Research Article

The Application of Restriction Landmark Genome Scanning Method for Surveillance of Non-Mendelian Inheritance in F_1 Hybrids

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We analyzed inheritance of DNA methylation in reciprocal F_1 hybrids (subsp. *japonica* cv. Nipponbare × subsp. *indica* cv. Kasalath) of rice (*Oryza sativa* L.) using restriction landmark genome scanning (RLGS), and detected differing RLGS spots between the parents and reciprocal F_1 hybrids. *MspI/Hpa*II restriction sites in the DNA from these different spots were suspected to be heterozygously methylated in the Nipponbare parent. These spots segregated in F_1 plants, but did not segregate in selfed progeny of Nipponbare, showing non-Mendelian inheritance of the methylation status. As a result of RT-PCR and sequencing, a specific allele of the gene nearest to the methylated sites was expressed in reciprocal F_1 plants, showing evidence of biased allelic expression. These results show the applicability of RLGS for scanning of non-Mendelian inheritance of DNA methylation and biased allelic expression.

1. Introduction

DNA methylation is very common in mammals and plants and plays an important role in the regulation of gene expression. For example, allele-specific DNA methylation regulates monoallelic expression, such as genomic imprinting [1–3], X-chromosome inactivation [4, 5], autosomal random monoallelic expression [6, 7], and allelic exclusion [8]. The methylation status in these phenomena is altered or inherited in a specific manner during development, growth, and reproduction. In mammals, DNA methylation patterns throughout the genome change dramatically during tumourigenesis [9], gametogenesis [10], or early development [11]. For example, imprinted genes are regulated by methylation of a differentially methylated region, and the allele-specific methylation pattern in the differentially methylated region

is established in the germ cell line after erasing imprinting memory by demethylation [11]. In contrast, in plants, the methylation status of some genes is stably inherited through meiosis [12, 13]. Recent studies [14–16] have shown that methylation patterns can be altered in plant hybrids by introgression, and in allopolyploids. However, generational changes in methylation status and its inheritance in plants have remained unclear.

Restriction landmark genome scanning (RLGS) employs two-dimensional electrophoresis (2DE) of genomic DNA, which allows visualization of thousands of loci [17–20]. This method is appropriate for genome-wide methylation surveys [21–24]. We analyzed the inheritance of DNA methylation in the first filial generation (F1) hybrid between Oryza sativa L. subsp. japonica cv. Nipponbare and subsp. indica cv. Kasalath by RLGS, and detected altered inheritance and

demethylation of specific RLGS spots in F1 plants [25]. In this study, we analyzed the appearance or disappearance of two altered spots in reciprocal F1 hybrids and selfed progeny, and detected an unexpected allelic expression bias.

2. Materials and Methods

2.1. Plant Materials and DNA Preparation. Seeds of *Oryza sativa* L. subsp. *japonica* cv. Nipponbare and subsp. *indica* cv. Kasalath were sown and grown in the field. Reciprocal hybrids were produced by crossing the same individual of each cultivar as the female parent on one culm and as the male parent on another culm. Crossing Nipponbare as the seed parent with Kasalath as the pollen parent gave F_1 hybrids designated NKF_1. The converse cross gave KNF_1 hybrids. We grew plants of Nipponbare, Kasalath, NKF_1 (nine individuals from the same parents), and KNF_1 (nine individuals from the same parents), and the selfed progeny of the parents for 2 months, and then isolated the genomic DNA of each from the leaf blade and sheath by a standard CTAB extraction method [26].

2.2. RLGS and Identification of Target Spots. The methylation status of the parental Nipponbare and Kasalath, 9 NKF_1 plants (NK1 to NK9), and 9 KNF_1 plants (KN1 to KN9) was analyzed by an RLGS method with combinations of *Not*I–*Msp*I–*Bam*HI (hereafter [*Msp*I] pattern) or *Not*I-*Hpa*II-*Bam*HI ([*Hpa*II] pattern) restriction enzymes [22, 25]. Briefly, $0.4\,\mu g$ of genomic DNA was treated with 2 U DNA polymerase I (Nippon Gene, Tokyo, Japan) in $10\,\mu L$ of blocking buffer (10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), $0.4\,\mu M$ dGTP, $0.2\,\mu M$ dCTP, $0.4\,\mu M$ ddATP, and $0.4\,\mu M$ ddTTP) at $37°C$ for 20 minutes. Next, to inactivate DNA polymerase I, the sample was incubated at $65°C$ for 30 minutes. Thereafter, the genomic DNA was digested with 20 U *Not*I (NEB, Beverly, MA, USA) in a volume of $20\,\mu L$, then the digested DNA was end-labeled by filling reaction with Sequenase ver. 2.0 (USB, Cleveland, OH, USA) in the presence of $0.33\,\mu M$ [α-^{32}P] dGTP (3,000 Ci/mmol), $0.33\,\mu M$ [α-^{32}P] dCTP (6,000 Ci/mmol), and 1.3 mM DTT at $37°C$ for 30 minutes. Thereafter, this reaction mixture was incubated at $65°C$ for 30 minutes to inactivate the enzyme. The sample was divided into two tubes. One was digested with 25 U *Msp*I (Toyobo, Tokyo, Japan), and the other was treated with 25 U *Hpa*II (Toyobo) at $37°C$ for 1 hour. Each sample was fractionated on an agarose disc gel (0.8% SeaKem GTG agarose, FMC Bioproducts, Rockland, ME, USA) in a 2.4 mm diameter $\times$ 63 cm long tube, and then electrophoresed in the 1st-dimensional (1-D) buffer (0.1 M Tris-acetate, pH 8.0, 40 mM sodium acetate, 3 mM EDTA, pH 8.0, 36 mM NaCl) at 100 V for 1 hour followed by 230 V for 23 hours. After 1-D electrophoresis, the gel was extruded from the tube and soaked for 30 minutes in the reaction buffer for *Bam*HI, and then the DNA in the gel was digested with 1500 U *Bam*HI for 2 hours. The gel was fused onto the top edge of a 50 cm (W) $\times$ 50 cm (H) $\times$ 0.1 cm (T) 5% vertical polyacrylamide gel

using melted agarose (0.8%) to connect the gels. The 2nd-dimensional (2-D) electrophoresis parameters were Tris-borate-EDTA (TBE) buffer (50 mM Tris, 62 mM boric acid, 1 mM EDTA), at 100 V for 1 hour followed by 150 V for 23 hours. An area of 35 cm $\times$ 41 cm of the original gel was excised and dried. Autoradiography was performed 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 for 1–3 days on an imaging plate (Fuji Photo Film, Tokyo, Japan). Finally, the imaging plate was analyzed by a BAS-2000 scanner (Fuji Photo Film). *Msp*I and *Hpa*II are restriction enzymes that recognize the same sequence, but have different methylation sensitivity. When the second C of the sequence CCGG is methylated (C^mCGG), *Msp*I, but not *Hpa*II, cleaves the site. Conversely, neither *Msp*I nor *Hpa*II digests $^mC^mCGG$ or ^mCCGG. Differences between [*Msp*I] and [*Hpa*II] patterns indicate a methylated CpG (C^mCGG) at an *Msp*I/*Hpa*II site.

Target spots were identified using *in silico* RLGS computer software [22, 25], which simulates RLGS analysis of genome sequence data. The software calculates the length and mobility of each DNA fragment from the *Not*I to *Msp*I end or to the next *Not*I end in a 1D gel, and the DNA fragment length from the *Not*I to *Bam*HI end in a 2D gel to produce a 2D pattern (*in silico* RLGS pattern). We compared autoradiographic RLGS patterns with corresponding *in silico* RLGS patterns and identified each RLGS spot. The spots unidentified by *in silico* RLGS analysis were cloned and sequenced as previously described [22, 25] with specific cloning linkers: *Not*I linker (5′-GGCCGCATGAATGGCGCGCCAAAGA-3′, 3′-CGTACTTACCGCGCGGTTTCT-biotin-5′) and *Bam*HI linker (5′-GATCCTGTACTGCACCAGCAAATCC-3′, 3′-GACATGACGTGGTCGTTTAGG-5′).

2.3. Confirmation of Restriction Enzyme Sites by Digestion and PCR-Based DNA Methylation Analysis of Target Spots. To compare methylation status among Nipponbare, Kasalath, and their F_1s, we confirmed the presence of restriction enzyme sites in the parents. We designed flanking primers for the *Not*I and *Msp*I/*Hpa*II sites of each RLGS spot. Using 1 ng Nipponbare or Kasalath genomic DNA as a template, PCR was carried out with 0.4 U KOD plus polymerase (Toyobo,), $1.5\,\mu L$ flanking primers (10 pmol/μL), 1 mM $MgSO_4$, 0.2 mM dNTPs, and KOD buffer (total volume $20\,\mu L$). PCR conditions were $94°C$ for 5 minutes followed by 30 cycles of $94°C$ for 15 s, $60°C$ for 30 s, and $68°C$ for 1 minutes. An aliquot of each PCR product was treated with *Not*I or *Msp*I. Then untreated and treated products were electrophoresed in an agarose gel (0.8%–3.0%), and the band sizes were compared to confirm that the sites were present and did not differ by any DNA size polymorphism. Next, we confirmed the methylation status of the *Not*I and *Msp*I/*Hpa*II sites of the RLGS spot. Genomic DNA (1 ng) of Nipponbare, Kasalath, or the reciprocal F_1s was digested with 30 U *Not*I, *Msp*I, or *Hpa*II, and used as a PCR template. Undigested genomic DNA was used as a positive control. PCR was performed as described above.
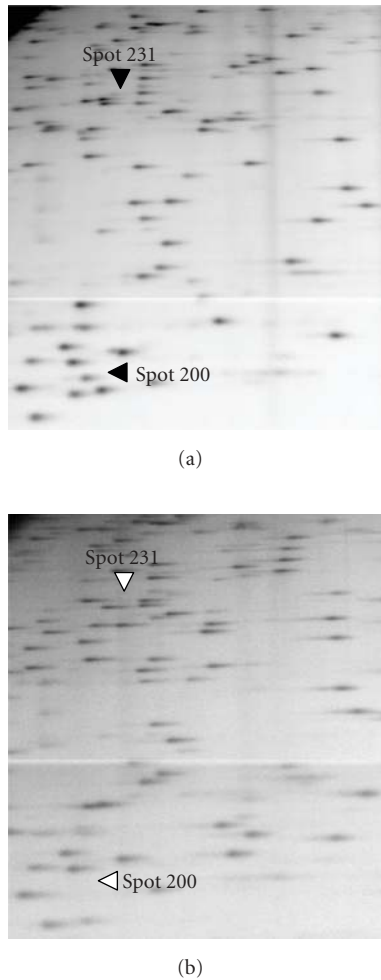
(a)

(b)

FIGURE 1: RLGS [*Msp*I] (*Not*I-*Msp*I-*Bam*HI) patterns of rice genomic DNA. Comparison of Nipponbare and Kasalath patterns revealed Nipponbare and Kasalath specific spots. (a) Nipponbare pattern. Spots 200 and 231 were detected at diminished spot intensities and are indicated by closed arrowheads. (b) Kasalath pattern. Neither spots 200 nor 231 were not detected.

2.4. Total RNA Isolation and Expression Analysis by RT-PCR.

Using an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan), total RNA was isolated from the leaf blade and sheath of the same parents, NK5, NK7, KN5, and KN10. The 4 reciprocal F_1 hybrids were chosen because two target RLGS spots (200 and 231) were detected in NK5 and KN5, but not in NK7 and KN10. First-strand cDNA was synthesized from 50 ng DNA-free samples with a ReverTra-Plus RT-PCR Kit (Toyobo, Osaka, Japan). The cDNA was used for RT-PCR analysis of each target gene. For spot 200, we used forward primer 5′-CACATCCTGATCACCGTCCA-3′ and reverse primer 5′-GTCCCAACCCGTGATCAAGTT-3′. For spot 231, we used forward primer 5′-ACTCAGGCTCAGATCGCCAT-3′ and reverse primer 5′-CCCGAGCTCCGTTTAGCATA-3′. Actin 1 was used as an internal standard (forward primer: 5′-TATGGTCAAGGCTGGGTTCG-3′, reverse primer: 5′-AACACAATACCTTGGGTACG-3′). PCR for each gene followed an initial denaturation for 2 minutes at 94°C, then
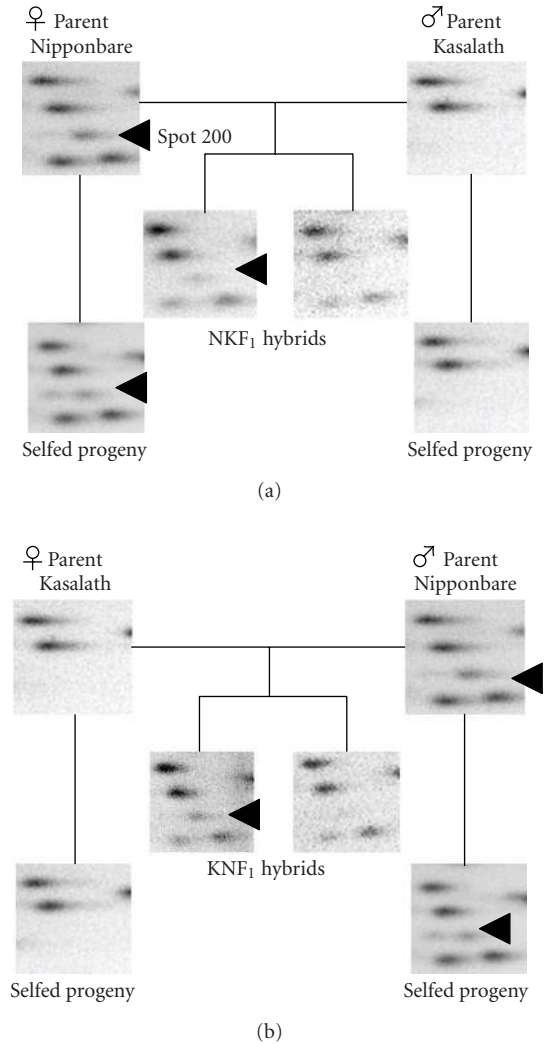

(a)

(b)

FIGURE 2: RLGS [*Msp*I] (*Not*I-*Msp*I-*Bam*HI) combination patterns of the parents, their selfed progeny, and their reciprocal F_1 hybrids. Spot 200 (arrowhead) was detected in the [*Msp*I] patterns (**Figure 1**) and [*Hpa*II] (*Not*I-*Hpa*II-*Bam*HI) patterns (data not shown) of Nipponbare and its selfed progeny. The presence or absence of the spot segregated in both F_1 populations (NKF_1 and KNF_1). The spot intensity of this spot was half that of the others.

37 cycles of 10 s at 98°C, 30 s at 60°C, and 20 s at 68°C. The PCR products were analyzed by electrophoresis followed by ethidium bromide staining.

3. Results and Discussion

Analysis of the RLGS patterns of the parents and the reciprocal hybrids showed variations in some spots between samples, reflecting changes in DNA methylation. One such altered spot was spot 200, which was detected in both the [*Msp*I] and [*Hpa*II] patterns of Nipponbare at a diminished spot intensity (half the intensity of the surrounding spots), but was absent in Kasalath (Figures 1 and 2). Cloning and sequencing of this DNA fragment placed it in the 5′ region of a non-protein coding transcript (Os11g0417300) (**Figure 3**).
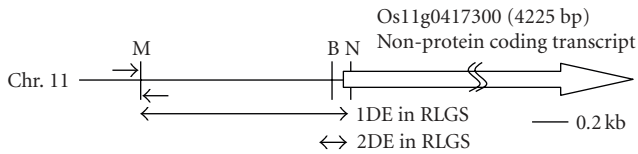
FIGURE 3: Location of spot 200. Schematic of the region of chromosome 11 containing the restriction enzyme sites located in the region 5′ to the transcription start site of the non-protein coding transcript (Os11g0417300). The DNA fragments were digested at the *Not*I (N) and *Msp*I/*Hpa*II (M) sites and fractionated by one dimensional electrophoresis. Next, the DNA fragments that were digested at the *Bam*HI (B) sites were fractionated by two dimensional electrophoresis, which allowed detection of the B-N fragment as an RLGS spot. Spot 200 corresponds to the fragment between the N and B sites. The N and M sites were identified in the parental Nipponbare and Kasalath, and in both reciprocal hybrids. The B site was only absent in Kasalath, resulting in the absence of spot 200 in the RLGS pattern.

Comparison of the relative spot positions between autoradiographic RLGS patterns of the parental Nipponbare and *in silico* RLGS pattern derived from Nipponbare genome sequence data revealed that the DNA fragments digested at the *Not*I (N) and *Msp*I (M) sites were fractionated by 1-D electrophoresis, and the DNA fragments digested at the N and *Bam*HI (B) sites were fractionated by 2-D electrophoresis as spot 200 (Figure 3). By restriction enzyme digestion and sequencing, we confirmed the existence of N, M, and B in the parental Nipponbare (data not shown). In the parental Kasalath, there were N and M sites, but no B site (data not shown). The results of RLGS analysis of the NKF$_1$ and KNF$_1$ hybrids showed that the presence or absence of spot 200 segregated 1:1 in both populations (Figure 2 and Table 1). The diminished spot intensity in the parental Nipponbare and its segregation in F$_1$ hybrids imply that the *Msp*I/*Hpa*II site of spot 200 is methylated heterozygously in Nipponbare. Accordingly, it was assumed that spot 200 was detected in the F$_1$ individuals that had a non-methylated M site, and not detected in the F$_1$ individuals that had a methylated M site. Additionally, spot 200 was detected in all selfed progeny (nine individuals) of Nipponbare at half intensity (Figure 2 and Table 1). In RLGS analysis, halved intensity of a spot indicates a heterozygote, which was confirmed theoretically and practically in earlier studies [27, 28]. From this observation, it was assumed that the M site was methylated heterozygously in the selfed progeny as well as the parental Nipponbare because of non-Mendelian inheritance of methylation.

We suspected that the methylation status correlated with expression of the nearest gene. Therefore, we analyzed the expression of the non-protein coding transcript (Os11g0417300) that is the nearest gene to the *Msp*I/*Hpa*II site of spot 200 (Figure 3). The cDNA (GenBank accession No. **AK109537**) of the non-protein coding transcript, which was previously isolated, is expressed in flower, leaf, and panicle (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene&cmd=search&term=AK109537). We analyzed expression of the gene by RT-PCR. Total RNA was isolated from the
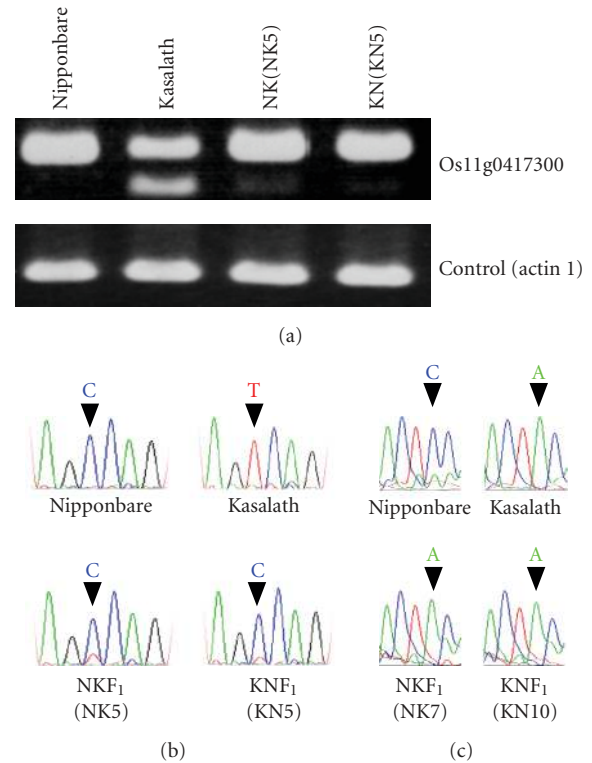

FIGURE 4: Expression analysis of the nearest gene to the methylated site. (a) RT-PCR showed that a non-protein coding transcript (Os11g0417300) was expressed in leaf blade and sheath of Nipponbare, Kasalath, NKF$_1$, and KNF$_1$ plants. (b) Sequence analysis of the RT-PCR products of the expressed Os11g0417300 allele. The single nucleotide polymorphism between Nipponbare (C) and Kasalath (T) is indicated in the RT-PCR products by arrowheads. Specific expression of the Nipponbare allele was confirmed by detection of base C in both NK5 and KN5 plants. (c) Sequence analysis of RT-PCR products of the expressed Os01g0327900 allele. The single nucleotide polymorphism in RT-PCR products between Nipponbare (C) and Kasalath (A) is indicated by arrowheads. Specific expression of the Kasalath allele was confirmed by detection of base A in both NK7 and KN10 plants.

leaf blade and sheath of the parental Nipponbare, parental Kasalath, two NKF$_1$ individuals (NK5 and NK7), and two KNF$_1$ individuals (KN5 and KN10). Spot 200 was detected in the patterns of NK5 and KN5, but not in the patterns of NK7 and KN10. The cDNAs were PCR-amplified and separated by agarose gel electrophoresis (Figure 4(a)). The non-protein coding transcript was expressed in the leaf blade and sheath of the parents, NKF$_1$s, and KNF$_1$s (data for NK7 and KN10 are not shown but gave the same result). Next, we sequenced the RT-PCR products to reveal the parental origin of the expressed sequence in the F$_1$ hybrids. The presence of a single nucleotide polymorphism (C/T) between Nipponbare and Kasalath allowed this distinction to be made. Sequence analysis of the RT-PCR products from NK5 and KN5, which had spot 200 in their RLGS patterns, showed allelic expression bias for the Nipponbare allele (Figure 4(b)); analysis of NK7 and KN10, which did not have spot 200, also showed bias (data not shown but gave the same

TABLE 1: Summary of RLGS pattern of spot 200.

Generation		RLGS pattern of spot 200	
		MspI patterns (intensity)	HpaII patterns (intensity)
Parent	Nipponbare	Present (1/2)	Present (1/2)
Parent	Kasalath	Absent	Absent
Selfed progeny (9 individuals)	Nipponbare	Present (1/2)	Present (1/2)
Selfed progeny (4 individuals)	Kasalath	Absent	Absent
NKF$_1$ (9 individuals)	Nipponbare × Kasalath	Segregated5 present: 4 absent (1/2: 0)	Segregated4 present: 5 absent (1/2: 0)
KNF$_1$ (9 individuals)	Kasalath × Nipponbare	Segregated7 present: 2 absent (1/2: 0)	Segregated4 present: 5 absent (1/2: 0)

result). The bias in the reciprocal hybrids was strong, and implied monoallelic expression of the Nipponbare allele. In addition, we detected a Kasalath-specific splicing variant as a smaller transcript with an expression level lower than that of the Nipponbare allele. This transcript was absent in NKF$_1$ and KNF$_1$. Sequencing this transcript revealed a splicing variant that leads to a 76-bp deletion at the 3′ end of exon 2.

The non-Mendelian spot 231 showed the same behavior as spot 200 on RLGS. The spot intensity was half that of the surrounding spots and the presence or absence of this spot also segregated 1 : 1 in NKF$_1$ and KNF$_1$. Additionally, spot 231, like spot 200, was detected in all selfed progeny of Nipponbare. We similarly analyzed the expression of the nearest gene (DUF295 family protein Os01g0327900) in two NKF$_1$ (NK5 and NK7) and two KNF$_1$ (KN5 and KN10) individuals. Sequence analysis of the RT-PCR products showed that only the Kasalath allele was expressed in NK5, NK7, KN5, and KN10 (Figure 4(c) shows the results for NK7 and KN10; data for NK5 and KN5 are not shown but gave the same result). In this study, we have given two examples of the nearest gene to a heterozygous methylated site showing allelic expression bias.

Recently, monoallelic expression in F$_1$ hybrids of plants has been reported. Zhuang and Adams [29] reported that in *Populus* interspecific hybrids, 17 out of 30 genes analyzed showed >1.5-fold expression bias for one of two alleles, with monoallelic expression of one gene [29], while intraspecific maize hybrids have shown unequal expression of parental alleles [30–32]. Therefore, histone modification or DNA methylation is considered one cause of allelic expression bias.

Elucidation of the significance and mechanism of regulation of monoallelic expression requires detection of more RLGS spots showing non-Mendelian inheritance along with the analysis of the methylation status of the corresponding DNA sequence and the expressed allele. Further expression analyses of genes in F$_1$s having different genetic backgrounds will support our findings for application to other genes. Moreover, revealing the function of the splicing variant of Kasalath in F$_1$ hybrids may provide better understanding of the mechanism of allelic exclusion inducing heterosis, hybrid weakness, and genome barriers.

4. Conclusion

Our findings clearly demonstrate that the RLGS method can be successfully applied to survey non-Mendelian inheritance of DNA methylation. Consequently, we detected two loci showing non-Mendelian inheritance and allelic expression bias in F$_1$ hybrids of rice. The systematic scanning has the following advantages: (1) easy detection of candidates for non-Mendelian inheritance of DNA methylation by simple comparison of spot patterns between parents and F$_1$ hybrids, (2) low cost and quick yield results in only 3 days, and (3) detection of potentially more non-Mendelian spot candidates using different restriction enzyme combinations in RLGS.
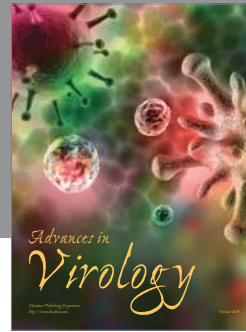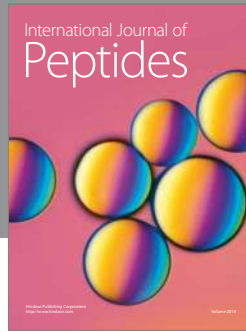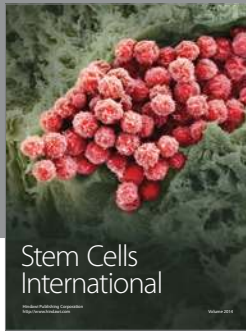
Acknowledgments

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