JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Novel Reference Gene, *PKABA1*, Used in a Duplex Real-Time Polymerase Chain Reaction for Detection and Quantitation of Wheat- and Barley-Derived DNA

SISSEL B. RØNNING, KNUT G. BERDAL, CHARLOTTE BØYDLER ANDERSEN, AND ARNE HOLST-JENSEN*

Section for Feed and Food Microbiology, National Veterinary Institute, Ullevålsveien 68, Post Office Box 8156 Dep., 0033 Oslo, Norway

We report the development of a duplex real-time Polymerase Chain Reaction (PCR) for the simultaneous detection and quantification of wheat- and barley-derived DNA. We used a single primer pair to amplify the single-copy gene *PKABA1* from wheat and barley, using minor-groove-binding probes to distinguish between the two cereals. The assay was fully specific, and different wheat and barley cultivars exhibited similar Ct values, indicating stability across cultivars with respect to allelic and copy number composition. The limits of detection were 5 and 10 PCR-forming units for wheat and barley, respectively, making the duplex assay as sensitive as other singleplex reference gene systems published. We were able to detect both wheat and barley simultaneously in real food samples, and the duplex assay is considered to be suitable as an endogenous reference gene system for the detection and quantification of wheat and barley in genetically modified organisms (GMO) and other food and feed analyses.

KEYWORDS: ***Triticum aestivum*; *Hordeum vulgare*; *PKABA1*; quantitative; endogenous reference system; duplex; real-time PCR**

INTRODUCTION

Labeling of genetically modified foods containing transgenic material is required according to Norwegian (*1*, *2*) and EU legislation [summarized by Miraglia et al. (*3*)]. The estimated global area of approved biotech crops for 2004 was 81.0 million hectares, and soybean, maize, cotton, and canola were the four dominating genetically modified crops grown commercially (*4*). The number of GM crops approved worldwide is increasing (*5*). For example, in July 2004 the glyphosate-tolerant (Roundup Ready) wheat containing a transformation event designated MON 71800 was approved in the United States. Barley, a major cereal crop used as feed, malt, and food, is often a target for genetic engineering and several reports describe successful stable transformation of barley, as summarized by Choi et al. (*6*). Presently, no genetically modified barley variety is approved on the world market (*5*), but the need for GMO barley varieties will probably increase in the future in order to increase yields and to combat diseases and pests.

Real-time Polymerase Chain Reaction (PCR) is the most commonly used DNA-based GMO detection technique today, because it is a fast and accurate concept for the quantitative detection of GMOs in processed food and feed samples. Several real-time PCR amplification systems targeting different regions of the introduced genes, by use of TaqMan chemistry (*7*), have been developed for a range of authorized GMOs (*8*). Simultaneous amplification of multiple sequences in a single PCR could potentially save considerable time and effort for the analysts, since the number of reactions required to assess the presence of possible GMOs in a food sample is reduced. Real-time multiplex detection systems that target several GMO targets in a single assay have been reported (*9*, *10*). Common to these assays is that each target has a separate primer/probe set and all the sets are mixed in the PCR. The presence of more than one primer pair in the multiplex PCR increases the chance of obtaining false amplification products, primarily due to primer-dimer formation (*11*). An alternative approach is to use a multiplex, single-tube approach with universal primers combined with species-specific probes, in which each probe is labeled with a different fluorescent reporter dye. The risk of primer-dimer formation decreases because only one primer pair is present in the reaction. This approach has been developed for the simultaneous identification of different fish species (*12*) and for the detection of *Plasmodium* species in blood (*13*), but as far as we know this approach has never been described for the simultaneous detection and quantification of different plant species.

In real-time PCR the quantity of product synthesized during the reaction is measured in real time by the detection of a fluorescence signal, and estimation of the initial quantity takes place in the logarithmic phase (*7*). An endogenous reference gene gives an estimate of the total amount of relevant species

* To whom correspondence should be addressed: phone +47 23 21 62 43; fax +47 23 21 62 02; e-mail arne.holst-jensen@vetinst.no.

10.1021/jf052328n CCC: $33.50
Published on Web 01/06/2006

Table 1. Oligonucleotides Used in This Study

name	orientation	sequence 5′–3′	T_m[a]	length
UnivF	sense	CAAGTATGTCATAGAGATTTGAA	48	23
UnivR	antisense	GTAACCGAAGTCACAAATCT	48	20
TAest	probe	5′-FAM-TCGCACCTCGGCT-MGBNFQ-3′	65	13
HVulg	probe	5′-VIC-TCGCTCCTCGACTC-MGBNFQ-3′	65	14
TaPK3	synthetic oligo	GACCTGCAGCAAGTATGTCATAGAGATTTGAAACTAAAAATACTCTCTTGGATGGTAGT-GTTGCGCCTGACTCAAGATTTGTGACTTCGGTTACTCCAAGGTA		105

[a] Calculated by use of Primer Express, version 2.0 (Applied Biosystems, Foster City, CA).

Figure 1. Sequence alignment of the exon 3 of the gene encoding the protein kinase PKABA1 from *Triticum aestivum* (accession number M94726), *Hordeum vulgare* (accession number AB058924), and *Secale cereale* (accession number DQ295068). Corresponding sequences from a selection of species are included in the alignment, such as *Oryza sativa* (accession number XM_470803), *Zea mays* (accession number AY108950), *Triticum aestivum* TaPK3 (accession number U29095), *Vitis vinifera* (accession number AF178575), *Fagus sylvatica* (accession number AJ586511), *Arabidopsis thaliana* (accession number AY081538), *Brassica napus* (accession number L12393), *Nicotiana tabacum* (accession number AY081175), and *Glycine max* (accession number L19360). Mismatches are presented with gray shading. The primers used are indicated with arrows, and the probes are boxed.

DNA in the sample, and GMO detection methods rely on the amplification of GM target specific sequences and their quantification relative to the reference gene (*14*). With this technology the amount of GMO is calculated as a function of total species-specific DNA in the food product. A reference gene has three requirements: it should be species-specific and exhibit low copy number (preferably single copy) and low heterogeneity among cultivars (*15*). Several reference gene systems have been published, for example, for the rice *SPS* (*15*) and *gos9* (*16*) genes, the rapeseed *BnACCg8* and HMG-I/Y genes (*14*, *17*), the soybean *lec* gene (*18*), the tomato *LAT52* gene (*19*), the sunflower *helianthinin* gene (*16*), the wheat *acc1* gene (*16*), the barley γ-hordein gene (*16*), the cotton *Sad1* gene (*20*) and the maize *Adh1*, *hmga*, *ivr1*, and *zein* genes (*21*). Real-time PCR methods have also been developed for detection and quantification of different cereals in food: these methods are based on SYBRGreen and melting curve analysis (*22*, *23*) and can be used as a confirmatory method to the protein assay for food sample analysis, but this method can only be used as reference gene system in a GMO analysis for single-ingredient (species) materials.

The serine/threonine protein kinase PKABA1 was first described in 1992 in wheat embryos (*24*) and later described in barley (*25*). The *PKABA1* gene belongs to the SnRK2 family and consists of nine exons and eight introns. The gene is reported to be single-copy for wheat, barley, and rice (*25*−*27*). It is suggested that PKABA1 is an intermediate in the abscisic acid (ABA) signal transduction pathway leading to the suppression of α-amylase gene expression in aleurone layers (*28*). The subcellular location of PKABA1 is unknown, but observations suggest that this kinase also has roles in the nucleus and cytosol (*29*). Here we report the common primer set and specific probes for the single-copy gene encoding the protein kinase PKABA1 in wheat and barley. We report the PCR cycling conditions suitable for using this sequence as an endogenous reference gene in both qualitative and quantitative duplex assay.

MATERIALS AND METHODS

Materials. Six wheat (*Triticum aestivum*) varieties, nine barley (*Hordeum vulgare*) varieties, and oat (*Avena sativa*) were provided by Aksel Bernhoft, National Veterinary Institute, while four wheat varieties, rye (*Secale cereale*), timothy (*Phleum pratense*), durra (*Sorghum bicolor*), cabbage (*Brassica oleraceae* subsp. *capitata*), cauliflower (*Brassica oleraceae* subsp. *botrytis*), and soybean (*Glycine max*) were provided by the gene bank at Institute für Pflanzengenetik and Kulturpflanzenforschung (IPK), Germany. Other samples of different species were obtained from local stores: rice (*Oryza sativa*), tomato (*Lycopersicon esculentum*), pepper (*Capsicum annuum*), potato (*Solanum tuberosum*), pea *(Pisum sativum*), bean (*Phaseolus vulgaris*), lens (*Lens culinaris*), peanut (*Arachis hypogeae*) and eggplant (*Solanum melongena*). Maize (*Zea mays*) and rape seed (*Brassica napus*) samples were provided by David Zhang, Laboratoire Biogeves, France. Additionally, 10 food samples obtained from the local store, The Norwegian Food Safety Authority, and GeMMA (Central Science Laboratory, U.K.) (cf. **Table 4**) were included in the study and analyzed according to the protocol. The samples from the Norwegian Food Safety Authority and GeMMA were provided with a detailed declaration, either as a label on the product or as a product attached document.

DNA Extraction. Total genomic DNA was extracted by use of a CTAB-based protocol (*30*) modified as described (*31*). DNA concentrations were measured in nanograms per microliter on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Primers and Probes. The primers and the probes (**Table 1**) were designed manually on the basis of an alignment containing both new sequences and sequences retrieved from EMBL/GenBank (**Figure 1**). Melting temperature (T_m) and possible hairpin and primer-dimer formation were tested theoretically by use of Primer Express, version 2.0 (Applied Biosystems, Foster City, CA). The primers were purchased from DNA Technology (Århus, Denmark). The minor-groove-binding (MGB) probes were labeled with fluorescent reporter dyes 5′- FAM and 5′-VIC and the nonfluorescent quencher MGBNFQ at the 3′-end. The probes were purchased from Applied Biosystems (CA).

PCR Conditions. The PCR reaction volume of 25 μL contained 5 μL of template DNA, 0.8 μM of each primer, 0.2 μM of each probe, and 12.5 μL of universal amplification mix (includes ROX as a passive reference) (Applied Biosystems). The PCR reactions were run on an

ABI Prism 7900HT Sequence Detection System device (Applied Biosystems), with the following program: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The fluorescence signals obtained were measured once for each cycle at the extension step. The PCR results were then analyzed by use of the Sequence Detection System software version 2.1 (Applied Biosystems).

Calibration Curves and Estimation of the Limit of Detection. The haploid genome sizes of wheat and barley have been estimated to be 15 966 and 4873 Mbp, respectively (*32*). The mass of a haploid genome was calculated from the formula $m = n[1.096 \times 10^{-21}$ g/bp], where m = mass and n = genome size (base pairs) (*33*). The mass was then divided by the copy number of the gene of interest, and in this way measured DNA concentrations (nanograms per microliter) were translated into estimates of the numbers of copies of the haploid wheat and barley genome, respectively. Calibration curves based on 3-fold dilution series for both wheat and barley were established by use of DNA copy numbers in the approximate range of 10 000−40 template copies per PCR reaction. To determine the limit of detection (LOD), the DNA was diluted in 3-fold dilution series and each concentration was analyzed in five parallel experiments. Notably, copy numbers assessed from mass do not necessarily correspond to PCR-forming units (PFU) that are the amplifiable copies.

RESULTS

Selection of a Suitable DNA Sequence for Qualitative and Quantitative PCR Detection of Wheat and Barley. To select a suitable endogenous reference gene for wheat and barley, we searched the public sequence databases [EMBL, GenBank (*34*)] for single-copy genes in the wheat and barley genome. After selection of several candidate genes, we chose a DNA fragment encoding the serine/threonine protein kinase PKABA1 in wheat (EMBL/GenBank accession number M94726) and barley (EMBL/GenBank accession number AB058924). The specific primers and the fluorogenic probes designed for real-time assays were based upon these DNA sequences and their positions are shown (**Figure 1**).

Qualitative and Real-Time Quantitative PCR Assay Detection. Different primer sets were subjected to a first qualitative PCR test, and the primer pair that produced the single band with highest intensity was selected to develop a quantitative real-time assay (**Table 1**), although this band probably was a result of coamplification of *TaPK3* and *PKABA1* in wheat (cf. **Figure 1**). We used fluorogenic 3′-minor-groove-binding (MGB) probes to be able to design short probes with greater differences in T_m values between matched and mismatched probes. According to the manufacturer, MGB probes discriminate between single mismatches, but we occasionally observed unspecific signals from closely related cereals, and in order to develop stringent PCR conditions we varied the $MgCl_2$ concentration within the range of 3−6 mM, the primer concentrations within the range of 0.5−0.9 μM, the probe concentrations within the range of 0.1−0.3 μM, and the synthesis temperature within the range of 60−67 °C. The optimal conditions, described under Materials and Methods, are those that produced the lowest *Ct* values and no detectable fluorescence signals in negative control samples, including samples containing DNA from nontarget species products.

Calibration Curves. Three-fold dilution series (corresponding to approximately 10 000−40 haploid genome equivalents of wheat and barley) were used to establish calibration curves (**Figure 2**). The squared regression coefficients (R^2) were 0.995 and 0.996 for the wheat and barley probes, respectively, showing good linearity between copy number and fluorescence values (*Ct*).

Specificity of the Assay and Allelic Variation between Cultivars. To test the specificity of the assay, real-time PCR

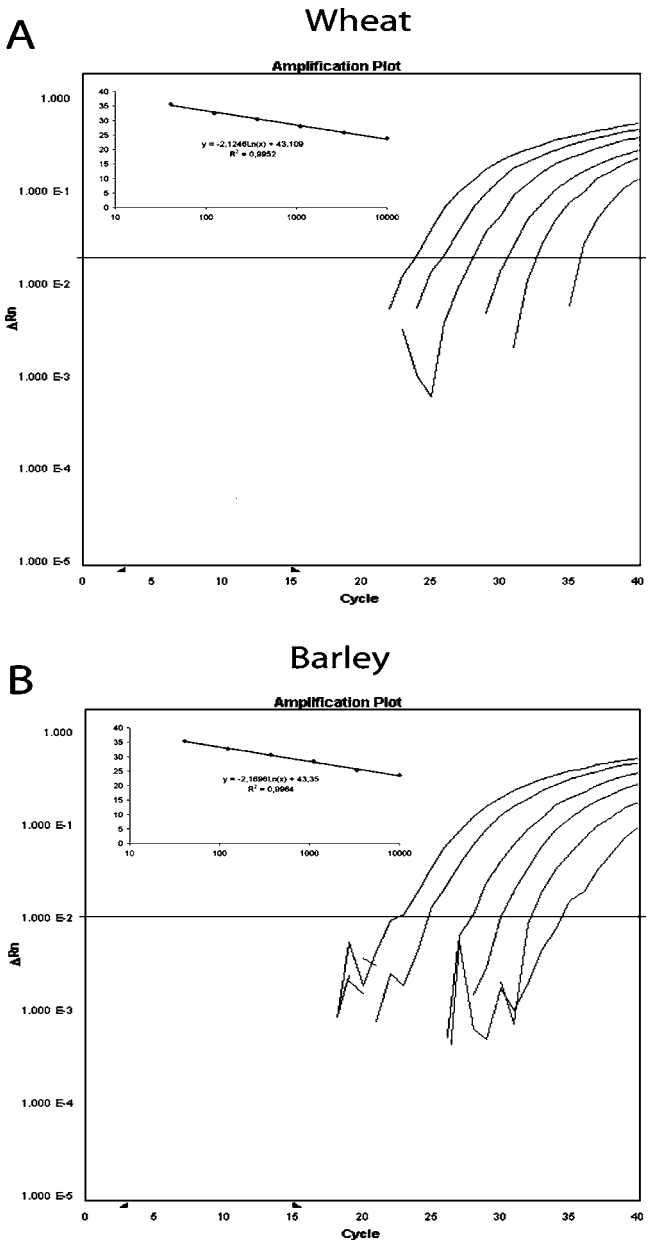

Figure 2. Amplification and calibration curves for the wheat and barley *PKABA1* gene. The dilution series contained approximately 10 000, 3333, 1111, 370, and 123 initial template copies of both wheat and barley in the PCR reaction. **(A)** Amplification curves for the wheat *PKABA1* gene and calibration curves plotting natural logarithm of the copy number of the input DNA versus *Ct.* **(B)** Amplification curves for the barley *PKABA1* gene and calibration curves plotting natural logarithm of the copy number of the input DNA versus *Ct.*

was set up with approximately 13 000 template copies per reaction from different plant species (**Figure 3**) that were either closely related to wheat and barley, such as oat (*A. sativa*), rye (*S. cereale*), maize (*Z. mays*), rice (*O. sativa*), timothy (*P. pratense*) and durra (*S. bicolor*), or distantly related but frequently found in food, such as cabbage (*B. oleraceae* subsp. *capitata*), cauliflower (*B. oleraceae* subsp. *botrytis*), soybean (*G. max*), pea (*P. sativum*), bean (*P. vulgaris*), lens (*L. culinaris*), peanut (*A. hypogeae*), tomato (*L. esculentum*), rape seed (*B.a napus*), potato (*S. tuberosum*), eggplant (*S. melongena*), and pepper (*C. annuum*). No unspecific amplification was observed with any of the species tested. To further test the assay, fixed amounts of approximately 2500 template copies from 10 different wheat cultivars and approximately 10 000

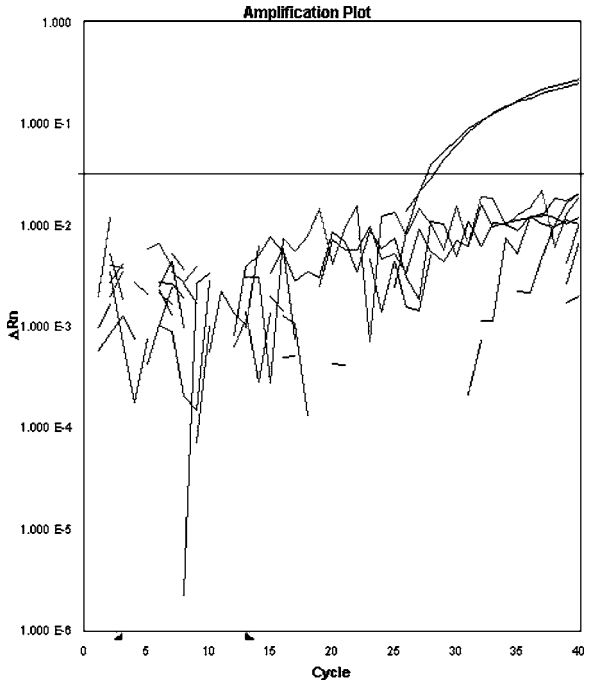

Figure 3. Specificity analysis of the wheat and barley probes in real-time PCR: Quantitative detection of DNA from wheat, barley, and 17 nontarget plant species. Amplification signals detected by the probes were observed only for wheat and barley.

template copies of nine different barley cultivars were analyzed in separated reactions. For wheat the observed *Ct* values varied between 25.4 and 27.1, and for barley the observed *Ct* values varied between 25.6 and 27.4.

A coamplification of *TaPK3* and *PKABA1* in wheat was expected because of high sequence similarity (**Figure 1**), although we did not expect to observe any amplification signals from *TaPK3* due to high specificity of the MGB probes. To verify this, we designed a synthetic *TaPK3* oligonucleotide corresponding to the *TaPK3* amplification product (cf. **Table 1**) and used it in high concentrations (10 000 target copies per PCR) as a template in a real-time PCR. No amplification signal was observed with either of the wheat or barley probes, although amplification had occurred according to the agarose gel electrophoresis (data not shown).

Since the wheat genome is not fully sequenced, there is the possibility that other unknown homologous DNA fragments could be amplified with the primers. These could eventually also affect the specificity. To assess this we sequenced the PCR products from both wheat and barley, and no other fragments but *PKABA1* were detected.

Limits of Detection. We assessed the sensitivity of the real-time PCR assay by analysis of a 3-fold dilution series of wheat and barley DNA, containing approximately 425, 141, 47, 16, 5, and 1.8 initial template copies of the haploid genomes of each of the species per reaction in five parallel experiments (**Tables 2** and **3**). The ability to detect wheat and barley decreased with decreasing copy number. We were able to detect wheat in all five parallels down to 5 copies and in four of the parallels for 1.8 copies. We were able to detect barley in all five parallels down to 16 copies, while only two of the parallels were positive for 5 copies and three parallels were positive for 1.8 copies. The system showed that the absolute LOD for wheat was approximately 5 PFU, while the LOD for barley was approximately 10 PFU. Using PFU as unit of measurement rather than target template copies is more correct, as some of the target copies may be damaged or by other means fail to function as templates for PCR (*35*). The lowest number we were able to detect was estimated at 1−2 PFU for both wheat and barley. The *Ct* variations among the parallels of the same template concentrations increased with decreasing copy number.

Table 2. Amplification Data Used To Determine the Absolute LOD for Wheat[a]

estimated template copies	signal rate (no. of positive signals)	mean *Ct* values[b]	SD of observed *Ct* values
425	5/5	27.3	0.3
141	5/5	29.1	0.1
47	5/5	30.9	0.8
16	5/5	33.4	1.7
5	5/5	35.3	1.6
1.8	4/5	37.1	1.4

[a] Five-fold dilution series of wheat, based on initial template copy number. [b] Mean *Ct* value of positive signals.

Table 3. Amplification Data Used To Determine the Absolute LOD for Barley[a]

estimated template copies	signal rate (no. of positive signals)	mean *Ct* values[b]	SD of observed *Ct* values
425	5/5	29.2	0.5
141	5/5	31.2	0.5
47	5/5	33.0	0.6
16	5/5	35.6	1.0
5	2/5	37.7	0.9
1.8	3/5	39.1	0.7

[a] Five-fold dilution series of barley, based on initial template copy number. [b] Mean *Ct* value of positive signals.

Table 4. Analysis of DNA Extracts from Food and Feed Samples

product	wheat[a]	barley[a]
oat biscuits with cornflakes	96	0
bread	65	0
Pringles sour cream and onion chips	10	17
GeMMA mixed wheat/maize flour	37	0
GeMMA mixed wheat/soybean flour	1090	0
grain feed sample 80	60	12
grain feed sample 83	40	36
grain feed sample 84	92	0
grain feed sample 85	0	709
grain feed sample 86	0	273

[a] Average number of PCR forming units (PFU) of the target sequence per nanogram of DNA, estimated from two dilution and calibration curves generated for wheat and barley.

Wheat and Barley Detection in Processed Food. Ten different food and feed samples were examined for presence of wheat- and barley-derived DNA (**Table 4**) by use of the developed duplex real-time PCR assay. Eight of the samples presumably contained wheat, yielding *Ct* values varying from 26 to 33 with the wheat probe. Five of the samples presumably contained barley, yielding *Ct* values varying from 25 to 32 with the barley probe.

DISCUSSION

Here we describe and present the first validation of the serine/threonine kinase encoding the gene *PKABA1* as endogenous reference gene for detection and quantification of wheat- and barley-derived DNA. This gene meet all the requirements that are necessary for a reliable reference gene system: it is species-specific, single−copy, and shows little heterogeneity among

different cultivars. *PKABA1* maps to only a single locus per haploid genome in wheat (*27*), barley (*25*), and rice (*26*). TaPK3 is another member of the wheat PKABA1 kinase subfamily, with high sequence homology to the PKABA1, probably with a different function (*36*). *PKABA1* maps to the same locus on all three chromosomes in wheat, while *TaPK3* maps to just a single locus on one of the chromosomes (K. Simmons, personal communication). In the real-time PCR assay presented here for wheat and barley, *TaPK3* and *PKABA1* will probably be coamplified and the PCR efficiency of *PKABA1* might be influenced. This is of modest importance when this assay is used as a reference system, as long as the sample to be tested is commutable with the reference sample, for example, by use of genomic DNA for both the reference sample and the unknown sample. There are several sequence differences in the probe regions (**Figure 1**), and the assay readily discriminates between *PKABA1* and *TaPK3*. With universal primers, targeting several cereals, a coamplification will obviously take place in mixed samples. However, use of 3′-minor-groove-binding probes (MGB) minimized specificity problems. These probes have higher melting temperatures and increased specificity, especially when a mismatch is located in the MGB region of the probes. The probes are more specific for single base mismatches and the fluorescence quenching is more efficient (*37*). The sensitivity of the assay (LOD) developed here was determined to be 5 PFU for wheat and 10 PFU for barley. The sensitivity is similar to that reported for, for example, the endogenous tomato gene *LAT52* (*19*) and for the maize *zein* and *Adh1* genes (*21*). MGB probes increase specificity (*37*), but when large quantities (ca. 13 000 haploid genome equivalents) of closely related cereal plant species were included in the PCR, unspecific signals were frequently observed. Extensive optimization was necessary in order to reduce this problem, and by using the universal amplification mix from Applied Biosystems we were able to obtain high specificity.

We did not perform a Southern blot in order to verify that *PKABA1* is a single-copy gene, since previous experiments performed by others have demonstrated this for rice, barley, and wheat (*25*−*27*). Single-copy genes are in general associated with low rates of mutation and changes in the copy number among different cultivars. Different cultivars for species-specific DNA quantification must exhibit identical efficiencies in analyses with suitable real-time PCR systems, thus avoiding over- or underestimation of DNA. A number of wheat and barley cultivars were analyzed with the real-time PCR developed, and these cultivars all produced similar *Ct* values. Hernandez et al. (*21*) experienced that *Ct* values varied up to 1.14 and 2.90 cycles for the maize endogenous genes *hmga* and *Adh1*, respectively. The observed small variation in *Ct* values observed may be ascribed to (i) differences in the quality of the extracted DNA (target copies failed to function as template in a PCR) (*35*), (ii) errors associated with the dilution of the DNA delivered, or (iii) imprecise measurements of DNA concentrations. The low level of variation of the *Ct* values in the real-time PCR indicate that the copy number of *PKABA1* per haploid genome was invariant among the tested wheat and barley cultivars and that this system is suitable for detection, identification, and quantification of wheat and barley.

The simultaneous amplification of multiple sequences in a single real-time PCR is expected to save considerable time and effort in a GMO analysis, as real-time multiplex assays are less labor-intensive and less expensive than real-time singleplex PCR reactions. The risk of intertube variation is reduced since the targets are coamplified within the same reaction tube. Multiplex conventional PCR systems that are able to detect up to seven different targets in a single assay reaction have been developed (*38*). Duplex and multiplex quantification methods for GMOs based on real-time PCR have been described in the literature (e.g., *10*, *39*), and common to these assays is that each target has a primer/probe set. We have developed a duplex quantitative real-time assay targeting both wheat and barley simultaneously, using a single primer set and different probes. Similar approaches have been developed for the simultaneous detection of *Plasmodium* species (*13*) and of three fish species (*12*). The duplex detection of *Plasmodium* obtained high sensitivity in experimental simulations of mixed infection with plasmid positives, although the authors could not confirm reliability of the multiplex PCR for real mixed infections. We were able to detect both wheat and barley simultaneously in the real food samples tested (**Table 4**) for Pringles sour cream and onion chips and grain feed samples 80 and 83. The latter are samples with known content of wheat and barley, while in Pringles sour cream and onion chips neither wheat nor barley was declared on the ingredient list. This may indicate that the chips product was botanically impure or contaminated, and this is quite normal. The risk of contamination is in general high with increased processing (*40*), and international trade allows some botanical impurity.

In conclusion, we have developed a duplex real-time PCR system that simultaneously detects and quantifies wheat and barley. The method is specific, shows no allelic variation between cultivars, and is as sensitive as other singleplex reference gene systems developed.

ACKNOWLEDGMENT

We thank David Zhang, Laboratoire BIOGEVES, France; Aksel Bernhoft, National Veterinary Institute, Norway; and the gene bank at Institute für Pflanzengenetik and Kulturpflanzenforschung (IPK), Germany, for kindly providing plant material. We also thank Ann-Kristin Knutsen for sequencing of the *S. cereale PKABA1* gene.

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Received for review September 21, 2005. Revised manuscript received November 20, 2005. Accepted November 28, 2005. This study was financially supported by a grant from the Research Council of Norway (136430/140), and the European Commission through the Integrated Project Co-Extra (contract no. 7158). This is gratefully acknowledged.

JF052328N