JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Real-Time Polymerase Chain Reaction Based Assays for Quantitative Detection of Barley, Rice, Sunflower, and Wheat

Marta Hernández,[†] Teresa Esteve, and Maria Pla*

Molecular Genetics Department, Institut de Biologia Molecular de Barcelona (IBMB), Consejo Superior de Investigaciones Científicas, Jordi Girona Salgado 18-26, 08034 Barcelona, Spain

Quality assurance is a major issue in the food industry. The authenticity of food ingredients and their traceability are required by consumers and authorities. Plant species such as barley (*Hordeum vulgare*), rice (*Oryza sativa*), sunflower (*Helianthus annuus*), and wheat (*Triticum aestivum*) are very common among the ingredients of many processed food products; therefore the development of specific assays for their specific detection and quantification are needed. Furthermore, the production and trade of genetically modified lines from an increasing number of plant species brings about the need for control within research, environmental risk assessment, labeling/legal, and consumers' information purposes. We report here the development of four independent real-time polymerase chain reaction (PCR) assays suitable for identification and quantification of four plant species (barley, rice, sunflower, and wheat). These assays target *γ-hordein*, *gos9*, *helianthinin*, and *acetyl-CoA carboxylase* sequences, respectively, and were able to specifically detect and quantify DNA from the target plant species. In addition, the simultaneous amplification of *RALyase* allowed bread from durum wheat to be distinguished. Limits of detection were 1 genome copy for barley, sunflower, and wheat and 3.3 copies for rice real-time PCR systems, whereas limits of quantification were 10 genome copies for barley, sunflower, or wheat and approximately 100 haploid genomes for rice real-time PCR systems. Real-time PCR cycling conditions of the four assays were stated as standard to facilitate their use in routine laboratory analyses. The assays were finally adapted to conventional PCR for detection purposes, with the exception of the wheat assay, which detects rye simultaneously with similar sensitivity in an agarose gel.

KEYWORDS: *Hordeum vulgare*; barley; *Oryza sativa*; rice; *Helianthus annuus*; sunflower; *Triticum aestivum*; wheat; PCR; real-time PCR; endogenous reference control; GMO

INTRODUCTION

By fraudulently labeling and selling low quality products as premium products, the food industry has lost significant amounts of money and consumer confidence. An essential prerequisite for quality control in the food industry is the availability of tests to confirm the origin of ingredients used in processed foodstuffs. Traceability is an important commercial topic, and it is necessary to preserve consumers' confidence and to satisfy mandatory regulations (*1*, *2*). Therefore, the ability of methods to discriminate the origin of feed and food ingredients and food sources is of prime importance. In particular, tracking genetically modified organisms (GMO) within the environment and the food chain is fundamental for environmental risk assessment and production and trade of genetically modified (GM) crops. Specific regulations in some countries establish the compulsory

labeling of foods containing authorized GMOs above determined thresholds (*3*).

Because DNA is more stable than other molecules, e.g., proteins, to physical and chemical food processing treatments, DNA-based tests have proven to be very useful to authenticate the species used in foodstuffs production. In particular, real-time (RTi) polymerase chain reaction (PCR) is a powerful specific, accurate, and sensitive technique for the quantification of nucleic acids by monitoring the increase of product through the fluorescence detection during the whole process (*4*). PCR and RTi-PCR have been used for the analysis of plant species, i.e., rice (*5*), maize (*6*−*9*), soybean (*10*, *11*), rapeseed (*12*−*14*), cotton (*15*), tomato (*16*, *17*), and potato (*16*, *18*), for the detection and quantification of many GMO events (*19*−*25*) and for the determination of transgene copy number especially in biolistic transformed plants (*26*, *27*). Quantification by RTi-PCR is based on extrapolation to a regression curve constructed with calibrators at known concentrations. For GMO analysis, both matrix-based and pure analyte reference materials (RM) have been used (*28*, *29*). Quantification of transgenic DNA requires the analysis of species-specific reference genes that

* Corresponding author. E-mail: mpsgmp@ibmb.csic.es.
† Current address: Laboratory of Molecular Biology, Instituto Tecnológico Agrario de Castilla y León, Subdirección de Investigación y Tecnología, Junta de Castilla y León, Ctra. Burgos km.119, Finca Zamadueñas, 47071 Valladolid, Spain. Tel.: +34 983 414328. Fax: +34 983 414780.

Table 1. Target Genes and Oligonucleotides

plant species	gene	acc. no.	ref	orientation	name	sequence (5′–3′)	amplicon (bp)
barley	*γ-hordein*	M36378	28	forward	hor1	AGACAAGGCGTGCAGATCG	73
				reverse	hor2	GACCCTGGACGAGCACACAT	
				probe[a]	horp	CCTCAGCCGCAACAGGTGGGTC	
rice	*oryzainβ*	D90407	43	forward	orb1	CGCCGCGTTCCTGCT	71
				reverse	orb2	CGTTGTAGGAGATGATCGACATG	
				probe[b]	orbp	CTCATCGTCGTTGGTCACCGCG	
	gos9	X51909	29	forward	org1	TTAGCCTCCCGCTGCAGA	68
				reverse	org2	AGAGTCCACAAGTGCTCCCG	
				probe[b]	orgp	CGGCAGTGTGGTTGGTTTCTTCGG	
wheat	*acc1*	AF029895	31	forward	tri1	TGCCCATTGTCGGCCTTA	93
				reverse	tri2	GCATTCCAACCATCTGCCC	
				probe[a]	tri12 p	TGCCTCGACAACACCATCGCTATCC	
	RALyase	AB032124	44	forward	tri3	GGAGCGGTTGCTGTTGGA	68
				reverse	tri4	GCCTCGGTGCATTAATAATCTTG	
				probe[b]	tri34 p	CGATTGGCGGTGCCTCTACTCACG	
sunflower	*hel*	M28832	30	forward	hel1	CTCGAGCACCTCCGGCT	60
				reverse	hel2	AGCGTGGAAAGAGGCGAACTCCG	
				probe[a]	help	GGATTGGATGGCATTCGG	
				forward	hel1	CTCGAGCACCTCCGGCT	289[c]
				reverse	hel3	GCCCTGCAAGGTTTGCTATC	

[a] Dual-labeled fluorescent TaqMan probe (FAM as reporter, TAMRA as quencher). [b] Dual-labeled fluorescent TaqMan probe (VIC as reporter, TAMRA as quencher). [c] Southern probe.

provide an estimation of the total amount of the DNA from that plant species; hence quantification can be calculated as GMO proportion. Target sequences for plant RTi-PCR assays should be species-specific, conserved in different cultivars, present in low copy number, and should not be subjected to genetic manipulation.

Species such as barley, rice, sunflower, and wheat are the subject of genetic manipulations for research and commercial purposes. For example, two rice (CL121, CL141, CFX51 from BASF Inc. and LLRICE06, LLRICE62 from Aventis Crop Science), one sunflower (X81359 from BASF Inc.), and two wheat (AP602CL from BASF Inc. and SWP965001 from Cyanamid Crop Protection) herbicide tolerant GM varieties have been authorized for commercialization (www.agbios.com).

Here we report four RTi-PCR systems for the quantitative identification of barley, rice, sunflower, and wheat DNA in food products. They target the barley *γ-hordein* gene which encodes a storage protein (*30*), the rice *gos9* gene (*31*), the sunflower *hel* gene encoding the HELIANTHIN or 11S storage protein (*32*), and the wheat acetyl-CoA carboxylase gene (*acc1*) (*33*). The assays were capable to determine the presence of small amounts of barley, rice, sunflower, and wheat DNA in commercial food samples and might be useful for use as species-specific reference controls for transgene quantification purposes.

MATERIALS AND METHODS

Materials. *Arabidopsis thaliana*, barley (*Hordeum vulgare*), cauliflower (*Brassica oleracea*), lentils (*Lens* esculenta), lupine (*Lupinus albus*), maize (*Zea mays*), millet (*Panicum miliaceum*), mung bean (*Phaseolus* aureus), oat (*Avena sativa*), potato (*Solanum tuberosum*), rapeseed (*Brassica napus*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*), soybean (*Glycine max*), sunflower (*Helianthus annuus)*, teosinte (*Zea diploperennis*), tomato (*Lycopersicon esculentum*), wheat (*Triticum aestivum*), and white bean (*Vicia faba*) plants were grown in green houses at the IBMB-CSIC.

Twenty-six sunflower varieties were kindly provided by Jose M. Fernández Martínez from the Instituto de Agricultura Sostenible (CSIC, Córdoba, Spain): Aitana, Albany, Arpon, Attila, Coban, Coriolis, Coro, Deborah, Espanil, Florasol, Indiana, Jalón, K-69, Kasol, Lanzador, Medallón, Megasun, Mikado, PAU-9701, Rodrigo, Sanbro, Saxo, Sofia, Sunko, Turbo, and Xistral. The 20 nontransgenic rice varieties were

provided by GeneScan (Freiburg, Germany): Arc 11960, BaSmati 370, Biron, Boewani, Bluebonnet, Chokoto, Dinorado, Gbitu and Takmaru Huk zo dou, Jarneri, Jempu-djempel, Khao ay, Na ma salay, Perurutong b, Santa amelia, Sudhubalawee, Taipei 309, Tieu be, and Tsou-yuen. Fifteen different barley varieties provided and cultivated in our green houses were also analyzed: Arlois, Barbarossa, Beka, Garbo, Germania, Graphic, Hispanic, Kym, Majestic, Orla, Orria, Trebon, Volga, Wisa, and Zaida. Finally, 18 bread wheat varieties (i.e., Adalid, Anza, Aragon-03, Astral, Babui, Bancal, Bonpain, Cartaza, Gazul, Isengrein, Marius, Pinzon, Recital, Rinconada, Sarina, Soisson, Tremie, and Yecora) and 10 durum wheat varieties (i.e., Alteraos, Antón, Bolido, Borla, Jabato, Mexa, Roqueño, Sula, Valira, and Vitron) were used.

Twelve different food products were purchased at the local market: plaited bread sticks, wheat germ, whole wheat bran, biscuits, multi-cereal cakes, rice-based drink, soluble extract of cereals and chicory, beer extra quality, refined sunflower oil, breakfast cereals, bread, and wholemeal bread.

DNA Extraction. Large-scale genomic DNA extraction from 30 g of leaves of sunflower and maize (cultivar W64) was done according to a method published by Dellaporta et al. (*34*). Small-scale DNA extraction was done using a CTAB-based method (*35*). DNA was quantified using the spectrophotometer GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). DNA from food samples was extracted using the CTAB-based method previously reported (*36*).

Oligonucleotides. Oligonucleotide primers and probes (**Table 1**) were designed using the Primer Express v1.5 software (Applied Biosystems Division of Perkin-Elmer Corp., Foster City, CA). They were synthesized by MWG-Biotech AG (Ebensburg, Germany). TaqMan probes were labeled on the 5' end with the fluorescent 6-carboxyfluorescein (FAM) or VIC reporter dye and with the quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) attached to its 3' end. Primer pairs can be used for qualitative PCR and in combination with the TaqMan probe for quantitative RTi-PCR.

PCR Conditions. Qualitative PCRs were run in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus Instruments, Emeryville, CA) using the following cycle conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 20 s at 95 °C, and 1 min at 60 °C for rice and wheat, 1 min at 62 °C for barley, 1 min at 65 °C for sunflower, followed by 5 min of elongation at 72 °C. Each reaction contained: $1\times$ buffer II; 200 μM each dATP, dCTP, and dGTP; 400 μM dUTP; 1 unit of AmpliTaq gold DNA polymerase (Applied Biosystems); and 0.2 units of AmpErase uracil N-glycosylase. Specific conditions were: 3 mM $MgCl_2$ (except for barley 1.5 mM) and 300 nM of each primer (see **Table 1**).

Barley, Rice, Sunflower, and Wheat Real-Time PCR

J. Agric. Food Chem., Vol. 53, No. 18, 2005 **7005**

Amplification products were analyzed by 2% agarose gel electrophoresis (in $1 \times$ TAE) and stained with ethidium bromide.

RTi-PCRs were run on an ABI PRISM7700 sequence detection system platform (Applied Biosystems division of Perkin-Elmer Corp., Foster City, CA) using the following standard program: 2 min at 50 °C, 10 min at 95 °C, 50 cycles of 15 s at 95 °C, and 1 min at 60 °C. Unless stated, all RTi-PCR were performed in triplicate using the TaqMan PCR core reagents (Applied Biosystems-Roche Molecular Systems Inc., Branchburg, NJ) in 20 μL final volume. Concentrations of primers and probes were optimized under a range of different $MgCl_2$ concentrations. Each reaction contained: $1 \times$ buffer A (including ROX as a passive reference dye); 200 μM each dATP, dCTP, and dGTP; 400 μM dUTP; 1 unit of AmpliTaq gold DNA polymerase; 0.2 units of AmpErase uracil *N*-glycosylase; and 2 μL of DNA solution. Specific conditions were: 6 mM $MgCl_2$ (except for sunflower 6.5 mM), 300 nM of each primer and 200 nM probe for sunflower and rice, and 150 nM probe for barley and wheat (see **Table 1**).

Duplex RTi-PCR assay combined in a single reaction tube the *RALyase* oligonucleotides with those specific for wheat based on the *acc1* gene by using the standard thermocycling program and the following PCR conditions: $1 \times$ buffer A, 6 mM $MgCl_2$, 200 μM dNTPs, 1 unit of AmpliTaq gold DNA polymerase, 0.2 units of AmpErase uracil *N*-glycosylase, 300 nM each *tri1* and *tri2*, 100 nM *tri12p*, 50 nM each *tri3* and *tri4*, 100 nM *tri34p* and 2 μL of DNA solution. TaqMan probes *tri12p* and *tri34p* were labeled with different reporter dyes, i.e., VIC and FAM, respectively, which allowed independent analysis of *RALyase* and *acc1* amplification.

RTi-PCR products were analyzed using the Sequence Detection System software 1.7 (Applied Biosystems). Fluorescence signals obtained were continuously measured once per cycle at the extension step.

Specificity, Limit of Detection (LOD), and Limit of Quantification (LOQ). The specificity was tested using 100 ng of each plant species and variety. The sensitivity of the PCR assays was tested using genomic DNA of the corresponding plant species serially diluted in 25 ng/μL BSA to give 10^4, 10^3, 10^2, 33, 10, 4, and 2 copies of genomic DNA per PCR reaction. Standard curves were constructed using the same DNA solutions. According to Arumuganathan and Earle (*37*), 5.05 pg of barley, 0.46 pg of rice, 3.14 pg of sunflower, or 16.55 pg of wheat genomic DNA were considered one haploid genome copy. Three replicates were performed for each reaction containing 10^5 to 10 initial genome copies, and at least 10 replicates were performed for each reaction containing 4 or 2 initial template copies. For rice, 10 replicates of 100, 33, and 10 copies per reaction were also tested.

Southern Blot. Since barley γ-*hordein*, wheat *acc1*, and rice *gos9* genes are reported as low copy number genes, the copy number of our sunflower target gene was tested by Southern blot. Sunflower genomic (10 μg) DNA and 13 μg of maize genomic DNA were digested to completion with *Eco*RI and *Hind*III. The fragments were separated by 0.9% w/v agarose gel electrophoresis at 60 V for 5 h 30 min and blotted onto Hybond-N+ filters (Amersham Pharmacia Biotech, Piscataway, NJ). Filter was incubated in hybridization buffer (1% BSA, 1 mM EDTA, 0.125 M $NaHPO_4$ pH 7.2, 7% SDS) at 65 °C for 16 h with the α^{32}P-radiolabeled *hel1*/*hel3* PCR 289-bp product (**Table 1**). PCR products were labeled using a Random Primed DNA kit (Roche Diagnostics GmbH, Mannheim, Germany). The filters were washed three times at 65 °C for 20 min with sufficient volumes of Church wash buffer (1 mM EDTA, 0.02 M $NaHPO_4$ pH 7.2, 1% SDS) and finally exposed to X-ray films (Kodak X-Omat AR film and intensifying screen) for one week.

RESULTS AND DISCUSSION

Selection of Suitable Genes for PCR- and RTi-PCR-Based Detection and Quantification of Barley, Rice, Sunflower, and Wheat. Suitable endogenous reference genes were selected from public databases (GenBank) by searching for sequences belonging to low copy number gene families and not subjected to genetic manipulation. Plant storage proteins are known to be highly species specific and are the target of various endogenous

reference RTi-PCR assays (e.g., *zein* for maize, *lectin* for soybean) (*6*). With this criterion, we considered barley γ-*hordein*, rice *orizainβ* gene, which encodes a cystein protease (*38*), sunflower *helianthinin*, and wheat *gliadin* as candidate targets. *In silico* analyses of these sequences were performed (using the BLASTN software) aiming at the selection of exonic gene regions with enough interspecific divergence to design suitable primers and probe sets. This was possible for γ-*hordein* (acc. no. M36378), *orizainβ* (acc. no. D90407), and *helianthinin* (acc. no. M28832); however *gliadin* was not conserved among wheat cultivars (especially durum vs bread), and therefore it was discarded. Instead, we selected an *in silico* wheat-specific region of the low copy-number *acetyl-CoA carboxylase* (*acc1*) gene.

We designed primers and probes on the selected sequences (**Table 1**) and conducted preliminary experiments for detection of target DNA from different cultivars in both conventional and RTi-PCR assays. In contrast to γ-*hordein*-, *helianthinin*-, and *acc1*-, the *orizainβ*-based RTi-PCR system was not capable of producing any fluorescent signal from a number of rice cultivars, whereas amplification was observed when using conventional PCR. Further sequencing of the *orizainβ* gene fragment corresponding to the *orbp* probe from a selection of rice cultivars showed strong nucleotide variability (i.e., up to 9 mismatches in 32 nt), which can explain the false negative RTi-PCR results observed. Therefore, the *orizainβ*-based assay was not suitable for our purposes. The low copy number *gos9* gene was then selected as an alternative rice target, which subsequently proved to fulfill the requirements.

Optimization of the PCR and RTi-PCR Assays. Once the suitability of the different targets was established, we optimized both conventional and RTi-PCR conditions for the following systems: γ-*hordein* for barley, *gos9* for rice, *helianthinin* for sunflower, and *acc1* for wheat. Optimal conditions are described in the Materials and Methods section. Standard cycling conditions for RTi-PCR as recommended by the manufacturer of the PCR device were used to facilitate multiplexing and routine quantification of different genes in the same PCR run.

Species Specificity and Allelic Variability of the Assays. The specificity of the assays was evaluated by running RTi-PCR reactions containing 100 ng of DNA from 20 different plant species either phylogenetically related to the target species or frequently found in food. No amplification was observed with any of the species tested other than the targets (i.e., 100% exclusivity). Conventional reactions were equally specific, with the only exception of wheat assays which also amplified rye genomic DNA (data not shown). Wheat, rye, and also barley are phylogenetically closely related species as they all belong to the *Triticeae* tribe. The scarce genetic information available from rye can explain the promising *in silico* results with the *acc1* gene. Additional conventional PCR experiments targeting other candidate wheat genes, e.g., *RALyase* (*39*) (**Table 1**), produced rye and also barley positive amplification (data not shown). As it occurred with *acc1*-based assays, *RALyase* RTi-PCR did not show any cross reaction with these or other species.

A reliable endogenous reference gene should not exhibit allelic variation, and it should ideally have a constant number of copies per haploid genome across different cultivars of the target species (*12*). We performed both conventional and RTi-PCRs based on the γ-*hordein*, *gos9*, *helianthinin*, and *acc1* genes on 100 ng of DNA from 15 barley, 20 rice, 26 sunflower, or 28 wheat (18 durum and 10 bread wheat) cultivars, respectively. For all four targets, a product of the expected size and similar intensity was obtained for all these cultivars after conventional PCR (data not shown). Likewise, RTi-PCRs produced similar
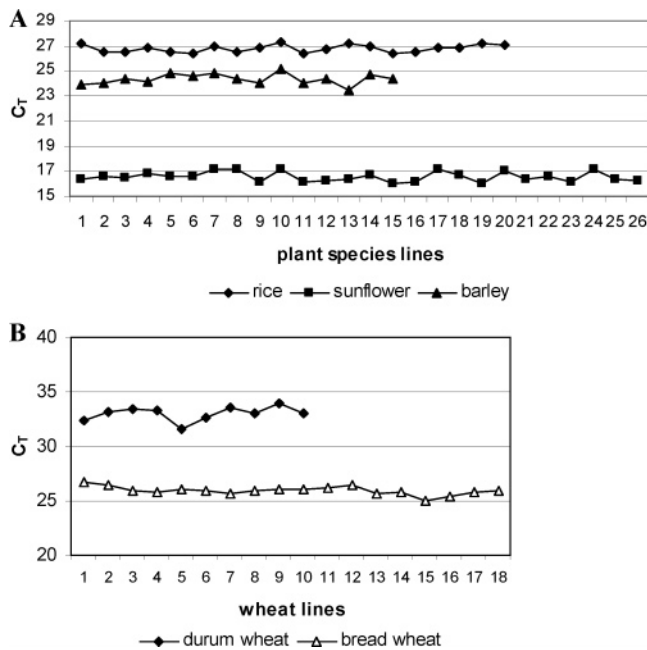
Figure 1. Average of C_T values obtained from triplicate RTi-PCRs performed using 100 ng of genomic DNA extracted from different lines of plant species. **(A)** From rice (20 varieties): 1, Perurutong b; 2, Huk zo dou; 3, Basmati 370; 4, Tieu be; 5, Tsou-yuen; 6, Sudhubalawee; 7, Jarneri; 8, Chokoto; 9, Boewani; 10, Arc 11960; 11, Khao ay; 12, Jempu-djempel; 13, Dinorado; 14, Biron; 15, Taipei 309; 16, Bluebonnet; 17, Santa amelia; 18, Na ma salay; 19, Gbitu; 20, Takmaru. From sunflower (26 varieties): 1, Aitana; 2, Albany; 3, Arpon; 4, Attila; 5, Coban; 6, Coriolis; 7, Coro; 8, Deborah; 9, Espanil; 10, Florasol; 11, Indiana; 12, Jalón; 13, K-69; 14, Kasol; 15, Lanzador; 16, Medallón; 17, Megasun; 18, Mikado; 19, PAU-9701; 20, Rodrigo; 21, Sanbro; 22, Saxo; 23, Sofia; 24, Sunko; 25, Turbo; 26, Xistral. From barley (15 varieties): 1, Arlois; 2, Barbarossa; 3, Beka; 4, Garbo; 5, Germania; 6, Graphic; 7, Hispanic; 8, Kym; 9, Majestic; 10, Orla; 11, Orria; 12, Trebon; 13, Volga; 14, Zaida; 15, Wisa. **(B)** From wheat (18 bread wheat varieties): 1, Adalid; 2, Anza; 3, Aragon-03; 4, Astral; 5, Babui; 6, Bancal; 7, Bonpain; 8, Cartaza; 9, Gazul; 10, Isengrein; 11, Marius; 12, Pinzon; 13, Recital; 14, Rinconada; 15, Sarina; 16, Soisson; 17, Tremie; 18, Yecora; (10 durum wheat varieties): 1, Alteraos; 2, Antón; 3, Bolido; 4, Borla; 5, Jabato; 6, Mexa; 7, Roqueño; 8, Sula; 9, Valira; 10, Vitron.

C_T values for γ-*hordein* (mean C_T, 24.36 ± 0.43), *gos9* (mean C_T, 26.80 ± 0.30), and *helianthinin* (mean C_T, 16.54 ± 0.39) in all tested barley, rice, and sunflower cultivars, respectively (**Figure 1A**). These variations could be attributed to either differences in the quality of the extracted DNA or especially to its quantification and were similar to those obtained for other previously reported RTi-PCR assays (*5, 9, 12, 16, 40–42*). These results indicate that the selected target gene regions exhibit similar copy number and sequence conservation among different cultivars. It should be noted that the target gene copy number per cell may vary with the ploidy level. Although no significant differences on C_T values could be detected among durum or bread wheat cultivars, the wheat *acc1*-based RTi-PCR assay produced statistically different (*p* < 0.01) results between durum (mean C_T, 32.98 ± 0.64) and bread (mean C_T, 25.93 ± 0.40) varieties, which results in the underestimation of DNA amount from durum cultivars (around 2 orders of magnitude) (**Figure 1B**). Especially for single-copy genes or small multigene families such as *acc1* (see later), this type of difference is indicative of mismatches within the target sequence (*40*). The combination of the *acc1* assay with another RTi-PCR system-capable to amplify genomic DNA from the two wheat genotypes
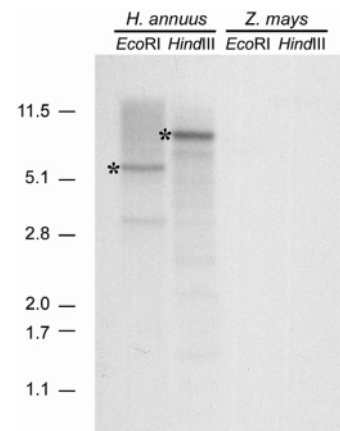

Figure 2. Southern blot analysis with genomic DNA from *Helianthus annuus* and *Zea mays*. Genomic DNA digested by *Eco*RI and *Hind*III was hybridized with a 289-bp *hel1/hel3* PCR product. Molecular-mass marker indicated on the left-hand side was run on the same gel. Note the absence of cross hybridization with maize DNA and the copy number of *hel* gene is very low.

with the same efficiency should allow distinguishing among durum and bread wheat. The *RALyase* RTi-PCR assay exhibited conserved C_T values for all tested bread and durum wheat lines (mean C_T, 25.24 ± 0.85). Therefore, the normalization of the *acc1* C_T values with those obtained by *RALyase* RTi-PCR assay should allow determining the genotype of wheat (bread vs durum) in a given sample. The combination of the *acc1* and the *RALyase* RTi-PCR assays was evaluated in duplex format with all 28 wheat lines and allowed clear distinction (*p* < 0.01) of the two wheat types (mean $\Delta C_T = 0 ± 0.39$ for bread wheat lines and mean $\Delta C_T = 6.25 ± 1.27$ for durum wheat lines).

Gene Copy Number. Single-copy genes are in general subjected to low rates of mutation and changes in the copy number among the different cultivars. Targeting a low copy number gene should make RTi-PCR assays less dependent on this type of putative intercultivar variation. Southern experiments previously reported establish barley γ-*hordein* and wheat *acc1* polypeptides as encoded by small multigene families (*30, 33*), and the rice genomic clone *gos*9 corresponds to 1–2 genes (*31*). We thus analyzed the copy number of the sunflower *hel* gene by Southern blot. After hybridization with a 289-bp PCR fragment (amplified with *hel1* and *hel3* primers, **Table 1**) which included the target sequence, a single major band was detected in the lanes corresponding to genomic DNA digested with *Eco*RI and *Hind*III (**Figure 2**). This is indicative of this sequence being either unique or present in low copy numbers within the haploid sunflower genome. As expected, no cross hybridization was detected with equivalent amounts of maize DNA.

LOD and LOQ. Decreasing amounts of genomic DNA from each plant species were subjected to RTi-PCR to assess the LOD and LOQ of the four developed RTi-PCR assays. Barley-, sunflower-, and wheat-specific RTi-PCR could detect down to two genomes, which suggests the targets are low copy number genes. The rice-specific assay was less performing i.e., it could consistently detect 100 haploid copies; being the lowest detectable amount 4 haploid copies in 10% of the replicates (**Table 2**). A linear regression among the starting amount of target DNA and C_T values was obtained for the four RTi-PCR systems, with regression coefficients (R^2) above 0.988 and slopes indicating efficiencies ($E = 10^{-1/s} - 1$) (*43*) above 80%. This demonstrates that there is a high correlation between the original amount of genomic DNA in the template and the RTi-PCR C_T value and

Barley, Rice, Sunflower, and Wheat Real-Time PCR

J. Agric. Food Chem., Vol. 53, No. 18, 2005 **7007**

Table 2. Determination of the Limits of Detection and Quantification of the Four RTi-PCR Systems

template haploid copy number	10000	1000	100	33	10	4	2
Oryza sativa ssp Indica var. IR36							
amount of DNA (pg)	4575	457.5	45.75	15.18	4.57	1.83	0.91
signal ratio[a]	3/3	3/3	10/10	8/10	2/10	1/10	0/10
mean C_T values[b]	29.43	33.33	37.24				
SD C_T values[c]	0.10	0.08	0.74				
Helianthus annuus var. Aitana							
amount of DNA (pg)	31 400	3140	314	103.6	31.4	12.56	6.28
signal ratio[a]	3/3	3/3	3/3	3/3	3/3	10/10	10/10
mean C_T values[b]	19.35	22.56	25.69	27.33	28.19	30.29	32.04
SD C_T values[c]	0.07	0.13	0.11	0.17	0.51	0.14	0.24
Hordeum vulgare var. Wisa							
amount of DNA (pg)	50 500	5050	505	166.65	50.50	20.20	10.10
signal ratio[a]	3/3	3/3	3/3	3/3	3/3	10/10	10/10
mean C_T values[b]	24.48	27.51	30.24	32.09	34.92	35.04	37.22
SD C_T values[c]	0.12	0.05	0.21	0.42	0.21	1.05	1.11
Triticum aestivum var. Astral							
amount of DNA (pg)	165 500	16 550	1655	546.15	165.50	66.20	33.10
signal ratio[a]	3/3	3/3	3/3	3/3	3/3	10/10	10/10
mean C_T values[b]	26.26	28.77	30.94	31.81	32.48	34.22	35.17
SD C_T values[c]	0.05	0.25	0.19	0.47	0.13	0.74	0.52

[a] Signal ratio corresponds to positive/total PCR reactions. [b] C_T refers to threshold cycle value. [c] SD is the standard deviation.

Table 3. Detection of Barley, Rice, Sunflower, and Wheat in Commercial Food Samples by RTi-PCR

food samples	RTi-PCR/label indication[a]			
	barley	rice	sunflower	wheat
beer	−/+	−/−	−/−	−/−
biscuits	−/−	+/+	−/−	+/+
bread	−/−	−/−	−/−	+/+
breakfast cereals	−/−	+/+	−/−	+/+
multicereal cakes	+/+	+/+	−/−	−/−
plaited bread sticks	−/−	−/−	−/−	+/+
refined oil	−/−	−/−	−/+	−/−
rice-based drink	−/−	+/+	−/−	−/−
soluble extract of cereals and chicory	−/+	−/−	−/−	−/+
wheat germ	−/−	−/−	−/−	+/+
whole wheat bran	−/−	−/−	−/−	+/+
wholemeal bread	−/−	−/−	−/−	+/+

[a] +, positive amplification; −, negative amplification; italicized are the food products for which the expected components could not be detected.

that the RTi-PCR performance of the four assays was adequate for our purposes.

The LOQ values were calculated based on the C_T values and confidence intervals, e.g., for barley, the 95% confidence interval for dilutions containing 10, 4, and 2 haploid genome copies overlap with each other meaning that a reliable quantification is not possible at such reduced levels of DNA. The estimation of the LOQ also requires taking into account the bias associated with serial dilutions of DNA samples for the particular experimental design, i.e., the probable range of haploid genome copies that would be sampled needs to be considered. This was performed by Monte Carlo simulations. The obtained LOD values were of 33 genome copies for *γ-hordein*, *hel*, and *acc1* and 100 genome copies for *gos9* with 95% confidence. These values are similar to previously reported RTi-PCR assays (*9*, *12*) and indicate that, in RTi-PCR analysis, the selected barley, rice, sunflower, and wheat target genes exhibit a reliable amplification linearity down to approximately 50, 100, 150, and 550 pg of template DNA, respectively.

Analysis of Food Samples. To ensure the practical use of these systems, we performed a number of assays to identify each of the four plant species in food products purchased at the local market (**Table 3**). Barley-, rice-, sunflower-, and wheat-specific RTi-PCR results totally agreed with the ingredients shown on the label of nine food products tested. Wheat DNA was detected in wheat germ, breakfast cereals, whole wheat bran, plaited bread sticks, biscuits, bread, and wholemeal bread; rice DNA was identified in breakfast cereals, multicereal cakes, biscuits, and rice-based drink; and barley DNA was detected in multicereal cakes. Remarkably, the target plant species was minor in the composition of some of these products (e.g., breakfast cereals, multicereal cakes), and some of these food samples were highly processed (e.g., bread, cakes, biscuits). From these, barley and wheat results have particular importance because of their gluten content, which is not tolerated by celiac patients (*44*). In contrast, determination of the presence of barley, rice, sunflower, or wheat could not be achieved in beer, refined oil, and soluble extract of cereals and chicory. These are three highly processed food matrixes from which sufficient quantities of DNA suitable for RTi-PCR could not be extracted. Spectrophotometrical determination of DNA concentration in these refined foods showed values below the limit of detection (approximately 5 ng/μL), further indicating that the paucity of target DNA extracted from these food products was the cause of the negative amplification results.

In conclusion, we have developed RTi-PCR systems suitable for detection, identification, and quantification of barley, rice, sunflower, and wheat DNA of unknown cultivars. They are specific, sensitive, and reliable and can be used to determine the presence of rice, wheat, sunflower, and barley ingredients in food samples. Therefore, these systems fulfill the requirements to be used as endogenous reference control for GMO analysis and determination of integrated copy number in transformed plants and can be considered as valuable tools for traceability purposes.

LITERATURE CITED

(1) Regulation (EC) no 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the european food safety authority and laying down procedures in matters of food safety. *Off. J. Eur. Communities: Legis.* **2003**, *L31*, 1−24.

(2) Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labeling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending directive 2001/18/EC. *Off. J. Eur. Communities: Legis.* **2003**, *L268*, 24−28.

(3) Regulation (EC) no 1829/2003 of the European Parliament and of the Council of 22 september 2003 on genetically modified food and feed. *Off. J. Eur. Communities: Legis.* **2003**, *L268*, 1−24.

(4) Hernández, M.; Rodríguez-Lázaro, D.; Ferrando, A. Current methodology for detection, identification and quantification of genetically modified organisms. *Curr. Anal. Chem.* **2005**, *1* (2), 203−221.

(5) Ding, J.; Jia, J.; Yang, L.; Wen, H.; Zhang, C.; Liu, W.; Zhang, D. Validation of a rice specific gene, sucrose phosphate synthase, used as the endogenous reference gene for qualitative and real-time quantitative PCR detection of transgenes. *J. Agric. Food Chem.* **2004**, *52*, 3372−3377.

7008 *J. Agric. Food Chem.,* Vol. 53, No. 18, 2005

Hernández et al.

(6) Vaitilingom, M.; Pijnenburg, H.; Gendre, F.; Brignon, P. Real-time quantitative PCR detection of genetically modified Maximizer maize and Roundup Ready soybean in some representative foods. *J. Agric. Food Chem.* **1999**, *47*, 5261−5266.

(7) Studer, E. I.; Dahinden, I.; Lüthy, J.; Hübner, P. Nachweis des gentechnisch veränderten "Maximizer" Mais mittels der Polymerase-Kettenreaktion (PCR). *Mitt. Geb. Lebensm. Hyg.* **1997**, *10*, 515−524.

(8) Vollenhofer, S.; Burg, K.; Schmidt, J.; Kroath, H. Genetically modified organisms in food-screening and specific detection by polymerase chain reaction. *J. Agric. Food Chem.* **1999**, *47*, 5038−5043.

(9) Hernandez, M.; Duplan, M. N.; Berthier, G.; Vaitilingom, M.; Hauser, W.; Freyer, R.; Pla, M.; Bertheau, Y. Development and comparison of four real-time polymerase chain reaction systems for specific detection and quantification of Zea mays L. *J. Agric. Food Chem.* **2004**, *52*, 4632−4637.

(10) Meyer, R.; Candrian, U. PCR-based analysis for the identification and characterization of food components. *Lebensmittel-Wissenschaft Technol.* **1996**, *29*, 1−9.

(11) Van Duijn, G. J.; van Biert, R.; Bleeker-Marcelis, H.; van Boeijen, I.; Adan, A. J.; Jhakrie, S.; Hessing, M. Detection of genetically modified organisms in foods by protein- and DNA-based techniques: bridging the methods. *J. AOAC Int.* **2002**, *85*, 787−791.

(12) Hernandez, M.; Rio, A.; Esteve, T.; Prat, S.; Pla, M. A rapeseed-specific gene, acetyl-CoA carboxylase, can be used as a reference for qualitative and real-time quantitative PCR detection of transgenes from mixed food samples. *J. Agric. Food Chem.* **2001**, *49*, 3622−3627.

(13) James, D.; Schmidt, A. M.; Wall, E.; Green, M.; Masri, S. Reliable detection and identification of genetically modified maize, soybean, and canola by multiplex PCR analysis. *J. Agric. Food Chem.* **2003**, *51*, 5829−5834.

(14) Weng, H.; Yang. L.; Liu, Z.; Ding, J.; Pan, A.; Zhang, D. A novel reference gene, high-mobility-group protein I/Y, can be used in qualitative and real-time quantitative PCR detection of transgenic rapeseeds. *J. AOAC Int.* **2005**, *88*, 577−584.

(15) Yang, L.; Chen, J.; Huang, C.; Liu, Y.; Jia, S.; Pan, L.; Zhang, D. Validation of a cotton-specific gene, Sad1, used as an endogenous reference gene in qualitative and real-time quantitative PCR detection of transgenic cottons. *Plant Cell Rep.* **2005**, February, epub.

(16) Hernandez, M.; Ferrando, A.; Esteve, T.; Puigdomenech, P.; Prat, S.; Pla, M. 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. *J. Food Prot.* **2003**, *66*, 1063−1070.

(17) Yang, L.; Pan, A.; Jia, J.; Ding, J.; Chen, J.; Huang, C.; Zhang, C.; Zhang, D. Validation of a tomato specific gene, LAT52, used as an endogenous reference gene in qualitative and real-time quantitative PCR detection of transgenic tomatoes. *J. Agric. Food Chem.* **2005**, *53*, 183−190.

(18) Rho, J. K.; Lee, T.; Jung, S.-I.; Kim, T.-S.; Park, Y.-H.; Kim, Y.-M. Qualitative and quantitative PCR methods for detection of three lines of genetically modified potatoes. *J. Agric. Food Chem.* **2004**, *52*, 3269−3274.

(19) Hernandez, M.; Pla, M.; Esteve, T.; Prat, S.; Puigdomenech, P.; Ferrando, A. A specific real-time quantitative PCR detection system for event MON810 in maize YieldGard based on the 3′-transgene integration sequence. *Transgenic Res.* **2003**, *12*, 179−189.

(20) Hernandez, M.; Esteve, T.; Prat, S.; Pla, M. Development of real-time PCR systems based on SYBR Green I, AmpliFluor and TaqMan technologies for specific quantitative detection of the transgenic maize event GA21. *J. Cereal Sci.* **2004**, *39*, 99−107.

(21) Berdal, K. G.; Holst-Jensen, A. Roundup Ready soybean event-specific real-time quantitative PCR assay and estimation of the practical detection and quantification limits in GMO analyses. *Eur. Food Res. Technol.* **2001**, *213*, 432−438.

(22) Terry, C. F.; Harris, N. Event-specific detection of Roundup Ready Soya using two different real time PCR detection chemistries. *Eur. Food Res. Technol.* **2001**, *213*, 425−431.

(23) Nielsen, C. R.; Berdal, K. G.; Holst-Jensen, A. Characterisation of the 5′ integration site and development of an event-specific real-time PCR assay for NK603 maize from a low starting copy number. *Eur. Food Res. Technol.* **2004**, *219*, 421−427.

(24) Ronning, S. B.; Vaitilingom, M.; Berdal, K. G.; Holst-Jensen, A. Event specific real-time quantitative PCR for genetically modified Bt11 maize (*Zea mays*). *Eur. Food Res. Technol.* **2003**, *216*, 347−354.

(25) Windels, P.; Bertrand, S.; Depicker, A.; Moens, W.; Bockstaele, E.; Loose, M. Qualitative and event-specific PCR real-time detection methods for StarLink maize. *Eur. Food Res. Technol.* **2003**, *216*, 259−263.

(26) Ingham, D. J.; Beer, S.; Money, S.; Hansen, G. Quantitative real-time PCR assay for determining transgene copy number in transformed plants. *Biotechniques* **2001**, *31*, 132−4, 136−40.

(27) Mason, G.; Provero, P.; Vaira, A. M.; Accotto, G. P. Estimating the number of integrations in transformed plants by quantitative real-time PCR. *BMC Biotechnol.* **2002**, *2*, 20.

(28) Taverniers, I.; Van Bockstaele, E.; De Loose, M. Cloned plasmid DNA fragments as calibrators for controlling GMOs: different real-time duplex quantitative PCR methods. *Anal. Bioanal. Chem.* **2004**, *378*, 1198−1207.

(29) Taverniers, I.; Windels, P.; Vaïtilingom, M.; Van Bockstaele, E.; De Loose, M. Event-specific plasmid standards and real-time PCR methods for transgenic Bt11, Bt176 and GA21 maize and transgenic GT73 canola. *J. Agric. Food Chem.* **2005**, *53*, 3041−3052.

(30) Cameron-Mills, V.; Brandt, A. A gamma-hordein gene. *Plant Mol. Biol.* **1988**, *11*, 449−461.

(31) de Pater, B. S.; Schilperoort, R. A. Structure and expression of a root-specific rice gene. *Plant Mol. Biol.* **1992**, *18*, 161−164.

(32) Vonder Haar, R. A.; Allen, R. D.; Cohen, E. A.; Nessler, C. L.; Thomas, T. L. Organization of the sunflower 11S storage protein gene family. *Gene* **1988**, *30*, 74, 433−443.

(33) Gornicki, P.; Faris, J.; King, I.; Podkowinski, J.; Gill, B.; Haselkorn, R. Plastid-localized acetyl-CoA carboxylase of bread wheat is encoded by a single gene on each of the three ancestral chromosome sets. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 14179−14184.

(34) Dellaporta, S. L.; Wood, J.; Hicks, J. B. A plant DNA minipreparation version II. *Plant Mol. Biol. Rep.* **1983**, *1*, 19−21.

(35) Rogers, S. O.; Bendich, A. J. Extraction of DNA from Milligram Amounts of Fresh, Herbarium and Mummified Plant-Tissues. *Plant Mol. Biol.* **1985**, *5*, 69−76.

(36) Meyer, R.; Jaccaud, E. Detection of genetically modified soya in processed food products: development and validation of a PCR assay for the specific detection of Glyphosate Tolerant Soybeans. *Proceedings of the 9th European Conference on Food Chemistry (EURO FOOD CHEM IX)* **1997**, FECS *1*, 23−28.

(37) Arumuganathan, K.; Earle. E. D. Nuclear DNA content of some important plant species. *Plant Mol. Biol. Rep*. **1991**, *9*, 208−218.

(38) Rodríguez-Lázaro, D.; Hernández, M.; Scortti, M.; Esteve, T.; Vázquez-Boland, J. A.; Pla, M. Quantitative detection of *Listeria monocytogenes* and *Listeria innocua* by real-time PCR: assessment of *hly*, *iap* and *lin02483* targets and AmpliFluor technology. *Appl. Environ. Microbiol.* **2004a**, *70*, 1366−1377.

(39) Rodríguez-Lázaro, D.; D'Agostino, M.; Herrewegh, A.; Pla, M.; Cook, N.; Ikonomopoulos, J. Real-time PCR-based methods for quantitative detection of *Mycobacterium avium* subsp. *paratuberculosis* in water and milk. *Int. J. Food Microbiol.* **2005**, *101*, 93−104.

Barley, Rice, Sunflower, and Wheat Real-Time PCR

J. Agric. Food Chem., Vol. 53, No. 18, 2005 **7009**

(40) Rodríguez-Lázaro, D., Hernández, M.; Pla, M. Simultaneous quantitative detection of *Listeria* spp. and *Listeria monocytogenes* using a duplex real time PCR-based assay. *FEMS Microbiol. Lett.* **2004b**, *233*, 257−267.

(41) Higuchi, R.; Fockler, C.; Dollinger, G.; Watson, R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology* **1993***, 11*, 1026−1030.

(42) Frick, T. J.; Olsen, W. A. Celiac disease and the spectrum of gluten sensitivity. *Gastroenterologist* **1994**, *2*, 285−92.

(43) Watanabe, H.; Abe, K.; Hosoyama, H.; Arai, S. Molecular cloning and gibberellin-induced expresion of multiple cysteine proteinases of rice seeds (oryzains). *J. Biological Chem.* **1991**, *266*, 16897−16902.

(44) Ogasawara, T.; Sawasaki, T.; Morishita, R.; Ozawa, A.; Madin, K.; Endo, Y. A new class of enzyme acting on damaged ribosomes: ribosomal RNA apurinic site specific lyase found in wheat germ. *EMBO J.* **1999**, *18*, 6522−31.