JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of Taxon-Specific Sequences of Common Wheat for the Detection of Genetically Modified Wheat

MAYU IIDA,[†] SATOMI YAMASHIRO,[†] HIROHITO YAMAKAWA,[†] KATSUYUKI HAYAKAWA,[†] HIDEO KURIBARA,[‡] TAKASHI KODAMA,[‡] SATOSHI FURUI,[§] HIROSHI AKIYAMA,[#] TAMIO MAITANI,[#] AND AKIHIRO HINO*,[§]

QE Center, Research and Development, Quality Assurance Division, Nisshin Seifun Group Inc., 5-3-1 Tsurugaoka, Oi-machi, Iruma-gun, Saitama 356-8511, Japan; Center for Food Quality, Labeling and Consumer Services, 2-1 Shintoshin, Chuo-ku, Saitama-shi, Saitama 330-9731, Japan; National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan; and National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Qualitative and quantitative Polymerase Chain Reaction (PCR) systems aimed at the specific detection and quantification of common wheat DNA are described. Many countries have issued regulations to label foods that include genetically modified organisms (GMOs). PCR technology is widely recognized as a reliable and useful technique for the qualitative and quantitative detection of GMOs. Detection methods are needed to amplify a target GM gene, and the amplified results should be compared with those of the corresponding taxon-specific reference gene to obtain reliable results. This paper describes the development of a specific DNA sequence in the *waxy-D1* gene for common wheat (*Triticum aestivum* L.) and the design of a specific primer pair and TaqMan probe on the *waxy-D1* gene for PCR analysis. The primers amplified a product (Wx012) of 102 bp. It is indicated that the Wx012 DNA sequence is specific to common wheat, showing homogeneity in qualitative PCR results and very similar quantification accuracy along 19 distantly related common wheat varieties. In Southern blot and real-time PCR analyses, this sequence showed either a single or a low number of copy genes. In addition, by qualitative and quantitative PCR using wx012 primers and a wx012-T probe, the limits of detection of the common wheat genome were found to be about 15 copies, and the reproducibility was reliable. In consequence, the PCR system using wx012 primers and wx012-T probe is considered to be suitable for use as a common wheat-specific taxon-specific reference gene in DNA analyses, including GMO tests.

KEYWORDS: ***Triticum aestivum* L.; common wheat; genetically modified; GMO detection; real-time PCR; endogenous reference gene; *waxy*; granule-bound starch synthase; quantitative analysis**

INTRODUCTION

Numerous genetically modified (GM) crops have been developed by recombinant DNA (r-DNA) techniques, and many of these crops are being marketed throughout the world. Marketed GM crops such as soy and maize have been authorized for use by the governments of many countries after safety assessment review.

Many consumers, however, are concerned about the use of GM crops and about consuming genetically modified foods. To provide information on the use of GM technologies and foster consumers' rights to select foods they want to eat, new labeling systems for foods derived from GM crops have been, or are being, introduced in the European Union (EU), Korea, Japan, Australia, and other countries. To enforce these labeling policies, analytical methods for GMOs are needed. Numerous DNA- and protein-based methods have been developed in the past decade (*1*−*6*). To determine the level of GM content in foods on the market, the DNA-based Polymerase Chain Reaction (PCR) technique is most often used. Real-time PCR methods must be able to reliably quantify the GMO content in different matrices at threshold levels of 5% in Japan, 3% in Korea, and 0.9% in the EU for authorized GMOs.

The number of authorized GM crop plants is growing rapidly worldwide (*7*). Fifty-nine GM crops, including soy, maize, canola, cotton, beets, and potatoes, all of which have novel traits such as a tolerance to herbicides and insect and virus resistance, have been authorized for use in food in Japan. GM wheat has not yet been introduced into the market, but herbicide-resistant GM wheat (*8*) and *Fusarium* disease-resistant GM wheat are now being developed (*9*), and the safety of the former has been

* Author to whom correspondence should be addressed (telephone +81-29-838-8079; fax +81-29-838-7996; e-mail akhino@nfri.affrcgo.jp).
[†] Nisshin Seifun Group Inc.
[‡] Center for Food Quality, Labeling and Consumer Services.
[§] National Food Research Institute.
[#] National Institute of Health Sciences.

10.1021/jf0505731 CCC: $30.25 © 2005 American Chemical Society
Published on Web 07/13/2005

evaluated (*8*). Thus, the development of detection methods for GM wheat is required, because GM wheat will likely appear on the market in the next decade.

A taxon-specific DNA sequence is essentially needed as a positive or comparative control for qualitative and quantitative PCRs when detecting target GM sequence (*5*, *6*). In GMO quantification using real-time PCR methods, the design of a taxon-specific DNA sequence to measure the ratio of copy numbers between a GMO and a taxon-specific DNA sequence is needed. An appropriate taxon-specific PCR system must not detect any other crop species and must be able to give the same quantitative result when equal amounts of DNA from different varieties in the same taxon are analyzed. Therefore, the design of a taxon-specific DNA sequence is one of the most important steps in designing a method of detecting GM wheat, because the sequence can then be used for any GM wheat detection systems. Furthermore, the taxon-specific DNA detection method could be a useful tool for examining products subject to GM labeling in countries concerned about evaluating surviving DNA in processed foods.

We searched for candidates for the endogenous reference gene DNA sequence using the DDBJ database first. The search conditions were that the candidates must be wheat (*Triticum aestivum* L.) specific, be a single copy or a stable low copy gene, and have low heterogeneity in *T. aestivum* L. The genome constitutions of polyploid wheat are AABBDD in common wheat (*T. aestivum* L.) and AABB in durum wheat (*Triticum durum* L.). We targeted hexaploid common wheat, because the amount of common wheat in the product is much greater than that of durum wheat (*10*), and the safety of GM wheat was evaluated prior to that of durum wheat. Several candidates that met the above requirements were selected as common wheat species-specific markers.

In this paper, we report the specific primer pair for the wheat (*T. aestivum* L.) *waxy* gene and the PCR conditions appropriate for the use of this sequence as a taxon-specific gene for quantitative and qualitative PCR assays. We used qualitative and real-time quantitative PCR assays to characterize its species specificity, test the detection sensitivity, and set up a reliable qualitative and quantitative system for GM wheat detection.

MATERIALS AND METHODS

Wheat and Other Plant Samples. Grain samples of common commercial wheat (*T. aestivum* L.) belonging to five classes, including hard red winter wheat, club wheat, soft white wheat, hard red spring wheat, and domestic wheat (Norin 61 and Shirogane) were collected by our laboratory. Nineteen kernels of non-GM common wheat, which were randomly sampled from the grain samples of these five classes of commercial wheat and identified as 19 different varieties from the polymorphism of the kernels by the randomly amplified polymorphic DNA (RAPD) technique (*11*), were used for the test of intraspecies variability of the target DNA sequence. The varieties of Norin 61 and/or single kernels derived from the hard red spring wheat sample are used as representative common wheat samples in this paper. Four kernels of durum wheat (*T. durum* L.), which were also randomly collected from commercial durum wheat samples from Canada and the United States and identified as four different varieties from the polymorphism by RAPD, are used in this paper. In addition, multiple kernels randomly sampled from a durum wheat class, Canadian amber durum (CAD), and other seed samples of different species such as *Hordeum vulgare* (barley), *Secale sereale* (rye), *Avena sativa* (oats), *Setaria italica* (Italian millet; awa), *Panicum miliaceum* L. (common millet; kibi), *Glycine max* (soy), *Fagopyrum esculentum* (buckwheat), *Brassica napus* (rapeseeds), and *Zea mays* (corn) were also collected by our laboratory. The samples of 11 non-GM rice varieties including 6 varieties of *Oryza sativa* L. ssp. *Japonica*, Koshihikari, Jaguary, Khau Nok, Khau Than Chiem, and Urasan 3, and 6 varieties of *Oryza sativa* L. ssp. *Indica*, Lemont, Amaroo, IR36, RD6, Dular, and Aijiaonante, were kindly provided by the Genebank of the Ministry of Agriculture, Forestry and Fisheries of Japan.

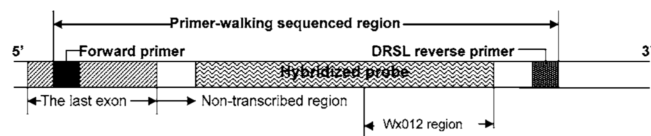

Figure 1. Schematic diagram of the sequenced region in the *Wx-D1* allele. The hatched box shows the last exon of the *Wx-D1* gene. The black and stippled boxes indicate the primers for the primer-walking sequencing, respectively. The wavy box indicates the probe (444 bp) used for Southern hybridization.

Extraction of Genomic DNA. All seed samples were ground in an electric mill (Yasui Kikai Co., Ltd., Osaka, Japan). The ground samples were then taken for DNA extraction. The genomic DNA samples from the kernels of four classes of common wheat and durum wheat used for RAPD and the qualitative and real-time quantitative PCR were extracted using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the standard Japanese method (*12*). The other plant genomic DNA samples used for qualitative and real-time quantitative PCR were extracted using the DNeasy Plant Maxi Kit (Qiagen GmbH, Hilden, Germany) according to methods reported previously (*5*).

The DNA concentration of solutions was determined by measuring the UV absorption at 260 nm. The quality of extracted DNAs was evaluated from the ratio of UV absorptions at 260/280 and 260/230 nm.

Oligonucleotide Primers and Probe. The oligonucleotide primers and TaqMan fluorescent dye-labeled probe between the primers were designed by Primer Express software [Applied Biosystems Co., Ltd. (ABI), Foster City, CA]. The oligonucleotides were synthesized and purified by Qiagen Co., Ltd. (Tokyo, Japan), then diluted with an appropriate volume of water to final concentrations of 50 μmol/L for the qualitative PCR and 5 μmol/L for the quantitative PCR, and stored at -20 °C until use. The TaqMan probe, which was labeled on the 5′-end with the 6-carboxy-fluorescein (FAM) reporter dye and with the 6-carboxytetramethylrhodamine (TAMRA) quencher dye attached to the 3′-end, was synthesized by Applied Biosystems Japan (Tokyo, Japan).

Sequence of *Wx-D1*. To obtain the corresponding DNA sequence for the design of the PCR primer pair and probe, we sequenced one region of the *Wx-D1* gene by primer-walking sequencing with the primers designed on the basis of the sequence information from DDBJ (accession no. AF113844) and the *Wx-D1* specific primer, DRSL (*13*), on ABI Prism 3100 (ABI). The sequenced region is shown in **Figure 1**. The direct DNA sequencings of PCR products were performed by Qiagen Co., Ltd. (Tokyo, Japan).

Conditions of Qualitative PCR. Qualitative PCRs were run in a 25 μL final volume on a GeneAmp PCR System 9600 (Perkin-Elmer/Applied Biosystems Division, Foster City, CA). Each reaction mixture contained 25 ng of sample DNA, 2.5 μL of PCR buffer II (ABI), 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.625 unit of AmpliTaq Gold Polymerase (ABI), and a 0.5 μM primer pair. The amplification reaction was performed in a thermal cycler according to the following PCR step-cycle program: preincubation at 95 °C for 10 min; 40 cycles consisting of denaturation of DNA at 95 °C for 30 s, annealing at 63 °C for 30 s, and extension at 72 °C for 30 s; followed by a final extension at 72 °C for 7 min. The amplification products were analyzed by 2% agarose gel electrophoresis (in 1× TBE) and stained with 0.5 μg/mL ethidium bromide. The gel was photographed with a CCD camera under UV irradiation at 312 nm.

To test the sensitivities of the qualitative assay, the extracted common wheat DNA was serially diluted to final concentrations of 60, 12, 3, 0.6, 0.2, 0.05, 0.01, 0.005, 0.0005, and 0 ng/μL. Thus, the amounts of extracted DNA per reaction tube were 150, 30, 7.5, 1.5, 0.5, 0.125, 0.025, 0.0125, 0.00125, and 0 ng.

Quantitative Real-Time PCR. PCR reactions were performed in a 25 μL final volume containing 50 ng of sample DNA, 12.5 μL of

Table 1. Primer Pairs and Fluorogenic Probes for Qualitative and Quantitative PCR and PCR for Production of the 444 bp Probe for Southern Blotting

name	sequence (5′–3′)	sense/antisense	amplicon size (bp)	purpose
wx012-5′	gTCgCgggAACAgAggTgT	sense	102	taxon-specific detection
wx012-3′	ggTgTTCCTCCATTgCgAAA	antisense		
wx012-T	CAAggCggCCgAAATAAgTTgCC	TaqMan probe		
wxs01-5′	AAAAggAAgTTCTggTgCATgg	sense	444	probe for Southern blotting
wx012-3′	same as above	antisense		

Universal Master Mix (ABI), 0.5 μM primer pair, and 0.2 μM probe. Real-time PCR reactions were run on ABI Prism 7700 (ABI) using the following program: 2 min at 50 °C, 10 min at 95 °C, and 45 or 50 cycles of 30 s at 95 °C and 1 min at 59 °C. The real-time PCR products were analyzed using Sequence Detection System software 1.6.3 (ABI). The no-template control (NTC) was also prepared as the negative control for analyses, which included 5 ng/μL salmon testis DNA (Sigma Chemical Co., St. Louis, MO). In the reaction plate, each sample was quantified in triplicates. The process to draw threshold lines was performed according to the method given in a previous paper (*5*).

For the generation of standard curves, extracted genomic DNA was diluted to final concentrations of 300, 60, 12, 2, 0.6, 0.1, 0.05, and 0 ng/μL. Thus, the amounts of extracted DNA per reaction tube were 750 000, 150 000, 30 000, 7500, 1500, 250, 125, and 0 pg.

To evaluate the amplification features of each DNA solution, extraction was performed by different methods, including the DNeasy Plant Maxi Kit (Qiagen), Genomic-Tip 20/G (Qiagen), CTAB method (*14*), and DNeasy Plant Mini Kit (Qiagen). These methods are standard Japanese methods described previously (*12*, *15*). All DNA extracted by these methods was evaluated by quantitative PCRs.

Southern Blotting Analysis. Thirty micrograms of genomic DNA extracted from the common wheat sample was fully digested with *Bam*HI, *Eco*RI, and *Fba*I. The fragments were separated by electrophoresis on 1% I.D.NA agarose (Cambrex Bio Science Rockland Inc. USA) gel at 30 V for 10 h and blotted onto nitrocellulose membrane, Hybond-N$^+$ (Amersham Biosciences UK Limited, Buckinghamshire, U.K.). A 444 bp DNA fragment of the *Wx-D1* gene was used as the hybridized probe (**Figure 1**). The probe was generated by PCR twice using the primers wxs01-5′ and wx012-3′ (**Table 1**). The first PCR was performed using genomic DNA extracted from common wheat varieties as a template, and the amplification product was purified by electrophoresis and the QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The second PCR was performed using the purified amplification product of the first PCR as a template, and the amplification product was purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). We labeled the probe by alkaline phosphatase enzyme and detected CDP-*Star* chemiluminescent detection reagents using the Gene Images Alkphos Direct Labeling and Detection System (Amersham Biosciences UK Limited), according to the kit's protocol. The hybridizing was performed at 53 °C overnight. The filter was then exposed to X-ray films for 2 h.

RESULTS AND DISCUSSION

Selection of a Taxon-Specific DNA Sequence for the Detection of Wheat Using PCR. To construct reliable GMO detection methods, it is necessary to design taxon-specific DNA sequences as well as GM target-specific sequences. The taxon-specific DNA has three requirements: that it be species-specific, have a single or a stable low copy number, and have low intraspecies variability. To select a suitable reference gene of wheat for PCR amplification, we searched for candidate DNA sequences that had a single-copy gene in *T. aestivum* L. using a public DNA database, the DNA Data Bank of Japan (DDBJ). We obtained several candidates for wheat-specific genes, such as *Wx-D1* (accession no. AF113844), *TaSUT* (accession no. AF408845), *CbpII* (accession no. J02817), and *Lr1* (accession no. S79983) genes and the wheat GSS region (accession no. AJ440705). The specificities of these genes and their copy numbers were investigated by PCR and Southern blot analyses, respectively, and all of the genes except for *waxy* cross-reacted with durum wheat (data not shown). The *waxy* genes encode the *waxy* protein (granule-bound starch synthase, EC 2.4.1.21), which plays a role in amylase synthesis in the plastid of plants (*16*). In common wheat, the *waxy* gene is organized in a triplicate set of single-copy homeoloci, *Wx-A1*, *Wx-B1*, and *Wx-D1*, derived from chromosomes 7A, 4A, and 7D, respectively (*17*−*19*), although durum wheat does not have the genome D. In fact, the *Wx-D1*-specific PCR primer (DRSL) was developed as a marker to classify partial waxy wheat (*13*). Consequently, we sequenced a region of the *Wx-D1* gene and obtained the 1323 bp DNA sequence (**Figure 2**). We designed several primer pairs from the sequence and selected the best primers (wx012-5′/3′). The TaqMan probe (wx012-T) was also designed in the sequence between the primers (**Table 1**), which were evaluated for taxon specificity by both qualitative and quantitative PCRs.

```
CgCgCCCCTggCTCACCAgTATgATggCCggCgCCgACgTgCTCgCCgTC 50
ACCAgCCgCTTCgAgCCCTgCggCCTCATCCAgCTCCAggggATgCgCTA 100
CggAACggTAAACTTTTCCTTCTTgCCAAgTCCTTACTTCCTgAgCAATC 150
ATgAgCCATgCCCATgACCgAAgTTTCTTCCAAATTTTCAgCCgTgCgCg 200
TgCgCgTCCACCggCgggCTTgTCgACACgATCgTggAgggCAAgACCgg 250
gTTCCACATgggCCggCTCAgTgTCgATgTAAgTTCATCAATCTCTTCAA 300
TAAATTCTTCATCTTgTTCATCCTgggAgCTCAggCAgATCATCAAACgg 350
gTTTCCTTTTTCCTCTTggTggCCAgTgCAACgTggTggAgCCggCCgAC 400
gTgAAgAAggTggTgACCACCCTgAAgCgCgCCgTCAAggTCgTCggCAC 450
gCCggCATACCATgAgATggTCAAgAACTgCATgATACAggATCTCTCCT 500
ggAAggTAAgTCAgTCTCTggTCTggTTTAggATgCATTTTCCAgAACAA 550
CTAAgAgTTAAgACTACAATggTgCTCTTgTTCgATgTATCCATTAATgg 600
TggCTTgCgCATATggTgCAggggCCAgCCAAgAACTgggAggACgTgCT 650
TCTggAACTgggTgTCgAggggAgCgAgCCgggggTCATCggCgAggAgA 700
TTgCgCCgCTCgCCATggAgAACgTCgCCgCTCCCTgAAgAgAgAAAgAA 750
AAggAAgTTCTggTgCATggAgCgTCCATCCAgTCTgCAgggTTCTCgTA 800
TggggAgATAgCCgCTTgTTgTAgCgAAgAAgggCCgATATATATAATAT 850
ATAgACTTATAAgTACTTAACTTTTgTTgTgCCgCTTgCCTCTTTTACAA 900
ACAAAAAAgAAgTTAggggTTgTgCTTgTTATAgTgTgCTgAACTgTgCT 950
TgCATTTTggTgTggTATATTgCAATAAACAAAggATTTgTTATgTgTTT 1000
TTgCTATTggTTCTCCgTgTTTgAgCCgAATCAAgTTATTTTgTggggT 1050
TTCAAAggTACATTTTTgTgTTCTTggAggTggCAgCTTCggTCgCAggA 1100
ACAgAggTgTTCAAggCggCCgAAATAggTTgCCgCCTgCggCggAATCg 1150
CCACCCACCgTgAAgTTCACCgTTTCgCAATggAggAACACCTAggTgTA 1200
AgTTTCAAAATggCggCgCgATgACCgCCAAgATCAATgCgACACAACCA 1250
ggAAATgACAgATgACCgCCAAgATCAACgCACACAACAAATgACgCAAg 1300
gggAgCgATCATggCTgAAACAg
```

Figure 2. Detected sequence of 3′-terminal of *Wx-D1*.

Specificity in the Qualitative and Quantitative PCR Detections. To evaluate the specificity of the Wx012 PCR system using the primers (wx012-5′/3′) and TaqMan probe (wx012-T), we ran qualitative and quantitative real-time PCRs using 25 and 50 ng of genomic DNA extracted from 11 different species of plant, including barley, rye, oats, Italian millet, common millet, soybeans, buckwheat, rice, rapeseed, maize, sesame, and a common wheat (Norin 61) as templates. The electrophoresis results of the qualitative PCR showed that a 102 bp band expected size was observed from the common wheat, and there were no amplification products observed from any of the species tested other than wheat (**Figure 3A**). The results of the DNA sequence from each PCR product corresponded to

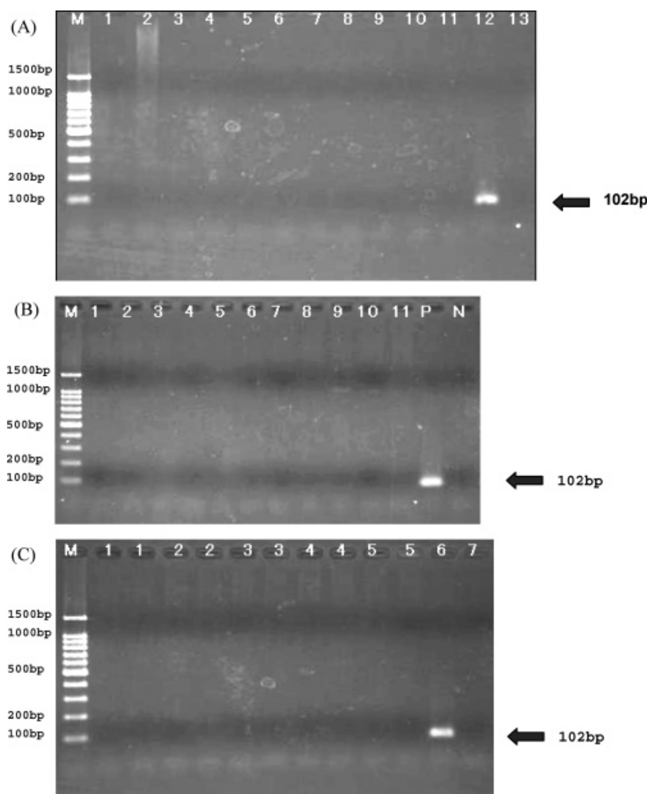

Figure 3. Specificity detection of the Wx012 DNA sequence by qualitative PCR with Wx012-5′/3′ primer pairs: (**A**) agarose gel electrophoresis of PCR products amplified from wheat and other plants genomic DNA [barley (1), rye (2), oats (3), Italian millet (awa) (4), common millet (kibi) (5), soybeans (6), buckwheat (7), rice (8), rapeseed (9), maize (10), sesame (11), and common wheat (12); the others are amplification of the no-template control (13) and the 100 bp ladder size standard (M)]; (**B**) agarose gel electrophoresis of the PCR product amplified from 11 varieties of rice genomic DNA [Koshihikari (1), Jaguary (2), Khao Nok (3), Khau Tan Chiem (4), Urasan No. 1 (5), Lemont (6), Amaroo (7), IR36 (8), RD6 (9), Dular (10), Aijiaonante (11), common wheat (P), and no-template control (N)]; (**C**) agarose gel electrophoresis of PCR products amplified from four varieties of durum wheat genomic DNA (1–4), Canadian amber durum (5), common wheat (6), and no-template control (7). Lane M indicates the 100 bp ladder size standard.

the expected genomic DNA sequence (data not shown). We also investigated the specificities of the PCR system to DNA extracted from 11 varieties of rice (**Figure 3B**), the 4 varieties of durum wheat, and multiple kernels of CAD durum wheat (**Figure 3C**). The DNA extracted from multiple kernels of CAD was used to widely confirm the specificity of the PCR system, because commercial wheat grain is usually a mixture of multiple varieties. In addition, as a result of the real-time PCR using the Wx012 to the DNAs extracted from the N61 and the other plant species, there were no amplifications detected with any of the species tested other than wheat (**Figure 4**). Similar amplification plots were also obtained from DNAs extracted from the other 18 varieties that were derived from the five classes of commercial common wheat described under Materials and Methods (data not shown). These results indicated that the Wx012 system provides a highly specific PCR condition to common wheat in the tested species.

Performance of the Qualitative and Quantitative Detection of Wx012 from Different Common Wheat Varieties. The appropriate taxon-specific DNA sequence should not exhibit allelic variation and should have a single or stable low copy number among different wheat varieties. To test the intraspecies variability of the Wx012 region among different common wheat varieties, we ran qualitative PCR using 25 ng of genomic DNA extracted from the 19 varieties that were derived from the five classes of commercial common wheat described under Materials and Methods. The electrophoresis analysis after qualitative PCR showed that PCR products of identical size and equivalent intensity were obtained, and no additional band was shown in any of these tested varieties (**Figure 5A**). The results indicate that the Wx012 system could stably amplify the designed DNA sequences with high specificity and that there were no major differences among the common wheat varieties.

We performed real-time PCR on 50 ng of genomic DNA extracted in parallel from 19 different common wheat varieties. The results of quantitative PCR exhibited similar amplification plots (data not shown), and a comparison of the Ct values obtained showed slight variability (**Figure 5B**) ranging from 27.56 to 28.50. These results suggest that the copy number of the Wx012 region was considered to be identical among the varieties tested. The copy number of the Wx012 region can be considered to be single when the results of the Southern blot analysis are combined.

Copy Number of the Wheat Wx012 Gene Confirmed by Southern Blot. Single-copy genes are usually related to low probability of mutation and changes in the copy number among different varieties. Targeting a single or stable low copy number DNA sequence should result in stable, real-time PCR assays. Thus, to estimate the copy number of the Wx012 region in the common wheat genome, we performed a Southern blot analysis in which the genomic DNA was digested with *Bam*HI, *Eco*RI, and *Fba*I and hybridized with the 444 bp DNA fragment of the *Wx-D1* gene, as described under Materials and Methods (**Figure 1**). A region longer than the amplified sequence was used as the probe in the Southern blot analysis to obtain clear results. Two hybridized bands were detected in every enzyme-digested DNA sample (**Figure 6**). The target Wx012 region was not amplified in durum wheat in the above PCR analysis. The results indicate that each homologous chromosome D has a single copy or two copies of the target fragment of Wx012, because the *Wx-D1* gene is located on diploid chromosomes in the D genome, and each gene may have different restriction sites of them. Therefore, we concluded that the Wx012 region should be single copy or two copies in common wheat genomic DNA.

Sensitivity of Qualitative and Quantitative PCR Assays. We assessed the sensitivity of the Wx012 system by performing PCR amplification three times at 10 levels (0−150 ng of DNA as template) of a diluting amount of wheat genomic DNA that was quantified by spectrophotometer. The qualitative PCR stably allowed detection of a fragment of the Wx012 region in 125 pg of common wheat genomic DNA in the PCR amplification five times (data not shown). The limit of detection could be in the range of 6.4−7.6 copies, because one haploid genomic copy size of common wheat was considered to be in the range of 16.5−19.5 pg (*20−23*), and the Wx012 system could theoretically detect up to 0.5% of target template DNA in one reaction tube.

In a quantitative PCR assay, the amplification plots could be stably obtained when the amount of the DNA template was lowered to 250 pg (**Figure 7**) and when the amount of DNA template corresponded to ∼15.1 genomic copies. In addition, to verify the accuracy of the quantification system for relative quantitative analysis, we drew a standard curve for the Wx012 quantitative PCR system three times. A linear relationship (coefficient counts were from 0.996 to 0.999) with a slope from −3.4 to −3.7 was obtained between the Ct value and the initial

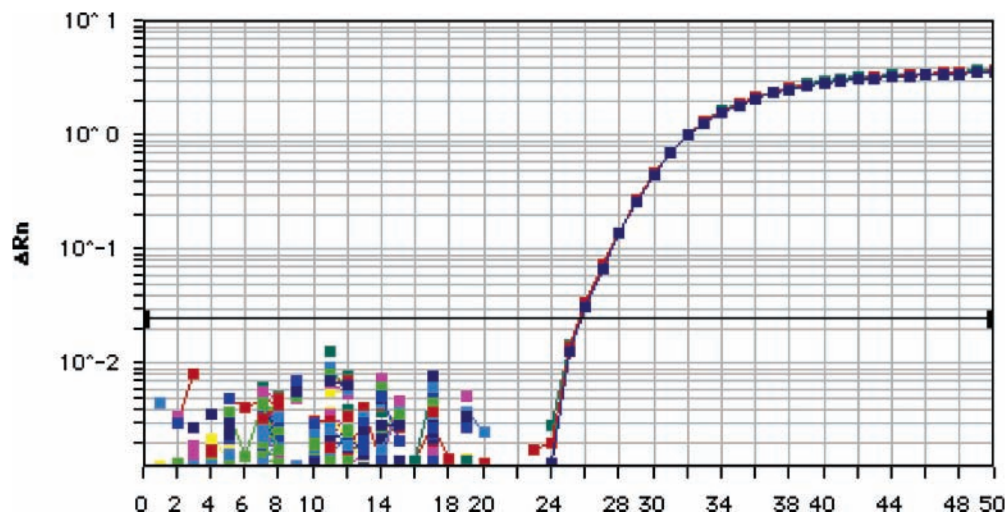

Figure 4. Specific quantitative detection of the Wx012 DNA sequence with wx012-5′/3′ primers and the wx012-T probe. The amplification plot was generated from 14 different plants [buckwheat, kidney beans, rapeseed, ceci, Italian millet (awa), common millet (kibi), soy, oats, rye, maize, rice, and barley], no-template control (salmon sperm), 10 varieties of rice, 4 varieties of durum wheat, no-template control (salmon sperm), and common wheat (Norin 61). No amplification was observed other than that for common wheat.

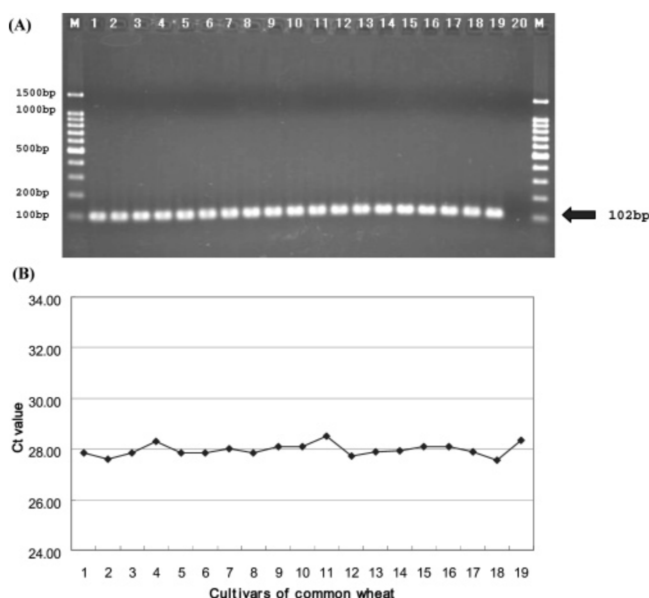

Figure 5. Performance of the qualitative and quantitative detection of Wx012 from different common wheat varieties: (**A**) agarose gel electrophoresis of the PCR products amplified from 19 varieties of common wheat (1–19) and no-template control (20) and 100 bp ladder size marker (M); (**B**) Ct values obtained from 19 different varieties of common wheat.

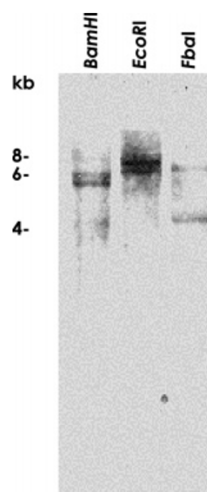

Figure 6. Estimation of copy number of Wx012 fragment as illustrated by genomic Southern blot of common wheat genomic DNA digested with *Bam*HI, *Eco*RI, and *Fba*I, hybridized to the 444 bp probe.

DNA quantity (picograms). This result indicates that the Wx012 quantitative PCR system is sufficiently efficient to quantify the genomic DNA of common wheat.

The threshold level of the accidental or technically unavoidable presence of authorized GMO for non-GM labeling has been defined as 0.9% (EU), 3% (Korea), and 5% (Japan) (*24*−*26*); the range of the quantification of GMO should be as a low as 0.5%. According to the quantification method of GM maize and soy in previous reports (*5*, *6*), the copy number of the taxon-specific gene is calculated using plasmid as reference molecules, and the limit of quantification (LOQ) of the molecules is 20 copies. The 20 haploid genomic copies of wheat in the 50 ng template DNA included in one PCR tube should, in theory, be in the range of 0.66−0.78%, which could be higher than the LOQ of the GM wheat. To develop a sensitive quantitative PCR method for detecting GM wheat that can be marketed soon, a PCR detection system with sufficient sensitivity must be designed with due consideration for genomic size and the copy number of target DNA sequences.

Reproducibility of Quantitative PCR. To further demonstrate the accuracy of the Wx012 quantification system, we ran the quantitative PCR in triplicate with five dilutions of common wheat genomic DNA. The coefficient of variation (CV%) values ranged from 1.015 to 2.475, and the standard deviation (SD) values ranged from 0.256 to 0.880, respectively (**Table 2**). Because these values were relatively small, this system was considered to be stable and reliable.

Effect of Extraction Methods of Templates DNA on Wx012 Real-Time PCR. To investigate the effects of the extraction methods of template DNAs on the quantification of the Wx012 region, we extracted common wheat genomic DNAs using four methods, the DNeasy Plant maxi kit, the DNeasy Plant mini kit, the Genomic-Tip 20/G (Qiagen), and the CTAB method (*14*). Quantitative real-time PCRs using the Wx012 system for each extracted DNAs exhibited equivalent amplification plots regardless of the extraction methods used (data not shown), with the CV% values ranging from 1.790 to 3.527 and the SD values ranging from 0.5132 to 1.009, respectively. The

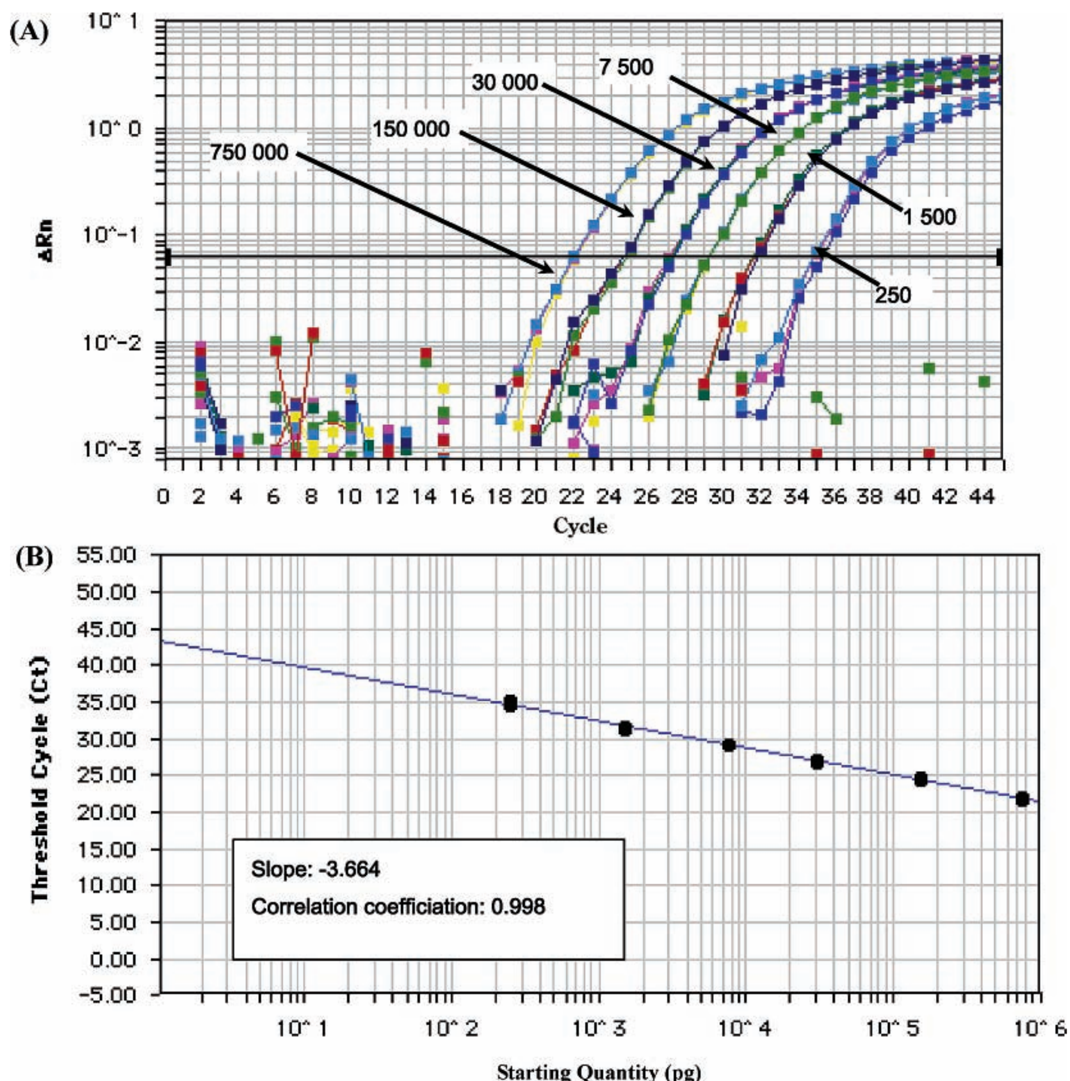

Figure 7. (**A**) Amplification plots of real-time PCR for detection of Wx012 DNA sequence generated by seven concentrations of genomic DNA from common wheat (0, 250, 1500, 7500, 30 000, 150 000, and 750 000 pg per reaction). (**B**) Standard curve generated from the amplification data given in (**A**).

Table 2. Reproducibility of the Ct Measurements Using Real-Time PCR for Five Levels of Concentration of Genomic DNA from Common Wheat

starting concn (ng/μL)	Ct value			av	SD[a]	CV[b] (%)
	1	2	3			
0.1	34.56	35.98	36.17	35.57	0.88	2.475
0.6	31.72	32.6	33.1	32.47	0.699	2.153
12	27.28	28.08	28.11	27.82	0.468	1.681
60	25.03	25.5	25.45	25.33	0.257	1.015
300	22.24	22.9	22.8	22.65	0.356	1.573

[a] Standard deviation. [b] Coefficient of variation.

results indicated that all four extraction methods are applicable to the detection method of Wx012.

In this paper, we report on our design of a specific primer pair (wx012-5′/3′) to be used in qualitative and quantitative PCR assays for common wheat. A PCR product with a 102 bp length and its related qualitative and quantitative PCR cycling conditions were found to be suitable for the detection and the cycling conditions were similar to those of other widely used endogenous genes, such as maize and soybeans, in GMO detection (*5*, *6*, *14*). In addition, the sensitivities of 125 pg of wheat genomic DNA for qualitative PCR and of 250 pg of DNA for quantitative PCR were acceptable for monitoring the GM crops. The results in this paper demonstrate that this Wx012 fragment met the requirements for a taxon-specific reference DNA sequence for PCR analyses without false results. The requirements for a taxon-specific DNA sequence are a single or stable two-copy number, common wheat specificity, and homogeneity among the suitable varieties. We will be able to develop good GM wheat detection systems using PCR by developing GM target-specific sequences when new GM wheat varieties are marketed. All of the data from the real-time PCR indicated that the Wx012 region plays a role in the taxon-specific reference gene for the quantitative detection of GM wheat. Because we have demonstrated that the Wx012 primers and wx012-T probe give the high specificity for common wheat and the homogeneity in different varieties of common wheat in PCR detections, the Wx012 must provide reliable data that are not influenced by other species of plants, including durum wheat, in the sample. In addition, quantitative PCR using the primer/probe system of Wx012 gave a highly linear coefficient among the Ct values, the initial amount of the wheat DNA, and the reproducible data. These results indicated that we would be able to design appropriate and stable quantitative methods for detecting GM wheat if there is a need. In the future, we will confirm whether

the wx012 system can play a role in various matrices made from wheat, such as bread and noodles.

LITERATURE CITED

(1) Matsuoka, T.; Kawashima, Y.; Akiyama, H.; Miura, H.; Goda, Y.; Sebata, T.; Isshiki, K.; Toyoda, M.; Hino, A. A detection method for recombinant DNA from genetically modified soybeans and processed foods containing them. *J. Food Hyg. Soc. Jpn.* **1999**, *40*, 149−157.

(2) Matsuoka, T.; Kawashima, Y.; Miura, H.; Kusakabe, Y.; Isshiki, K.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. A method of detecting recombinant DNAs from four lines of genetically modified maize. *J. Food Hyg. Soc. Jpn.* **2000**, *41*, 137−143.

(3) Matsuoka, T.; Kuribara, H.; Takubo, K.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. Detection of recombinant DNA segments introduced to genetically modified maize (*Zea mays*). *J. Agric. Food Chem.* **2002**, *50*, 2100−2109.

(4) Akiyama, H.; Sugimoto, K.; Matsumoto, M.; Isuzugawa, K.; Shibuya, N.; Goda, Y.; Toyoda, M. A detection method of recombinant DNA from genetically modified potato (New Leaf Plus Potato) and detection of New Leaf Plus potato in snacks. *J. Food. Hyg. Soc. Jpn*. **2002**, *43*, 24−29.

(5) Kuribara, H.; Yoichiro, S.; Matsuoka, T.; Takubo, K.; Futo, S.; Aoki, N.; Hirao, T.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Novel reference molecules for quantitation of genetically modified maize and soybean. *J. AOAC Int.* **2002**, *85*, 1077−1089.

(6) Shindo, Y.; Kuribara, H.; Matsuoka, T.; Futo, S.; Sawada, C.; Shono, J.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Validation of real-time PCR analysis for line-specific quantitation of genetically modified maize and soybean using new reference molecules. *J. AOAC Int*. **2002**, *85*, 1119−1126.

(7) James, C. Preview: Global status of commercialized transgenic crops. *ISAAA Briefs* **2003**, *30*.

(8) Obert, C. J.; Ridley, P. W.; Schneider, W. R.; Riordan, G. S.; Nemeth, A. M.; Trujillo, A. W.; Breeze, L. M.; Sorbet, R.; Astwood, J. D. The composition of grain and forage from glyphosate tolerant wheat MON7180 is equivalent to that of conventional wheat (*Triticum aestivum* L.). *J. Agric. Food Chem.* **2004**, *52*, 1375−1384.

(9) Anand, A.; Zhou, T.; Trick, H. N.; Gill, B. S.; Bockus, W. W.; Muthukrishnan, S. Greenhouse and field testing of transgenic wheat plants stably expressing genes for thaumatin-like protein, chitinase and glucanase against *Fusarium graminearum*. *J. Exp. Bot.* **2003**, *54* (384), 1101−1111.

(10) Koo, W. W.; Taylor, R. D. *2005 Outlook of the U.S. and World Wheat Industries, 2004−2013*; Center for Agricultural Policy and Trade Studies, Department of Agribusiness and Applied Economics, North Dakota State University: Fargo, ND, 2005; pp 2−7.

(11) Sun, G.; Bond, M.; Nass, H.; Martin, R.; Dong, Z. RAPD polymorphisms in spring wheat cultivars and lines with different level of *Fusarium* resistance. *Theor. Appl. Genet.* **2003**, *106* (6), 1059−1067.

(12) Testing for Foods Produced by Recombinant DNA Techniques by Ministry of Health, Labor and Welfare; Ministry of Health, Labour and Welfare, Tokyo, Japan, 2002.

(13) Nakamura, T.; Vrinten, P.; Saito, M.; Konda, M. Rapid classification of partial waxy wheats using PCR-based markers. *Genome* **2002**, *45*, 1150−1156.

(14) Matsuoka, T.; Kawashima, Y.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. Method of detecting recombinant DNAs from four lines of genetically modified maize. *J. Food Hyg. Soc. Jpn.* **2000**, *41*, 137−143.

(15) *Instruction Manual for Testing and Analyzing Genetically Modified Food—Quantitative PCR*; Japanese Agricultural Standard Testing and Analysis Handbook Series; Centre for Food Quality, Labeling and Consumers Services: Saitama, Japan, 2002.

(16) Vos-Scheperkeuter, G. H.; de Boer, W.; Visser, R. G. F.; Feenstra, W. J.; Witholt, B. Identification of granule-bound starch synthase in potato tubers. *Plant Physiol.* **1986**, *82*, 411−416.

(17) Chao, S.; Sharp, P. J.; Worland, A. J.; Warham, E. J.; Koebner, R. M. D.; Gale, M. D. RFLP-based genetic maps of wheat homeologous group 7 chromosomes. *Theor. Appl. Genet.* **1989**, *78*, 495−504.

(18) Ainsworth, C.; Clerk, J. Expression, organization and structure of the genes encoding the *waxy* protein (granule-bound starch synthase) in wheat. *Plant Mol. Biol.* **1993**, *22*, 67−82.

(19) Vrinten, P.; Nakamura, T.; Ymamori, M. Molecular characterization of *waxy* mutations in wheat. *Mol. Gen. Genet.* **1999**, *261*, 463−471.

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

(21) Bennet, M. D.; Smith, J. B. Nuclear DNA amounts in angiosperms. *Philos. Trans. R. Soc. London Ser. B−Biol. Sci.* **1976**, *274*, 227−274.

(22) Bennet, M.; Leitch, I. Nuclear DNA amounts in angiosperms; progress, problems and prospects. *Ann. Bot.* **2005**, *95*, 45−90.

(23) Bennet, M.; Leitch, I. Nuclear DNA amounts in angiosperms. *Ann. Bot.* **1995**, *76*, 113−176.

(24) European Commission (EC) Regulation 49/2000, January 10, 2000. *Off. J. Eur. Communities* **2000**, *L6*, 13−14.

(25) Notification 2000-31 (April 22, 2000), Ministry of Agriculture and Forestry of Korea, Seoul, Korea.

(26) Notification 1775 (June 10, 2000), Food and Marketing Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan, Tokyo, Japan.

Received for review March 15, 2005. Revised manuscript received June 14, 2005. Accepted June 15, 2005.

JF0505731