



Roles of histone lysine methyltransferases in morphological changes during infection processes of *Magnaporthe oryzae*

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Doctoral Dissertation

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processes of *Magnaporthe oryzae***

PHAM THI MINH KIEU

The Graduate School of Agricultural Science
KOBE UNIVERSITY

July, 2014

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*Magnaporthe oryzae***

イネ科植物いもち病菌の感染器官形成におけるヒストンメチル基
転移酵素の役割の解析

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List of abbreviation

Abbreviations used in this thesis are listed below in alphabetical order.

Abbreviation	Description
KMT	Histone lysine methyltransferase
KDM	Histone demethylase
SET	Su(var)3-9, Enhancer of Zeste, Trithorax
MLL	Myeloid/lymphoid or mixed- lineage leukemia
Dim-	Defective in methylation-
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation sequencing
mRNA-seq	Messenger RNA sequencing
me	Methylation
ac	Acetylation
H3K4me	Histone 3 Lysine 4 methylation
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
GNAT	General Control Non-Derepressible 5-related N-Acetyltransferases
PTM	Posttranslational modification
PRMT	Protein arginine methyltransferase
HYG	Hygromycin phosphotransferase cassette
PDA	Potato dextrose agar
EDTA	Ethylendiamin tetraacetic acid
PEG	Polyethylene glycol
HR	hypersensitive reaction
cAMP	Cyclic adenosine monophosphate
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
Bp	Base pair
cDNA	Complementary DNA
GH	Glycosyl hydrolase
MM-C	Carbohydrate-limited minimal medium

DEGs	Differentially expressed genes
KOG	euKaryotic Orthologous Group
FC	Fold change
GO	Gene ontology
DSB	DNA double strand break

CHAPTER I

Background information

4. General introduction

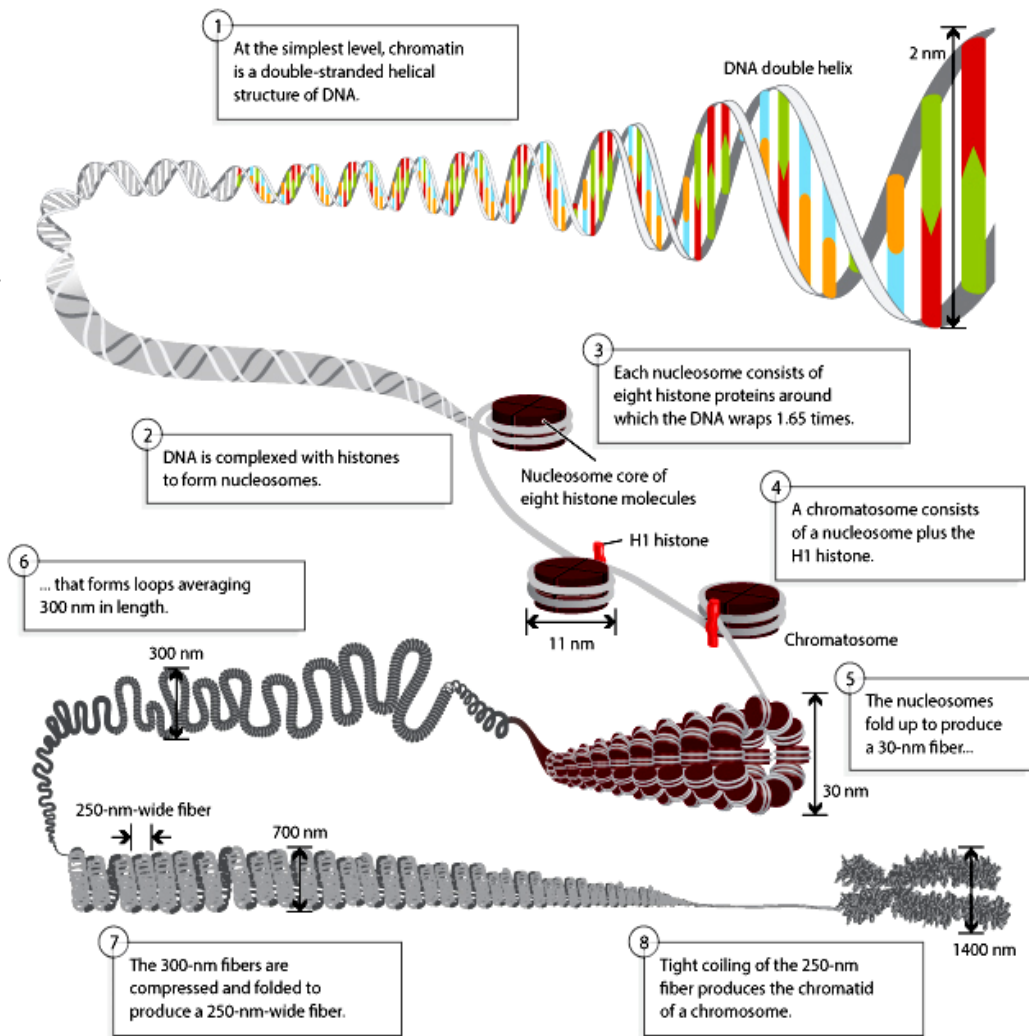
In recent years, epigenetics has been very popular and became a newly emerging trend in the molecular biotechnology field. Epigenetics was originally defined as the study of heritable changes in gene activity that are not caused by changes in the DNA sequence. For example, chromatin modifications are mechanisms that produce such changes without altering the underlying DNA sequence. Chromatin modification includes chromatin-remodeling, histone modification, DNA methylation, insertion of histone variants, and the effects of non-coding RNAs (ncRNAs), known to affect chromatin structure (Dulac C., 2010). Chromatin structure and function can also be affected by other post-translational modifications of the amino-terminal tails of nucleosomal histones. DNA methylation, chromatin remodeling, and histone modification do not act independently from each other but strongly interrelated *in vivo* (Steinfeld et al., 2007). These mechanisms could regulate gene expression, leads to different types and functions of organism cells. The understanding of the influence of chromatin on gene regulation suggests that DNA methylation and histone post-translational modifications lead to the recruitment of protein complexes that regulate transcription. Previous studies revealed that transcription occurs when favored by mixtures of complex modification proteins that can have several roles in cells (reviewed by Berger, 2007).

5. Chromatin structure and nucleosomes

Chromatin is the combinations or complexes of DNA and proteins. In eukaryotic cell, chromatin is packed into the nucleus of living cells, and contains several hundred centimeters of DNA. The primary function of chromatin are to package DNA into smaller

volume to fit in the cell; to strengthen the DNA to allow mitosis; to prevent DNA damage and to control gene expression and DNA replication. The major proteins of chromatin are the histones, small proteins containing a high proportion of basic amino acids (arginine and lysine) that facilitate binding to the negatively charged DNA molecule (Cooper GM., 2000). There are five basic types of histone molecules, including H1, H2A, H2B, H3, and H4. Histone H1 is called the linker histone as it binds to the linker DNA, the DNA between two nucleosomes. Histone H2A, H2B, H3 and H4 are the core histones present in the nucleosome (Fig 1A). Histones and DNA form the smallest subunit of chromatin called the nucleosome. The nucleosomes are portions of double-stranded DNA that are wrapped around protein complexes called histone core. There are about ~147 base pairs of DNA wound around an octamer of core histone proteins of nucleosome (Hansen JC., 2012). All core histones contain N-terminal tails protruding out of nucleosomes. Nucleosomes are formed as a beginning step for DNA compaction that also contributes to structural support as well as serves functional roles (Verdone L., *et al.*, 2006). Every chromosome contains hundreds of thousands of nucleosomes, and these nucleosomes are joined by the DNA that runs between them (an average of about 20 base pairs). This joining DNA is referred to as linker DNA. Each chromosome is thus a long chain of nucleosomes, which gives the appearance of a string of beads when viewed under an electron microscope (Reviewed by Annunzito AT., 2008). Chromatin structure can be directly affected by epigenetic changes such as DNA methylation, histone methylation, histone acetylation, histone phosphorylation and chromatin remodeling. The ability to regulate chromatin structure is essential in eukaryotic organisms in order to organize the DNA into compact arrangement and for defining which pieces of DNA are available for transcription (reviewed by Zamudio NM., *et al.*, 2008). The

chromatin proteins associated with DNA may be activated or silenced. Epigenetic modifications associated with gene activation are DNA demethylation (May L.N. *et al*, 2012), histone acetylation (reviewed by Kuo and Allis, 1998), H3K4 methylation (Stein *et al*, 2011), H3K36 methylation (Pelechano V., *et al*, 2010), H3K9 demethylation (Klose RJ., *et al*, 2007), and H3K27 demethylation (Lee S., *et al*, 2012) while those related to gene suppression are DNA methylation (Gohlke J, *et al*, 2013), histone deacetylation (Ding SL, *et al*, 2010), H3K4 demethylation (Chicas A., *et al*, 2012), H3K36 demethylation, H3K9 methylation (Tsurumi A, *et al*, 2013), and H3K27 methylation (reviewed by Yoo and Hennighausen, 2012).



Reviewed by Annunzito AT., 2008

Figure IA. Chromosomes are composed of compact DNA tightly-wound around nucleosomes. Chromosomal DNA is packed inside nuclei with the help of nucleosomes, complexes of positively-charged histone proteins that strongly adhere to negatively-charged DNA. DNA winds around a single nucleosome, composed of eight histone proteins. Nucleosomes fold up to form a 30-nm chromatin fiber, which form loops averaging 300 nm in length. The 300 nm fibers are compressed and fold to produce 250nm-wide fiber, which is tightly coiled into the chromatid of a chromosome.

6. Chromatin remodeling and histone modifications

3.1. Chromatin remodeling

Chromatin remodeling factors comprise an ATPase subunit along with other polypeptides that are responsible for transcriptional regulation. Chromatin remodeling is important for cell function because remodelers work with other chromatin factors such as chromatin modification to control packaging and un-packaging states of chromatin as the DNA elements that control chromosomal processes (enhancers, promoters, replication origins) must be exposed in a regulated manner to execute various processes such as gene transcription, DNA replication, DNA repair, and DNA recombination. Thus, chromatin structure not only provides a packaging solution, but also an opportunity for gene expression regulation (Cedric and Bradley, 2009). The extremely accurate consequence of remodeling of nucleosome structure is dependent on the circumstances of nucleosome at a given promoter and can lead to either: activation, or repression of transcription. Therefore, that induces structural changes to the chromatin occurring throughout cell division cycle. These changes range from limited changes necessary for transcriptional regulation to comprehensive changes.

3.2. Histone modifications

Histone modifications include addition or removal of various molecular moieties on histone proteins. Histone can be modified at many residues on histone tails. There are more than 60 different residues on histone tails where modifications have been detected either by specific antibodies or by mass spectrometry. There are numbers of modifications can take place on histone. At least eight distinct types of modifications are known; methylation, acetylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, deimination,

and Proline isomerization (Table A; reviewed by Kouzarides T., 2007). The most information regards the small covalent modifications of acetylation, methylation, and phosphorylation. The complexity comes from the fact that methylation at lysines or arginines may be one of three different forms: mono-, di-, or trimethyl for lysines and mono- or di- (asymmetric or symmetric) for arginines. Phosphorylation often occurs on multiple distinct sites on a given protein. Phosphorylation on serine is the most common, followed by threonine. Tyrosine phosphorylation is relatively rare but can be at the origin of protein phosphorylation signaling pathways in most of the eukaryotes (Cohen P., 2002). This array of modifications gives enormous potential for functional responses, but it has to be remembered that not all these modifications will be on the same histone at the same time. The timing of the appearance of a modification will depend on the signaling conditions within the cell

Table A. Different classes of modifications identified on histone		
Chromatin Modifications	Residues Modified	Functions Regulated
Acetylation	K-ac	Transcription, Repair, Replication, Condensation
Methylation (lysines)	K-me1 K-me2 K-me3	Transcription, Repair
Methylation (arginines)	R-me1 R-me2a R-me2s	Transcription
Phosphorylation	S-ph T-ph	Transcription, Repair, Condensation
Ubiquitylation	K-ub	Transcription, Repair
Sumoylation	K-su	Transcription
ADP ribosylation	E-ar	Transcription
Deimination	R > Cit	Transcription
Proline Isomerization	P-cis > P-trans	Transcription

Reviewed by Kouzarides T., 2007

3.2.1 Histone acetylation

Beyond methylation, acetylation is one of the most common modifications on histone code. The main role of histone acetylation is its involvement in the regulation of

transcriptions. Recent studies have revealed the role of histone acetylation in other important processes regulating the structure and function of chromatin, and hence, the eukaryotic genome. These reactions are typically catalyzed by histone acetyltransferase (HAT) or histone deacetylase (HDAC) activity. HATs catalyze the transfer of acetyl groups from acetyl-CoA to defined lysine residues of histone. Lysine site specificity thereby largely depends on the corresponding type of enzyme (Kolle *et al.*, 1998). HATs are divided into several families. Major families identified so far are General Control Non-Derepressible 5 (Gcn5) –related N-Acetyltransferases (GNAT) family, MYST family, and p300/CBP family. GNAT is the most well-studied families with acetylation abilities (Yang, 2007). The yeast ortholog of p55, Gcn5, had been identified as an important transcriptional co-activator. This protein was recruited to gene promoters prior to transcription and necessary for activation. Further ossifying the connection between histone acetylation and gene transcription, this suggested that the HAT activity of Gcn5 is required for its ability to activate transcription (Candau *et al.*, 1997; Kuo *et al.*, 1998). Biochemical assays of proteins and protein complexes involved in transcriptional activation led to the identification of a large number of histone acetyltransferases, strengthening the hypothesis that histone acetylation is tightly connected to transcriptional activation (Roth and Denu, 2001)

HDACs catalyze the removal of acetyl groups from lysine residues of core histone tails. Classical HDACs are currently divided into four classes: Class I includes HDACs , 2, 3 and 8. Class II is divided into two subgroups, Class IIA and Class IIB. Class IIA includes HDACs 4, 5, 7, and 9 while Class IIB includes HDACs 6 and 10. Class III contains the Sirtuins and Class IV contains only HDAC11 (Gallinari P., 2007). HDACs can affect DNA-dependent events, such as transcription, recombination, replication and repair. Running

parallel to the advances made with HATs, HDACs and HDAC-containing protein complexes were identified and linked to the repression of transcription (Yang and Seto, 2003). However, members of HDCA were also suggested to be involved in gene activation. Deletion of the class I HDAC, HDAC1, led to strongly reduced virulence of *Cochiliolus carbonum* on corn plant and a reduced expression of extracellular depolymerases as well (Baidyaroy *et al.*, 2001).

3.2.2 Histone methylation

Histone methylation is a process that histone methyltransferase catalyzes the transfer of methyl groups into amino acids of histone proteins. Methylation is one of the most common histone modifications. Methylation can occur on several lysine (K) and arginine (R) residues in the N-termini of histone core (Lachner *et al.*, 2003). Depending on the target site, methylation can modify histone so that different portions of chromatin are activated or inactivated. Histone methylation is crucial for proper programming of the genome during development. Misregulation of histone methylation can lead to diseased state such as cancer in mammals (Li X and Zhao X, 2008). Until recently, methylation was believed to be stable epigenetic mark for cell division, maintaining a gene in an active or inactive state.

Arginine methylation is a common posttranslational modification (PTM). This type of PTM occurs on both nuclear and cytoplasmic proteins, and is particularly abundant on shuttling proteins. The diverse roles that arginine methylation of the core histone tails play in regulating chromatin function. Histone arginine methylation is involved in both gene activation and suppression (reviewed by Berger, 2002). A family of nine protein arginine methyltransferases (PRMTs) catalyzes methylation of a subset target histones. Arginine methylation of histone tails can promote or prevent the docking of key transcriptional

effector molecules, thus playing a central role in the orchestration of the histone code (reviewed by Lorenzo and Bedford, 2010). In addition, members of the protein arginine methyltransferase family can also methylate histones *in vitro* (Gary and Clarke, 1998). Earlier studies demonstrated that a nuclear receptor coactivator-associated protein, CARM1, is a H3-specific arginine methyltransferase and that the human homolog of the *Drosophila* heterochromatic protein Su(var)3-9, is a H3-specific lysine methyltransferase, provided substantial evidence for the involvement of histone methylation on transcriptional regulation (Chen *et al.*, 1999; Rea *et al.*, 2000).

Several lysine residues, including lysines 4, 9, 27, 36 and 79 of H3 and lysine 20 of H4, are preferred sites of methylation (reviewed by Black JC *et al.*, 2012; Strahl et al, 1999). Methylation of lysine residues within histone is tightly regulated by KMTs and KDMs to maintain genomic stability, and to be a major determinant for formation of active and inactive regions of the genome. Methylation at lysine residues have been demonstrated to be involved in both transcriptional activation, and silencing. Lysine can be in mono-, di-, or trimethylated forms. Generally, methylation of histone H3 at lysine 4, 36, 79 (H3K4me, H3K36me, H3K79me) is associated with gene activation. In contrast, H3K9, H3K27, and H4K20 methylation correlates with gene repression.

Lysine methylation coordinates the recruitment of chromatin modifying enzymes that regulate chromatin condensation and nucleosome mobility in order to maintain local regions of active or inactive chromatin. In addition, lysine methylation can block binding of proteins that interact with unmethylated histone or directly inhibit catalysis of other regulatory modifications on neighbor residues. Histone lysine methyltransferase are known to contain a conserved catalytic SET domain.

3.2.3 SET domain proteins

Many histone lysine methyltransferases (KMTs) contain a SET domain, named after regions shared by three *Drosophila* proteins: Su(var)3-9, Enhancer of zeste, and Trithorax (Jenuwein, 2006). The SET domain, which is thought to be involved in protein-protein interactions, includes conserved N- and C- terminal regions (SET-N and SET-C) and an intervening insert region (SET-I). The SET domain is a 130 amino acid catalytic domain, typically required for full KMT activity. SET domain-containing enzymes currently comprise of the two classes of KMTs. There are dozens of human KMT proteins (SUV proteins), homologs of the *Drosophila* Su(var). There are two homologs, SUV39H1 and SUV39H2, that catalyze tri-methylation at H3K9 after it has already been mono-methylated. Both proteins contain N-terminal chromatin organization modifier (Chromo) domains, which facilitate the condensation of heterochromatin. They function mainly in these condensed heterochromatin regions for suppressing gene expression. Two additional Su(var) homologs, SUV420H1 and SUV420H2, specifically trimethylate H4K20. Like the SUV39 homologs, these proteins are targeted to heterochromatin and are involved in epigenetic transcriptional repression (Dillon SC., *et al.*, 2005).

In the *Saccharomyces cerevisiae*, Set1 histone H3K4 methyltransferase complex interact with RNA polymerase II (Pol II) during early elongation. Set1 is important for the appropriate recruitment of Nrd1 in budding yeast (Terzi N *et al.*, 2011). The *S. cerevisiae* SET1 and SET2 complexes are involved in transcriptional elongation as part of the RNA polymerase II holoenzyme (Ng HH, *et al.*, 2003; Krogan NJ *et al.*, 2003). Tri-methylation of H3K4 catalyzed by SET1 is associated with regions of genes that are transcribed early, whereas the SET2-mediated methylation of H3K36, which is associated with downstream

regions that are transcribed in the later stages of transcriptional elongation. In *Neurospora crassa* and *Aspergillus nidulans*, there are seven SET domain proteins. The *N. crassa* SET2 protein has been identified as an H3K36 specific protein, and was essential for growth and development in this fungus. It was explained that methylation of H3K36 is required for the transcription of genes involved in asexual and sexual differentiation because mutants showed slow growth, sparse conidiation and female sterility (Adhveryu *et al.*, 2005). Methylation of H3K36 by SET2 was associated with transcribed regions of Pol II-regulated genes implied a conserved role for this modification in the transcription elongation process (Morris *et al.*, 2005). Similar to *Neurospora* protein, fission yeast *S. pombe* SET2 has been showed to be a nucleosome-selective H3 methyltransferase. In addition, the SET2 of *S. cerevisiae* also showed specificity for H3K36 and related to transcriptional activation (Strahl *et al.*, 2002). The mammalian nuclear-receptor-binding SET-domain-containing protein (NSD1, a member of the SET2 family) has been found to play a crucial role in post-implantation development, methylating H3K36 and H4K20 (Rayasam GV *et al.*, 2003).

CHAPTER II

Split-marker strategy, a useful technique for disrupting histone lysine methyltransferase (KMT) genes of the rice blast fungus, *M. oryzae*

4. Introduction

Magnaporthe oryzae is an excellent model organism for studying the basic molecular mechanisms underlying plant-fungus interactions. During the last decade, RNA silencing has become a wonderful technique for functional genomics studies (Nakayashiki *et al.*, 2005; Murata *et al.*, 2007; Nguyen *et al.*, 2008). However, complicated interpretation of data generated from siRNA-mediated knock-down studies has been sometimes observed, especially being derived from “off-target” effects (Jackson and Bartz, 2003). Conventionally gene knock-out methods are more commonly used for fungal genetics. For disrupting a targeted gene in the *M. oryzae* genome, a homologous recombination method with a plasmid carrying the full-length of a hygromycin phosphotransferase cassette (HYG) and DNA fragments adjacent to the targeted genes was used (Wu *et al.*, 1997; Zhang *et al.*, 2011; Xu and Hamer, 1996). This strategy was successfully applied to gene disruption in many fungal species such as *Saccharomyces cerevisiae* (Rothstein, 1983) and *Candida albicans* (Kelly *et al.*, 1987). However, in contrast to yeast and *Candida*, the efficient disruption was generally low in some other fungal pathogens. There are two major factors that contribute to the low deletion efficiency in the pathogenic fungi. First, promiscuous integration systems occur which result in ectopic integration at high frequency, while the preferred homologous integration event occurs at low to barely detectable frequencies. This problem can be compounded by the next occurrence of multiple integration or insertions at the integration site. A second problem, which is rarer happened but still problematic, is the possibility that the transforming DNA does not integrate at all.

A split-marker gene disruption strategy which provides a higher efficiency of homologous integration (Catlett *et al.*, 2002; Fu *et al.*, 2006) was applied to create disrupted

mutations in *M. oryzae*, *S. cerevisiae* and other fungi (Mehrabi *et al.*, 2008; Fairhead *et al.* 1996, Fu *et al.*, 2006). In the split-marker disruption method, two constructs each of which contains a 5' or 3' flank of the target gene and overlapping two third of the selection marker (HYG) cassette. The homologous recombination occurs at three positions, one site is between the overlapping regions of the HYG cassette and the other two sites are between vectors and genomic sequences at the upstream and downstream flanking regions of the target gene. Therefore, ectopic integration was reduced and the expected homologous integrations appear in high frequency. In this study, we applied and developed this split marker transformation approach to generate disruption constructs of histone lysine methyltransferase genes in rice blast fungus, *M. oryzae*.

5. Materials and methods

5.1. Fungal strain and plasmid extraction

A *Magnaporthe oryzae* strain used in this study to delete HMT gene is wheat-infected wild type Br48 (Urashima *et al.*, 1999). The wild type strain and mutated transformants constructed in this study were cultured on barley seeds soaked with sucrose 0.5% solution in test tube and then dried in a plastic box with silica gel at 4°C for long-term storage (Nakayashiki *et al.*, 1999). For working culture of fungi, a barley grain from the stock culture was placed on a PDA (potato dextrose agar) plate and incubated at 25°C. Plasmid pSP72-HPH containing hygromycin phosphotransferase (*hph*) gene was used to create disruption vectors. A single bacterial colony was transferred into LB (yeast extract 0.5%, tryptone 1%, NaCl 1%) liquid then incubated at 37°C with vigorous shaking (220 – 250 rpm) for 16 hours. The plasmid was extracted by following Genopure Plasmid Midi Kit (Roche).

5.2. Construction of disruption vectors and fungal transformations

First, PCR products of upstream and downstream of targeted genes amplified from genomic DNA of wild type strain were cloned separately into the multiple cloning site of plasmid pSP72-hph which containing the HYG cassette. For screening, a colony cracking method was applied to identify positive clones. Bacterial colonies were cultured on LB agar-ampicillin plates. A small quantity of each colony was spun into 20µl cracking buffer (NaOH 50mM, SDS 0.5%, EDTA 5mM) and incubated at 65°C for two hours. The plasmid DNA released in the solution was directly checked by gel electrophoresis for screening positive clones. Then, plasmids containing upstream of target gene was amplified with pair

of primer F/HY to create 5' end of disruption construct, 3' end of construct was amplified from plasmid containing downstream of target gene by using primer YG/R. The resulting PCR products were purified by using DNA purification KIT (Winzard SV Gel and PCR Clean-up system, Promega, USA), then used for next fungal transformation steps.

For protoplast preparation, *M. oryzae* was cultured in CM liquid (0.3 % casamino acid, 0.3% yeast extract, 5% sucrose) for 5 days at 25°C on shaker with 100 rpm. Mycelia of *M. oryzae* wild type Br48 growth in CM liquid were digested cell walls by shaking in lysing enzymes solution from *Trichoderma harzianum* (Sigma). The PCR fragments amplified from upstream and downstream of target genes fusing to hygromycin selectable marker cassette were mixed and introduced into wild type fungal protoplasts by a polyethylene glycol (PEG)-mediated method as previously described (Kadotani *et.al.*, 2003). For screening disrupted transformants, fungal colonies PCR were performed with appropriate sets of primers for each gene.

5.3. Southern blot analysis

Fungal genomic DNA was extracted from transformants by using Plant Genomic DNA Extraction Miniprep System (Viogene U.S.A) following the manufacturer's instruction. Genomic DNA was concentrated by ethanol precipitation, and adjusted to 5µg/µl. The southern blot analysis was performed using the DIG DNA Labeling and Detection KitTM (Roche Applied science). Twenty micrograms of genomic DNA were digested by appropriate restriction enzymes to each gene. The digests were separated by agarose gel electrophoresis and transferred to Hybond N⁺ membrane (Amersham biosciences). The hybridization procedures were carried out according to the manufacturer's instructions. Probes used in genomic southern analysis were generated by a PCR DIG labeling system.

The hybridization signals were detected using anti-dioxigenin-AP Fab Fragment (Roche) and CDP-star Ready to use (Roche Molecular Biochemicals).

6. Result and discussion

3.1 Construction vectors for disrupting histone methyltransferase genes

Linear DNA consisting of a full selectable marker gene flanked on both sides by short stretches of DNA that target a gene of interest was used to generate disruption mutants of histone lysine methyltransferases. In particular, the split-marker deletion technique was used for deleting the targeted genes. This method was first developed by *Fairhead C.*, et al., 1996 on budding yeast. In this strategy, two constructs are required per transformation, each containing a 5' flank or 3' flank of the histone methyltransferase genes and roughly two thirds of a hygromycin selectable marker cassette. In previous reports, they used both fusion PCR-based and plasmid-based deletion methods (*Turgeon et al*, 1985), and 5' and 3' flanks of targets were constructed less than 1000 bp. In this study, to increase efficiency of expected homologous integration, the length of 5' and 3' flanks were improved up to approximately 2000 bp, then each flank were cloned separately into pSP-72HYG. The length of homologous sequence within the hygromycin gene was up to 120 bp. (Fig 1). With improvement of this split marker method, it saved much of the time for generating mutations of histone lysine methyltransferase and others genes in *M. oryzae*, and it may easily apply to disrupt a gene in other filamentous fungi as well.

3.2 The split-marker homologous replacement strategy is useful to generate the *M. oryzae* deletion mutants of KMT genes

In the *M. oryzae* genome (http://www.broad.mit.edu/annotation/genome/magnaporthe_grisea/multihome.html), seven putative histone lysine methyltransferase (KMT) genes were reported based on sequence similarity and domain

structure of known KMTs in the KEGG database (<http://www.genome.ad.jp/kegg>). They were named MoKMT1 (Mgg_06852.6), MoKMT3 (Mgg_01661.6), MoKMT4 (Mgg_05254.6), MoKMT5 (Mgg_07393.6), MoSET1 (Mgg_015053.6), MoSET3 (Mgg_02937.6), and MoSET6 (Mgg_10842.6) (Table S1). MoKMT1, MoKMT3, MoKMT4, and MoKMT5 likely belong to corresponding KMT families (Allis, *et al.*, 2007). MoSET1 is a member of the KMT2 family, typified by *S. cerevisiae set1*. MoSET3 and MoSET6 showed amino acid identity with *Neurospora crassa* SET3 and *Schizosaccharomyces pombe* SET6.

The frequency of homologous integration by the split-marker recombination method was high enough to easily obtain KMT mutants. In most cases, positive candidates were obtained by screening 50 to 100 independent transformants. The resulting *mokmt* mutants were selected on hygromycin-containing media and confirmed by DNA gel Southern blot hybridization with the DIG DNA labeling system. For each gene, we applied an appropriate restriction enzyme to digest genomic DNA and a specific probe corresponding to the KMT gene. The structure and putative functional domains of KMT genes and their deletion constructs were showed in the Fig 2 to Fig 8.

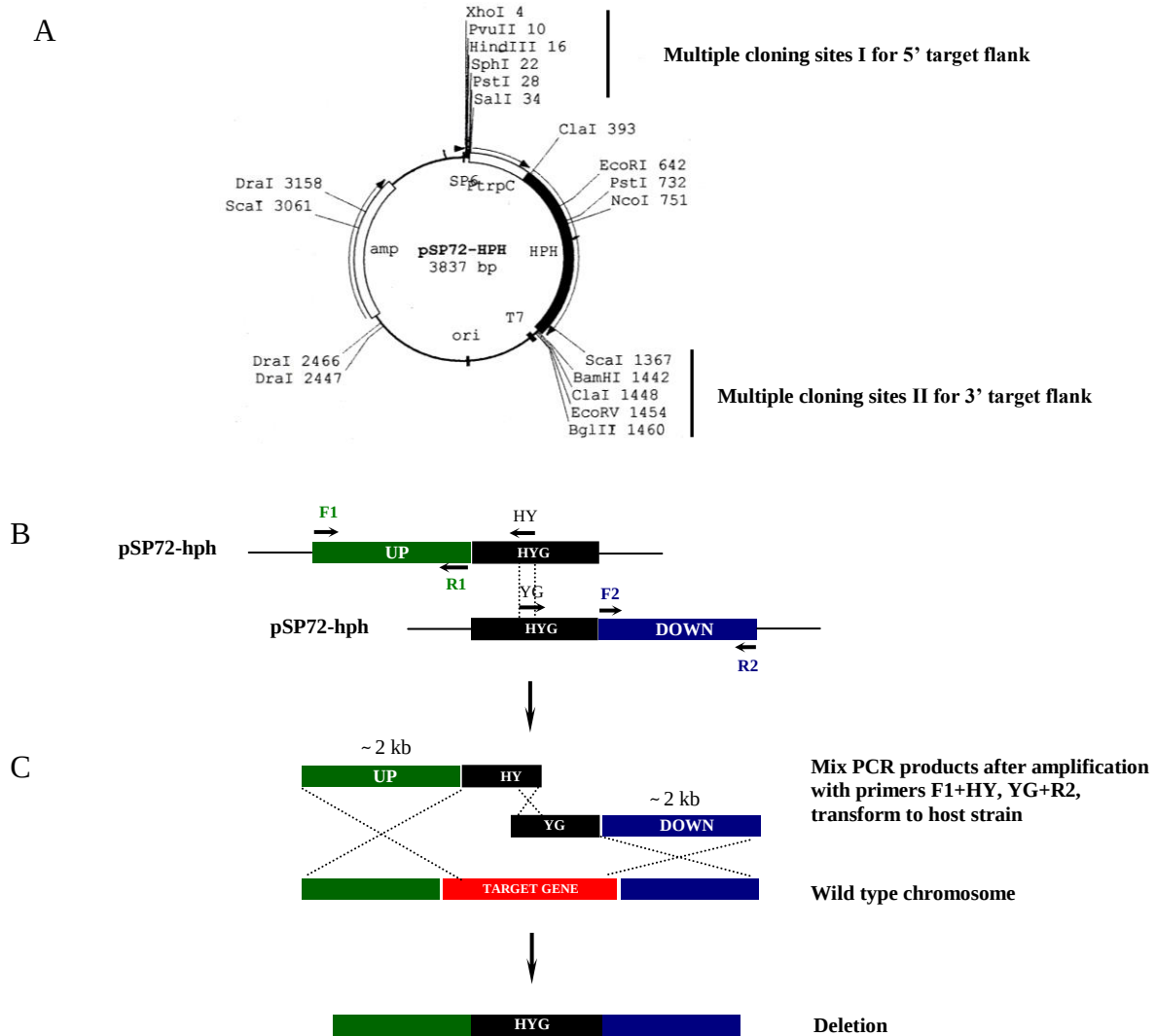


Figure 1. **Schematic representation of split-marker strategy for gene disruption.** (A) pSP72-HPH vector circle map and sequence reference points. (B) Primers F1/R1 and F2/R2 amplify 5'flanking (upstream) and 3'flanking (downstream) sequences of targeted genes, upstream fragments were cloned into multiple cloning sites (MCS) I, and downstream fragments were cloned into MCS II. Pair of primers F1/HY amplify upstream fusing with a first part of HYG marker and YG/R2 amplify downstream fusing with a last part of HYG marker. (C) Two separate PCR fragments were used directly for transformation. Homologous recombination occur between the flanking regions and wild type chromosome DNA, and between regions of overlapping regions of selection marker HYG cassette in a deletion.

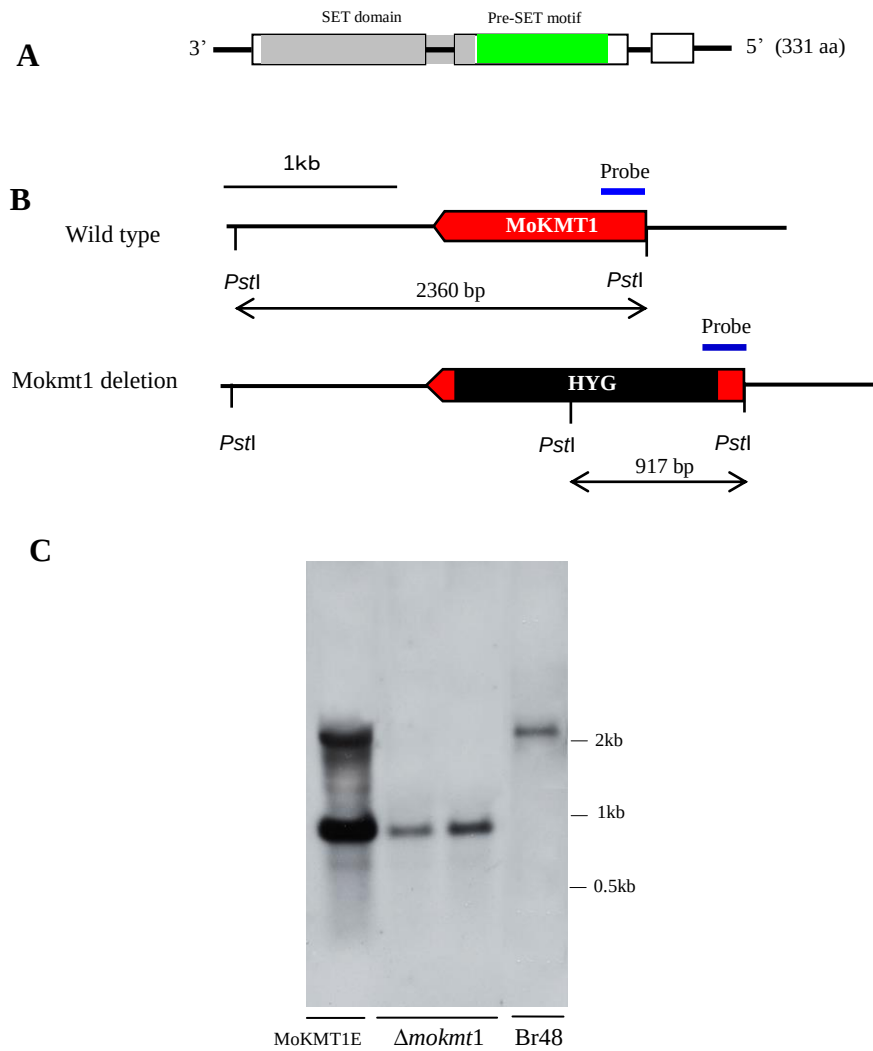


Figure 2. Construction of MoKMT1 deletion strains ($\Delta mokmt1$). **A**, Gene structure of MoKMT1 (Mgg-06853.6) allele with introns (lines) and exons (boxes): The colored regions show the conserved protein motifs, SET (grey box) and pre-SET (green box) domains. **B**, Genome structures of MoKMT1 locus with the wild type (Br48) and $\Delta mokmt1$ strains. Deletion was constructed by using the split-marker system with primers *Hmt18up-XhoI-F/HY* and *YG/Hmt18down-BglII-R*. A probe 214-bp (blue bar) was amplified by polymerase chain reaction with *Hmt18down-KpnI-F/Hmt18-sre-R*, then used for Southern blot analysis. PstI-digestion of genomic DNA from wild type and mutants is expected to generate 2360-bp and 917-bp DNA fragments, respectively. **C**, Southern blot analysis using the probe and PstI-digested genomic DNA. MoKMT1E indicates an ectopic mutant.

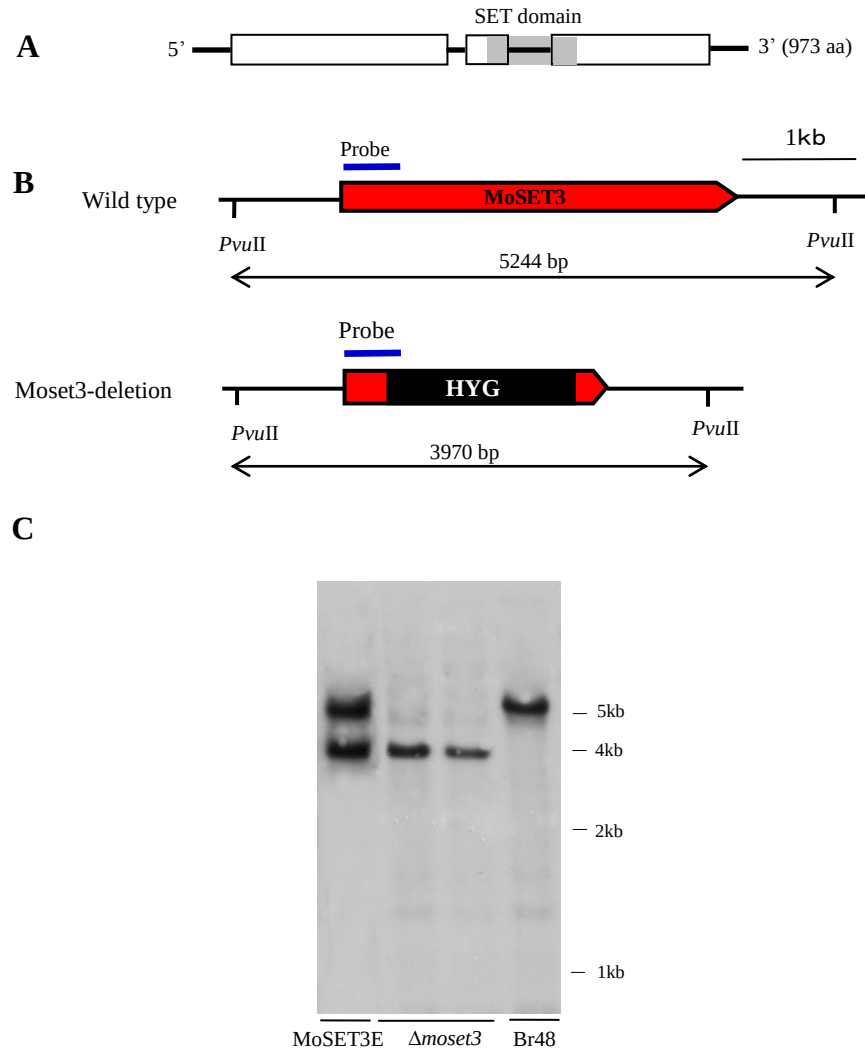


Figure 3. Construction of MoSET3 deletion strains ($\Delta moset3$). (A) Gene structure of MoSET3 (MGG_02937) with introns (lines) and exons (boxes): The colored region represents the conserved motif, SET domain (grey box). (B) Presumed genome structure of the MoSET3 locus in the wild type (Br48) and $\Delta moset3$ strains. Deletion strains were constructed using the split-marker system with primers *23up-HindIII-F/HY* and *YG/22down-BglIII-R*. A 508-bp probe (blue bar) was amplified by polymerase chain reaction with *Hmt23-srce-F/23up-SphI-R* and used for Southern blot analysis. *PvuII* digestion of genomic DNA from Br48 and $\Delta moset3$ strains is expected to generate 5244-bp and 3970-bp DNA fragments, respectively. (C) Southern blot analysis using the probe and *PvuII*-digested genomic DNA. MoSET3E indicates an ectopic mutant

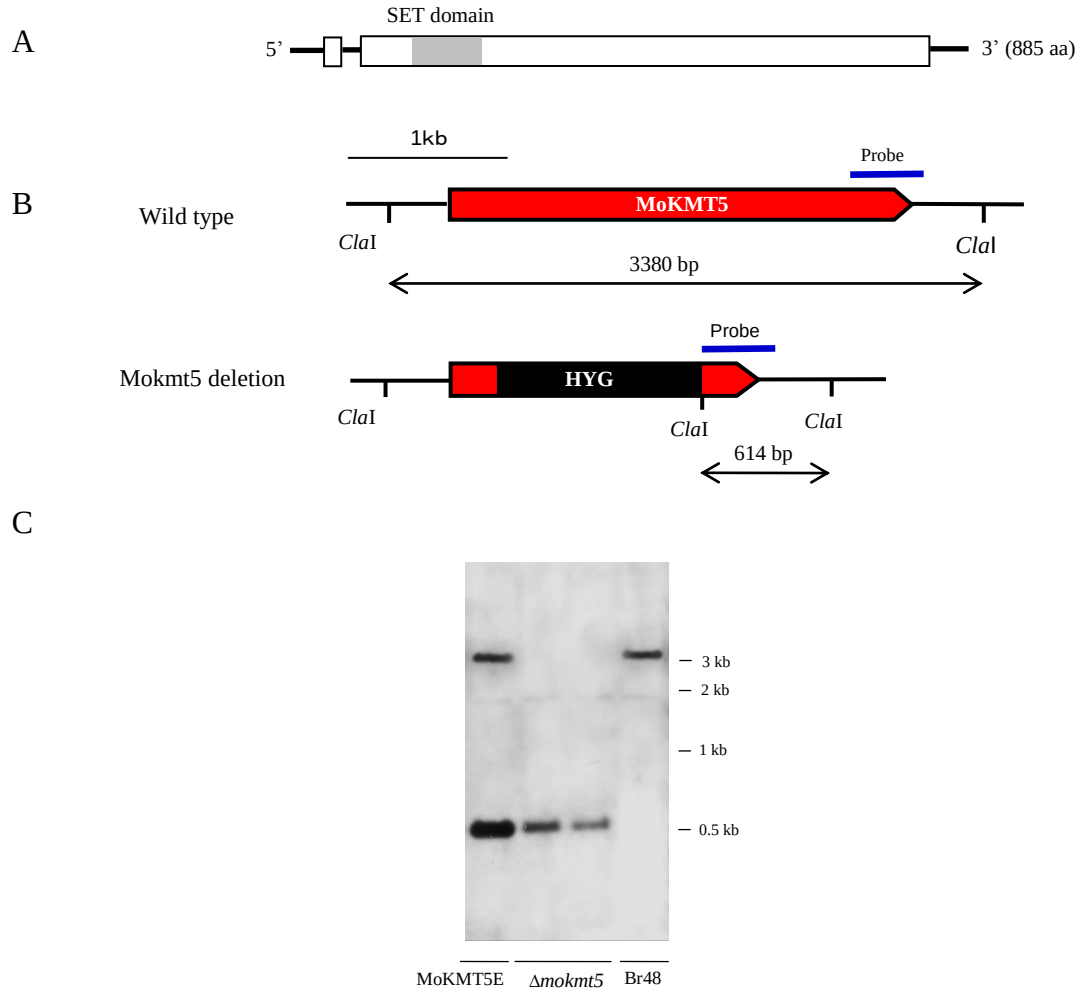


Figure 4. Construction of MoKMT5 deletion strains ($\Delta mokmt5$). (A) Gene structure of MoKMT5 (MGG_07393) with introns (lines) and exons (boxes): The colored region represents the conserved motif, SET domain (grey box). (B) Presumed genome structure of the MoKMT5 locus in the wild type (Br48) and $\Delta mokmt5$ strains. Deletion strains were constructed using the split-marker system with primers *Set9up-F/HY* and *YG/Set9down-R*. A 391-bp probe (blue bar) was amplified by polymerase chain reaction with *Set93down-F/Set9scree-R* and used for Southern blot analysis. *ClaI*-digestion of genomic DNA from Br48 and $\Delta mokmt5$ strains is expected to generate 3380-bp and 614- bp DNA fragments, respectively. (C) Southern blot analysis using the probe and *ClaI*digested genomic DNA. MoKMT5E indicates an ectopic mutant.

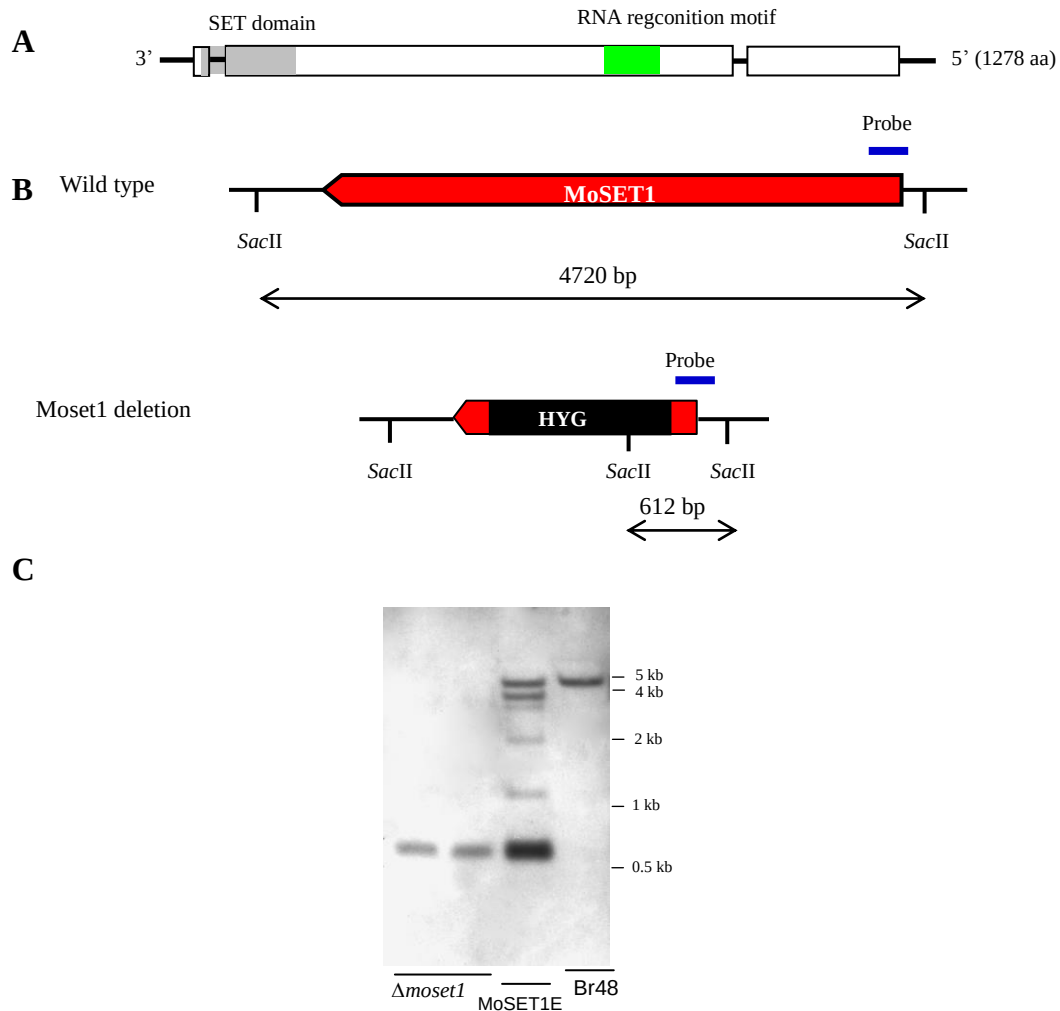


Figure 5. Construction of MoKMT5 deletion strains ($\Delta moset1$). (A) Gene structure of MoSET1 (Mgg-15053.6) with introns (lines) and exons (boxes): The colored region represents the conserved motif, SET domain (grey box) and RNA-regconition motif (green box). (B) Genome structure of the MoSET1 locus in the wild type (Br48) and $\Delta moset1$ strains. Deletion strains were constructed using the split-marker system with primers 29up-F/HY and YG/29down-R. A 364-bp probe (blue bar) was amplified by polymerase chain reaction with 29down-F/scree29-R and used for Southern blot analysis. *SacII*-digestion of genomic DNA from Br48 and $\Delta moset1$ strains is expected to generate 4720-bp and 612- bp DNA fragments, respectively. (C) Southern blot analysis using the probe and *SacII* digested genomic DNA. MoKMT5E indicates an ectopic mutant.

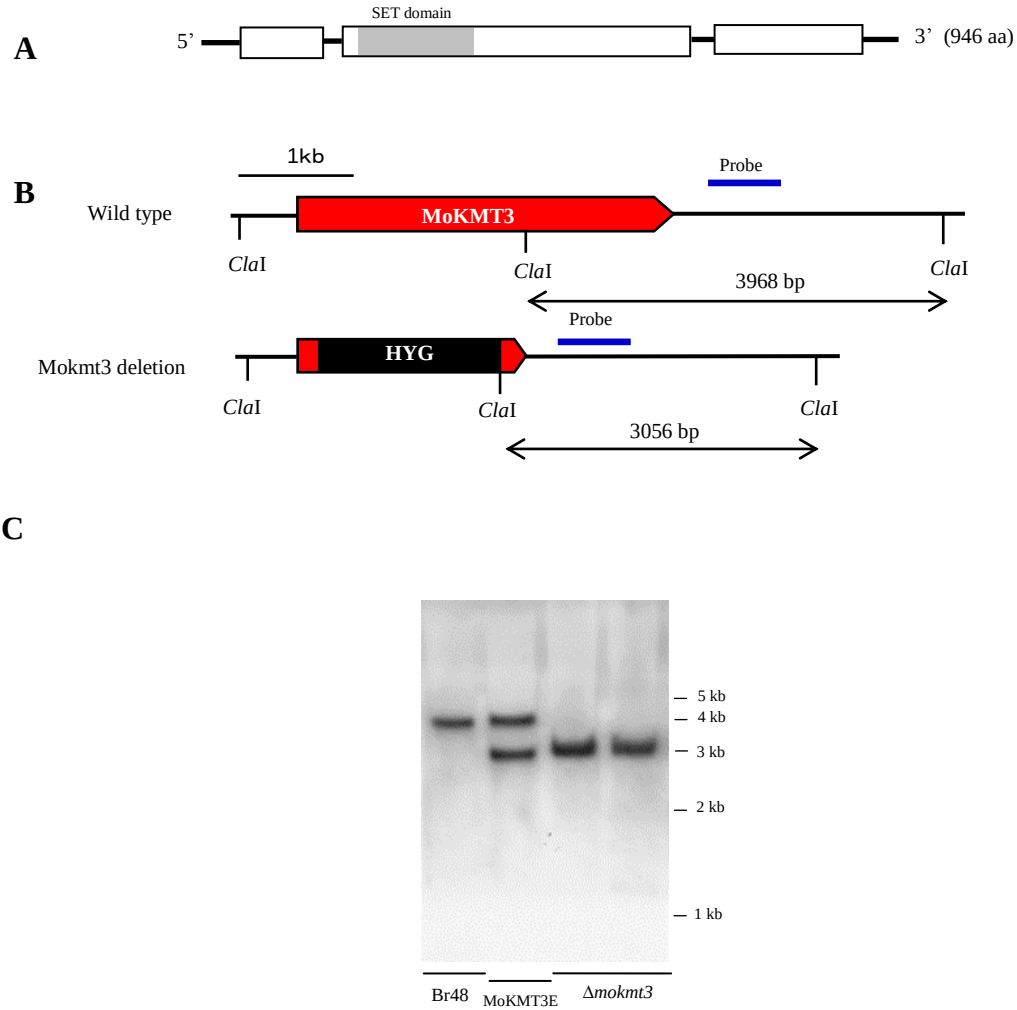


Figure 6. Construction of MoKMT3 deletion strains ($\Delta mokmt3$). (A) Gene structure of MoKMT3 (MGG_01661) with introns (lines) and exons (boxes): The colored region represents conserved the protein motif, SET domain (grey box). (B) Genome structure of the MoKMT1 locus in the wild type (Br48) and $\Delta mokmt3$ strains. Deletion strains were constructed using the split-marker system with primers *27up-F/HY* and *YG/Hmt27-1661down-BglIII-R*. A 429-bp probe (blue bar) was amplified by polymerase chain reaction with *Probe27-F/Probe27-R* and used for Southern blot analysis. *ClaI* digestion of genomic DNA from Br48 and $\Delta mokmt3$ is expected to generate 3968-bp and 3056-bp DNA fragments, respectively. (C) Southern blot analysis using the probe and *ClaI*-digested genomic DNA. MoKMT3E indicates an ectopic mutant.

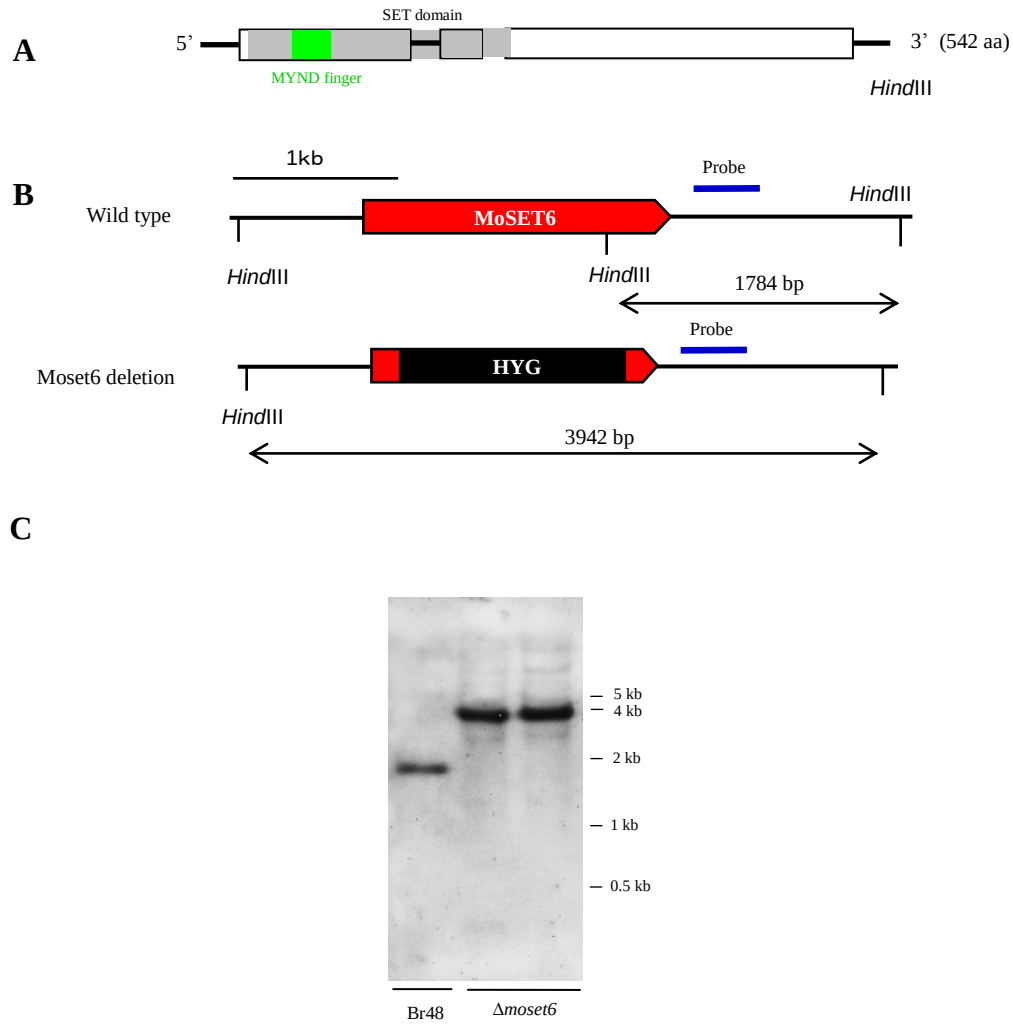


Figure 7. Construction of MoSET6 deletion strains ($\Delta moset6$). (A) Gene structure of MoSET6 (MGG_10842) with introns (lines) and exons (boxes): The colored regions represent the conserved motifs, SET domain (grey box) and MYND finger (green box). (B) Genome structure of the MoSET6 locus in the wild type (Br48) and $\Delta moset6$ strains. Deletion strains were constructed using the split-marker system with primers *Hmt10842up-F/HY* and *YG/Hmt10842down-R*. A 500-bp probe (red bar) was amplified by polymerase chain reaction with *Hmt6probe-F/Hmt6probe-R* and used for Southern blot analysis. *HindIII*-digestion of genomic DNA from Br48 and $\Delta moset6$ is expected to generate 1784-bp and 3942-bp DNA fragments, respectively. (C) Southern blot analysis using the probe and *HindIII*-digested genomic DNA.

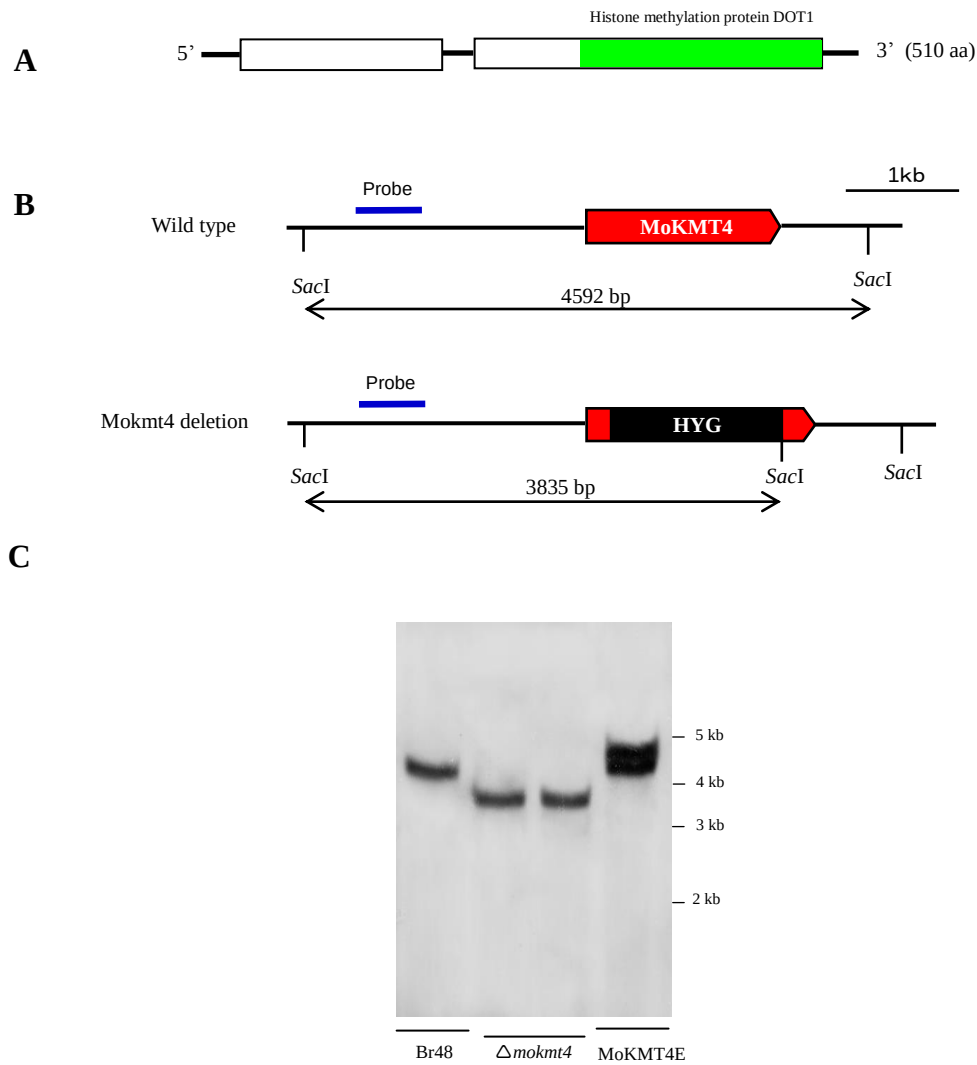


Figure 8. Construction of MoKMT4 deletion strains ($\Delta mokmt4$). (A) Gene structure of MoKMT4 (MGG_05254) with introns (lines) and exons (boxes): The colored region shows sequence similarity to DOT1 histone methyltransferase (green box). (B) Genome structure of the MoKMT4 locus in the wild type (Br48) and $\Delta mokmt4$ strains. Deletion strains were constructed using the split-marker system with primers *Hmt22-05254-SphI-up-F/HY* and *YG/22down-R*. A 491-bp probe (blue bar) was amplified by polymerase chain reaction with *H22probe-F/H22probe-R* and used for Southern blot analysis. *SacI*-digestion of genomic DNA from Br48 and $\Delta mokmt4$ is expected to generate 4592-bp and 3835-bp DNA fragments, respectively. (C) Southern blot analysis using the probe and *SacI*-digested genomic DNA. MoKMT4E indicates an ectopic mutant.

CHAPTER III

Roles of histone lysine methyltransferase genes in infection-related morphogenesis and pathogenesis in *M. oryzae*

5. Introduction

KMTs are conserved in a wide range of eukaryotes, playing roles in cellular signaling pathways related to the cell cycle, cell motility, transcription, apoptosis, and cancer. In human, there were many studies about correlation of chromatin remodeling complexes and human disease. Human BRG1 gene, a component between the evolutionarily conserved SWI-SNF chromatin remodeling complexes, is mutated in multiple tumor cell lines (Wong AK, *et al.*, 2000). The NSD3L isoform carrying the catalytic SET-domain histone methyltransferase regulated genes controlling cellular signaling pathways related to cell cycle, cell motility, transcription, apoptosis, and breast cancer in human, and linked to different kinds of cancer (Zhou, *et al.*, 2010; Mareike *et al.*, 2010). In filamentous fungi, the H3K9-specific histone lysine methyltransferase Dim-5 (defective in methylation 5), was proved to be necessary for normal growth and full fertility in *Neurospora* (Tamaru and Selker, 2001). DIM-5 is recruited to A:T-rich regions in the *Neurospora* genome to establish the heterochromatic state by H3K9 methylation. However, the activity of DIM-5 depended on DIM-7, an interactor of DIM-5 that is essential for recruitment of DIM-5 to form heterochromatin (Zachary *et al.*, 2010). Beside DIM-5, in *N. crassa* SET2 has been shown to be essential for normal morphology, since the SET2 mutant showed defects in growth and development likely due to a lack of methylated H3K36 (Keyur *et al.*, 2005). Similar to DIM-5 in *N. crassa*, SET1 in *S. cerevisiae* is implicated in the control of transcription, and chromosome structure and actually plays a role in many aspects of growth and developmental regulation (Corey *et al.*, 1997). In *A. nidulans*, chromatin-level regulation plays a role in the gene expression of SM gene clusters (Bok, *et al.*, 2009).

In *Magnaporthe oryzae*, the function of histone lysine methyltransferase (MoKMTs) and their putative roles as transcriptional regulators remains poorly understood. It is interesting to know whether MoKMT proteins affect pathogenesis and morphogenesis during infection processes of the rice blast fungus. Here we showed that *Mokmt* mutants showed defects in vegetative growth, sporulation, appressorium formation and pathogenicity at variable levels. Especially, the MoSET1 gene was crucial for appressorium formation and infection-related development in this fungus.

6. Materials and methods

2.1. Plant materials

We used three wheat cultivars, *T. aestivum* “Thatcher”, *T. aestivum* “Chinese spring”, *T. aestivum* “Norin 4”, and two barley cultivar *Hordeum vulgare* “Nakaizumi-zairai” and “Nigrate”. Thatcher was resistant to Br48 whereas Chinese spring, Norin 4, and Nakaizumi-zairai were susceptible (Zhan, *et al.*, 2008; Hyon *et al.*, 2012). Nigrate was super-susceptible to Br48 (Hyon *et al.*, 2012).

2.2. Assays for growth rate, conidiation, conidial germination and appressorium formation

A piece of mycelia of each strain was cultured on PDA dishes for 14 days. The growth rate was estimated based on the average of diameter of colony sizes from three replications. For conidiation, conidial germination and appressorium formation assays, all strains were grown on oatmeal agar and sporulation was induced under BLB light. After incubation for 3 days, conidia were harvested by suspending them with 20ml sterile distilled water per plate, and spore concentration was adjusted to 10^5 spores per ml. The conidial suspension dropped on slide glasses was incubated in humidity boxes at 25°C for 5 hours for accessing conidial germination, appressorium formation were observed under microscope after 5 and 24 hr incubation, respectively.

2.3. Plant infection tests

Wheat and barley seedlings were grown in vermiculite supplied with liquid fertilizer in plastic pots (5.5cm×15cm×10cm) at 22°C in a controlled-environment incubator with a 12h photoperiod for 8 days. For preparing conidial suspension, fungal mycelia cultured on a

PDA medium were transferred to oatmeal agar plates (40g of oatmeal, 17g of agar in 1000 ml water) and incubated in darkness at 25°C for 5 days. Then, aerial mycelia were removed by rubbing surface of mycelia with a sterile eppendorf tube, and further incubated under BLB light for 3 days at 25°C to induce conidiation (Murakami *et al.*, 2000). The conidia were suspended in sterile distilled water and diluted to $1-2 \times 10^5$ spores per milliliter. The primary leaves of 8-day-old wheat and barley seedlings were sprayed with the spore suspension containing 0.01% Tween 20. The inoculated seedlings were maintained under high humidity and dark conditions for 24 hours then moved to an incubator at 22°C with a 12h photoperiod for 5 days. Symptoms appeared were assessed based on the size and color of lesions to determine infection type. The size of a lesion was rated from 0 to 5: 0, no visible evidence of infection; 1, pinpoint spots; 2, small lesion (<1.5 mm); 3, lesion with an intermediate size (<3 mm); 4, large and typical lesion; 5, complete blighting of leaf blades. The color was classified with two basic colors brown (B, b) and green (G, g) (Zhang *et al.*, 2008). For example, the infection type 4bG means a brown and green lesion with a large size and brown lesions appear less than green lesions.

2.4. Genetic complementation of the *mokmt* deletion mutants

The full-length of MoKMT genes and 5'flanking and 3'flanking regions were amplified with specific pairs of primers and KOD FX Neo (Toyobo). The resulting constructs were cloned into the *SmaI* or *EcoRV* site of pBluescript SK(+/-), then introduced into protoplasts of mutants via a PEG-mediated co-transformation method with plasmid pII99 bearing the geneticin-resistance gene (Kadotani *et al.*, 2003). The resulting protoplasts were grown on geneticin containing CM 20% sucrose agar plates for 5 to 7 days. Colonies formed on the

selecting plates were picked up and screened by PCR analysis and verified by southern blot hybridization.

2.5. Cytological response assays

For microscope observation of host cell response to the deletion mutants, inoculated leaves were picked up at 48 hours post inoculation (hpi) and deeply boiled in alcoholic lactophenol (lactic acid/phenol/glycerol/distilled water/ethanol = 1:1:1:1, vol/vol/vol/vol/vol) for 2 minutes as described previously (Murakami *et.al.*, 2000). Samples were observed using a fluorescent microscope under bright and fluorescent fields. The observation was focused on appressorium-formed spores during infection processes into epidermal host cell. Host response was classified into four types: no reaction, papilla formation, hypersensitive reaction (HR), and hyphal growth.

2.6. Western blotting analysis

Western blot analysis was performed with a SDS-PAGE electrophoresis system. Fungal mycelia powder was suspended in 1×TBS buffer contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Nonidet P-40. The homogenates were then centrifuged (12,000 rpm, 2 minutes), and supernatants were collected to new tubes. Equal amount of suspension protein were subjected to 15% SDS_polyacrylamide gel electrophoresis, blotted to PVDF membrane; and probed with primary antibodies against methylation of histone H3 and H4 at several lysine residues such as anti-H3K4me1 (Active Motif # 39298), anti-H3K4me2 (Active Motif # 39141), anti-H3K4me3 (Active Motif # 39159), anti-H3K4ac (active motif #39381), anti-H3K9me3 (Active Motif # 39161), anti-H3K27me3 (Active motif # 39158), anti-H3K36me3 (Active motif # 61102), anti-H3K79me2 (Active motif # 39144), anti-

H4K20me3 (Active Motif # 39181), anti-H3K14me2 (Active motif # 39350), and anti-H3 (Active Motif #39163). Membranes were washed with TBS-Tween 20 three times for 10 minutes. Proteins reacting with the antibodies were visualized by appropriate peroxidase (HRP)-conjugated secondary antibody and ECL plus western blotting detection reagents.

2.7. RNA isolation and quantitative RT-PCR (qRT-PCR)

Total RNA extraction and cDNA preparation were carried out as described previously (Vu *et al.*, 2012) with a few modification. Total RNA was isolated from 72 hip-infected leaves and mycelia powder using Sepasol RNA I Super (Nakalai Tesque, Kyoto). Total RNA was then applied to cDNA synthesis using ReverTra Ace qPCR RT master mix with genomic DNA remover KIT (Toyobo, Japan). qRT-PCR assay was carried out using qPCR Thunderbird SYBR qPCR Mix (Toyobo) according to the manufacturer's instructions with specific primers for targets and internal control gene (actin) (Table S3). The level of target mRNA, relative to the mean of the reference house keeping gene, was calculated by the comparative Ct method.

3. Results

3.1. Changes of histone lysine methylation levels in *mokmt* deletion mutants

Histone lysine methylation levels in the KMT deletion mutants were assessed using western blotting with specific antibodies. H3K9me3 was reduced to an undetectable level in the $\Delta mokmt1$ mutant. In the $\Delta moSET1$ mutant, the levels of H3K4me2 and H3K4me3 were strongly reduced, while H3K4me1 moderately decreased. Similarly, H4K20me3 abundance was strongly reduced in the $\Delta mokmt5$ mutant (Fig 9A). These target sites for *Magnaporthe* KMTs were consistent with known target sites for corresponding KMT family proteins in other organisms. Western blotting results were also used to test antibodies against H3K4ac, H3K14me2, H3K27me3, H3K36me3, and H3K79me2, however, no specific signal reduction in any KMT deletion mutants was detected (Fig 9A). The decreased levels of histone methylation in the deletion mutants were completely recovered in gene complementation strains (Figure 9B). These results indicated that MoKMT1, MoSET1, and MoKMT5 catalyze methylation of H3K9, H3K4, and H4K20, respectively, in *M. oryzae*.

3.2. *M. oryzae* KMT deletion mutants showed defects in infection-related morphogenesis

To investigate the roles of the MoKMT genes in pathogenesis of this model filamentous fungus, we first examined vegetative growth of *mokmt* mutants. The growth rate of *mokmt* mutants were generally lower than that of the wild type strain (Fig 10) on PDA medium at 25°C. Especially, $\Delta moSET1$ showed the most severe reduction in vegetative growth. The rate of conidiation, germination, and appressorium formation were assessed for phenotypical characterization of the KMT-mutants with regards to infection. To determine whether the

MoKMT genes control other morphogenesis, *mokmt* mutants was cultured on oatmeal agar for 10 days and induce conidia under BLB light for germination, conidiation and appressorium formation tests. Similar to growth phenotype, conidiation and appressorium formation of the $\Delta moSet1$ mutant displayed the most severe defects, and $\Delta mokmt3$ and $\Delta moSet3$ mutants exhibited moderate reductions (Table 1). Compared with the wild-type strain, the rates of conidiation and appressorium formation were reduced to less than 10% in the $\Delta moSet1$ mutants, and to 20–50% in the $\Delta mokmt3$ and $\Delta moSet3$ mutants (Table 1). In contrast, only $\Delta mokmt3$ and $\Delta moSet3$, and not $\Delta moSet1$, exhibited defects in germination. The rate of germination was decreased by 40–50% in the $\Delta mokmt3$ and $\Delta moSet3$ mutants. Interestingly, while the $\Delta moSet1$ mutants germinated at levels comparable to the wild-type, the conidia of $\Delta moSet1$ mutants often appeared to be malformed. The wild-type strain produced three-celled, tear-drop-shaped spores. Conidia of the $\Delta moSet1$ mutants were also three-celled, but were more elongated than the wild-type spores (Figure 12A). All phenotypic defects observed in the $\Delta moSet1$, $\Delta mokmt3$ and $\Delta moSet3$ mutants recovered to wild-type levels in the corresponding complement strains, indicating that the KMT mutant phenotypes were caused by the corresponding KMT genes. Taken together, the results indicated that the MoSET1 gene holds an important role in conidial and appressorium formation, it maybe leads to impairing pathogenicity of rice blast fungus.

3.3. *M. oryzae* KMT deletion mutants showed defects in pathogenicity to host plants

To prove the roles of MoKMT genes in during infection into host plant cells, we performed infection assays using three wheat and two barley cultivars with different levels of resistance/susceptibility to wheat blast fungus Br48. The order of susceptibility of the

cultivars to the wild-type strain Br48 was as follows: barley, Nigrata (super susceptible) > barley, Nakazumi-zairai $\approx$ wheat, Norin 4 (susceptible) > wheat, Chinese spring (moderate susceptible) > wheat, Thatcher (moderate resistant). When seedlings of wheat and barley cultivars were spray inoculated with conidia from wild type Br48, $\Delta mokmt4$, $\Delta mokmt5$, $\Delta moset6$ and ectopic transformants, abundant lesions formed (Fig 11). $\Delta mokmt1$, $\Delta moset3$, $\Delta mokmt3$ mutants mutants showed significantly reduction in pathogenicity on all tested plants cultivars except Nigrata (Table 2). On leaves inoculated with the $\Delta mokmt1$, $\Delta moset3$, $\Delta mokmt3$ mutants, few lesions were still observed but infection type of $\Delta mokmt1$, $\Delta moset3$, $\Delta mokmt3$ showed 3bg, 2bg, 3b on wheat and 2bg, 2B, 2BG on barley, whereas infection type of wild type on wheat and barley are 4BG (Fig 11A, B). But, the degree of disease index was not different between the wild-type and these three mutant strains on Nigrata, full blast symptoms appear even with a low concentration of conidia suspension as inoculum (data not shown). These results showed that the MoKMT1, MoSET3, and MoKMT3 gene is necessary for cell growth of *M. oryzae* and its full pathogenicity on wheat cultivars during infection. However, the *mokmt* mutants of these genes retained virulence enough to cause full disease on barley cultivar Nigrata but not on wheat cultivars. Especially, consistent with the rates of appressorium formation, pathogenicity to the wheat and barley cultivars was most defective in the $\Delta moset1$ mutants. There are no any lesions were observed on seedling leaves at 5 dpi (Fig 9C). On super-susceptible seedling Nigrata, the $\Delta moset1$ mutants were still virulent even though the number of lesions were reduced compared to those on leaves infected with the wild-type strain. The lesions produced by the $\Delta moset1$ mutants were classified to the infection type 3bG. However cellular responses of Barley14 to the $\Delta moset1$ mutants were mostly regarded to be resistant reaction (data not shown). The $\Delta moset1$

mutants were most severely impaired in hyphal growth, sporulation and appressorium formation, and pathogenicity to susceptible wheat and barley cultivars. Using protein BLAST search, we identified that *M. oryzae* MoSET1 is homologous to the SET1 gene in *S. cerevisiae*. *Set1* mutant revealed changed phenotypes including morphological abnormalities, stationary defects, and growth and conidiation defects (Corey *et al.*, 1997). Taken together, these results indicated that MoSET1 holds an extremely important role in infection processes of *M. oryzae*.

To confirm that whether the phenotypic defects observed in the $\Delta mokmt$ mutants were caused by the gene replacement event, the wild-type MoKMT genes were re-introduced into the $\Delta mokmt$ mutants, and examined if the wild type phenotype could be restored. All four complemented transformants of $\Delta moset1$, $\Delta moset3$, $\Delta mokmt1$, and $\Delta mokmt3$ exhibited full virulence to wheat and barley (Fig 11D), and recovered all morphological changes (Table 1, and 2), respectively.

3.4. Cytological response of wheat cv. Norin 4 to infection with the *mokmt* mutants

To further examine which stage in the infection processes of *mokmt* deletion mutants was blocked, cytological assay of inoculated leaves of the wheat cultivar Norin 4 were performed. Cytological responses were classified into four types A, B, C, and D. In Type A, no reaction of the host cells was observed. In Type B, fungal infection was blocked by the formation of papilla with yellow fluorescence around the sites of the papilla. Type C describes the hypersensitive reaction of epidermal cells at the sites of fungal penetration. Types A to C are resistance responses of host cells, whereas Type D describes a susceptible response in which development of infection hypha was clearly observed (Murakami *et al.*,

2000). At least 100 spores with an appressorium were assessed in cytological sections of infected leaves.

In leaves infected with the wild type strain, the incidence of susceptible response Type D was predominant (63.9%) (Table 3). Similarly, Type D was predominant in leaves infected with the $\Delta mokmt4$, $\Delta mokmt5$, and $\Delta moset6$ mutants at levels similar to the wild-type strain. The $\Delta mokmt1$ mutant showed a slight reduction in compatible interaction rate (Type D), and slight increases in incompatible interaction rates (Type A–C) (Table 3). In contrast, the rate of resistant responses (Types A + B + C) was the majority in leaves infected with $\Delta moset1$, $\Delta moset3$, and $\Delta mokmt3$ mutants (Table 3). The $\Delta moset3$ and $\Delta mokmt3$ mutants induced cytological responses at very similar rates in the host cells. Infection by the two mutants was mostly prevented by the HR (~60%), and partly blocked at the papilla (~20%). Only a small percentage of germlings successfully formed invasive hyphae in infected cells (Table 3). In leaves inoculated with the $\Delta moset1$ mutant, Type A (no reaction) was predominant, (77.3%), and about 20% of germlings were stopped at the papilla stage. This suggested that this mutant mostly failed to penetrate plant cuticle and/or cell walls. Consistent with the other assays, these results suggested that the MoSET1 gene plays an extremely critical role during plant infection of *M. oryzae*. Interestingly, however, the $\Delta moset1$ mutant still retained pathogenicity to super-susceptible Nigrata cultivar cells. Hyphal growth was observed in some Nigrata cells infected with the $\Delta moset1$ mutant. This was consistent with the results of the previous infection tests.

The order of the degrees of reduction in KMT-mutant pathogenicity was as follows: $\Delta moset1 > \Delta moset3 > \Delta mokmt3 > \Delta mokmt1 > \text{Wild-type}$. The *mokmt* mutants $\Delta mokmt4$, $\Delta mokmt5$, and $\Delta moset6$ showed no detectable differences from the wildtype strain in all

infection assays in this study. Therefore, we concluded that *MoSET1* played the most important role in infection-related morphogenesis and pathogenesis in *M. oryzae*, and focused on the *MoSET1* gene for further studies.

3.5. Exogenous signal molecules restored the defects of infection-related morphogenesis in $\Delta mo set1$ mutants but not its infectivity to the host plant

Pharmacological examination was performed to gain an insight into which stage in the signaling pathway leading to appressorium formation was blocked in $\Delta mo set1$ mutants. Chemical and physical signals from host plants can trigger infection-related morphogenesis in *M. oryzae*. One such chemical signal is 1, 16-hexadecanediol, a plant cutin monomer released from the plant cuticle by degradation enzymes produced by the fungus (Gilbert *et al.*, 1996). After perception of external signals, the secondary messenger cyclic AMP (cAMP) plays a crucial role in the signaling pathway leading to appressorium formation in *M. oryzae* (Lee *et al.*, 1993, Mitchell & Dean, 2005). Therefore, the effects of 1,16-hexadecanediol and cAMP on infection-related morphogenesis in the $\Delta mo set1$ mutant were examined. In the presence of cAMP and cutin monomer 1,16-hexadecanediol, the rates of appressorium formation were greatly restored (over 80%) in the $\Delta mo set1$ mutant, but still lower than observed in the wild type strain (Fig 12 D). This suggested that the $\Delta mo set1$ mutant may have defects in the production and perception of external signals from host plants.

To determine whether the addition of 1, 16-hexadecanediol and cAMP can restore the infectivity of $\Delta mo set1$. We next suspended conidia of $\Delta mo set1$ mutants in 5 mM cAMP, and 5 μ M 1, 16-hexadecanediol as inoculum to perform spot inoculation on susceptible wheat cultivar Norin 4. No symptom was observed on leaves inoculated with both treatments

(Fig 12 B), suggesting that defects in appressorium formation were not only cause making the *Δmoset1* mutant noninfectious on wheat. The importance of appressoria is to create penetration peg to attach into host cells, to elucidate if germ tubes can penetrate into host cells via wounds on surface of leaves without appressorium and penetration peg formation, wound inoculation test were performed on Norin 4 cultivar. The *Δmoset1* mutant failed to cause disease on the wounded leaves (Fig 12 C). These results indicated that the *Δmoset1* mutant had some deficits in its ability to develop disease, even after entering into plant cells. Taken together, results suggested that *MoSET1* is involved in the regulation of genes required for external signaling perception and disease development in plant cells.

3.6. Histone H3K4 methyltransferase regulates substrate-dependent activation of cell wall degradation enzymes in *Magnaporthe oryzae*

To penetrate into host cells, plant pathogens secrete an array of cell wall degrading enzymes (CWDEs) which hydrolyze the components of host epidermis such as wax, cuticle and cell walls (Walton, 1994). To examine the involvement of *MoSET1* in substrate-dependent transcriptional activation of a wide range of CWDEs, we determined expression level of 36 CWDE genes including 11 putative endoxylanase belong to glycosyl hydrolase families 10 and 11 (GH10, and GH11) and 25 putative cellulase (cellobiohydrolases, endoglucanases, and β -glucosidases) during infection in comparison with those in vegetative mycelia on rich media. In infected leaves, 16 of the 36 CWDEs were up-regulated compared to in vegetative mycelia at statistically significant levels (Fig 13). The up-regulated genes included CWDEs belonging to GH6 (MGG_05520), GH7 (MGG_07908, MGG_10712, MGG_14954), GH10 (MGG_01542, MGG_15430 MGG_07868, MGG_02245), GH11 (MGG_08401, MGG_08331, MGG_07955), and AA9 (MGG_07575, MGG_08409,

MGG_04547, MGG_11948, MGG_12696), suggesting their potential roles in the pathogenicity of the fungus. In contrast, the transcripts of MGG_05364, MGG_06593, and MGG_14243 were relatively abundant in the vegetative mycelia (Fig 13). To address whether the activation of the CWDEs during infection was dependent on their substrate in plant cells, we compared the expression levels of the up-regulated CWDE genes in mycelia cultured in the presence (1% CM-cellulose or 2% xylose) or absence of their substrates. The results indicated that most of the up-regulated CWDE genes with exception of MGG_02245 (GH10) were also activated by adding their substrates in the medium (Fig 14), suggesting that their up-regulation during infection was due to, at least partly, the presence of their substrates in plants. In general, 2% xylose was not as effective for inducing xylanases as was 1% CM-cellulose for inducing cellulases (Fig 14). Thus, the induction of the xylanase genes in host plants should involve some factors other than xylose.

We next examined the involvement of H3K4 methylation in the substrate-dependent activation of CWDEs in *Δmoset1* mutant. The qRT-PCR analysis showed that in the *Δmoset1* strain, the substrate-dependent up-regulation was highly impaired in most of the CWDE genes with the exception of MGG_05520 (Fig 14). In some cases, the induction ratio was less than 1, indicating that their transcript levels under inductive conditions were even lower than those under non-inductive conditions in the *Δmoset1* strain. Overall, these results suggested that MoSET1 directly or indirectly regulated a wide range of CWDEs in *M. oryzae*.

7. Discussion

Chromatin structure can be vigorously changed by covalent modifications that take place on histones. This kind of modifications display a level of diversity and complexity, histone H3 and H4 have long amino-terminal tails that protrude from nucleosome and are subject to an array of post-translational modifications, typically acetylation and methylation (Strahl and Allis, 2000). In the rice blast fungus, *M. oryzae*, the histone deacetylase *Tig1* has been demonstrated to be required for infectious growth and conidiogenesis in *M. oryzae*, highlighting that chromatin modification is an essential regulatory mechanism during plant infection process (Ding, *et al*, 2010). In the current experiments, we elucidated the roles of the seven histone lysine methyltransferase (KMT) genes in infection processes of *M. oryzae*. The *MoKMT1*, *MoSET1*, *MoKMT3*, and *MoSET3* played important roles in infection-related morphogenesis and pathogenicity at varying degrees, while *MoKMT4*, *MoKMT5*, and *MoSET6* did not. By western blotting analysis we found that *MoKMT1* homologous to *N. crassa* DIM-5 is responsible for H3K9 methylation. In *N. crassa*, H3K9me3 catalyzed by DIM-5 is recognized by HP1 that forms a complex with DIM-2 DNA methyltransferase. Consistent with our results in $\Delta mokmt1$ mutants, Dim-5 is required for normal growth and full fertility in *N. crassa* (Tamaru & Selker, 2001). The $\Delta mokmt3$ and $\Delta moset3$ mutants showed a significant reduction in vegetative growth, germination, appressorium formation, conidiation, and pathogenicity to host plants. The *M. oryzae* *MoKMT3* is a homologue of *N. crassa* SET-2 protein. *N. crassa* SET-2 is a nucleosome-selective lysine specific methyltransferase for H3K36 methylation and associated with transcribed regions of active genes (Adhvaryu K., *et al.*, 2005). *MoKMT3* is essential for normal growth and development, and thus could affect the correct expression of a number of genes involved in

the phenotypic defects (Adhvaryu K., *et al.*, 2005). NCBI BLAST with protein sequence shows that MoSET3 is homologous to human and *Drosophila* ASH1L that binds to the 5'-transcribed region of actively transcribed genes and required for maximal gene expression and H3K4 methylation (Nakamura T *et al.*, 2000; Gregory GD *et al.*, 2007). Therefore, if MoSET3 is a functional homolog of ASH1L, MoSET3 may also contribute to gene activation in *M. oryzae*.

Among the seven KMTs genes in *M. oryzae*, MoSET1 was the most important gene for infection-related morphogenesis and pathogenicity. MoSET1 is an ortholog of *S. cerevisiae* SET1. SET1 is associated with H3K4 methylation and conserved in a wide range of eukaryotes (Mersman DP. *et al*, 2012). SET1 is implicated in the control of transcription and chromosome structure, and actually plays a role in many aspects of growth and developmental regulation (Corey *et al.*, 1997). Following the results of morphological and pathogenic assays, the $\Delta moset1$ mutants showed severe defects in vegetative growth, appressorium formation, conidiation, and pathogenicity to host plants, but not in the rate of germination. Consistent with previous knowledge, the $\Delta moset1$ mutants lost di- and tri-methylation at histone H3 lysine 4 (Fig 9), an evolutionary conserved epigenetic mark for gene activation. Therefore, our results suggested that MoSET1 largely affected expression of infection-related genes in *M. oryzae* via histone methylation.

The deletion of MoHMT4 led to severe defects in appressoria formation, and therefore in the pathogenicity of the mutants on the host plants (Fig S12, Fig 1). However, the appressorium defect in $\Delta moset1$ was completely restored when $\Delta moset1$ conidia were incubated in the presence of cAMP or cutin monomer 1,16-hexadecanediol. In *M. oryzae*, cAMP signaling is known to regulate appressorium initiation and development. The cAMP-

dependent protein kinase is necessary for infection-related morphogenesis and pathogenesis in several phytopathogenic fungi (Mitchell and Dean, 1995). The addition of cAMP to *M. grisea* conidia induced appressorium formation (Lee and Dean, 1993). Our results suggested that MoSET1 may control activation of genes involved in the cAMP pathway. Interestingly, infectivity of the $\Delta moSet1$ mutants to host plants was, however, not recovered by the addition of cAMP, indicating that $\Delta moSet1$ mutants had defects in the pathogenicity other than appressorium formation.

To our knowledge, no information is so far available about biological roles of histone lysine methyltransferase in fungal pathogenicity. The knock-out mutants of histone lysine methyltransferase genes in our research provided novel findings on involvement of histone methyltransferase proteins in infection processes of *M. oryzae*. In addition to their involvement in infection-related morphogenesis, KMT genes were implicated in producing a series of cell wall degrading enzymes (CWDEs) such as cellulases, hemicellulase, and endo- β -1,4 xylanase (Vu *et al.*, 2013). Cytological analysis of CWDE knock down mutants showed that xylanases play significant roles in both penetration and horizontal expansion of rice blast in infected plants (Vu *et al.*, 2012, Nguyen *et al.*, 2011). Recently, a novel *M. oryzae* endotransglucosylase, a member of the glycoside hydrolase 7 family (GH7), was identified and shown to degrade β -glucans and decrease the physical strength of plant cell walls (Takahashi *et al.*, 2013). Therefore, the severe reduction of virulence in the $\Delta moSet1$ mutant could be attributed to low expression levels of CWDEs during infection in the mutant (Table 3). This suggested that H3K4 methylation may be contributed to fungal virulence by up-regulating CWDE genes in *M. oryzae*.

Table 1. Comparison of phenotypes among mutants, ectopic transformants and wild type

Fungal strains	Germination (%)	Appressorium formation (%)	Conidiation ($\times 10^6$ spores/plate)
Br48	89.35 $\pm$ 1.36	91.13 $\pm$ 2.15	88.16 $\pm$ 4.05
$\Delta mokmt1.9$	94.27 $\pm$ 2.12	91.05 $\pm$ 0.86	109.85 $\pm$ 18.26
$\Delta mokmt1.13$	94.55 $\pm$ 3.65	ND	101.63 $\pm$ 26.75
MoKMT1E	91.74 $\pm$ 2.51	85.77 $\pm$ 6.96	125.56 $\pm$ 5.86
$\Delta moaset3.8$	59.32 $\pm$ 2.05**	43.26 $\pm$ 2.09**	23.18 $\pm$ 3.09**
$\Delta moaset3.13$	47.65 $\pm$ 1.83**	48.53 $\pm$ 2.27**	23.69 $\pm$ 5.47**
MoSET3E	92.40 $\pm$ 3.09	95.33 $\pm$ 1.09	96.20 $\pm$ 9.15
$\Delta mokmt5.2$	96.93 $\pm$ 1.62	93.80 $\pm$ 2.98	63.83 $\pm$ 7.97
$\Delta mokmt5.4$	97.60 $\pm$ 1.1	90.60 $\pm$ 3.14	62.33 $\pm$ 16.07
MoKMT5E	97.20 $\pm$ 1.63	91.43 $\pm$ 2.3	64.17 $\pm$ 6.99
$\Delta moaset1.35$	93.67 $\pm$ 0.97	5.3 $\pm$ 0.62**	1.76 $\pm$ 0.16**
$\Delta moaset1.36$	96.17 $\pm$ 0.31	3.83 $\pm$ 0.3**	1.51 $\pm$ 0.28**
MoSET1E	95.73 $\pm$ 1.31	90.43 $\pm$ 1.42	135.33 $\pm$ 17.96
$\Delta mokmt3.22$	56.79 $\pm$ 2.91**	44.91 $\pm$ 2.69**	21.30 $\pm$ 5.25**
$\Delta mokmt3.26$	61.15 $\pm$ 9.61**	43.99 $\pm$ 4.43**	22.50 $\pm$ 4.78**
MoKMT3E	91.08 $\pm$ 3.10	87.40 $\pm$ 2.52	80.50 $\pm$ 3.2
$\Delta moaset6.8$	91.08 $\pm$ 3.10	88.59 $\pm$ 1.45	84.17 $\pm$ 1.53
$\Delta moaset6.13$	90.80 $\pm$ 2.45	88.53 $\pm$ 2.27	84.13 $\pm$ 3.75
MoSET6E	91.18 $\pm$ 2.50	90.00 $\pm$ 4.32	78.30 $\pm$ 6.61
$\Delta mokmt4.17$	91.44 $\pm$ 4.83	89.25 $\pm$ 2.10	79.68 $\pm$ 4.49
$\Delta mokmt4.19$	89.28 $\pm$ 1.32	90.80 $\pm$ 2.45	80.76 $\pm$ 5.22
MoKMT4E	90.54 $\pm$ 1.50	87.33 $\pm$ 1.56	79.11 $\pm$ 4.46
MoKMT1C	87.13 $\pm$ 2.7	85.43 $\pm$ 4.4	98.77 $\pm$ 5.69
MoSET3C	91.43 $\pm$ 1.77	89.16 $\pm$ 2.10	121.33 $\pm$ 12.22
MoSET1C	ND	91.93 $\pm$ 2.48	83.53 $\pm$ 11.91
MoKMT3C	90.72 $\pm$ 1.97	91.33 $\pm$ 1.56	82.20 $\pm$ 3.36

Means and standard deviations were calculated from three independent replications. $p < 0.01$, ND: not determine

Table 2. Infection types in wheat and barley cultivars inoculated with wild type and disrupted mutants

Fungal strain	Infection type (22°C)				
	Wheat cultivar			Barley cultivar	
	Thatcher	Chinese spring	Norin 4	Nakaizumi-zairai	Nigrate
Br48	2B	4bg	4bG	4bG	5G
$\Delta mokmt1.9$	1b	2B	3bg	3bG	5G
$\Delta mokmt1.13$	1B	3Bg	3bG	3bG	5G
MoKMT1E	2B	4bg	4bG	4bG	5G
$\Delta moset3.8$	1b	1B	2bg	2B	5G
$\Delta moset3.13$	1b	1b	2bg	2B	5G
MoSET3E	2b	4bG	4bG	4bG	5G
$\Delta mokmt5.2$	2B	4Bg	4bG	4Bg	5G
$\Delta mokmt5.4$	2B	4bg	4bG	4Bg	5G
MoKMT5E	2B	4bg	4bG	4bG	5G
$\Delta moset1.35$	0	0	0	0	3BG
$\Delta moset1.36$	0	0	0	0	3BG
MoSET1E	3b	4bG	4bG	4bG	5G
$\Delta mokmt3.22$	2b	2B	3b	2BG	5G
$\Delta mokmt3.26$	1b	2B	3Bg	3bg	5G
MoKMT3E	2B	3bG	4bG	4bG	5G
$\Delta moset6.8$	2B	4bg	4bG	4bG	5G
$\Delta moset6.13$	2B	3bG	4bG	4bG	5G
MoSET6E	2B	3bG	4bG	4bG	5G
$\Delta mokmt4.17$	2B	3bG	4bG	4bG	5G
$\Delta mokmt4.19$	2B	3bG	4bG	4bG	5G
MoKMT4E	2B	3bG	4bG	4bG	5G
MoKMT1C	2b	4Bg	4bG	4bG	5G
MoSET3C	3b	4bG	4bG	4bG	5G
MoSET1C	2B	3bG	4bG	4bG	5G
MoKMT3C	2B	3bG	4bG	4bG	5G

- The size of lesion: 0, no visible evidence of infection; 1, pinpoint spots; 2, small lesion (<1.5 mm); 3, lesion with an intermediate size (<3 mm); 4, large and typical lesion; 5, complete blighting of leaf blades.

- The color of lesion: G: green, B: brown. 0 to 3B: resistant; 3G to 5G: susceptible

Table 3. Cellular response of wheat leaves cultivar Norin 4 to different *mokmt* disrupted mutants and parent strain

Fungal strains	Percentage of cytological reaction				HR ratio (C/C+D)
	No reaction (A)	Papilla formation (B)	HR (C)	Hyphal growth (D)	
Br48	3.6	5.8	26.7	63.9	29.5
$\Delta mokmt1.9$	9.9	6.9	34.65	48.51	41.67 *
$\Delta moset3.13$	16.7	19.2	60.3	3.8	94.1**
$\Delta mokmt5.4$	3.21	4.87	26	67.31	27.86
$\Delta moset1.36$	77.3	21.27	1.4	0	100 **
$\Delta mokmt3.22$	20.7	18.0	55.3	5.8	90.5**
$\Delta moset6.8$	7.1	6.4	28.1	59.4	32.59
$\Delta mokmt4.19$	5.3	5.2	25.3	61.1	29.28

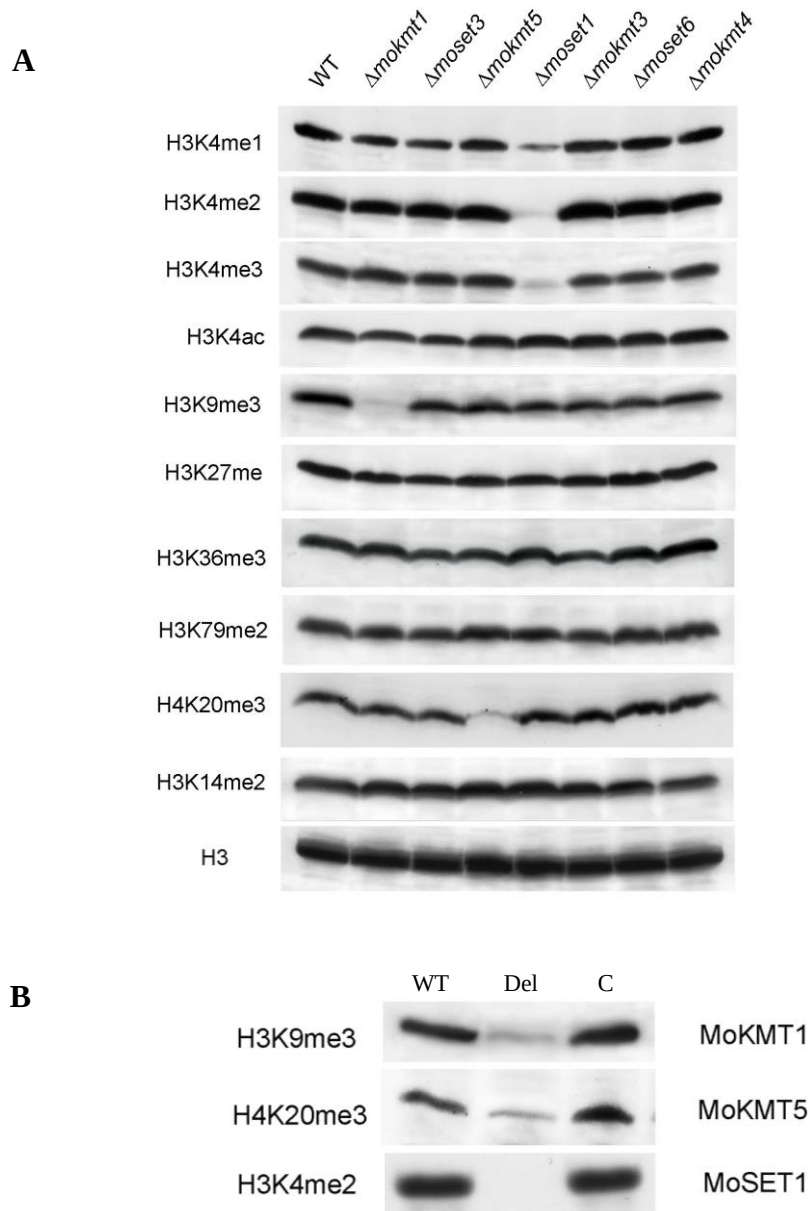
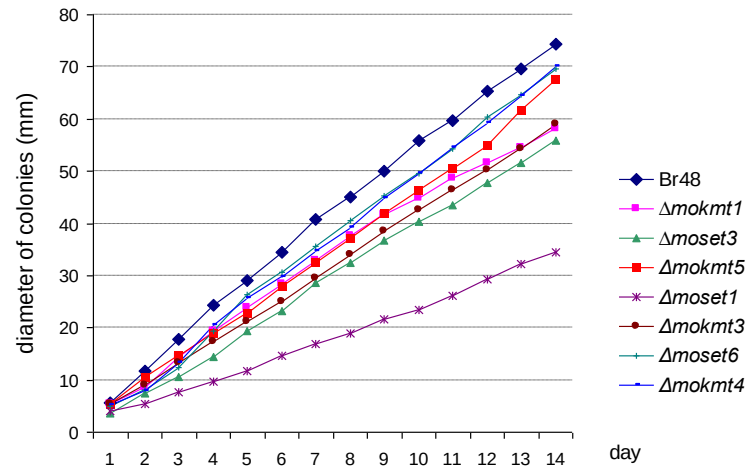


Figure 9. Western blot annalysis of histone modification in *mokmt* mutants (A) and genetic complementation (B) strains. Total protein extracted from *M. oryzae* cells, wild type (WT), *mokmt* deletion mutants [Del] and complemented strains [C] was subjected to 15% SDS-polyacrymide gel electrophoresis, and probe with antibodies against hisotne methylation H3K4me1 (Active Motif #39298), H3K4me2 (Active Motif #39141), H3K4me3 (Active Motif #39159), H3K4ac (active motif #39381), H3K9me3 (Active Motif #39161), H3K27me3 (Active motif # 39158), H3K36me3 (Active motif # 61102), H3K79me2 (Active motif # 39144), H4K20me3 (Active Motif #39181), H3K14me2 (Active motif # 39350), and normal histone 3 (Active Motif #39163).

A



B

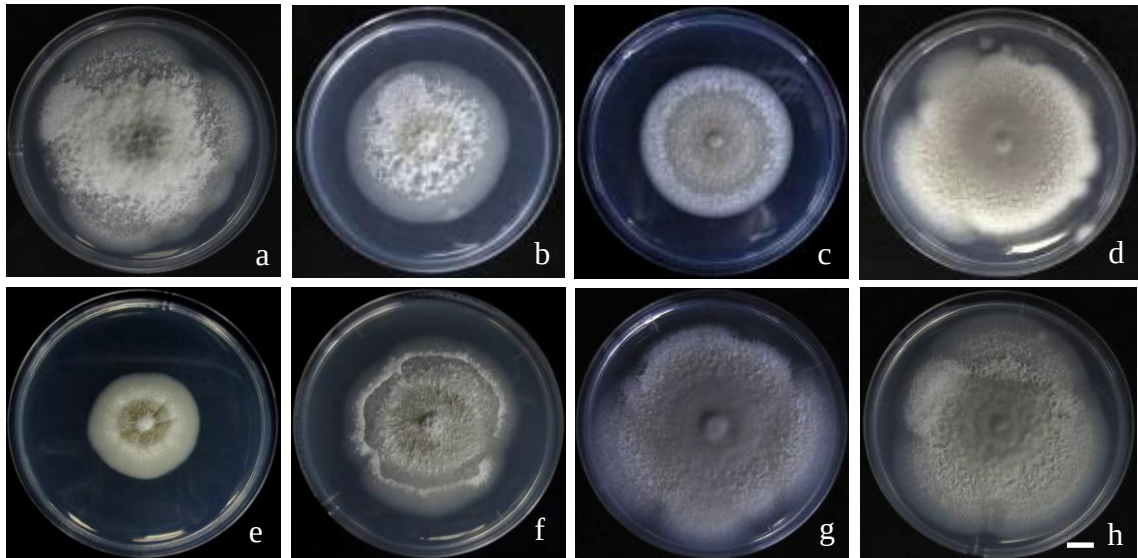


Figure 10. Growth rate of the *mokmt* mutants of *M. oryzae*. (A) Vegetative growth was measured for 14 days on PDA medium. The graphs show average of three independent replications. (B) Mycelium growth at 14 days after incubation at 25°C; (a) Wild type Br48 strain, (b) $\Delta mokmt1$, (c) $\Delta moset3$, (d) $\Delta mokmt5$, (e) $\Delta moset1$, (f) $\Delta mokmt3$, (g) $\Delta moset6$, and (h) $\Delta mokmt4$ mutants. Bar = 10 cm.

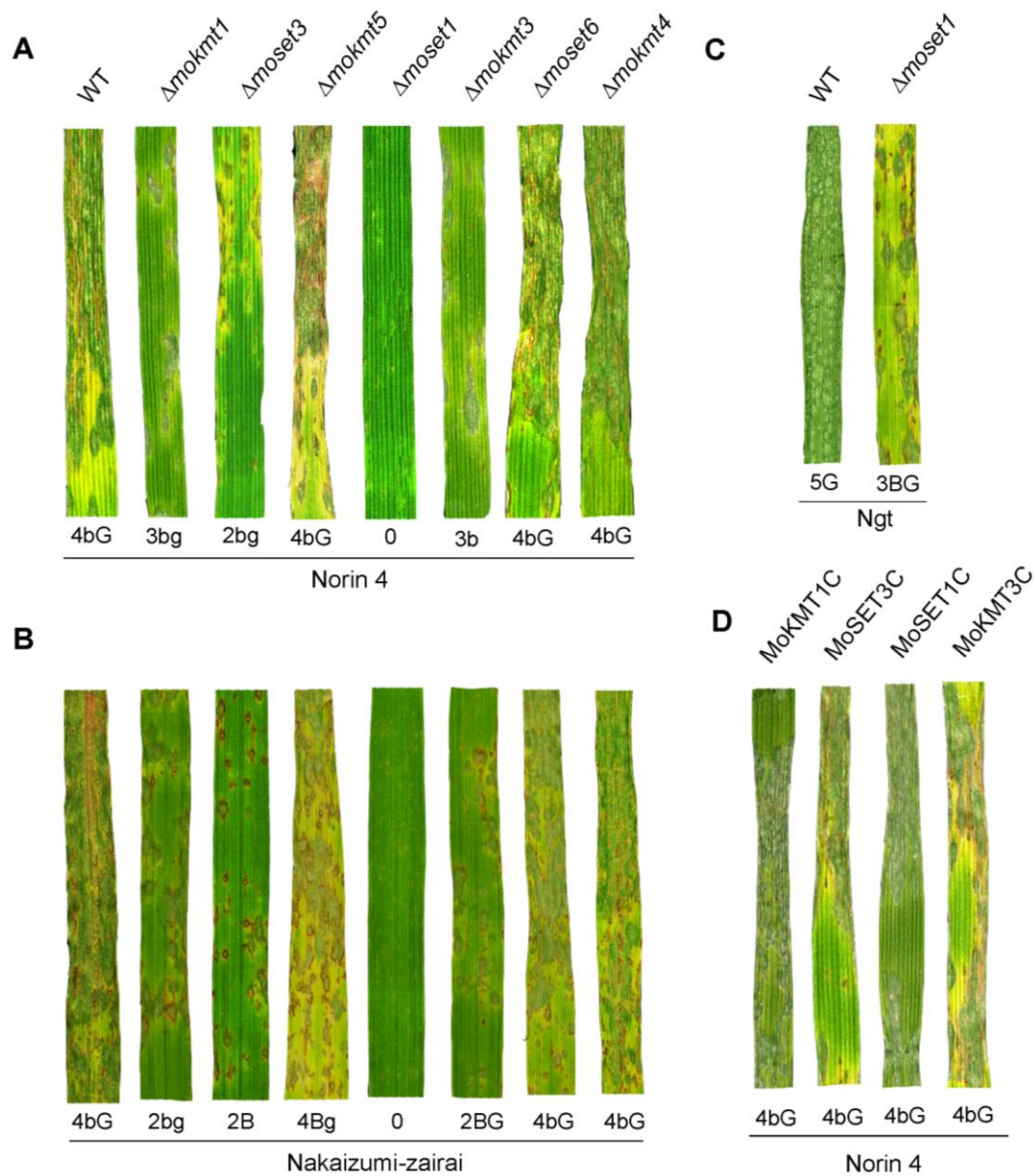


Figure 11. Infection assays of the *mokmt* deletion mutants. (A) Disease development after spraying sporal suspension of wild type (WT) and *mokmt* mutants onto wheat leaves Norin 4 cultivar. (B) and barley Nakaizumi-zairai cultivar. (C) Pathogenesis of the $\Delta moset1$ mutant and wild type strains on the super susceptible barley cultivar Nigrate (Ngt). Conidia ($1-2 \times 10^5$ spores/ml) was sprayed onto the leaves and incubated at 22°C with a 12h photoperiod for 5 days. (D) Disease development on wheat leaves Norin 4 cv. inoculated with complemented strains of *mokmt* mutants MoKMT1C, MoKMT3C, MoSET1C and MoSET3C.

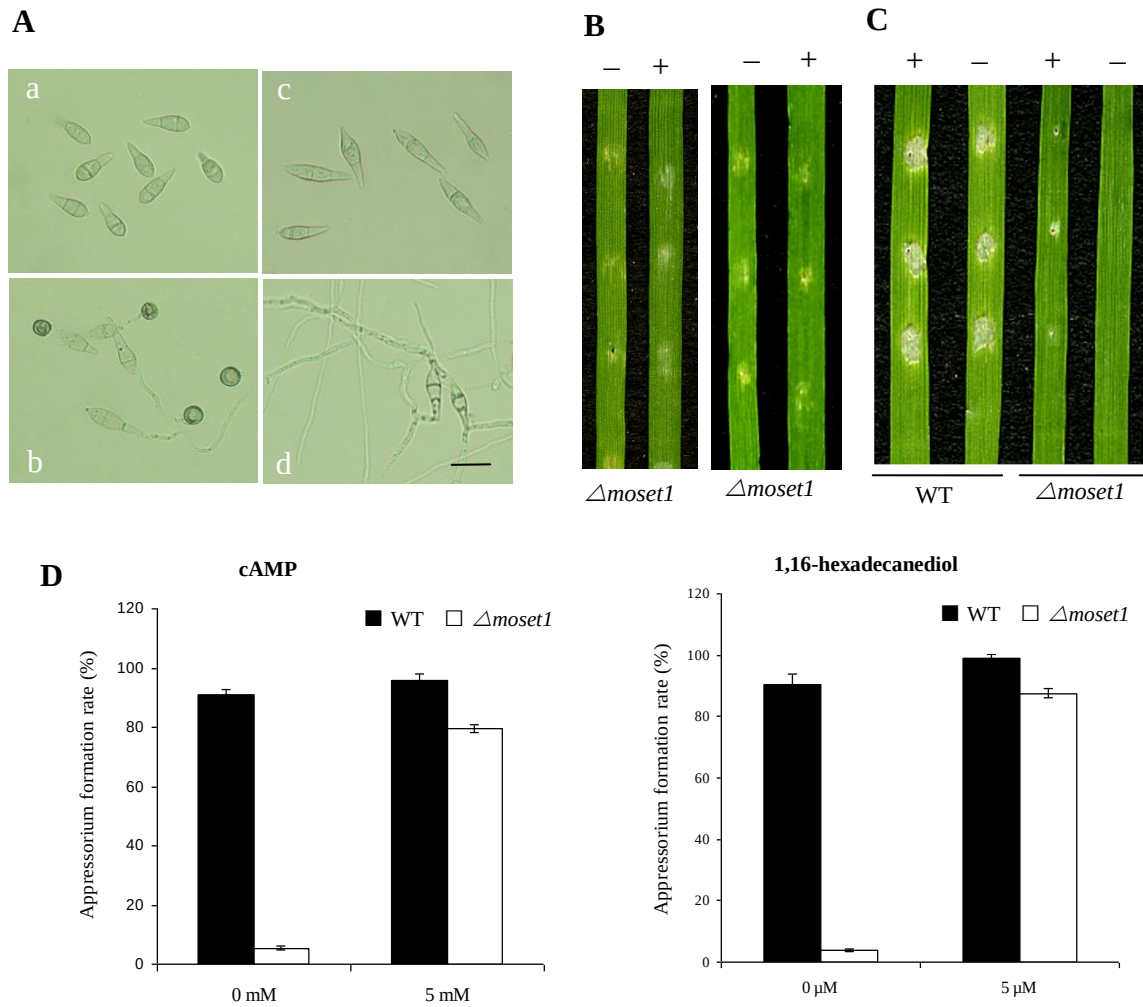


Figure 12. The defect of $\Delta moset1$ mutant in appressorium formation was restored by exogenous signal molecules. (A) Effect of MoSET1 deletion on conidial morphology and appressorium formation. Conidia (a) and appressoria (b) of *M. oryzae* wild-type strain Br48. Conidia of the $\Delta moset1$ mutant showed an elongated shape (c) and seldom formed an appressorium (d). Bar = 10 μ m. (B,C) Inoculation test of the $\Delta moset1$ mutant on wheat. Infection assay was performed using the wheat cultivar Norin4 at 22°C. (B) Spore suspension at a concentration of 1-2 $\times 10^5$ spores/ml was with (+) or without (-) 5mM cAMP or 5 μ M 1, 16-hexadecanediol (cutin monomer) was dropped onto intact leaf surface. (C) Spore suspension was dropped to leaves with (+) or without (-) a wound created by breaching the cuticle with a needle. Inoculated leaves were incubated under dark and humid conditions for 24 h, and moved to an incubator at 22°C. Five days after inoculation, symptoms on the inoculated plants were evaluated. (D) Exogenous addition of 5mM cAMP (B) and 5 μ M 1,16-hexadecanediol restored appressorium formation in the $\Delta moset1$ mutant. Error bars represent standard deviation.

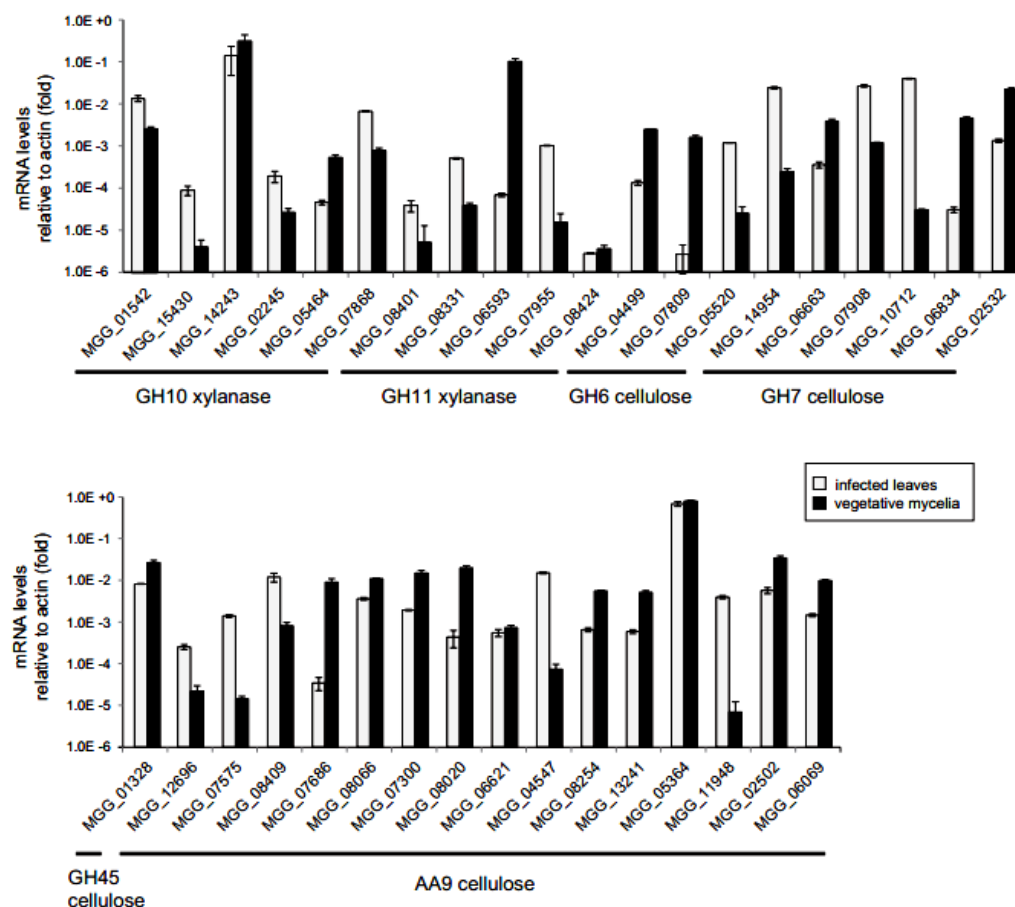


Figure 13. Expression profiles of thirty-six *M. oryzae* cell wall-degrading enzyme (CWDE) genes in infected wheat leaves relative to those in vegetative mycelia. Transcript levels of six GH10 xylanases, five GH11 xylanases, three GH6 cellulases, six GH7 cellulases, one GH45 cellulases, and fifteen AA9 cellulases were examined by quantitative RT-PCR (qRT-PCR) with the internal control gene actin (MGG_03982). Relative transcript quantities of the CWDE genes to the actin gene were measured in vegetative mycelia and infected wheat leaves collected at 72 hpi. This experiment was performed with two biological replicates with duplicate technical replicates. Error bars represent standard deviations

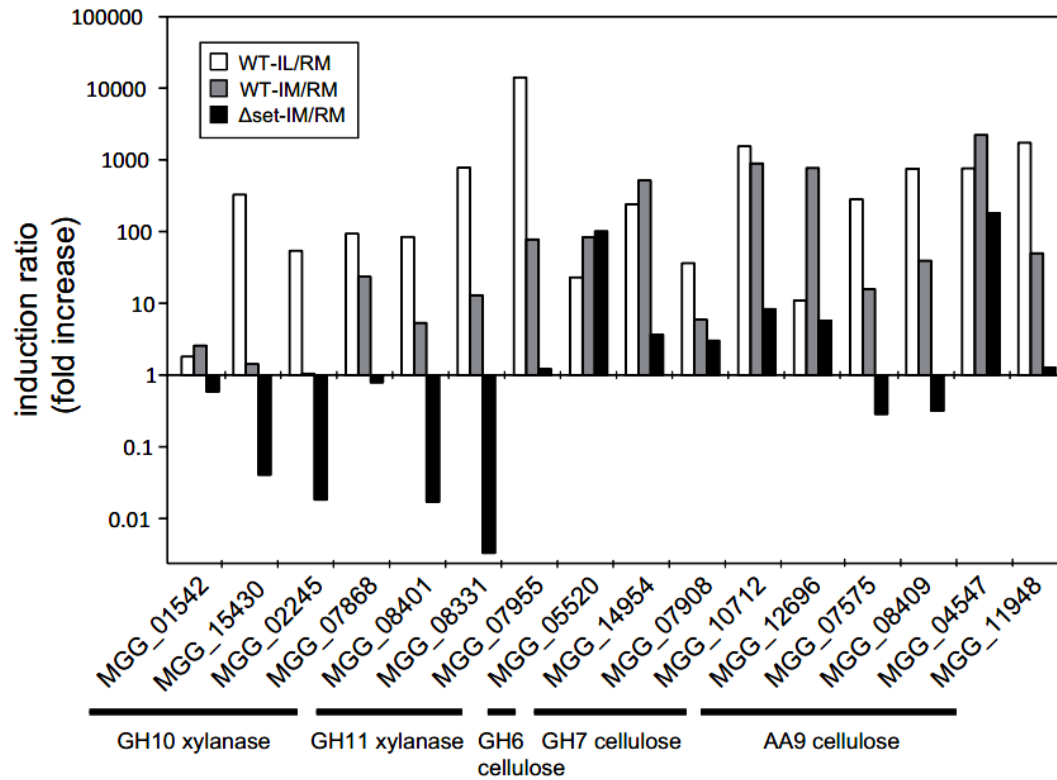


Figure 14. Comparison of fold induction of CWDE genes in substrate containing media between Δ moset1 and wild-type (WT) strains of *M. oryzae*. Quantitative RT-PCR (qRT-PCR) analysis was performed to examine transcript levels of the 16 infection-induced CWDE genes in infection leaves, induction media containing 1% CMC-cellulose or 2% xylose, and rich media. cDNA quantities were normalized to the actin gene (MGG_03982). Values in the graph indicate fold induction ratios of the CWDE genes under the following three combinations; WT-infection leaves/WT-rich media (WT-IL/RM), WT-induction media/WT-rich media (WT-IM/RM), and Δ moset1-induction media/ Δ moset1-rich media (Δ set-IM/RM). This experiment was performed with three biological replicates with duplicate technical replicates.

CHAPTER IV

Roles of MoSET1 in regulating global gene expression during infection-related morphogenesis in *M. oryzae*

1. Introduction

In eukaryote cells, histone methyltransferase complexes have been shown to hold an important role in the activation or repression of transcription through modulating the structure of chromatin and ability to modify a variety of proteins (Shilatifard, 2006). Chromatin state can be analyzed by chromatin immunoprecipitation (ChIP), in which an antibody is used to enrich DNA from genomic regions carrying a specific epitope. Enrichment at individual loci is commonly assayed by polymerase chain reaction (PCR), but this method does not reveal global binding site of protein to the genome. Recently, the ChIP-seq and ChIP-chip techniques allow researchers to investigate the distribution of the histone marks (and other chromatin binding proteins) on the genome at a high resolution, which led to the discovery that the binding patterns of the proteins and histone marks over the genome are also highly informative in predicting genomic functions and annotations (Robertson AG, *et al.*, 2008). Therefore, ChIP-seq is used to determine how transcription factors and other chromatin-associated proteins interact with DNA to regulate gene expression, which is essential for fully understanding many biological processes and disease states. ChIP-seq analyses in model organisms showed that H3K4 tri-methylation and H3K9 acetylation marks are concentrated globally near promoters, and that their distribution was related to Pol II occupancy and gene activity (Barski *et al.*, 2007). Both activating marks potentiate recruitment of the general transcriptional factor and antagonize transcriptional silencing by chromatin regulation (Klymenko and Muller, 2004).

RNA-Seq is a recently developed approach to transcriptome profiling that uses deep-sequencing technologies. The key aims of transcriptomics are: to catalogue all species of transcript, including mRNAs, non-coding RNAs and small RNAs; to determine the

transcriptional structure of genes, in terms of their start sites, 5' and 3' ends, splicing patterns and other post-transcriptional modifications; and to quantify the changing expression levels of each transcript during development and under different conditions (Wang Z *et al.*, 2009).

M. oryzae displays dramatic morphological changes during infection (Wilson & Talbot, 2009). To penetrate into host plant cells, *M. oryzae* spores germinate and form appressorium at the tip of the germ tube. The appressorium generates enormous turgor pressure and physical force to breach the host cuticle, and the fungus eventually develops invasive hyphae to colonize host cells. These morphological changes are accompanied with global transcriptional alterations (Gowda M *et al.*, 2006). My study showed that MoSET1 essential for H3K4 methylation hold the most important role in infection processes. In this chapter, we focus on the role of MoSET1 in global gene regulation related to the formation of infection structures in *M. oryzae*. While it is generally believed that H3K4 di- and tri-methylation are epigenetic marks for gene activation in higher eukaryotes, involvement of H3K4 methylation in gene repression is also reported in fungi and other organisms. RNA-seq and ChIP-seq technology with di- and tri-methylation of H3K4 antibodies were used to investigate the effect of MoSET1 on transcriptome profiling in *M. oryzae* during infection processes. Then, the relationship between H3K4 enrichment and corresponding transcript expression will be clarified.

2. Materials and methods

2.1. Fungal strains and growth conditions

The *M. oryzae* wheat-infection wild-type strain Br48 and the $\Delta moset1$ mutant were used in this experiment. For working culture of fungi, a barley grain from the stock culture was placed on a PDA (potato dextrose agar) plate and incubated at 25°C. Fungal plugs were transferred to flasks containing complete medium (5% sucrose, 3% casamino acids, and 3% yeast extract) and incubated in a shaker at 120 rpm and 25°C for 4 days. The mycelia were collected using Whatman filter paper and washed with distilled water. The mycelia were used as a starting material for all *in vitro* experiments (temperature up shift, oxidative, nutritional induction and limitation). The temperature up shift experiment was done by incubating fungal mycelia in CM liquid in a shaker (120 rpm) at 42°C for 45 minutes. The oxidative experiment was carried out using fungal mycelia in CM liquid plus methyl viologen (Paraquat; 10 mM) in a shaker at 25°C for 24 hours. Carbon limitation experiment was performed in minimal medium (MM: 6g/L NaNO₃, 0.5g/L MgSO₄, 0.5g/L KCL, 1.5g/L KH₂PO₄, 10g/L D-Glucose) without adding carbon source (MM-C: 6g/L NaNO₃, 0.5g/L MgSO₄, 0.5g/L KCL, 1.5g/L KH₂PO₄) (Mathioni, *et.al*, 2011). The medium for controlling in Carbon limitation experiment, we used minimal medium plus 3% yeast extract and 3% casamino acid. For nutritional induction experiment, yeast nitrogen base without amino acids (Difco BD, Franklin Lakes, NJ) was used with 1% xylose or an other carbon source.

2.2. Chromatin immuno-precipitation (ChIP) assays

Chromatin immuno-precipitation (ChIP) experiments were performed with germinating conidia, appressorium formation and vegetative mycelia of wild type (Br48) strain using the ChIP-IP® Express kit (Active motif # 53008) according to manufacturer's instruction using

sonication as the method for chromatin shearing. Extracts containing fragments for ChIP assay were prepared as follows: 100mg samples were treated with 1% formaldehyde by shaking gently (100rpm) for 30 minutes at room temperature and washed with 1×PBS. Formaldehyde was quenched with 2.5M Glycine and washed with 1×PBS. The nuclei pellets were collected by filtration and re-suspended in 350µl Shearing buffer supplemented with 2 µl PIC + 2 µl PMSF and placed the samples in ice. The DNAs were sheared on ice by sonication using Bioruptor apparatus (Diagenode) for 3 cycles of 1 min on at high intensity (200 W) and 30 sec off, followed by 4 cycles of 1 min on at medium intensity (160 W) and 30 sec off, and then centrifuged the sheared chromatin samples at 15000rpm at 4°C for 10 minutes. The size of the sheared chromatin was around 200 to 1,000 bp as determined by agarose gel electrophoresis. For immunoprecipitation, the reactions were set up by mixing 50 µl sheared chromatin, 10 µl ChIP buffer 1, and 25 µl Protein G magnetic beads, 1 µl Protease inhibitor cocktail and the appropriate antibodies. Then, the ChIP incubation was performed on an end-to-end rotator at 4°C for overnight. The beads were wash one time with 800 µl ChIP buffer 1 and two times with 800 ChIP buffer 2. To elute chromatin, 50 µl Elution buffer was added to the beads and incubated at room temperature on an end-to-end rotator for 15 minutes, then added 50 µl Reverse cross-linking buffer and placed the tubes in magnetic stand to allow beads to pellet to side of tubes, then quickly transferred the supernatant which contains the chromatin to the fresh tubes. The ChIP and Input DNA samples were incubated at 95°C for 15 minutes in a thermocycler. The tubes were then returned to room temperature and treated with 2 µl Proteinase K by incubating at 37°C for 1 hour. The samples were incubated again to inactivate the Proteinase K with 2 µl Proteinase K Stop Solution. The supernatant was kept at -20°C for qPCR analysis. Indexed ChIP-seq

libraries were prepared with the Illumina-compatible NEXTflex ChIP-seq Kit (Bioo Scientific) according to the manufacturer's instructions. Fragment size selection of ChIP-seq libraries was done using Agencourt AMPure XP beads (Beckman Coulter). The products are purified and enriched with PCR to create the final double stranded cDNA library. The MiSeq system (Illumina) was used to sequence the cDNA library.

2.3. rRNA depletion and RNA-sequencing library preparation

Depletion of rRNA from total RNA was done by using Ribo-ZeroTM Magnetic Kit (Human/Mouse/Rat) as following manufacturer's instructions. Indexed RNA-seq libraries were prepared with the NEBNext UltraTM RNA Library Prep Kit for Illumina kit (New England Biolabs) or NEXTflexTM Directional RNA-Seq Kit (BIOO Scientific Corp.) according to the manufacturer's instructions. Fragment size selection of RNAseq libraries was done using Agencourt AMPure XP beads. The products were purified using Agencourt AMPure XP Magnetic beads and enriched with PCR amplification to create the final double stranded cDNA library. Finally, the cDNA library was sequenced in accordance with the manufacture's instructions (Illumina, USA) using Illumina Miseq System.

2.4. NGS data analysis

The RNA-seq and ChIP-seq reads (75-120 bp) were mapped to the genome of the *Magnaporthe oryzae* strain 70-15 (release 8.0, <http://www.broadinstitute.org/>) using TopHat v2.0.10 (Kim *et al.*, 2011) and bwa v0.6.2-r126 (Li, 2009), respectively. RNA-Seq and ChIP-seq data were visualized in the Integrative Genomics Viewer genome browser (Robinson, *et al.*, 2009). The edgeR package (Robinson *et al.*, 2010) for R v3.0.1 (Core, 2013) was used for TMM normalization (Robinson, 2010), identification of differentially expressed genes (DEGs) from RNA-seq data, and detection of genes differentially enriched

for histone modifications from ChIP-seq data with the corrected p-value (Benjamini, 1995) cutoffs of 0.01 (ChIP-seq analysis) or 0.001 (RNA-seq analysis). The GOstats package (Falcon, 2007) was used to identify statistically significant enriched Gene Ontology (GO) categories.

3. Results

3.1 Methylation of histone 3 lysine 4 (H3K4) is associated with transcriptionally active genes

To test the possibility that the transcriptional activities of infection-related genes was controlled by MoSET1, using an antibody against H3K4me2 we carried out ChIP-qPCR assay of 25 genes involved in host cell-wall attaching such as cAMP-dependent kinase, Ca^{2+} and CAM binding protein Ca^{2+} pumps, Ca^{2+} transporters, and cellulase genes belong to glycosyl hydrolase (GH) family 6 and family 7 (Vu, *et al*, 2012; Nguyen, *et al*. 2008). Scatter plots were produced to see a correlation between levels of gene expression and histone modification. However, the correlation coefficients between methylation of histone H3 lysine 4 and gene expression were under significant levels (Fig 15).

We next performed qPCR with the Δmoset1 mutant to monitor the expression levels of the genes that are known to be transcriptional up-regulated under stress conditions in the wild-type strain (Mathioni, *et al*, 2011; Nguyen, *et al*, 2011). The mRNA expression of an endo 1,4- β xylanase (Mgg_07868.6) gene involved in plant cell wall degradation (Wu, *et al*, 1997), showed only 1-fold change in the Δmoset1 mutant while the wild type strain displayed an approximately 33-fold increase on inducing medium containing 1% xylose (Fig 16A). The mRNA expression abundance of the endoxylanase gene also exhibited a similar result in carbohydrate-limited minimal medium (MM-C) where only approximately 4-fold increase was found in the mutant while about 18-fold increase in the wild type (Fig 16B). Under high temperature, starvation (MM-C), and oxidative stress (Paraquat: PQ) conditions, the expression levels of DUF427 domain-containing protein (Mgg_04164.6) were suppressed in the Δmoset1 mutant compared to those in the wild type strain (Fig 16B, C, D).

Similarly, the N-acyl-L-amino acid amidohydrolase (Mgg_05912.6) gene was induced up to 192-fold in the wild type strain while only 12-fold increase was observed in the $\Delta moset1$ mutant under oxidative stress condition (Fig 16D). In general, under non-inducing conditions, gene expression in the $\Delta moset1$ mutant was higher than in the wild type strain, but under inducing conditions levels of gene expression in the mutant were often lower compared to those in the wild-type strain. Taken together, the MoSET1 gene can control gene transcriptional by up-regulating target genes under inducing conditions while by down-regulating them under non-inducing conditions.

For further determining the relationships of H3K4 methylation and global gene expression and genome-wide H3K4 methylation patterns during infection-related morphogenesis, we performed chromatin immunoprecipitation sequencing (ChIP-seq) and RNA sequencing (RNA-seq) analyses using chromatin and RNA samples extracted from mycelia and germination tubes of wild type and $\Delta moset1$ strains (Table S4). ChIP-seq and RNA-seq data were visualized by showing a representative chromosomal region (Figure 17). The results showed that DNA from immunoprecipitates with normal histone H3 antibodies distributed relatively evenly throughout the genome, while DNA immunoprecipitated with H3K4me2 and H3K4me3 antibodies predominantly localized to coding regions in the *M. oryzae* genome. H3K4me3 accumulated relatively more in the 5' gene regions. The number of genes showing differences in normalized mean coverage of H3K4me2 and H3K4me3 enrichment between vegetative mycelia and germination tubes is presented in Table 4. Changes in the H3K4me3 ChIP coverage were more frequently detected than those in the H3K4me2 coverage, suggesting that H3K4me3 could be a mark for gene regulation during infection-related morphogenesis in *M. oryzae*.

To determine relationship between H3K4 methylation and gene expression in the whole genome, we categorized the *M. oryzae* genes into groups of 100 genes based on the expression levels and analyzed the levels of H3K4 methylation. In general view, levels of H3K4me2 and H3K4me3 decreased as RNA levels decreased in both mycelia and germination tubes (Figure 18A, B). This suggested that H3K4 methylation was associated with active gene expression in *M. oryzae*.

We next examined whether changes in the H3K4me2/me3 patterns were associated with gene activation/suppression during infection-related morphogenesis in *M. oryzae* at a global scale. RNA-seq analysis revealed that a total of 4,077 genes showed significant increases (1,936 genes) or decreases (2,141 genes) ($P < 0.001$) in expression levels in germination tubes. We divided these genes into five up-regulated and five down-regulated gene groups, and changes in H3K4me2/me3 ChIP enrichment in the groups were plotted (Figure 18C). The median H3K4me2 levels slightly decreased as the magnitude of transcript reductions increased. The H3K4me3 profile showed a more dynamic correlation with the transcript levels compared with the H3K4me2 levels, and the medians of the H3K4me3 levels were higher in the up-regulated gene groups and lower in the down-regulated gene groups. It is noteworthy that the transcriptional activity of a gene was not always associated with local enrichment of H3K4 methylation. Higher H3K4me2/me3 coverage in mycelia than in germination tubes was accompanied with higher gene expression in some cases such as MGG_11149 in Figure 17. The up-regulation of the MGG_11149 gene in mycelia was significantly diminished in the $\Delta moset1$ mutant, supporting the idea that H3K4me2/me3 contributes to gene activation. However, transcriptional activation of the neighbor genes (MGG_01755, MGG_01756, and MGG_01757) in germination tubes did not concomitantly

occur with apparent H3K4me2/me3 enrichment at their loci (Figure 17). In addition, with the MGG_11148 gene, H3K4me2/me3 enrichment in the wild-type strain appeared to be accompanied with gene activation but a similar change in gene expression also occurred in the $\Delta moset1$ mutant (Figure 17). Such apparent discrepancies were observed in many other cases. Therefore, in general tendency, H3K4 methylation seems to be associated with transcriptionally active genes, but the mechanism of gene regulation by H3K4 methylation is quite complicated.

3.2 MoSET1 directly or indirectly plays a role in gene activation and suppression

RNA-seq analysis of $\Delta moset1$ during infection-related morphogenesis resulted that a total of 2,572 genes were differentially expressed in $\Delta moset1$ mycelia compared with the wildtype strain ($P < 0.01$), with 1,491 genes up-regulated and 1,081 genes down-regulated. In germination tubes, there were 2429 genes differentially expressed in $\Delta moset1$ with 1,385 genes were up-regulated and 1,044 genes down-regulated in the $\Delta moset1$ mutant (Table 5). These results indicated that a significant amount of *M. oryzae* genes were affected by the *MoSET1* mutation. Interestingly, the number of genes up-regulated in the $\Delta moset1$ mutant was higher than the number of down-regulated genes, suggesting that *MoSET1* directly or indirectly plays a role in gene suppression, as well as in gene activation. To analyze the characteristics of differently expressed genes between the wild type and $\Delta moset1$, we examined the frequency distribution of genes belonging to these gene groups in mycelia (Figure 19A) and germination tubes (Figure 19B), based on the expression levels in the wild-type strain. In both mycelia and germination tubes, genes down-regulated in $\Delta moset1$ were highly biased to the high expression gene groups in the wild-type strain, while those up-regulated in $\Delta moset1$ strain were more frequently distributed in medium and low

expression level gene groups (Figure 19A, B). We next addressed how *Δmoset1* affected gene regulation by comparing the fold change (FC) in gene expression during infection-related morphogenesis between the wild-type and *Δmoset1* strains. A log2-scale scatter plot showed a positive linear correlation between the FC values in the wild-type and *Δmoset1* strains ($p = 6.8E-06$) (Figure 19C). However, regression analysis gave the equation, $y = 0.51x - 0.04$ with the correlation coefficient, $r^2 = 0.49$, indicating that the correlation was only moderate. The slope lower than one indicated that gene expression changes were generally more marked in the wild-type strain (x-axis) than in the *Δmoset1* mutant (y-axis) for both up- and down-regulated genes (Figure 19C). This supported the conclusion that MoSET1 contributed to bilateral and global gene regulation during infection-related morphogenesis in *M. oryzae*.

3.3 Characterization of differentially expressed genes (DEGs) during infection-related morphogenesis in a MoSET1-dependent or -independent manner

To assess the effect of MoSET1 on gene induction and suppression during infection-related morphogenesis, we focused on a subset of 4,077 genes that showed a significant change in expression levels between wild-type mycelia and germination tubes in the RNA-seq analysis (Table 4). There were less up-regulated genes in the subset (1,936 genes) than down-regulated genes (2,141 genes). To understand the dependency of their gene expression on MoSET1, we defined the criterion of “MoSET1-dependent genes” based on a comparison of FC values (germination tubes/mycelia) between wild-type and *Δmoset1* strains. When the FC of a gene in the *Δmoset1* strain was less than 50% of the wild-type strain, the gene was categorized as a MoSET1-dependent gene. Genes not meeting the criterion were classified as “MoSET1-independent genes”. Based on these criteria, 1,201 and 735 genes were

categorized as MoSET1-dependent and -independent up-regulated genes, respectively; and 883 and 1,259 genes were grouped as MoSET1-dependent and -independent down-regulated genes, respectively. Therefore, approximately half of the transcriptional changes during infection-related morphogenesis were directly or indirectly dependent on MoSET1 in *M. oryzae*. Dependency on MoSET1 was more evident with the up-regulated genes. MoSET1-dependent and -independent genes were further classified using euKaryotic Orthologous Group (KOG) functional categories (Figure 20). The KOG category “signal transduction mechanisms” was highly over-represented in the MoSET1-dependent up-regulated gene set. Consistently, several GO categories related to “signal transduction mechanisms” were significantly over-represented in the gene set. In this category, we found 41 kinases and 13 GTPase regulators, indicating that a large number of key signal mediators were transcriptionally regulated by MoSET1. Interestingly, the GO category “active transmembrane transporter activity” was also over-represented in this gene set. Twenty-four active transmembrane transporters were regulated by MoSET1. The KOG categories over-represented in the MoSET1-dependent down-regulated gene set were “translation, ribosomal structure and biogenesis” and “RNA processing and modification”. Interestingly, in this MoSET1-dependent down-regulated gene set, sixty-four structural constituents of the ribosome were found. This indicated that down-regulation of ribosome-related genes was highly associated with MoSET1. In addition, various nucleic acid binding proteins, especially those that bind RNA, were down-regulated in a MoSET1-dependent manner. These included proteins homologous to nuclear ribonucleoprotein, RNA helicase, tRNA synthetase, rRNA biogenesis protein, poly(A) polymerase, and poly(A)-binding protein.

Over all, MoSET1-dependent and independent genes tended to be enriched in different gene categories.

4. Discussion

Histone methylation is known to be involved in both gene activation and repression (Shilatifard, 2008). H3K4 methylation, one of the prominent histone modification marks, is proposed to be associated with activation of transcription (Rando, 2007; Santos-Rosa *et al.*, 2002; Schubeler *et al.*, 2004) in various eukaryotic organisms. ChIP-seq and RNA-seq analyses revealed that enrichment for H3K4me2 within the gene body and around the TSS is a characteristic trait of expressed genes in yeast (Bernstein *et al.* 2002; Li *et al.* 2007), and in mammals (Bernstein *et al.* 2005; Barski *et al.* 2007). In *M. oryzae*, substrate-induced gene expression of GH6 and GH7 cellulases was associated with enrichment of H3K4me2. (Vu VB, *et al.*, 2013). Similarly, MoSET1 was required for inducing xylanase (Mgg_07868), DUF427 domain-containing protein (Mgg_04164), N-acyl-L-amino acid amidohydrolase (Mgg_05912) genes. Interestingly, the expression levels of Mgg_07868, Mgg_04164 and Mgg_05912 were increased under non-inducing conditions in the Δ moSet1 mutant (Fig 16), suggesting that a possible role of H3K4 methylation in gene suppression or inactivation. This result is also consistent with the reactivation of *GAL1* in Δ set1 mutant in yeast (Zhou, 2011). The SET1 in *S. cerevisiae* was originally identified as a gene required for transcriptional silencing of silent mating-type loci in the subtelomeric region (Nislow C, *et al.*, 1997). In addition, knocking down MLL5, a trithorax homolog, resulted in reducing H3K4 methylation at the cell cycle regulated element (CCRE), and repressing the expression of Pax7, Myf5 and myogenin (Sebastian *et al.*, 2009). Wang *et al.*, 2011 proved that H3K4 tri-methylation was also associated with the repressed *PHO5* gene and remains essentially unchanged during *PHO5* activation and inactivation. Subsequently, SET1 was demonstrated to play a role in silencing rDNA and the retrotransposon Ty1 in *S. cerevisiae* (Briggs SD, *et*

al., 2001; Berretta J, *et al.*, 2008). Consistently, contribution of *MoSET1* to the repression of ribosome-related genes was shown in this study (Figure 20). Moreover, the recently research on fission yeast revealed that the lack of the H3K4 methyltransferase *Set1* generally increased DSB (DNA double strand break)-inducing protein Rec12 binding to chromatin but partially reduced DSB formation at some loci (Yamada *et al.*, 2013). Thus, SET1 orthologs in fungi are involved in gene silencing in addition to gene activation. Our RNA-seq analysis revealed that significant numbers of *M. oryzae* genes were up- or down-regulated in the $\Delta moSET1$ mutant in comparison with the wild-type strain, supporting that H3K4 methylation is directly or indirectly involved in both gene activation and suppression. *MoSET1*-dependent gene up-regulation was largely detected in highly-expressed genes in the wild-type strain, whereas *MoSET1*-dependent gene suppression was more frequently observed in genes with middle or low expression levels (Figure 19). It therefore appears that *MoSET1* contributes to proper gene expression, activating genes when required, and suppressing them when not. Apparent differences in the roles of *MoSET1* among organisms can be attributed to the available experimental approaches.

Table 4. Numbers of genes diferentially enriched for H3K4me2/H3K4me3 in ChIP-seq analysis and differentially expressed in RNA-seq analysis between mycelia and germination tubes.

Strain	Histone modification	Myc>GT	Myc<GT
ChIP-seq			
WT	H3K4me2	293	133
WT	H3K4me3	223	399
RNA-seq			
WT	-	2,141(883)*	1,936(1,201)*
<i>Δmoset1</i>	-	1,926	1,817

WT, wild type strain Br48; Myc, mycelia; GT, germination tubes

*, numbers in parentheses represent the number of MoSET1-dependent genes

Table 5. Numbers of genes differentially expressed in mycelia and germination tubes between the wild type and $\Delta moset1$ strains.

Stage	WT > $\Delta moset1$	WT < $\Delta moset1$
Myc	1,081	1,491
GT	1,044	1,385

WT, wild type Br48; Myc, mycelia; GT, germination tubes

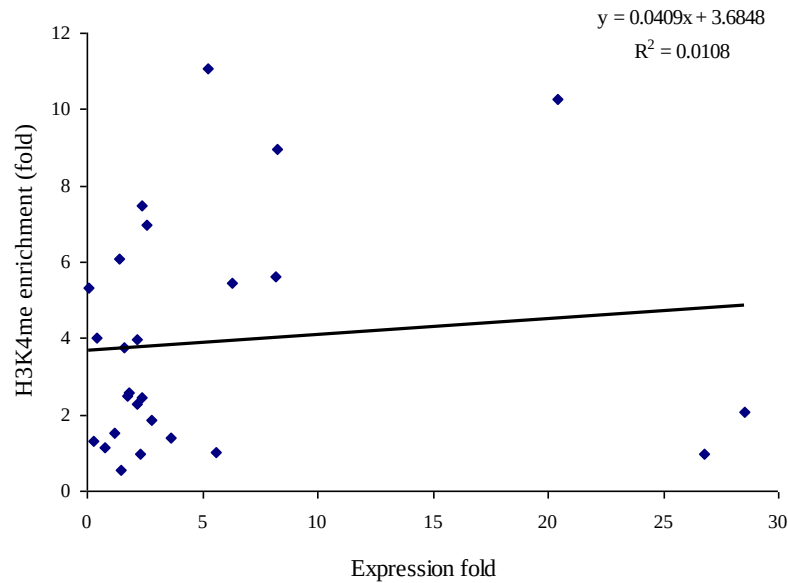


Figure 15. Relationship between H3K4me2 and infection-related gene expression in *M. oryzae*. H3K4me2 enrichment was examined by ChIP-qPCR, and the fold was calculated by dividing the value of germinating conidia by the value of vegetative mycelia. Each dot in the plots represents the means of three replicates of CPKA, Ca²⁺ and CAM binding protein Ca²⁺ pumps, Ca²⁺ transporters, and GH6 and GH7 cellulase genes. The mRNA expressions were not correlated with the amounts of H3K4me2 in the naive genome with $R^2 = 0.0108$.

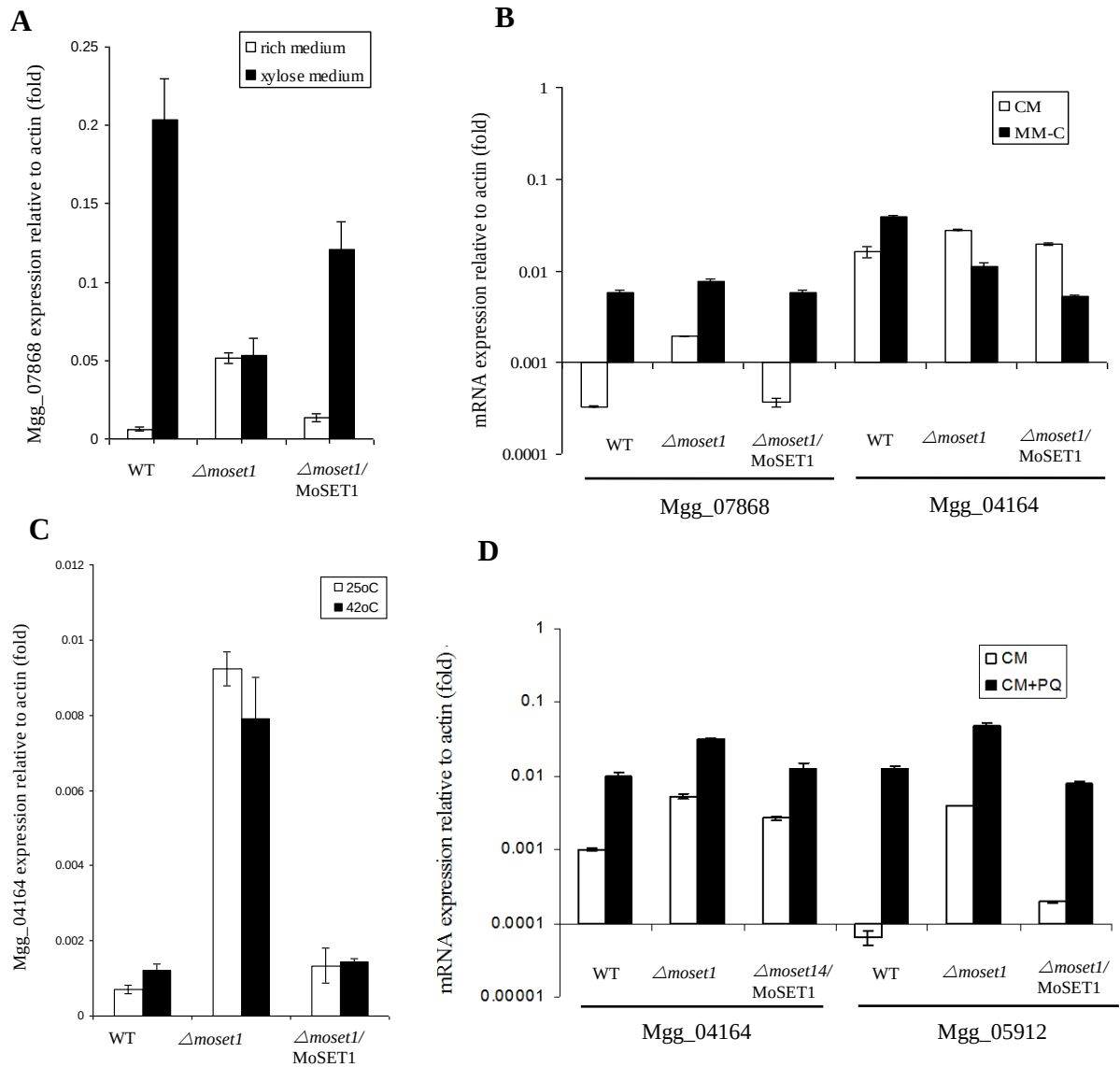


Figure 16. Gene expressions under different environment conditions. qPCR analysis of (A) endo-1,4 β -xylanase (Mgg_07868) expression in response to 1% xylose; (B) Mgg_04164 and Mgg_07868 expression in response to carbohydrate-limited minimal medium (MM-C); (C) DUF427 domain-containing protein (Mgg_04164) expression in response to temperature shift up condition 42°C; (D) Mgg_04164 and N-acyl-L-amino acid amidohydrogenase (Mgg_05912) expression in response to oxidative stress condition (Paraquat; 5mM). The wild type (WT), $\Delta moset1$, $\Delta moset1/MoSET1$ were cultured in liquid CM for 4 days before applying to appropriate inducing conditions. Values are relative expression levels relative to actin gene. These experiments were performed two biological and technical replications, and error bars represent standard deviations (SD).

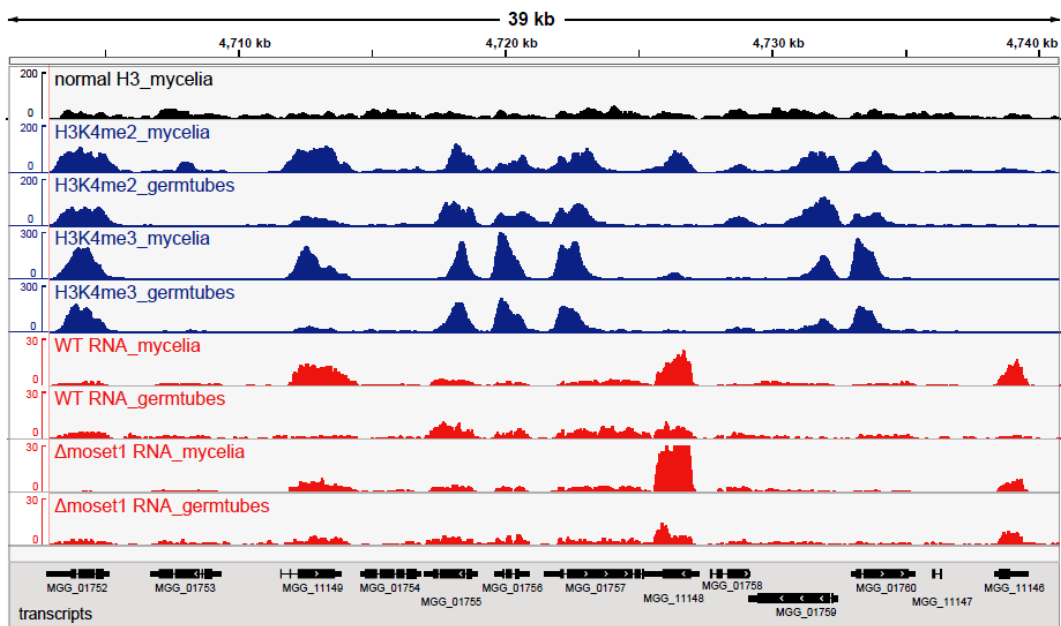


Figure 17. Representative view of ChIP-seq and RNA-seq results in *Magnaporthe oryzae* Chromosome 2. Blue and red peaks in the wiggle plot represents the normalized ChIP-seq and RNA-seq read coverage, respectively. Black peaks are for the control ChIP-seq library with anti-normal H3.

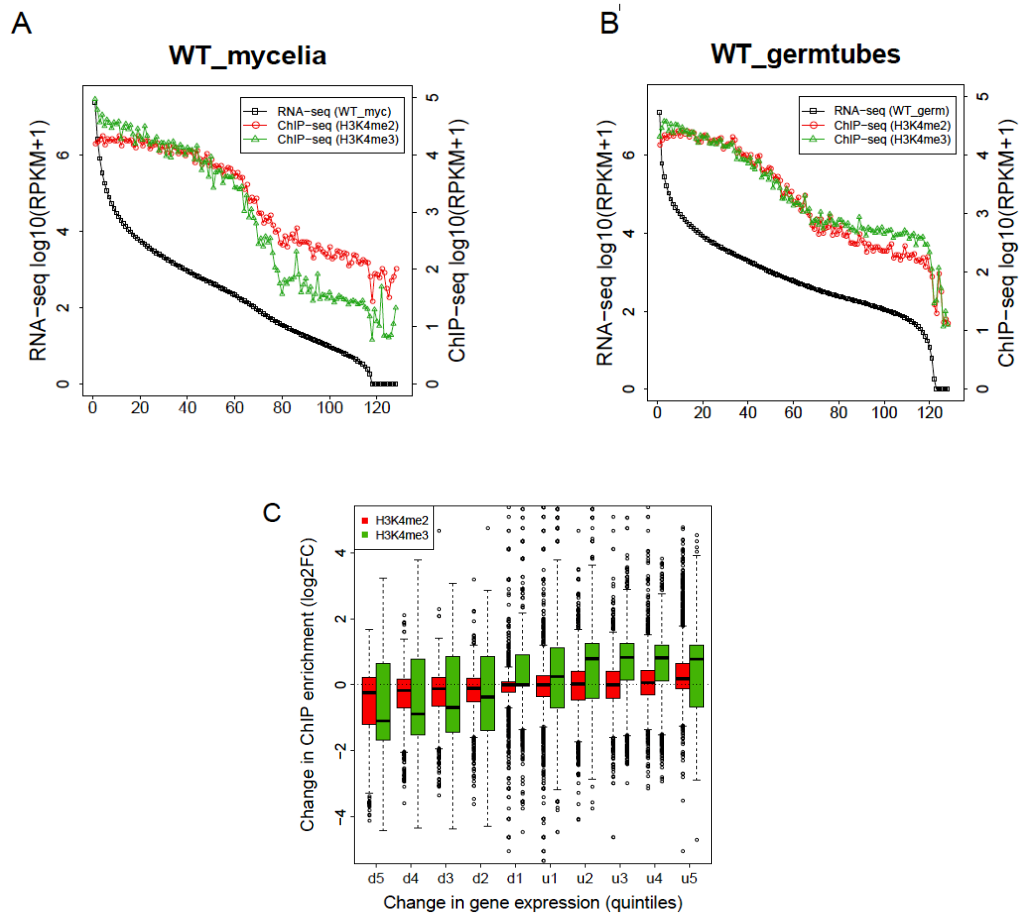


Figure 18. H3K4me2 and H3K4me3 abundance was generally associated with active transcription. (A)-(B) Genes were separated into groups of 100 genes according to their expression levels from high to low (left to right on the X axis) in mycelia (A) and germtubes (B). The average expression level of each gene group is given by an open black box. Open red circles and green triangles indicate the levels of H3K4 me2 and H3K4me3 in the gene groups, respectively. (C) Genes that were up- or down-regulated during infection-related morphogenesis were categorized into 10 groups (5 groups each in up-regulated [u1-u5] or down-regulated genes [d1-d5]) according to their change in transcript levels. The change in the H3K4 methylation (H3K4me2 or H3K4me3) in each group is represented as a box plot. In box plots, the central black line indicates the median value, and the top and bottom edges of the box are the 75th and 25th percentiles. The whiskers extend to the most extreme data points which are no more than 1.5 times the interquartile range from the box.

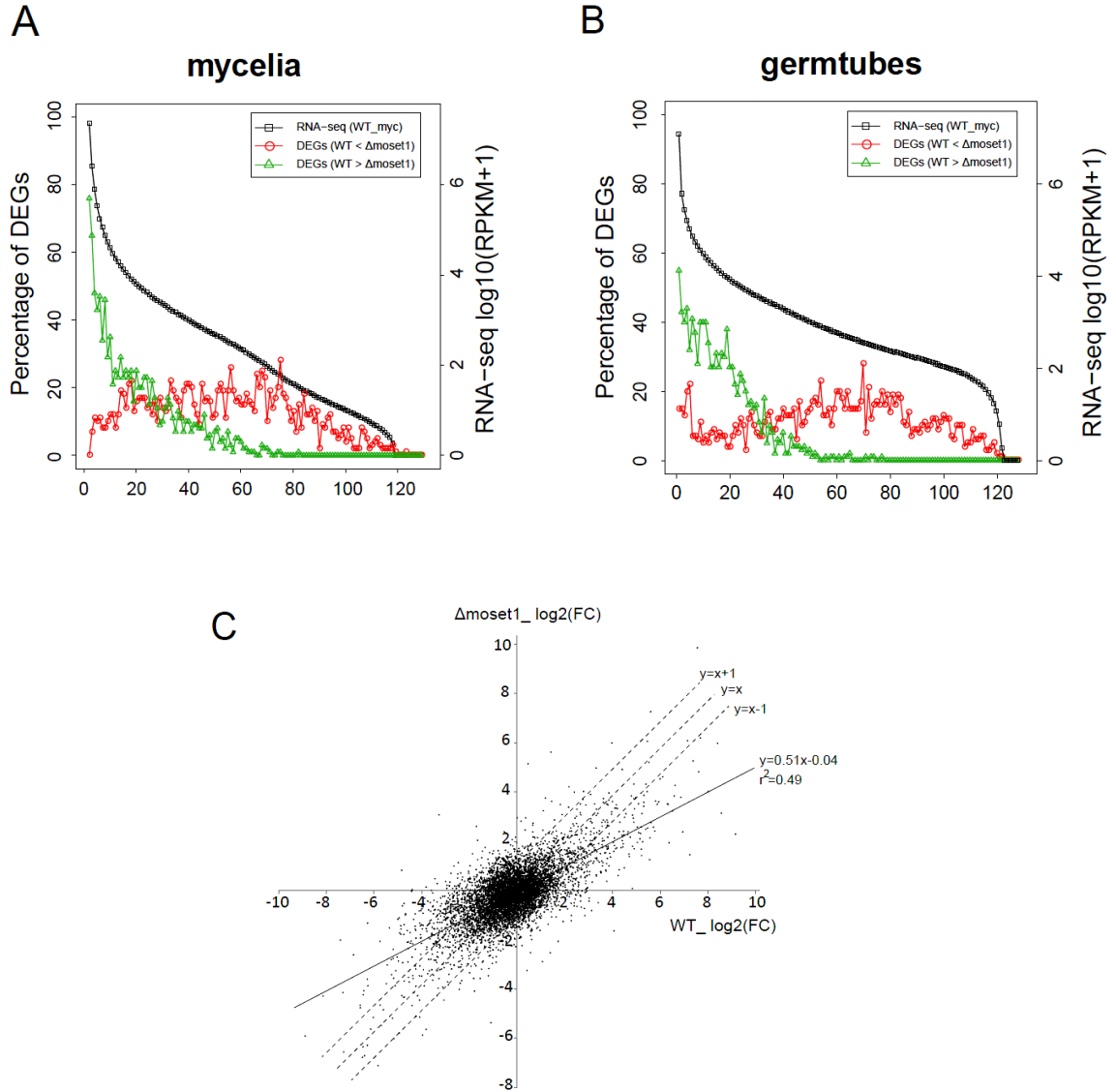


Figure 19. MoSET1 contributed to bilateral and global regulation of differentially expressed genes (DEGs). (A-B) Genes were separated into groups of 100 genes according to their expression levels from high to low (left to right on the X axis) in wild-type mycelia (A) and germtubes (B). Red open circles and green open triangles indicate the percentages of the down- and up-regulated DEGs between the wild-type (WT) and *ΔmoSet1* strains in the gene groups, respectively. (C) A log2 scale scatter plot comparison of fold changes (FC) in gene expression (germtubes/mycelia) between the WT and *ΔmoSet1* strains. A subset of 6,254 genes transcribed above 15.0 RPKM either in mycelia or germtubes of WT were plotted in the graph.

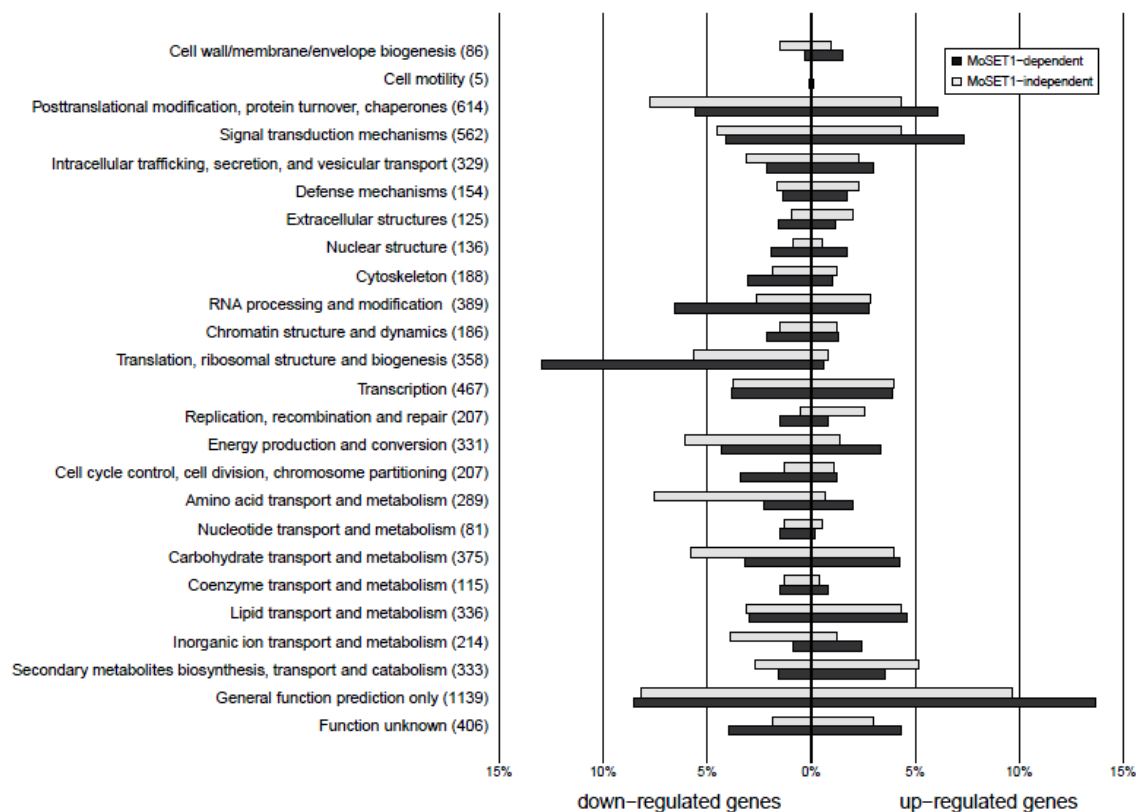


Figure 20. KOG functional classification of differentially expressed genes (DEGs) between vegetative mycelia and germtubes with regard to dependency on MoSET1. A total of 4,077 DEGs identified in the RNA-seq analysis were assigned to KOG functional categories. The assigned DEGs were further subdivided into four groups based on the direction of regulation (up or down) and the dependency on MoSET1. The percent distribution of DEGs in the subgroup by KOG category is presented. The total number of *M. oryzae* genes per KOG is shown in parentheses.

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Supplementary

Table S1. Histone lysine methyltransferase (KMTs) genes and functions

Gene No.	Name	Pre-name	Protein domain
MGG-06852.6	MoKMT1	MoHMT1	Pre-SET motif and SET domain
MGG-02937.6	MoSET3	MoHMT2	SET domain
MGG-07393.6	MoKMT5	MoHMT3	SET domain
MGG-15053.6	MoSET1	MoHMT4	RNA recognition motif (a.k.a. RRM, RBD, or RNP domain) and SET domain
MGG-01661.6	MoKMT3	MoHMT5	SET domain
MGG-10842.6	MoSET6	MoHMT6	SET domain
MGG-05254.6	MoKMT4	MoHMT7	Histone methylation protein DOT1

Table S2. Wild type and mutant strains of *Magnaporthe oryzae* used in this study

Strain	Genotype Description	Reference
Br48	Wild type (wheat infecting isolate)	Urashima <i>et al.</i> , 1999
$\Delta mokmt1.9$	<i>Mokmt1</i> deletion mutant of Br48	This study
$\Delta mokmt1.13$	<i>Mokmt1</i> deletion mutant of Br48	This study
MoKMT1E	Ectopic transformant for $\Delta mokmt1$ deletion mutant	This study
MoKMT1C	Complementation strain of $\Delta mokmt1.9$ mutant	This study
$\Delta moset1.35$	<i>Moset1</i> deletion mutant of Br48	This study
$\Delta moset1.36$	<i>Moset1</i> deletion mutant of Br48	This study
MoSET1.19E	Ectopic transformant for $\Delta moset1$ deletion mutant	This study
MoSET1C	Complementation strain of $\Delta moset1.36$ mutant	This study
$\Delta mokmt3.22$	<i>Mokmt3</i> deletion mutant of Br48	This study
$\Delta mokmt3.26$	<i>Mokmt3</i> deletion mutant of Br48	This study
MoKMT3E	Ectopic transformant for $\Delta mokmt3$ deletion mutant	This study
MoKMT3C	Complementation strain of $\Delta mokmt3.26$ mutant	This study
$\Delta mokmt4.17$	<i>Mokmt4</i> deletion mutant of Br48	This study
$\Delta mokmt4.19$	<i>Mokmt4</i> deletion mutant of Br48	This study
MoKMT4E	Ectopic transformant for $\Delta mokmt4$ deletion mutant	This study
$\Delta mokmt5.2$	<i>Mokmt5</i> deletion mutant of Br48	This study
$\Delta mokmt5.4$	<i>Mokmt5</i> deletion mutant of Br48	This study
MoKMT5E	Ectopic transformant for $\Delta mokmt5$ deletion mutant	This study
MoKMT5C	Complementation strain of $\Delta mokmt5.2$ mutant	This study
$\Delta moset3.8$	<i>Moset3</i> deletion mutant of Br48	This study
$\Delta moset3.13$	<i>Moset3</i> deletion mutant of Br48	This study
MoSET3E	Ectopic transformant for $\Delta moset3$ deletion mutant	This study
MoSET3C	Complementation strain of $\Delta moset3.13$ mutant	This study
$\Delta moset6.14$	<i>Moset6</i> deletion mutant of Br48	This study
$\Delta moset6.16$	<i>Moset6</i> deletion mutant of Br48	This study
MoSET6E	Ectopic transformant for $\Delta moset6$ deletion mutant	This study

Table S3. Primers used in this study

Primer	Sequence
Hmt18up-XhoI-F	5'-CTCGAGGTGCAAGGTTGAGCATCTGA-3'
Hmt18up-SalI-R	5'-GTCGACCCCTCTCCGAGAATCCTTTC-3'
Hmt18down-KpnI-F	5'-GGTACCCCCGTTTCTTCAACCATAGC-3'
Hmt18down-BglII-R	5'-AGATCTATCGCCAGAGAGTGGTGAAC-3'
Hmt18-sre F	5-ACATGGAGGGCAGGATGTAG-3'
Hmt18-sre R	5'-CCCCTGCAGTTAGTGCTTTT-3'
18-Full-F	5'-TTGAGCACTGAGATCCAACG-3'
18-Full-R	5'-CTGGCACACGGAATTATCCT-3'
Hmt23up-HindIII-F	5'-ACGAAGCTTCATCGAGAACTCAAGCGTCA-3'
Hmt23up-SphI-R	5'-TAGGCATGCTGGTACGTCTCCTCCTGTCC-3'
Hmt23down-KpnI-F	5'-CTAGGTACCAGACCAAGCAAGGGAAAACA-3'
Hmt23down-BglII	5'-GGCAGATCTGGCAGCAGGAAGTACCAGAC-3'
Hmt23-srce-F	5'-TACATCCAGACGCGTCATTC-3'
Hmt23-Scre-R	5'-CCATTTGCCCAGATACTCGT-3'
Hmt23comple-F	5'-CTGTGCGGTTTGTGACACTC-3'
Hmt23comple-R	5'-ACACCGCAAACCTGAAATGT-3'
Set9up-F	5'-CGGTGCTCTGTCTCTAGTTTCA-3'
Set9up-R	5'-GCGCATCTGTCAGTATGTCATC-3'
Set9down-F	5'-CTAAGGGCAAGATCGACGAG-3'
Set 9down-R	5'-CGAACTGAATTCGAAATCGGAACG-3'
Set9scre-F	5'-CCGACTGTACGACTCAGATGG-3'
Set9scre-R	5'-ACCCATCACGTCTTCACCTC-3'
Hmt3full F	5'-ATCCCTTCGTCGCTAGTTTG-3'
Hmt3full R	5'-ATATCACTCTGATACGAAACCCATC-3'
29up-F	5'-GCCGCAACCTCTACCAATGC-3'
29up-R	5'-TCCGGTATCCAACCTAGTGC-3'
29down-F	5'-CCCTGCCTGTGTGGAACCTAC-3'
29down-R	5'-GTCCGTTCTCGGTGGTCTCT-3'
Scre29- F	5'-TGAGAGACATTGCACGGAGT-3'
Scre29- R	5'-GTCATCAAAGCCGAATCGAG-3'
29 full-F	5'-CAATTACCTCTTAACAACGACACC-3'
29 full-R	5'-TGACATGCGACTTCGGACT-3'
27up-F	5'-GCAATCTGTTTGCCTATGAAAT-3'
27up-R	5'-TGGGTCTCCATCTTGACGA-3'
Hmt27-1661down BamHI-F	5'-GGATCCGAACCTGGACGATGCCGTTAT-3'
Hmt27-1661down BglI-R	5'-AGATCTCCCAATCGGATACAACAACC-3'
27screening-F	5'-CGCGTCTGATTATGGACGAC-3'
27screening-R	5'-GATGCAGTCCGAGTCTTCG-3'
Probe27-F	5'-GGGACGAGAACTGCTGAAAG-3'
Probe27-R	5'-TTGTAGACGAGGTGCAGACG-3'
27full-F	5'-AAGCGTACCCTCCCTTTGTC-3'
27full-R	5'-GGTGAAAGTATGTCAAACTCAAGC-3'
Hmt10842up-F	5'-GTGGCAAGCGAAGACTCATT-3'

Hmt10842up-R	5'-AGTGGTGACATGTCGTCGAG-3'
Hmt610842own-F	5'-TGAGGAGGTGGGTGAAGAAC-3'
Hmt10842down-R	5'-GCAGGCTTGGTTTGAAGTAC-3'
Scree10842-F	5'-ATGACTGGGAGGTCTTGCAC-3'
Scree10842-R	5'-CCTCGATGCATACGAATCCT-3'
Hmt6probe-F	5'-GGTACCTACCTGGTTACGGTGAG-3'
Hmt6probe-R	5'-CCTGAGTGGTTCAATACCACCTAC-3'
Hmt22-05254up-SphI-F	5'-GCATGCTACCCCTTTCCTACCGCTTT-3'
Hmt22-05254up-SalI-R	5'-GCATGCGCTTGCGCTGATACTGTTGT-3'
Hmt22-05254down-BamHI-F	5'-CGACCTAGTCCGAATATTCCTC-3'
22down-R	5'-CTACTATCTATCTCACCGCCAGCA-3'
H22 probe-F	5'-GCGGTTACTCACCGTAAAGC-3'
H22 probe-R	5'-AAGCAGGCAACCAACCATAC-3'
Mgg_07868 RT2-F	5'-CAGCAGAAGCAGGTGTACAAGA-3'
Mgg_07868 RT2-R	5'-AGCTGAAGGGGTCGTACAGAT-3'
Mgg_06164 RT 100 F	5'-TTTTCCCACGATGCCTACCT-3'
Mgg_06164 RT 100 R	5'-AAAGTATACGTTGCCCTCGAC-3'
Mgg_05912 RT F	5'-GTCGAGGGGCTGTTGGAC-3'
Mgg_05912 RT R	5'-TCTTCTCCTCAACCTCGCA-3'
HY	5'-GGCTGCAGAACAGCGGGCAGTTCGG-3'
YG	5'-GCCGTGCACAGGGTGTACGTTGC-3'
Actin - F	5'-GCGGTTACACCTCTCTACCAC-3'
Actin - R	5'-AGTCTGGATCTCCTGCTCAAAG-3'
MGG_01542.6-F	5'-TCGACAGCAACTACAACCCTAA-3'
MGG_01542.6-R	5'-GGTCAGGGGGTAAAATCTGAG-3'
MGG_15430.6-F	5'-CCTTCATTGGCGCACTCT-3'
MGG_15430.6-R	5'-GCACAACCTTGCCCGAAC-3'
MGG_14243.6-F	5'-GACTTGTCACTGGGTGGACAT-3'
MGG_14243.6-R	5'-GGGGGAAGAACAAGGTTCC-3'
MGG_02245.6-F	5'-CTATCGTCCTCGCCCTTG-3'
MGG_02245.6-R	5'-AGTCTTGGCACCAGTCCATC-3'
MGG_05464.6-F	5'-TAGCGTTACGACGTCCAAGA-3'
MGG_05464.6-R	5'-CTCAAGGCCAGTGTGAGACC-3'
MGG_07868.6-F	5'-AGGCGGGTCTCGAGTACTTT-3'
MGG_07868.6-R	5'-CGGCTTCGTACTGAGGGTAG-3'
MGG_08401.6-F	5'-AACTATCCCTGGCAGTGGTG-3'
MGG_08401.6-R	5'-TGATTGCCCTTGGTACCTCA-3'
MGG_08331.6-F	5'-GCGCCGACGTCACCTA-3'
MGG_08331.6-R	5'-GCCCTGGGGGCTGTAG-3'
MGG_06593.6-F	5'-CGCTCGCTTCTGTGTTAGC-3'
MGG_06593.6-R	5'-CTCATTGCCCCAGTTCTGA-3'
MGG_07955.6-F	5'-GGTTCCAAGAGCATCACGTACT-3'
MGG_07955.6-R	5'-GGTTGTACTCGCCGTAGTTCTC-3'
MGG_08424.6-F	5'-ACGACGGCTTCTACTATTCCTG-3'
MGG_08424.6-R	5'-GACCAGGTGATGCTGTAGGAC-3'
MGG_10712.6-F	5'-GCAGTTCTGCACAGACCAGTT-3'

MGG_10712.6-R	5'-AGGACCATTGGGATTTTCGTAG-3'
MGG_02532.6-F	5'-GAGACGCACGAGAAGCTCAC-3'
MGG_02532.6-R	5'-ATGGAGTCCAGGACGATGTAGT-3'
MGG_05520.6-F	5'-ATGGCTAGCAAGCTGTTCTC-3'
MGG_05520.6-R	5'-AGTCCTGTCCACCACATTGAC-3'
MGG_14954.6-F	5'-TTCATCAGGCAGGTTGAG-3'
MGG_14954.6-R	5'-ACCTCTTCTTGACACACCTC-3'
MGG_06663.6-F	5'-GCAAGGTTGGCTGCTAATTC-3'
MGG_06663.6-R	5'-AGAGGTGTACAAGGGCAAGC-3'
MGG_07908.6-F	5'-GTGAGGAGTGGGGAGAGTGG-3'
MGG_07908.6-R	5'-CCTAAAGTACTCCTCGAACCAAGC-3'
MGG_04499.6-F	5'-GGCATGTCTGATGCCTACAAG-3'
MGG_04499.6-R	CGACTCTTAGAACTGGGGCTTA-3'
MGG_06834.6-F	5'-TGATTCGCAAGATCACAACTCT-3'
MGG_06834.6-R	5'-GCACTTTTGCCAGGTAAGAGAC-3'
MGG_07809.6-F	5'-GAGTACTGCACCAACGTCTTCA-3'
MGG_07809.6-R	5'-AGCATGTTGGAGTAGTGGTCGT-3'
MGG_12696.6-F	5'-GCGTCAGGGTCGACATCTA-3'
MGG_12696.6-R	5'-CTACTTGAAGCCCGACCAGA-3'
MGG_01328.6-F	5'-GGACAGAACCACTTTGACATTG-3'
MGG_01328.6-R	5'-GAATCGCCAGTAACAACCTTTT-3'
MGG_07575.6-F	5'-ACCAAGAGCATTGCGACTG-3'
MGG_07575.6-R	5'-CACAAATGTCACTCCCATCG-3'
MGG_08409.6-F	5'-CCTTCACCAACGACATAGTCAA-3'
MGG_08409.6-R	5'-CTCGATGAACTCGTTGTACCAG-3'
MGG_07686.6-F	5'-AGGCACTGGAACCAAGACC-3'
MGG_07686.6-R	5'-GCATCCCTCACAGGGAGTAA-3'
MGG_08066.6-F	5'-CCTTCAGCGAGCATGGTGTC-3'
MGG_08066.6-R	5'-CCCGGACCCTTGTAAGTTGGTC-3'
MGG_07300.6-F	5'-GCGTACACTGCCAACGAC-3'
MGG_07300.6-R	5'-GGTAGTATACGCCTCCGGAAC-3'
MGG_08020.6-F	5'-ATGCAAACGACCCAGGTATC-3'
MGG_08020.6-R	5'-CATCAATTACAGCCGGTCCT-3'
MGG_06621.6-F	5'-CGGACCCCTTGTTTCATTC-3'
MGG_06621.6-R	5'-TGAAGTTGCCACAGTGTTCA-3'
MGG_04547.6-F	5'-GTCAAGATTCCCGGCTTCAT-3'
MGG_04547.6-R	5'-GGGGACGGTGTAGTTCTTGA-3'
MGG_08254.6-F	5'-TAGTGAGTTCAGGCCAGCTGTA-3'
MGG_08254.6-R	5'-AGGCAGTACAAAACCCCATAGA-3'
MGG_13241.6-F	5'-GCTACGACGTAACCAAATACCC-3'
MGG_13241.6-R	5'-GATGTCTGGTGAGCCAAAGTT-3'
MGG_05364.6-F	5'-ACAGCACTGGCGGTGGTAAC-3'
MGG_05364.6-R	5'-GGCAGGCGTCGTTGTTGT-3'
MGG_11948.6-F	5'-CCGGCATCAAGATCAACATC-3'
MGG_11948.6-R	5'-AGGTTTGTGGTGTGGTCCTC-3'
MGG_02502.6-F	5'-CAACCCGACCAACAAGGTC-3'

MGG_02502.6-R	5'-ATGGGCCAGTTGATGTTGAC-3'
MGG_06069.6-F	5'-ACAGTGTTTTTCACCACCTTGTT-3'
MGG_06069.6-R	5'-ATTCAAAGGTCATCTTGCTACC-3'

Table S4. Summary of next generation sequencing (NGS) analyses.

^{*1} NGS_ID	^{*2} Strain	^{*3} Stage	Antibody	^{*4} Source	Parsed reads	Mapped reads	^{*5} %
a. RNA-seq							
RS4WM	WT	myc	N/A	N/A	16,631,292	7,581,678	45.59
RS5WM	WT	myc	N/A	N/A	12,120,620	5,638,237	46.52
RS4WG	WT	GT	N/A	N/A	15,432,828	5,479,815	35.51
RS5WG	WT	GT	N/A	N/A	9,791,812	3,549,629	36.25
RS2SM	Δ moset1	myc	N/A	N/A	4,995,254	2,141,518	42.87
RS2SM-r	Δ moset1	myc	N/A	N/A	6,643,250	2,808,947	42.28
RS4SM	Δ moset1	myc	N/A	N/A	15,352,676	6,586,377	42.90
RS5SM	Δ moset1	myc	N/A	N/A	13,423,494	5,781,722	43.07
RS4SG	Δ moset1	GT	N/A	N/A	22,067,774	8,275,442	37.50
RS5SG	Δ moset1	GT	N/A	N/A	9,784,324	3,972,780	40.60
b. ChIP-seq							
CS2WM	WT	myc	H3K4me2	AM 39141	5,5417,88	5,160,056	93.11
CS2WG	WT	GT	H3K4me2	AM 39141	5,145,166	4,946,169	96.13
CS2WG-r	WT	GT	H3K4me2	AM 39141	7,281,384	7,009,233	96.26
CS3WM	WT	myc	H3K4me3	AM 39159	8,467,364	8,261,375	97.57
CS3WM-r	WT	myc	H3K4me3	AM 39159	8,499,116	8,299,671	97.65
CS3WG	WT	GT	H3K4me3	AM 39159	7,779,596	7,360,835	94.62
CS3WG-r	WT	GT	H3K4me3	AM 39159	12,249,390	11,589,122	94.61
CS0WM	WT	myc	Normal H3	AM 39163	4,048,838	3,689,903	91.13
CS0WM-r	WT	myc	Normal H3	AM 39163	5,518,302	5,042,660	91.50

Samples were subjected to 75-nt or 120-nt pair-end sequencing.

*1, “-r” in NGS_ID indicates a technical replicate.

*2, WT, wild-type Br48 strain

*3, myc, vegetative mycelia; GT, germination tubes

*4, AM, active motif

*5, relatively low percentages of mapped reads in RNA-seq analyses were due to the presence of cDNA derived from mitochondrial RNA species that were not mapped to the genome sequence we used.

Curriculum vitae

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ABSTRACTS AND PRESENTATIONS

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