Running title: HDA6 interacts with MET1 and silences transposons

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HDA6 directly interacts with DNA methyltransferase MET1 and maintains transposable elements silencing in Arabidopsis

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FOOTNOTES

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Abstract

The molecular mechanism of how histone deacetylase HDA6 participates in maintaining transposable elements (TEs) silencing in Arabidopsis is not yet defined. In the present study, we show that a subset of TEs was transcriptional reactivated, and TE reactivation was associated with elevated histone H3 and H4 acetylation as well as increased H3K4Me3 and H3K4Me2 in hda6 mutants. Decreased DNA methylation of the TEs was also detected in hda6 mutants, suggesting that HDA6 silences the TEs by regulating the histone acetylation and methylation as well as DNA methylation status of the TEs. Similarly, transcripts of some of these TEs were also increased in the met1 mutant, with decreased DNA methylation. Furthermore, H4 acetylation, H3K4Me3, H3K4Me2 and H3K36Me2 were enriched at the co-regulated TEs in the met1 and hda6 met1 double mutants. Protein-protein interaction analysis indicated that HDA6 physically interacts with MET1 in vitro and in vivo, and further deletion analysis demonstrated that the C-terminal region of HDA6 and the BAH domain of MET1 were responsible for the interaction. These results suggested that HDA6 and MET1 interact directly and act together to silence TEs by modulating DNA methylation, histone acetylation and histone methylation status.
Introduction

Transposable elements (TEs) constitute a major part of the complex genomes of plants and animals. The genome sequence analysis of *Arabidopsis* has revealed that TEs are enriched in the centromeric region of chromosomes, which is highly methylated and packed into heterochromatin (Initiative, 2000). TEs are classical models for epigenetic inheritance and silent transposons can be activated and inherited in the active state (Lippman et al., 2003). Recent studies revealed that DNA methylation, histone deacetylation, histone methylation and RNA interference are involved in activation or silencing of TEs (Murfett et al., 2001; Johnson et al., 2002). DNA methylation in mammalian genome occurs predominantly in the context of CG sequence and is maintained by the DNMT1 methyltransferase. In *Arabidopsis*, the DNMT1 homolog, *MET1*, plays vital roles in maintaining cytosine methylation (Murfett et al., 2001). Antisense suppression or mutation of *MET1* in *Arabidopsis* causes a global reduction in cytosine methylation, particularly at CG sites (Finnegan et al., 1996; Ronemus et al., 1996), and induces the release of TEs and transcriptional gene silencing (Kankel et al., 2003; Saze et al., 2003). In addition to DNA methylation, TEs are also subjected to the regulation mediated by histone deacetylation and methylation and RNA interference (RNAi) (Lippman et al., 2003).

DNA methylation and histone deacetylation are two major epigenetic marks that contribute to the stability of gene expression status (MacDonald and Roskams, 2009). In several cases, gene silencing has been reported to be relieved by treatment with either histone deacetylase (HDAC) inhibitors or an inhibitor of DNA methylation (Chen and Pikaard, 1997; Pikaart et al., 1998; Selker, 1998). Inhibiting cytosine methylation induces histone acetylation, whereas inhibiting histone deacetylation causes the loss of cytosine methylation (Lawrence et al., 2004). In mammals, HDACs and DNMTs were suggested to act in same protein complexes. HDACs can be recruited by high DNA methylation levels, via association with methyl-DNA binding domain (MBD) containing proteins (Nan et al., 1998), or via
direct recruitment by the DNA methyltransferase DNMT1 (Fuks et al., 2000),
suggesting a tight interplay between histone deacetylation and DNA methylation.

In Arabidopsis, the Histone Deacetylase 6 (HDA6), a class I RPD3-like HDAC,
is required for TE and rRNA gene silencing and cytosine methylation maintenance
(Lippman et al., 2003; Earley et al., 2006; Earley et al., 2010). HDA6 was first
identified to be involved in transgene silencing through an
auxin-responsive-element mutant screening (Murfett et al., 2001). HDA6 mutant
alleles axe1-1 to axe1-5 displayed increased expression of the auxin-responsive
reporter genes in the absence of auxin treatment, suggesting a role of HDA6 in gene
silencing (Murfett et al., 2001). Another hda6 mutant allele, sill, was also
identified in a screen for mutations releasing transgene silencing, indicating that
HDA6 is required for maintenance of transcriptional gene silencing (Probst et al.,
2004). Furthermore, HDA6 was also identified as an essential component in
RNA-directed DNA methylation (RdDM) (Aufsatz et al., 2002; Aufsatz et al.,
2007). The HDA6 mutant allele rts1 exhibits reactivation of RdDM-silenced
promoters and results in reduced cytosine methylation in symmetric sequence
contexts, highlighting a function for HDA6 in methylation maintenance (Aufsatz et
al., 2002; Aufsatz et al., 2007). More recently, To et al. (2011) reported that HDA6
regulates locus-directed heterochromatin silencing in cooperation with MET1. In
addition, HDA6 and MET1 co-target to the heterochromatin sites and maintain
heterochromatin silencing (To et al., 2011). However, the molecular mechanism
underlying the function of HDA6 in gene silencing is still unclear.

In the present study, we found that a subset of TEs was reactivated in the hda6
mutants, axe1-5 and sill. In addition, the histone acetylation, histone methylation
and DNA methylation status of these TEs were affected in hda6 mutants. Direct
protein-protein interaction between HDA6 and MET1 was detected by yeast
two-hybrid, bimolecular fluorescence complementation (BiFC) and GST pull
down assays. Furthermore, the landscapes of histone modification and DNA
methylation at the TEs were also studied in met1-3 and axe1-5 met1-3 plants. Our
results indicate that HDA6 and MET1 interact directly and act together to maintain
TEs silencing by modulating their histone acetylation, methylation and DNA methylation status.

Results

_HDA6_ maintains TEs silencing through modulating histone H3 and H4 acetylation as well as histone H3K4 methylation

Our previous study revealed that a subset of TEs was up-regulated in _axe1-5_ and _HDA6_-RNAi plants (Yu et al., 2011). An additional _hda6_ mutant allele, _sil1_, in Col background (Probst et al., 2004) was also obtained and the expression of these transposons in the _sil1_ and _axe1-5_ were compared. As shown in Fig. 1, all eight transposons were remarkably activated in both _axe1-5_ and _sil1_ mutants, further supporting the notion that HDA6 is required for the silencing of TEs.

To investigate whether the reactivation of TEs was caused by histone acetylation, we measured the histone H3 and H4 acetylation levels of the TEs surrounding their transcription starting sites in _axe1-5_ and _sil1_ mutants by chromatin immunoprecipitation assay (ChIP) using antibodies specific for acetylated histone H3K9K14 and H4K5K8K12K16, respectively (Koch et al., 2008). As shown in Fig. 2A and B, the histone H3 and H4 acetylation levels of these TEs were elevated in _axe1-5_ and _sil1_ mutants compared to the wild-type, suggesting that HDA6 may silence TEs by histone deacetylation.

Since an increase in histone acetylation is often correlated with the methylation at lysine 4 of histone H3 (H3K4) (Strahl and Allis, 2000), we further analyzed H3K4Me3 and H3K4Me2 levels of the up-regulated TEs in _axe1-5_ and _sil1_ plants. As shown in Fig. 2C, in _axe1-5_ and _sil1_ mutants, the H3K4Me3 levels of _AT5G19015_, _AT2G20460_, _AT4G09480_, _AT2G26630_ and _AT2G04460_ were highly enriched, while no substantial changes were detected at _AT2G04770_, _AT5G59620_ and _AT4G09540_ loci. Furthermore, the H3K4Me2 levels of _AT2G04770_, _AT5G59620_, _AT2G20460_, _AT4G09480_ and _AT4G09540_ were elevated in _axe1-5_ and _sil1_ mutants (Fig. 2D). These data demonstrated that either H3K4Me3 or
H3K4Me2 was accumulated in axe1-5 and sill plants. Therefore, the hyper-acetylation of the TEs in hda6 mutants is correlated with an increase in H3K4 methylation. Taken together, our results suggested that HDA6 silences the TEs by modulating the histone H3 and H4 acetylation as well as H3K4 methylation levels.

To investigate whether HDA6 directly regulates TEs in vivo, Arabidopsis plants over-expressing HDA6-MYC (35S:HDA6-TAP) were used to perform a ChIP assay with an anti-MYC antibody. Real-time PCR was used to analyze the relative abundance of HDA6, surrounding the transcription starting sites and coding regions of the representative TEs (AT2G04770 and AT2G09540). As seen in Fig. 3, HDA6 was recruited to the transcription starting sites and coding regions of AT2G04770 and AT2G09540, suggesting that these TEs are direct targets of HDA6.

**HDA6 functions in maintaining CG, CHG and CHH methylation of TEs**

To assess whether the reactivation of the TEs in hda6 mutant is associated with changes in DNA methylation, McrBC-PCR assay was used to analyze the DNA methylation status of TEs. McrBC is a DNA methylation sensitive enzyme which preferentially cuts methylated DNA, and high levels of methylation result in increased McrBC digestion and consequently reduced amplification by PCR.

As shown in Fig. 4A, in wild-type, no obvious DNA bands were detected in McrBC-digested samples of AT2G04770, AT5G19015, AT2G20460, AT4G09480, AT4G09540, AT2G26630 and AT2G04460, indicating that these HDA6-regulated TEs are highly methylated. In contrast, a strong DNA band was detected in digested sample of AT5G59620, suggesting that this TE is relatively hypo-methylated in the wild-type. In the axe1-5 mutant, strong bands were scored in McrBC-digest samples of all the TEs, indicating loss of cytosine methylation of the TEs. Taken together, these data demonstrate that HDA6 affects the DNA methylation status of the TEs.

The DNA methylation status of three representative TEs, AT5G59620, AT2G04460 and AT2G04470, were further analyzed by bisulfite sequencing. As
shown in Fig. 4B and Supplemental Fig. S1, the CG sites at these loci were heavily methylated in the wild-type. In comparison, methylation levels at CHG and CHH sites in these loci are much lower than those at CG sites in the wild-type (Fig. 4B). An obvious decrease of CG methylation in AT2G04460 was detected in the axe1-5 mutant. Furthermore, substantial decreases of CHG and CHH were detected in AT2G04460 and AT5G59620, and the CHG methylation of AT2G04770 was also lost in the axe1-5 mutant. These data demonstrate that loss of function of HDA6 results in loss of cytosine methylation in CG, CHG and CHH sites in TEs, further suggesting that HDA6 plays an important role in maintaining CG, CHG and CHH methylation.

To explore the effect of HDA6 on genomic cytosine methylation, we employed methylation sensitive amplification polymorphism (MSAP) to analyze the landscapes of this DNA modification in axe1-5 mutants. MSAP assay is a modified version of AFLP analysis incorporating methylation-sensitive restriction enzymes in an efficient procedure to reveal genome-wide DNA methylation alterations in a locus-specific manner (Zhang et al., 2009). Hpa II and Msp I recognize the same restriction site (5'-CCGG) but have different sensitivities to methylation of the cytosines. Hpa II does not cut if either of the cytosine is fully (double strand) methylated, whereas Msp I does not cut if the external cytosine is fully or hemi (single) methylated. Thus, full methylation of the internal cytosine, or hemi-methylation of the external cytosine at the assayed CCGG sites can be unequivocally identified by MSAP. For clarity, we hereby refer to these two types of patterns as CG and CHG methylation, respectively (Dong et al., 2006). By using 8 pairs of selected EcoR I + Hpa II/Msp I primer combinations (Supplementary Table 1), 101 and 80 clear and reproducible bands were scored for the wild-type and axe1-5 mutant, respectively (Fig. 4C and Supplemental Fig. S2). 37.6% (38) of the bands were detected to be CG methylated in the wild-type. In contrast, only 22.5% (18) CG methylation sites were found in the axe1-5 mutant, suggesting that the hda6 mutation induces a decrease in genomic cytosine methylation. This data further enforces the role of HDA6 in maintaining cytosine
Interactions of HDA6 with MET1 both in \textit{vivo} and \textit{in vitro}

In \textit{Arabidopsis}, the \textit{DNMT1} homolog \textit{MET1} plays vital roles in maintaining cytosine methylation (Finnegan et al., 1996). The finding that HDA6 is required for CG, CHG and CHH methylation of TEs prompted us to investigate the interaction between HDA6 and MET1. Using the yeast two hybrid assay, we found that HDA6 can interact with MET1 in yeast cells (Fig. 5B). Deletion analysis identified that the first BAH (bromo-adjacent homology) domain (amino acids 735-869) of MET1 and the C-terminal region (amino acids 333-471) of HDA6 were responsible for their interaction (Fig. 5A, 5B and 5C).

The interaction of HDA6 and MET1 was further confirmed by the bimolecular fluorescence complementation (BiFC) assay. MET1 and HDA6 were fused to the N-terminal 174 amino acid portion of YFP in the pEarleyGate201 vector (pEarleyGate201-YN) and the C-terminal 66 amino acid portion of YFP in the pEarleyGate202 vector (pEarleyGate202-YC), respectively (Lu et al., 2010). The corresponding fused constructs were co-delivered into tobacco leaves by infiltration with \textit{Agrobacterium} (GV3101) (Kerppola, 2006), and fluorescence was observed using a confocal microscope. As shown in Fig. 5D, strong signal was observed in the nuclei of tobacco epidermal cells, suggesting a tight interaction between the HDA6 and MET1 proteins. In addition, the constructs were also co-delivered into \textit{Arabidopsis} protoplasts. The direct interaction was observed in the nucleus of \textit{Arabidopsis} protoplasts (Supplemental Fig. S3).

Furthermore, we determined the interaction between HDA6 and MET1 by using \textit{in vitro} pull-down assay. C-terminal region of HDA6 (amino acids 333-471) and the double BAH domain of MET1 (amino acids 735-1049) were fused with glutathione-S-transferase and His-tag, respectively (Eckner et al., 1994). The purified proteins were mixed and incubated in \textit{vitro}, and the elution was detected with an anti-His antibody. As illustrated in Fig. 5E, GST-HDA6-C-terminal was pulled down by His-MET1-BAH, suggesting a direct association of HDA6 with
MET1. Taken together, we conclude that HDA6 and MET1 interact both in vitro and in vivo, and the first BAH domain of MET1 and the C-terminal of HDA6 are responsible for the interaction.

HDA6 and MET1 act together to manipulate DNA methylation, histone acetylation and histone methylation of TEs

We further examined the genetic interaction between HDA6 and MET1 in regulating transposon silencing. We analyzed the expression of TEs in a met1-null mutant, met1-3 (Saze et al., 2003). Among the eight TEs been up-regulated in axe1-5 and sill, four TEs including AT5G19015, AT2G26630, AT2G20460 and AT2G04770 were highly activated in met1-3 plants (Fig. 6A), indicating that they are regulated by both HDA6 and MET1. Three of these TEs, AT2G04770, AT5G19015 and AT2G26630, were also identified by To et al. (2011) to be coregulated by HDA6 and MET1. McrBC-PCR analysis shows that the DNA methylation levels of AT5G19015, AT2G26630, AT2G20460 and AT2G04770 were obviously decreased in met1-3 plants (Fig. 6B). These TEs were also hypomethylated in axe1-5 (Fig. 4A), suggesting that MET1 and HDA6 may act together to maintain the DNA methylation of these TEs.

We further analyzed the axe1-5met1-3 double mutant and the histone landscapes of the four co-regulated TEs by ChIP assay. Increased expression of co-regulated TEs was observed in the hda6met1 double mutant (Supplemental Fig. S5). As shown in Fig. 7A, histone H4 acetylation levels of AT2G04770, AT5G19015 and AT2G20460 were elevated in met1-3 mutant and axe1-5met1-3 plants. In addition, histone H4 acetylation levels of AT2G04770 and AT5G19015 were additively accumulated in axe1-5met1-3. These data indicate that HDA6 acts additively with MET1 to regulate the H4ac of TEs.

Furthermore, H3K4Me3 and/or H3K4Me2 of these four TEs were also enriched in met1-3 mutant and axe1-5 met1-3 plants (Fig. 7B and 7C). H3K4Me3 of AT2G20460 and AT2G26630 in axe1-5met1-3 were higher than that of axe1-5 and met1-3 single mutants. Moreover, H3K36Me2 of AT2G04470, AT5G19015
and AT2G20460 were elevated in axe1-5 and met1-3 plants and additively increased in axe1-5met1-3 plants. These data suggest that HDA6 and MET1 co-regulate the histone methylation profiles of TEs. Taken together, our results suggest that HDA6 and MET1 act together to regulate DNA methylation, histone acetylation and histone methylation of the TEs in Arabidopsis.

**Discussion**

**HDA6 silences TEs by modulating histone acetylation, histone methylation and DNA methylation**

Plant genomes contain many transposable elements, most of which are inactivated or “silenced” by chromatin-remodeling and DNA methylation factors (Okamoto and Hirochika, 2001). A subset of TEs is transcriptional reactivated in hda6 mutants, suggesting that HDA6 is required for silencing of TEs. Activation marks, such as histone H3 and H4 acetylation, H3K4 tri- or di-methylation and H3K36 di-methylation are increased, whereas cytosine methylation is decreased at the TEs in hda6 plants, suggesting that HDA6 silences the TEs by regulating these modifications. HDA6 may therefore play important roles in the interplay among histone deacetylation, histone demethylation and DNA methylation in transcriptional regulation.

HDA6 is a Trichostain A sensitive HDAC capable of removing acetyl groups from multiple lysines of histone (Earley et al., 2006; Earley et al., 2010). HDA6 may therefore repress the TEs through direct deacetylation of their chromatin. Accumulation of H3K4Me3, H3K4Me2 and H3K36Me2 marks at the TEs in hda6 mutants also suggests a role for HDA6 in repression of active histone methylation. The crosstalk between histone deacetylation and demethylation has previously been implicated to modulate gene expression in mammalian cells (Shi et al., 2005; Lee et al., 2006; Shiekhattar et al., 2006). The histone demethylase, LSD1, is an integral component of histone deacetylase corepressor complexes in which HDACs and LSD1 may cooperate to remove activating acetyl and methyl histone
modifications (Lee et al., 2006). More recently, we found that HDA6 physically associates with Flower Locus D (FLD), a plant homolog of mammalian LSD1 (Yu et al., 2011). HDA6 may therefore recruit a histone demethylase such as FLD to regulate histone methylation of TEs.

Cytosine methylation levels in CHG and CHH sites were reduced in axe1-5 plants, which is associated with the loss of the H3K9Me2 mark (Supplemental Fig. S4). Previous studies revealed that methylation maintained by CMT3 is dependent on H3K9Me2 (Johnson et al., 2002; Lindroth et al., 2004), indicating that HDA6 may act indirectly to maintain CHG and CHH methylation through regulation of H3K9Me2 of TEs.

**HDA6 directly interacts with MET1**

The interplay between histone deacetylation and DNA methylation in gene silencing has been documented (Nan et al., 1998; Fuks et al., 2000; Lippman et al., 2003; To et al., 2011). In mammal cells, DNA methyltransferase DNMT1 represses gene expression via association with the methyl-CG-biding domain protein (MBD), which in turn recruits histone deacetylase activities (Nan et al., 1998). Based on this model, MBD proteins act as a bridge on the DNA methylation and histone deacetylation in gene silencing. A more direct connection between the DNA methylation and deacetylation has also been demonstrated by the finding that the mammalian DNMT1 binds to HDAC1 using the N-terminal non-catalytic domain to repress gene transcription through histone deacetylase activity (Fuks et al., 2000; Rountree et al., 2000). Furthermore, DNMT1 also interacts directly with HDAC2 to form a repression complex at replication foci during late S-phase (Rountree et al., 2000). Two de novo methyltransferases, DNMT3A and DNMT3B interact with HDACs through their PHD-like motif (Bachman et al., 2001). These associations may help to establish the chromatin in late S-phase and maintain the repressive heterochromatin state throughout the cell cycle (Rountree et al., 2000; Bachman et al., 2001).

In the present study, we demonstrated that the *Arabidopsis* DNMT1 homolog
MET1 interacts with HDA6, suggesting a direct connection between the DNA methylation and deacetylation in plants through the association of MET1 and HDA6. The direct interaction of HDA6 and MET1 may be necessary for the establishment and maintenance of proper maturation of heterochromatin in *Arabidopsis* cells. Deletion analysis by yeast two hybrid and GST-pull assay showed that the BAH domain of MET1 and the C-terminal region of HDA6 were responsible for this interaction. The C-terminal region of HDA6 contains a SANT-like domain (Aufsatz et al., 2007), which is known to mediate DNA binding as well as protein-protein interactions (Aasland et al., 1996). Thus, the C-terminal of HDA6 is an important region in maintaining cytosine methylation through interaction with MET1.

The interaction between HDA6 and MET1 in *Arabidopsis* was previously inferred based on the genetic evidence (Lippman et al., 2003; To et al., 2011). By characterizing the chromatin modifications of a subset of transposons in mutants deficient for DNA methylation, histone deacetylation and methylation, it was proposed that MET1, DDM1 (Deficient in DNA methylation 1) and HDA6 proteins may act together in a protein complex (Lippman et al., 2003). Gain of histone H3K4 methylation in *met1* mutants and loss of DNA methylation in *ddm1* and *hda6* was found in representative transposons (Lippman et al., 2003). In this study, we also demonstrated increases of histone H4 acetylation and H3K4me in *met1*-3 and a decrease of DNA methylation in *axe1*-5 in the TEs that are co-regulated by MET1 and HDA6. More recently, To el al. (2011) identified a number of loci silenced by MET1 and/or HDA6. It was found that 81 and 733 AGI loci were identified to be released in *axe1*-5 and *met1*-3, respectively, and 47 loci were common targets of HDA6 and MET1 (To et al., 2011). Far more loci show reactivation in the *met1* mutant than those in the *hda6* mutant, suggesting that HDA6 is only required to silence some MET1 targets. The direct interaction of HDA6 and MET1 suggests that they can form a repression complex to regulate these common targets. Besides HDA6, a plant-specific histone deacetylase HDT1 is also report to be required for rRNA gene silencing. Blocking histone
deacetylation by *HDT1*-RNAi induces a loss of cytosine methylation (Lawrence et al., 2004). Further research is required to determine whether MET1 may also associate other HDACs such as HDT1 to maintain gene silencing of the other targets.

**HDA6 and MET1 act together to repress TEs**

DNA methylation is associated with gene repression and plays a key role in protecting the genome from “selfish” DNA elements such as TEs (Chan et al., 2005). In *Arabidopsis*, DNA methylation occurs at both symmetric (CG and CHG) and asymmetric (CHH) sites. MET1 is responsible for the maintenance of symmetric CG cytosine methylation, while the plant specific CMT3 (CHROMOMETHYLASE 3) is responsible for the maintenance of the CHG and asymmetric sites (Bartee et al., 2001; Cao et al., 2003). Loss of cytosine methylation in CG, CHG and CHH sites of TEs in *axe1-5* suggests that HDA6 may functionally associate with a DNA methyltransferase to maintain DNA methylation.

DNA methylation inhibitor aza-dC treatment induces H3K9 acetylation, and the loss-of-function mutant of *MET1* accumulates H4 acetylation of *Ta2* retrotransposon (Tariq et al., 2003; Lawrence et al., 2004). Furthermore, combined treatment with 5-AzaC and histone deacetylation inhibitor TSA induces a more accessible chromatin structure of *metallothionein I* gene (*MT-I*) (Majumder et al., 2006), revealing a tight interplay between HDACs and DNMTs. In present work, we demonstrate that *HDA6* mutations induce a reduction of DNA methylation, and a loss-of-function mutation of *MET1* causes an increase in histone H4 acetylation of TEs, suggesting that the enzymatic activities of HDACs and DNMTs are closely linked in *Arabidopsis*. In addition, *axe1-5met1-3* double mutant displayed increases in histone H4 acetylation, H3K4Me2, H3K4Me3 and H3K36Me2 compared with wild-type, supporting a scenario that HDA6 and MET1 act cooperatively to silence TEs. The additive increase of these histone modifications of some loci in *axe1-5met1-3* double mutant suggests that HDA6 and MET1 may
collaborate with other chromatin remodeling factors to modulate the chromatin landscapes. In the present study, we demonstrated that HDA6 directly associates with MET1 to silence TEs by modulating DNA methylation, histone acetylation and histone methylation status. Our study provides a new prospective to understand the interplay between histone deacetylation and DNA methylation in gene silencing.

In addition to interacting with MET1 to maintain gene silencing, HDA6 was characterized as an essential component in RdDM pathway (Aufsatz et al., 2002; Probst et al., 2004). Among the eight up-regulated TEs in axe1-5 and sil1, AT2G04460, AT4G09540, AT4G09480 and AT5G59620 were not activated in met1 mutant (Fig. 6A), implying that these TEs may not be regulated by MET1. A recent study revealed that rdm4 (RNA-directed DNA Methylation 4, a component in RdDM pathway) mutant releases the transcripts of AT2G04460 (He et al., 2009), suggesting this TE may be silenced by RdDM pathway.

Materials and methods

Plant materials

Arabidopsis wild-type and hda6 mutants (axe1-5 and sil1) in Columbia background were grown under long-day condition (16-hr light, 8-h darkness). axe1-5 was originally isolated based on deregulated expression of auxin-responsive transgenes and was outcrossed to Col wild type 3 times (Yu et al., 2011). The sil1 mutant Col background was isolated by genetic screening for mutants, showing ectopic expression of seed storage proteins in leaves, which carry a point mutation in the 46 base after the ATG initiation codon leading to the replacement of Gly16 by Arg (Probst et al., 2004).

Real-time RT-PCR analysis

0.1 to 0.2 g of Arabidopsis thaliana leaves were ground up using liquid nitrogen in a mortar and pestle and mixed with 1 ml TRIZOL Reagent (Invitrogen)
to isolate total RNA. One microgram of total RNA was used for the first-strand cDNA synthesis after DNase treatment. cDNA was synthesized in a volume of 20 μl that contained the M-MLV reverse transcriptase buffer (Promega), 10 mM dithiothreitol, 1.5 μM poly(dT) primer, 0.5 mM dNTPs, 25 U RNasin ribonuclease inhibitor, 200 U M-MLV reverse transcriptase at 37 °C for 1 h.

cDNAs obtained from reverse-transcription were used as templates to run real-time PCR. The following components were added to a reaction tube: 9 μL of iQ™ SYBR Green Supermix solution (Catalogue no. 170-8882, Bio-Rad), 1 μL of 5 μM specific primers and 8 μL of the diluted template. Thermocycling conditions were 95 °C for 3 minutes followed by 40 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 20 seconds, with a melting curve detected at 95 °C for 1 minute, 55 °C for 1 minute and detected the denature time from 55 °C to 95 °C. Each sample was quantified at least in triplicates and normalized using Ubiquitin 10 (UBQ10) as an internal control. The primer pairs for quantitative RT-PCR are listed in Supplemental Table S1.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay was carried out as described (Gendrel et al., 2005). Chromatin was extracted from 18-day old plants growing in a long-day condition. After fixed with formaldehyde, the chromatin was sheared to an average length of 500 bp by sonication, and then immunoprecipitated with specific antibodies including anti-acetyl-histone H3K9K14 (Catalogue no. 06-599, Millipore), anti-acetyl-histone H4K5K8K12K16 (Catalogue no. 06-866, Millipore), anti-trimethyl-histone H3K4 (Catalogue no. 04-745, Millipore), anti-dimethyl-histone H3K4 (Catalogue no. 17-677, Millipore), anti-dimethyl-histone H3K9 (Catalogue no. 05-1250, Millipore) and anti-dimethyl-histone H3K36 (Catalogue no. 07-369, Millipore). The DNA cross-linked to immunoprecipitated proteins was analyzed by real-time PCR. Relative enrichments of transcriptional starting sites of transposons in axe1-5, met1-t3 and axe1-5/met1-3 over Col were calculated after normalization to
Each of the immunoprecipitations was replicated three times with different sets of plants, and each sample was quantified at least in triplicates during real-time PCR analysis. The primers used for real-time PCR analysis in ChIP assays were listed in Supplemental Table S2.

**McrBC-PCR and bisulfite sequencing**

McrBC-PCR analysis was performed as previously described (Ding et al., 2007). 100 ng genomic DNA from 18-day old Col and *axel-5, met1-3* plants, growing in a long-day condition, was digested with 20 U of McrBC endonuclease (Catalogue no. M0272S, New England Biolabs) for 8 h at 37 °C. Following the McrBC treatment, subsequent PCR was used to analyze methylation status of the transposons.

Bisulfite sequencing analysis was carried out with the EZ DNA methylation-Gold Kit (Catalogue no. D5005, Zymo Research). Briefly, 500 ng genomic DNA samples were treated with conversion reagents as described in the manufacture’s instruction. The treated DNA was cleaned up and served as templates for subsequent PCR. The PCR products were cloned into pGEM®-T Easy Vector and then transformed into *E. coli*. More than 15 individual clones for each genotype were sequenced and analyzed with the web-based tool as described (Gruntman et al., 2008). The primers used for McrBC-PCR and bisulfite sequencing assays were listed in Supplemental Table S3.

**Methylation sensitive amplification polymorphism (MSAP)**

MSAP assay was carried out as described in (Zhang et al., 2009). Purified DNA was digested with enzyme combinations *EcoR I / Hpa II* and *EcoR I / Msp II*. Appropriate adapters (Supplemental Table S3) were ligated to the restricted ends. Each sample was subjected to primary and secondary amplification using the primers as listed in Supplemental Table S3. After denaturing, the PCR products were electrophoresed on 12% denaturing polyacrylamide gel and visualized by silver staining. Only clear and completely reproducible bands were
Yeast two-hybrid assay

Yeast two-hybrid assays were performed according to the instructions for the Matchmaker GAL4-based two hybrid system 3 (Clontech). Constructs were generated by cloning full length or different regions of MET1 and HDA6 cDNA fragments into pGADT7 and pGBKT7 vectors. All constructs were transformed into yeast strain AH109 by the lithium acetate method and yeast cells were grown on minimal medium/-Leu-Trp according to the manufacture’s instructions (Clontech). Transformed colonies were plated onto minimal medium/-Leu/Trp-His/x-α-gal containing 3AT (3-Amino-1, 2, 4-triazole) to test for possible interactions between MET1 and HDA6.

BiFC assay

Full length cDNA fragments of MET1 and HDA6 were subcloned into the pCR8/GW/TOPO vectors, and then recombined into the YN (pEarleyGate201-YN) and YC (pEarleyGate202-YC) Vectors (Lu et al., 2010). Constructed vectors were transiently transformed into Arabidopsis protoplasts (Yoo et al., 2007). Transfected cells were imaged using TCS SP5 (Leica) Confocal Spectral Microscope Imaging System.

To detect the interaction in tobacco, leaves of 2 - 4-week-old tobacco plants (Nicotiana benthamiana) are infiltrated with Agrobacterium strains (GV3101) containing HDA6 and MET1 BiFC construct pairs. Epidermal cell layers are examined, 3-4 days after infiltration, using the Yellow fluorescent (YFP) filter.

In vitro GST pull down assay

GST pull-down assay was performed as previously described (Yang et al., 2008) with some modifications. GST and GST-HDA6 C-terminal recombinant proteins were incubated with 30 μl GST resin in a binding buffer (50 mM Tris-Cl,
pH7.5, 100 mM NaCl, 0.25% Triton X-100, 35 mM β-mercaptoethanol) for 2 h at 4°C. After the washing step with the binding buffer, the MET1 BAH-domain-His recombinant protein was added and incubated for additional 2 h at 4°C. Following extensive washing, the pulled-down proteins were eluted by boiling, then separated on 10% SDS-PAGE and detected by Western blotting using an anti-His antibody.

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Figure legends

Fig. 1 Gene expression analysis of transposons in axe1-5 and sill mutants. Total RNA samples were extracted from 18-day-old seedlings growing under long-day condition.

Fig. 2 ChIP analysis of histone acetylation and methylation levels of the up-regulated transposable elements in axe1-5 and sill mutants. The immunoprecipitated DNA was quantified by real-time PCR. H3K9K14Ac (A), H4K5K8K12K16Ac (B), H3K4Me3 (C) and H3K4Me2 (D) levels of the regions surrounding the transcription starting sites were analyzed by normalization to an internal control (ACTIN2).

Fig. 3 HDA6 directly targets to TEs. Transgenic plants expressing HDA6-MYC were subjected to ChIP analysis using an anti-MYC antibody. Wild type plants were used as negative controls. The relative fold of enrichment of a target surrounding the transcription starting sites was calculated by dividing the amount of DNA immunoprecipitated from the HDA6-MYC transgenic plants by that from the negative control plants and compared with input DNA. “S” means transcriptional starting site and “E” means coding region.

Fig. 4 Cytosine methylation analyses of the up-regulated TEs in axe1-5. (A) McrBC-PCR analysis of DNA cytosine methylation status of the TEs in wild-type
and *axe1-5* plants. “d” means McrBC digested DNA input and “u” means undigested DNA input. (B) Bisulfite sequencing analysis of three representative TEs in *axe1-5*. N represents A, T or C. (C) Comparison of CG methylation and CHG methylation levels in CCGG sites in wild-type and *axe1-5* by MSAP.

**Fig. 5** HDA6 interacts with MET1 both in *vitro* and in *vivo*

(A) A diagram of MET1 and HDA6 protein domains. (B) HDA6 interacts with MET1 and the first BAH domain of MET1 in yeast cells. (C) The BAH domain of MET1 interacts with the C-terminal region of HDA6 in yeast cells. (D) HDA6 interacts with MET1 in tobacco epidermal cells. (E) HDA6 C-terminal interacts with MET1 BAH domain *in vitro*. GST-HDA6 C-terminal and GST was incubated with MET1 R2 (BAH)-His and GST affinity resin, the bound proteins were then eluted from resin and probed with the anti-His antibody.

**Fig. 6** Gene expression (A) and cytosine methylation (B) analyses of HDA6-regulated TEs in *met1-3* plants. “d” means McrBC digested DNA input and “u” means undigested DNA input.

**Fig. 7** Histone modification landscapes of the TEs co-regulated by HDA6 and MET1. ChIP analysis of histone H4K5K8K12K16Ac (A), H3K4Me3 (B), H3K4Me2 (C) and H3K36Me2 (D) levels of the TEs in *axe1-5, met1-3* and *axe1-5met1-3* double mutant.
Fig. 1 Gene expression analysis of transposons in *axe1-5* and *sil1* mutants. Total RNA samples were extracted from 18-day-old seedlings growing under long-day condition.
Fig. 2 ChIP analysis of histone acetylation and methylation levels of the up-regulated transposable elements in *axe1-5* and *sil1* mutants. The immunoprecipitated DNA was quantified by real-time PCR. H3K9K14ac (A), H4K5K8K12K16ac (B), H3K4Me3 (C) and H3K4Me2 (D) levels of the regions surrounding the transcription starting sites were analyzed by normalization to an internal control (*ACTIN2*).
**Fig. 3** HDA6 directly targets to TEs. Transgenic plants expressing HDA6-MYC were subjected to ChIP analysis using an anti-MYC antibody. Wild type plants were used as negative controls. The relative fold of enrichment of a target surrounding the transcription starting sites was calculated by dividing the amount of DNA immunoprecipitated from the HDA6-MYC transgenic plants by that from the negative control plants and compared with input DNA. “S” means transcriptional starting site and “E” means coding region.
Fig. 4 Cytosine methylation analyses of the up-regulated TEs in *axe1-5*. (A) McrBC-PCR analysis of DNA cytosine methylation status of the TEs in wild-type and *axe1-5* plants. “d” means McrBC digested DNA input and “u” means undigested DNA input. (B) Bisulfite sequencing analysis of three representative TEs in *axe1-5*. H represents A, T or C. (C) Comparison of CG methylation and CHG methylation levels in CCGG sites in wild-type and *axe1-5* by MSAP.
Fig. 5 HDA6 interacts with MET1 both in vitro and in vivo

(A) A diagram of MET1 and HDA6 protein domains. (B) HDA6 interacts with MET1 and the first BAH domain of MET1 in yeast cells. (C) The BAH domain of MET1 interacts with the C-terminal region of HDA6 in yeast cells. (D) HDA6 interacts with MET1 in tobacco epidermal cells. (E) HDA6 C-terminal interacts with MET1 BAH domain in vitro. GST-HDA6 C-terminal and GST was incubated with MET1 R2 (BAH)-His and GST affinity resin, the bound proteins were then eluted from resin and probed with the anti-His antibody.
Fig. 6 Gene expression (A) and cytosine methylation (B) analyses of HDA6-regulated TEs in *met1-3* plants. “d” means McrBC digested DNA input and “u” means undigested DNA input.
Fig. 7 Histone modification landscapes of the TEs co-regulated by HDA6 and MET1. ChIP analysis of histone H4K5K8K12K16ac (A), H3K4Me3 (B), H3K4Me2 (C) and H3K36Me2 (D) levels of the TEs in *axe1-5*, *met1-3* and *axe1-5 met1-3* double mutant.