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M. Kawase

# Application of the restriction landmark genomic scanning (RLGS) method to rice cultivars as a new fingerprinting technique

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**Abstract** The restriction landmark genomic scanning (RLGS) method was applied to rice, using two Japanese cultivars, 'Nipponbare' and 'Koshihikari', and a Chinese landrace, 'Liu'Zhou'Bao'Ya'Zao'. More than 3000 landmarks were detected as spots on the individual autoradiograms of each cultivar. 'Nipponbare' and 'Liu'Zhou' Bao'Ya'Zao' showed apparently different RLGS profiles, from which the genetic similarity (GS) between them was estimated as 0.344. Although the two Japanese cultivars, 'Nipponbare' and 'Koshihikari' showed quite similar RLGS profiles, they were easily distinguished on the basis of the presence or absence of specific spots; the GS value between them was calculated as 0.980. The RLGS method is shown to be a powerful fingerprinting technique, especially for the classification and identification of cultivars in rice and probably in other crops as well.

**Key words** RLGS · Fingerprinting · Genetic similarity · Cultivar identification · Rice

## Introduction

The restriction landmark genomic scanning (RLGS) method is a new technique developed for analyzing genomic DNA of higher organisms (Hatada et al. 1991). This method is based on the principle that restriction enzyme sites can be used as landmarks. It employs direct labeling of the genomic DNA at the restriction sites and two-dimensional (2-D) electrophoresis to resolve and identify these landmarks. This procedure gives a 2-D pattern with thousands of scattered spots of landmarks after autoradiography.

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M. Kawase (✉)
Laboratory of Plant Biotechnology,
Department of Crop Improvement,
Shikoku National Agricultural Experiment Station,
3–1, Sen'yu-cho 1, Zentsuji, Kagawa 765, Japan

In the study presented here, the RLGS method was applied to rice cultivars as a new fingerprinting technique. Distantly related and closely related cultivars were compared in order to assess the possibility of using the RLGS method for further analyses.

## Materials and methods

### Rice cultivars used

Two Japanese improved cultivars, 'Nipponbare' and 'Koshihikari', and a Chinese landrace, 'Liu'Zhou'Bao'Ya'Zao', were used. 'Nipponbare' and 'Liu'Zhou'Bao'Ya'Zao' were provided by the Laboratory of Plant Germplasm Introduction, the National Institute of Agrobiological Resources, Tsukuba, Ibaraki, Japan, and 'Koshihikari' was obtained from the Laboratory of Crop Eco-physiology, Shikoku National Agricultural Experiment Station, Zentsuji, Kagawa, Japan.

### DNA extraction

DNA was extracted from the leaves of 14-day-old seedlings using cetyltrimethylammonium bromide (CTAB) as described by Murray and Thompson (1980).

### RLGS method

RLGS was carried out basically according to the procedure described by Hatada et al. (1991), which consists of a blocking reaction, landmark cleavage, labeling, the first fractionation and the second fractionation. (1) Blocking reaction: 5 μg extracted DNA was incubated at 37°C for 30 min with 2 units of DNA polymerase I (TaKaRa) in 10 μl of 50 m*M* TRIS-HCl (pH 7.4) containing 10 m*M* $MgCl_2$, 100 m*M* NaCl, 20 m*M* dithiothreitol (DTT), 0.4 μ*M* 2′-deoxyguanosine 5′-[α-thio]-triphosphate (dGTPαS, Amersham), 0.2 μ*M* 2′-deoxycytosine 5′-[α-thio]-triphosphate (dCTPαS, Amersham), 0.4 μ*M* 2′,3′-dideoxy-adenosine 5′-[α-thio]-triphosphate (ddATPαS, TOYOBO) and 0.4 μ*M* 2′,3′-dideoxythymidine 5′-[α-thio]-triphosphate (ddTTPαS, TOYOBO). This blocking reaction was performed to reduce the nonspecific incorporation of radioactivity in the labeling step by means of the preceding incorporation of nucleotide analogues into damaged sites, such as nicks, gaps and/or double-strand breakage in the DNA, that might have occurred through the DNA extraction process (Hatada et al. 1991). The deoxynucleotide analogues

(dGTPαS and dCTPS) which can be incorporated into a cleaved site of *Not*I and the dideoxynucleotide analogues (ddATPαS and ddTTPαS) which can not be incorporated into it were chosen. The incorporated dideoxy-[α-thio]-nucleotide analogues are difficult to eliminate by the 3′ exonuclease activity of polymerase in the labeling step and also prevent an additional incorporation of the radioactive nucleotides at the blocked ends. After the treatment, the enzyme was inactivated at 65°C for 30 min. (2) Landmark cleavage: the treated DNA was digested with 20 units of *Not*I (TOYOBO) at 37°C for 2 h. (3) Labeling: the cleaved ends were filled in with 20 units of Sequenase ver.2 (USB) in the presence of 0.17 μ*M* α-[$^{32}$P]dGTP (~110 TBq; Amersham) and 0.17 μ*M* α-[$^{32}$P]dCTP (~222 TBq; Amersham) at 37°C for 30 min. (4) First fractionation: the labeled DNA was then digested with 20 units of *Eco*RV (TOYOBO) at 37°C for 60 min to reduce the fragment sizes for the following fractionation. Following the above-mentioned treatments, the solution containing 1.5 μg of the DNA was applied onto a 0.8% agarose vertical gel (SeaKem GTG; FMC) of ø2.4 mm × 61 cm. The first dimension (1-D) electrophoresis was conducted in an electrophoresis buffer (100 m*M* Tris, 40 m*M* Sodium acetate, 36 m*M* NaCl, 31 m*M* EDTA; pH 8.0) at 100 V for 2 h and then at 230 V for 24 h. (5) Second fractionation: after the 1-D electrophoresis, the gel portion (33 cm long) containing DNA fragments of approximately 9 kbp–500 bp was equilibrated twice for 10 min each in 40 ml of HB (50 m*M* Tris-HCl, 10 m*M* $MgCl_2$, 100 m*M* NaCl, 10 m*M* DTT). Then, the DNA fragments fractionated in the gel were treated in situ with 500 units of *Mbo*I (TaKaRa) in 500 μl of HB containing 0.01% BSA at 37°C for 2 h. The gel was fused with a 36 × 43 × 0.1 cm polyacrylamide gel (5%; acrylamide/bisacrylamide, 29:1) by adding melted agarose to fill up the gap. The 2-D electrophoresis was conducted in a TBE buffer (89 m*M* Tris, 89 m*M* boric acid, 2 m*M* EDTA) at 100 V for 2 h and then 200 V for 24 h. The gel was dried and exposed by autoradiography for 6 to 24 h on an X-ray film (X-OMAT AR2; Kodak) at –80°C using an intensifying screen.

### Estimation of genetic similarity (GS)

Genetic similarities between cultivars were calculated by the measure devised by Dice (1945), which was suggested for DNA polymorphism by Nei and Li (1979):

$$GS(i,j)=2N(i,j)/[N(i)+N(j)],$$

where GS(i,j) is the measure of genetic similarity between cultivars i and j, N(i,j) is the total number of spots common to i and j, and N(i) and N(j) are the numbers of spots for cultivars i and j, respectively. A GS value reflects the proportion of RLGS spots that cannot be distinguished between two cultivars.

## Results

### RLGS profiles obtained by autoradiography

More than 3000 landmarks were detected as scattered spots on the individual autoradiograms of each cultivar (Fig. 1). A high resolution with a low background was achieved by the blocking reaction. The relative position and the intensity of each spot were highly reproducible when the same DNA sample was analyzed repeatedly. A RLGS profile, which is the pattern of the spots, was unique to each cultivar used. A poorer resolution was sometimes observed in the area where DNA fragments of more than 6 kbp were distributed throughout the 1-D electrophoresis. An exposure of autoradiography for 12 h was adequate to obtain a clear RLGS profile when 1.5 μg of the treated DNA was applied onto the 1-D gel.

### Differences between cultivars

The RLGS profiles of 'Nipponbare' and 'Liu'Zhou' Bao'Ya'Zao' showed quite different patterns, which suggested a wide genetic difference between those two cultivars. A limited area shown in Fig. 1 could be compared, although the different patterns made it rather difficult to identify each spot between them. 'Nipponbare' and 'Liu'Zhou'Bao'Ya'Zao' had 387 and 403 spots in the area, respectively, of which 136 spots were scored as common or indistinguishable spots. Therefore, the GS value could be calculated as 0.344.

On the other hand, the two Japanese cultivars, 'Nipponbare' and 'Koshihikari', showed quite similar patterns. An area showing the clearest resolution was chosen for precise comparison (Fig. 2). This area contained 1156 spots that were common to both cultivars. Additionally, 24 and 25 spots were found to be unique to 'Nipponbare' and 'Koshihikari', respectively. The GS value between them was calculated as 0.980.

## Discussion

### RLGS as a new fingerprinting technique in rice

The haploid genome size of rice chromosomal DNA is about $4 \times 10^8$ bp (Moore et al. 1993), and the average size of *Not*I fragments has been suggested to be about 100 kbp (Umehara et al. 1994). The approximate number of spots was expected to be 8000, and more than 3000 were observed in the present analysis. This fact indicates that at least one-third of the total landmarks can be scanned and therefore that RLGS can be an efficient fingerprinting technique based on the genetic information from the whole genome.

The distinct profiles obtained by the RLGS method clearly show a wide genetic difference between 'Nipponbare' and 'Liu'Zhou'Bao'Ya'Zao', providing a GS value of 0.344. The difficulties in identifying each spot between such distantly related cultivars may be overcome by using some appropriate internal markers in the DNA samples and/or by obtaining an additional RLGS profile for a mixed DNA sample to provide the total spots. Oka (1953, 1958) classified rice cultivars into two major groups, the Indica type and the Japonica type. Restriction fragment length polymorphism (RFLP) analysis of nuclear DNA clearly supported his classification and also indicated a wide genetic difference between 'Nipponbare' (Japonica type) and 'Liu'Zhou'Bao'Ya'Zao' (Indica type) (Kawase et al. 1991). Most of the RFLP probes used in variation studies have, however, been previously selected to show polymorphism, which may result in underestimated GS values. In comparison to RFLP analysis, the RLGS method provides unbiased information on genetic polymorphism throughout the whole genome and an accurate estimation of genetic similarity.

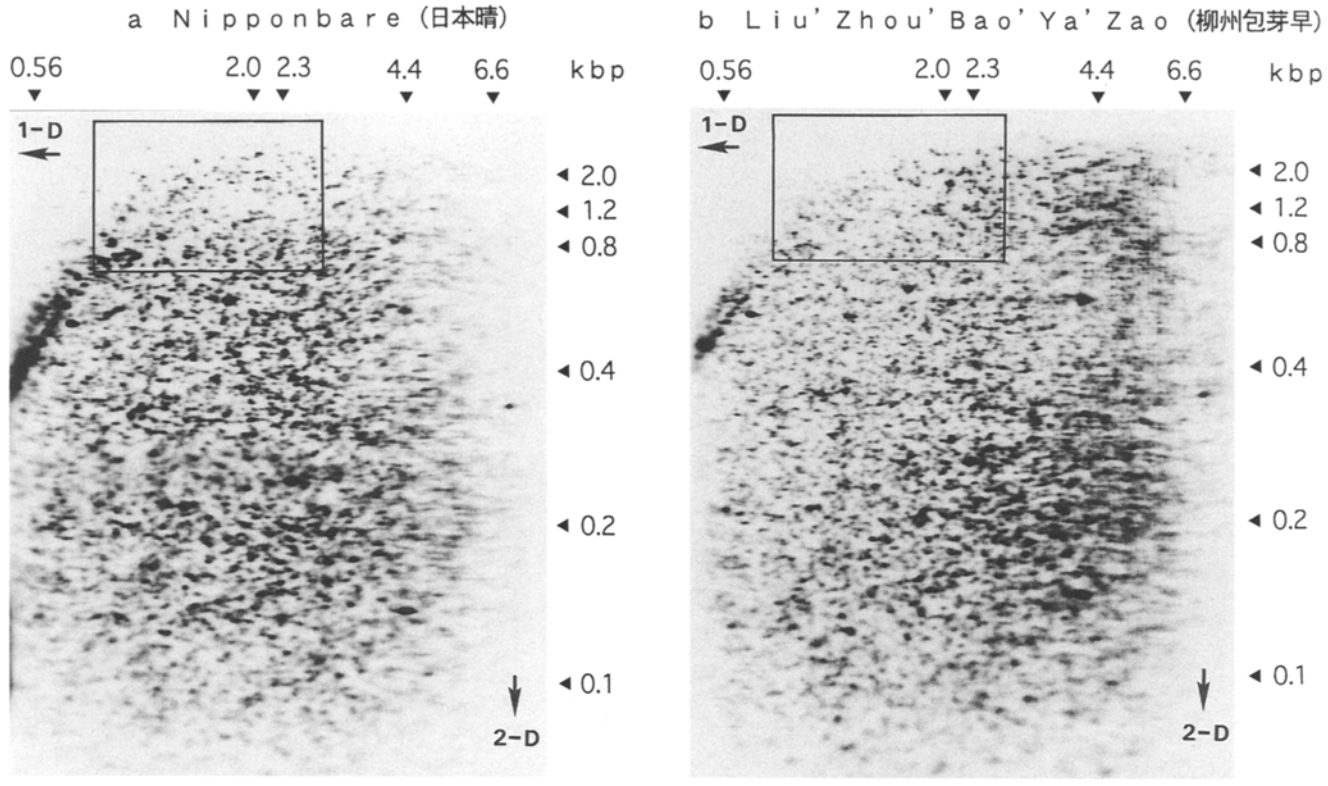


**Fig. 1a, b** More than 3000 spots were scattered on the RLGS profile obtained by autoradiography. 'Nipponbare' (**a**) and 'Liu'Zhou' Bao'Ya'Zao' (**b**) showed quite different patterns. The *enclosed area* was used for estimation of the GS value between the two cultivars

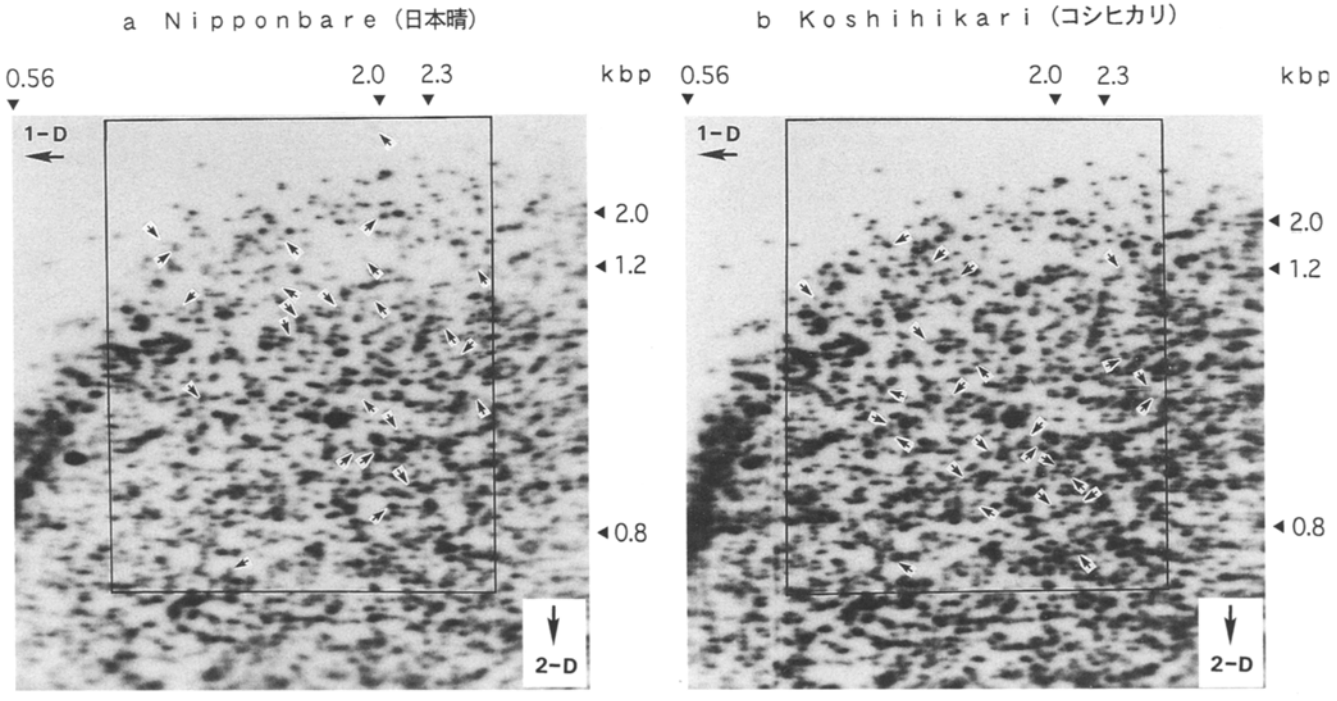


**Fig. 2** The RLGS profiles of 'Nipponbare' and 'Koshihikari' showed a high similarity, but they could be distinguished on the basis of the presence or absence of particular spots in the *enclosed area* that was chosen for precise comparison. *Arrowheads* indicate the spots unique to each of the two cultivars compared

A considerable number of spots were detected to be different between 'Nipponbare' and 'Koshihikari', although they showed a high similarity in the RLGS profiles. RLGS clearly distinguished between these two cultivars and estimated the GS value between them as 0.980. Therefore, the RLGS method is concluded to be a powerful fingerprinting technique that is applicable for the classification and identification of cultivars in rice and probably in other crops.

In general, Japanese modern rice cultivars have been bred on the basis of rather limited genetic resources, which has resulted in making it difficult to detect polymorphism by random amplified polymorphic DNA (RAPD) and/or RFLP analysis. The utilization of molecular markers has been prevented by the low availability of informative markers among Japanese cultivars. For example, at most 23% of the 106 RFLP probes that have been informative between Indica and Japonica types have shown some polymorphism in any one pair of eight landraces that have been frequently used as breeding materials in the pedigrees of modern Japanese cultivars (Kawase, unpublished data). RLGS can be a new tool for genetic analysis, even on such a narrow genetic base.

## Further utilization of RLGS

RLGS will be used for fingerprinting, linkage mapping and phylogenetic studies, since the whole genome can be scanned simultaneously. The changes in the combination of three restriction enzymes may enable the further detection of polymorphism. This method needs only a small amount of DNA, the actual amount depending perhaps on the genome size, while neither probe nor primer is necessary. In the present study, 1.5 μg of rice DNA needs a 12-h exposure to autoradiography, which implies that the amount may be reduced to 0.1 μg if the exposure period is lengthened to 1 week. The cloning method of the DNA fragments corresponding to the spots on the RLGS profile has already been reported (Hirotsune et al. 1993). Accordingly, RLGS will be of great use as a powerful tool in plant genome studies.

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