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Development of a New Cultivar-Discrimination Method Based on DNA Polymorphism in a Vegetatively Propagated Crop

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Abstract

Restriction landmark genome scanning (RLGS) is a high-speed genome analysis method to detect DNA polymorphism, and is suitable to develop useful DNA markers for cultivar-discrimination of agricultural plants. The mat-rush, *Juncus effusus* is a vegetatively propagated crop that has been cultivated for over 500 years in Japan as the material of "tatami-omote," a surface of Japanese conventional mats. In recent years, Japanese mat-rush cultivars are grown in foreign countries and reimported to Japan in violation of the Seeds and Seedlings Law, economically damaging domestic production. The largest problem of illegal mat-rush reimportation has been with cv. Hinomidori. We developed a cultivar-discrimination DNA marker of mat-rush using RLGS, and detected the RLGS spot markers that didn't exist specifically in Hinomidori. The spot marker can be used with the RLGS method to identify Hinomidori accurately among cultivars.

Discipline: Biotechnology
Additional key words: DNA marker, mat-rush, RLGS, Seeds and Seedlings Law

Introduction

Mat-rush (*Juncus effusus* L.) is a perennial plant that grows on damp ground of uncultivated fields. It is distributed widely in warm temperature zones in Eurasia, Oceania and the American continent. In Asia, mat-rush grows wild and is cultivated in Japan, China, Taiwan, and Korea. In these countries, only one subspecies, *J. effusus* L. var. *decipiens* Buchen is cultivated. Though mat-rush is propagated by seed or vegetative growth in the wild, cultivated plants have been reproduced vegetatively in paddy fields since the 16th century. Genetic variability maintained in cultivated populations is thought to be extremely low.

In Japan, mat-rush is utilized as a raw material for tatami-omote, for many kinds of designed-mat products and for Japanese sandals. The domestic price of mat-rush has hovered at low levels and farm management has become difficult since the rapid increase in the quantity of imported tatami-omote made of mat-rush. One of the reasons for this rapid increase is thought to be due to an excellent Japanese cultivar of mat-rush, i.e., Hinomidori that was taken out, grown outside of Japan, and illegally reimported as products of high-quality and low-price. In particular, mat-rush production in Kumamoto Prefecture that has a 90% share of Japanese production has been economically damaged to a great extent.

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The Customs Tariff Law was amended in 2003 to cope with the problem of reimported products that violate breeder's rights. Furthermore, the Seeds and Seedlings Law was amended in 2003 in order to reinforce the protection of breeder's rights and strengthen penal regulations for violation of harvests. The establishment of DNA-based discrimination technology in such vegetatively propagated crop cultivars is expected to provide practical methods for the protection of breeders' rights; it is therefore an urgent problem to establish this new discrimination technology.

In mat-rush and other crops, amplified fragment length polymorphism (AFLP)[4], simple sequence repeat (SSR)[10], single nucleotide polymorphism (SNP)[14], and inter-simple sequence repeat (ISSR)[2] have been used to develop DNA markers for cultivar-discrimination. Some of the techniques have been applied for various species[3,5,8,11,12]. These are time consuming trial and error methods to find polymorphisms, so that, we tried to use another method that surveys many loci at once to develop a DNA marker in a short period.

Restriction landmark genome scanning (RLGS)[7] employs direct end labeling of the genomic DNA digested with a restriction enzyme and two-dimensional electrophoresis. This method enables one to visualize thousands of loci on a single autoradiogram. Taking advantage of the high-speed scanning ability of RLGS, the RLGS spots/loci were subjected to analysis to develop DNA polymorphic markers. The RLGS spot/loci were used in genetic analysis in mammals[17–19], plants[9] and fungi[20]. The RLGS method has various applications[1,6,15], because it can be used to scan for physical genomic DNA status such as amplification, deletion and methylation.

In the present study, we report the development of DNA cultivar-discrimination of a vegetatively propagated crop, such as mat-rush, using RLGS.

Materials and methods

We used 18 mat-rush cultivars, including 3 major cultivars, i.e., Hinomidori, Kiyonami and Okayama-3, which have a 28.0%, 20.3% and 19.5% share of the planted acreage (in 2003), respectively in Japan (Fig. 1). Only these 18 cultivars have been planted as the material of mat-rush products for at least the last 10 years. These plant materials of mat-rush were provided by the Kumamoto Prefectural Agricultural Research Center (Kagami, Kumamoto). Shoots of mat-rush were collected and ground under liquid nitrogen. Total genomic DNA was isolated by the cetyl trimethyl ammonium bromide (CTAB) extraction method[13] with modification accord-

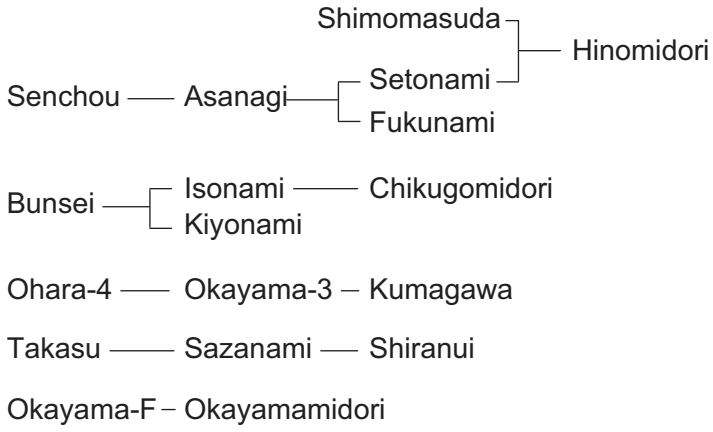

Fig. 1. The genealogical tree of major mat-rush cultivars

The relationships of 18 mat-rush cultivars are shown. Asanagi was separated from the population of Sencho (landrace), Setonami and Fukunami were isolated from somatic mutants generated by gamma-ray irradiated Asanagi. Hinomidori was isolated from the crossed population of Shimomasuda × Setonami. Isonami and Kiyonami were separated from the population of Bunsei (landrace). Chikugomidori was isolated from tissue cultured Isonami. Okayama-3 was separated from the population of Ohara-4, and Kumagawa was from Okayama-3. Sazanami was separated from the population of Takasu (landrace), and Shiranui was isolated from the progeny of natural crossing Sazanami. Okayamamidori was separated from the population of Okayama-F (landrace). All the above native cultivars are not used for production of tatami-omote, but the cultivars that were isolated and fixed with characteristics of superior quality are commercially produced.

ing to Kawase (1994)[9]. Briefly, frozen shoots were ground with mortar and pestle. Shoot powder, 10 mg sodium lauryl sulfate (SDS) and 10 mg polyvinylpyrrolidone were mixed and suspended in 0.4 mL CTAB extraction buffer: 1% CTAB, 0.1 mol/L Tris-HCl (pH 8.0), 50 mmol/L ethylene diamine tetraacetic acid (EDTA), 1.4 mol/L NaCl, and 0.1% β-mercaptoethanol. One μL of proteinase K solution (40 mg/mL) was added to the lysate. The lysate was incubated at 56ºC for 30 min, followed by extraction with phenol and chloroform, and DNA was precipitated with equal volume of 2-propanol.

The RLGS procedure, which was reported elsewhere[7,16], is described as follows: (*i*) 0.4 μg of mat-rush genomic DNA was treated with 10 units of DNA polymerase I in 10 μL of blocking buffer: 50 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, 10 mmol/L dithiothreitol (DTT), 0.33 mmol/L dGTP, 0.33 mmol/L dCTP, 33 mmol/L dATP, and 33 mmol/L dTTP. Thereafter, the enzyme was inactivated at 65ºC for 30 min. (*ii*) The DNA was then digested with 20 units of *Bsp*EI in a volume of 20 μL. (*iii*) The cleavage ends were filled in and labeled with ^{32}P in the presence of 13 units of Sequenase Ver. 2.0™ (Amersham, Buckinghamshire, England), 0.33 mmol/L [α-^{32}P] dGTP (3,000 Ci/mmol) and 0.33 mmol/L [α-^{32}P] dCTP (6,000 Ci/mmol) at 37ºC for 30 min in 22.5 μL of 50 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl and 10 mmol/L DTT. To inactivate the enzyme, this reaction mixture was then, incubated at 65ºC for 30 min. (*iv*) One microgram of the DNA from the step (*iii*) was fractionated on an agarose disc gel (0.8% Seakem GTG™ agarose: FMC BioProducts, Maine, USA) in a 2.4 mm diameter × 63 cm long tube, and then electrophoresed in the first-dimension (1D) buffer: 50 mmol/L Tris-acetate (pH 7.5), 0.7 mmol/L magnesium acetate, at 100 V for 1 h followed by 230 V for 23 h. (*v*) The noodle-like gel containing size fractionated genomic DNA was extruded from the tube and soaked for 30 min in the reaction buffer for *Eco*RI. Thereafter, DNA was digested in the gel with 1,500 units of *Eco*RI for 2 h. (*vi*) The gel was fused on the top edge of a 50 (W) × 50 (H) × 0.1 (thick) cm, 5% polyacrylamide vertical gel by adding melted agarose to fill up the gap between each gel. Second-dimensional electrophoresis was carried out in TBE buffer: 50 mmol/L Tris, 62 mmol/L boric acid, 1 mmol/L EDTA, at 100 V for 1 h followed by 150 V for 23 h. (*vii)* An area 35 × 41 cm of the original gel was excised and dried. Autoradiography was performed for 3–10 days on a film (XAR-5; Kodak, New York, NY, USA) at –80ºC using an intensifying screen (Quanta III; Sigma-Aldrich, St. Louis, MO, USA) or analyzed by the BAS-2000™ (Fuji Photo Film Co, Ltd., Tokyo, Japan) imaging system with 1–3 days exposure of the imaging plate (Fuji Photo Film).

Results and discussion

Mat-rush genomic DNA was digested with various restriction enzymes to decide the landmark enzyme site. It was considered that *Bsp*EI is a suitable landmark enzyme for RLGS analysis, because we should take low complexity genome information in RLGS for the first trial of non-model organisms analysis. For example, Fig. 2 shows the digestion with typical 6-base cutters, *Eco*RI (recognizing GAATTC) and *Bsp*EI (TCCGGA). The major population of *Eco*RI-digested DNA was about 9–23 kb (lane 3). On the other hand, that of *Bsp*EI-digested DNA was over 23 kb (lane 4) indicating *Bsp*EI was a rare cutter for mat-rush genomic DNA. Simultaneously, there is a risk that some of the methylated loci in the genome were not digested, because the sensitivity of methylation on *BspE*I is not known well.

The RLGS analysis of 18 cultivars with the enzyme combination of *Bsp*EI (for the 1st dimension) and *Eco*RI (for the 2nd dimension in the 2 dimensional electrophoresis) detected about 40 RLGS spots in each cultivar as shown in Fig. 3. RLGS patterns of cv. Shimomasuda and Hinomidori are shown in Fig. 3-a and 3-b, respectively. The areas shown in rectangle in Fig. 3, a and b, were magnified as c and d, respectively. The same areas in the

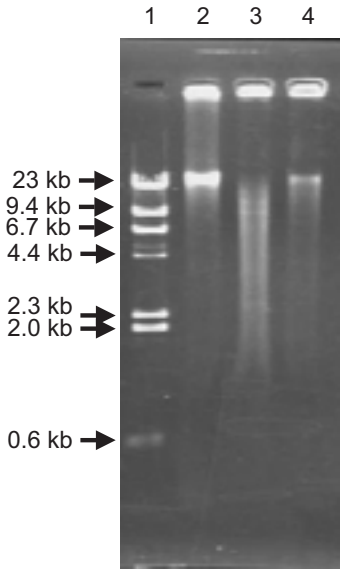

Fig. 2. Agarose gel electrophoresis pattern of mat-rush DNA, digested with *Bsp*EI or *Eco*RI

Lanes 1 to 4 correspond to the following DNA samples. Lane 1: λ DNA digested with *Hin*dIII as a size marker, 2: 0.2 μg of cv. Okayama-3 genomic DNA undigested, 3: 0.2 μg of Okayama-3 DNA digested with *Eco*RI, and 4: 0.2 μg of Okayama-3 DNA digested with *Bsp*EI.

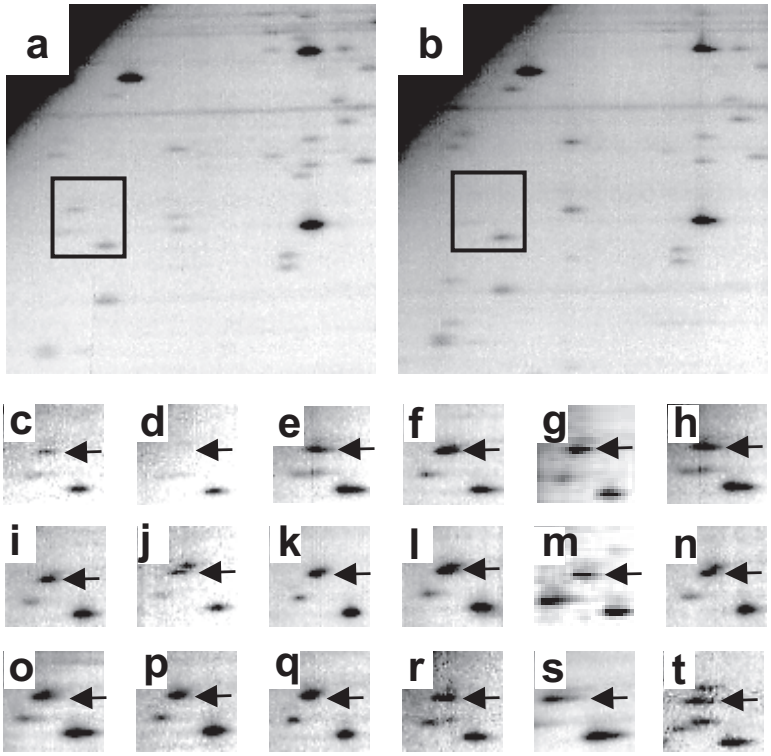

Fig. 3. The RLGS spot marker showing cv. Hinomidori specific DNA polymorphism
RLGS spot patterns with about 40 spots of cv. Shimomasuda (a) and Hinomidori (b) are shown. The areas shown in rectangle in a and b were magnified as c and d, respectively. The same areas of the other 16 cultivars are shown in e to t (e, Senchou; f, Asanagi; g, Setonami; h, Fukunami; i, Bunsei; j, Isonami; k, Kiyonami; l, Chikugomidori; m, Ohara-4; n, Okayama-3; o, Kumagawa; p, Takasu; q, Sazanami; r, Shiranui; s, Okayama-F; and t, Okayamamidori.). Arrowheads show the location of the null spot specifically in Hinomidori.

other 16 cultivars are shown in e to t (e, Senchou; f, Asanagi; g, Setonami; h, Fukunami; i, Bunsei; j, Isonami; k, Kiyonami; l, Chikugomidori; m, Ohara-4; n, Okayama-3; o, Kumagawa; p, Takasu; q, Sazanami; r, Shiranui; s, Okayama-F; and t, Okayamamidori.). Based on the comparison of 18 cultivars, we found that a spot (arrowhead) was absent specifically in Hinomidori (Fig. 3-d) but present in the other 17 cultivars.

RLGS spot polymorphism could be used as a DNA marker to identify Hinomidori among almost all cultivars in this study. All three samples of Hinomidori that were cultivated in different places in Kumamoto Prefecture were identical concerning the absence of the spot (data not shown). We concluded that this RLGS spot marker should be a very reliable cultivar-discriminating marker.

As the RLGS method could survey the polymorphism among about 40 (or more) of restriction enzyme sites at a single experiment, precise DNA-based discrimination would be possible if raw material of tatami-omote could be supplied from foreign countries. It is an effective way to develop DNA markers in a short period for vegetatively propagated crops such as mat-rush that have not been genetically analyzed. It was thought that our technology is applicable to cultivar-discrimination for various vegetatively propagated crops such as strawberry, potato, konnyaku, etc. We are now cloning the RLGS spot DNA for an STS marker. That STS marker from the RLGS spot marker will be used as well as an SSR marker that has been applied to practical cultivar-discrimination in mat-rush.

Conclusion

1. We developed the technology for cultivar-discrimination using RLGS.
2. We obtained a RLGS spot marker applying the technology to mat-rush.
3. The DNA marker identifies the most important mat-rush cultivar, Hinomidori.

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