Is the Fungus *Magnaporthe* Losing DNA Methylation?

Ken-ichi Ikeda,* Ba Van Vu,[†] Naoki Kadotani,[†] Masaki Tanaka,[†] Toshiki Murata,[†] Kohta Shiina,[†]
Izumi Chuma,[†] Yukio Tosa,[†] and Hitoshi Nakayashiki*^{†,1}

*Laboratory of Cell Function and Structure and [†]Laboratory of Plant Pathology, Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan

ABSTRACT The long terminal repeat retrotransposon, *Magnaporthe* gypsy-like element (MAGGY), has been shown to be targeted for cytosine methylation in a subset of *Magnaporthe oryzae* field isolates. Analysis of the F₁ progeny from a genetic cross between methylation-proficient (Br48) and methylation-deficient (GFS11-7-2) isolates revealed that methylation of the MAGGY element was governed by a single dominant gene. Positional cloning followed by gene disruption and complementation experiments revealed that the responsible gene was the DNA methyltransferase, *MoDMT1*, an ortholog of *Neurospora crassa* *Dim-2*. A survey of MAGGY methylation in 60 *Magnaporthe* field isolates revealed that 42 isolates from rice, common millet, wheat, finger millet, and buffelgrass were methylation proficient while 18 isolates from foxtail millet, green bristlegrass, Japanese panicgrass, torpedo grass, Guinea grass, and crabgrass were methylation deficient. Phenotypic analyses showed that *MoDMT1* plays no major role in development and pathogenicity of the fungus. Quantitative polymerase chain reaction analysis showed that the average copy number of genomic MAGGY elements was not significantly different between methylation-deficient and -proficient field isolates even though the levels of MAGGY transcript were generally higher in the former group. *MoDMT1* gene sequences in the methylation-deficient isolates suggested that at least three independent mutations were responsible for the loss of *MoDMT1* function. Overall, our data suggest that *MoDMT1* is not essential for the natural life cycle of the fungus and raise the possibility that the genus *Magnaporthe* may be losing the mechanism of DNA methylation on the evolutionary time scale.

CYTOSINE methylation is a chemical modification of DNA and is at the heart of an epigenetic mechanism involved in the control of a variety of biological processes, such as embryogenesis, cell differentiation, X-chromosome inactivation, genomic imprinting, and gene silencing (Laird and Jaenisch 1996). Cytosine methylation occurs in all three domains of life, namely, *Eubacteria*, *Archaeobacteria*, and *Eukaryota*, and is regarded as an ancestral mechanism that antedates the divergence of eukaryotes and prokaryotes (Tajima and Suetake 1998). Consistent with this, the catalytic enzymes of cytosine methylation, the cytosine DNA methyltransferases (DMTs), are conserved in eukaryotes and prokaryotes and contain nine well-conserved motifs arranged in the same order along

their primary structure (Kumar *et al.* 1994; Jurkowski and Jeltsch 2011).

Interestingly, however, the pattern and extent of genomic DNA methylation shows considerable diversity among eukaryotes (Yoder *et al.* 1997; Colot and Rossignol 1999; Rabinowicz *et al.* 1999). While cytosine DNA methylation is common in higher plants and vertebrates, it is virtually absent in certain lower organisms, such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Caenorhabditis elegans* (Antequera *et al.* 1984; Proffitt *et al.* 1984; Hodgkin 1994). Consistent with this observation, no gene related to a functional DMT has been detected in the genomes of the above-mentioned organisms (Ponger and Li 2005).

In filamentous fungi, cytosine methylation has been extensively studied in *Neurospora crassa* and *Ascobolus immersus*. Several genes involved in cytosine methylation have been identified in these organisms. *Dim-2* in *N. crassa* (Kouzminova and Selker 2001) and *Masc2* in *A. immersus* (Chernov *et al.* 1997) are involved in DNA methylation and transcriptional silencing in vegetative cells. *Masc1* in *A. immersus* is responsible for

Copyright © 2013 by the Genetics Society of America

doi: 10.1534/genetics.113.155978

Manuscript received July 31, 2013; accepted for publication August 13, 2013

Supporting information is available online at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.155978/-/DC1>.

¹Corresponding author: Laboratory of Cell Function and Structure, Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan.

E-mail: hnakaya@kobe-u.ac.jp

methylation induced premeiotically (MIP) during the sexual stage (Malagnac *et al.* 1997). *Rid*, the *N. crassa* ortholog of *Masc1*, is required for repeat-induced point mutation (RIP) during the sexual phase (Freitag *et al.* 2002) but whether it is an actual DNA methyltransferase is not yet known. Interestingly, the RIPed sequences appear to be recognized by an unidentified factor, which promotes methylation of histone H3 at Lys9 by histone methyltransferase, *Dim-5*, and subsequently cytosine DNA methylation by *Dim-2* (Tamaru and Selker 2001; Tamaru *et al.* 2003).

Magnaporthe oryzae is the causal agent of blast disease on many gramineous plants. Taxonomically, it belongs to the class Ascomycetes and family Sordariaceae, as does *N. crassa*. *Magnaporthe* isolates can be classified into several subgroups according to their host plants (Kato *et al.* 2000; Couch and Kohn 2002). Interestingly, we previously found that the DNA methylation status of the long terminal repeat (LTR) retrotransposon, *Magnaporthe* gypsy-like element (MAGGY), differed among the *M. oryzae* subgroups. For example, MAGGY elements were severely targeted for DNA methylation in isolates from rice and common millet but not in isolates from foxtail millet and torpedo grass (Nakayashiki *et al.* 2001). The methylation status of the MAGGY elements was unlikely to be associated with their copy number in the genome or the transpositional activity of the elements (Nakayashiki *et al.* 2001).

In this study, we addressed the mechanism underlying the difference in MAGGY methylation among *Magnaporthe* isolates. Our results show that a single dominant gene, *MoDMT1*, encoding a DIM-2-like DMT, was responsible for the difference among *Magnaporthe* isolates. The evolutionary implications of sporadic loss-of-function mutations in *MoDMT1* among *Magnaporthe* isolates are discussed.

Materials and Methods

Fungal strains and cultures

We used 56 *M. oryzae* isolates from nine host plants, 3 *M. grisea* isolates from crabgrass, and 1 *Magnaporthe* sp. isolate from buffelgrass (Table 1). All isolates were obtained by monoconidial isolation. Mating was performed on oatmeal agar media as described previously (Murakami *et al.* 2000). After 4 weeks of incubation, perithecia were formed and 80 isolates from 10 complete sets of tetrad asci were obtained.

Cosmid and plasmid construction

Genomic cosmid libraries of GFSI1-7-2 (foxtail millet isolate) and Br48 (wheat isolate) were constructed in the cosmid vector pMLF2 (An *et al.* 1996). Fifty micrograms of total DNA was partially digested with *Sau3AI* and ligated into the pMLF2 vector at the *Bam*HI site. The complementation plasmid pMoDMT1 was constructed as follows. An *Xho*I-*Not*I DNA fragment (6.2 kb) containing the *MoDMT1* gene was excised from a cosmid clone of Br48 and subcloned into pBluescript SK-. The disruption vector pMoDMT1Δ was constructed by

replacing a 2.4-kb *Bgl*II fragment in the *MoDMT1* gene with the Blasticidin S resistance gene cassette driven by the *TrpC* promoter.

Fungal transformation

Fungal transformation was carried out by a polyethylene glycol (PEG)-mediated method as described previously (Nakayashiki *et al.* 1999), with minor modifications. Fungal protoplasts were produced by incubating the mycelia in cell-wall digestion buffer (10 mM Na₂HPO₄, 1.2 M MgSO₄) containing 20 mg/ml lysing enzymes (Sigma, St. Louis, MO) for 3 hr. The protoplasts were collected by centrifugation at 800×g for 10 min and then washed with STC solution (20% sucrose, 20 mM Tris-HCl (pH 7.5, 25 mM CaCl₂). Plasmid DNA was added to 200 μl of the protoplast suspension and mixed. After 10 min of incubation on ice, 200 μl, 400 μl, and 800 μl of PEG solution (60% PEG 3350 in 25 mM Tris-HCl (pH 7.5, 25 mM CaCl₂) were added in a step-by-step manner, and the suspensions were incubated on ice for 10 min. The PEG solution was removed by centrifugation at 2000×g. Collected protoplasts were suspended in 1 ml of STC solution and mixed with 40 ml of regenerated agar media (20% sucrose, 0.3% yeast extract, 0.3% Casamino acids, 1.5% agarose). After overnight culture at 25°, selective agar media containing 100–400 μg/ml of hygromycin B or blasticidin S was overlaid. After 4–7 days of incubation at 25°, antibiotic-resistant regenerants on the plates were transferred to new potato dextrose agar (PDA) media containing antibiotics for secondary screening.

Extraction of fungal genomic DNA and Southern hybridization

Fungal mycelia were grown in CM liquid broth (0.3% Casamino acids, 0.3% yeast extract, 0.5% sucrose) at 25° for 1 week. Total genomic DNA was isolated as described previously (Nakayashiki *et al.* 1999) and digested with appropriate restriction enzymes. The digests were fractionated on a Tris-acetate-EDTA (TAE) agarose gel and transferred to Hybond N⁺ (GE Healthcare, Buckinghamshire, UK). Southern blot analysis was performed using an enhanced chemiluminescence (ECL) direct system (GE Healthcare) according to the manufacturer's instructions. For transposition assay by Southern analysis, a specific MAGGY probe (SB probe) and *Bam*HI digestion were used to generate a signal per MAGGY copy in the genome (Eto *et al.* 2001).

Extraction of fungal RNA and qPCR analysis

RNA isolation and cDNA synthesis were performed as described previously with slight modifications (Vu *et al.* 2012). Total RNA was isolated from mycelia using Sepasol RNA I Super (Nacalai tesque, Kyoto, Japan). One microgram of total RNA was subjected to cDNA synthesis using the ReverTra Ace qPCR RT master mix with gDNA remover kit (TOYOBO, Osaka, Japan). qRT-PCR assay was carried out using FastStart SYBR Green Master (Roche Applied Science, Mannheim, Germany) according to the manufacturer's

Table 1 *Magnaporthe* field isolates used in this study

Host plant	Isolate ^a	Methylation of MAGGY	Locality
<i>M. oryzae</i>			
<i>Oryza sativa</i> (rice)	Br13	+	Brazil
	CHNOS59-6-11	+	China
	Ishi5-1	+	China
	YUN05-2	+	China
	CD141G	+	Cote d'Ivoire
	CD180	+	Cote d'Ivoire
	CD262	+	Cote d'Ivoire
	GUY11	+	Guyana
	IN77-46-1-3	+	India
	IN77-32-1-3	+	India
	PO-02-7303	+	Indonesia
	PO-02-7305	+	Indonesia
	PO-02-7307	+	Indonesia
	PO-02-7402	+	Indonesia
	PO-02-7501	+	Indonesia
	PO-12-7301	+	Indonesia
	PO-12-7302	+	Indonesia
	88A	+	Japan
	117.4	+	Japan
	1601-3	+	Japan
	1836-1 ^b	+	Japan
	5A	+	Japan
	2201.1	+	Japan
	2303.1	+	Japan
	2403.1	+	Japan
	3103.2	+	Japan
	4411.2	+	Japan
	INA168 ^b	+	Japan
	KEN53-33	+	Japan
	P2b	+	Japan
	VTB6.1	+	Vietnam
<i>Setaria italica</i> (foxtail millet)			
	IN77-16-1-1 ^b	—	India
	IN77-17-1-1	—	India
	IN77-22-1-1	—	India
	GFS11-7-2 ^b	—	Japan
	NNS12-1-1	—	Japan
	NNS13-2-1	—	Japan
	NRS13-1-1 ^b	—	Japan
	NRS13-1-3 ^b	—	Japan
<i>Setaria viridis</i> (green bristlegrass)			
	KANSV1-4-1 ^b	—	Japan
	NI913 ^b	—	Japan
	NI1078 ^b	—	Japan
<i>Panicum miliaceum</i> (common millet)			
	NNPM1-2-4 ^b	+	Japan
	NNPM3-1-1	+	Japan
	STPM4-3-3	+	Japan
	SZPM1-1-1	+	Japan
	YNPM2-2-1	+	Japan
<i>Panicum bisulcatum</i> (Japanese panicgrass)			
	NI922	—	Japan
<i>Panicum maximum</i> (Guinea grass)			
	IN77-33-1-1 ^b	—	India
<i>Eleusine coracana</i> (finger millet)			
	IN77-31-1-1	+	India
	G10-1 ^b	+	Japan
	Z2-1	+	Japan
<i>Triticum aestivum</i> (wheat)			
	Br48 ^b	+	Brazil

(continued)

Table 1, continued

Host plant	Isolate ^a	Methylation of MAGGY	Locality
<i>Panicum repens</i> (torpedo grass)	Br116.5	+	Brazil
	IN77-28-1-1 ^b	—	India
	IN77-45-1-2 ^b	—	India
<i>Magnaporthe</i> sp. <i>Cenchrus ciliaris</i> (buffelgrass)	NI981 ^b	+	Japan
<i>Magnaporthe grisea</i> <i>Digitaria sanguinalis</i> (crabgrass)	Dig4-1	—	Japan
	IBDS4-1-1 ^b	—	Japan
	NI907	—	Japan

^a Isolates used for sequence analysis of the *MoDMT1* gene.

^b The list of *Magnaporthe* isolates were made on the basis of phylogenetic relatedness to rice isolates (Hirata *et al.* 2007 and this study).

instructions with specific primers for targets and an internal control gene (actin; MGG_03982). The primer sequences used are as follows: MoDMT1 (MGG_00889) (5'-gaacaaga tgcgggagaggctgtctc-3', 5'-ctccatgatgtcaaagtcggtgtctca-3'), MAGGY (5'-ctgctatccactgtctcacca-3', 5'-gaagccaacttcccaat ca-3'), and actin (5'-gcggttacacctctctaccac-3', 5'-agtctggatc tctgtctcaaag-3'). Fluorescence from DNA-SYBER Green complex was monitored by Thermal Cycler Dice Realtime System (Takara Bio, Otsu, Japan) throughout the PCR reaction. The level of target mRNA, relative to the mean of the reference actin gene, was calculated by the comparative Ct method.

To assess genomic copy number of MAGGY by qPCR, a pair of primers for a single-copy genomic region (5'-ccttct cgaccgcatacctgt-3', 5'-gcaatccaaaggggtgaagagt-3') with the MAGGY primers above were used. Genomic copy number of MAGGY was estimated by dividing the quantity value obtained with the MAGGY primer pair by the value with the single genomic region pair.

DNA sequencing and analysis

DNA sequencing was performed using either a plasmid or PCR product as a template. The PCR products were amplified directly from genomic DNA or cDNA and purified by gel extraction using Wizard SV Gel and PCR Cleanup System (Promega, Madison, WI). Large PCR fragments were sequenced by the primer-walking method to bridge entire sequences with various primers. DNA sequencing reaction was performed using the BigDye Terminator Cycle Sequencing Ready Reaction kit v. 3.1 (Applied Biosystems, Carlsbad, CA) and analyzed by the ABI Prism 3100 genetic analyzer. Primers to amplify rDNA, actin, and β -tubulin sequences were described previously (Hirata *et al.* 2007). Nucleotide sequences were aligned using CLUSTAL W (Thompson *et al.* 1994). The phylogenetic tree was constructed by the neighbor-joining method. The robustness of branching patterns was tested by 1000 bootstrap pseudo replications.

Pathogenicity test

Two cultivars of foxtail millet (*Setaria italica*), Aka-awa, and O-oke-awa, and a cultivar of wheat (*Triticum aestivum*),

Norin No. 4, were used for pathogenicity test. Twelve foxtail millet or wheat seeds were sown in Sakata super-mix soil (Sakata Seed Corp., Yokohama, Japan) or vermiculite, respectively, in a seedling case (5.5 × 15 × 10 cm) and grown at 20°–26° for 5–7 days. The conidia of *M. oryzae* strains were suspended in water, and their concentration was adjusted to 1 × 10⁵ conidia/ml with 0.01% Tween 20. Twenty milliliters of the conidial suspension was sprayed on the adaxial surface of leaves with a glass sprayer connected to an air compressor. The inoculated seedlings were kept in a humid and dark box at 20°–22° for 20 hr. They were then transferred to a controlled-environment room with a 10-hr photoperiod of fluorescent lighting (4000 lx) and incubated further at 20°–22°. Five days after inoculation, the symptoms were evaluated. The criteria of pathogenicity were those adopted by Murakami *et al.* (2000, 2003). The affected area was rated using six progressive grades from 0 to 5: 0, no visible evidence of infection; 1, expansion of lesions up to 5%; 2, expansion of lesions up to 20%; 3, expansion of lesions up to 50%; 4, expansion of lesions up to 80%; 5, expansion of lesions to >80% of the whole leaf.

Results

The DMM1 locus, encoding a DNA methyltransferase ortholog of Neurospora Dim-2, is responsible for the differences in MAGGY methylation status between the M. oryzae isolates, Br48 and GFSI1-7-2

We have previously shown that the retrotransposon MAGGY is methylated in the genome of *M. oryzae* isolates from rice, wheat, and common millet, but not in those from foxtail millet and torpedo grass (Nakayashiki *et al.* 2001). To gain genetic information concerning the difference in the methylation status of MAGGY among the *M. oryzae* isolates, we first crossed a methylation-proficient wheat isolate (Br48) with a methylation-deficient foxtail millet isolate (GFSI1-7-2).

The genomic DNA of 80 F₁ progeny (obtained from 10 complete sets of tetrad asci) from a cross between the foxtail millet and wheat isolates was analyzed for DNA methylation of MAGGY using the isoschizomers HapII (sensitive to CpG

Figure 1 Methylation of MAGGY DNA in F₁ progeny from a cross between wheat and foxtail millet isolates. (A) Fungal genomic DNA was digested with the isoschizomers *MspI* (M) and *HapII* (H) and probed with MAGGY. Parental isolates: wheat isolate Br48 and foxtail millet isolate GFSI1-7-2. F₁ progeny: one of the complete tetrad progeny sets from a single ascus, designated by numbers from 8K1 to 8N2. (B) Genetic mapping of *dmm1*. The genetic map was constructed using MAPMAKER on the basis of the segregation of DNA methylation of MAGGY in 80 F₁ progeny obtained from a cross between Br48 and GFSI1-7-2.

methylation) and *MspI* (insensitive to CpG methylation). Methylation-proficient and -deficient progeny were segregated in a 1:1 ratio in every tetrad ascus (Figure 1A), indicating that a single locus, which we named deficiency in methylation of *mAGGY* (*dmm1*), was involved in the difference in the methylation status of MAGGY among the *M. oryzae* isolates. Mapping of the *dmm1* locus using amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), and cosmid markers revealed that *dmm1* was located in linkage group 4 and closely linked to the AFLP marker, A0402AB, and to cosmid markers, T1-C6 and T1-E11 (Figure 1B). The end sequences of the cosmid markers indicated that the *dmm1* locus was closely linked to a homolog of *Neurospora dim-2* methyltransferase. Thus, we obtained a partial fragment of the *Magnaporthe* homolog of *dim-2* (*MoDMT1*, MGG_00889) by PCR and performed genomic Southern analysis. *EcoRV* digestion generated polymorphic bands in the *MoDMT1* locus between the parental GFSI1-7-2 and Br48 isolates (Supporting Information, Figure S1). A 4.5-kb RFLP marker perfectly cosegregated with a functional allele of *dmm1* in the 10 sets of tetrad progeny (Figure S1).

We then screened a genomic cosmid library of the wheat isolate, Br48, for the *MoDMT1* gene. From a resulting cosmid clone, a *XhoI*-*NotI* fragment, which contained the complete *MoDMT1* ORF and upstream (1511 bp) and downstream (892 bp) sequences, was further subcloned into pBluescript SK(-), establishing pMoDMT1. The methylation-deficient foxtail millet isolate, GFSI1-7-2, was transformed with

pMoDMT1 and subjected to the isoschizomer analysis for DNA methylation of MAGGY. The results showed that the MAGGY sequences were methylated in the pMoDMT1 transformants (Figure 2), suggesting that the *MoDMT1* gene was responsible for the methylation of MAGGY and, thus, that *dmm1* was a nonfunctional (recessive) allele of *MoDMT1*. To confirm these results, we also constructed a *MoDMT1* disruptant of the Br48 strain (Br48- $\Delta dim2$) using the conventional double crossing over gene replacement method (Figure S2) and introduced the cloned MAGGY element into Br48- $\Delta dim2$. As expected, no DNA methylation of the MAGGY sequences was detected in Br48- $\Delta dim2$ (Figure 2). Thus, we concluded that the *MoDMT1* gene is *Dmm1*.

Sequencing the *MoDMT1* gene from Br48 revealed that the entire ORF spanned 3948 bp and contained a single intron (138 bp). The deduced 1294 amino acid product possessed two conserved domains of the Dmmt1 methyltransferase family, a methyltransferase domain and a bromo-adjacent homology (BAH) domain (Callebaut *et al.* 1999) (Figure 3). The methyltransferase domain contains eight conserved motifs in most organisms (Kumar *et al.* 1994). The amino acid sequence of *MoDMT1* showed 52.2% identity and 83.6% similarity to that of *N. crassa Dim-2*. Methyltransferases belonging to the Dmmt1 family, such as Dnmt1, Masc2, and Met1, are somewhat diverged from the Dim-2 group (Smith *et al.* 2012; Figure S3).

The *MoDMT1* sequence of the methylation-deficient strain, GFSI1-7-2, exhibited 19 point mutations, a 30-bp insertion, and a 42-bp deletion compared with that of the

Figure 2 Gene disruption and complementation of *MoDMT1* in methylation-proficient and -deficient *M. oryzae* isolates. *MoDMT1* in the methylation-proficient Br48 isolate was disrupted by homologous recombination, resulting in the Br48- $\Delta dim2$ strain. Conversely, a functional *MoDMT1* gene was introduced into the methylation-deficient GFSI1-7-2 isolate. Methylation status of MAGGY DNA was examined by isoschizomer analysis with CpG methylation-insensitive *MspI* (M) and CpG methylation-sensitive *HpaII* (H). In the case of Br48 and Br48- $\Delta dim2$, a cloned MAGGY element was introduced prior to the isoschizomer analysis.

methylation-proficient strain, Br48 (Table 2). Among the sequence changes between Br48 and GFSI1-7-2, only one single-nucleotide substitution at nucleotide 3112 in the conserved methyltransferase domain caused a nonsense mutation in GFSI1-7-2 (Table 2 and Figure 3).

Genetic variation in *MoDMT1* among *Magnaporthe* isolates

To examine genetic variations in *Magnaporthe MoDMT1* genes in association with MAGGY methylation, we first evaluated the methylation status of MAGGY in various field isolates obtained from 11 different host plants by isoschizomer analysis (some examples are given in Figure S4). The wheat, finger millet, and crabgrass isolates did not possess endogenous MAGGY elements. Therefore, we examined MAGGY methylation in those isolates by introducing a cloned copy of

MAGGY. The results are summarized in Table 1. Interestingly, methylation-proficient and -deficient isolates were clearly segregated on the basis of the host-specific pathotypes. The methylation-proficient pathotypes were rice (31 isolates), common millet (5 isolates), finger millet (3 isolates), wheat (2 isolates), and buffelgrass (1 isolate), while the methylation-deficient pathotypes were foxtail millet (10 isolates), green bristlegrass (3 isolates), torpedo grass (2 isolates), Guinea grass (1 isolate), Japanese panicgrass (1 isolate), and crabgrass (3 isolates). We then sequenced the entire ORF of *MoDMT1* in 14 representative methylation-proficient and -deficient *Magnaporthe* isolates using a cosmid or PCR fragment as a template (Table 1 and Table 2) and performed sequence comparison among the isolates focusing on mutations that could cause a possible malfunction of *MoDMT1*. In addition, to know their phylogenetic relationship, a dendrogram of the 14 isolates was constructed using multilocus analysis of conserved genes (Figure 4).

Nonsense and frameshift mutations: The nonsense mutation at nucleotide 3112 found in the foxtail millet isolate, GFSI1-7-2, was also detected in another foxtail millet isolate, NRSI3-1-1 (Table 2 and Figure 3). This nonsense mutation appeared to be responsible for the loss of function of the *MoDMT1* protein in the isolates since the other changes found in GFSI1-7-2 and NRSI3-1-1 were also detected in methylation-proficient strains. Interestingly, however, the nonsense mutation at nucleotide 3112 was not conserved in the other methylation-deficient isolates, including foxtail millet isolates. Instead, in four of the methylation-deficient isolates, a 2-bp deletion at nucleotide 1654 shifted the reading frame for 10 residues before introducing a stop codon, causing a premature truncation of the *MoDMT1* protein (Table 2 and Figure 3). Thus, the frameshift mutation appeared to cause a loss of function of the *MoDMT1* protein in these isolates.

Insertions/deletions: Insertion/deletion events were detected at four sites in the *MoDMT1* gene among *M. oryzae* isolates. Except for the one described above, all the other insertion/deletion events did not cause a frameshift of the reading frame. Interestingly, the region from nucleotide 2465 to 2540 in the standard strain, 70-15, was composed of five repeats of a 15-nucleotide sequence (AGATGGCTCTGTCCT). In the other isolates, the copy number of this

Figure 3 Schematic representation of *MoDMT1* with amino acid substitutions in *M. oryzae* field isolates. Amino acid substitutions detected in methylation-deficient *M. oryzae* field isolates were presented in a scheme of *MoDMT1* protein. Numbers indicate the positions of amino acids in *MoDMT1* protein. The letters on the left side of the arrows show amino acids in the *MoDMT1* protein of the reference strain 70-15. The letters on the right side of them indicate the corresponding amino acids or stop codon in the field isolates. The names of the

M. oryzae field isolates were given above or below the amino acid substitutions found in the isolates. BAH, bromo-adjacent homology domain. *, a stop codon occurs 10 amino acids downstream from a 2-bp deletion at the position.

[illegible]

² Nucleotide polymorphic sites in the nucleotide sequence of the standard *M. oryzae* strain 70-15.

The nucleotide letters indicate missense mutations. The nucleotide letters indicate nonsense mutations.

THE INDICATED LETTERS INDICATE NONSENSE MUTATIONS.

In the more distantly related buffelgrass and crabgrass isolates, insertion and deletion events were more frequently found. Compared to the *MoDMT1* sequence of strain 70-15, five insertions (6, 6, 6, 12, and 45 bp) and five deletions (3, 3, 3, 6, and 45 bp) were observed in buffelgrass strains and three insertions (21, 45, and 93 bp) and 5 deletions (3, 3, 6, 12, and 60 bp) were observed in crabgrass strains (Table 2). Notably, despite the frequent insertion and deletion events, no frameshift mutations were detected in these isolates.

The buffelgrass and crabgrass isolates (*Magnaporthe* sp. and *M. grisea*, respectively) showed 399 (methylation-proficient buffelgrass isolate) and 559 (methylation-deficient crabgrass isolate) nucleotide substitutions (Figure S5). The rate of nucleotide substitution in the *MoDMT1* gene was considerably higher in the buffelgrass and crabgrass isolates than in the *M. oryzae* isolates. Interestingly, between the 70-15 strain and IBDS4-1-1 isolates, the nucleotide substitution rate in the *MoDMT1* gene was 15.6%, while that in the rDNA-ITS and β -tubulin sequences was 5.9 and 6.6%, respectively. As a reference, we also sequenced IBDS4-1-1 SET1-like histone lysine methyltransferase (MGG_15053), a gene involved in histone modification. The rate of nucleotide substitutions in the SET1-like histone lysine methyltransferase between the 70-15 and IBDS4-1-1 strains was 8.1%. Thus, the mutation rate in the *MoDMT1* gene was higher than that in the other genes examined.

Mutations in DNA Methyltransferase 851

Figure 4 Phylogenetic relationships of the *Magnaporthe* isolates used in the sequence analysis of *MoDMT1*. The phylogenetic relationships of the *Magnaporthe* isolates used in the sequence analysis of *MoDMT1* were analyzed using rDNA (internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2), actin, and β -tubulin sequences. A combined gene phylogenetic tree was constructed by the neighbor-joining method with 1000 bootstrap repetitions. Scale bar indicates genetic distance (substitution/site). Methylation-proficient isolates are shown in outline characters on a black background. The sites of collection and host plants of the *Magnaporthe* isolates are given in Table 1.

methylation-proficient isolates (1836-1, INA168, CHNOS59-6-11, and Br48) and five methylation-deficient isolates (GFSI1-7-2, IN77-20-1-1-1, IN7745-1-3, NI913, and IBDS4-1-1). Total RNA was extracted from vegetative mycelia and subjected to the analysis. *MoDMT1* expression was detectable in all isolates and the difference among them was within ~ 4.5 -fold (Figure S6). In addition, isolates with relatively high and low levels of *MoDMT1* expression were detected in both the methylation-proficient and -deficient groups (Figure S6), suggesting that the loss of *MoDMT1*-mediated DNA methylation of MAGGY in the *Magnaporthe* isolates was not due to defects in transcription.

Complementation test: In the four methylation-deficient isolates, NI913, IN77-28-1-1, IN77-45-1-2, and IBDS4-1-1,

no-nonsense or frameshift mutation was found in the *MoDMT1* gene (Table 2). To know whether the *MoDMT1* locus in these methylation-deficient isolates was responsible for the phenotype, p*MoDMT1* was introduced into NI913, IN77-45-1-2, and IBDS4-1-1 strains together with the cloned MAGGY element when the isolate possessed no or only a few copies of the MAGGY element. IN77-28-1-1 was also used in the transformation experiment. However, no transformant with p*MoDMT1* was obtained due to low transformation efficiency. Methylation of MAGGY DNA sequences was detected in all the p*MoDMT1*-transformants, indicating that mutation (s) in the *MoDMT1* locus caused the loss of MAGGY DNA methylation in those isolates (Figure S7). However, the rate of MAGGY methylation appeared to be lower in the IBDS4-1-1 strain compared to that in the NI913 and IN77-45-1-2 strains, suggesting that *MoDMT1* isolated from *M. oryzae* (wheat isolate) might not function as effectively in *M. grisea* (crabgrass isolate) as in *M. oryzae*. Overall, these results suggest that at least three independent loss-of-function mutations can occur in the *MoDMT1* locus in *Magnaporthe* species.

Phenotypic effects of the *MoDMT1* mutation in *M. oryzae*

To address the roles of *MoDMT1* in the life cycle of *M. oryzae*, we examined phenotypic differences between two sets of *MoDMT1* dominant and recessive strains, Br48 and Br48- $\Delta dim2$, and GFSI1-7-2 and its transformant with p*MoDMT1*. Characteristics of mycelial growth, mating, and pathogenicity on host plants were compared with those of the parent strains. However, no detectable *MoDMT1*-dependent difference was observed for any of the characteristics examined (Table 3). Viable ascospores were normally produced, even in the cross between two methylation-deficient strains (Table 3).

Finally, we examined the effect of *MoDMT1* on the control of the MAGGY element in the genome. Five isolates from methylation-proficient and five isolates from methylation-deficient natural MAGGY carriers were subjected to qRT-PCR analysis of the element to determine its transcript levels and genomic copy number. The MAGGY transcript level in the methylation-deficient isolates was 3.7 times higher on average than that in the methylation-proficient isolates (Figure 5A), suggesting that DNA methylation contributed significantly to repressing the transcription of MAGGY in *M. oryzae*.

Table 3 Phenotypic comparison between methylation-proficient and -deficient *M. oryzae* strains

Strain	Methylation of MAGGY	Growth on rich media ^a	Pathogenicity on host plants	Cross with Br48	Cross with Br48- $\Delta dim2$
GFSI1-7-2 ^b	—	6.40 $\pm$ 0.12	+++	Fertile	Fertile
GFSI1-7-2+ <i>MoDMT1</i>	+	6.57 $\pm$ 0.13	+++	Fertile	Fertile
Br48 ^c	+	6.18 $\pm$ 0.10	+++	n.t.	n.t.
Br48- $\Delta dim2$	—	6.15 $\pm$ 0.06	+++	n.t.	n.t.

n.t., not tested.

^a Colony diameter (cm) after 10 days culture on CM agar media.

^b Mating type, MAT1-2.

^c Mating type, MAT1-1.

However, interestingly, the average genomic copy number of MAGGY in the methylation-deficient isolates did not differ significantly from that in the methylation-proficient isolates (Figure 5B), suggesting that DNA methylation did not function effectively as a defense mechanism against MAGGY proliferation in the genome on the evolutionary time scale. To further address this point, transpositional activity of the MAGGY element was also examined in methylation-proficient and -deficient F1 progeny from a cross between GFSI1-7-2 and Br48. New transposition events were detected as additional bands on Southern blots between two sister progeny. The results showed that there was no statistically significant difference in the transposition activity between the two progeny groups (Table 4), supporting our previous conclusion that DNA methylation was not the main force repressing MAGGY proliferation in the *M. oryzae* genome, although it did have suppressive effects on MAGGY transcription (Nakayashiki *et al.* 2001).

Discussion

Ponger and Li (2005) reported that eukaryotic DMTs can be grouped into at least six subfamilies, *dnmt1* to *dnmt6*. In the *M. oryzae* genome, two putative DMT-like genes were identified and designated as *MoDMT1* and *MoDMT2* (Y. H. Lee, personal communication). *MoDMT1* and *MoDMT2* are orthologous to *Dim-2* and *Rid* in *N. crassa* and belong to the *dnmt1* and *dnmt4* subfamilies, respectively (Ponger and Li 2005). *MoDMT2* is closely related to *Rid*, which is responsible for RIP in *N. crassa*. Since RIP also operates in *M. oryzae* (Ikeda *et al.* 2002), it is tempting to speculate that *MoDMT2* has a role in RIP in *M. oryzae*.

In this study, we show that the lack of DNA methylation of the MAGGY elements in certain *M. oryzae* field isolates was caused by at least three independent mutations in the *MoDMT1* gene. The loss of DNA methylation in *M. oryzae* did not cause any detectable phenotypes with regard to hyphal growth, fertility, or pathogenicity. In addition, it did not significantly affect transpositional activity of the MAGGY element despite its suppressive effect at the transcriptional level. In *N. crassa*, *Dim-2* is also dispensable for normal growth and fertilization, including RIP. In contrast, *mascl* and *dmtA*, the homologs of *rid*, in *A. immersus* and *Aspergillus nidulans*, respectively, are essential for fertility (Malagnac *et al.* 1997; Lee *et al.* 2008). Interestingly, *mascl*, a *dnmt1*-like methyltransferase in *A. immersus*, was dispensable for fertility and DNA methylation of natural genomic retroelement-like repeats and tandemly repeated rDNA despite showing methyltransferase activity *in vitro* (Malagnac *et al.* 1999). Thus, in fungal taxa, *rid*-like genes may have more biological roles than do *dnmt1* family members.

The results of this study are curious with regard to the significance of DNA methylation in *Magnaporthe*. Our survey of MAGGY methylation status among *Magnaporthe* field isolates favored the hypothesis that DNA methylation by *MoDMT1* is dispensable because methylation-deficient

Figure 5 Quantitative analysis of genomic copy number and mRNA levels of the MAGGY element in methylation-proficient and -deficient *M. oryzae* isolates. Five methylation-proficient [Me (+)] and five methylation-deficient [Me (-)] *M. oryzae* isolates were grown in rich liquid media for 5 days. Genomic DNA and total RNA were extracted from mycelia and subjected to qPCR analysis of MAGGY DNA and qRT-PCR analysis of MAGGY transcript, respectively. (A) Values indicate average MAGGY transcript levels in methylation-deficient *M. oryzae* isolates (open bar) relative to those in methylation-proficient isolates (solid bar). *Actin* (MGG_03982) was used to normalize the data. This experiment was performed with two biological replicates with triplet technical replicates. Error bars represent standard deviations. *, significant difference (two-tailed *t*-test, *P* < 0.05). (B) Values indicate average genomic copy numbers of the MAGGY element in methylation-proficient (solid bar) and -deficient (open bar) *M. oryzae* isolates. The genomic copy number of MAGGY was estimated by fold amplification relative to a single copy gene. This experiment was performed with triplet technical replicates. Error bars represent standard deviations.

Magnaporthe isolates were as common as methylation-proficient isolates in the field (Table 1). In addition, the rate of mutation in the *MoDMT1* gene was considerably higher than that in other genes, such as β -tubulin and SET1-like histone lysine methyltransferase. Dhillon *et al.* (2010) reported that in certain field isolates of the wheat pathogen, *Mycosphaerella graminicola*, *dim-2*-like MgDNMT, which is responsible for all detectable cytosine methylation in this fungus, is amplified in the genome, but subsequently inactivated due to RIP mutations. This also suggests that *Dim2*-like genes are dispensable for hemibiotrophic ascomycete pathogens to become established in their niches under natural conditions.

On the other hand, two lines of evidence in this study argue against the above hypothesis. First, while methylation-deficient and -proficient isolates were sporadically found among *Magnaporthe* isolates, only a single type (methylation-deficient or -proficient) was observed within a particular pathotype. For example, all the foxtail millet isolates tested in this study were deficient in MAGGY methylation, while all the rice isolates were methylation proficient regardless of their sites of collection (Table 1). Intriguingly, there were at least three different types of loss-of-function mutation in the isolates from foxtail millet and related *Setaria* plants. This suggested that these mutations occurred independently in these isolates after diversification from the ancestral *Setaria*-infecting strain. Recently, it has been shown that epigenetic

Table 4 Transposition of MAGGY in methylation-proficient and -deficient F₁ progeny of *M. oryzae*

Methylation-deficient progeny	Number of transpositions ^a	Methylation-proficient progeny	Number of transpositions
1-K1	9	1-M1	9
1-K2	8	1-M2	4
1-L1	3	1-N1	8
1-L2	8	1-N2	3
2-K1	3	2-M1	3
2-K2	0	2-M2	2
2-L1	12	2-N1	12
2-L2	9	2-N2	7
Average ^b	6.5		6

^a Number of additional MAGGY bands observed in only one of the two sister progeny obtained from a cross between Br48 and GFS11-7-2.

^b No significant difference between methylation-proficient and -deficient F₁ progeny by *t*-test (*P* < 0.05).

regulation plays an important role in secondary metabolite production in various fungi, including *Aspergillus* and *Fusarium* (Lee *et al.* 2009; Reyes-Dominguez *et al.* 2012). Thus it seems possible that DNA methylation-mediated gene regulation could influence the competitiveness of the fungus on a specific host plant species through the production of secondary metabolites including fungal effectors.

Second, even though several *Magnaporthe* isolates appeared to have lost the ability to methylate MAGGY DNA, the entire *MoDMT1* ORF was retained in most cases with the exception of the *Setaria*-infecting isolates. The fact that the *MoDMT1* ORF has been maintained despite the high rate of mutations (including deletions and insertions) suggests that the gene plays a beneficial role in *Magnaporthe* other than methylation of transposable elements (TEs). However, this role may not be easily detectable experimentally.

DNA methylation is believed to be a potent defense mechanism against TEs in a wide range of eukaryotic organisms. In this study, DNA methylation actually functioned effectively to suppress the MAGGY element at the transcriptional level (Figure 5A). However, the rate of transposition and genomic copy number of MAGGY did not differ significantly between the methylation-deficient foxtail millet isolates and methylation proficient rice isolates (Figure 5B and Table 4). These results were consistent with our previous observation; the level of MAGGY transcripts did not always correlate with the rate of its transposition (Nakayashiki *et al.* 2001; Murata *et al.* 2007). In this regard, it is interesting that *S. cerevisiae*, which possesses no DNA methylation or RNAi, is one of the most successful eukaryotes at protecting the genome from TE proliferation. The transcript of the Ty1 LTR-retrotransposon is known to be very abundant (0.1–0.8% of total RNA) in the cell; however, Ty1 transposition seldom occurs (Elder *et al.* 1981; Curcio *et al.* 1990). Thus, as suggested previously, there is likely to be an effective post-transcriptional defense against TE transposition into the genome at least in certain organisms including *M. oryzae* (Garfinkel *et al.* 2003; Murata *et al.* 2007; Matsuda and Garfinkel 2009).

The importance of DNA methylation in gene regulation and development seems to differ considerably among eukaryotic organisms. In some higher eukaryotes such as mouse and *Arabidopsis*, mutants deficient in DNA methylation show embryonic lethality or severe developmental defects (Li *et al.*

1992; Kakutani *et al.* 1996; Okano *et al.* 1999; Saze *et al.* 2003), suggesting that DNA methylation is essential for normal growth and development. In contrast, other eukaryotes, including well-studied model organisms such as *S. cerevisiae*, *S. pombe*, and *C. elegans*, are devoid of DNA methylation. It seems that there are two different evolutionary strategies for DNA methylation. Some organisms are evolving to utilize DNA methylation for gene regulation while others are discarding DNA methylation. Overall, our data in *M. oryzae* indicate that *MoDMT1*-mediated DNA methylation plays only a very minor, if any, role in normal growth, pathogenicity, and transposon control and, thus, is not essential for the natural life cycle of the fungus. Sporadic loss-of-function mutations of the *MoDMT1* gene may indicate that *Magnaporthe* is losing DNA methylation on the evolutionary time scale.

Acknowledgments

We are grateful to Dr. H. Kato, formerly a Professor at Kobe University, for providing the isolates, detailed information on them, and his valuable suggestions. This research was partly supported by a Grant-in-Aid for Young Scientists B (No. 19780036) and by Grant-in-Aid for Scientific Research (B) (No. 22380031) from the Japan Society for the Promotion of Science.

Literature Cited

- An, Z., M. L. Farman, A. Budde, S. Taura, and S. A. Leong, 1996 New cosmid vectors for library construction, chromosome walking and restriction mapping in filamentous fungi. *Gene* 176: 93–96.
- Antequera, F., M. Tamame, J. R. Villanueva, and T. Santos, 1984 DNA methylation in the fungi. *J. Biol. Chem.* 259: 8033–8036.
- Callebaut, I., J. C. Courvalin, and J. P. Mornon, 1999 The BAH (bromo-adjacent homology) domain: a link between DNA methylation, replication and transcriptional regulation. *FEBS Lett.* 446: 189–193.
- Chernov, A. V., P. Vollmayr, J. Walter, and T. A. Trautner, 1997 Msc2, a C5-DNA-methyltransferase from *Ascobolus immersus* with similarity to methyltransferases of higher organisms. *Biol. Chem.* 378: 1467–1473.
- Colot, V., and J.-L. Rossignol, 1999 Eukaryotic DNA methylation as an evolutionary device. *BioEssays* 21: 402–411.
- Couch, B. C., and L. M. Kohn, 2002 A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. *Mycologia* 94: 683–693.

- Curcio, M. J., A. M. Hedge, J. D. Boeke, and D. J. Garfinkel, 1990 Ty RNA levels determine the spectrum of retrotransposition events that activate gene expression in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 220: 213–221.
- Dhillon, B., J. R. Cavaletto, K. V. Wood, and S. B. Goodwin, 2010 Accidental amplification and inactivation of a methyltransferase gene eliminates cytosine methylation in *Mycosphaerella graminicola*. *Genetics* 186: 67–77.
- Elder, R. T., T. P. St. John, D. T. Stinchcomb, R. W. Davis, S. Scherer *et al.*, 1981 Studies on the transposable element Ty1 of yeast. *Cold Spring Harb. Symp. Quant. Biol.* 45: 581–591.
- Eto, Y., K. Ikeda, I. Chuma, T. Kataoka, S. Kuroda *et al.*, 2001 Comparative analyses of the distribution of various transposable elements in *Pyricularia* and their activity during and after the sexual cycle. *Mol. Gen. Genet.* 264: 565–577.
- Freitag, M., R. L. Williams, G. O. Kothe, and E. U. Selker, 2002 A cytosine methyltransferase homologue is essential for repeat-induced point mutation in *Neurospora crassa*. *Proc. Natl. Acad. Sci. USA* 99: 8802–8807.
- Garfinkel, D. J., K. Nyswander, J. Wang, and J. Y. Cho, 2003 Post-transcriptional cosuppression of Ty1 retrotransposition. *Genetics* 165: 83–99.
- Hirata, K., M. Kusaba, I. Chuma, J. Osue, H. Nakayashiki *et al.*, 2007 Speciation in *Pyricularia* inferred from multilocus phylogenetic analysis. *Mycol. Res.* 111: 799–808.
- Hodgkin, J., 1994 Epigenetics and the maintenance of gene activity states in *Caenorhabditis elegans*. *Dev. Genet.* 15: 471–477.
- Ikeda, K., H. Nakayashiki, T. Kataoka, H. Tamba, Y. Hashimoto *et al.*, 2002 Repeat-induced point mutation (RIP) in *Magnaporthe grisea*: implications for its sexual cycle in the natural field context. *Mol. Microbiol.* 45: 1355–1364.
- Jurkowski, T. P., and A. Jeltsch, 2011 On the evolutionary origin of eukaryotic DNA methyltransferases and Dnmt2. *PLoS ONE* 6: e28104.
- Kakutani, T., J. A. Jeddeloh, S. K. Flowers, K. Munakata, and E. J. Richards, 1996 Developmental abnormalities and epimutations associated with DNA hypomethylation mutations. *Proc. Natl. Acad. Sci. USA* 93: 12406–12411.
- Kato, H., M. Yamamoto, T. Yamaguchi-Ozaki, H. Kadouchi, Y. Iwamoto *et al.*, 2000 Pathogenicity, mating ability and DNA restriction fragment length polymorphisms of *Pyricularia* populations isolated from Gramineae, Bambusideae and Zingiberaceae plants. *J. Gen. Plant Pathol.* 66: 30–47.
- Kouzminova, E., and E. U. Selker, 2001 *dim-2* encodes a DNA methyltransferase responsible for all known cytosine methylation in *Neurospora*. *EMBO J.* 20: 4309–4323.
- Kumar, S., X. Cheng, S. Klimasauskas, S. Mi, J. Posfai *et al.*, 1994 The DNA (cytosine-5) methyltransferases. *Nucleic Acids Res.* 22: 1–10.
- Laird, P. W., and R. Jaenisch, 1996 The role of DNA methylation in cancer genetics and epigenetics. *Annu. Rev. Genet.* 30: 441–464.
- Lee, D. W., M. Freitag, E. U. Selker, and R. Aramayo, 2008 A cytosine methyltransferase homologue is essential for sexual development in *Aspergillus nidulans*. *PLoS ONE* 3: e2531.
- Lee, I., J. H. Oh, E. K. Shwab, T. R. Dagenais, D. Andes *et al.*, 2009 HdaA, a class 2 histone deacetylase of *Aspergillus fumigatus*, affects germination and secondary metabolite production. *Fungal Genet. Biol.* 46: 782–790.
- Li, E., T. H. Bestor, and R. Jaenisch, 1992 Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69: 915–926.
- Malagnac, F., B. Wendel, C. Goyon, G. Faugeton, D. Zickler *et al.*, 1997 A gene essential for *de novo* methylation and development in *Ascombolus* reveals a novel type of eukaryotic DNA methyltransferase structure. *Cell* 91: 281–290.
- Malagnac, F., A. Grégoire, C. Goyon, J. L. Rossignol, and G. Faugeton, 1999 *Masc2*, a gene from *Ascombolus* encoding a protein with a DNA-methyltransferase activity *in vitro*, is dispensable for *in vivo* methylation. *Mol. Microbiol.* 31: 331–338.
- Matsuda, E., and D. J. Garfinkel, 2009 Posttranslational interference of Ty1 retrotransposition by antisense RNAs. *Proc. Natl. Acad. Sci. USA* 106: 15657–15662.
- Murakami, J., Y. Tosa, T. Kataoka, R. Tomita, J. Kawasaki *et al.*, 2000 Analysis of host species specificity of *Magnaporthe grisea* toward wheat using a genetic cross between isolates from wheat and foxtail millet. *Phytopathology* 90: 1060–1067.
- Murakami, J., R. Tomita, T. Kataoka, H. Nakayashiki, Y. Tosa *et al.*, 2003 Analysis of host species specificity of *Magnaporthe grisea* toward foxtail millet using a genetic cross between isolates from wheat and foxtail millet. *Phytopathology* 93: 42–45.
- Murata, T., N. Kadotani, M. Yamaguchi, Y. Tosa, S. Mayama *et al.*, 2007 siRNA-dependent and -independent post-transcriptional co-suppression of the LTR-retrotransposon MAGGY in the phytopathogenic fungus *Magnaporthe oryzae*. *Nucleic Acids Res.* 35: 5987–5994.
- Nakayashiki, H., K. Kiyotomi, Y. Tosa, and S. Mayama, 1999 Transposition of the retrotransposon MAGGY in heterologous species of filamentous fungi. *Genetics* 153: 693–703.
- Nakayashiki, H., K. Ikeda, Y. Hashimoto, Y. Tosa, and S. Mayama, 2001 Methylation is not the main force repressing the retrotransposon MAGGY in *Magnaporthe grisea*. *Nucleic Acids Res.* 29: 1278–1284.
- Okano, M., D. W. Bell, D. A. Haber, and E. Li, 1999 DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell* 99: 247–257.
- Ponger, L., and W. H. Li, 2005 Evolutionary diversification of DNA methyltransferases in eukaryotic genomes. *Mol. Biol. Evol.* 22: 1119–1128.
- Proffitt, J. H., J. R. Davie, D. Swinton, and S. Hattman, 1984 5-Methylcytosine is not detectable in *Saccharomyces cerevisiae* DNA. *Mol. Cell. Biol.* 4: 985–988.
- Rabinowicz, P. D., K. Schutz, N. Dedhia, C. Yordan, L. D. Parnell *et al.*, 1999 Differential methylation of genes and retrotransposons facilitates shotgun sequencing of the maize genome. *Nat. Genet.* 23: 305–308.
- Reyes-Dominguez, Y., S. Boedi, M. Sulyok, G. Wiesenberger, N. Stoppacher *et al.*, 2012 2012 Heterochromatin influences the secondary metabolite profile in the plant pathogen *Fusarium graminearum*. *Fungal Genet. Biol.* 49: 39–47.
- Saze, H., O. M. Scheid, and J. Paszkowski, 2003 Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. *Nat. Genet.* 34: 65–69.
- Smith, K. M., P. A. Phatale, E. L. Bredeweg, L. R. Connolly, K. R. Pomraning *et al.*, 2012 Epigenetics of filamentous fungi, pp. 1–43 in *Encyclopedia of Molecular Cell Biology and Molecular Medicine*, Ed. 2, edited by R. A. Meyers. Wiley-VCH, Weinheim, Germany.
- Tajima, S., and I. Suetake, 1998 Regulation and function of DNA methylation in vertebrates. *J. Biochem.* 123: 993–999.
- Tamaru, H., and E. U. Selker, 2001 A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. *Nature* 414: 277–283.
- Tamaru, H., X. Zhang, D. McMillen, P. B. Singh, J. Nakayama *et al.*, 2003 Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in *Neurospora crassa*. *Nat. Genet.* 34: 75–79.
- Thompson, J. D., D. G. Higgins, and T. J. Gibson, 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.
- Vu, V. B., K. Itoh, Q. B. Nguyen, Y. Tosa, and H. Nakayashiki, 2012 Cellulases belonging to glycoside hydrolase families 6 and 7 contribute to the virulence of *Magnaporthe oryzae*. *Mol. Plant Microbe Interact.* 25: 1135–1141.
- Yoder, J. A., C. P. Walsh, and T. H. Bestor, 1997 Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet.* 13: 335–340.

Communicating editor: E. U. Selker

GENETICS

Supporting Information

<http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.155978/-/DC1>

Is the Fungus *Magnaporthe* Losing DNA Methylation?

Ken-ichi Ikeda, Ba Van Vu, Naoki Kadotani, Masaki Tanaka, Toshiki Murata, Kohta Shiina,
Izumi Chuma, Yukio Tosa, and Hitoshi Nakayashiki

Figure S1 Segregation of a MoDMT1 RFLP fragment in 80 F1 progeny obtained from a cross between wheat (Br48) and foxtail millet (GFSI1-7-2) isolates

(A) RFLP analysis between wheat (W) and foxtail millet (F) isolates. Genomic DNA was digested with ten restriction enzymes (shown above the lanes) and probed with a fragment of the MoDMT1 gene. Arrowheads indicate polymorphic bands. (B) Southern blots of *EcoRV*-digested DNA of 80 F1 progeny. The polymorphic band at 4.5 kb was segregated in a 1:1 ratio. Parental isolates: foxtail millet isolate (F) and wheat isolate (W). F1 progeny: 10 complete sets of tetrad asci (SET1–SET10). K to N are representative tetrad in each set. A and B indicate negative and positive DNA methylation of MAGGY, respectively.

Figure S2 Disruption of the MoDMT1 gene in the wheat isolate Br48

A, Schematic representation of wild-type and disrupted loci of MoDMT1 (MGG 00889) . The MoDMT1 disruptant, Br48- Δ dim2 was constructed through targeted gene disruption by homologous recombination using the disruption vector pMoDMT1 Δ . pMoDMT1 Δ was made by replacing a 2.4-kb internal *Bgl*II fragment in the MoHMT1 gene with the Blasticidin S resistance gene cassette driven by the TrpC promoter. B, Genomic Southern analysis of Br48 (WT), Br48- Δ dim2 (1) and an ectopic mutant (2). Fungal genomic DNA was digested with *Xho* I, and probed with the entire MoDMT1 coding sequence.

Figure S3 Phylogenetic relationship of MoDMT1 with other eukaryotic DNA methyltransferases

(A) A phylogenetic tree of eukaryotic DNA methyltransferases (DMT). The unrooted phylogenetic tree was constructed by neighbor-joining method after alignment of deduced amino acid sequences of DMT protein. Values at the interior nodes are bootstrap percentages derived from 1000 replications. The scale bar indicates an evolutionary distance of 0.1 amino acid substitution per position in the sequence.

(B) Multiple alignment of the amino acid sequences of the Dnmt1 methyltransferase family proteins. Eight conserved domains were indicated as I, IV, V, VI, VII, VIII, IX, and X.

Figure S4 DNA methylation of MAGGY in *Magnaporthe* field isolates from different host plants

Methylation status of MAGGY DNA in the *Magnaporthe* field isolates was examined by isoschizomer analysis with methylation-insensitive *MspI* (M) and methylation-sensitive *HpaII* (H). In the cases of G10-1 and IBDS4-1-1, a cloned MAGGY element was introduced in them prior to the isoschizomer analysis.

[illegible]

Figure S6 Transcription levels of MoDMT1 in methylation-proficient and -deficient *M. oryzae* isolates

Four methylation-proficient (+) and five methylation-deficient (-) *M. oryzae* isolates were grown in rich liquid media for five days and subjected to quantitative RT-PCR (qRT-PCR) analysis of the MoDMT1 transcript. Values indicate expression levels of MoDMT1 relative to the internal control gene, actin (MGG_03982).

Figure S7 Genetic complementation of the MoDMT1 gene in the methylation deficient isolates, NI913, IN77-45-1-2 and IBDS4-1-1.

The plasmid, pMoDMT1 carrying a functional MoDMT1 from the Br48 strain was introduced into the methylation deficient isolates, NI913, IN77-45-1-2 and IBDS4-1-1. In the cases of IN77-45-1-2 and IBDS4-1-1, a cloned MAGGY element was simultaneously introduced at transformation. Methylation status of MAGGY DNA was then examined by isoschizomer analysis with CpG methylation-insensitive *MspI* (M) and methylation-sensitive *HapII* (H).