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4

Inheritance of DNA Methylation in Plant Genome

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1. Introduction

1.1 The role of DNA methylation in mammals and plants

Genomic DNA contains not only information of DNA sequence, but also epigenetic information that is the direct DNA modification by methylation (the addition of methyl group to the 5th carbon of pyrimidine ring of cytosine) and histone modifications (acetylation, methylation, etc). Epigenetic information is closely related to regulation of gene expression. If a methyl group is dislocated to position 5 of the pyrimidine ring of cytosine, the hydrogen bond between complementary GC bases will not be inhibited, but this methyl group is positioned so as to be exposed in the major groove of the double-helix structure of DNA, and according to the genome region/sequence undergoing modification of methylation, gene expression is inhibited by the interaction between the genome and DNA-binding molecules.

Methylated cytosine is very common in plant and mammalian genomes, and plays an important role in the regulation of many cellular processes including X inactivation, chromosome stability, chromatin structure, embryonic development and transcription. DNA methylation in most mammals occurs at cytosine on the CG sequence, a 2-base sequence lined up in the order of cytosine-guanine. In plants, on the other hand, methylation of a non-CG sequence (CNG and CHH, where N is A, G, C or T, and H is A, C or T) frequently exists in addition to methylation of the CG sequence. Moreover, there is a large difference between mammals and plants in methylation dynamics throughout the life cycle [Law & Jacobsen 2010]. In mammals, methylation patterns change dramatically during gametogenesis and early development [Monk *et al*. 1987, and Tada *et al*. 1997]. In mice, the methylation level of the genome decreases after fertilization to the lowest level at implantation, which is rapidly induced at the time of tissue differentiation after implantation. In reproductive cells,

moreover, methylation of genomic DNA is eliminated once and then methylation occurs again, but the sex-specific methylation pattern is written according to the gene and is adjusted so that a specific uniparental allele may be expressed. The gene showing sex-specific expression is called the imprinted gene and plays an important role in development and differentiation [Obata *et al*. 1998, Ueda *et al*. 2000, and Davis *et al*. 2000].

In contrast, generational changes in methylation status and inheritance in plants have been unclear. The methylation statuses of some genes are stably inherited [Bender *et al*. 1995, Jacobsen *et al*. 1997, Kakutani *et al*. 1999], but, recent studies show that DNA methylation patterns are altered in F1 hybrids from interspecific or intraspecific crossing [Matzke *et al*. 1999, Wendel 2000, Shaked *et al*. 2001, Pikaard 2001, Madlung *et al*. 2002, Comai *et al*. 2003, Liu *et al*. 2004, Dong *et al*. 2006, and Akimoto *et al*. 2007]. These alterations might be caused by the interaction among alleles, and/or the change of epigenetic regulation. For example, imprinted genes that have sex-dependent methylation patterns in endosperm have been identified in plants, and they play an important role in the control of flowering or seed development [Grossniklaus *et al*. 1998, Kiyosue *et al*. 1999, Kinoshita *et al*. 1999, Choi *et al*. 2002, Xiao *et al* 2003, Kohler *et al*. 2003, Scott *et al*. 2004, Kohler *et al*. 2005, Baroux *et al*. 2006, and Gehring *et al*. 2006]. Another example, paramutation is an allele-dependent transfer of epigenetic information, which results in the heritable silencing of one allele by another [Brink 1956, and Coe 1959]. In recently, Shiba *et al*. (2006) suggested that tissue-specific monoallelic *de novo* methylation in F1 involved in determining the dominance interactions that determine the cruciferous self-incompatibility phenotype. These analyses of DNA methylation inheritance may help to identify new important genes, such as imprinted gene, and to further clarify the biological significance of DNA methylation.

1.2 Concept of RLGS as a tool for DNA methylation analysis

Restriction Landmark Genome Scanning (RLGS) [Hayashizaki *et al*. 1993, Okazaki *et al*. 1995] is a unique quantitative approach well suited for simultaneous assay of methylation status [Costello *et al*., 2002]. The other genome wide methylation analyze methods *viz*., Tiling microarrays [Zhang *et al*. 2006] and methylation-sensitive amplification polymorphism (MSAP) [Reyna-Lopez *et al*. 1997, Xiong *et al*. 1999] are the comprehensive or easily applied method, respectively, but RLGS excels these methods because of its reduced cost and short span of experimental time (3 days). In RLGS, intensity of the RLGS spots directly reveals the methylation level and partial methylation such as imprinting, whereas in other methods the methylation levels are not inferred directly from the results. These important advantages of RLGS rank it as an appropriate method for genome wide methylation survey. This method had been used for development of genetic linkage maps [Okuizumi *et al*. 1995A, Okuizumi *et al*. 1995B], methylation analysis in tumor tissue [Ohsumi *et al*. 1995, Miwa *et al*. 1995, Wang *et al*. 2009], and identification of imprinted genes in mammals [Hayashizaki *et al*. 1994A, Shibata *et al*. 1995, Plass *et al*. 1996] and based on which several interesting research in epigenetics and genetics such as alteration of genomic DNA methylation [Takamiya *et al*. 2008B, Takamiya *et al*. 2009] and genetic diversity study [Okuizumi *et al*. 2010] had been carried out. The "*in silico* RLGS", is a software originally developed and named by us, and now the name and the concept are spread to other researchers [Matsuyama 2008]. This software can be utilized for the RLGS analysis of the organisms for those the whole genome sequence is available. One among such organism is Rice which enables the utilization of "*in silico* RLGS" analysis

[Takamiya *et al.* 2006] because of the availability of its whole genome sequence [International Rice Genome Sequencing Project, 2005].

2. Methodology of RLGS to detect DNA methylated sites efficiently in the plant genome

2.1 Method development to use isoschizomer restriction enzyme such as *Msp*I and *Hpa*II
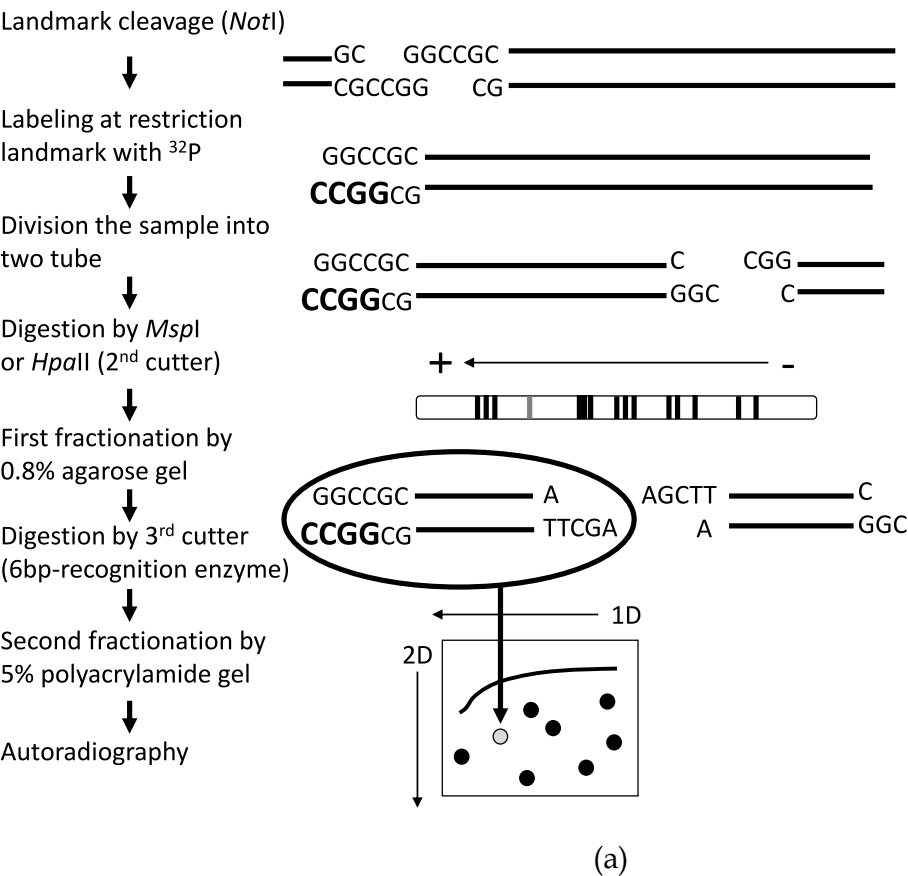
RLGS is a high-speed genome scanning system. It employs direct end-labeling of the genomic DNA digested with rare-cutting restriction enzyme such as *Not*I, followed by high-resolution two-dimensional (2-D) electrophoresis. Thousands of loci with high reproducibility can be detected as spots on the 2-D pattern in this method. A lot of methylated sites can be analyzed using the conventional RLGS method because the recognition site (GCGGCCGC) of the first cutter *Not*I [Hayashizaki *et al.* 1993, Watanabe *et al.* 1995], which is a methylation sensitive restriction enzyme (Fig. 1A), is often located in the CpG islands [Bird 1992]. But, this conventional method has bottle-neck in distinguishing methylation polymorphism from sequence polymorphism.

This bottle-neck of the conventional RLGS urges for the development of improved RLGS method (Figs. 1A and1B). In this improved method, isoschizomer restriction enzymes (*Msp*I and *Hpa*II) were used for the direct detection of methylated sites, that we produce 2 patterns; the "[*Msp*I] pattern" employs the restriction enzyme combination of *Not*I-*Msp*I-*Bam*HI as the 1st-2nd-3rd cutter, and another "[*Hpa*II] pattern" uses *Not*I-*Hpa*II-*Bam*HI as the 1st-2nd-3rd cutter. Rationale to utilize the isoschizomer is, they recognize the same sequence (CCGG) but with difference in methylation sensitivity. For example, the *Msp*I cleaves the CCGG at 2nd C which is methylated ($C^{5m}CGG$) but *Hpa*II doesn't cleave because of its differential sensitivity for the methylation. The different methylation sensitivity between *Msp*I and *Hpa*II is reflected in difference of RLGS spot patterns (Fig 1B). Briefly, the spot which is only detected in either [*Msp*I] or [*Hpa*II] pattern show the *Msp*I/*Hpa*II site of the RLGS spot is methylated. We called this spot as "methylated spot." The additional qualities of this improved RLGS method were (1) easy identification of methylated sites and their location in genome, (2) methylation of coding regions are surveyed efficiently and (3) its ability to scan methylation sites in an individual or a tissue. Furthermore, in an un-sequenced species and even in a cloned organism, this improved method distinguishes DNA mutation from the methylation changes.
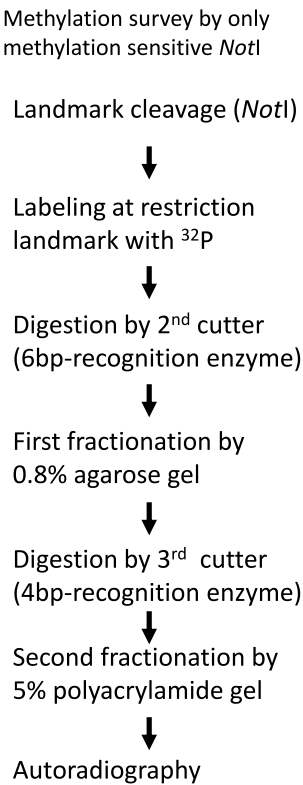
Genomic DNA was isolated from 8-week old leaf blades and leaf sheath of Nipponbare, Kasalath, and F1 plants (Crossing subsp. *japonica* cv. Nipponbare as the seed parent with subsp. *indica* cv. Kasalath as the pollen parent gave F_1 hybrids designated NKF_1) grown for 8 weeks. Isolated genomic DNA (0.2 µg) was treated with 2 U of DNA polymerase I (NIPPON GENE, Tokyo, Japan) in 10 µL of blocking buffer (10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.4 µM dGTP, 0.2 µM dCTP, 0.4 µM ddATP and 0.4 µM ddTTP) at 37°C for 20 min. Thereafter, the polymerase I was inactivated by incubating at 65 °C for 30 min. Then the DNA was digested with 20 U of *Not*I (NEB, Beverly, MA, USA) in volume of 20 µL. The cleavage ends were filled in and labeled with ^{32}P in the presence of 1.3 U of Sequenase ver. 2.0™ (USB, Cleveland, OH, USA), 0.33 µM [α-^{32}P] dGTP (3,000 Ci/mmol), 0.33 µM [α-^{32}P] dCTP (6,000 Ci/mmol) and 1.3 mM DTT at 37 °C for 30 min in 22.5 µL. Next,

this reaction mixture was incubated at 65 ˚C for 30 min to inactivate the enzyme. The processed sample was divided into two tubes. One was digested with 25 U of *Msp*I (Toyobo, Tokyo, Japan) and the other was treated with 25 U of *Hpa*II (Toyobo) and incubated at 37oC for 1 h. Each sample was fractionated on an agarose disc gel (0.8% Seakem GTG™ agarose, FMC Bioproducts, Rockland, Maine, USA) in the 2.4 mm diameter × 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 h followed by 230 V for 23 h. The size fractionated genomic DNA was carefully extruded from the tube and soaked for 30 min in the reaction buffer for *Bam*HI. Thereafter, DNA was digested in the gel with 1500 U of *Bam*HI for 2 h. The gel was fused into the top edge of a 50 cm (W) × 50 cm (H) × 0.1 cm (thickness) 5% polyacrylamide vertical gel by adding melted agarose (0.8% at 60-65˚C) to connect each gel. The 2nd-dimensional (2-D) electrophoresis was carried out in Tris borate EDTA (TBE) buffer (50 mM Tris, 62 mM boric acid, 1 mM EDTA), at 100 V for 1 h followed by 150 V for 23 h. An area 35 cm × 41 cm of the original gel was excised and dried. Autoradiography was performed for 3-10 days on a 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 the BAS-2000™ (Fuji Photo Film).

(a)

Genomic DNA digested by *Not*I
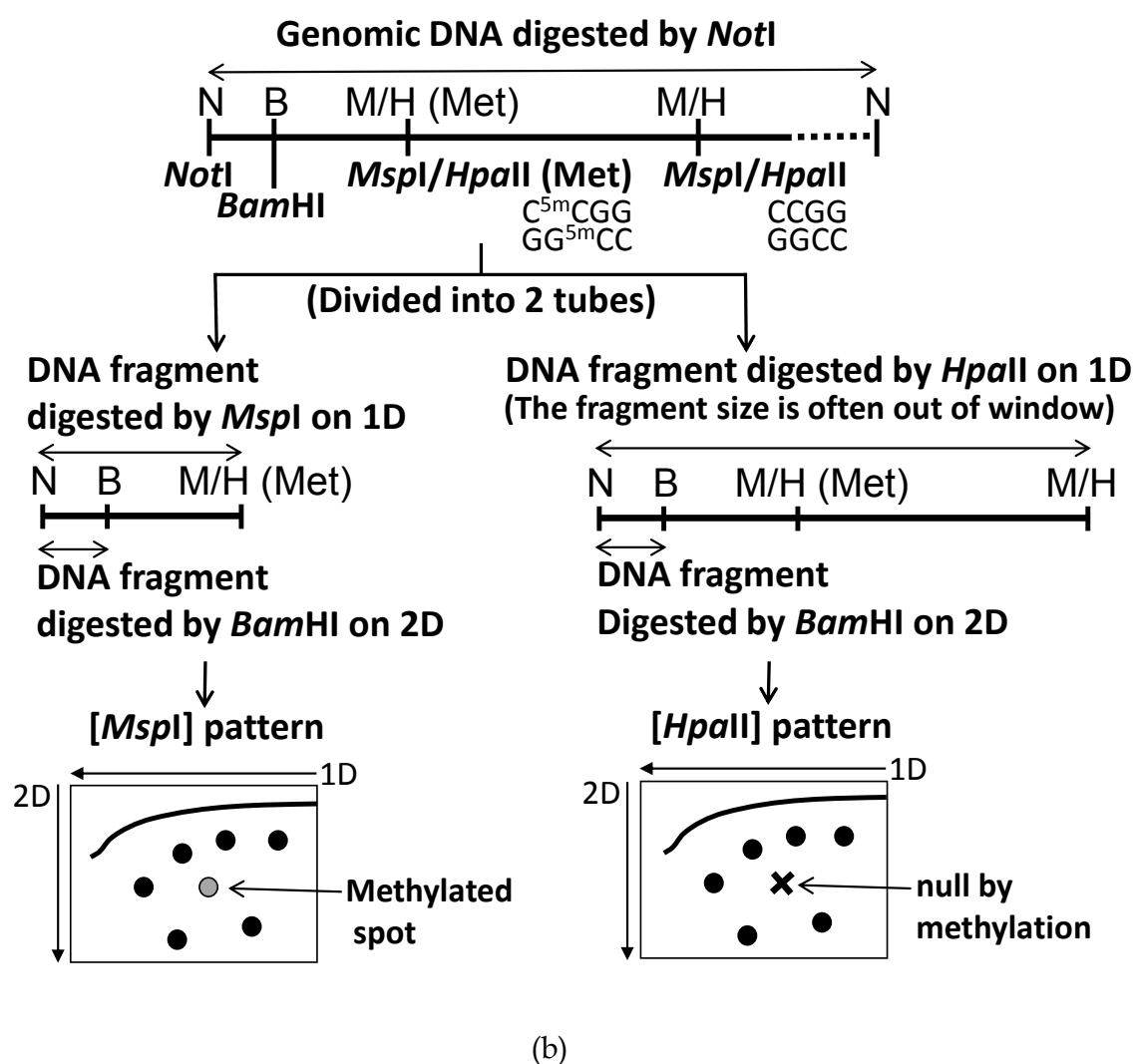
N   B   M/H (Met)   M/H   N

*Not*I | *Msp*I/*Hpa*II (Met)   *Msp*I/*Hpa*II
       *Bam*HI   C⁵ᵐCGG   CCGG
                            GG⁵ᵐCC   GGCC

(Divided into 2 tubes)

DNA fragment   **DNA fragment digested by *Hpa*II on 1D**
digested by *Msp*I on 1D   **(The fragment size is often out of window)**

N   B   M/H (Met)   N   B   M/H (Met)   M/H

DNA fragment   **DNA fragment**
digested by *Bam*HI on 2D   **Digested by *Bam*HI on 2D**

[*Msp*I] pattern   **[*Hpa*II] pattern**

2D | ———————1D   2D | ———————1D

← Methylated spot   ← null by methylation

(b)

Fig. 1. Conventional (right side) and improved (left side) RLGS procedures. (a)The conventional RLGS method is a tool that uses landmarks to directly label restriction enzyme sites scattered on genomic DNA, expand them on a single image by high-resolution two dimensional electrophoresis, and detect several thousands of spots at once. In the conventional method, the differences of methylation status among samples are surveyed using NotI methylation sensitivity. However, it has been difficult to distinguish methylation polymorphism from sequence polymorphism, and can't adopt for methylation surveillance of one sample. In improved method (left flow chart), isoschizomers (*Msp*I and *Hpa*II) are used as second cutter. We are possible to detect methylated sites directly in even if an individual or a tissue by comparison between [*Msp*I] pattern and [*Hpa*II] pattern. (b) *Msp*I and *Hpa*II both recognize CCGG site, but have different methylation sensitivity. We show one example of methylated spot: *Msp*I can digest the methylated *Msp*I/*Hpa*II site (C⁵ᵐCGG), which is the nearest to *Not*I site, and the DNA fragment (from *Not*I end to *Msp*I/*Hpa*II end) is electrophoresed on detectable first dimensional area. On the other hand, *Hpa*II cannot digest the methylated *Msp*I/*Hpa*II site, and digests non-methylated *Msp*I/*Hpa*II site at the downstream. Therefore, longer DNA fragment (from *Not*I end to non-methylated *Msp*I/*Hpa*II end) is electrophoresed at out of window on the first dimension. This figure was cited from Takamiya, T. 2007.
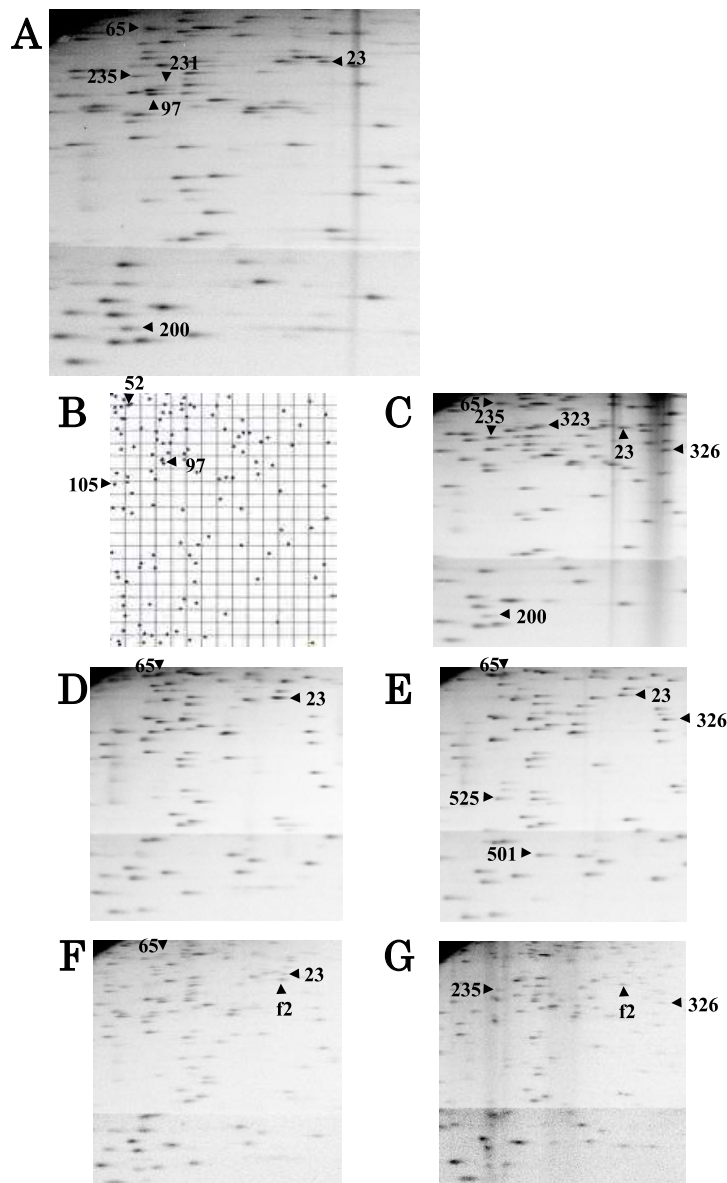
Fig. 2. RLGS patterns for detection of methylated sites. (A) RLGS pattern of Nipponbare genomic DNA with restriction enzyme combination *Not*I-*Msp*I-*Bam*HI ([*Msp*I] pattern). (B) *in silico* RLGS pattern predicted from rice genome sequence data. (C) Nipponbare *Not*I-*Hpa*II-*Bam*HI ([*Hpa*II]) pattern. In comparison with [*Msp*I] pattern, 25 spots were specific to [*Msp*I] and 18 to [*Hpa*II]. (D) Kasalath [*Msp*I] pattern. (E) Kasalath [*Hpa*II] pattern from the genomic DNA in (D): 19 spots were specific to [*Msp*I], and 13 to [*Hpa*II]. (F) F1 hybrid [*Msp*I] pattern. In comparison to its parents, spots 200 and 235 were absent, and spot f2 was new. (G) F1 hybrid [*Hpa*II] pattern from the genomic DNA in (F): 29 spots were specific to [*Msp*I], and 26 to [*Hpa*II]. Compared with the parental patterns (C, E), spots 23, 65, 200, 231, 323, 501, and 525 were absent, and F1-specific spot f2 was new (G). These figures were cited from Takamiya, T., Hosobuchi, S., Noguchi, T., Asai, K., Nakamura, E., Habu, Y., Paterson, A. H., Iijima, H., Murakami, Y., Okuizumi, H. (2008). Inheritance and alteration of genome methylation in F1 hybrid rice. Electrophoresis. 29, 4088-4095. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

2.2 *In silico* RLGS analysis oriented by whole genome sequence

A software named "*in silico* RLGS" was developed that simulates RLGS spots based on known sequence data to identify each spot in actual RLGS. The software searches for restriction enzyme sites in the entire genome sequence that were used in an actual RLGS experiment, and calculates fragment length between the restriction sites and mobility for simulating a 2-D spot pattern. In our experiment the whole rice genome sequence data was obtained from http://rgp.dna.affrc.go.jp/J/IRGSP/Build3/build3.html. *Not*I sites and *Msp*I sites that were near to the *Not*I sites were searched through the whole sequence and given ID numbers. Length of identified fragments between restriction sites (from *Not*I end to the nearest *Msp*I end or from *Not*I end to next *Not*I end) and mobility of each DNA fragment in the 1-D electrophoresis were calculated by the software according to Southern E. (1979). The exact mobility of RLGS spots were confirmed from electrophoresis of λ DNA fragments with known sequences. In addition, the *Bam*HI sites were also surveyed and DNA fragment (from *Not*I end to the nearest *Bam*HI end) length in 2-D was estimated. Based on this, a 2-D graph (*in silico* RLGS pattern) was drawn. The *in silico* RLGS pattern was compared to its corresponding autoradiographic (actual) RLGS pattern with relative spot positions. This comparison leads to the identification each RLGS spot immediatelyand precisely.

3. Results of DNA methylation analysis using improved RLGS

3.1 Detection of methylated spots in F1 hybrid and the parents

To detect the DNA methylation in plant genome, improved RLGS and *in silico* RLGS were used [Takamiya *et al*. 2008A]. Two rice individuals, *Oryza sativa* L. var Nipponbare and *O. sativa* L. var Kasalath as parent and its F1s were used as the experimental material to analyze the pattern of the DNA methylation and its inheritance in the F1 hybrid. F1 were obtained from crossing Nipponbare as the seed parent with Kasalath as the pollen parent.

The RLGS pattern with *Not*I-*Msp*I-*Bam*HI combination ([*Msp*I] pattern) and *Not*I-*Hpa*II-*Bam*HI combination ([*Hpa*II] pattern) were obtained for the parents (Nipponbare and Kasalath) and F1. The [*Msp*I] pattern showed 85 spots with Nipponbare, 77 spots with Kasalath and 111 spots with F1 hybrid. In the same way, [*Hpa*II] pattern showed 78, 71 and 108 spots with Nipponbare, Kasalath and F1 hybrid, respectively. The genome sequence of the Nipponbare was analyzed in *in silico* software and it showed 117 spots. The spot pattern obtained was compared with actual RLGS pattern and 56 spots were found to be in common. For example the spot 97 was detected in both patterns (Fig 1A and 1B),, with a locus on chromosome 9.

To detect the RLGS spots differed in methylation ("methylated spot"), the [*Msp*I] pattern and [*Hpa*II] pattern were compared. This comparison showed that 43 methylated spots in Nipponbare and 32 methylated spots in Kasalath. Next, we compared Nipponbare and Kasalath patterns to detect methylation polymorphysms between parents. Thirty five spots of [*Msp*I] pattern and 42 spots of [*Hpa*II] pattern were specific to Nipponbare and similarly the Kasalath also had 27 and 35 spots in specific with [*Msp*I] pattern and [*Hpa*II] pattern respectively. Moreover, 50 spots of [*Msp*I] pattern and 36 spots of [*Hpa*II] pattern were common between Nipponbare and Kasalath. The spots which were not identified by *in silico* RLGS were cloned from the 2D polyacrylamide gel.

In F1, though most of the spots followed the Mendelian law of inheritance, still 8 parental spots (spot numbers: 23, 65, 200, 231, 235, 323, 501 and 525) disappeared and one new spot (f2) was detected. These 9 spots (8 disappeared spots and 1 new spot) indicate the abnormal inheritance and the details were summarized in Table 1. The pattern of spot 323 in parents, F1 and selfed progenies were shown Figure 3c.

Spot No.	in silico	Nipponbare		Kasalath		F1	
		*Msp*I	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	*Hpa*II
23	x	o	o	o	o	o	x
65	o	o	o	o	o	o	x
200	x	o	o	x	x	x	x
231	x	o	o	x	x	x	x
235	x	o	o	x	x	x	o
323	x	x	o	x	x	x	x
501	x	x	x	x	o	x	x
525	x	x	x	x	o	x	x
f2	x	x	x	x	x	o	o
52	o	x	x	x	x	x	x
105	o	x	x	x	x	x	x

Table 1. Altered inheritance of RLGS and *in silico* RLGS spots. o, spot present; x, spot absent. One RLGS spot (f2) was newly detected in F1 hybrids, and 8 RLGS spots were absent in the NKF1. Two *in silico* RLGS spots (52 and 105) were found to be altered methylation inheritance by PCR analysis. This table was cited from Takamiya, T., Hosobuchi, S., Noguchi, T., Asai, K., Nakamura, E., Habu, Y., Paterson, A. H., Iijima, H., Murakami, Y., Okuizumi, H. (2008). Inheritance and alteration of genome methylation in F1 hybrid rice. Electrophoresis. 29, 4088-4095. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

3.2 Mapping of methylation status of rice genome using RLGS

The mapping of methylation status of rice genome using RLGS was carried out as following steps. (1) Methylated spot was detected by comparison [*Msp*I] and [*Hpa*II] patterns, and its locus was identified by *in silico* RLGS. (2) The presence of restriction site of the *Not*I and the *Msp*I/*Hpa*II of methylated spot was confirmed using PCR analysis. (3)The methylation status of restriction sites of methylated spot was confirmed by another PCR analysis. (4) The parental origin of the methylated alleles in F1 was determined by using the sequence analysis. These steps are explained in detail as follows.

The presence of the restriction sites of *Not*I and *Msp*I/*Hpa*II was confirmed by PCR analysis by designing the flanking primers for the restriction sites of RLGS spots that were identified by *in silico* RLGS or spot cloning. The presence of restriction sites was confirmed in the RLGS spot 97 of Nipponbare and Kasalath (Fig. 4) as an example. The Figure 4A shows the various PCR products in lane 1 to 4. The lane 1 and 2 was the amplified genomic DNA of parents, Nipponbare and Kasalath using the flanking primers and lane 3 and 4 were the PCR products, obtained from the each parent after treatment with *Msp*I. The DNA fragments of lane 3 and 4 are smaller than the fragments of lane 1 and 2, this is because the *Msp*I/*Hpa*II site were digested and divided into 456 bp (detected) and 123 bp (estimated). This difference in the fragment size confirms the presence of *Msp*I/*Hpa*II sites in both Nipponbare and Kasalath.
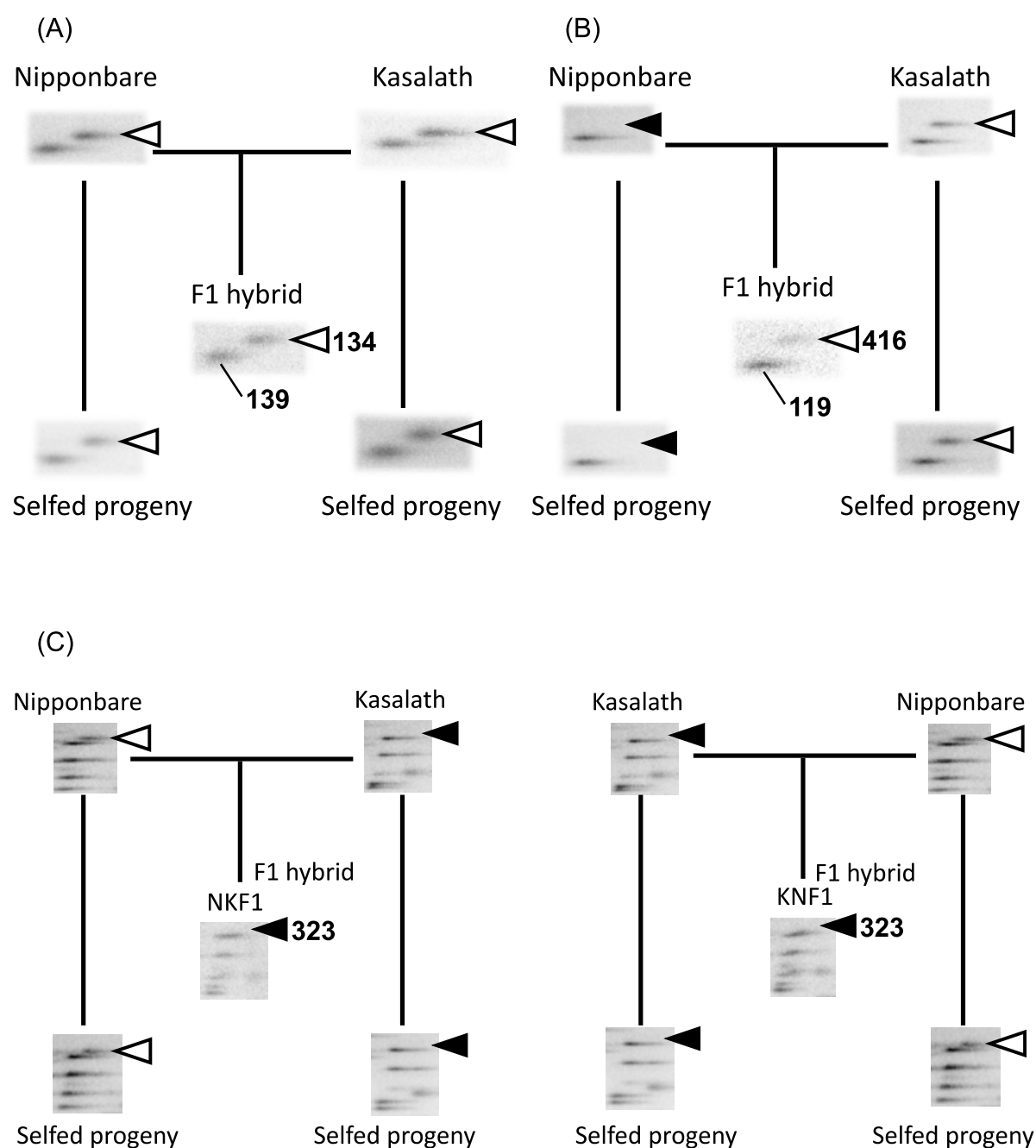
Fig. 3. Mendelian and non-Mendelian inheritance of RLGS spots pattern. (A) Spot 134 (arrowhead) was detected in both parents viz., Nipponbare and Kasalath and the same pattern of the spot was also detected in the F1. (B) Spot 416 (arrowhead) was detected in only one parent (Kasalath). This spot on the RLGS pattern of F1 was detected at diminished (half) intensity of the normal spots such as spot 119. The spots 134 and 416 showed Mendelian inheritance. (C) There was a spot 323 (arrowhead) in the pattern of parental Nipponbare, but not in parental Kasalath. It was expected to be at half intensity in F1 patterns, however, the spot 323 disappeared in all 9 patterns of NKF1 individuals and 8 patterns of KNF1s. In the patterns of all 9 selfed progenies of parental Nipponbare, the spot 323 was detected similar to parental Nipponbare. This spot 323 was indicating non-Mendelian inheritance. These figures were cited fromTakamiya, T. 2007.
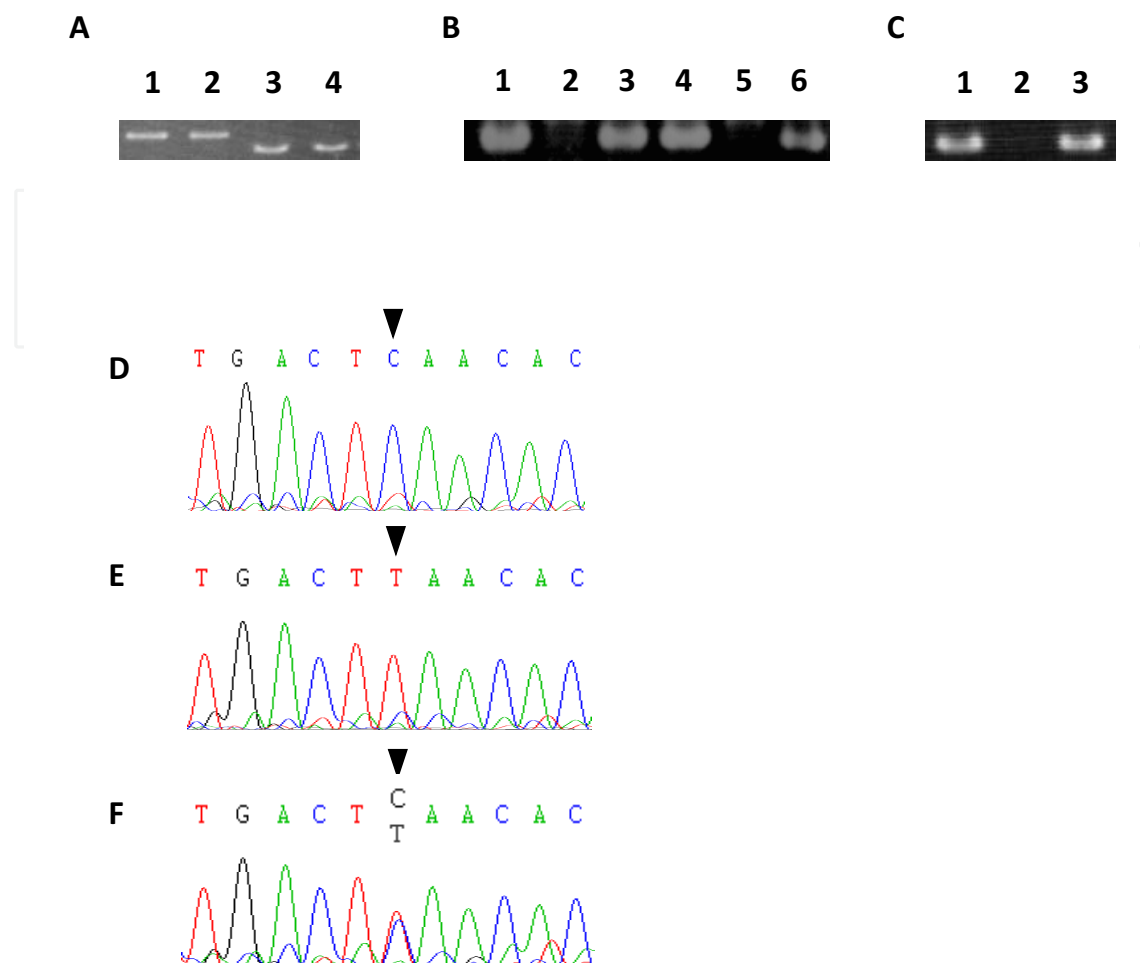
Fig. 4. Confirmation of methylation status. (A) Confirmation of restriction enzyme sites with flanking primers for the *Msp*I/*Hpa*II site of spot 97. Lanes 1 and 2 are PCR products amplified from genomic DNA of Nipponbare (N) and Kasalath (K), respectively. The PCR products were purified, and treated with *Msp*I, then, loaded into lanes 3 and 4, respectively. (B) PCR-based DNA methylation analysis of spot 97 of the parents (Nipponbare and Kasalath). Lanes 1, 2, and 3 for Nipponbare or 4, 5, and 6 for Kasalath are the PCR products for the templates (un-, *Msp*I-, and *Hpa*II-digested genomic DNA, respectively). (C) PCR-based DNA methylation analysis of spot 97 in the F1. Lane 1 is the uncut positive control. Lanes 2 and 3 are PCR products from F1 genomic DNA that was treated with *Msp*I and *Hpa*II, respectively. (D) Part of the nucleotide sequence of the DNA fragment in lane 1 shown in Fig 4B (Nipponbare). (E) Part of the nucleotide sequence of the DNA fragment in lane 4 shown in Fig 4B (Kasalath). One SNP (shown by arrow-head) was detected in this region.(F) Part of the nucleotide sequence of the DNA fragment in lane 3 shown in Fig 4C. This DNA fragment had C/T heterozygously. These figures were cited from Takamiya, T., Hosobuchi, S., Noguchi, T., Asai, K., Nakamura, E., Habu, Y., Paterson, A. H., Iijima, H., Murakami, Y., Okuizumi, H. (2008). Inheritance and alteration of genome methylation in F1 hybrid rice. Electrophoresis. 29, 4088-4095. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

To confirm the methylation status of spots with restriction enzyme site in Nipponbare and Kasalath, different PCR products were obtained (Fig. 4B). The PCR product loaded in lane 1 genomic DNA of Nipponbare and the lane 2 and 3 were loaded with the product obtained by using *Msp*I or *Hpa*II treated genomic DNA of Nipponbare as template, respectively. The lanes 4 to 6 are loaded with the PCR products of Kasalath obtained in similar manner to that of Nipponbare. The lane 1 and 4 are used as the positive control for Nipponbare and Kasalath, respectively. The lane 2 and 5 do not have any bands where as the lane 3 and 6 had the band sizes similar to that of their positive controls. This result showed that the *Msp*I/*Hpa*II site was methylated (C5mCGG), and it was correspondence with RLGS result. Similar to the parents, the *Msp*I/*Hpa*II site was methylated in F1 (Fig. 4C).

In Nipponbare, the methylation status of 90 *Not*I and 92 *Msp*I/*Hpa*II sites were checked. Out of 182 sites, 60 sites (33%) were methylated and 4 Nipponbare specific sites were identified. Similarly in Kasalath, 82 *Not*I and 84 *Msp*I/*Hpa*II sites were tested for methylation status resulted in identification of 59 methylation sites (36%) and 10 Kasalath specific sites.

Finally, to determine the parental origin of the methylated allele, the bands were subjected for sequence analysis. The bands of Nipponbare in lane 1 and bands of Kasalath in lane 4 (Fig 4B) were purified and sequence analysis was done. This analysis detects an SNP for spot 97 showing C in Nipponbare (Fig 4D) and T in Kasalath (Fig 4E). Similarly the band in lane 3 (Fig. 4C), which was amplified from *Hpa*II digested genomic DNA of F1, was sequenced and it was found to be heterozygous with both C and T (Fig. 4F). The sequence analysis shows that methylation prevented digestion of the *Msp*I/*Hpa*II sites of both the alleles, and the methylation status of the parents was inherited to F1 following Mendelian law. Similarly, we examined the other 25 methylated spots that showed the same appearance or absence between the parents and F1, and confirmed that all methylation status were inherited to F1.

Some of the *Not*I and *Msp*I/*Hpa*II sites that were specifically found in the *in silico* RLGS were also checked based on the PCR and sequence analysis. This analysis identified some new altered methylations in F1. The identified spots were demethylations at *Not*I of spot 52 (Chr.11) and the *Msp*I/*Hpa*II site of spot 105 (Chr.3) in F1, and their details are given in the Table 1 and Figure 5. The specific occurrence of the spots only in *in silico* RLGS is due to the methylation in *Not*I sites.

In the entire process of analysis, a total of 103 RLGS spots were identified and most of these spots were analyzed for methylation status by RLGS and PCR. The result of this analysis was summarized in methylation map (Fig 5). In the map, the numbered spot depict that those spots were methylated at one or more *Not*I or *Msp*I/*Hpa*II sites of Nipponbare, Kasalath or F1. In total, seven altered spots were mapped (shown as 'AI' in Fig 5). The other spots *viz.*, 23, 501, 525 and f2 are not yet to cloned. Out of this 103 *Not*I sites, 17 and 14 sites were located within 2.0 kb upstream and downstream of a gene respectively, and 63 sites located within the gene. The remaining 9 sites were in the intergenic regions. Similarly, 25 *Msp*I/*Hpa*II sites were in 5′ upstream region, 48 within gene and 15 sites in 3′ downstream region. Thus, 182 sites (88%) out of 206 were located between 2.0 kb upstream and downstream of genes.
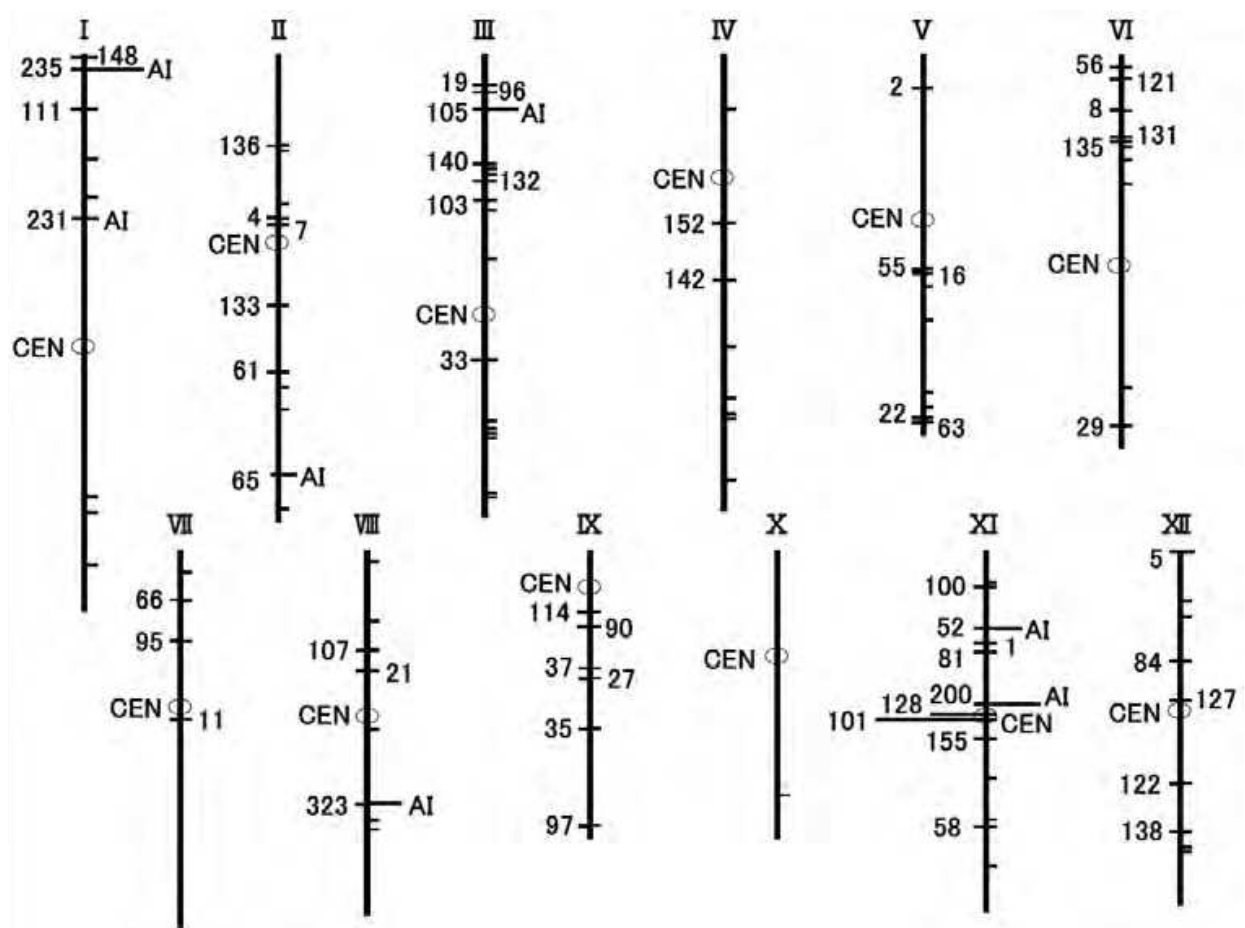
Fig. 5. Map of methylation sites. Numbered loci had at least 1 methylation in *Not*I or *Msp*I/*Hpa*II digests of Nipponbare, Kasalath, or F1s. "AI" indicates 7 loci with altered inheritance. Inheritance at the other loci appeared to be consistent. Centromeres (CEN) are indicated by ellipses. This map was cited from Takamiya, T., Hosobuchi, S., Noguchi, T., Asai, K., Nakamura, E., Habu, Y., Paterson, A. H., Iijima, H., Murakami, Y., Okuizumi, H. (2008). Inheritance and alteration of genome methylation in F1 hybrid rice. Electrophoresis. 29, 4088-4095. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

3.3 Non-Mendelian inheritance of DNA methylation

To prove non-Mendelian inheritance of DNA methylation in altered spot loci, we analyzed reciprocal F_1 hybrids (subsp. *japonica* cv. Nipponbare × subsp. *indica* cv. Kasalath) of rice (*Oryza sativa* L.). 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 inverse cross gave KNF_1 hybrids. The seeds 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 were grown for 2 months, and the genomic DNA was isolated from the leaf blade and sheath of each individual, and the RLGS analysis was performed.
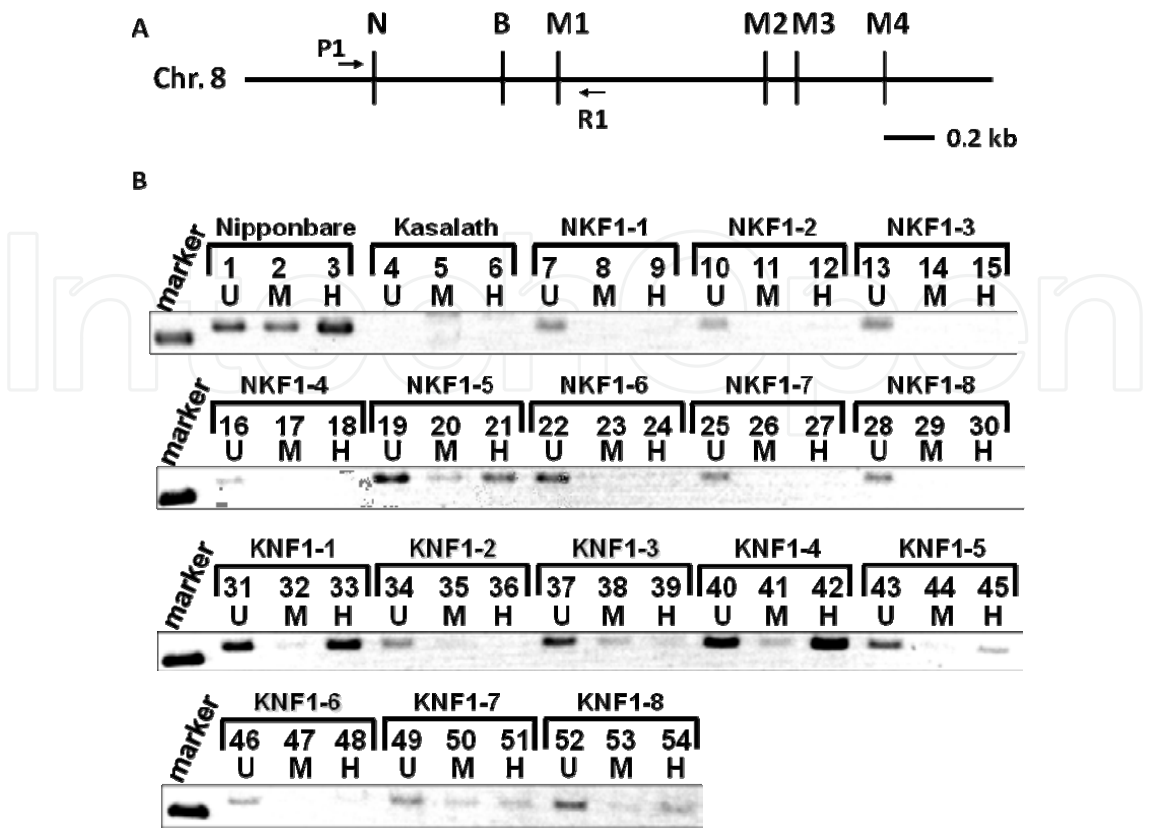
Fig. 6. Methylation analysis of an abnormally inherited RLGS spot 323. (A) DNA of spot 323 was located on Chr. 8. Arrows P1 and R1 indicate flanking primers for PCR-based methylation analysis. (B) PCR-based methylation analysis of M1 site of spot 323 in the parents and 8 NKF1 (NKF1-1~NKF1-8) and 8 KNF1 (KNF1-1~KNF1-8) hybrids. Lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, 37, 40, 43, 46, 49, and 52 are the PCR products from genomic DNA of each line as positive controls (U = uncut). M and H indicates *MspI* and *HpaII* digests of each line. The methylation status of M2 and M3 sites were also checked (data not shown). m: size marker, 1.0 kb band. These figures were cited from Takamiya, T., Hosobuchi, S., Noguchi, T., Asai, K., Nakamura, E., Habu, Y., Paterson, A. H., Iijima, H., Murakami, Y., Okuizumi, H. (2008). Inheritance and alteration of genome methylation in F1 hybrid rice. Electrophoresis. 29, 4088-4095. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

Consider spot 323 of Nipponbare as an example which is a *HpaII* specific spot but not detected in the F1. In most of the F1, the demethylation of the M1/M2 site of spot 323 was detected based on the PCR analysis (Fig 6 shown the result of M1 site). The PCR product of Kasalath genomic DNA was not amplified by the primer P1 and R1. This non-amplification of these regions by the primers may be due to some difference in base sequence between Nipponbare and Kasalath. The spots 200, 231, 235, 501 and 525 were specific to Nipponbare or Kasalath and these spots do not appeared in F1. Therefore, the regions of these spots may have some differences in DNA sequence or methylation status between Nipponbare and Kasalath. Alternatively, these polymorphic regions might have altered methylation status in the F1 hybrid [Takamiya *et al.* 2009]. The altered methylation of spot 323 suggests possible sequence-dependent demethylation, for example as a result of paramutation induced by allelic exclusion (Chandler *et al.*, 2000). For better understanding of the methylation behavior of spot 323, detailed analyses like expression analysis of the gene near to the spot may be required.

The spot 200 was detected in both the [*Msp*I] and [*Hpa*II] patterns of Nipponbare at a diminished spot intensity but disappeared in Kasalath (Fig 7). The spot is another non-Mendelian example [Takamiya *et al.*, 2009]. In RLGS analysis halved/diminished spot intensity indicates the heterozygote which was confirmed theoretically and practically in earlier studies (Hayashizaki *et al.* 1994B., Okuizumi *et al.* 1997). The DNA fragment of spot 200 was cloned and sequence analysis was carried out, which place it in the 5′ region of a non-protein coding transcript (Os11g0417300) (Fig 8A). The spot position of autoradiographic RLGS pattern of Nipponbare was compared with *in silico* RLGS pattern. Then, the DNA fragments digested at *Not*I (N) and *Msp*I (M) was separated by 1-D electrophoresis, and the N and *Bam*HI (B) sites were fractionate by 2-D electrophoresis (Fig 8A). Restriction enzyme digestion and sequencing was employed to confirm the existence of N, M and B in the parental Nipponbare, whereas in Kasalath the N and M site exist but the B site was absent. The presence or absence of spot 200 obtained based on the RLGS analysis of NKF_1 and KNF_1 shows that this spot segregated as 1:1 in both the population (Fig 7 and Table 2). The diminished spot intensity and its segregation in F1 hybrids show that the *Msp*I/*Hpa*II site of spot 200 is methylated heterozygously. Similarly, it was assumed that the detection and absence of spot 200 in F1 was due to the non-methylated and methylated M site, respectively. Moreover in all selfed progenies (9 individuals) of Nipponbare, the spot 200 was detected with halved intensity (Fig 7, Table 2). From this, it was concluded that in the selfed progeny and in parental Nipponbare, the M site was methylated heterozygous because of non-Mendelian inheritance of methylation.

Generation (Cultivar) [Sample size]	RLGS pattern of spot 200		RLGS pattern of spot 231	
	*Msp*I patterns (intensity)	*Hpa*II patterns (intensity)	*Msp*I patterns (intensity)	*Hpa*II patterns (intensity)
Parent (Nipponbare)	Present (1/2)	Present (1/2)	Present (1/2)	Present (1/2)
Parent (Kasalath)	Absent	Absent	Absent	Absent
Selfed progeny (Nipponbare) [9 individuals]	9 present (1/2)	9 present (1/2)	9 present (1/2)	9 present (1/2)
Selfed progeny (Kasalath) [4 individuals]	4 absent	4 absent	4 absent	4 absent
NKF_1 (Nipponbare × Kasalath) [9 individuals]	Segregated 5 present : 4 absent (1/2 : 0)	Segregated 4 present : 5 absent (1/2 : 0)	Segregated 2 present : 6 absent* (1/2 : 0)	Segregated 5 present : 4 absent (1/2 : 0)
KNF_1 (Kasalath × Nipponbare) [9 individuals]	Segregated 7 present : 2 absent (1/2 : 0)	Segregated 4 present : 5 absent (1/2 : 0)	Segregated 2 present : 7 absent (1/2 : 0)	Segregated 3 present : 6 absent (1/2 : 0)

This table was cited from Takamiya *et al.* 2009.

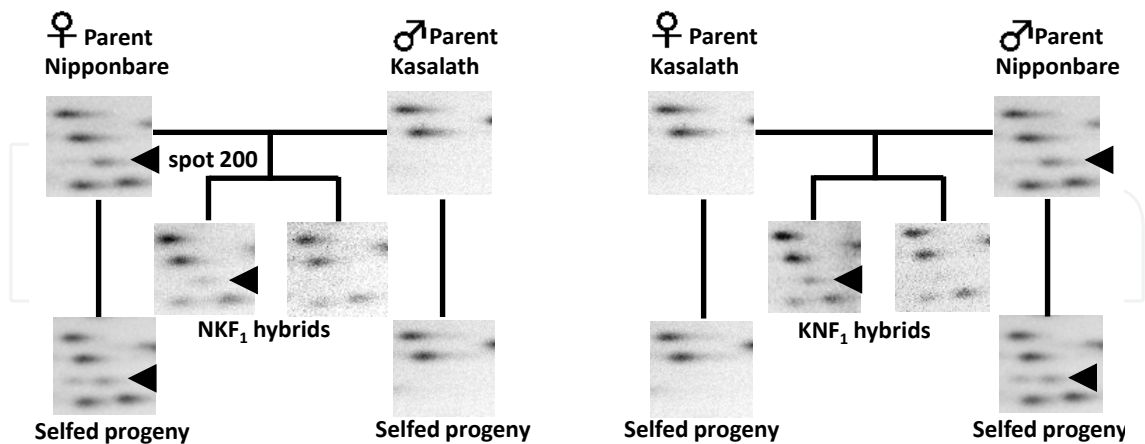Table 2. Summary of RLGS pattern of spot 200 and 231.

Fig. 7. Non-Mendelian pattern of spot 200. A part of RLGS [*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 and [*Hpa*II] 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. This figure was cited from Takamiya *et al.* 2009.

Expression analysis of the non-protein coding transcript (Os11g0417300), which is the nearest gene to the *Msp*I/*Hpa*II site of spot 200 (Fig 8A) [Takamiya et al., 2009], was done to clarify whether the methylation status in correlated with expression of nearest gene. The cDNA (Genebank accessions No: AK 109537) of the non protein coding transcript expressed in flower, leaf and panicle (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene& cmd=search&term=AK109537). Total RNA was isolated from the leaf blade and sheath of the parental Nipponbare, Kasalath, two individuals each in NKF$_1$ (NK5 and NK7) and KNF$_1$ (KN5 and KN10) hybrids. The pattern of NK5 and KN5 alone detected the spot 200 whereas in the pattern NK7 and KN10 the spot was not detected. The cDNAs' of both NK5 and KN5 were PCR amplified and separated by agarose gel electrophoresis (Fig 8B). The gene was expressed in the parents, NK5, and KN5 (Fig. 8B). The single nucleotide polymorphism (C/T) between Nipponbare and Kasalath was found and the RT-PCR products were sequence to reveal the parental origin of the expressed sequence in F_1 hybrid (Fig 8C). The RT-PCR products of NK5 and KN5 which had spot 200 in their RLGS pattern were subjected to sequence analysis. Allelic expression bias for the Nipponbare allele was found in NK5 and KN5 (Fig 8C). Similarly, the bias for Nipponbare allele was found in KN7 and NK10 which do not have spot 200 (data not shown). Strong allele bias was found in the reciprocal hybrids and this shows the monoallelic expression of the Nipponbare allele. In addition, a splicing variant (smaller transcript with lower expression than Nipponbare allele) specific to Kasalath was detected (Fig 8B), but this was absent in NKF$_1$ and KNF$_1$. This transcript was sequenced and a splicing variant that leads to a 76 bp deletion at the 3' end of exon 2 was revealed. The reason is unknown, but, it implies that some effect of methylation is influencing the variant expression.
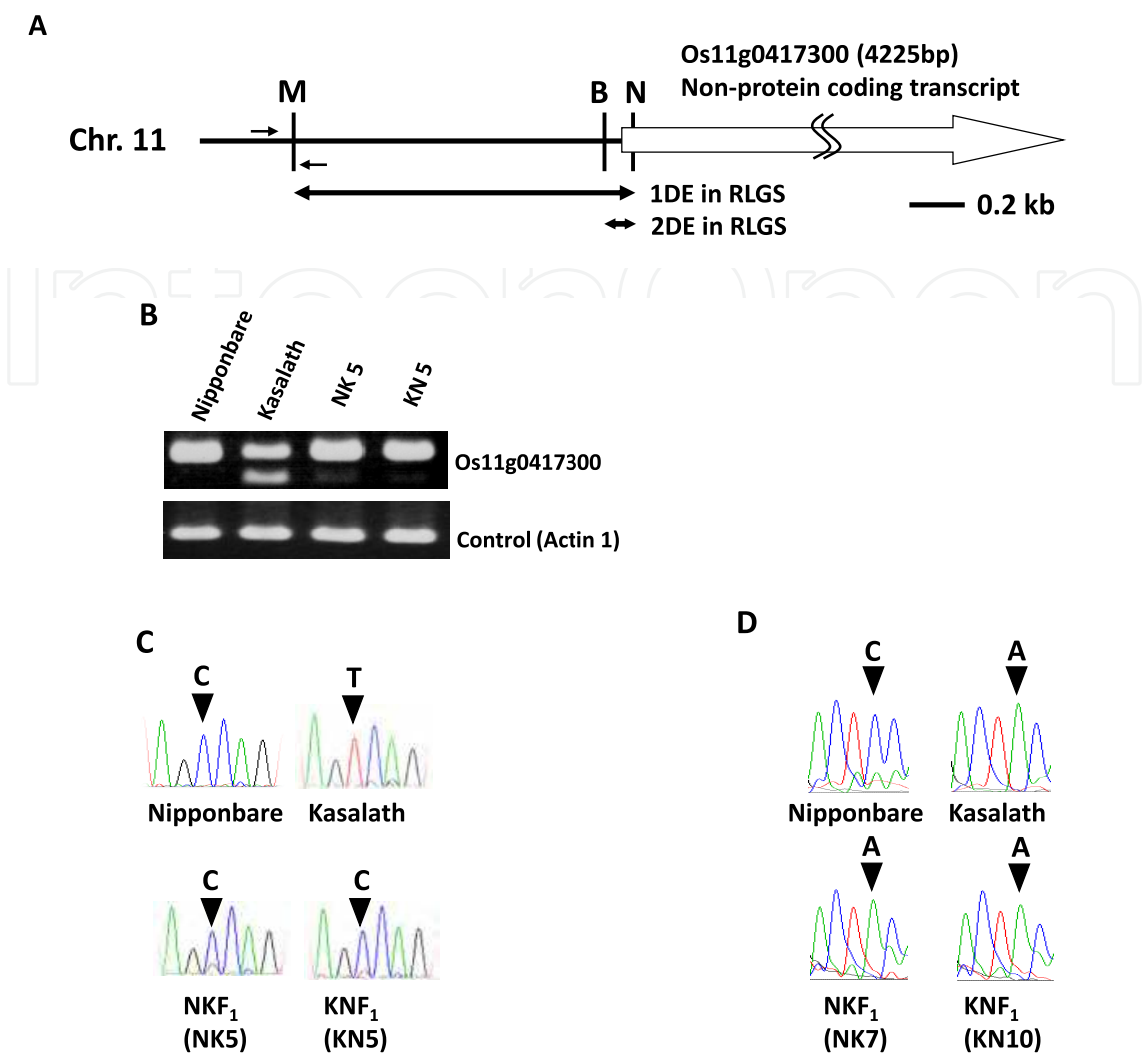
Fig. 8. Analysis of another abnormally inherited RLGS spot 200. (A) Schematic representation of the region of chromosome 11 containing the restriction enzyme sites located in the region 5′ to the transcription start site of the non-protein coding transcript (Os11g0417300). The DNA fragments were digested 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. (B) 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. (C) Sequence analysis of the RT-PCR products of the expressed Os11g0417300 allele. The single nucleotide polymorphism between Nipponbare (Cytosine) and Kasalath (Tymine) is indicated in the RT-PCR products by arrowheads. Specific expression of the Nipponbare allele was confirmed by detection of base Cytosine in both NK5 and KN5 plants. (D) Sequence analysis of RT-PCR products of the expressed Os01g0327900 allele. The single nucleotide polymorphism in RT-PCR products between Nipponbare (Cytosine) and Kasalath (Adenine) is indicated by arrowheads. Specific expression of the Kasalath allele was confirmed by detection of base Adenine in both NK7 and KN10 plants. These figures were cited from Takamiya *et al*. 2009.

The spot 231, which behaved similar to that of non-Mendelian spot 200, had diminished spot intensity and 1:1 segregation in NKF_1 and KNF_1 (Table 2), and also it was detected in all the selfed progeny of Nipponbare. For this spot 231, the expression of the nearest gene (DUF 295 family protein Os01g0327900) was analyzed 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 (Fig 8D shown the result of NK7 and KN10). In this study, two examples (spot 200 and 231) have been given for the nearest gene to a heterozygous methylated site showing allelic expression bias.

Monoallelic expression in F_1 hybrids of plants has been reported in several crops. Intraspecific maize hybrids have shown unequal expression of parental alleles [Guo *et al*. 2004, Springer *et al*. 2007A, and Springer *et al*. 2007B]. Besides, 17 out of 30 genes analyzed showed >1.5-fold expression bias for one of two alleles, with monoallelic expression of one gene in *Populus* interspecific hybrids [Zhuang *et al*. 2007]. Therefore, it is considered that allelic expression bias was caused by epigenetic status of DNA methylation and/or histone modification.

In plants, identification of more RLGS spots exhibiting non-Mendelian inheritance and simultaneously studying their methylation status of the corresponding DNA sequence and their expressed allele will explain the importance and better understanding of gene regulatory mechanisms such as monoallelic expression. Extensive and detailed expression analysis of genes in F1s with different genetic backgrounds is very much essential because the findings can be applied to other genes as well. The mechanism of allelic exclusion inducing heterosis, hybrid weakness and genome barriers might be better understood by revealing the regulation and function of the splicing variant of Kasalath (Fig. 8B) in F_1 hybrids.

3.4 Various aspects on DNA methylation roles

The RLGS method is very powerful for methylation analysis other than that for genetic analysis of DNA methylation. In the Takamiya *et al*. 2006, the methylated status was compared among 3 ecotypes of Arabidopsis using the RLGS method. Methylation at a total of 17 sites (*Not*I: 9 sites and *Hpa*II: 8 sites) was detected in the 3 ecotypes. Among them, there were 8 common methylation sites among the 3 ecotypes, and the 9 residual sites (53%) showed methylation polymorphism. Among all restriction enzyme sites analyzed (37 non-methylated sites and 17 methylated sites), the sites showing a different methylated status among the 3 ecotypes accounted for 17% of the total sites (9/54). In the studies so far, it has been reported that methylation is involved in inactivation and metastasis inhibition of transposon and retrotransposon [Hirochika *et al*. 2000]. In the pseudogene and gypsy-like retrotransposon family-like region in centromeres, both the *Not*I site and *Msp*I/*Hpa*II site were methylated in the 3 ecotypes, which may suggest the relation between the pseudogene in the centromere region and inactivation of the movable element.

Moreover, the RLGS method can be applied to the detection of genomic variation in plant tissue culture [Takamiya *et al*. 2008B]. The genome DNA of 2 ramets obtained from one seed of rice was extracted and analyzed for RLGS with the combination of *Not*I-*Hpa*II-*Bam*HI and with *Not*I-*Msp*I-*Bam*HI to compare their pattern. As a result, 10 different spots (6%) were detected between ramets. One spot among the 10 different spot was cloned to confirm the methylated status by PCR, it was found that the methylated status at the restriction enzyme (*Hpa*II) site was different between the 2 ramets.

In our recent RLGS analysis, we tried to detect the tissue-specific methylated status by conducting RLGS analysis of endosperm, embryo, leaf blade and leaf sheath and comparing the methylated status of each. As a result, there were 35 shared methylated spots in 3 defferent tissues among 58 methylated spots in total (Fig. 9). Fifty-six methylated spots detected in endosperm and embryo, including 19 tissue-specific spots. That is, a 34% difference was observed between endosperm and embryo. Next, 56 methylated spots were detected in endosperm and the leaf blade/sheath, including 20 tissue-spcific spots which showed a 36% difference between endosperm and leaf blade/sheath. Next, 49 methylated spots were detected in embryo and leaf blade/sheath, including 7 tissue-specific spots. That is, a 14% difference was observed between embryo and leaf blade/sheath. The result of comparison between endosperm and embryo (34%) showed a 2.4-fold larger difference with the comparison between embryo and leaf blade/sheath (14%), and the result of comparison between endosperm and blade/sheath (36%) showed a 2.6-fold larger difference than the comparison between embryo and leaf blade/sheath (14%). The leaf blade/sheath is differentiated from the embryo, but because the endosperm and embryo are independent tissues and have different functions and gene expression, the difference in methylated status is also considered large. Two recent studies show that endosperm DNA methylation is reduced genome-wide, and this reduction is likely to originate from demethylation in the central cell nucleus of the female gametophyte [Hsieh *et al.* 2009, and Gehring *et al.* 2009].
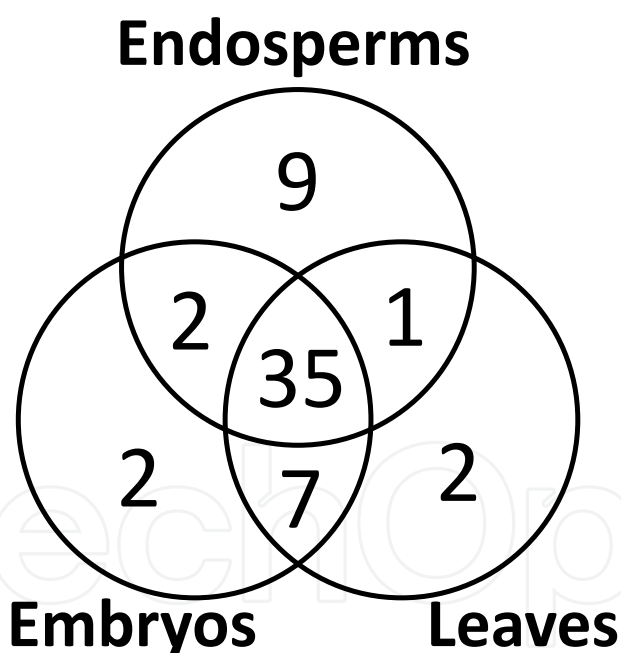

Fig. 9. Comparison of methylated spot among endosperm, embryo, and leaf blade/sheath of rice. We compared detection of methylated spots among 3 tissues. The numerals in circles indicate the number of methylated spots. This figure was cited from Hosobuchi, S. 2007.

Among the spots showing tissue-specific methylated status, expression analysis of spot 226 was conducted by RT-PCR. The *Msp*I/*Hpa*II site of spot 226 was positioned in the 5′ region of the Chr. 5 gene Zn-finger, C-x8-C-x5-C-x3-H type domain-containing protein (Os05g0497500) (Fig. 10A). When RT-PCR analysis (primer sets: rtpcr226-F, 5′-CTGGTGGAGATATGAAGAACAA-3′; rtpcr226-R: 5′-TATGTTTAACAACGGGATGTGT-3′)
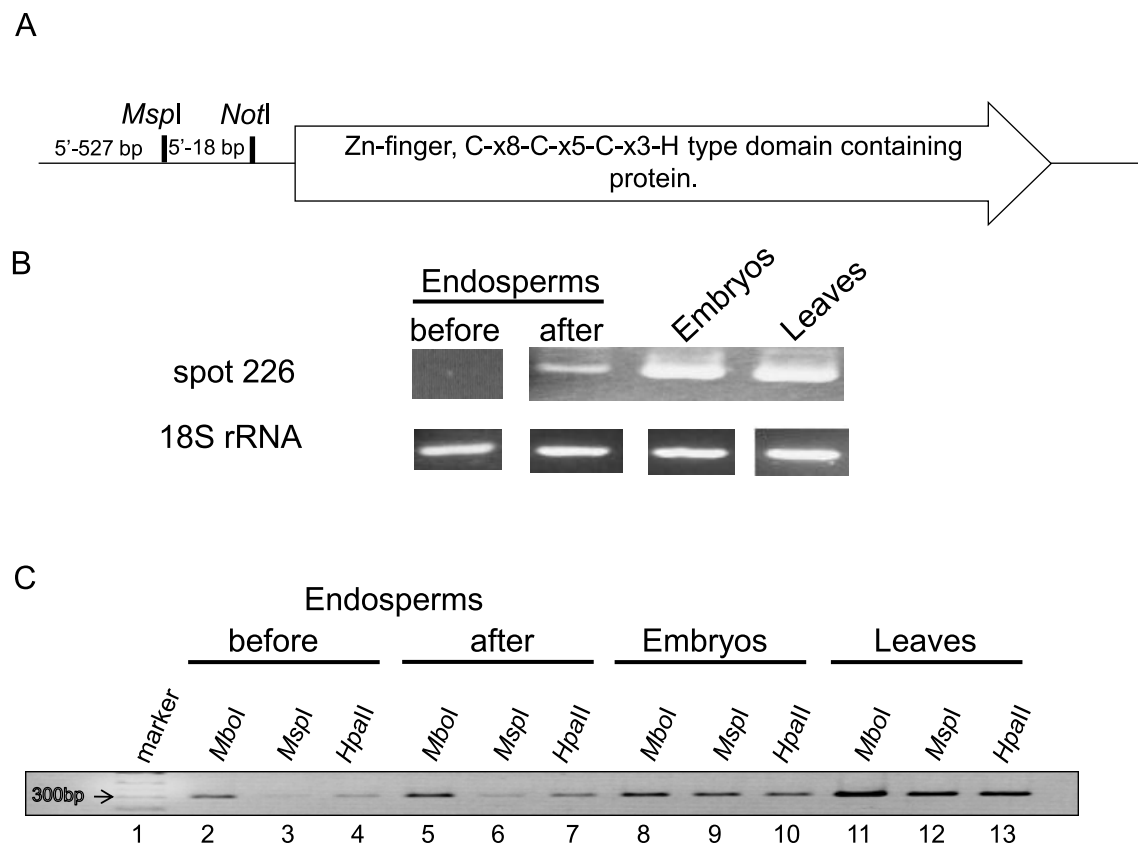
Fig. 10. Analysis of tissue-specific methylated spot 226. (A) Positions of *Not*I site and *Msp*I site toward a nearby gene are shown. Both sites are located on the 5' side of the Zn-finger, C-x8-C-x5-C-x3-H type domain-containing protein (Os05g0497500). (B) The results of RT-PCR analysis of Zn-finger, C-x8-C-x5-C-x3-H type domain-containing protein (Os05g0497500). This gene was not expressed in endosperm before water absorption, but it was expressed in embryos and leaf blades/sheaths, and weakly in endosperm after water absorption. 18S rRNA is a control. (C) In regard to the *Msp*I/*Hpa*II site of spot 226, we analyzed the methylation status in the endosperm before and after water absorption and in embryos and leaf blades/sheaths by the PCR method. We used *Mbo*I-treated DNA as a positive control. The *Msp*I/*Hpa*II site of spot 226 was methylated in embryos and leaf blades/sheaths. These figures were cited from Hosobuchi, S. 2007.

of this gene was conducted, this gene was expressed strongly in the embryo and leaf blade /sheath in which methylation was detected at the *Msp*I/*Hpa*II site (Fig. 10B) by RLGS analysis. Moreover, this gene never expressed in the endosperm before absorption of water, but weak expression was confirmed in the endosperm after absorption of water for 10 minutes (Fig. 10B). Next, when the methylated status at the *Msp*I/*Hpa*II site was analyzed by the PCR method, methylation was confirmed in the embryo and leaf blade/sheath (Fig. 10C). When comparing the endosperm before and after absorption of water, more PCR products were amplified in the endosperm after absorption of water (Fig. 10C. Comparison between Lanes 4 and 7). In the future, quantitative analysis will be required, but from the present results, it is assumed that the partial methylation rate of the *Msp*I/*Hpa*II site is higher in the endosperm after absorption of water than in the endosperm before the absorption of water. That is, there is the possibility that induction of DNA methylation and

gene expression may begin within as very a short time as 10 minutes. When the 5′ region is methylated, gene expression is usually inhibited, but our results are inconsistent with the general role. However, because the methylation analysis was conducted only at the *Msp*I/*Hpa*II site of spot 226, it is necessary to analyze the DNA methylated status in the promoter region of this gene widely and quantitatively.

4. Conclusion

RLGS is useful for genome wide surveillance of epigenetic alterations which effects to gene regulation and unknown phenomena with DNA methylation, because RLGS is suitable for exploratory studies on account of its low cost, and short set-up time. It can analyze any un-sequenced living things. As mentioned above, RLGS analysis of reciprocal hybrids in rice provided new interesting observations, and this strategy will apply to study of mammalian epigenetics.

5. Acknowledgments

6. References

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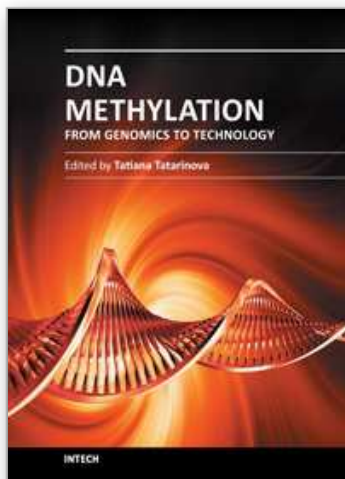
Epigenetics is one of the most exciting and rapidly developing areas of modern genetics with applications in many disciplines from medicine to agriculture. The most common form of epigenetic modification is DNA methylation, which plays a key role in fundamental developmental processes such as embryogenesis and also in the response of organisms to a wide range of environmental stimuli. Indeed, epigenetics is increasing regarded as one of the major mechanisms used by animals and plants to modulate their genome and its expression to adapt to a wide range of environmental factors. This book brings together a group of experts at the cutting edge of research into DNA methylation and highlights recent advances in methodology and knowledge of underlying mechanisms of this most important of genetic processes. The reader will gain an understanding of the impact, significance and recent advances within the field of epigenetics with a focus on DNA methylation.

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