Search for Cell-Wall-Degrading Enzymes of World-Wide Rice Grains by PCR and Their Effects on the Palatability of Rice

Sumiko Nakamura, Keisuke Machida, and Ken'ichi Ohtsubo

Faculty of Agriculture, Niigata University, 8050 Ikarashi-ninocho, Nishi-ku, Niigata 950-2181, Japan

Received February 28, 2012; Accepted May 28, 2012; Online Publication, September 7, 2012

[doi:10.1271/bbb.120147]

Such rice cultivars as Japonica, Japonica-Indica hybrid, Javanica and Indica, were evaluated for their main chemical components (amylose content and protein content), pasting property of rice flour (consistency), physical property of the cooked rice grains (adhesion, L3), and enzyme activities (cellulase and xylanase). The amylose content, cellulase activity and xylanase activity showed significant positive or negative correlation with the pasting property (consistency) of rice flour ($r = 0.89$, $r = 0.58$, $r = 0.70$, respectively) and with the physical property of the cooked rice grains (adhesion, L3: $r = -0.51$, $r = -0.61$, $r = -0.71$, respectively) at the level of 1%. Endogenous xylanase and cellulase played important roles to determine the texture of the cooked rice grains similarly to the amylose content. Part of the DNA sequences of the α-glucosidase gene differed among the Japonica, Japonica-Indica hybrid and Indica subspecies. We found discriminative DNA bands appearing by PCR, corresponding to 1,4-β-xylanase and endo-1,4-β-glucanase 13 in the case of Indica rice, Indica-Japonica hybrid rice, and Javanica rice (non-Japonica subspecies). The equation for estimating the physical property (adhesion) of cooked rice grains by PCR was improved by adding novel primers related to the cell-wall-degrading enzymes.

Key words: PCR; rice; cellulase; xylanase; texture

Rice (Oryza sativa L.) is one of the most important cereal crops throughout the world and is the staple food for about half of the world’s population. Rice is widely grown in over 100 countries, about 90% of the world’s rice being grown and consumed in Asia.1 Cultivated rice is classified into Japonica, Javanica and Indica subspecies, of which the palatability is markedly different with each. Cooked rice grains of Japonica are soft and sticky, while those of Indica are hard and non-sticky.2,3 Various physico-chemical measurements,4,5 near-infrared spectroscopy,6 chemical components and the Japanese Taste Analyzer7 have been reported for evaluating rice palatability.

It is well known that rice palatability is affected by the variety or cultivar and the variety characteristics are mainly determined by DNA. The properties intrinsic to each rice cultivar are determined by the DNA sequence, and the present authors have developed formulae for evaluating the palatability based on a multiple regression analysis using the results of physico-chemical measurements and PCR.8 We have improved these by adding starch-related PCR primers and protein-related primers.9 Lestari et al. have used our primers and their original primers to develop an estimation formula for the results of the Mido-value of cooked rice grains.10

Cooked rice is softened by the decomposition of the cell-wall, suggesting the important role of cell-wall-degrading enzymes in the physical properties of the endosperm cell wall. Softening of the texture upon cooking by the addition of cellulase11 and xylanase12 has been reported. Tsuiji et al. have reported that endogenous poly-galacturonase activities affected the texture of the cooked rice grains.13 The extent of decomposition of pectin has been correlated with the hardness of cooked rice ($r = -0.800$, $p < 0.05$) and the polygalacturonase activities of milled rice ($r = 0.677$, $p < 0.05$).13 α-Glucosidase hydrolyzes maltose and soluble starch to glucose, and it has been reported to affect the eating qualities of rice grains.14 Iwata has reported that the α-glucosidase activity showed a positive correlation with the GBSS activity and amylose content.5 This can be explained by a relationship between α-glucosidase and starch accumulation in developing rice. α-Glucosidase may be involved, to some extent, in amylopectin synthesis.15,16 A correlation ($R^2 = 0.86$) has also been reported between the viscosity of cereals (foodstuffs) and their arabinoxylan and β-glucan contents.17 Nakai et al. have reported that there were three mRNAs encoding function-unknown hydrolase family 31 homologous proteins in rice (Oryza sativa L., var Nipponbare) seeds whose mRNAs were expressed in the ripening and germinating stages.18 One of these purified mRNAs showed high α-xidosidase activity in particular when using xyloglucan oligosaccharides.19

We measured in the present study the main components (amylose and protein), enzyme activities, pasting property and physical property of Japonica, Indica-Japonica hybrid, Javanica and Indica subspecies and searched for the novel PCR primers related to cell-wall-degrading enzymes.

We found novel PCR primers related to the cell-wall-degrading enzymes and attempted to improve the equation for estimating the palatability of cooked rice grains. These primers were added to a multiple regression analysis8,9 against the results of the physico-chemical measurements.

---

1 To whom correspondence should be addressed. Tel./Fax: +81-25-262-6360; E-mail: ohtsubok@agr.niigata-u.ac.jp
Materials and Methods

Material. Forty two cultivars of rice were harvested or purchased in 2008. *Japonica* rice of the glutinous type (Hakuko-mochi, Habutae-mochi, Cam 101, and Chigonomoto), and short-grain *Japonica* rice of the non-glutinous type (Asanohikari, Akihikari, Koshihikari, Kirara 397, Nipponbare, Akigumo, Natsugumo, the non-glutinous type (Asanohikari, Akihikari, Koshihikari, Kirara 397, Nipponbare, Akigumo, Natsugumo, the non-glutinous type (Asanohikari, Akihikari, Koshihikari, Kirara 397, Nipponbare, Akigumo, Natsugumo, the non-glutinous type (Asanohikari, Akihikari, Koshihikari, Kirara 397, Nipponbare, Akigumo, Natsugumo, the non-glutinous type (Asanohikari, Akihikari, Koshihikari, Kirara 397, Nipponbare, Akigumo, Natsugumo, the non-glutinous type (Asanohikari, Akihikari, Koshihikari, Kirara 397, Nipponbare, Akigumo, Natsugumo, the non-glutinous type (Asanohikari, Akihikari, Koshihikari, Kirara 397, Nipponbare, Akigumo, Natsugumo, the non-glutinous type (Asanohikari, Akihikari, Koshihikari, Kirara 397, Nipponbare, Akigumo, Natsugumo, the non-glutinous type (Asanohikari, Akihikari, Koshihikari, Kirara 397, Nipponbare, Akigumo, Natsugumo, the non-glutinous type). Long-grain rice, and Takanari).

Each sample was stored at 4°C and milled into flour by a coffee mill (Mills IFM-100, Iwatani, Japan). Asanohikari, Akihikari, Hinohikari, Kinhikari, Kirara 397, Nipponbare IR2061, and Milyang 23 were kindly provided by the National Food Agency, and Koshikihari, Kareimai and Yumetoiro were cultivated in an experimental field of Hukoku University, Joetsu. Hoshiyutaka was cultivated in an experimental field of Kyushu University and Hakuko-mochi, Habutae-mochi and Sari-queen were cultivated in an experimental field of Crop Science Institute, Tsukuba. Akihikari, Natsugumo, Koshino-menjiman, Chigonomoto, Ekkakou and Kaoruko were kindly provided by Niigata Prefectural Agricultural Research Institute. IR2061 and Milyang 23 were provided by the Japanese Agricultural Cooperative, while Hanakui 409, Tsuba 68, Kyotoku 52, and Shiotoyo 47 were kindly provided by Dr. Anthony Blakeny of Australia. Kalijira, Long grain rice, Medium grain rice, Jasmin rice, Basmati, Asahi, Carnaloli, Arborio Texamati, Tamnxoa, and Masuri were purchased in a local market.

Chemical and physical properties related to the textural characteristics. The amylose content of the milled rice was estimated by the iodine colorimetric method of Juliano. Potato amylose (type III, Sigma Chemical Co., St. Louis, MO, USA) and waxy rice starch (Shimada Chemical Industry Co., Japan) were used as the amylose and amylopectin standards for amylose determination. The nitrogen content was determined by a nitrogen combustion analyzer (modified Dumas method; FP-528 model, Leco, USA) and the protein content was then calculated by multiplying the nitrogen content by the nitrogen protein conversion coefficient of 5.95. The pasting properties of each rice flour sample was evaluated by an RVA-3D, rapid visco analyzer (Newport Scientific, Warwird, Australia) by the method of Toyoshima et al. The RVA test for rice was adopted as an approved method (AAC OFFicial Method 61-02, 1995). The physical properties of the cooked rice were determined with a Myboy Tensipresser (Taketomo Electric Corp., Tokyo, Japan) by the method of Okadome et al. The adhesive properties of cooked rice grains were measured with the Tensipresser using low-compression (25%) test program for adhesion (L3).

Xylanase activity. The xylanase activity of milled rice was enzymatically determined with a kit (Megazyme International Ireland, Wicklow, Ireland). A rice flour sample (0.5 g) was suspended for 20 min in a 0.1 M MES buffer containing SDS (1% w/v) at pH 6.0 at room temperature, before being centrifuged for 10 min at 1,500 x g. A 0.5 mL amount of the supernatant was used for the assay. Xylose AX (a dyed xylan substrate tablet) was added to the supernatant, and the tube was immediately placed in a water bath at 40°C and incubated for exactly 30 min. The solution was then added to 5 mL of a Tris–HCl buffer solution (pH 9.0), the mixture then being vigorously stirred with a vortex mixer and kept at room temperature for 5 min. The sample solution was then passed through a Whatman no. 1 (9 cm) filter paper and the absorbance of the filtrate was measured at 590 nm against the reaction blank.

Cellulase activity. The cellulase activity of milled rice was determined with a kit (Megazyme International Ireland). A rice flour sample (1.0 g) was suspended for 15 min in 5 mL of a 0.04 M acetate/phosphate buffer at pH 4.6 and room temperature (lower than 30°C) before being centrifuged for 10 min at 1,000 x g. A 0.5 mL aliquot of an Azo-Barley glucan substrate solution and 0.5 mL aliquot of the enzyme extract were mixed and pre-warmed to 30°C for 5 min. The mixture was vigorously agitated and then incubated at 30°C for exactly 10 min, before being combined with 3.0 mL of precipitant solution A and vigorously stirred. The tube was allowed to stand at room temperature for 5 min, stirred again, and then centrifuged for 10 min at 1,000 x g. The absorbance of the supernatant of each sample solution was measured at 590 nm against the reaction blank of distilled water.

Extraction and purification of template DNA. DNA was extracted from 0.4 g of milled rice flour by the CTAB method. Briefly, rice powder (0.4 g) was suspended in 0.8 mL of 1.5% cetyl trimethyl ammonium bromide (CTAB) in a 0.1M Tris–HCl buffer solution (pH 8.0), 2 mM EDTA, and 1.4 mM NaCl. The mixture was incubated at 65°C for 30 min in a water bath. Chloroform and iso-amyl alcohol (24:1, v/v; 0.8 mL) were added, and the solution stirred gently for 15 min by using a rotating shaker. The solution was then centrifuged (8,000 x g for 15 min) with a refrigerated Hi-mac CR21F (Hitachi) and the upper layer was transferred to another microtube. A CTAB solution (10%, 0.8 mL) and chloroform/iso-amylalcohol (24:1, v/v; 5 mL) were added to the microtube, and the mixture was gently stirred for 15 min and then centrifuged (8,000 x g, 15 min). The upper layer was transferred to another tube and stood for 5 min in a freezer (−80°C) after adding 2.5 times the volume of the precipitation buffer (50 mM Tris–HCl buffer solution (pH 8.0), 10 mM EDTA, and 1% CTAB). The resulting precipitate was collected by centrifugation (6,000 x g for 15 min), dissolved in 0.2 mL of TE (a 1 mM Tris–HCl buffer solution (pH 8.0), and 0.1 mM EDTA), decomposed by adding 1 mL of RNase A, bovine pancreas, 10 mg/mL, Nippon-gene, Tokyo, Japan) and incubated for 30 min at 55°C. A neutral phenol solution was then added, and the upper layer was transferred to another tube after mixing and centrifugation (8,000 x g for 15 min). The same volume of a solution of phenol/chloroform (1:1, v/v) was added to the mixture, which was followed by mixing and centrifugation (8,000 x g for 15 min), and the upper layer was transferred to another tube. To the solution was added 0.2 mM NaCl and 2 times the volume of cold ethanol to precipitate the DNA. The resulting DNA was washed with 30μL of 70% ethanol and dissolved in 30μL of a TE solution. This purified DNA was subjected to PCR as a template.

DNA amplification by PCR. Taq-DNA polymerase (Takara-Bio Inc., Otsu, Japan) was used for DNA amplification. A 3 μL amount of template DNA (400 ng/μL by absorption at 260 nm) was used for each PCR run. DNA was denatured for 1 min at 94°C, annealed for 1 min at 58–70°C, and elongated for 2 min at 72°C with a Dice thermal cycler (Takara-Bio, Otsu, Japan). This procedure was conducted 35 times.

Electrophoresis of the amplified DNAs. Proliferated DNAs were analyzed by electrophoresis (Mupid-2, Cosmo, Bio, Tokyo, Japan) for 40 min on 2% agarose gel at 100V DC. The DNA was stained by ethidium bromide after electrophoresis and detected by irradiating with UV light.

Development of sequence-tagged site (STS) primers for sequence characterized amplified region (SCAR) markers. STS primers for PCR were developed according to a rapid analysis to differentiate the rice cultivars by PCR as described in our previous report. Discriminative band DNAs were extracted from the agarose gel after electrophoresis of the PCR products by using Easy T (Takara Bio, Japan), DNA cloning was then carried out by using a Topo XL PCR cloning kit (Invitrogen, Carlsbad, CA, USA). The DNA sequence was determined by using a QIAprep Spin Miniprep DNA preparation kit (Qiagen, Tokyo, Japan) and VI-1 BigDye Terminator Cycle sequencing kit (Applied Biosystems, Tokyo, Japan) with an ABI PRISM 310 automatic DNA sequencing system (Applied Biosystems, Tokyo, Japan).

Multiple regression analyses using PCR to estimate the physico-chemical properties. The discriminative DNA bands were expressed as 0 (not observed) or 1 (observed) for digitizing the results of PCR and these data were subjected to a multiple-regression analysis against the
results of physico-chemical measurements.\textsuperscript{8,9} We usually used all the available objects in cross validation, subsequently making models of parts of the data and testing the other parts. The simplest alternative was to divide the data set into two parts A and B. We first made a model of A and ran a test on B before making a model of B and running a test on A.\textsuperscript{5,9} We used the results of PCR with primers for cellulase, xylanase, G22, GBSS and glutelin and the template DNAs of 21 rice cultivars (Hakicho-mochi, Habatae-mochi, Chigono-hoho, Hinohakki, Arborio, Nipponbare, Aki-gumo, Natsugumo, Pedle, Medium-grain rice, Kaoruko, Tugawa 1, Kitsu 88, Kaireimai, Tamxoan, Kalijira, Masuri, Long-grain rice, IR 2061, Yumetoiro, and Takanari), to develop estimating formulae by a multiple-regression analysis against the physical property parameter, adhesion. We then validated the multiple-regression formulae by using template DNAs from the other 21 rice cultivars (Cam 101, Asahonikari, Akihikari, Koshikihakki, Kinohakki, Kirara 397, Vaccab, Carnalolli, Dongarga, Haniku 409, Ryoku 25, Shiotoyo 47, Ekkakou, Hoshiyutaka, Sari-queen, Koshinomenjiman, Jasmin rice, Basmati, Nanjing 11, Milyang 23, and Takanari). The adaptability of the formula for estimating adhesion to the unknown rice samples was then validated.

\textbf{Results and Discussion}

\textbf{Starch and protein composition}

The amylose content of rice is probably the most important factor in determining the basic quality or utilization of the grain.\textsuperscript{3,5,20} Milled rice is classified as glutinous according to the amylose content: 0–5%, very low; 5.1–10.0%, low; 10.1–20.0%, intermediate; 20.1–25%, high; greater than 25%, very high.\textsuperscript{25} The amylose contents of the short-grain \textit{Japonica} rice of non-glutinous type were 7.16–24.62%, those of the \textit{Javanica} rice (Troical \textit{Japonica}) were 17.60–28.90%, those of the \textit{Japonica-Indica} hybrid rice were 17.14–28.38%, and those of the long-grain non-glutinous \textit{Indica} rice were 15.40–32.10% (Tables 1 and 2). The low-amylose rice grains generally become soft and sticky after cooking, while those containing high amylose become hard after cooking.\textsuperscript{25,26}

Rice grains with higher protein content generally tend to be lower in palatability to the Japanese after cooking.\textsuperscript{27} The distribution of crude protein in milled rice is generally 5–15%, the amount of protein affecting the physical properties of the cooked rice grains; the higher the protein content, the harder and less sticky the rice becomes upon cooking.\textsuperscript{23} The protein content of the \textit{Japonica} type of non-glutinous rice was usually lower than that of the \textit{Indica} type. The protein content of the short-grain \textit{Japonica} rice of the non-glutinous type was 5.30–8.90%, that of the \textit{Javanica} rice (Troical \textit{Japonica}) was 6.10–8.80%, that of the \textit{Japonica-Indica} hybrid rice was 5.90–6.90%, and that of the long-grain non-glutinous \textit{Indica} rice was 6.50–11.00% (Tables 1 and 2).

\textbf{Pasting properties of the milled rice flour samples}

The viscosity change during a gelatinization test\textsuperscript{5,21} is a useful predicctor of rice textural properties, since pasting properties influence the eating quality of rice. The properties of interest in the present study were consistency, \textit{i.e.}, the difference between the final viscosity and the minimum viscosity. Rice with a high amylose content generally shows high consistency. The consistency (RVU) of short-grain \textit{Japonica} rice of the non-glutinous type was 64.60–144.50, that of the \textit{Javanica} rice (troical \textit{Japonica}) was 110.00–185.00, that of the \textit{Japonica-Indica} hybrid rice was 129.00–165.00, that of long-grain \textit{Indica} rice of the non-glutinous type was 120.80–245.00, and that of \textit{Japonica} rice of the glutinous type was 15.00–44.00 (Tables 1 and 2). The consistency was positively correlated with both the amylose content and protein content, but negatively correlated with adhesion.

\textbf{Physical properties of the cooked rice grains}

The palatability and acceptability of rice grains are greatly affected by such physical properties, as the hardness and stickiness.\textsuperscript{5,22} Adhesion (L3) as related to the determined sensory stickiness of the surface layer of a cooked rice grain by the low-compression test is an important instrumental index of the stickiness of cooked rice grains.\textsuperscript{21} We have previously determined the adhesion (L3) of cooked world-wide rice (Asahi, 1.07; Hitomebore, 1.62; Koshikihakki, 1.78; Hakicho-mochi, 2.12; Nakatokishinbon, 1.04; Hoshiyutaka, 0.38; IR2061, 0.11; Kalijira, 0.37; Motooboi, 0.21; Sari-queen, 0.62; Wita7, 0.22; and Yumetoiro, 0.29) measured by the low-compression test (25\%) with a Tensipresser.\textsuperscript{9} The adhesion (L3) range of the soft type of \textit{Indica} rice was higher than that of the hard type of \textit{Indica} rice. The adhesion (L3) values for short-grain \textit{Japonica} rice of the non-glutinous type were 1.14–3.02, for \textit{Javanica} rice were 0.32–1.50, for \textit{Japonica-Indica} hybrid rice were 0.38–2.85, for long-grain \textit{Indica} rice of the non-glutinous type were 0.09–2.53, and for \textit{Japonica} rice of the glutinous type were 2.00–3.13 (Tables 1 and 2). Adhesion (L3) was negatively correlated with the amylose content, protein content and consistency.

\textbf{Cellulase and xylanase activity}

In addition to the amylose content, the composition of the cell wall of the rice endospem also affects the adhesiveness and hardness/softness. Cellulose in the cell wall can be hydrolyzed by cellulase (endo-1,4-\beta-D-glucanase) resulting in increased softness. The strength and adhesion of the cell wall affects the physical properties of the rice endospem.\textsuperscript{11–13} Short-grain \textit{Japonica} rice of the non-glutinous type showed cellulase activity (U/g) of 93.71–136.94, \textit{Javanica} rice showed 122.36–144.96, \textit{Japonica-Indica} hybrid rice showed 107.32–131.16, long-grain \textit{Indica} rice of the non-glutinous type showed 121.89–138.90, \textit{Japonica-Indica} hybrid rice of non-glutinous type were 129.00–89.00, and \textit{Japonica} rice of the glutinous type showed 98.50–112.02 (Tables 1 and 2).

Cooked rice is softened by decomposition of the cell wall, suggesting that the hemicellulosic polysaccharides play an important role in the physical properties of the endospem cell walls.\textsuperscript{11,12} The xylanase activity (U/g) of short-grain \textit{Japonica} rice of the non-glutinous type was 196.06–300.80, \textit{Javanica} rice was 298.33–449.66, \textit{Japonica-Indica} hybrid rice was 296.69–350.60, long-grain \textit{Indica} rice of the non-glutinous type was 310.00–559.26 and \textit{Japonica} rice of the glutinous type was 230.47–275.02 (Tables 1 and 2). The \textit{Indica} rice varieties have been characterized as high in amylose content, high in protein content, high in consistent viscosity, and low in adhesion. We found that the xylanase activity was also high for \textit{Indica} rice (Tables 1 and 2).

As expected, a high positive correlation was shown between the consistent viscosity and amylose content ($r = 0.89$) at the level of 1%. The xylanase activity was
Adhesion (L3) was negatively correlated with the adhesion (\(r = -0.51\)), consistency (\(r = -0.65\)), xylanase activity (\(r = -0.71\)) and cellulase activity (\(r = -0.61\)) at the respective level of 1% (Table 3). It was found that endogenous xylanase and cellulase played important roles in determining the texture of the cooked rice grains, similarly to the amylase content.

Table 2. Average of Main Chemical Components, Pasting and Physical Properties and Cell-Wall-Digesting Enzyme Activities of World-Wide Rice

<table>
<thead>
<tr>
<th>Amylese content (%)</th>
<th>SD</th>
<th>Protein content (%)</th>
<th>SD</th>
<th>Cellulase activity (U/g)</th>
<th>SD</th>
<th>Xylanase activity (U/g)</th>
<th>SD</th>
<th>Consistency (RVU) SD</th>
<th>SD</th>
<th>Adhesion (L3) SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutinous rice</td>
<td>0.63a 0.16</td>
<td>6.15a 0.05</td>
<td>27.95a 12.76</td>
<td>105.13a 6.90</td>
<td>253.13a 21.75</td>
<td>2.35a 0.53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Japonica rice</td>
<td>18.39b 4.70</td>
<td>6.72a 1.06</td>
<td>114.02b 26.03</td>
<td>107.15a 14.29</td>
<td>259.24a 37.42</td>
<td>2.04a 0.71</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Japonica-Indica hybrid rice</td>
<td>21.99bc 5.07</td>
<td>6.44a 0.31</td>
<td>144.94bc 16.55</td>
<td>112.20b 7.68</td>
<td>241.96ac 1.95</td>
<td>1.62ab 1.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indica rice</td>
<td>21.30bc 4.65</td>
<td>7.37ab 1.00</td>
<td>141.67 35.92</td>
<td>128.01b 13.02</td>
<td>382.25bc 60.68</td>
<td>0.67b 0.43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Correlation significant at 5% by the method Tukey. Means with same letter are not significantly different (\(p < 0.05\)).

positively correlated with the amylase content (\(r = 0.58\)), protein content (\(r = 0.53\)), consistency (\(r = 0.70\)), and cellulase activity (\(r = 0.62\)) at the respective level of 1%, and negatively correlated with the adhesion (\(r = -0.71\)) at the level of 1% (Table 3). Adhesion (L3) was negatively correlated with the amylase content (\(r = -0.51\)), consistency (\(r = -0.65\)), xylanase activity (\(r = -0.71\)) and cellulase activity (\(r = -0.61\)) at the respective level of 1% (Table 3). It was found that endogenous xylanase and cellulase played important roles in determining the texture of the cooked rice grains, similarly to the amylase content.
Table 3. Correlation between Results of PCR, Chemical Components, Pasting and Physical Properties and Cell-Wall-Digesting Enzyme Activities of Various Rice Samples

<table>
<thead>
<tr>
<th></th>
<th>Result of PCR for xylanase</th>
<th>Result of PCR for RNA-binding protein</th>
<th>Result of PCR for retrotransposon protein</th>
<th>Result of PCR for endo-1,4-β-glucanase</th>
<th>Amylose content (%)</th>
<th>Protein content (%)</th>
<th>Consistency (RVU)</th>
<th>Cellulase activity (U/g)</th>
<th>Xylanase activity (U/g)</th>
<th>Adhesion (L3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Result of PCR for xylanase</td>
<td>0.49</td>
<td>0.53</td>
<td>0.27</td>
<td>0.49</td>
<td>0.62</td>
<td>0.53</td>
<td>0.62</td>
<td>0.44</td>
<td>0.44</td>
<td>0.53</td>
</tr>
<tr>
<td>Result of PCR for RNA-binding protein</td>
<td>0.41**</td>
<td>0.46**</td>
<td>0.48**</td>
<td>0.48**</td>
<td>0.54**</td>
<td>0.58**</td>
<td>0.58**</td>
<td>0.58**</td>
<td>0.58**</td>
<td>0.58**</td>
</tr>
<tr>
<td>Result of PCR for retrotransposon protein</td>
<td>0.39**</td>
<td>0.37**</td>
<td>0.37**</td>
<td>0.37**</td>
<td>0.37**</td>
<td>0.37**</td>
<td>0.37**</td>
<td>0.37**</td>
<td>0.37**</td>
<td>0.37**</td>
</tr>
<tr>
<td>Result of PCR for endo-1,4-β-glucanase</td>
<td>0.49</td>
<td>0.49</td>
<td>0.49**</td>
<td>0.49**</td>
<td>0.49**</td>
<td>0.49**</td>
<td>0.49**</td>
<td>0.49**</td>
<td>0.49**</td>
<td>0.49**</td>
</tr>
<tr>
<td>Amylose content (%)</td>
<td>0.44**</td>
<td>0.37**</td>
<td>0.37**</td>
<td>0.37**</td>
<td>0.37**</td>
<td>0.37**</td>
<td>0.37**</td>
<td>0.37**</td>
<td>0.37**</td>
<td>0.37**</td>
</tr>
<tr>
<td>Protein content (%)</td>
<td>0.62**</td>
<td>0.53**</td>
<td>0.53**</td>
<td>0.53**</td>
<td>0.53**</td>
<td>0.53**</td>
<td>0.53**</td>
<td>0.53**</td>
<td>0.53**</td>
<td>0.53**</td>
</tr>
<tr>
<td>Consistency (RVU)</td>
<td>0.48**</td>
<td>0.48**</td>
<td>0.48**</td>
<td>0.48**</td>
<td>0.48**</td>
<td>0.48**</td>
<td>0.48**</td>
<td>0.48**</td>
<td>0.48**</td>
<td>0.48**</td>
</tr>
<tr>
<td>Cellulase activity (U/g)</td>
<td>0.58**</td>
<td>0.58**</td>
<td>0.58**</td>
<td>0.58**</td>
<td>0.58**</td>
<td>0.58**</td>
<td>0.58**</td>
<td>0.58**</td>
<td>0.58**</td>
<td>0.58**</td>
</tr>
<tr>
<td>Xylanase activity (U/g)</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
</tr>
<tr>
<td>Adhesion (L3)</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Correlation significant at 5% (*) or 1% (**) by t test.

Characteristics of each group of rice sub-species

In amylose content and pasting property (consistency), Indica rice had higher values as reported by Shimizu et al. and Tran et al. and glutinous rice had lower values than the other rice samples as shown in Table 2. In respect of the protein content, Indica rice had higher values than the other rice samples. In respect of the physical properties (adhesion, L3), glutinous rice and Japonica rice had lower values, while for the xylanase and cellulase activities, glutinous rice, Japonica rice and Japonica-Indica hybrid rice had lower values than Indica rice and Javanica rice.

Development of STS primers for PCR

α-Glucosidase is one of the enzymes related to the palatability of rice. The α-glucosidase genes have been identified by Nakai et al. during the ripening and germinating stages in rice. We designed primers (5′-ggt aca acg tgc cgt cgg-3′, sense) by using from number 128 nucleotide to number 146 nucleotide, and primer b (5′-gcc ggc gac gac ggc ggc ggc cag-3′, antisense) by using from number 687 nucleotide to number 705 nucleotide of α-glucosidase reported by Nakai et al. (Fig. 1). These primers partially cover the probe sequence used for southern or northern blotting. PCR amplification by using these primers, showed that two discriminative DNA bands were useful to differentiate Japonica rice from Indica, and Indica-Japonica hybrid rice (Fig. 2A). The sequences of DNA amplified by PCR with primers (a and b) using template DNA of Indica rice are shown in Fig. 1. There is a wide difference between this sequence and that identified by Nakai et al. (Fig. 2A). The nucleotide sequence, in Fig. 3A, indicated by dotted line A, suggests a 59 amino acid signal sequence. The deduced amino acid sequence showed high similarity (95%) with that of the 1,4-β-xylanase enzyme.

Using Template DNA of Indica Rice.

The nucleotide sequence, indicated by dotted line A, suggested a 59 amino acid signal sequence. The deduced amino acid sequence showed high similarity (95%) with that of the 1,4-β-xylanase enzyme.
that of enzyme RNA-binding protein. The electrophoregrams after PCR when using primer d (5'-att gag att gac gac aac gac gac gac aac gat cct cct ctg-3', sense) and primer e (5'-ctc cat taa tgt ctc gtt ttg tgg c-3', anti-sense) in Table 4 are shown in Fig. 4B. It was found that discriminative DNA bands appeared specifically in glutinous rice (Hakucho-mochi and Habutaemochi), Japonica rice (Asanohikari, Akihikari, Koshihikari, Hinohikari, Kinuhikari, Kirara 397, Nipponbare, Natsugumo, Tsugawa 1, Kitsu 88, Ryuko 25, Shiotoyo 47, and Ekkako), Japonica-Indica hybrid rice (Sari-queen, Kareimai, and Koshinomenjiman), Javanica rice (Medium-grain rice, Carnalori, Pelde, and Doongara) and Indica rice (Jasmine rice, Tamxoan, Nanjing 11, Karijira, Long-grain rice, and Takarani).

Fig. 2. DNA Sequence Amplified by PCR with Primers a and b to Detect SNPs Characteristic of Japonica, Indica and Japonica-Indica Hybrid Rice Cultivars.

A. One gene difference between the nucleotide sequence of the Koshihikari line (Koshihikari and Hitomebore) and non-Koshihikari line (Asahi, Hakuo-mochi, and Nakateshinsenbon) is shown in one region of this nucleotide sequence. B. One gene difference between the nucleotide sequence of soft-type Indica rice (Hoshiyutaka, Sari-queen, and Kalijira) and hard-type Indica rice (IR2061, Motoboi, Wita7, and Yumetoiro) is shown at two points of this nucleotide sequence. The DNA sequence is shown from 3' end and is expressed as a complementary one. C. One gene difference between the nucleotide sequence of the Hoshiyutaka, Sari-queen, Kalijira, IR2061, Motoboi, Wita7 and Yumetoiro cultivars of Indica rice and that of the Koshihikari, Hitomebore, Asahi, Hakuo-mochi and Nakateshinsenbon cultivars of Japonica rice is shown at many points of this nucleotide sequence. The DNA sequence was shown from the 3' end and is not expressed as a complementary one. g. AAAAAAAAAAAAG (identical sequence to that in Fig. 5g). f. ATTGAGATTGCGTACCTGG (identical sequence of Fig. 5f).

Fig. 3. Homology of the Deduced Amino Acid Sequences Indicated by Dotted Lines.

A. Nucleotide sequence indicated by dotted line A (1,4-β-xylanase). B. Nucleotide sequence indicated by dotted line B (RNA-binding protein). C. Nucleotide sequence indicated by dotted line C (endo-1,4-β-glucanase 13). D. Nucleotide sequence indicated by dotted line D (retrotransposon protein).

Difference in 1,4-β-xylanase gene among the different rice samples

After PCR amplification with primers a and b using template DNA of Indica rice, the DNA bands that appeared were extracted from the agarose gel, before cloning and sequencing of DNA as shown in Fig. 2B. A single nucleotide difference between the nucleotide sequence of the soft type of Indica rice (Kalijira) and Japonica-Indica hybrid rice (Hoshiyutaka and Sari-queen), and the nucleotide sequence of the hard type of Indica rice (IR2061, Motoboi, Wita7, and Yumetoiro) was found in two regions. After PCR amplification with primers a and b using template DNA of Japonica rice (Asahi, Hitomebore, Koshihikari, Hakuo-mochi, and...
Nakateshinsenbon), the DNA bands that appeared were extracted from the agarose gel, before cloning and sequencing of DNA as shown in Fig. 2C. A single nucleotide difference between the nucleotide sequence of the Koshihikari line (Koshihikari and Hitomebore) and non-Koshihikari line (Asahi, Hakucho-mochi, and Nakateshinsenbon) was found in one region. The discriminative DNA bands contained the gene of endo-1,4-\(\beta\)-glucanase 13 enzyme (cellulase). The nucleotide sequence indicated by dotted line C might therefore be the particular gene for non-Japonica rice similar to the xylanase gene.

**Discrimination of Japonica and Indica rice based on the retro-transposon gene**

In order to produce a novel DNA marker, the PCR conditions were changed as reported in our previous paper.\(^9\) PCR amplification was carried out using cellulase-related primers (5'-aag tact tct tct aac gag ttt ccg ca-3', forward: and 5'-aca atg atc atc aag cac ccc gac cag cc-3', reverse). The result of PCR enabled us to find, a novel discriminative DNA band specific to Indica rice and Javanica rice, in addition to the original cellulase-related band. The discriminative DNA bands were extracted from the agarose gel, and the DNA sequences were determined. The nucleotide sequence suggested an 86 amino acid sequence as shown in Fig. 3D. This

<table>
<thead>
<tr>
<th>Table 4. Sequences of STS Primers and PCR Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylanase primer</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>F</td>
</tr>
<tr>
<td>R</td>
</tr>
<tr>
<td>RNA-binding protein</td>
</tr>
<tr>
<td>R</td>
</tr>
<tr>
<td>Endo-1,4-(\beta)-glucanase</td>
</tr>
<tr>
<td>R</td>
</tr>
<tr>
<td>Retrotransposon protein</td>
</tr>
<tr>
<td>R</td>
</tr>
</tbody>
</table>

F, forward; R, reverse
Fig. 5. DNA Sequence of Amplified by PCR with Primers f and g Using Template DNA of Japonica Rice.

The nucleotide sequence, indicated by dotted line C, suggested an 80 amino acid signal sequence. The deduced amino acid sequence was identical (100%) with that of the endo-1,4-β-glucanase 13 enzyme.

deduced amino acid sequence showed high homology (95%) with that of the retro-transposon protein deposited in the DDBJ/Gen Bank nucleotide sequence databases under accession no. AB033547. The electrophoregrams after PCR using primer j (5'-aag tac tac tct aac gag agt aca ag-3', sense) and primer k (5'-atg atc atc aga ccc agg cat ctt g-3', anti-sense) listed in Table 4 are shown in Fig. 4D. Discriminative DNA bands appeared in Indica rice (IR2061, Yumetoiro, Jasmine rice, Basmati, and Javanica rice) (Vachal, Carnaroli, Arborio, and Medium-grain rice). The nucleotide sequence indicated by PCR amplification using primers j and k might therefore be a particular gene sequence for Indica rice and Javanica rice. Nakamura et al. have reported starch synthase IIa as a key-enzyme to discriminate Japonica and Indica rice. We propose the following multiple-regression equation based on 21 rice cultivars which had not been used for calibration.

Improving the formula for estimating the palatability of cooked rice grains

We have previously reported the relationship between the physicochemical properties and the DNA analysis by PCR, and we improved these by adding starch-related and protein-related PCR primers. Equations for estimating the palatability were developed in this present study by using the aforementioned independent variables based on the results of PCR against the dependent variables related to such a palatability factor, as the adhesiveness of cooked rice grains.

We attempted to improve the equations for estimating the palatability by applying the results of this study that identified genes by PCR, using the novel primers related to the cell-wall-degrading enzymes, xylanase and cellulase, retro-transposon, and the RNA-binding protein. We propose the following multiple-regression equation for adhesion (L3) as a function of seven PCR-derived genes:

$$L3 = -0.259 \times \text{xylanase} - 0.033 \times \text{RNA-binding protein} - 0.601 \times \text{endo-1,4-β-glucanase} - 0.469 \times \text{retrotransposon} + 0.296 \times \text{G22} - 0.260 \times \text{GBSS} + 0.295 \times \text{Glu} + 1.359$$

We carried out a prediction by using template DNAs from the other 21 rice cultivars which had not been used for calibration.

The results using these novel primers improved the multiple-regression coefficients of the palatability equation. The determination coefficient ($R^2$) for adhesion (L3) was improved from 0.67 in our previous paper to 0.80 in the present paper as shown in Fig. 6. These results suggest that the equation for estimating rice palatability based on the DNA analysis have probable application to the selection of desirable textural characteristics to that would facilitate plant breeding or the evaluation of cooked rice quality.
Influential observations and cellulase among the such rice cultivars as Indica and cellulase-related primer showed significant correlation, example, Indica negative correlation with the adhesion (r = 0.49) and the actual cellulase activity (r = 0.49). The result of PCR using the retrotransposon-related primer also showed significant correlation with the actual cellulase activity (r = 0.49), although the amylose content and protein content showed the second highest correlation (r = 0.71), and the cellulase activity showed the second highest correlation (r = 0.53) for xylanase and cellulase and xylanase activities. Endogenous xylanase and cellulase were found to play important roles in determining the texture of the cooked rice grains, similarly to the amylose content. We found high-homology in the DNA sequences encoding 1,4-β-glucanase and endo-1,4-β-glucanase 13 for Indica rice, Indica-Japonica hybrid rice, and Javanica rice (non-Japonica rice). The equations for estimating the palatability of the cooked rice grains based on a multi-variate analysis were improved by applying the results of PCR, using primers related to the cell-wall-degrading enzymes.

We investigated Japonica and non-Japonica subspecies by PCR, using cellulase-related and xylanase-related primers. The result of PCR using the xylanase-related primer showed significant correlation with the actual xylanase activity (r = 0.49, p < 0.01), and the result of PCR using the cellulase-related primer also showed significant correlation with the actual cellulase activity (r = 0.44, p < 0.01) as indicated in Table 3. The result of PCR using the retrotransposon-related primer also showed significant correlation with the actual xylanase activity (r = 0.49, p < 0.01) and with the actual cellulase activity (r = 0.48, p < 0.01), and negative correlation with the adhesion (r = −0.48, p < 0.01). We found more SNIPs concerning xylanase and cellulase among the such rice cultivars as Japonica, Indica and the Japonica-Indica hybrid. We will therefore continue our investigation of these SNIPs; for example, Japonica-specific xylanase and Japonica-specific cellulase.

The results of PCR using the xylanase-related primer and cellulase-related primer showed significant correlations (p < 0.01) with the textural property of adhesion (L3; r = −0.65 for xylanase and r = −0.56 for cellulase) and pasting property (consistency; r = 0.53 for xylanase and r = 0.53 for cellulase).

We investigated in the present study the relationship between the chemical or biological properties of raw rice and the physical properties of rice flour or cooked rice grains. In respect of the pasting property (consistency of rice flour), amylose showed the highest correlation (r = 0.89, p < 0.01), and xylanase activity showed the second highest correlation (r = 0.70, p < 0.01). In respect of the physical property of adhesion (L3) of the cooked rice grains, the xylanase activity showed the highest correlation (r = −0.71), and the cellulase activity showed the second highest correlation (r = −0.61, p < 0.01), although the amylose content showed only low correlation (r = −0.51, p < 0.01). The effect of endogenous cell-wall-degrading enzymes on the physical properties of the cooked rice grains is in accordance with their results.

We investigated Japonica and non-Japonica subspecies by PCR, using cellulase-related and xylanase-related primers. The result of PCR using the xylanase-related primer showed significant correlation with the actual xylanase activity (r = 0.49, p < 0.01), and the result of PCR using the cellulase-related primer also showed significant correlation with the actual cellulase activity (r = 0.44, p < 0.01) as indicated in Table 3. The result of PCR using the retrotransposon-related primer also showed significant correlation with the actual xylanase activity (r = 0.49, p < 0.01) and with the actual cellulase activity (r = 0.48, p < 0.01), and negative correlation with the adhesion (r = −0.48, p < 0.01). We found more SNIPs concerning xylanase and cellulase among the such rice cultivars as Japonica, Indica and the Japonica-Indica hybrid. We will therefore continue our investigation of these SNIPs; for example, Japonica-specific xylanase and Japonica-specific cellulase.

The results of PCR using the xylanase-related primer and cellulase-related primer showed significant correlations (p < 0.01) with the textural property of adhesion (L3; r = −0.65 for xylanase and r = −0.56 for cellulase) and pasting property (consistency; r = 0.53 for xylanase and r = 0.53 for cellulase).
Acknowledgments

We would like to express our gratitude to Dr. Wallace Yokoyama, Professor Cui Jing for valuable cooperation. And we thank the former National Food Agency, Hokuriku Center, Japanese Agricultural Cooperative, Kyushu University and Crop Science Institute for presenting the rice samples. Part of this research was supported by grant-in aid for Scientific Research (C) by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the research and development project for promoting new policy in agriculture by the Ministry of Agriculture, Forestry and Fisheries of Japan.

References