JARQ 42 (3), 151 – 155 (2008) http://www.jircas.affrc.go.jp

Application of RLGS Method for Detection of Alteration in Tissue Cultured Plants

Tomoko TAKAMIYA[1,2,4†], Yuhko OHTAKE[3†], Saeko HOSOBUCHI[1,2], Tomotsugu NOGUCHI[1,2], Makoto KAWASE[1], Yasufumi MURAKAMI[2] and Hisato OKUIZUMI[1*†]

[1] Genebank, National Institute of Agrobiological Sciences (Tsukuba, Ibaraki 305–8602, Japan)
[2] Faculty of Industrial Science & Technology, Tokyo University of Science (Noda, Chiba 278–8510, Japan)
[3] Division of Plant Sciences, National Institute of Agrobiological Sciences (Tsukuba, Ibaraki 305–8602, Japan)

Abstract

Tissue culture is used as a tool to propagate seedlings massively in industry. However, unfavorable genomic alteration that affects phenotypic characters occurs in the procedure. A technique which scans these genetic changes genome-wide can enables us to discriminate the clone that has genomic alteration at early stages. In this study, we used restriction landmark genome scanning (RLGS) to detect alterations among ramets of rice, which redifferentiated from a callus that had been cultured for 4 weeks. As a result, 6% (10 spots) of the total (161 spots examined) were different between two ramets. One spot out of ten was cloned and was located on a predicted gene. Application of a PCR-based method to the RLGS spot region detected one methylation alteration among the ramets. Accordingly, RLGS was successful in surveying methylation alteration events during tissue culture in plants and it should be possible to use this method for monitoring of alteration levels.

Discipline: Biotechnology
Additional key word: DNA methylation

Introduction

In rice, a convenient transformation method via callus has been established[5]. However, genomic alterations often occur during tissue culture. For example, changes of methylation status[7] and transposition of transposable elements[9] induce morphological changes. The longer a callus is cultured, the more alterations occur[6]. These alterations could cause unfavorable phenotypic character changes in plantlets when propagated massively as a clone. On this point, we need a reliable and convenient technology that can evaluate the stability of the genome in a cloned population.

The restriction landmark genome scanning (RLGS) method employs direct end-labeling of the genomic DNA digested with restriction enzymes and two-dimensional electrophoresis[3,4]. This method enables one to visualize thousands of loci on a single autoradiogram, and detect DNA polymorphism by comparing RLGS patterns. The RLGS method also enables a genome-wide survey for alteration of methylation status, using a methylation sensitive restriction enzyme, and has been used to detect methylation changes in tumorigenesis[10].

In this study, we analyzed ramets redifferentiated from a callus that was derived from an identical rice seed using RLGS, and detected genomic alterations between ramets.

Materials and methods

1. Tissue culture

The process of tissue culture was according to Hiei et al.[5]. Briefly, a single seed of *Oryza sativa* L. cv. Nipponbare was sown on N6 medium[2] containing 2 μg/ml 2,4-dichlorophenoxyacetic acid to induce a callus. After

† The authors contributed equally to this paper.
Present address:
[4] College of Pharmacy, Nihon University (Funabashi, Chiba 274–8555, Japan)
*Corresponding author: e-mail okuizumi@affrc.go.jp
Received 18 April 2007; accepted 25 December 2007.

4 weeks, the callus was transferred into N6 medium without 2,4-dichlorophenoxyacetic acid, and redifferentiated. We selected 4 ramets (ramet 1, 2, 3, and 4) and cultivated the ramets for 4 weeks (at 15 cm high with 3–4 leaves). The four ramets had no difference in morphology and growth.

2. Preparation of genomic DNA and RLGS

Leaf blades and sheaths of ramets 1, 2, 3, and 4 were collected and ground by mortar and pestle with liquid nitrogen. Total genomic DNA was isolated by the cetyl trimethyl ammonium bromide (CTAB) extraction method with modification according to Kawase[8] and Okamoto et al.[11]. We carried out RLGS with restriction enzyme combination, *Not*I–*Hpa*II–*Bam*HI (NEB, Beverly, MA, USA), described in Okamoto et al.[11] and Takamiya et al.[12].

3. RLGS spot cloning

We employed the improved spot cloning method[12] based on conventional methods[3,4]. Briefly, the genomic DNA (0.1 µg) was digested by *Not*I and *Hpa*II at 37ºC for 1 h. Then, the sample was mixed with DNA fragments labeled at the *Not*I sites and digested with *Hpa*II. Thereafter, 2-D electrophoresis was carried out as described by Okamoto et al.[11] and Takamiya et al.[12]. The target RLGS spots were punched out from the dried gel and soaked in Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The DNA fragments in the gel were electroeluted and purified. The purified DNA fragment was ligated with 1 µL *Not*I linker (0.5 pmoles/µL: 5'- GGCCGCATGAATGGCGCGCCAAAGA-3', 3'-CG TACTTACCGCGCGGTTTCT-Biotin-5'), 1 µL *Bam*HI linker (0.5 pmoles/µL: 5'-GATCCTGTACTGCACCAG CAAATCC-3', 3'-GACATGACGTGGTCGTTTAGG-5') and 2 µL of ligation high solution in Ligation High™ (Toyobo, Tokyo, Japan). Ligation reaction was carried out at 16ºC for 2 h according to the manufacture's protocol. After ligation, target DNA was purified using Dynabeads™ M-280 Streptavidin (Dynal Biotech ASA, Oslo, Norway) according to the manufacturer's instruction. The Dynabeads were resuspended in 4 µL TE, and 1 µL was used as a PCR template. PCR was performed with 0.4 U of polymerase kit, KOD plus™ (Toyobo), 2 µL *Not*I primer (10 pmoles/µL: 5'-GCGCGCCATTCATGC GGCCG-3'), 2 µL *Bam*HI primer (10 pmoles/µL: 5'-TT GCTGGTGCAGTACAGGATCC-3'), 1 mM $MgSO_4$, 0.2 mM dNTPs and KOD buffer (total volume: 20 µL). PCR conditions were: 94ºC for 5 min followed by 30 cycles of 94ºC for 15 sec, 60ºC for 30 sec, and 68ºC for 1 min. Nucleotide sequences of the PCR products were directly determined by CEQ™2000XL (Beckman Coulter, Fullerton, CA, USA) using CEQ-DTCS quick start kit™ (Beckman Coulter).

4. PCR method for methylation analysis

Fifty nanograms of genomic DNA were digested by 50 U *Msp*I or *Hpa*II, and used as a PCR template. Undigested genomic DNA was also used as a positive control to indicate existing target sites. PCR was carried out with 0.4 U of KOD plus™ polymerase (Toyobo), 1.5 µL forward primer (10 pmoles/µL), 1.5 µL reverse primer (10 pmoles/µL), 1 mM $MgSO_4$, 0.2 mM dNTPs and KOD buffer (total volume: 20 µL). PCR conditions were: 94ºC for 10 min followed by 30 cycles of 94ºC for 15 sec, 60ºC for 30 sec and 68ºC for 1 min.

Primers used for PCR amplifications were; C4F(M1): CGAGTCGAGCAGTTGGGAG and C4R(M1): GGGA CATCTATGCCATGTGGG.

Results and discussion

RLGS profiles of ramets 1 and 2 that were derived from an identical single seed were analyzed with the enzyme combination of *Not*I-*Hpa*II-*Bam*HI. As a result, 157 and 155 spots were detected on areas of the RLGS profiles of ramet 1 (Fig. 1-a) and ramet 2 (Fig. 1-b), respectively. In total, 161 unique spots were observed in these patterns. One hundred and fifty-one spots out of 161 were common in the two ramets. On the other hand, we observed 10 different spots (6% of 161 spots) between these cloned plants' profiles. Six spots were detected specifically in ramet 1, and 4 spots were specific in ramet 2. In Fig. 1, ramet-specific spots are indicated by closed arrowheads, conversely, disappearance of spots are indicated by open arrowheads.

We cloned one ramet 2 specific spot (C4) from the pattern (Fig. 1-b), and obtained the sequence shown in Fig. 2. As a result of a blast search of NCBI (http://www.ncbi.nlm.nih.gov/) and RAP-DB (http://rapdb.lab.nig.ac.jp/), the nucleotide sequence of spot C4 was mapped on the 3' region of a predicted gene (Os01g0894400 shown in Fig. 3).

We thought that the differences of spot patterns might be caused by alteration of DNA methylation status, because *Not*I and *Hpa*II are methylation sensitive enzymes, and the polymorphic spot regions between ramets might be altered often during tissue culture. At this point, we examined methylation status of restriction enzyme sites using a PCR-based method with the genomic DNA of ramets 3 and 4 that were derived from the identical seed as the ramets 1 and 2. When the alteration occurs in the early stage of culture, the probability is that the same genome change event is supposed to be shared in some ramets. It is also possible that the same methylation

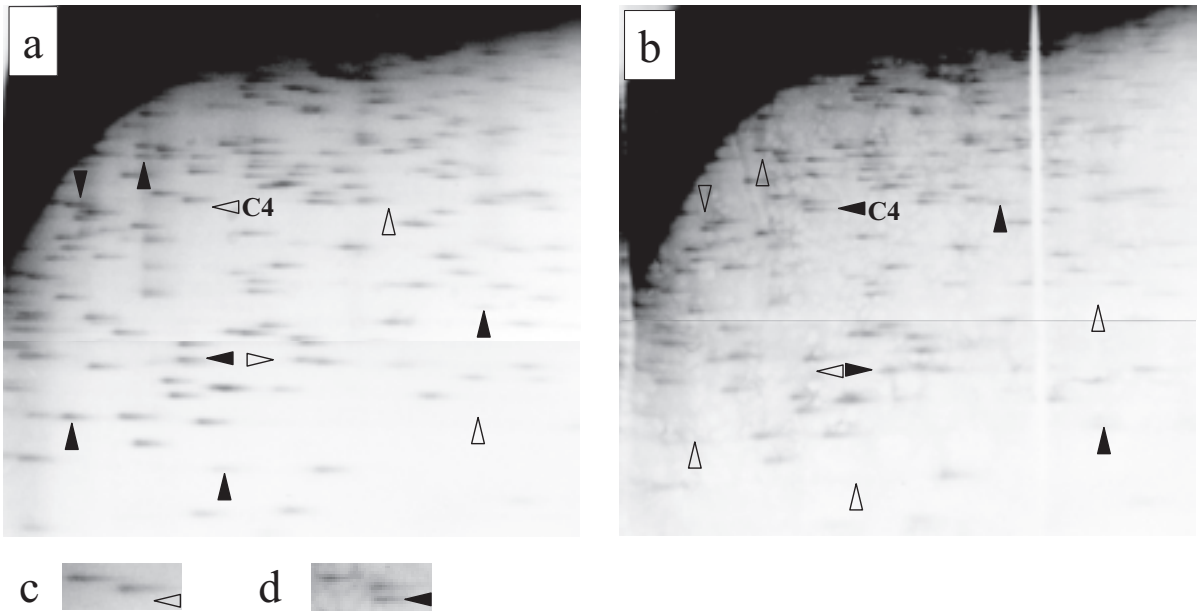

Fig. 1. RLGS spot patterns

a: RLGS pattern of ramet 1, with the restriction enzyme combination, *Not*I-*Hpa*II-*Bam*HI. There are 157 spots in the profile.

b: RLGS pattern using *Not*I-*Hpa*II-*Bam*HI for ramet 2. The pattern has 155 spots. In comparing a and b, ten spots are different. Six spots are specific in Fig. 1-a, and four are detected specifically in Fig. 1-b. Spot C4 indicated by closed arrowhead is specific in Fig. 1-b and null in Fig. 1-a (open arrowheads). Figures 1-c and 1-d are magnified images of the spot C4 in ramets 1 (Fig. 1-a) and 2 (Fig. 1-b), respectively.

GGATCCTTTTCCAGTTATATCGATGAAAAACTTTTTTCGTTATGTGTTAAACAAGTTATA
GCATTGACATTAGAAAAAGAAAGTTGAGAGGAATGCTCCTAATATACTGGCATAGCTGCC
ATTACGCCACATGAAAAACACGATCAATCACGAATTCACAACACTCACCATGTGATACGT
GTGATGGCAATCCCCAGCGGTTCAACATCCACGGCTGAGGATTCGCGGGTAGCAACCTCG
GTTTGAAGCCAAAAGGCAACCACTACCGTCCAGCTCTACTAGGACGGGCCCACCAAGCTG
GGTCCGACGTGTCATCGACGAGTGGGCCCCACACAGCAGGACAACGAGGACGAGTGGGCC
CCAGCATTCACCCCCACGAGGCCCACCGACAGCGACCCAGGCGGCCGC

Fig. 2. Sequence of spot C4

The spot C4 was detected in the pattern of ramet 2, and located on chromosome 1. Underlines show *Not*I (GCGGCCGC) and *Bam*HI (GGATCC) sites.

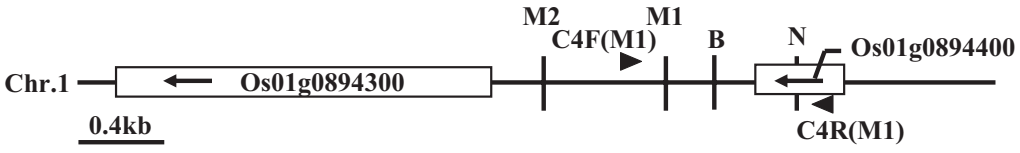

Fig. 3. Location of spot C4

The nucleotide sequence obtained from the spot C4 is located from Os01g0894400 (predicted gene) to its 3' region. The gene is shown by a box. Arrows are the direction of the ORFs. Arrowheads show the primers used for methylation analysis. N, B and M1–M2 indicate *Not*I, *Bam*HI and *Msp*I/*Hpa*II sites of the RLGS spot, respectively.

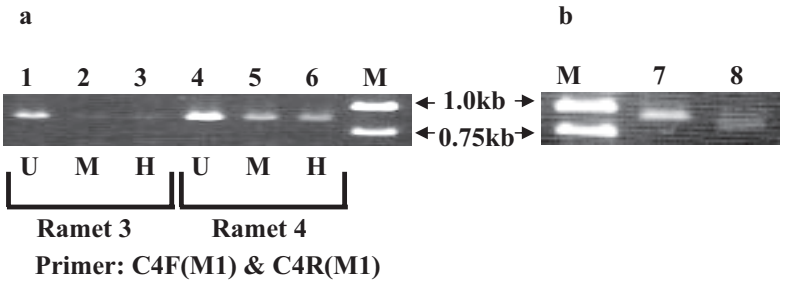

Fig. 4. Methylation status of restriction enzyme sites of spots C4

a: Analyzing methylation status of M1 site of spot C4 with primer C4F(M1) and C4R(M1).
Lane M is a size marker. Lanes 1 to 3 are PCR products that were amplified from genomic DNA of ramet 3. U, M and H indicate the treatment of PCR templates; U: undigested genomic DNA (positive control); M: *Msp*I-digested; H: *Hpa*II-digested. There is no band on lane 2 (M) and lane 3 (H). The M1 site of ramet 3 is not methylated. Lanes 4 to 6 are PCR products amplified from genomic DNA of ramet 4. On lanes 5 (M) and 6 (H), faint bands were detected.

b: Reconfirming M1 site of ramet 4.
PCR products of lanes 5 and 6 were purified and mixed. It was divided into two samples; one was loaded on lane 7 directly. Another one was treated with *Msp*I (lane 8).

alteration can occur independently among ramets, if the region is unstable in its methylation status. Then, an analysis of the C4-spot region using ramets 3 and 4 was carried out. First of all, the genomic DNA of ramets 3 and 4 were treated with *Msp*I or *Hpa*II, and used as a PCR template. *Hpa*II has different methylation sensitivity, it doesn't digest in the case that internal cytosine is methylated ($C^{5m}CGG$), whereas *Msp*I digests $C^{5m}CGG$. The site of *Msp*I/*Hpa*II next to *Not*I (N) was named "M1" (Fig. 3), and its methylation status was analyzed using primers C4F(M1) and C4R(M1). As for ramet 3, no band was detected from genomic DNA digested with *Msp*I or *Hpa*II (Fig. 4-a, lanes 2 and 3, respectively). The results show that the M1 site of ramet 3 was not methylated. On the other hand, regarding ramet 4, faint bands were detected in the case of *Msp*I or *Hpa*II digestion (Fig. 4-a, lanes 5 and 6, respectively). These results indicated that the M1 site was methylated $^{5m}C^{5m}CGG$, $^{5m}CCGG$ or the DNA sequence was altered at the site in ramet 4. First, we confirmed that the M1 site of spot C4 was on ramet 4's genome. The band on lane 7 is the PCR product (880 bp) which was purified from the mixture of lanes 5 and 6. The mixed PCR product was digested with *Msp*I, and loaded on lane 8. The PCR product was expected to be divided into 771 bp and 109 bp fragments, and the 771 bp fragment was detected in lane 8. These results showed the nucleotide sequence at the M1 site of ramet 4 was not altered, but the site was methylated. As to *Not*I site (N) of spot C4, no methylation was detected in either ramets 3 or 4 by the same method (data not shown).

From the results mentioned above, we detected one methylation alteration among the ramets, which redifferentiated from an identical seed. As shown by Cheng et al., *Tos*17 was demethylated in tissue culture and remethylated during redifferentiation[1]. Similarly, the regions which we analyzed in the present study might be affected frequently during callus induction or redifferentiation.

The systematic scanning method as shown in this research has advantages as follows. (1) The method can detect a little alteration between samples. Although we analyzed the ramets which were derived from an identical single seed and had the same genetic background, we detected some alterations on RLGS spots. (2) It has a possibility to detect genomic alteration that could affect phenotypic characters because RLGS surveys methylation of *Not*I and *Msp*I/*Hpa*II in the vicinity of a gene[12]. (3) RLGS is applicable to almost all plants. Consequently, it is conceivable that this method enables the detection of regions which are easily altered in tissue culture.

Conclusion

Our experiments clearly demonstrate that the RLGS method can be successfully applied to detect alterations in DNA methylation during tissue culture in plants. A callus derived from an identical single seed was cultured for 4 weeks, and redifferentiated. The 2 ramets were analyzed by RLGS analysis (*Not*I- *Hpa*II- *Bam*HI), and these patterns were compared. As a result, the 10 (6%) out of 161 RLGS spots indicated certain genome alterations on DNA sequence or methylation status among the ramets. At least one spot (C4) suggested the alterations of methylation status in *Msp*I/*Hpa*II sites.

The RLGS method is useful for genome wide surveillance of epigenetic alterations in tissue culture. The markers of an alteration's hot spot (the region whose

methylation status is susceptible to artificial and/or environmental stress), which will be established using the RLGS method in a further study, are possible and suitable for monitoring the epigenetic stability of the genome in tissue culture and micro-propagation.

References

1. Cheng, C., Daigen, M. & Hirochika, H. (2006) Epigenetic regulation of the rice retrotransposon *Tos*17. *Mol*. *Genet*. *Genomics*, **276**, 378–390.
2. Chu, C. C. et al. (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Sci*. *Sin*., **18**, 659–668.
3. Hatada, I. et al. (1991) A genomic scanning method for higher organisms using restriction sites as landmarks. *Proc. Natl. Acad. Sci. USA*, **88**, 9523–9527.
4. Hayashizaki, Y. et al. (1993) Restriction landmark genomic scanning method and its various applications. *Electrophoresis*, **14**, 251–258.
5. Hiei, Y. et al. (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *Plant J*., **6**, 271–282.
6. Hirochika, H. et al. (1996) Retrotransposons of rice involved in mutations induced by tissue culture. *Proc. Natl. Acad. Sci. USA*, **93**, 7783–7788.
7. Kaeppler, S. M. & Phillips, R. L. (1993) Tissue culture-induced DNA methylation variation in maize. *Proc. Natl. Acad. Sci. USA*, **90**, 8773–8776.
8. Kawase, M. (1994) Application of the restriction landmark genomic scanning (RLGS) method to rice cultivars as a new fingerprinting technique. *Theor*. *Appl*. *Genet*., **89**, 861–864.
9. Liu, Z. L. et al. (2004) Activation of a rice endogenous retrotransposon Tos17 in tissue culture is accompanied by cytosine demethylation and causes heritable alteration in methylation pattern of flanking genomic regions. *Theor. Appl. Genet*., **109**, 200–209.
10. Ohsumi, T. et al. (1995) Loss of heterozygosity in chromosomes 1, 5, 7 and 13 in mouse hepatoma detected by systematic genome-wide scanning using RLGS genetic map. *Biochem. Biophys. Res. Commun*., **212,** 632–639.
11. Okamoto, H. et al. (2006) Development of a new cultivar-discrimination method based on DNA polymorphism in a vegetatively propagated crop. *JARQ*, **40**, 65–69.
12. Takamiya, T. et al. (2006) Restriction landmark genome scanning method using isoschizomers (*Msp*I/*Hpa*II) for DNA methylation analysis. *Electrophoresis*, **27**, 2846–2856.