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BMT/8/2
ORIGINAL: English
DATE: August 15, 2003

UPOV

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

WORKING GROUP ON BIOCHEMICAL AND MOLECULAR TECHNIQUES AND DNA-PROFILING IN PARTICULAR

Eighth Session
Tsukuba, Japan, September 3 to 5, 2003

CULTIVAR IDENTIFICATION OF RICE BY PCR METHOD AND ITS APPLICATION TO PROCESSED RICE PRODUCTS

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As the cultivars of rice affect eating quality, processing suitability and price markedly, identification of rice cultivar is very important. It is obliged to display the name of cultivar on its package under the Japan Agricultural Standard Law. It became possible to identify rice cultivar by PCR and electrophoresis method using DNA extracted and purified from rice grains. Two kinds of multiplex kit for identification of Koshihikari, dominant cultivar in Japan, have been developed. Application of cultivar identification method by DNA polymorphism to processed rice products was investigated. The present authors developed "enzyme treatment method", in which starch is decomposed by the heat stable alpha-amylase, followed by hydrolysis by proteinase K with SDS and purification of extracted DNAs by PCI. We showed that it is possible to identify rice cultivars of commercial precooked rice, rice cake and rice crackers by PCR method using template DNA prepared by the "enzyme treatment method".

Introduction

1. Rice is one of the most important arable crop with wheat and corn in the world. There are more than several tens of thousands of varieties or cultivars of rice in the world and rice is a staple food for more than half of people in the world. Rice is markedly diversified from the viewpoint of genetics, morphology and properties. There are tall or dwarf, high-yielding or low-yielding, pathogen-resistant or pathogen susceptible, insect-resistant or insect susceptible, purple leaf or green leaf, long-grain or short grain, big-grain or fine grain. On the contrary, high-quality rice is closely related by inbred breeding to attain high-palatability, high processing suitability or characteristic aroma, etc.

2. Rice grains of the famous cultivars are traded or distributed at higher price as "Premium rice" because of their high palatability, processing suitability or special aroma, etc. As those premium rices sell at high price, some dishonest rice wholesalers or retailers blend low quality cheap rice to high quality premium rice and mislabel to be "high-quality premium rice". Furthermore, rice consumers demand information about rice which they purchase. Therefore, technology to identify rice cultivar is very important for breeders, farmers, inspectors, wholesalers, retailers, food industries and consumers.

3. Rice cultivar has been identified based on morphological characteristics of rice plant or grains, ripening ratio after crossing, or the isozyme patterns, etc. Recently, novel cultivar identification method based on DNA polymorphism has been developed accompanied with the progress in molecular biology. As rice genome has as many as 430,000,000 basepairs in total, there are huge amount of information in DNA useful for cultivar identification.

4. DNA fingerprinting was developed by Jeffreys in 1985(Jeffreys, 1985) and is used for criminal investigation and trial at court. Cultivated rice (*Oryza sativa* L.) is one of the most polymorphic crop species in the world. On the contrary, many high-quality rice or premium rice is closely related because inbred lines are often used for their development. Therefore, it is necessary to develop a technology to differentiate rice cultivars clearly and in detail.

5. Major collections of rice germplasm are held in international centers around the world. Useful information can be obtained using molecular markers which afford many benefits for

identifying variation and estimating biological diversity (Virk et al, 1995). DNA-based markers have the obvious advantage of sampling the genome directly and RFLP analysis has been widely used for assessing variation of plants (Helentjaris et al., 1985, Tanksly et al., 1989, Parminder et al, 1995). RFLP analysis has been used to distinguish between species of *Oryza* (Wang et al., 1992) and particularly between indica and japonica type of Oryza sativa (Zang et al., 1992)

6. Recently, PCR-based marker system has been developed by Williams et al (Williams et al., 1990). In this RAPD (Random Amplified Polymorphic DNA) method, short oligonucleotides of arbitrary sequence are used singly to support the amplification of regions of the test plant genome and amplification products are separated by gel electrophoresis. Differences between genotypes are reflected as difference in the banding patterns.

7. There are some reports on RAPD analysis of rice germplasm including Indica and Japonica types, to identify suitable parents for linkage map construction, and for gene tagging for drought resistance (Fukuoka et al., 1992, Yu and Nguyen, 1994). It was shown that RAPD analysis is a useful tool in determining the genetic relationships among rice cultivars (Yu and Nguyen, 1994). RAPD analysis was revealed to be reproducible and amenable for identification of each single plant line of F1 hybrid rice in China (Wang et al, 1994) and Australian rice cultivars (Ko et al., 1994).

8. In addition to RAPD markers, microsatellites or simple sequence repeats (SSRs) are DNA sequences with repeat lengths of a few basepairs and variation in the number of nucleotide repeats can be detected with PCR by selecting the conserved DNA sequences flanking the SSR as primers. In 1993, SSR markers were developed for rice (Wu and Tanksley, 1993, Zhao and Kochert, 1993). SSRs are useful not only characterize the relationship between heterosis and marker genotype heterozygosity but also to identify chromosome segments that may have significant effects on yield and its component traits in rice (Zhang et al., 1994).

9. Investigation were carried out to quantify genetic diversity with random amplified polymorphic DNA (RAPD) markers in a sample of 134 predominantly japonica cultivars and two wild species. The indica and japonica cultivars were classified into separate groups by cluster analysis. In conclusion, RAPDs are useful for classification of japonica cultivars, but many primers will be needed to resolve closely related japonica cultivars (D. J. Mackill, 1995)
In order to clarify the phylogenetic relation ships in A-genom species of rice, RAPD analysis was carried out using 29 accessions and it was demonstrated that O sativa and O. glaberrima have probably originated from the Asian form of O. perennis and from O. breviligulata, respectively (Ishii et al., 1996).

10. Traditional pedigree and RAPD data obtained from 26 elite cultivars and line s of rice recommended for commercial production in Louisiana and elsewhere in USA were compared (Cao and Oard, 1997). Seven primers among 69 primers were identified that could differentiate all cultivars and lines. Genetic distances obtained from RAPD data were correlated with estimated kinship coefficient.

11. In case of RAPD markers, many other DNA bands than the target DNA for cultivar differentiation appear in the electrophoregram after PCR. Therefore, it is recommended to develop the STS (sequence tagged site) markers or SCAR (sequence characterized amplified region) markers based on RAPD markers.

12. Genetic diversity among 42 Indian elite rice varieties, which is important for selection of parents for conventional breeding and hybrid program, was evaluated using three different types of DNA markers and parentage analysis (Davierwala et. al., 2000). Three molecular marker systems, random amplified polymorphic DNA (RAPD), inter-simple single sequence repeat (ISSR), and sequence tagged microsatellite site (STMS), provided wider genome coverage and, therefore, would be a better indicator of the genetic relationships among the 42 elite rice cultivars than those revealed using individual molecular markers.

13. RAPD, RFLP, nuclear SSLP and chloroplast SSLP analyses were carried out to clarify the phylogenetic relationships among A-genome species of rice (Bautista et al., 2001). Their banding patterns were scored and compared to evaluate the similarity between accessions. Genetic differentiation within and between taxa was examined based on the average similarity indices. Dendrograms for RAPD, RFLP, and nuclear and chloroplast SSLP analyses were constructed to reveal the overall genetic relationship among A-genome species.

14. In the present study, SCAR markers were developed, based on RAPD analysis, to differentiate Japanese rice cultivars by PCR. By the combination of these SCAR markers, "multiplex primer set" was developed to identify Koshihikari, most dominant rice cultivar in Japan. It was shown that these DNA analyses can be applied to the differentiation of material rice cultivars using processed rice products, such as boiled rice or rice cake, as samples.

Materials and Methods

15. Thirty three original Koshihikari rice seeds, produced in 1999, 2000 and 2001, were used for DNA extraction and purification. All the rice samples including other 49 different cultivar rice samples than Koshihikari were kindly provided by National Food Agency, Japan.

16. Rice flours were prepared by a coffee mill (Millser IFM-100, Iwatani, Japan) after whitening by the experimental rice mill (VP-31-T, Yamamoto Seisakusho, Japan) to the milling yield of 90%. According to the CTAB method (Rogers and Bendigh, 1988), DNAs of the milled rice flours were extracted.

17. In case of processed rice products, such as boiled rice grains or rice cake, different DNA extraction/purification method was developed. Processed rice product was subjected to the decomposition of starch by heat-stable alpha-amylase (Sigma, USA) for 60 min at 80 degrees C., followed by the hydrolysis of proteins by proteinase K (Takara-bio, Japan) for 2 hrs at 37 degrees. Thereafter, DNAs were extracted by phenol solution and purified by the phenol/chloroform/*iso*-amyl alcohol (PCI, 25/24/1, v/v/v) and ethyl alcohol.

18. DNAs were proliferated by the PCR method using 600 commercial random primers (10 mers or 12 mers) as primers. Taq-DNA polymerase was used for amplification of DNAs. Each DNA was denaturated for 1 min at 94 degrees C., annealed for 1 min at 36 degrees C. and elongated for 2 min at 72 degrees C and these procedures were repeated for 45 times. As a PCR apparatus, Thermal Cycler MP (Takara-bio, Japan) was adopted.

19. Proliferated DNAs were electrophoresed for 30 min through the agarose gel (2%) using a Mupid-2 electrophoresis system (Cosmobio, Japan) at the charge of direct current of 100 V. After the electrophoresis, the DNAs were stained by the ethydium bromide and detected by the irradiation of UV light.

Results and Discussion

20. In case of trade contract, inspection or grading or the survey of traceability of rice grains, PCR method must be applied using rice grains as samples. Shoot or leaves are not suitable for sample specimen. Therefore, milled rice flour, single kernel of polished rice or even boiled rice is used as materials for PCR in the inspection carried out in post-harvest inspection. CTAB method can be applied to milled rice flour for extraction and purification of template DNA for PCR (Ohtsubo et al, 1998).

21. In Japan, leading variety Koshihikari shares more than one thirds of total cultivation area of rice in Japan because it is palatable and traded at higher price than other rice cultivars. It is obliged, in Japan, that the name of rice cultivar, location of cultivation, and year of rice production are labeled on the package of rice by the Japan Agricultural Standard (JAS) Act. Therefore, it was necessary to develop the technology to identify Koshihikari cultivar by DNA analysis. The combinations of several STS primers were developed and commercialized (Ohtsubo et al, 2002) through Takara-bio Inc., Japan. Furthermore, for the purpose of detection of rice grains of other cultivars which were blended into Koshihikari, "Detection-kit" was developed. In case of detection-kit, no DNA is proliferated in case of Koshihikari and more than one DNA bands never fail to increase by PCR in case of rice grains of other cultivars. National Food Agency used these two kinds of primer sets for their inspection of rice retailers.

22. As shown in figure 1, Koshihikari can be discriminated from any other cultivars using the "primer set for identification" making three specific DNA bands. There are no other cultivar rice which show the same three band pattern with Koshihikari among 50 dominant rice cultivars in Japan. And the blended rice can be detected by the "primer set for detection" because Koshihikari shows no DNA bands although other cultivar rice reveal at least one DNA band.

23. In case of boiled white rice grain, decomposition of gelatinized rice starch and heat denatured proteins is necessary using heat stable alpha-amylase and protease K (Ohtsubo, 1999). It became possible to proliferate specific DNAs by PCR using template DNA prepared by the "enzyme treatment method." As shown in figure 2, It is possible to differentiate Hitomebore or Hinohikari from Koshihikari, their parental cultivar by PCR using the template DNA prepared from each single grain of boiled rice by the "enzyme treatment method."

24. "Enzyme treatment method" is also useful for preparation of template DNAs from rice cake. The results of PCR using template DNAs prepared from each rice cake by "enzyme treatment method" were same with those using template DNAs from material rice flour directly. It was possible to differentiate each waxy rice by the single PCR and electrophoresis by the development of primer set for "multiplex PCR" to identify waxy rice cultivars.

25. In conclusion, it became possible to identify or differentiate the rice cultivar by PCR. Practical primer set for Koshihikari, dominant cultivar in Japan, was developed. National Food Agency adopted this primer set for monitoring of rice retailers. It became possible to use not only raw rice but also processed rice products, such as boiled rice or rice cake, as materials for cultivar identification by PCR method.

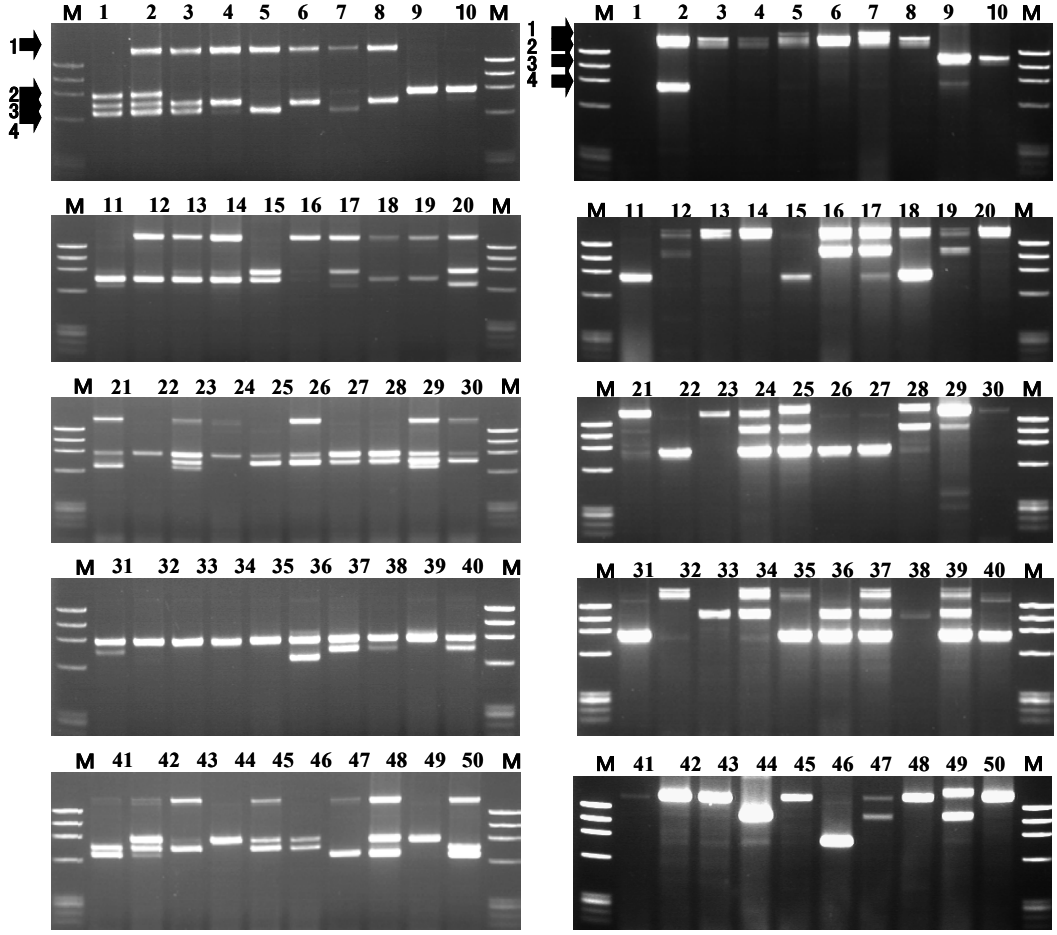

Figure 1. Primer sets for identification of Koshihikari or detection of other cultivar rice grains
A: Primer set for identification of Koshihikari, No.1: Koshihikari, No.2-50: Other cultivars
B: Primer set for detection of blending other cultivar rice to Koshihikari, No.1: Koshihikari (No DNA is proliferated by PCR.), No2-50: Other cultivars (DNA band appears by PCR)

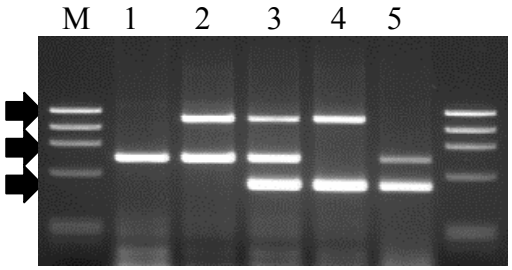

Figure 2. Differentiation of Japanese rice cultivars by PCR using template DNA extracted from a single boiled rice grain
Arrows: DNAs proliferated by PCR,
1: Koshihikari, 2: Hitomebore, 3: Hinohikari, 4: Mutsuhomare, 5: Kinuhikari

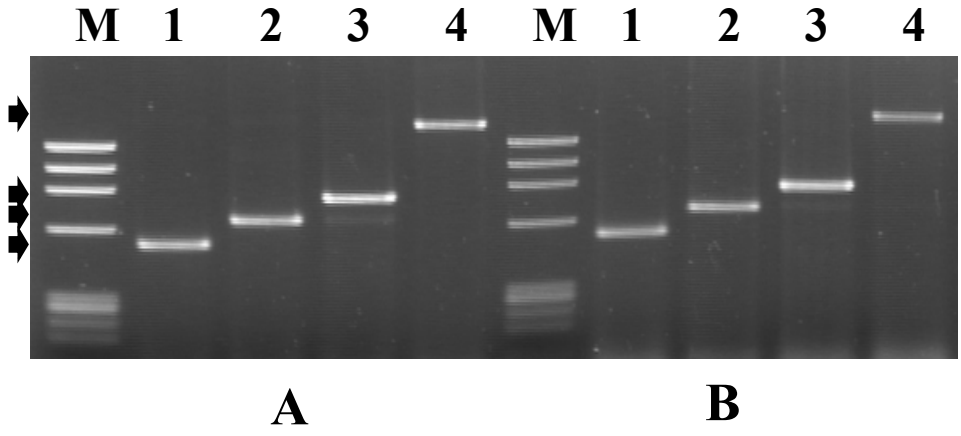

Figure 3. Differentiation of waxy rice cultivars by PCRM: DNA molecular weight marker,
1. Hiyokumochi, 2: Koganemochi, 3: Hakuchomochi, 4: Wataboushi
A: template DNAs were prepared from milled rice flours
B: template DNAs were prepared from rice cake

References

Alec J. Jeffreys, Annette MacLeod, Keiji Tamaki, David L. Neil and Darren G. Monckton, 1985, Minisatellite repeat coding as a digital approach to DNA typing, Nature, 316, 76-79.

Bautista, N.S., Solis, R., Kamijima, O., and Ishii T., 2001, RAPD, RFLP and SSLP analyses of phylogenetic relationships between cultivated and wild species of rice, Genes Genetics Syst., 76, 71-79.

Parminder S. Virk, Brian V. Ford-Loyd, Michael T. Jackson and H. John, 1995, Use of RAPD for the study of diversity within plant germplasm collections, Heredity, 74, 170-179.

Helentjaris, T, King, G., Slocum, M., Siedenstrang, C., and Wegman, S, 1985, Restriction fragment polymorphism as probes for plant diversity and their development as tools for applied plant breeding, Plant Molecular Biology, 5, 109-118.

Tanksley, S.D., Young, N.D., Paterson A. H., and Bonierbale, M.W.,1989, RFLP mapping in plant breeding: new tools for an old science. Bio/Technology, 7, 258-264.

Wang, Z. Y. and Tanksley, S. D., 1992, Polymorphism and phylogenetic relationships among species in the genus Oryza as determined by analysis of nuclear RFLPs, Theoretical and Applied Genetics, 83, 565-581.

Zang, Q., Saghai Maloof, M.A., Lu T.Y. , and Shen, B.Z., 1992, Genetic diversity and differentiation of Indica and Japonica rice detected by RFLP analysis, Theoretical and Applied Genetics, 83, 495-459.

Fukuoka, S., Hosaka, K., and Kamijima, O, 1992, Use of random amplified polymorphic DNAs for identification of rice accessions, Japanese Journal of Genetics, 67, 243-252.

Yu, L-X, and Nguyen, H.T., 1994, Genetic variation detected with RAPD markers among upland and lowland rice cultivars., Theoretical and Applied Genetics, 87, 668-672.

Takashige Ishii, Toshitsugu Nakano, Hideo Maeda, and Osamu Kamijima, 1996, Phylogenetic relationships in A-genom species of rice as revealed by RAPD analysis, Genes Genet. Syst., 71, 195-201.

John G. K. Williams, Anne R. Kubenik, Kenneth J. Livak, J. Antoni Rafalski, and Scott V. Tingey, 1990, DNA polymorphisms amplified by arbitrary primers are useful as genetic markers, Nucleic Acids Research, 18, 6531-6535.

L.-X. Yu, H.T. Nguyen, 1994, Genetic variation detected with RAPD markers among upland and lowland rice cultivars (Oryza sativa L.), Theoretical and Applied Genetics, 87, 668-672.

Mackill, D.J., 1995, Classifying Japonica rice cultivars with RAPD markers, Crop Science, 35, 889-894.

Zhao, X. and Kochert, G., 1993, Phylogenetic distribution and genetic mapping of a $(GGC)_n$ microsatellites from rice (Oryza sativa L.), Plant Molecular Biology, 21, 607-614.

Cao, D and Oard, J.H., 1997, Pedigree and RAPD-based DNA analysis of commercial U.S. Rice cultivars, Crop Science, 37, 1630-1635.

G. Wang, S. Castiglione, J. Zhang, R. Fu, J. Ma, W. Li, Y. Sun, and F. Sala, 1994, Hybrid rice (Oryza sativa L.): identification and parentage determination by RAPD fingerprinting, Plant Cell Reports, 14, 112-115.

H. L. Ko, D. C. Cowan, R. J. Henry, G. C. Graham, A. B. Blakeney, and L. G. Lewin, 1994, Random amplified polymorphic DNA analysis of Australian rice (Oryza sativa L.) varieties, Euphytica, 80, 179-189.

K. Ohtsubo, H. Toyoshima, and H. Okadome, 1998, Quality assay of rice using traditional and novel tools,, Cereal Foods World, 43, 203-206.

K. Ohtsubo, S. Nakamura, H. Morooka, T. Fujii, T. Fuse, and S. Kawasaki, 1999, Cultivar identification of rice using a single kernel of boiled rice, Nippon Shokuhin Kagakukougaku Kaishi (in Japanese language with English summary), 46, 262-267.

K. Ohtsubo, S. Nakamura, and T. Imamura, 2002, Development of the primer sets for identification of a rice cultivar, Koshihikari, by PCR (in Japanese language with English summary), Nippon Nogeikagaku Kaishi, 76, 388-397.

Davierwala, A. P., Chowdari, K. V., Kumar, S., Reddy, A. P. K., Ranjekar, P. K., and Gupta, V. S., 2000, Use of three different marker systems to estimate genetic diversity if Indian elite rice varieties, Genetica, 108, 269-284.

Yang, G.P., Saghai Maroof, M. A., Xu, C. G., Zhang, Q., and Biyashev, R. M., 1994, Comparative analysis of microsatellite DNA polymorphism in landraces and cultivars of rice, Molecular and General Genetics, 245, 187-194.

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