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A Sequence-tagged Site Marker for Identifying the Japanese Mat Rush (*Juncus effusus*) Cultivar 'Hinomidori'

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Abstract

We have developed a genetic marker that can identify a registered variety of mat rush in Japan. A vegetatively propagated plant, mat rush is cultivated and used as the material for the surface layer of tatami mats in Japan. Because it has been difficult to detect DNA polymorphism among mat rush cultivars, we applied restriction landmark genome scanning (RLGS) to discriminate mat rush cultivars. RLGS is a genome analysis technique that can detect many DNA polymorphisms as spots separated by two-dimensional electrophoresis. By cloning the DNA of spots specific to the superior mat rush cultivar 'Hinomidori' detected by RLGS, we developed a sequence-tagged site (STS) marker for polymerase chain reaction (PCR) analyses. This STS marker makes it possible to distinguish 'Hinomidori' specifically from other mat rush cultivars. The strategy of developing the STS marker in this study is applicable to other vegetatively propagated plants that are characterized by difficult DNA polymorphism detection.

Discipline: Biotechnology
Additional key words: DNA polymorphism, PCR, RLGS, spot cloning, vegetatively propagated plant

Introduction

Mat rush (*Juncus effusus*) is a perennial plant distributed widely throughout warm temperate zones in Eurasia and the American continents. The plants grow naturally in Asia, including in Japan, China, Taiwan, and Korea. *J. effusus* is bred and cultivated to make tatami omote, rug mats, wicks for oil lamps, and industrial art objects. Breeding is mainly based on bud mutations in the crossing parent plants. Consequently, the genetic diversity among the domesticated groups is considered to be low.

There have been frequent cases of a superior mat rush cultivar ('Hinomidori') being illegally imported back to Japan for use as a raw material to make tatami omote, mats, and sandals. 'Hinomidori' is the first cultivar in mat rush improved by artificial crossing, and the products exhibit more attractive surfaces owing to thin traces without flower buds (Nakazawa et al. 1999). It was registered in 2001 and sold for a high price (Noguchi & Kako 2006). Given the high quality of 'Hinomidori,' cultivar identification methods have become more important. However, differentiation by visual inspection is difficult. Therefore, a technique for DNA cultivar identification is required for the protection of intellectual rights.

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DNA polymorphism marker development in plants uses simple sequence repeat (Sarri et al. 2006), amplified fragment length polymorphism (Hanada et al. 2003), and single nucleotide polymorphism methods (Cabezas et al. 2011). Microarrays (Kilian et al. 2012) are used in a part of crops. For mat rush, the sequence tagged site (STS) marker method should be used because it is easy to operate, as well as being time- and cost-effective. Furthermore, it is applicable to DNA extracted from mat rush that has been processed by drying, heating, and/or dyeing. However, few STS markers for mat rush have been reported.

Restriction landmark genome scanning (RLGS) (Ando & Hayashizaki 2006, Okuizumi et al. 2011) is a method of scanning the entire genome using restriction enzyme sites on genome DNA as a landmark. Accordingly, RLGS does not require genome information beforehand and can detect many DNA polymorphisms at once. The RLGS method is understood to be effective for the detection of polymorphism in vegetatively propagated plants that have either not been subjected to advanced genome analysis or have very low genetic diversity (Jacob 1996). We applied the RLGS method to mat rush (Okamoto et al. 2006) and detected polymorphic spots among 18 cultivars, which are well known as genetic resources in Japan.

In the present study, we aimed to develop a cultivar identification STS marker specific to 'Hinomidori' using the polymorphic spot "A2" detected by the RLGS method (hereafter, "spot A2") to distinguish it among other mat rush cultivars (Okamoto et al. 2006).

Materials and methods

1. Plant materials and DNA preparation

Eighteen Japanese mat rush (*J. effusus*) cultivars including 'Hinomidori' were analyzed in the present study as well as in our previous study (Okamoto et al. 2006). The relationships between the cultivars ('Shimomasuda,' 'Hinomidori,' 'Senchou,' 'Asanagi,' 'Setonami,' 'Fukunami,' 'Bunsei,' 'Isonami,' 'Kiyonami,' 'Chikugomidori,' 'Ohara-4,' 'Okayama-3,' 'Kumagawa,' 'Takasu,' 'Sazanami,' 'Shiranui,' 'Okayama F,' and 'Okayamamidori') are described in Okamoto et al. (2006). Needle leaves of all these cultivars were collected from the NARO Kyusyu Okinawa Agricultural Research Center. Genomic DNAs were isolated from the leaves by the cetyltrimethylammonium bromide (CTAB) extraction method with some modifications according to Kawase (1994) and Okamoto et al. (2006). Briefly, frozen leaves were ground with a mortar and pestle. A total of 100 mg of leaf powder, 10 mg of sodium lauryl sulfate, and 10 mg of polyvinylpyrrolidone were suspended in 0.4 mL of CTAB extraction buffer [1% CTAB, 0.1 M Tris-HCl (pH 8.0), 50 mM EDTA, 1.4 M NaCl, and 0.1% 2-mercaptoethanol]. Then 1 µL proteinase K solution (Merck, Darmstadt, Germany) (40 mg/mL) was added to the lysate. The lysate was incubated at 56°C for 30 min, followed by extraction with phenol and chloroform, with DNA then being precipitated with an equal volume of 2-propanol and finally resuspended in 10 µL of Tris EDTA (TE) buffer (10 mM Tris-HCl and 1 mM EDTA).

2. Restriction landmark genome scanning

We performed RLGS with combinations of the *Bsp*EI and *Eco*RI (*Bsp*EI- *Eco*RI) restriction enzymes as described in Okamoto et al. (2006) and Takamiya et al. (2008). Briefly, (1) blocking nicks and gaps in mat rush genomic DNA (0.4 µg) were treated with 10 units of DNA polymerase I (Nippon Gene, Tokyo, Japan) in 50 µL of 1 × blocking buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM dithiothreitol (DTT)), 0.33 µM dGTP [αS], 0.33 µM dCTP [αS], 33 µM ddATP, and 33 µM ddTTP). Next, the enzyme was inactivated at 65°C for 30 min. (2) The DNA was then digested with 20 units of *Bsp*EI in a volume of 20 µL. (3) The cleavage ends were filled in with 13 units of Sequenase version 2.0™ (Amersham, Buckinghamshire, England) in the presence of 0.33 µM [α-^{32}P]dGTP (3,000 Ci/mmol), and 0.33 µM [α-^{32}P]dCTP (6,000 Ci/mmol) for 30 min. at 37°C in 22.5 µL of 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM DTT, 0.16 µM dGTP [αS], 0.16 µM dCTP [αS], 33 µM ddATP [αS], and 33 µM ddTTP [αS]. To inactivate the enzymes, the reaction mixture was incubated at 65°C for 30 min. (4) One microgram of the DNA from step (3) was fractionated on a Φ2.4 mm × 63 cm agarose disc gel (0.8% Seakem GTG™ agarose; Lonza, Walkersville, Maryland, USA) and then electrophoresed in a 1 × 1D buffer (50 mM Tris-acetate, pH 7.5/0.7 mM magnesium acetate) at 230 V for 23 h. (5) The portion of gel containing the DNA was excised as a strip and soaked for 30 min. in a reaction buffer for *Eco*RI. The DNA was then digested in the gel with 1,500 units of *Eco*RI for 2 h. (6) The gel was fused with a 50 × 50 × 0.1 cm polyacrylamide gel (5% polyacrylamide to acrylamide/bisacrylamide, 29:1) by the addition of melted agarose to fill the gap. Two-dimensional electrophoresis was performed in 1 × TBE buffer at 150 V for 24 h. (7) The gel was dried, and then a 35 × 41 cm area of the original gel was excised and autoradiographed for 3-10 days on film (XAR-5; Kodak, Rochester, NY, USA) at -80°C using an intensifying screen (Quanta III; Sigma-Aldrich, St. Louis, MO, USA), or with the BAS-2000™ (Fuji Film Co., Ltd., Tokyo, Japan) imaging system with 1-3 days exposure on an imaging plate (Fuji Film).

3. RLGS spot cloning

RLGS spot cloning was conducted according to Takamiya et al. (2008) and Watanabe & Hayashizaki (1997). Briefly, a target RLGS spot was punched out of the dried gel of the mat rush cultivar 'Shimomasuda,' which is the mater-

nal parent of 'Hinomidori,' and soaked with TE (pH 8.0). DNAs were electroeluted for 20 min. at 200 V into 1 × TBE buffer (50 mM Tris base, 62 mM boric acid, and 1 mM EDTA) in an apparatus with a V-shaped well (between the anode and cathode buffers) that was filled with 100 µL of the elution dye (8 M ammonium acetate in 1 × loading buffer: 6 × loading buffer contains 0.04% bromophenol blue, 0.04% xylene cyanol, 4 mM EDTA, and 5% glycerol). Electrophoresis was performed at 200 V for 20 min. in the TBE buffer. Then, 300 µL of the eluted solution was drawn out from the bottom of the V-shaped well and treated with phenol and chloroform, and DNA fragments were precipitated by ethanol with Ethachinmate™ (Nippon Gene). After centrifugation of the DNA, 70% ethanol washing and drying were performed. Then, a 1 µL *Bsp*EI linker (0.5 pmol/µL: 5′-pCCGGAGTCGTGACTGGGAAAACCCTGGCGT-3′, 3′-TCA GCACTGACCCTTTTGGGACCGCA-biotin-5′), 1 µL *Eco*RI linker (0.5 pmol/µL: 5′-pAATTCTGTACTG CACCAGCAAATCC-3′, 3′-GACATGACGTGGTCGTT TAGG-5′), and 2 µL of Ligation High™ (Toyobo, Tokyo, Japan) were added to the precipitated DNA. The ligation reaction was performed at 16°C for 2 h as per the manufacturer's instructions. After ligation, the target DNA was purified using Dynabeads™ M-280 streptavidin (Dynal Biotech, Oslo, Norway), as per the manufacturer's instructions.

Briefly, 10 µL of washed Dynabeads™ M-280 streptavidin and 6 µL of TE were added to the ligated DNA solution. After incubation at room temperature for 30 min., the Dynabeads™ M-280 streptavidin was separated with a magnet, and beads were washed four times with 150 µL of 2 × B&W washing buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 2 M NaCl]. DNA fragments were ligated to a biotinylated *Not*I linker and trapped with Dynabeads™ M-280 streptavidin. Washed DNA was resuspended in 4 µL of TE, and 1 µL was used as the polymerase chain reaction (PCR) template. PCR was performed with 0.2 U of the polymerase kit, KOD plus™ (Toyobo), 2 µL *Bsp*EI primer (10 pmol/µL: 5′-TTCCCAGTCACGACTCCGG-3′), 2 µL *Eco*RI primer (10 pmol/µL: 5′-TTGCTGGTGCAGTA CAGAATTC-3′), 1 mM $MgSO_4$, 0.2 mM dNTPs, and KOD buffer (total volume: 20 µL). The PCR conditions were 95°C for 10 min., followed by 35 cycles of 95°C for 15 s, 60°C for 30 s, and 68°C for 30 s.

The nucleotide sequence of the PCR product was determined by the sequencer CEQ™2000XL (Beckman Coulter, Fullerton, CA, USA) using a CEQ-DTCS quick start kit™ (Beckman Coulter).

4. PCR analysis

A total of 50 ng of genomic DNA for each of the 18 cultivars was used as the PCR template. PCR was performed with 0.2 U of KOD plus polymerase (Toyobo), 1.5 µL forward primer (10 pmol/µL), 1.5 µL reverse primer (10 pmol/µL), 1 mM $MgSO_4$, 0.2 mM dNTPs, and KOD buffer (total volume: 20 µL). PCR conditions were 95°C for 5 min., followed by 30 cycles of 95°C for 15 s, 60°C for 30 s, and 68°C for 20 s.

Primers used for PCR amplifications were P-Forward (A2-*Bsp*EI): 5′-TCCGGATTTAACTATTCGTCC-3′ and P-Reverse (A2-*Eco*RI): 5′-GAATTCATTTGCAAATG CAGACCG-3′.

Results and discussion

1. RLGS polymorphism spot cloning

We specifically cloned spot A2 that was not detected in 'Hinomidori' (Fig. 1). We punched out the spot from the gel and analyzed the sequence (Fig. 2). The length of the DNA fragment was 541 bp, and it matched the RLGS spot patterns. A homology search revealed that the region showed an identity of 69% with part of the probable inactive receptor kinase (At4g23740). The spot cloning method used in the present study was simpler and more effective than conventional methods, and practically applied to

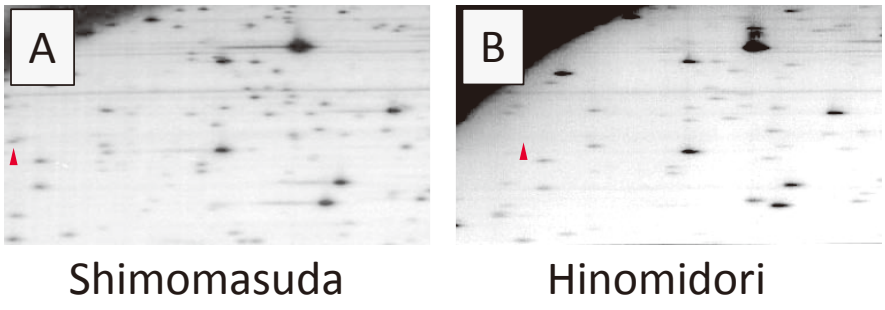

Fig. 1. Location of spot A2 in the RLGS spot patterns.
(A) RLGS profile of 'Shimomasuda' DNA. Arrowhead indicates spot A2. (B) RLGS profile of 'Hinomidori' DNA. Note that spot A2 is missing.

obtain polymorphic DNA fragments in the mat rush.

2. Primer design and polymorphism detection by PCR

A sequence-specific primer pair was designed at the 3′ and 5′ ends of spot A2 based on the analyzed sequence. Genomic DNA was extracted from the needle leaves of each of the 18 mat rush cultivars. PCR was then performed using the DNA as a template. As a result, a single band of approximately 500 bp was amplified in 'Hinomidori,' and three bands of approximately 500 bp were amplified in the other 17 mat rush cultivars (Fig. 3). From this result, it was possible to identify polymorphism in 'Hinomidori' among the 18 mat rush cultivars.

The following may explain why the other two longer DNA fragments were not amplified in 'Hinomidori': (1) deletion of a sequence in the primer binding region, (2) substitution of a sequence in the primer annealing region, and (3) insertion of a long sequence. The three bands of 'Shimomasuda' were thought to lie at the same locus or to represent a pseudogene, given that all three bands were amplified using the same primer. Further sequencing of those bands may provide more detailed insight on this point. In either case, three bands were detected in 'Shimomasuda' and one in 'Hinomidori' using this STS marker. This finding indicates that the STS method revealed specific PCR non-amplification in 'Hinomidori,' a finding that will be useful for practical cultivar identification.

Conclusions

In the present study, we performed RLGS on 18 mat rush cultivars with a combination of *Bsp*EI-*Eco*RI restriction enzymes. By cloning spot A2, which reveals polymorphism among mat rush cultivars, it was possible to develop a cultivar identification STS marker specific to 'Hinomidori' based on PCR from the RLGS analysis.

Cultivar improvement requires a considerable amount of time, money, and valuable genetic resources. Thus, the development of a cultivar identification method that protects breeders' rights is desirable. The STS polymorphic marker using the RLGS method, as described in this study, is applicable to other vegetatively propagated plants such as konjac (*Amorphophallus konjac* K. Koch) and potatoes.

5′-
TCCGGATTTAACTATTCGTCCCCTCCTTAAGCCGCCATTTTCTCCTCTCTCTCCCTCTCCCTCGAGTTCCTCATGCTTCAAGCAAAGCAA
AGCCCACTCTCTCTTCTTCATTTACCCCCTCTCTCTTCTCTGAACTCTTCTCTTTCTCCTTCTAAATAAACCCACCAACAATGGCTCTACC
ACCACCATTCTTCCTCGTCCTGCTCGTTCTGTTGCCATTCACTTCACCTGAGCCAATTTCAGACAAGTTCGCGCTTCTCGACTTCCTCTC
TCAAACGCCCCACTCGCGGGACCTGAACTGGGACGTTTCCCTCCCGGTGTGCAGCAACTGGACTGGAGTTACGTGCTCGCCGGAC
GGATCTCGCGTCACCGCCGTGCATCTGCCGGCCTTCGGGCTCACCGGCCAGATCCCGCCCAACACTCTCAGCCGCTTGTCCGCTCTT
CAGATCCTCAGCCTCCGTTCCAACGGACTCACCGGCCTGTTTCCGGCGGATTTTGGGAATCTCAGCTCGCTTACCGGTCTGCATTTGC
AAATGAATTC-3′

Fig. 2. Nucleotide sequence of spot A2. The sequence of RLGS polymorphic spot A2 in 'Shimomasuda' is available in DDBJ/EMBL/GenBank databases under accession number LC132965. Underlined portions indicate the positions of the primer pair.

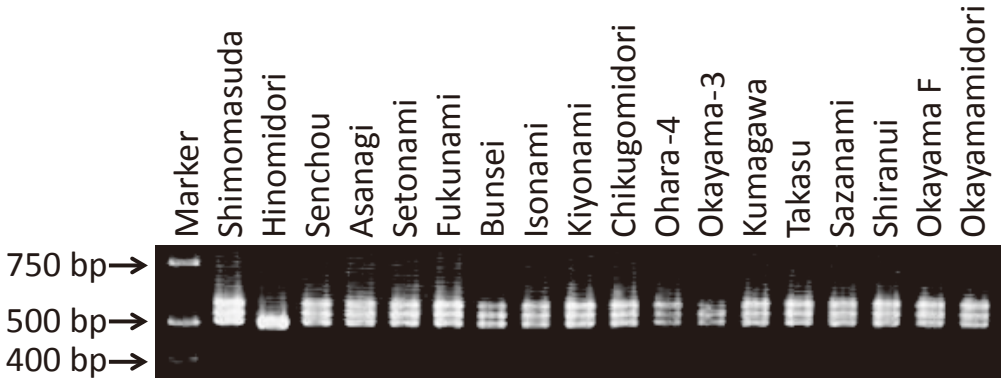

Fig. 3. PCR results for 18 mat rush cultivars. A single DNA band was detected in 'Hinomidori', whereas three DNA bands were detected in the other cultivars. As a result, null bands specific to 'Hinomidori' were detected.

The use of STS markers may greatly contribute to the efficiency of cultivar identification on the management site of the appropriate cultivar to protect the breeders' rights. It has also been useful for diversity analysis in rice, sorghum (Strelchenko et al. 2010), and dendrobium (Takamiya et al. 2011).

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