Application of AFLP Technique That Uses Non - radioactive Fluorescent Primers to the Detection of Genetic Diversity in Japanese Rice Cultivars and Cloning of DNA Sequences Derived from an Indica Genome

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Summary

To perform rapid amplified fragment length polymorphism (AFLP) analysis and efficiently clone AFLP fragments with non-radioactive primers, we modified the current AFLP protocol. When the modified procedure was applied to the rice genome, 30 to 80 AFLP fragments (~40 to 400 bp in length) could be detected in a single PCR reaction. To assess the suitability of the technique for the analysis of the rice genome, we first applied it to quantify genetic diversity in a sample of 55 predominantly Japanese modern and domestic cultivars. In this analysis, more than 75% of the Japanese rices were differentiated by the combination of six primer pairs. Then we used the technique to identify and clone AFLP fragments unique to the Japanese variety Kinuhikari compared to those found in the closely related cultivar Koshihikari. In this cloning, we obtained sequences derived from chromosomal segments of an Indica genome, which had introgressed into the Kinuhikari genome. These two studies demonstrated that the modified AFLP technique is suitable for the genome analysis in rice.

Key Words : AFLP, rice genome, genotyping, Japanese rice cultivars.

Introduction

In recent years, DNA marker techniques have played an essential role in plant genetics and breeding studies (Mohan *et al.* 1997). Among the techniques, restriction fragment length polymorphism (RFLP) has been commonly used for the construction of linkage maps. In addition to RFLP, a number of polymerase chain reaction (PCR)-based markers, such as random amplified polymorphic DNA (RAPD) (Williams *et al.* 1990), simple sequence repeat (SSR), and microsatellite (Tautz 1989) and anchored SSR (Zietkiewicz *et al.* 1994), have been developed and used in genetic mapping and biodiversity studies. Compared with RFLP, these PCR-based methods are technically simple and require only a small amount of DNA. Recently, a new technique,

amplified fragment length polymorphism (AFLP), has been developed (Vos *et al.*, 1995). This PCR-based technique which involves selective amplification of restriction fragments can generate a DNA fingerprint of as many as 100 DNA fragments in each reaction. The technique combines both the reliability of RFLP and the efficiency of RAPD. Thus, AFLP is potentially very useful for genetic mapping, genotyping of cultivars, and marker-assisted breeding.

Rice has become a model plant for genetic and genomic studies. Previous studies (Cho *et al.* 1996, Mackill *et al.* 1996, Maheswaran *et al.* 1997, Zhu *et al.* 1998) had demonstrated that AFLP enables to efficiently detect polymorphisms among cultivars, suggesting the potential application of this technique to genotyping of rice accessions. However, it remained to be determined whether the AFLP markers were also suitable for detecting differences in the genomes of closely related rice cultivars, such as Japanese rices. In the present study, we applied the AFLP method to the analysis of the genomes of Japonica rices, predominantly Japanese cultivars. For this study, we modified the current AFLP procedure so that we could detect and clone AFLP fragments non-radioactively. In the modified method, AFLP products are amplified with fluorescent primers and the resulting fragments are detected using a gel scanner/analyzer. This method enables to obtain an AFLP fingerprint rapidly and to clone specific AFLP fragments reliably. To assess the suitability and efficiency of our method for the analysis of the rice genome, we applied the method to the following two projects: 1) we investigated genetic variation and similarity in a sample of 55 rice cultivars, most of which consisted of Japanese cultivars, and 2) we cloned AFLP fragments specific to the genome of Kinuhikari and not found in the closely related Japanese elite cultivar Koshihikari.

Materials and Methods

Plant materials

To examine the resolution and reproducibility of AFLP fingerprints with our method, we analyzed three Japonica varieties (Koshihikari, Akihikari and Dinalaga) and two Indica varieties (Milyang 23 and Nanjing 11). For genotyping analysis of a large sample of rice cultivars, we used 55 rice varieties. This collection includes 13 Japanese modern cultivars, 33 Japanese

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domestic cultivars, 7 Japonica cultivars planted outside of Japan and 2 Indica cultivars. For the detection and cloning of Kinuhikari-specific DNAs, we used Koshihikari, Kinuhikari, and IR8. To compare the segregation patterns of an AFLP fragment with the Southern hybridization fragment probed by the cloned AFLP band, a subset of 22 lines from a recombinant inbred (RI) population derived from a cross between Akihikari and Milyang 23 (Fukuta *et al.*, unpublished results) was used. The DNA of each variety was isolated from pooled leaf tissues collected from a large number of seedlings by using the CTAB method (Murray and Thompson 1980). DNA of *Arabidopsis thaliana* ecotype Columbia was supplied by Gibco BRL Inc. (AFLP Core Reagent Kit).

AFLP protocol

We used the AFLP protocol described by Vos *et al.* (1995) with modifications. Briefly, 500 ng of genomic DNA was digested with *EcoRI* and *MseI*, then *EcoRI* and *MseI* adapters were ligated to the ends of the restriction fragments (AFLP Core Reagent Kit; Gibco BRL, Inc.). To generate large quantities of template for subsequent selective amplification, the adapter-ligated fragments were amplified with a non-selective *EcoRI* primer (E00; 5'-GACTGCGTACCAATTC-3') and an *MseI* primer containing one selective nucleotide at the 3' end (M00 + C; 5'-GATGAGTCCTGAGTAAC-3'). Selective amplification was then performed using an *EcoRI* selective primer that contained two selective nucleotides at the 3' end of E00 and an *MseI* selective primer containing a total of three selective nucleotides at the 3' end of M00. In this step, instead of 5 ng of a radioactively labeled *EcoRI* primer as described by Vos *et al.* (1995), 15 to 30 ng of an *EcoRI* selective primer labeled with rhodamine at the 5' end was used. For detecting Kinuhikari-specific AFLP fragments, a total of 89 selective primer combinations were used. Primers used for non-selective and selective amplifications were synthesized by Takara, Inc. All the amplification reactions were performed in a Takara TP3000 thermocycler.

The reaction products were analyzed on 6% denaturing polyacrylamide gels containing 6.5 M urea and 1x TBE buffer. Each gel was run at 30 W for 2 h. After electrophoresis, the AFLP fragments were visualized by using a gel scanner/analyzer (FMBIO-100; Takara, Inc.), which detects the fluorescence emitted by the amplified fragments. The AFLP fingerprints obtained were saved as image files.

Cloning of AFLP fragments

After we obtained an AFLP fingerprint, the polyacrylamide gel (which was laid on a glass plate) was placed over a printout of the AFLP fragment pattern; the areas containing fragments of interest were identified and excised from the gel. Each gel slice was placed with 10 μ l water in a microcentrifuge tube, then crushed with a micropipet tip. The AFLP fragment was PCR-amplified

in a volume of 25 μ l containing 5 μ l of the resuspended gel slice, 0.5 μ M of each primer used for the selective amplification, 2.5 μ l 10x PCR buffer, 200 μ M each dNTP, and 1U Taq polymerase (Takara, Inc). The thermocycler was programmed for 30 cycles at 94 °C (60 s), 55 °C (60 s), and 72 °C (120 s). The amplified products were ligated into the TA cloning vector (pCR; Invitrogen), and then transformed into *Escherichia coli* (JM109). The cloned AFLP fragments were subjected to Southern hybridization analysis on blots of rice DNAs. Labeling and detection of the probes were performed using the ECL system (Amersham).

Data Analysis

Each polymorphic AFLP fragment was given a score of 1 for presence or 0 for absence. To reveal the level of polymorphisms between a pair of two varieties, the genetic distance between two entries was computed as

$$GD = -\ln(2N/(N_i + N_j))$$

where N is the number of shared fragments and N_i and N_j are the total number of fragments for entries i and j (Lynch 1990, Zhou and Gustafson 1995). The score of polymorphic fragments was used to construct a cluster diagram by the UPGMA method with the software STATISTICA (version 5j).

Results and Discussion

Detection of AFLP fragments

To determine whether the modified AFLP method offers a sufficient sensitivity and resolution for the analysis of rice DNAs, we employed DNAs from various rice cultivars for the detection of AFLP fragments (Fig. 1). We also included DNA from *A. thaliana* ecotype Columbia as a reference. Using a selective primer pair, E00+AC/M00+CTG, we obtained clear fragment patterns for all the samples; reactions in which rice DNA was the template yielded 40 to 50 easily discernible bands (each ~40 to 400 bp in length) as well as many fainter bands. With other primer combinations, as many as 80 distinct bands were obtained per sample (data not shown). Our protocol is characterized by its simplicity in obtaining AFLP fingerprints; clear band patterns were obtained without difficulty only 30 minutes after electrophoresis. This modification is also characterized by its suitability for rapid and efficient cloning of AFLP fragments without the use of radioactive labeling. One example is illustrated in Fig. 2, where a fragment shows a polymorphism between the Japonica variety Koshihikari and the Indica variety IR8. Reliability of its cloning is demonstrated by the fact that identical segregations were observed for the AFLP fragment and the Southern hybridization band with the cloned DNA as a probe in 22 segregating lines from the RI mapping population (data not shown).

Identification and classification of Japanese cultivars

Fig. 1. shows that the use of only one primer pair

Fig. 1. AFLP patterns obtained from five rice cultivars and *A. thaliana* by using the selective primers E00+AC and M00+CTG. Lane 1, *A. thaliana* ecotype Columbia; lane 2, Koshihikari (cultivar group Japonica); lane 3, Akihikari (Japonica); lane 4, Milyang 23 (Indica); lane 5, Nanjing 11 (Indica); lane 6, Dinalaga (Javanica).

(E00+AC/M00+CTG) was sufficient to reveal genetic variations among the groups of rice cultivars, and about one-fourth of the resulting fragments were polymorphic among Japonica and Indica rices. This high efficiency of the AFLP method for detecting polymorphic fragments among rice cultivars had already been reported by several authors (Mackill *et al.* 1996, Maheswaran *et al.* 1997, Zhu *et al.* 1998). We then applied the modified AFLP method to survey the genetic diversity in Japanese cultivars. Domestic cultivars grown in Japan display a low genetic variation (Glaszmann and Arraudeau 1986). Modern Japanese cultivars have been bred by crossing these domestic cultivars. As a result, the modern Japanese cultivars may have a higher genetic similarity than domestic cultivars, which hindered the classification of these rices with a DNA fingerprinting method (Fukuoka *et al.* 1992). It is important to determine whether the AFLP technique offers a sufficient resolution for identifying and classifying these closely

related cultivars. The development of an identification system, which enables to distinguish one cultivar from other closely related ones, would be essential for managing germplasm collections of Japanese rices.

A total of 55 rice cultivars, consisting mostly of Japanese modern and domestic cultivars, were DNA-typed with AFLP. The use of six primer pairs (E00 + AA / M00 + CTA, E00 + AA / M00 + CAA, E00 + AC / M00 + CTG, E00 + TA / M00 + CAC, E00 + TT / M00 + CTC and E00 + GA / M00 + CAG) produced a total of 303 AFLP fragments, of which 89 (29%) showed polymorphism within the cultivars. On the average, 9.9 polymorphic fragments were detected between a pair of cultivars tested in the study. To estimate the genetic diversity within a subgroup or between two subgroups in our sample, we calculated the genetic distance (GD) from the number of shared and polymorphic fragments (Table 1). The averaged GD values in the table represent the averaged genetic distance between a pair of two cultivars within a subgroup or between two subgroups. Thus, this procedure can be considered to be useful to determine genetic heterogeneity in a subgroup or a distance between two subgroups. The lowest genetic diversity was observed in Japanese modern cultivars, followed by Japanese domestic cultivars. Japonica rices grown outside of Japan exhibited a much larger genetic variability than Japanese rices.

Fig. 3. shows the dependence of the percentage of the Japanese cultivars differentiated with AFLP markers on the number of the selective primer pairs used in this analysis. Combination of the six primer pairs identified more than 75 % of the Japanese cultivars used for this experiment. Although complete identification of these cultivars could not be achieved with this number of primer combinations, the results indicated the suitability of the AFLP method for differentiating closely related rice cultivars.

The polymorphism revealed in the AFLP fingerprints was used to produce the dendrogram shown in Fig. 4. Most of the Japanese cultivars were classified into a group consisting of small clusters and were distinct from other Japonica rices planted outside of Japan. Although some cultivars characterized by a parent-offspring relationship, such as Reimei and Akihikari, tended to be clustered together, no pronounced grouping was observed in the Japanese cultivars; no boundary was found between the modern cultivars and the domestic cultivars, or among cultivars grown in distinct regions in Japan, except for the cultivar Akamai-shinbon, a red-kerneled rice. The averaged GD value of Akamai-shinbon to other Japanese cultivars was calculated to be 0.039, indicating the genetic distinctness of this cultivar among Japanese rices. Akagi *et al.* (1997), using microsatellite markers, reported the results of genotyping analysis of Japanese rices. They successfully differentiated individual cultivars. However, cluster analysis did not reveal any distinct grouping in their sample of Japanese cultivars. The results obtained with our AFLP method and

Fig. 2. Cloning of an AFLP fragment by using the modified AFLP technique. (A) Partial AFLP fingerprints from IR8 (1) and Koshihikari (2) obtained by using the selective primers E00+AC and M00+CTG (*left*). We tried to clone one polymorphic fragment (*arrow*) detected between IR8 and Koshihikari. After excision of the band, the gel was rescanned to confirm that the correct portion was excised (*right*). (B) The product following PCR amplification of the excised AFLP fragment with the primer combination used during selective amplification. (C) Southern hybridization pattern of *Eco*RI-digested DNA from Koshihikari and IR8 that was probed with the cloned AFLP fragment.

Table 1. Averaged genetic distance calculated for each pair of two rice cultivars within a subgroup or between subgroups in our sample

Cultivars	Genetic distance
Japanese cultivars ¹⁾	0.015 ± 0.010
Japanese modern cultivars	0.010 ± 0.006
Japanese domestic cultivars	0.017 ± 0.010
Japonica cultivars grown outside of Japan	0.071 ± 0.017
Indica/Japonica	0.133 ± 0.015
Indica cultivars ²⁾	0.090

¹⁾ Japanese cultivars include modern and domestic cultivars.

²⁾ Indica cultivars include IR8 and Dular.

their microsatellite markers demonstrate the presence of a genetic uniformity in Japanese modern and domestic cultivars.

Detection and cloning of variety-specific AFLP fragments

Cloning of a DNA segment unique to one cultivar among related cultivars (the premise of genomic subtraction techniques) would be the first step for elucidating the genetic mechanisms underlying phenotypic differences among cultivars. For example, Kajiya *et al.* (1996) applied the representational difference analysis (RDA) technique to clone Akihikari-specific DNA

Fig. 3. Dependence of the percentage of differentiated Japanese rice cultivars on the number of primer combinations. A sample of 46 Japanese modern and domestic cultivars was DNA-typed by AFLP fingerprinting analysis.

markers from the genomes of Koshihikari and Akihikari. In the present study, we used the modified AFLP technique to detect differences between the genomes of Kinuhikari and Koshihikari and to clone DNA sequences specific to the Kinuhikari genome.

The genome of Kinuhikari predominantly consists of Koshihikari-like DNA sequences, with additional chro-

Fig 4. Dendrogram showing a cluster analysis of 55 rice cultivars with six combinations of primers. Cultivar names are as follows, 1. IR 8, 2. Dular, 3. CPSLO-17, 4. Ketan Nangka, 5. Lemont, 6. Dinalaga, 7. Sensho, 8. Akamai-shinbon, 9. Italica Livorno, 10. Texas Fortuna, 11. Tsushima-akamai, 12. Tsushima, 13. Nakate-shinsenbon, 14. Kinmaze, 15. Ishijiro, 16. Shinshu, 17. Aikoku, 18. Bozu, 19. Akage, 20. Akamai-4, 21. Ohba, 22. Senichi, 23. Kamenoo, 24. Takenari, 25. Fujisaka 5, 26. Akamai-3, 27. Sekiyama, 28. Sekitori, 29. Shinriki, 30. Tanshin-wase, 30. Shiratama, 31. Fukubozu, Omachi, Kokuryomiyako, Tamanishiki, 33. Banzai, 34. Kogyoku, 35. Toyokuni, Kameji, 36. Reimei, Akihikari, 37. Akitakomachi, 38. Norin 29, Jyukoku, 40. Koshihikari, 41. Rikuu 132, 41. Koganebare, Asahi, Aichi-asahi, 43. Ginbozu, Morita-wase, Toyonishiki, Sasanishiki, 44. Gohyakumangoku, Nipponbare.

mosomal segments derived from other Japonica varieties as well as the Indica variety IR 8 (Fig. 5). The phenotypic traits of the cultivars Koshihikari and Kinuhikari are quite similar; both are characterized by their high-quality grain. However, the culm of Kinuhikari is shorter than that of Koshihikari, and Koshihikari exhibits a stronger seed dormancy than Kinuhikari (Koga *et al.* 1989). These slight differences are likely to have originated from their respective unique genome regions. In particular, IR8-derived segments (not found in Koshihikari) may be responsible for some of the distinct characteristics of Kinuhikari.

Using a total of 89 selective primer combinations, we generated AFLP fingerprints of the genomes of Koshihikari, Kinuhikari, and IR8. A total of 634 polymorphisms were detected between Koshihikari and IR8, and 36 polymorphisms were observed between Koshihikari and Kinuhikari. Among the Kinuhikari-specific fragments, 24 originated from the IR8 genome (Fig. 6), suggesting that about 4% of the Kinuhikari genome is derived from that of IR8.

We then cloned 12 of the IR8-derived Kinuhikari fragments. The cloned sequences ranged in size from 60

Fig. 5. Pedigree showing the close genetic relatedness of Kinuhikari to Koshihikari.

Fig. 6. Kinuhikari-specific AFLP fragment revealed by using the primers E00+CA and M00+CAC. The presence of a corresponding band in IR8 indicates that this AFLP fragment is a segment of the Kinuhikari genome that had introgressed from IR8.

to 150 bp, and were classified into 10 distinct clones based on their Southern hybridization patterns. Southern analyses of DNAs from Kinuhikari, Koshihikari, and IR8 probed with these 10 groups revealed that in 4 sequences multiple bands are detected; the remaining 6 inserts hybridized to single bands. In general, PCR amplification using random anonymous sequences tends to generate fragments that reflect repeated sequences in the genome, making these products unsuitable for mapping or further studies. In contrast, more than half of the sequences isolated by AFLP were single-copy sequences. Although the number of cloned DNAs examined in this study was small, the results obtained suggest that the AFLP method would be suitable for tagging a gene of interest using the bulked-segregant method (Michelmore *et al.* 1991).

Concluding remarks

Based on the results obtained from the two studies described in this report, the modified AFLP technique enables to generate AFLP fingerprints with a sufficient degree of resolution and is a suitable alternative to conventional AFLP which involves radiolabeled primers. Compared to another fluorescent method, in which an autosequencer is used to detect fragments, our method offers the following advantages: specific AFLP fragments can be cloned, and primers used during selective amplification are not limited to those commercially available.

A problem inherent to the AFLP technique that remained to be solved in the present study is the small size of the cloned AFLP fragments; the cloned Kinuhikari-specific fragments were 60 to 150 bp long. We were unable to map these fragments by Southern hybridization using the non-radioactive labeling and detection method. Furthermore, attempts to screen a rice YAC library (Umehara *et al.* 1995) with our cloned DNAs were also unsuccessful (data not shown). To overcome this shortcoming, we are currently developing methods whereby longer fragments could be cloned. After obtaining longer Kinuhikari-specific sequences, we will map them on the Kinuhikari chromosomes and survey genes that make this cultivar distinct from Koshihikari.

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