Original Paper

An Endogenous Reference Gene of Common and Durum Wheat for Detection of Genetically Modified Wheat

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To develop a method for detecting GM wheat that may be marketed in the near future, we evaluated the proline-rich protein (PRP) gene as an endogenous reference gene of common wheat (Triticum aestivum L.) and durum wheat (Triticum durum L.). Real-time PCR analysis showed that only DNA of wheat was amplified and no amplification product was observed for phylogenetically related cereals, indicating that the PRP detection system is specific to wheat. The intensities of the amplification products and Ct values among all wheat samples used in this study were very similar, with no nonspecific or additional amplification, indicating that the PRP detection system has high sequence stability. The limit of detection was estimated at 5 haploid genome copies. The PRP region was demonstrated to be present as a single or double copy in the common wheat haploid genome. Furthermore, the PRP detection system showed a highly linear relationship between Ct values and the amount of plasmid DNA, indicating that an appropriate calibration curve could be constructed for quantitative detection of GM wheat. All these results indicate that the PRP gene is a suitable endogenous reference gene for PCR-based detection of GM wheat.

Key words: endogenous gene; genetically modified organism; proline-rich protein (PRP); real-time PCR; wheat

Introduction

Many GM crops have been developed worldwide. In several countries, the commercial use of GM crops, such as soybean and corn, requires government approval after a safety assessment, and the number of authorized GM crops has been increasing rapidly1. As of June 2012 in Japan, 186 GM crops, including soybean, corn, canola, cotton, beet, alfalfa, potatoes, and papaya had undergone safety assessment and been authorized for distribution and consumption as food. Although safety assessment is required for obtaining marketing authorization of GM crops, many consumers remain concerned about the safety of GM crops and processed foods containing them. To provide information regarding the GM technologies used and to foster consumers’ rights to select foods of their choice, labeling systems for GM crop-derived foods have been or are being introduced in the European Union (EU), Korea, Japan, and Australia, among other countries.

Analytical methods are required to assist in the enforcement of these labeling policies of GM organisms (GMOs). Numerous PCR-based methods have been developed in recent years for the detection of GMOs6–13. Almost all PCR-based GMO detection methods include GMO-specific PCR as well as endogenous reference gene-specific PCR.

An endogenous reference gene is a taxon-specific DNA sequence that plays an important role in the detection of GMOs. For qualitative detection, endogenous reference gene-specific PCR is used as an experimental control to confirm successful amplification of each DNA sample. For quantification, copy numbers of GMO-specific DNA and an endogenous reference gene are measured by real-time PCR and then the GMO content (%) is calculated as the ratio of these copy numbers. Therefore, selection of an appropriate endogenous reference gene is essential for the development of detection methods for GM crops. In general, an endogenous reference gene should fulfill the following three requirements: species specificity, sin-
gle or stable low-copy-number, and low intraspecific variability of nucleotide sequences.

Although no GM wheat has been commercialized as yet, many types of GM wheat, such as herbicide-tolerant and Fusarium-resistant wheat, have been developed using recombinant DNA techniques. Climate change events such as dry weather have resulted in lean harvests and a resulting rise in crop prices, which are likely to lead to an increased demand and supply of GM wheat in the near future. Thus, the development of detection methods for GM wheat would be required. We previously proposed the Waxy-D1 (Wx-D1) gene as a candidate endogenous reference gene for wheat, since it fulfilled the three requirements stated above. However, the Wx-D1 gene is not present in durum wheat (Triticum durum L.), an important wheat species widely used as a main ingredient of pasta. Therefore, we sought a candidate gene other than Wx-D1 gene. Here we describe the results of testing of the proline-rich protein (PRP) gene for suitability as an endogenous reference gene for common wheat (Triticum aestivum L.) and durum wheat.

Materials and Methods

Plant materials

Eight kinds of common wheat samples, No. 1 Canada Western Red Spring (1CW); Hard Red Spring (HRS); Hard Red Winter (HRW); Prime Hard (PH); Australian Standard White (ASW); and Western White (WW), two domestic wheat varieties (Norin 61 and Kitahonami) and durum wheat, were provided by Nisshin Flour Milling Inc. (Tokyo, Japan).

Other seed samples of different species were obtained from local stores: barley (Hordeum vulgare), rye (Secale cereale), rice (Oryza sativa), oats (Avena sativa), Italian millet (Awa; Setaria italica), common millet (kibi; Panicum milaceum L.), sorghum (Sorghum bicolor), buckwheat (Fagopyrum esculentum), rapeseed (Brassica napus), and corn (Zea mays).

DNA extraction

To remove debris, seed samples were washed with 1% sodium dodecyl sulfate (SDS), rinsed with distilled water, and dried. The seeds were ground using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) and the flour was used for DNA extraction. For common and durum wheat, DNA was extracted using the DNeasy Plant Maxi kit (QIAGEN, Hilden, Germany). The other plant DNAs were extracted using the GM quicker 2 kit (NIPPON GENE, Tokyo, Japan) according to the manufacturer’s manual. DNA concentration and quality were evaluated in terms of UV absorbance measured with a spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE, U.S.A.).

Sequencing of the downstream region of the PRP gene

For the purpose of retrieving the genomic DNA sequence adjacent to the wheat PRP gene (GenBank accession X52472), a wheat (Kitahonami) DNA library was constructed. Gene-specific primers 1 and 2 (GSP1 and GSP2) for the PRP gene were designed and synthesized by Operon Biotechnologies (Tokyo, Japan). GSP1: 5’-CATAGTATCCGGTTAAGGATGTA-3’. GSP2: 5’-GCACCCATGATGACTACTATTCTGTA-3’. GSP1 and GSP2 were coupled with the adaptor primers 1 and 2 (AP1 and AP2) for nested PCR, which was performed using the Genome Walker™ Universal Kit (Clontech, CA, U.S.A.) according to the manufacturer’s manual. After purification using a ChargeSwitch™-Pro PCR Cleanup Kit (Life Technologies, CA, U.S.A.), direct sequencing was performed by Operon Biotechnologies.

Oligonucleotide primers and probes

Oligonucleotide primers and probes were designed for conventional PCR and real-time PCR analysis using Primer Express 3 software (Life Technologies). The primers were synthesized by Operon Biotechnologies. The oligonucleotide probes, labeled with 6-carboxyfluorescein (FAM) at the 5’ end and 6-carboxytetramethylrhodamine (TAMRA) at the 3’ end, were synthesized by Life Technologies.

Conventional PCR analysis

The PCR reaction solution (25 µL) contained 1× PCR buffer II, 0.2 mmol/L dNTP, 1.5 mmol/L MgCl2, 0.5 µmol/L primers, 0.625 U AmpliTaq Gold® (Life Technologies), and 50 ng genomic DNA. PCR runs were run on a C1000 Thermal Cycler (Bio-Rad Laboratories, CA, U.S.A.). The primer pairs are listed in Table 1. Cycling conditions were 10 min at 95°C and 40 cycles of 30 s at 95°C, 30 sec at 60°C, and 30 sec at 72°C followed by a final extension at 72°C for 7 min.

<table>
<thead>
<tr>
<th>Name</th>
<th>Size (mer)</th>
<th>Sequence</th>
<th>Amplicon size (bp)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRPSF</td>
<td>29</td>
<td>5’-GCA CCC ATG AGT ACT ACT ATT CTG TA-3’</td>
<td>117</td>
<td>Taxon-specific detection</td>
</tr>
<tr>
<td>PRPds6R</td>
<td>22</td>
<td>5’-TGC AAA CGA ATA AAA GCA TGT G-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wx012F</td>
<td>20</td>
<td>5’-GTT CGC AGG AAC AGA GGT GT-3’</td>
<td>102</td>
<td>Taxon-specific detection</td>
</tr>
<tr>
<td>Wx012R</td>
<td>20</td>
<td>5’-GTT CCT CCA TTT CGA AA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRP-Taq5</td>
<td>28</td>
<td>5’-CTG TGC ACA TGA TCT TCT TCT GTC -3’</td>
<td></td>
<td>TaqMan Probe</td>
</tr>
<tr>
<td>Wx-Taq 1</td>
<td>23</td>
<td>5’-CAA GGC GGC CGA AAT AGG TTG CC-3’</td>
<td></td>
<td>TaqMan Probe</td>
</tr>
</tbody>
</table>
Specificity of the proposed detection method was evaluated with DNAs of 12 samples [one sample each of common wheat (Kitahonami) and durum wheat and 10 other species of plants], and nucleotide sequence stability was evaluated with DNAs of durum wheat and eight common wheat samples.

PCR products and a 100-bp DNA ladder standard (TaKaRa Bio, Otsu, Japan) were electrophoresed on 1.5% agarose gel. The gels were stained with SYBR® Green I (TaKaRa Bio).

Real-time PCR analysis

For real-time PCR analysis, the reaction solution (25 μL) contained 50 ng of sample DNA, 12.5 μL of Universal Master Mix (Life Technologies), 0.5 μmol/L primer pairs, and 0.2 μmol/L probe (Life Technologies). Real-time PCR analysis was performed on an ABI PRISM 7900HT (Life Technologies) thermal cycler according to the following step-cycle program: preincubation at 50°C for 2 min and 95°C for 10 min, 45 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 1 min. Specificity and nucleotide sequence stability were tested using the same DNA samples described in the preceding section.

Limit of detection

Limit of detection (LOD) was evaluated by amplifying a known amount of wheat DNA. DNA extracted from the Kitahonami variety of common wheat was used for evaluating LOD. The haploid genome size and mass of wheat were estimated to be 15,966 Mbp and 16.55 pg, respectively. The measured DNA concentrations were converted to numbers of haploid wheat genomes and diluted to make a series of 50, 25, 10, 5, 2.5, and 0.5 haploid genomes/μL. Real-time PCR analysis was performed with a reaction solution (10 μL) containing 0.5–50 haploid genomes, 5.0 μL of Universal Master Mix, 0.5 μmol/L primer pairs, and 0.2 μmol/L probe. The thermal cycling program was the same as that for real-time PCR.

Data analysis was performed using the “Amplification Plot” feature; the threshold was set at 0.256 and the baseline was set from 3 to 15. Amplification lines that crossed the threshold line were classified as positive.

LOD is defined as the lowest concentration of analyte giving a false negative rate of ≤5% (ISO 24276). Accordingly, 21 parallel PCR amplifications were performed on each dilution, and when the number of positives was ≥20, we considered the concentration to be detectable.

Plasmid construction

To connect the amplicons of the Wx-D1 and PRP genes, we performed PCR by the overlap extension method as described elsewhere. The integrated fragments were ligated into a pCR2.1 plasmid vector using a TOPO TA Cloning® Kit (Life Technologies) (Fig. 1). Cells of Escherichia coli strain TOP 10 (Life Technologies) were transformed using the plasmids. Plasmids were extracted and purified using the Purelink quick plasmid extraction kit (Life Technologies). To confirm the sequence of the inserted fragments, direct sequencing was performed by Operon Biotechnologies.

Results and Discussion

Selection of the candidate gene and its target region

We first searched the DDBJ database (http://www.ddbj.nig.ac.jp/) for taxon-specific DNA sequences that might be endogenous reference gene candidates. Follow-

![Figure 1](image1.png)

**Fig. 1.** Construction of the standard plasmid used for quantification

The schematic indicates the sequenced regions of the PRP and Wx-D1 genes.

![Figure 2](image2.png)

**Fig. 2.** Sequences of the downstream regions of the PRP genes of wheat and barley

Solid arrows indicate the sequences of the PRP8F/PRPds6R primer pairs. The bold box indicates the site of the Taq-Man probe.
Fig. 3. Cereal-crop specificity test using PRPSF/ds6R primer pairs

(A) Conventional PCR analysis: (M) 100-bp DNA ladder, (1) common wheat (Kitahonami), (2) durum wheat, (3) barley, (4) rye, (5) oats, (6) rice, (7) Italian millet, (8) common millet, (9) sorghum, (10) buckwheat, (11) rapeseed, (12) corn, and (13) no template (control).

(B) Real-time PCR analysis: Qualitative analysis of DNA from common wheat, durum wheat, and 10 non-target plant species. Amplification signals are observed only for common and durum wheat.
ing previous reports\(^{14,15}\), the criteria for the reference gene were set as follows: common and durum wheat specificity, presence of a single copy or stable low-copy-number, and low heterogeneity in wheat. We obtained several candidates for wheat-specific genes including \(Lr1\) (accession ST9983), \(gss\) (AJ440705), and \(PRP\) (X52472). For \(Lr1\) gene and \(gss\) gene, three primer pairs for either sequence were designed and PCR was performed\(^{15}\). None of these three primer pairs allowed common and durum wheat-specific detection without cross-reaction with other plants, including rye and oats\(^{15}\). Therefore, we selected the \(PRP\) gene for further evaluation.

**Specificity of the PRP gene in qualitative analyses**

The upstream region of the \(PRP\) gene contains many repeated sequences\(^{15}\). Therefore, we selected a candidate region for the design of primer pairs in the downstream region of wheat \(PRP\) gene. This region was found to have similarity to that of barley. We sequenced the corresponding regions in wheat and barley. They showed sufficient differences to distinguish wheat from barley (Fig. 2). We designed several primer pairs in this region and selected the best (PRP8F/PRPds6R) (Table 1).

To determine whether amplification with the primer pair PRP8F/PRPds6R was wheat-specific, we performed conventional PCR using DNAs extracted from barley, rye, oats, Italian millet, common millet, sorghum, buckwheat, rice, rapeseed, corn, common wheat (Kitahonami), and durum wheat as templates. Amplification products with the expected size of 117 bp were observed from common and durum wheat, but not from any of the other species (Fig. 3A). This result indicates that the primer pair PRP8F/PRPds6R is specific to wheat. We then designed a TaqMan probe (PRP-Taq5) (Fig. 2) using the sequence between the primer pair PRP8F/PRPds6R. To confirm the specificity of the PRP detection system comprising the newly designed primer pair and probe and real-time PCR conditions described in Materials and Methods, we performed real-time PCR analyses with DNA of common wheat, durum wheat and other plant species. Only DNAs extracted from common and durum wheat gave amplified products (Fig. 3B), demonstrating that the PRP detection system yielded amplified products from only the DNAs of common and durum wheat among all the grains tested. Therefore, we concluded that the PRP detection system was specific to wheat.

**Stability of the PRP detection system among wheat varieties**

To test the intraspecific variability of the PRP region among the eight common wheat and durum wheat samples, we performed conventional PCR analysis using genomic DNAs extracted from these wheat varieties as described in Materials and Methods. On electrophoretic separation, the amplification products all showed the expected size of 117 bp and their intensities appeared to be equal, with no additional band in any wheat sample (Fig. 4A). These results indicated that the primer pair PRP8F/PRPds6R stably amplified the corresponding

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**Table 1**

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Mean Ct values*</th>
<th>SD of Ct values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1CW</td>
<td>28.04</td>
<td>0.06</td>
</tr>
<tr>
<td>HRS</td>
<td>28.00</td>
<td>0.03</td>
</tr>
<tr>
<td>HRW</td>
<td>28.14</td>
<td>0.08</td>
</tr>
<tr>
<td>PH</td>
<td>28.12</td>
<td>0.07</td>
</tr>
<tr>
<td>ASW</td>
<td>28.24</td>
<td>0.05</td>
</tr>
<tr>
<td>WW</td>
<td>28.18</td>
<td>0.02</td>
</tr>
<tr>
<td>Norin 61</td>
<td>28.15</td>
<td>0.16</td>
</tr>
<tr>
<td>Kitahonami</td>
<td>28.15</td>
<td>0.06</td>
</tr>
<tr>
<td>Durum</td>
<td>27.75</td>
<td>0.10</td>
</tr>
</tbody>
</table>

*Mean is obtained from 3 replicates
DNA sequences with high specificity and that no major differences were present among the tested wheat samples. In addition, to evaluate the stability of the PRP detection system, we performed real-time PCR with genomic DNAs extracted from these wheat samples. The Ct values ranged from 27.75 to 28.24, and no significant difference was observed (Fig. 4B). These results suggested that the PRP detection system performed with high stability among these wheat samples and that the copy numbers and sequences of the PRP region were almost identical.

**Linearity and PCR efficiency in quantitative analysis**

We performed real-time PCR analysis with five copy number levels (500,000, 50,000, 5,000, 500, and 50) of plasmid DNAs (Fig. 1) and constructed a standard curve for the PRP detection system (Fig. 5). A linear relationship was obtained between the Ct value and the initial plasmid DNA quantity with $R^2$ from 0.998 to 1.000, slopes ranging from $-3.40$ to $-3.36$ and PCR efficiencies ($E=10^{-\frac{1}{slope}}-1$) from 0.97 to 0.98. These results indicate that the PRP detection system can reliably quantify wheat genomic DNAs.

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**Fig. 5.** Results of linearity test of copy-number detection by the PRP detection system applied to common wheat

(A) Amplification plot generated with five serial dilutions of the plasmid containing 500,000, 50,000, 5,000, 500, and 50 copies and no template (control). (B) Standard curve of the system.
Copy number of the PRP region

Single-copy genes are generally associated with a low rate of mutation and change in copy number among varieties. A DNA sequence with a single or stable low copy number should yield similar Ct values among varieties. We previously concluded that common wheat genomic DNAs have either single or double copies of the Wx012 region\(^1\). To estimate the copy number of the PRP region in the common wheat genome, we performed quantitative analyses of the PRP and Wx012 regions. The copy numbers of each region were obtained by real-time PCR analysis using a calibration curve drawn with the plasmid diluted in series to 500,000, 50,000, 5,000, 500, and 50 copies per 2.5 µL. We calculated and compared the copy number of the Wx012 region and that of the PRP region. In this experiment, durum wheat was not included because the Wx012 system does not work on durum wheat\(^2\).

The ratio of the copy number of the Wx012 region to that of the PRP region averaged between 1.03 and 1.24 (Fig. 6). This result strongly suggests that the PRP and Wx012 regions have the same copy number in 50 ng of genomic DNA extracted from the eight common wheat samples. The copy numbers of the PRP and the Wx012 regions were almost identical, indicating that the PRP region represents a single- or double-copy gene in the common wheat haploid genome.

LOD evaluation

To further assess the accuracy of the PRP detection system, we performed real-time PCR analysis with six serial dilutions of DNA from the common wheat Kitahonami containing 50, 25, 10, 5, 2.5, and 0.5 haploid genome copies with 21 replicates per dilution. The S.D. of the Ct values ranged from 0.22 to 1.06 (Table 2). Since these values are relatively low, the precision of the system was concluded to be stable and reliable. While all 21 replicates were positive for the dilutions of 50, 25, and 10 haploid genome copies, 95% (20/21), 90% (19/21), and 43% (9/21) of the 21 replicates were positive for 5, 2.5, and 0.5 copies, respectively (Table 2). False negative rates of less than 5% were obtained for 50, 25, 10, and 5 copies, suggesting that the LOD value was 5 haploid genome copies.

Conclusion

We have demonstrated that the PRP detection system has the characteristics required for use as an endogenous reference gene of common and durum wheat: species specificity, high homogeneity among wheat samples, and single or stable low copy number. By qualitative analysis, we confirmed that the PRP detection system is specific to wheat and has high nucleotide sequence stability. Comparison of the copy number of the PRP region and the Wx012 region indicated that the PRP region is a single or double copy gene in the common wheat haploid genome. Furthermore, we were able to detect common wheat at levels as low as five haploid genome copies with the PRP detection system. In addition, we constructed a calibration curve for the quantitative detection of GM wheat. Real-time PCR analysis using the PRP detection system showed a highly linear relationship between the Ct values and the amount of plasmid DNA, as well as high PCR efficiency. These results suggest that the PRP gene would be a suitable endogenous reference gene for the PCR-based detection of GM wheat.

Acknowledgment

The authors thank Nissin Flour Milling Inc., for providing wheat seed.

### References


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**Fig. 6.** Quantification of the PRP gene using a wheat DNA template.

The graph indicates the ratio of the number of Wx012 genes to the number of PRP genes.


**Table 2.** Determination of the limit of detection

<table>
<thead>
<tr>
<th>Template copy number</th>
<th>Signal ratio(^a)</th>
<th>Mean Ct values(^b)</th>
<th>SD of Ct values</th>
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<tbody>
<tr>
<td>50</td>
<td>21/21</td>
<td>32.62</td>
<td>0.22</td>
</tr>
<tr>
<td>25</td>
<td>21/21</td>
<td>34.11</td>
<td>0.61</td>
</tr>
<tr>
<td>10</td>
<td>21/21</td>
<td>35.77</td>
<td>1.06</td>
</tr>
<tr>
<td>5</td>
<td>20/21</td>
<td>36.63</td>
<td>0.74</td>
</tr>
<tr>
<td>2.5</td>
<td>19/21</td>
<td>37.28</td>
<td>0.79</td>
</tr>
<tr>
<td>0.5</td>
<td>9/21</td>
<td>37.82</td>
<td>0.64</td>
</tr>
</tbody>
</table>

\(^a\)Signal ratio corresponds to positive/total PCR reactions.  
\(^b\)Mean Ct value of positive signals.


