intensity reaches a preestablished threshold) for all reactions were within 1 cycle of one another, indicating the absence of PCR inhibitors.

PCR reactions. PCR oligonucleotides (Table 1) were designed with Primer Express 1.5 software (Applied Biosystems). Probes labeled with the fluorescent 6-carboxy-4′,5′-dichloro-2′, 7′-dimethoxy-fluorescein (JOE) reporter dye at the 5′ end and with the quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) at the 3′ end were purchased from Eurogentec. Primers were obtained from MWG-Biotech AG.

PCR reactions were carried out with TaqMan PCR core reagents (Applied Biosystems-Roche). Conventional PCR reactions were carried out with 20 μl of PCR mixture containing 1× buffer II (100 mM Tris-HCl [pH 8.3] and 500 mM KCl), 3 mM $MgCl_2$, 200 μM dNTPs, 0.3 μM each primer, 1 U of AmpliTaq Gold DNA polymerase, 0.2 U of AmpErase uracil *N*-glycosylase, and 2 μl of the DNA template and run in a GeneAmp PCR System 9600 (Perkin-Elmer) for 2 min at 50°C, then for 10 min at 95°C, and then for 40 cycles of 20 s at 95°C and 1 min at 60°C, with a final extension for 5 min at 72°C.

Real-time PCR reactions were carried out with a 20-μl reaction volume containing 1× PCR TaqMan buffer A (which includes ROX as a passive reference dye); 6 mM $MgCl_2$; 200 μM (each) dATP, dCTP, and dGTP; 400 μM dUTP; 300 nM each primer; 200 nM probe; 1 U of AmpliTaq Gold DNA polymerase; 0.2 U of AmpErase uracil *N*-glycosylase; and 2 μl of the DNA solution. Reactions were run with an ABI PRISM 7700 Sequence Detection System device (Applied Biosystems) for 2 min at 50°C, then for 10 min at 95°C, and then for 50 cycles of 15 s at 95°C and 1 min at 60°C. Unless otherwise indicated, all real-time PCR reactions were carried out in triplicate.

The combination of primers and probes used in each PCR system are shown in Table 1. Conventional PCR products were detected in agarose gels. Real-time PCR reactions were analyzed with the Sequence Detection System software (Version 1.7, Applied Biosystems). Quantification was performed by interpolation on a standard regression curve of C_T values generated from DNA samples of known concentrations. Real-time PCR reactions showing no amplification curves were considered to indicate a negative result or a lack of amplification.

Construction of plasmids for the standard curves. Potato and tomato PCR products were cloned with the pGEM-T Easy vector (Promega) according to the manufacturer's instructions to generate *pPAT* and *pTOM,* respectively. The presence of a single copy of the appropriate insert was assessed by sequencing with the Big Dye Terminator Cycle Sequencing kit and an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Plasmid DNA was purified with a Qiagen Plasmid Midi Kit (Qiagen) and quantified by 260-nm absorbance and agarose gel electrophoresis. The concentration was further assessed with the use of the PicoGreen kit (Molecular Probes) and a Luminescence Spectrometer LS50B (Perkin-Elmer). Plasmid DNA was serially diluted in a solution containing 50 ng of bovine serum albumin per μl to yield 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10, 4, 2, and 1 molecules per PCR reaction. On the basis of plasmid size (*pPAT,* 3,119 bp; *pTOM,* 3,129 bp) 1 molecule corresponds to 3.44 ag of *pPAT* and 3.45 ag of *pTOM* DNA, respectively. Plasmid DNA dilutions were used as external standards for all PCR systems. Statistical analyses were carried out for each serial dilution, and the confidence interval at the given probability was calculated. The calculations assumed a binomial distribution *(20)* and were carried out with the SAS System for Windows (Version 8.0, SAS Institute Inc., Cary, N.C.). Results were also confirmed by Monte Carlo simulations with 1,000,000 repetitions.

Extraction of DNA from food samples. DNA was extracted from the different food products by a CTAB-based method. Solid foodstuffs were minced and homogenized at room temperature in a mill. Liquid products such as tomato juice or beef stock were concentrated by serial centrifugations. Different starting amounts of products (5 g of pizza, 20 g of precooked meatballs, 50 ml of tomato juice, 50 ml of Spanish gazpacho, 50 ml of beef stock, 15 g of tomato sauce, 5 g of instant green soup, 2 g of instant potato flakes, 10 g of tomato ketchup, 1 g of instant cream of seafood soup, 10 g of sardines in tomato sauce, 10 g of squid in cocktail sauce, 2 g of Spanish omelet, 2 g of frozen French fries, and 2 g of potato croquettes) were incubated with CTAB buffer at 65°C for 45 min. Extracts were clarified by centrifugation (5,000 × *g* for 30 min), and the aqueous phase was transferred to a fresh tube and precipitated with 0.3 M sodium acetate and 1 volume of isopropanol. Pellets were washed with 70% ethanol and resuspended in 1 ml of TE buffer (10 mM Tris-HCl [pH 8] and 1 mM EDTA). After RNase A incubation at 37°C for 15 min, the DNA was further purified and concentrated in a silica column (QIAquick PCR Purification kit, Qiagen) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Selection of a gene-specific fragment suitable for specific identification and quantification of potato and tomato DNA: design of potato- and tomato-specific conventional and real-time PCR systems. Through a thorough scrutiny of genome databases, we retrieved and aligned different potato and tomato DNA sequences that

were encoded by low-copy-number gene families, showed enough variability to produce tomato- or potato-specific products, and were not targets of any genetic modification, thus being suitable for use as endogenous reference genes for the quantification of GM potatoes or tomatoes. From the sequences we retrieved, we chose those encoding the potato carboxypeptidase inhibitor (*PCI,* accession no. AF060551) *(30)* and its tomato homolog (*MCPI,* accession no. X59282) *(21).* These sequences correspond to two small proteic metallo-carboxypeptidase inhibitors induced to accumulate in potato and tomato leaves in response to mechanical wounding or methyl jasmonate treatment and thought to be part of their respective plants' defense mechanisms against insect and pest attack *(14, 25).* The full-length cDNA-encoding potato *PCI* is 309 bp long *(30),* and in *S. tuberosum* it was found to be encoded by a small multigene family *(23).* A 234-bp cDNA sequence that codes for tomato *MCPI* and shares an average overall homology of 84.9% with the potato cDNA has also been reported *(21).* These inhibitors are encoded as pre-proproteins *(30),* and although they share a high percentage of homology in the region corresponding to the mature inhibitor, the region encoding the N-terminal prodomain (nucleotides 96 through 294 of the potato cDNA and nucleotides 58 through 173 of the tomato cDNA, respectively) is much less conserved, showing only 61.8% identity between the two genes (Fig. 1). The lower percentage of homology observed in this region essentially results from three small in-frame insertions of 27, 9, and 3 bp in the potato *PCI* gene. Such insertions allowed the design of specific primers and TaqMan probes for both the tomato and the potato sequences (Fig. 1 and Table 1). None of the designed primers or the TaqMan probes matched any plant DNA sequence other than the corresponding tomato or potato *MCPI* or *PCI* genes when they were compared with the GenBank databases with the use of the *blastn* software. The primer pair *PATF-PATR* amplifies a fragment of 104 bp from the potato *PCI* gene, whereas the primer pair *TOMF-TOMR* amplifies a fragment of 114 bp from the tomato *MCPI* homolog. Both amplification products are in a size range considered optimal for real-time PCR amplification and are easy to detect in agarose gels.

We developed conventional PCR systems based on the primer pairs *PATF-PATR* and *TOMF-TOMR* for the specific detection and identification of potato and tomato DNA. In addition, we developed real-time quantitative PCR systems based on the same primer pairs in combination with the TaqMan probes *PATP* and *TOMP,* respectively, for potato and tomato DNA quantification.

Conventional PCR systems were optimized for primer concentrations within a range of $MgCl_2$ concentrations and annealing temperatures. Optimal PCR conditions (i.e., the conditions yielding the largest amount of amplification product for potato or tomato DNA and no detectable amplification product in no-template controls) were those described in "Materials and Methods." Also, we optimized both real-time PCR systems for primer and TaqMan probe concentrations with different $MgCl_2$ concentrations. The optimal conditions described in "Materials and Methods" are those that produced the smallest C_T together with the largest final increase in normalized fluorescence at the lowest concentration of primers and probe.

Species specificity and intraspecies conservation of the reference gene fragment. Once PCR conditions had been established, it was important to assess the ability of the developed PCR systems to detect potato and tomato DNA without cross-reacting with DNA from other plant species. With this aim, we evaluated conventional and real-time PCR reactions for 100 ng of DNA template obtained from 20 different plant species, including potatoes and tomatoes, 10 dicot species (*Arabidopsis thaliana,* cabbage, cauliflower, lentils, mung beans, rapeseed, soybeans, sunflowers, sweet potatoes, white beans, and lupines), and 6 monocot species (maize, millet, rice, rye, sorghum, and wheat). Considering the sensitivity of the method (see below) and the genome sizes for the different species assayed *(4),* 100 ng was deemed to be enough template DNA to detect any existing cross-amplification in the other plant species tested. Conventional and real-time PCR systems for both potatoes and tomatoes detected only potato and tomato DNAs, respectively, and did not amplify the other DNA templates. Also, the PCR systems developed for potatoes detected only potato DNA and did not amplify DNA from tomatoes. Conversely, the PCR systems developed for tomatoes did not recognize the potato DNA to any extent (data not shown).

To further assess the suitability of these methods, we tested their effectiveness in amplifying DNA from different potato and tomato cultivars. We observed that the lack of cross-reactivity between the potato and tomato genomes was preserved for the different cultivars, a finding that further corroborates the specificity of the methods. Conventional and real-time potato- and tomato-specific PCR analyses with 100 ng of template DNA extracted from 18 potato cultivars or 14 tomato cultivars were evaluated. The PCR systems developed for potatoes detected all of the potato cultivars but did not detect any of the tomato cultivars (data not shown). Likewise, the tomato PCR systems detected all of the tomato cultivars and did not amplify any of the potato DNAs, thus confirming the lack of cross-reactivity of these PCR systems. These results demonstrate that the PCR systems developed in the present study are highly species specific, with the specificity of the real-time PCR systems relying not only on the primers (as is the case for conventional PCR), but also on the probe.

Amplification products of the same size and intensity were obtained for all conventional PCR reactions for potatoes or tomatoes (data not shown). These results indicate that the amplified *PCI* or *MCPI* fragments do not exhibit major differences between cultivars at the nucleotide sequence level. In addition, we investigated whether the copy numbers of the *PCI* and *MCPI* genes vary among potato or tomato cultivars. If these copy numbers did vary, amplification efficiencies would vary among cultivars, thus compromising the suitability of the methods developed in the present study for quantification. Potato and tomato real-time PCR reactions were performed for four replicates of

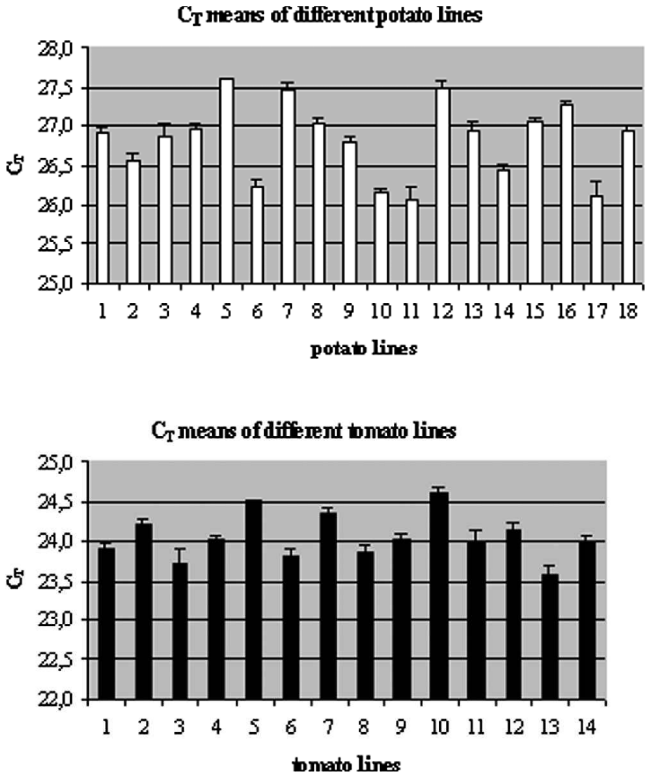

FIGURE 2. *Mean* C_T *values for four replicate real-time PCR reactions carried out for 18 potato and 14 tomato cultivars. Potato lines: (1) Ackensegen, (2) Bamberger Hörnchen, (3) Roseval, (4) Jubel, (5) Edzell Blue, (6) Aura, (7) Shetland Black, (8) Hermanns Blaue, (9) Pink fir Apple, (10) Naglerner Kipfler, (11) Vitelotte, (12) Sharon Blue, (13) Reichskanzler, (14) Piroschka, (15) Highland Burgundy Red, (16) Flava, (17) Hindenburg, and (18) Mamantambo. Tomato lines: (1)* L. hirsutum, *(2) Corbarino, (3) Orange Queen, (4) Yellow Stuffer, (5) Momor, (6) Noire de Crimée, (7)* L. pimpinellifolium, *(8) Valparaiso, (9)* L. peruvianum, *(10) Marmande V.R., (11) Naines Pomor, (12) Médine, (13) Flora Dade, and (14) Ohmiya Suncherry.*

each of the different cultivars, and mean C_T values were calculated for each of the cultivars. As can be seen in Figure 2, mean C_T values for the 18 potato cultivars were within a range of 1.5 cycles, and the overall standard deviation was 0.48 cycles. Likewise, mean C_T values for the 14 tomato cultivars were within a range of 0.9 cycles, with a standard deviation of 0.30 cycles. These results demonstrate that copy number does not sensibly vary among cultivars and that similar rates of amplification are obtained for the different template DNAs. Therefore, we can conclude that both PCR systems are suitable for the accurate identification and quantification of potato or tomato DNA irrespective of the particular cultivar or variety involved.

Determination of the detection and quantification limits of the assays. To determine the detection limits of the conventional and real-time PCR systems developed for potatoes and tomatoes, we obtained two plasmids, designated *pPAT* and *pTOM,* that contained, respectively, inserted single copies of the *PCI* potato and *MCPI* tomato PCR products (see "Materials and Methods"). These plasmid constructs were later used to build calibration curves for quantification for real-time PCR systems and were used as external quantification standards in the amplification procedures. On the basis of plasmid size (3,119 bp for *pPAT* and 3,129 bp for *pTOM*), one plasmid molecule corresponds to 3.44 and 3.45 ag of the *pPAT* and *pTOM* plasmid DNAs, respectively, thus allowing a very accurate determination of the number of molecules present in each PCR reaction. We prepared serial dilutions of both plasmid DNA templates to yield 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10, 4, 2, and 1 target DNA molecules per PCR reaction and ran conventional and real-time PCR reactions across the entire range of DNA dilutions.

With conventional PCR reactions, we were able to accurately detect the corresponding potato *PCI* or tomato *MCPI* templates with as few as 10 molecules per reaction, with amplification products being consistently obtained at this DNA dilution for all reaction replicates (data not shown). Dilutions down to 2 target DNA molecules per PCR reaction yielded amplification products for only one of three replicates, while the detection of 1 molecule was largely inaccurate.

For quantitative analyses, several sample replicates were prepared for each DNA dilution, and a standard curve was constructed by plotting the C_T values obtained in each real-time PCR reaction against the known amount of template present in the sample. Our experimental design resulted in a very efficient rate of amplification, as indicated by the slopes for the linear regression analysis (-3.409 for potatoes and -3.234 for tomatoes) and the very good correlation coefficients (1 and 0.998, respectively) obtained, thus demonstrating good precision and accuracy across a wide range of concentrations of target molecules. As can be seen in Table 2, real-time PCR systems for both tomatoes and potatoes allowed the detection of as few as 10 molecules of target DNA for all nine replicate reactions carried out in three independent experiments. Moreover, 1 molecule was detected in seven of nine replicate reactions (78% of the replicates) with the two real-time PCR systems. Thus, similar results were obtained with real-time PCR systems for both potatoes and tomatoes in terms of absolute detection limits, with a threshold value of 10 target DNA molecules being detected in all replicate reactions and with 1 molecule being detected in about 80% of the replicate reactions.

As expected, fluctuations among replicate PCR reactions increased with decreasing amounts of target DNA, with the confidence intervals for dilutions containing 10, 4, 2, and 1 copies of target DNA overlapping with each other (Table 2). Accordingly, we can conclude that up to >10 copies of target DNA molecules can be reliably quantified at the 95% confidence level for both potato and tomato plasmid standards. Taken together, these results confirm two advantages of the real-time PCR method over the conventional one, i.e., higher absolute detection limits and quantification across a wider range of DNA concentrations.

Identification of potatoes and tomatoes in food products. To assay the suitability of the PCR systems developed in the present study for the analysis of processed

TABLE 2. *Real-time PCR potato and tomato assays: detection and quantification limits obtained with the corresponding plasmid standards after statistical analysis for the determination of practical quantification limits*[a]

Parameter	Value of parameter for no. of template molecules								
	10^7	10^6	10^5	10^4	1,000	100	10	4	2	1
Lower confidence limit	9,993,623	998,023	99,380	9,804	938	80	4	1	0	0
Upper confidence limit	10,006,026	1,001,945	100,619	10,196	1,062	120	16	8	5	3
Plasmid *pPAT*										
Signal ratio (positive signal/total reactions)	9/9	9/9	9/9	9/9	9/9	9/9	9/9	7/9	6/9	7/9
Mean C_T	17.60	20.76	23.74	27.74	31.28	35.10	38.42	41.83	43.17	41.98
SD for C_T	0.06	0.08	0.08	0.15	0.08	0.41	1.16	4.79	5.13	4.73
Plasmid *pTOM*										
Signal ratio (positive signal/total reactions)	9/9	9/9	9/9	9/9	9/9	9/9	9/9	8/9	8/9	7/9
Mean C_T	17.07	20.43	23.22	27.57	30.90	34.33	36.20	37.83	37.38	39.75
SD for C_T	0.11	0.13	0.08	0.24	0.20	0.49	1.35	4.76	5.15	5.66

[a] C_T, threshold cycle value. Lower and upper confidence limits were calculated for a 95% confidence level.

food samples, we carried out both conventional and quantitative assays of DNA extracted from different food products available at the local market. Potato-derived food products like instant potato flakes, instant green soup, frozen French fries, Spanish omelets, and potato croquettes and tomato products like tomato juice, tomato ketchup, tomato sauce, and Spanish gazpacho were selected for analysis along with food products containing potato starch as a thickener, such as beef stock, precooked meatballs, and instant cream of seafood soup, and food products containing tomatoes as an ingredient, such as pizza, squid in cocktail sauce, instant cream of seafood soup, sardines in tomato sauce, precooked meatballs, and beef stock. Data presented in Table 3 indicate that the use of conventional and real-time PCR reactions enabled us to amplify a *PATF-PATR*–specific PCR product in all PCR reactions run on DNA extracted from potato food products with the exception of processed food containing potato starch. Likewise, a specific *TOMF-TOMR* PCR product was obtained in many of the PCR reactions run on DNA extracted from tomato food products (e.g., tomato juice) or from food products containing tomatoes as an ingredient (e.g., pizza, instant cream of seafood soup, and beef stock). We were unable to obtain amplification products from food samples subjected to harsh cooking conditions like those used for the preparation of tomato sauce or precooked meatballs.

Interestingly, we were also unable to detect any amplification product for food samples including vinegar as a component whether these food products had been subjected to precooking conditions (squid in tomato cocktail sauce and tomato ketchup) or were intended for direct consumption uncooked (Spanish gazpacho). To test whether the inability of our method to generate PCR products from these processed food samples was due to the presence of PCR inhibitors in the DNA solutions, we ran real-time PCR reactions specific for the control plasmid *pMON3 (18)*; these reactions involved 100 copies of the target plasmid DNA together with either water or a solution containing DNA extracted from the food. In these assays, all reactions showed very similar C_T values (mean C_T = 31.60 cycles, with a standard deviation of 0.64 cycles) except for the reaction involving DNA extracted from sardines in tomato sauce. The C_T value obtained for this DNA was 9 cycles above the mean, which indicates the presence of PCR inhibitors in this sample. Therefore, whereas inhibition was not observed in the rest of the reactions, a component with PCR-inhibitory activity appears to copurify with the DNA from the sample of sardines in tomato sauce. We further assessed these results by running real-time PCR reactions specific for tomatoes and potatoes for these problematic template DNAs in the presence of 5,000 copies of the *pPAT* or *pTOM* plasmid. Again, similar C_T values (mean C_T = 28.82 cycles, with a standard deviation of 0.70) were obtained for all samples with the exception of sardines in tomato sauce (mean C_T = 37.44 cycles). These results suggest that the negative results obtained for food samples such as precooked meatballs, tomato ketchup, tomato sauce, Spanish gazpacho, and squid in cocktail sauce resulted not from the presence of inhibitors in the extracted DNA, but

TABLE 3. *Analysis of food products with the described potato and tomato PCR systems*

Food products	Results for conventional amplification		Results for real-time amplification	
	Potato	Tomato	Potato	Tomato
Products containing potatoes				
Instant potato flakes	+	−	+	−
Instant green soup	+	−	+	−
Instant cream of seafood soup	−	+	−	+
Precooked meatballs	−	−	−	−
Beef stock	−	+	−	+
Spanish omelet	+	−	+	−
Potato croquettes	+	−	+	−
Frozen French fries	+	−	+	−
Products containing tomatoes				
Pizza	−	−	−	±
Tomato ketchup	−	−	−	−
Tomato sauce	−	−	−	−
Spanish gazpacho	−	−	−	−
Tomato juice	−	+	−	+
Squid in cocktail sauce	−	−	−	−
Instant cream of seafood soup	−	+	−	+
Sardines in tomato sauce	−	−	−	−
Precooked meatballs	−	−	−	−
Beef stock	−	±	−	+

from an inability to extract sufficient DNA from these samples. These results agree with those obtained by other laboratories *(2)* and indicate a low tolerance of DNA for an acidic pH, which leads to rapid depurination and fragmentation of the DNA molecule, rendering it unsuitable for use as a template for PCR amplification.

Aside from these particularly difficult samples, we were able to amplify tomato and potato DNAs from multiple processed food samples. The small amplicon size required for TaqMan chemistry is in part responsible for the successful outcomes of these analyses. Moreover, the high sensitivity of the real-time PCR methods we developed further contributed to the adequacy of these methods for the identification of potato and tomato DNA in food samples expected to contain very tiny amounts of DNA, which explains, for example, why we were able to detect DNA in pizza and in beef stock by real-time PCR reactions but not by conventional PCR reactions. In conclusion, these methods proved to work well even for food samples that are expected to contain large amounts of fragmented DNA resulting from high-pressure and high-temperature processing. Therefore, under the specified conditions, the sequences we describe are highly suitable for use as potato and tomato endogenous reference genes and, in combination with GMO event-specific primers, for use in PCR analysis aimed at the detection and quantification of genetically modified potato and tomato DNA in processed food samples.

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