Genetic dissection of DNA hypermethylation induced by *ddm1* mutation.

Taku SASAKI

Doctor of Philosophy

Department of Genetics
School of Life Science
The Graduate University for Advanced Studies (SOKENDAI)

Department of Integrated Genetics
National Institute of Genetics

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Summary

DNA methylation is an epigenetic mark important for heterochromatin silencing and regulation of gene expression. In plants, DNA methylation is observed in cytosines in all of contexts, CG, CHG, and CHH. DNA methylation in all contexts of cytosines depends on a SWI2/SNF2 chromatin remodeling factor called DDM1 (decrease in DNA methylation 1). In the *ddm1* mutant, DNA methylation was reduced globally, and silent repetitive elements were de-repressed. Although *ddm1* mutant initially grows normally, various developmental abnormalities were induced during repeated self-pollinations. Most of the abnormalities were induced by de-repression of silent element caused by the DNA hypomethylation. However, one abnormal phenotype named *bonsai* (*bns*) was associated with DNA hyper-methylation and silencing of the responsible gene, *BNS*, which encodes a protein similar to Anaphase Promoting Complex 13 (APC13). *BNS* locus is hyper-methylated locally in the background of global hypomethylation. To understand this enigmatic phenomenon, here I examined the factors involved in the *BNS* hyper-methylation. In addition, I analyzed effect of the *ddm1*-induced DNA hyper-methylation in genome-wide level.

To understand the factors required for this phenomenon, I first generated double mutants between *ddm1* and various factors involved in the epigenetic modifications. After repeated self-pollinations, I examined DNA methylation states in *BNS* locus. Previously-characterized components of RdDM pathway were dispensable for *ddm1*-induced *BNS* methylation. On the other hand, mutation in plant-specific DNA methyltransferase, CHROMOMETHYLASE 3 (CMT3) suppressed the induction of DNA hyper-methylation.

CMT3 was considered as a DNA methyltransferase for maintenance of non-CG methylation. The mutation in KRYPTONITE (KYP), a histone H3K9 methyltransferase which directs non-CG methylation by CMT3, also suppressed the induction of *BNS* methylation. These results indicate that *ddm1*-induced *BNS* methylation was mediated by KYP and CMT3, rather than RdDM pathway.

Next, to understand the genome-wide effect of *ddm1*-induced DNA hyper-methylation, I examined global states of DNA methylation in *ddm1* mutant by MeDIP-chip analysis. Consistent with previous reports, drastic reduction of DNA methylation in transposable elements was observed as a rapid effect in *ddm1* mutant. During self-pollinations, DNA methylation states changed in many loci. Some loci kept decreasing DNA methylation during self-pollinations. I also found numerous loci which behave in *BNS*-like manner. Induction of DNA methylation was observed in both genes and transposable elements. The slow DNA hyper-methylation was induced in all the three contexts, but it was generally most frequent at CHG sites. Compared to genic regions, induction of DNA hyper-methylation in transposable elements tend to be moderate. In most loci, as was the case for the *BNS* locus, induction of DNA hyper-methylation was suppressed in the *ddm1 kyp* double mutant.

In this study, I showed KYP and CMT3 mediated induction of DNA methylation in *ddm1* mutant. Interestingly, although KYP and CMT3 were considered as components required for the maintenance of DNA methylation in non-CG contexts, this study revealed that they are also involved in induction of DNA methylation including CG context in *ddm1* background. KYP has a SRA domain, which can bind to methylated DNA, and CMT3 has a

chromo-domain, which can bind to methylated histone, so they could be recruited to epigenetic modifications catalyzed by the other factor. This self-reinforcement mechanism may be involved in the spreading of DNA methylation in *ddm1* mutant.

Introduction

DNA methylation is an important epigenetic modification found in genomes of eukaryotes, including fungi, vertebrates, and plants. In plants, DNA methylation occurs in all contexts of cytosins: CG, CHG, and CHH (H is A, T, or C). Methylations in CG sites are maintained by Dnmt1 class DNA methyltransferase METHYLTRANSFERASE 1 (MET1) (Finnegan & Dennis 1993, Finnegan *et al*. 1996), and those in non-CG sites are maintained by CHROMOMETHYLASE 3 (CMT3), a plant-specific DNA methyltransferase (Bartee *et al*. 2001, Lindroth *et al.* 2001). Function of CMT3 depends on histone H3K9 methylation, so histone H3K9 methyltransferase KRYPTONITE (KYP)/SUVH4 is also required for the maintenance of non-CG methylation (Jackson *et al.* 2002). A Dnmt3 class DNA methyltransferase, DOMAINS REARRANGED METHYLASE 2 (DRM2) mediates *de novo* DNA methylation (Cao & Jacobsen 2002, Cao *et al*. 2003). In plant, *de novo* methylation is triggered by 24-nt small RNA and this pathway is called RNA-directed DNA methylation (RdDM) (Wassenegger *et al*. 1994, Aufsatz *et al*. 2002). In addition to conserved components in RNAi, such as RNA-dependent RNA polymerase 2 (RDR2), ARGONAUTE 4 (AGO4), and DICER-like 3 (DCL3), plant-specific RNA polymerases, Pol IV and Pol V are involved in this RdDM for biogenesis and guiding of small RNA (Cao *et al*. 2003, Zilberman *et al.* 2003, Chan *et al.* 2004, Xie *et al*. 2004, Onodera *et al.* 2005, Kanno *et al.* 2005, Herr *et al*. 2005).

In addition to these factors, DDM1 (decrease in DNA methylation 1), a SWI2/SNF2 subfamily chromatin remodeling factor, is required for the maintenance of DNA methylation in all of context (Vongs *et al.* 1993, Jeddeloh *et al.* 1999). DDM1 is conserved protein, and it's

homolog in mouse, *Lsh* (lympanoid specific helicase), is essential for normal DNA methylation and histone methylation (Dennis *et al.* 2001, Yan *et al.* 2003). Main target of DDM1 is heterochromatic sequences including transposable elements and repetitive sequences. In *ddm1* mutant, DNA methylation in heterochromatic region decreased and heterochromatic histone H3K9 methylation is replaced to H3K4 methylation (Gendrel *et al*. 2002). Such epigenetic changes causes de-repression of transposable elements, and transposition is observed in some of them (Miura *et al*. 2001, Singer *et al*. 2001, Tsukahara *et al*. 2009).

Although mouse *lsh* mutation results in perinatal lethality, *Arabidopsis ddm1* mutant develops normally. However, by proceeding repetitive self-pollinations, various types of developmental abnormalities were induced (Kakutani *et al.* 1996, Kakutani 1997). Interestingly, in most cases, induction of developmental abnormality is involved in transposable elements in some way. For example, *clam* and *wavy sepal* were caused by insertion of transposable elements into *DWF4* and *FAS1* gene, respectively (Miura *et al.* 2001, Tsukahara *et al.* 2009). Late-flowering phenotype *fwa* (flowering wageningen) is caused by ectopic expression of *FWA* gene, which has SINE-like sequence within promoter region (Soppe *et al.* 2000, Kinoshita *et al*. 2007, Fujimoto *et al*. 2008). The SINE is heavily methylated and *FWA* is silent in vegetative tissue, but in *ddm1* mutant, DNA methylation in SINE-like element is removed and *FWA* is ectopically activated (Soppe *et al*. 2000).

The *bonsai* (*bns*) is another example of the *ddm1*-induced developmental abnormalities. Counterintuitively, *bns* syndrome was caused by hypermethylation and silencing of the responsible gene. The responsible gene, *BNS*, encodes a protein similar to Anaphase Promoting Complex 13 (APC13) (Saze & Kakutani 2007). *BNS* locus has a

characteristic structure that is franked to LINE sequence, and this LINE sequence is essential for induction of DNA hyper-methylation into *BNS* locus (Saze & Kakutani 2007). Thus, *BNS* locus is hyper-methylated locally in the background of global hypomethylation. To understand this enigmatic phenomenon, here I examined the factors involved in the *BNS* DNA hyper-methylation. In addition, by genome-wide DNA methylation analyses, I found other loci hyper-methylated in *ddm1* mutant. Furthermore, I examined controlling mechanisms of DNA methylation in these loci.

Results

BNS methylation is independent from RdDM pathway

To dissect the mechanism of *ddm1*-induced DNA hyper-methylation in *BNS* locus, I combined *ddm1* mutation with a mutation in genes involved in epigenetic regulation, such as DNA methyltransferase (*met1*, *cmt3*, and *drm2*), RdDM pathway component (*rdr2*, *dcl3*, *ago4*, *nrpd1*, *nrpe1*/*nrpd1b*, and *nrpd2a*/*nrpe2a*), RNAi component (*ago1*, *rdr1*, *rdr6*, *dcl2*, and *sde3*), and histone modifier (*kyp/suvh4*). In the fourth generation (4G) of the double-mutant (three-times of self-pollinations after becoming homozygous for both mutations), DNA methylation states in *BNS* locus were analyzed by restriction digestion and subsequent PCR (Figure 1). In *ddm1* and *bns* epi-mutant, *BNS* locus-derived 24-nt siRNA, which is known as a trigger of *de novo* DNA methylation, is accumulated (Saze & Kakutani, 2007). This observation implied that *BNS* gene is *de novo* methylated by RdDM pathway. However, in double mutants for *DDM1* and RdDM components such as *RDR2*, *DCL3*, *AGO4*, *NRPD1*, *NRPE1*, and *NRPD2a*, *BNS* locus was methylated (Figure 1B). Bisulfite-sequencing analysis showed that *BNS* gene is methylated in all contexts in both of *ddm1 nrpd1* (upstream factor in RdDM pathway that is required for siRNA biogenesis) and *ddm1 drm2* (downstream factor in RdDM pathway that is required for *de novo* DNA methylation) double mutants. The methylation patterns of these double mutants were similar to that of *ddm1* single mutant lines, which were generated from siblings of the same F_2 population (Figure 2). These results show that *BNS* methylation does not depend on these previously-characterized components of RdDM pathway. Moreover, mutation in RNAi components such as *ago1*, *rdr1*, *rdr6*, *dcl2*, and

sde3, and combined mutation of *rdr1 rdr2 rdr6*, which has mutations in all of known active *RDR* (RNA-dependent RNA polymerase) genes, did not suppress *ddm1*-induced *BNS* methylation (Figure 3), suggesting that *ddm1*-induced *BNS* methylation may be independent of small RNA.

Establishment of BNS methylation is mediated by KYP and CMT3

Arabidopsis genome encodes three active DNA methyltransferases: MET1, CMT3, and DRM2 (Chan *et al*. 2005). From result described above, as DRM2 is not essential for *ddm1*-induced *BNS* hyper-methylation, either or both of remaining DNA methyltransferases may be required.

Recent studies indicate that there are many loci hyper-methylated at non-CG contexts in hypomethylated *met1-3* mutant, and *BNS* gene is also hyper-methylated in *met1* mutant (Mathieu *et al.* 2007, http://epigenomics.mcdb.ucla.edu/BS-Seq/, http://neomorph.salk.edu/epigenome.html). Consistent with previous studies, *BNS* gene was hyper-methylated in non-CG contexts in *met1-3* mutant (data not shown). *ddm1 met1-3* double mutant plants showed severe phenotypes, and they became sterile in the second generation. Therefore the *ddm1*-induced *BNS* methylation could not be analyzed in *ddm1 met1* double mutant. In second generation of *ddm1 met1-3* double mutant, pattern of DNA methylation was similar to that of the *met1* single mutant, although the extent of the DNA methylation was slightly decreased.

I then analyzed *CMT3*. By the restriction digestion and subsequent PCR, induction of *BNS* methylation was not found in any of *ddm1 cmt3* double mutant lines even after five

times of self-pollinations (sixth generation, 6G) (Figure 4). The *BNS* methylation was induced in all *ddm1 CMT3* lines self-pollinated in parallel. Bisulfite-sequencing analysis confirmed that *BNS* methylation was not induced in *ddm1 cmt3* double mutant. In addition, almost all non-CG methylation in franking LINE sequence were erased in the *ddm1 cmt3* double mutant (Figure 4).

CMT3-mediated non-CG methylation depends on a histone H3K9 methyltransferase KYP (Jackson *et al*. 2002). I therefore tested if *KYP* is also necessary for the *ddm1*-induced *BNS* methylation. Indeed, *kyp* mutation also suppressed *BNS* methylation (Figure 4).

KYP and CMT3 function together for the maintenance of non-CG methylation, especially at CHG context (Bartee *et al*. 2001, Lindroth *et al.* 2001, Jackson *et al*. 2002). I then examined if these proteins are necessary for maintenance of the *ddm1*-induced *BNS* methylation. The *ddm1 KYP*/*kyp* and *ddm1 CMT3*/*cmt3* plants were self-pollinated several times in the heterozygous state to induce the *BNS* methylation. Then, in the self-pollinated progeny of those plants, DNA methylation states of the double mutants were examined (Figure 5). DNA methylation in all contexts was lost in the *ddm1 kyp* and *ddm1 cmt3* double mutants, suggesting that KYP and CMT3 are necessary for the maintenance of *ddm1*-induced *BNS* methylation.

Genome-wide analysis of ddm1 induced DNA hyper-methylation

To detect other loci hyper-methylated in self-pollinated *ddm1* mutant, genome-wide DNA methylation states in 2G *ddm1* and 8G *ddm1* were determined by MeDIP (methylated

DNA immunoprecipitation)-chip analysis. In consideration of previous observations, two types of effects of *ddm1* mutation were hypothesized: rapid effect and slow effect. Rapid effect is change in DNA methylation observed relatively immediately after the *ddm1* mutation becomes homozygous, and calculated as a ratio of DNA methylation in WT to that in 2G *ddm1* (2G / WT). Slow effect is change in DNA methylation observed during self-pollination of *ddm1* mutant, and calculated as a ratio of DNA methylation in 2G to that in 8G *ddm1* mutant (8G / 2G).

As previously reported, great amount of reduction in DNA methylation was observed in 2G *ddm1* as a rapid effect, especially in transposable elements (Lippman 2004, Figure 6 A). In addition, DNA methylation states changed during self-pollinations in many loci. Some loci kept decreasing DNA methylation during self-pollinations. There are numerous loci which behave in *BNS*-like manner also: they are hyper-methylated during the self-pollinations (Figure 6 B-F). Consistent with previous reports, hyper-methylation in *BNS* and *SUPERMAN* was detected in this MeDIP-chip analysis (Saze & Kakutani 2007, Jacobsen & Meyerowitz 1997, Figure 6 C, D). Induction of DNA methylation was observed in both of genes and transposable elements (Figure 6 E, F). Slow effect was relatively stochastic compared to rapid effect: some of the slow effects were line-specific. On loci reproducibly hyper-methylated in both of two independent strains of 8G *ddm1*, DNA methylation states in more details were analyzed by bisulfite-sequencing analysis. The slow DNA hyper-methylation was induced into all the three contexts, but it was generally most frequent at CHG sites (Figure 7). Compared to genic region, induction of DNA hyper-methylation in transposable elements tend to be moderate (Figure 7). Although they were re-methylated during self-pollinations, these

transposable elements were still transcriptionally active in 8G *ddm1* (data not shown).

KYP-CMT3 mediated DNA hyper-methylation in target loci other than BNS

To see whether methylation of these loci also depends on KYP and CMT3 like *BNS* locus, genome-wide DNA methylation states in 2G *ddm1 kyp* and 8G *ddm1 kyp* double mutants were determined (Figure 6). In most loci with the slow hyper-methylation in the *ddm1* single mutant background, induction of DNA methylation was not detected in the self-pollinated *ddm1 kyp* double mutant (Figure 6 C-G). Bisulfite-sequencing analysis indicated that suppression of ectopic DNA methylation in *ddm1 kyp* double mutant (Figure 7), and similar results were observed in *ddm1 cmt3* double mutant (Figure 8). Interestingly, although KYP and CMT3 are required for maintenance of non-CG methylation, ectopic induction of DNA methylation in all contexts including CG was suppressed in *kyp* and *cmt3* mutant background (Figure 7, Figure 8).

Although *ddm1*-induced DNA hyper-methylation depends on KYP and CMT3 in most loci, induction of DNA methylation independent of KYP and CMT3 was also found. One example is At5g35510, which was preferentially methylated at CG contexts in the self-pollinated *ddm1*. In *ddm1 kyp* double mutant, DNA methylation in CG contexts was induced and similar pattern of DNA methylation to *ddm1* single mutant was observed (Figure 7 B, F).

To know whether RdDM pathway is involved in this phenomenon, DNA methylation in target loci of *ddm1*-induced DNA hyper-methylation was analyzed using self-pollinated *ddm1 dcl3* double mutant. As was the case for the *BNS* locus, DNA methylation was induced

in *ddm1 dcl3* double mutant in some target sequences of *ddm1*-induced DNA hyper-methylation (Figure 9). Interestingly, DNA methylation in all contexts, including CHH, was induced in *ddm1 dcl3* mutant, although CHH methylation is known as a hallmark of RdDM. These results implying that they are methylated by RdDM-independent mechanism.

IBM1-independent induction of DNA methylation

BNS locus was methylated in some mutants other than *ddm1*, such as *met1* and *ibm1*. In *ibm1*mutant, which encodes jmjC domain containing putative histone demethylase, *BNS* gene is methylated especially in CHG contexts and this induction is mediated by KYP and CMT3 (Saze *et al*. 2008). The fact that *ddm1*-induced DNA hyper-methylation occurred most frequently in CHG contexts and depend on KYP and CMT3 motivated me to analyze the relationship between slow-effect of *ddm1* and effect of *ibm1* mutation. Interestingly, previous study revealed that *ddm1 ibm1* double mutant show severe developmental abnormality (Saze *et al*. 2008). However, there were no correlation of target loci of ectopic hyper-methylation between self-pollinated *ddm1* and *ibm1* mutants (Figure 10A). Although it was shown that the long, moderately transcribed and constitutively expressed genes were most affected by *ibm1* mutation (Miura *et al*. 2009), patterns of transcriptional preference were different between *ibm1* and *ddm1* (Figure 10). Moreover, *ibm1* mutation induces DNA methylation preferentially in genes, *ddm1* mutation induced in TEs also (Figure 6, Figure 7). These results indicate that mechanisms of DNA hyper-methylation in *ddm1* and *ibm1* are different, although KYP and CMT3 are commonly involved.

Discussions

RdDM-independent induction of DNA methylation

For the *ddm1*-induced *BNS* methylation, previously characterized components of RdDM pathway are dispensable (Figure 1). Interestingly, Dnmt3 class *de novo* methyltransferase, *DRM2* was also dispensable for *ddm1*-induced *BNS* methylation, indicating that other DNA methyltransferase(s) might be involved in *de novo* DNA methylation. Not only components in RdDM pathway, but also other factors which are required for small RNA pathway such as *SDE3* (post-transcriptional gene silencing), *AGO1* (miRNA biogenesis), *RDR6* (tasiRNA biogenesis), *DCL2*, and *RDR1* are also dispensable. Instead, that requires KYP and CMT3 (Figure 4). These results indicate that there is alternative mechanism to induce DNA methylation by KYP and CMT3, other than RdDM pathway.

Possible model of ddm1-induced BNS methylation

Previous study hypothesized two models of *ddm1*-induced *BNS* methylation: small RNA mediated model and spreading model (Saze & Kakutani 2007). As I mentioned above, the *ddm1*-induced *BNS* methylation may be small RNA-independent. On the other hand, requirement of KYP and CMT3 is consistent with the spreading model, because self-reinforcement mechanism of H3K9 methylation and DNA methylation was indicated between KYP and CMT3 (Johnson *et al*. 2007). KYP has a SRA domain which can interact with methylated cytosine and CMT3 has a chromo-domain which can interact with methylated histone, so they could be recruited to epigenetic modifications catalyzed by the

other factor. On *BNS* locus, franking LINE sequence is essential for induction of DNA hyper-methylation (Saze & Kakutani, 2007). One possibility is that remaining epigenetic mark(s) within this LINE sequence functions as a start point of spreading of DNA methylation, by recruiting KYP and CMT3 into *BNS* locus. Consistent with this hypothesis, cytosines in the LINE sequence were still methylated in self-pollinated *ddm1* mutant (Figure 2, Figure 4), and this remaining epigenetic modification might recruit KYP and CMT3, and DNA methylation spread by self-reinforcement mechanism. Interestingly, small RNA corresponding to *BNS* was accumulated in *ddm1* mutant (Saze & Kakutani 2007), although it might be not necessary for induction of *BNS* methylation (Figure 1, Figure 2). One possibility is that hyper-methylated states in *BNS* locus triggered small RNA biogenesis. Although activity of Pol IV, a RNA polymerase for small RNA biogenesis, is completely unknown, Onodera *et al*. (2005) suggest a possibility that Pol IV transcribes RNA from methylated DNA regions. According to this hypothesis, small RNA might be accumulated as a result of *ddm1*-induced *BNS* hyper-methylation, and this accumulation of small RNA may be subsidiarily promote or reinforce the epigenetic states established by KYP and CMT3 mediated mechanism.

Dependency on KYP-CMT3, rather than RdDM

Although some reports suggested that CMT3 might function as a *de novo* DNA methyltransferase (Malagnac *et al*. 2002), KYP and CMT3 were considered as components required for maintenance of non-CG methylation, especially for CHG context. In this study, I showed that they are essential for induction of DNA methylation in all contexts (Figure 4, Figure 7, Figure 8), indicating that they might have new functions other than maintenance of

non-CG methylation. Compared to RdDM pathway, KYP and CMT3 mediated induction of DNA methylation is less efficient, and it requires several generations as shown in *BNS* locus (Saze & Kakutani 2007). In addition, because RdDM and KYP-CMT3 regulate redundant sequences (Chan *et al*. 2005), *de novo* methylation of KYP-CMT3 might be masked by that of RdDM. DDM1 is involved in maintenance of DNA methylation (Johnson *et al.* 2002). So, if RdDM induced *de novo* DNA methylation in *ddm1* background, induced DNA methylation would not be maintained efficiently. In such situation, because DNA methylation induced by RdDM could not be maintained efficiently, KYP and CMT3 mediated slow induction of DNA methylation might be manifested.

Epigenetic backup system in ddm1 mutant

In *ddm1* mutant, many hyper-methylated loci other than *BNS* locus were identified. As a possible role of this slow induction of DNA methylation, recovery of DNA methylation could be considered. DNA methylation is required for inactivation of sequences including genes and transposable elements, and loss of DNA methylation activate silent elements. Mis-regulation of silent elements caused by loss of DNA methylation induces activation of transposable elements (Mirouze *et al*. 2009, Tsukahara *et al*. 2009) and various developmental abnormalities (Soppe *et al*. 2000, Henderson & Jacobsen 2008). So, induction of DNA hyper-methylation in *ddm1* mutant might be a compensation system of DNA methylation against the unfavorable results caused by drastic reduction of DNA methylation.

Recently, similar phenomenon was reported in *met1* mutant (Mathieu *et al*. 2007). In *met1* mutant, almost all methylated cytosines in CG contexts are erased and histone

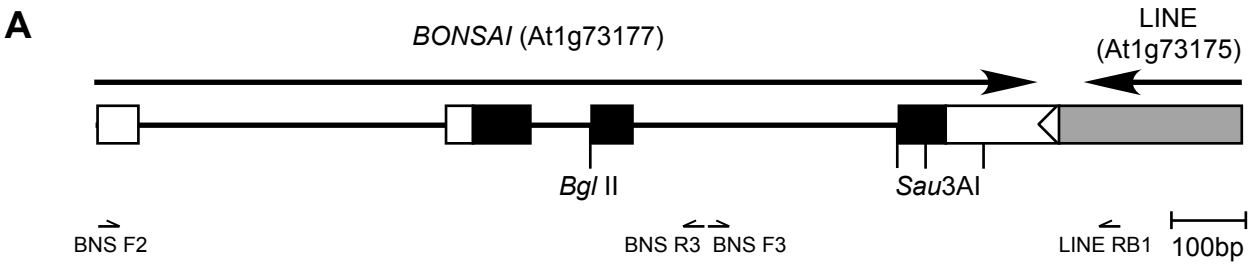
H3K9me2 signal is dispersed. However, after self-pollinations, non-CG methylation is induced and H3K9me2 signal was re-assembled (Mathieu *et al*. 2007). Phenomena observed in *met1* and *ddm1* look similar on the point of local hyper-methylation in genome-wide hypomethylated background, but there are some differences. For example, *ddm1*-induced DNA methylation occurs in the all contexts including CG, CHG, and CHH, but in *met1* mutant, induction of DNA methylation was limited to non-CG sites (Mathieu *et al*. 2007, Cokus *et al*. 2008, Lister *et al*. 2008). Targets of DNA hyper-methylation in these two mutants are also different (data not shown), so the trigger of the hyper-methylation may be different.

Although similar epigenetic “backup” systems were observed in *ddm1* and *met1* mutants, these “backup” systems could not recover the epigenetic states completely. In the case of *ddm1* mutant, for example, transposable elements which are activated by *ddm1* mutation were still active even after the induction of DNA methylation (data not shown). And like silencing of *BNS* gene, sometimes this system causes worse result. So, the biological significance of this epigenetic “backup” system is unclear.

Slow effect of *ddm1* mutation was stochastic, and I could not determine the target specificity of *ddm1*-induced DNA hyper-methylation. In RdDM pathway, small RNA determines the target specificity of *de novo* methylation. However, KYP and CMT3 mediated DNA methylation may be independent of small RNA, so target specificity may be decided in different way. In consideration of self-reinforcement mechanism between KYP and CMT3, H3K9 methylation and/or DNA methylation might be a target of DNA hyper-methylation. In the case of *BNS* locus, remaining epigenetic mark(s) might be a target of recruitment of KYP and CMT3. However, such structural feature was not always observed in other targets of

ddm1-induced DNA methylation, so somehow they might be *de novo* modified. Interestingly, in *ddm1* mutant, total amount of histone H3K9me2 is not changed although that in pericentromeric transposable elements is replaced to H3K4me2 (Gendrel *et al*. 2002), implying that distribution of H3K9me2 change in *ddm1* mutant. It could be considered that H3K9 methylation was redistributed in *ddm1* mutant, and mis-localized in euchromatic regions, and this epigenetic modification may recruit KYP and CMT3, and self-reinforcement mechanism may induce DNA methylation in target loci (Figure 11). In *ddm1* mutant, DNA methylation does not disappeared completely although in heterochromatic region. In target TEs of *ddm1*-induced DNA methylation, epigenetic mark(s) remaining also in *ddm1* background might be target(s) of KYP-CMT3 (Figure 11).

As I shown in this study, *ddm1* mutation has diverse effect, such as rapid reduction of DNA methylation and slow induction of DNA methylation, and from the researches on *ddm1*-induced developmental abnormalities, various phenomena which can not be observed in wild-type plant have been revealed. Although *ddm1* is well-used mutant as a genome-wide hypomethylated mutant, it is still unclear how defect in this chromatin remodeling factor induce drastic change in DNA methylation. More research on the functional mechanism of DDM1 may bring us the sturdy understanding on enigmatic phenomena induced by *ddm1* mutation.


A
BONSAI (At1g73177)
LINE
(At1g73175)
Bgl II
Sau3AI
BNS F2
BNS R3
BNS F3
LINE RB1
100bp

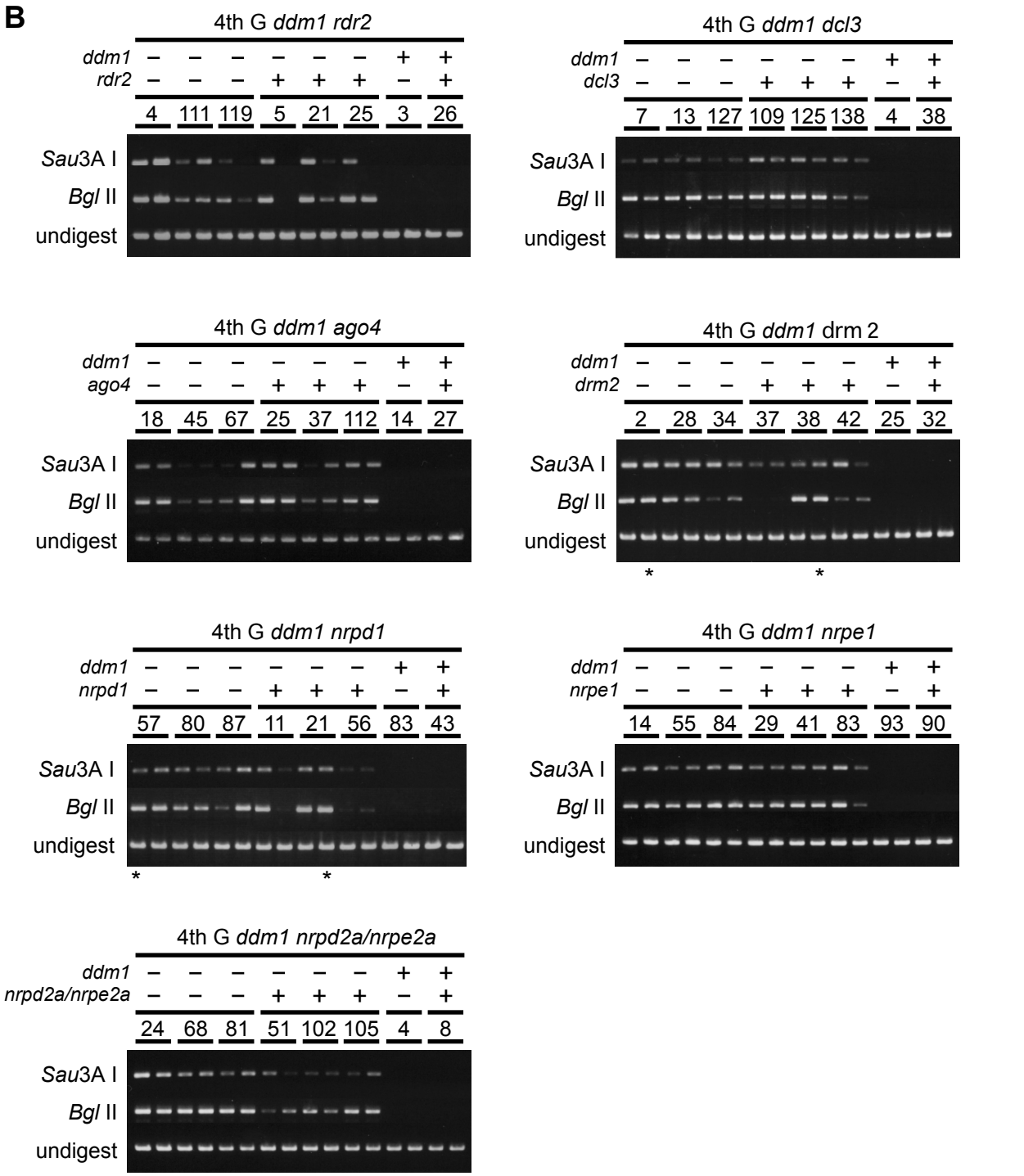
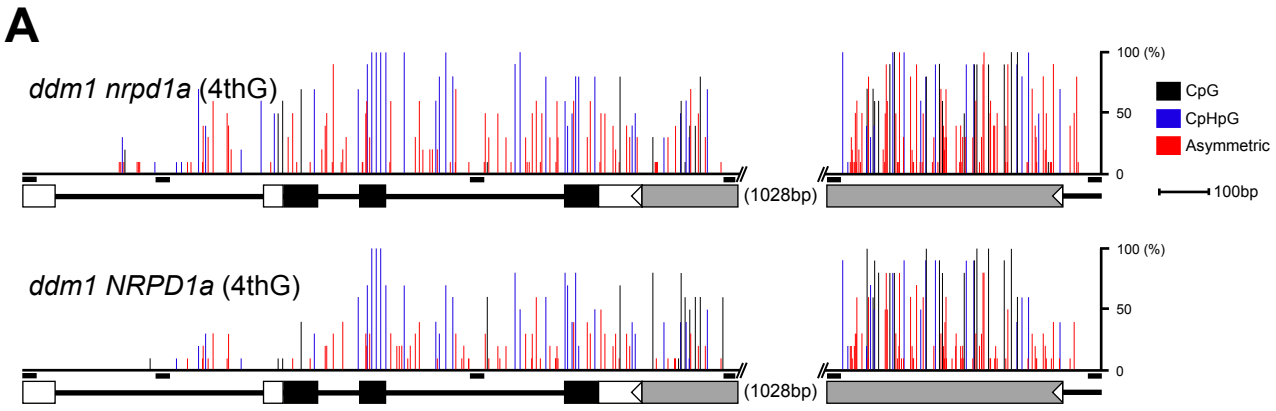

B
4th G ddm1 rdr2
ddm1 – – – – – – + +
rdr2 – – – + + + – +
4 111 119 5 21 25 3 26
Sau3A I
Bgl II
undigest
4th G ddm1 dcl3
ddm1 – – – – – – + +
dcl3 – – – + + + – +
7 13 127 109 125 138 4 38
Sau3A I
Bgl II
undigest
4th G ddm1 ago4
ddm1 – – – – – – + +
ago4 – – – + + + – +
18 45 67 25 37 112 14 27
Sau3A I
Bgl II
undigest
4th G ddm1 drm 2
ddm1 – – – – – – + +
drm2 – – – + + + – +
2 28 34 37 38 42 25 32
Sau3A I
Bgl II
undigest
* *
4th G ddm1 nrpd1
ddm1 – – – – – – + +
nrpd1 – – – + + + – +
57 80 87 11 21 56 83 43
Sau3A I
Bgl II
undigest
* *
4th G ddm1 nrpe1
ddm1 – – – – – – + +
nrpe1 – – – + + + – +
14 55 84 29 41 83 93 90
Sau3A I
Bgl II
undigest
4th G ddm1 nrpd2a/nrpe2a
ddm1 – – – – – – + +
nrpd2a/nrpe2a – – – + + + – +
24 68 81 51 102 105 4 8
Sau3A I
Bgl II
undigest

Figure 1 *ddm1*-induced *BNS* methylation in RdDM mutant. (**A**) Schematic structure of *BNS* gene (At1g73177) and franking LINE sequence (At1g73175). White and black boxes indicate UTRs and exons, respectively. Large and small arrows indicate annotated units and primers used in this analysis. (**B**) DNA methylation in *BNS* locus detected by restriction amplification. Genomic DNA digested by methylation-sensitive restriction enzyme *Sau3A*I (5'-GATC-3') or *Bgl* II (5'-AGATCT-3') was used as a template for PCR. 4G (F_5 generation) plants were used for this analysis. Samples indicated as * were used for bisulfite-sequencing analysis shown in **Figure 2**. Primer pairs BNS F2+R3 and BNS F3+LINE RB1 were used after *Bgl*II and *Sau*3AI digestion, respectively.


A
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100 (%)
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0
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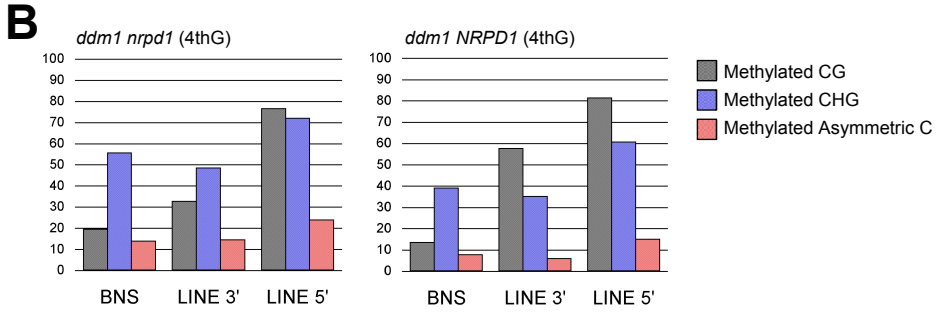
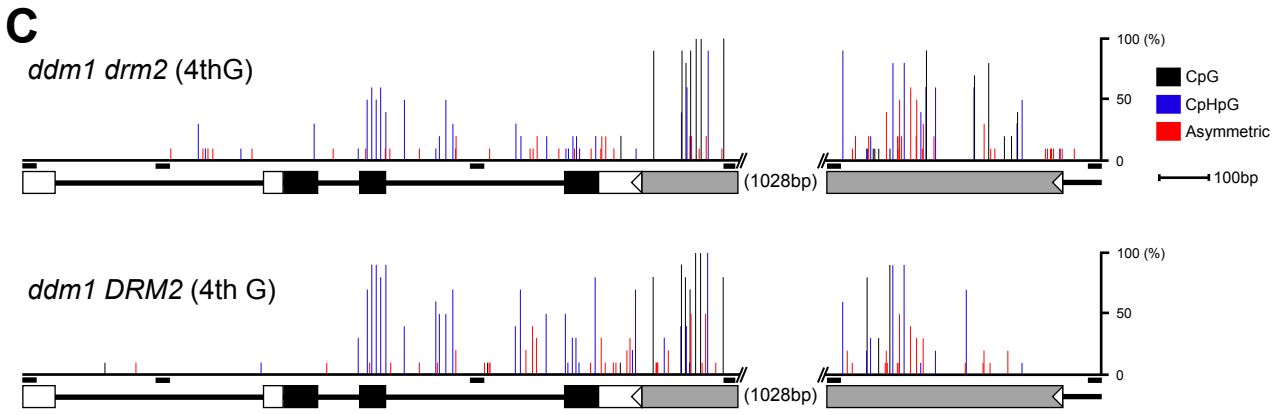
CpHpG
Asymmetric
100bp
(1028bp)


B
ddm1 nrpd1 (4thG)
ddm1 NRPD1 (4thG)
BNS
LINE 3'
LINE 5'
Methylated CG
Methylated CHG
Methylated Asymmetric C


C
ddm1 drm2 (4thG)
ddm1 DRM2 (4th G)
100 (%)
50
0
CpG
CpHpG
Asymmetric
100bp
(1028bp)

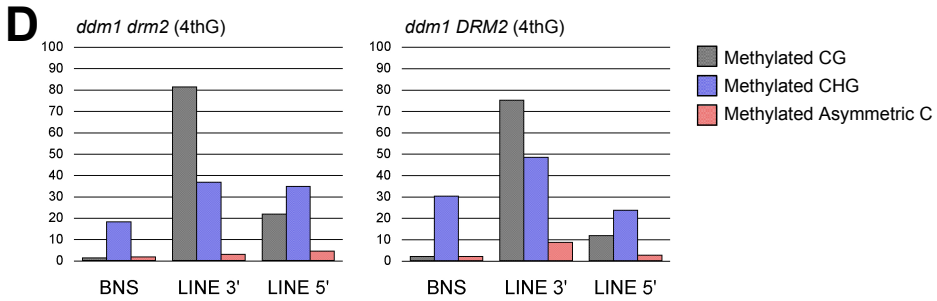

D
ddm1 drm2 (4thG)
ddm1 DRM2 (4thG)
BNS
LINE 3'
LINE 5'
Methylated CG
Methylated CHG
Methylated Asymmetric C

Figure 2 Detection of *BNS* methylation in RdDM mutant. DNA methylation states in *BNS* locus detected by bisulfite-sequencing analysis (**A**, **C**), and proportion of methylated cytosines in the *BNS* locus based on bisulfite-sequencing analysis (**B**, **D**). Analyzed samples were 4G *ddm1 nrpd1* (**A**, **B**) and 4G *ddm1 drm2* (**C**, **D**) double mutants with 4G *ddm1* single mutant generated from each F_2 populations. *NRPD1* and *DRM2* are required for small RNA biogenesis and DNA methylation, respectively.

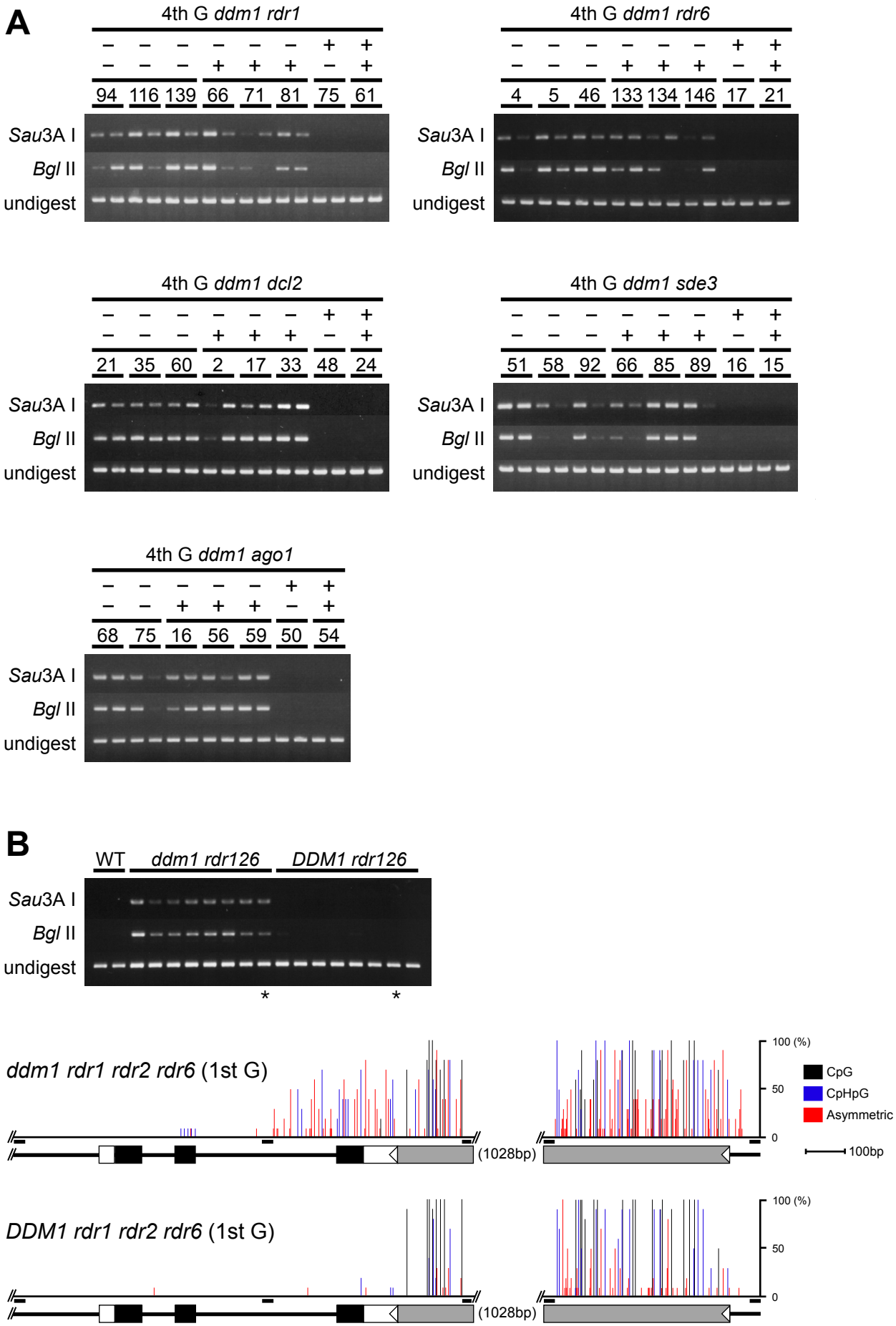
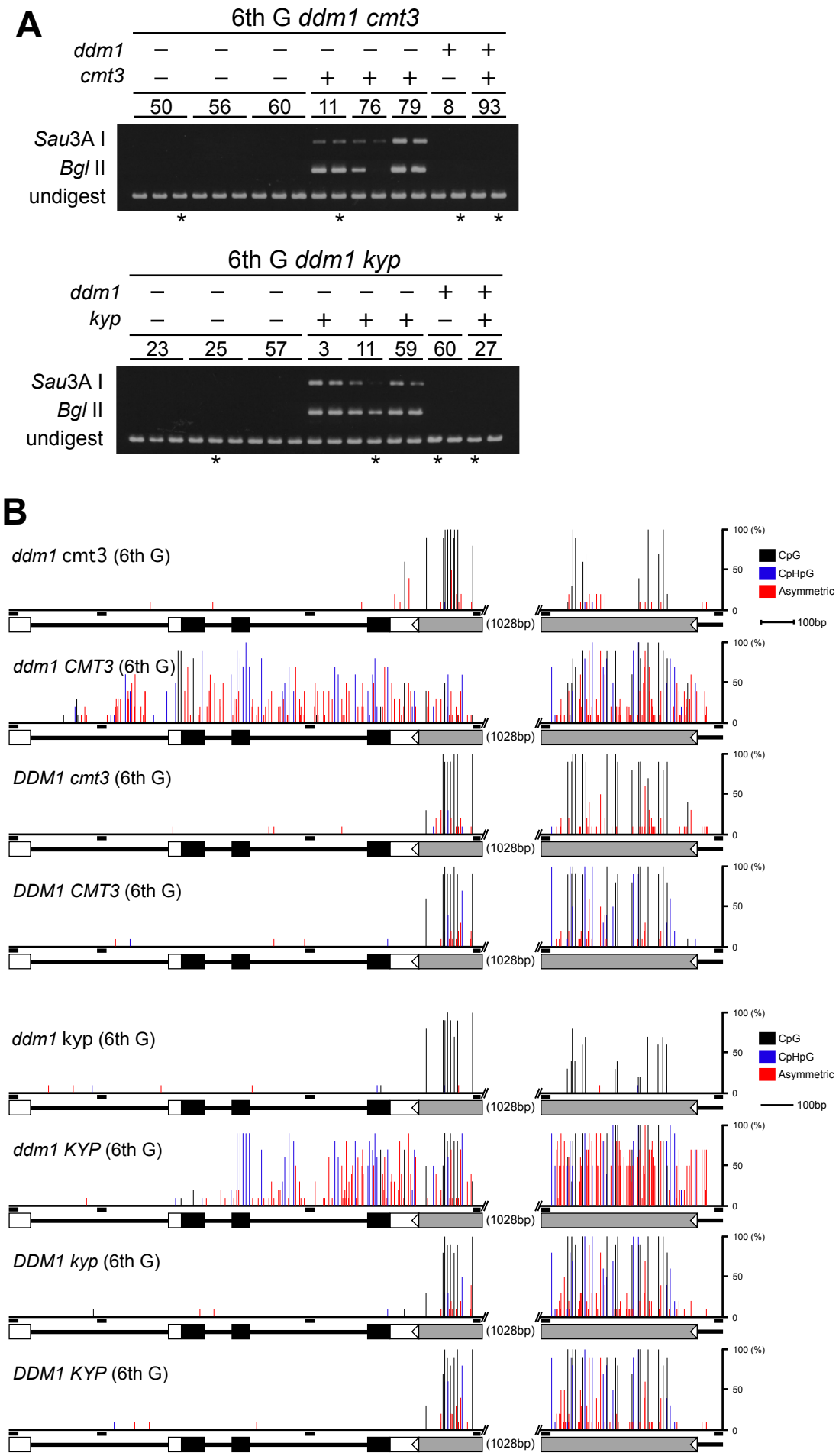

A
4th G ddm1 rdr1
4th G ddm1 rdr6
4th G ddm1 dcl2
4th G ddm1 sde3
4th G ddm1 ago1
Sau3A I
Bgl II
undigest
B
WT
ddm1 rdr126
DDM1 rdr126
ddm1 rdr1 rdr2 rdr6 (1st G)
DDM1 rdr1 rdr2 rdr6 (1st G)
(1028bp)
100 (%)
50
0
CpG
CpHpG
Asymmetric
100bp

Figure 3 *ddm1*-induced *BNS* methylation in RNAi pathway mutants. (**A**) DNA methylation states of *BNS* locus were detected by restriction digestion and subsequent PCR in RNAi pathway mutants. (**B**) DNA methylation states of *BNS* locus in 1G *ddm1 rdr1 rdr2 rdr6* quadrople mutant detected by restriction digestion and subsequent PCR (upper) and bisulfite-sequencing analysis (lower). Samples indicated as * were used for bisulfite-sequencing analysis.


A
6th G ddm1 cmt3
ddm1 – – – – – – + +
cmt3 – – – + + + – +
50 56 60 11 76 79 8 93
Sau3A I
Bgl II
undigest
6th G ddm1 kyp
ddm1 – – – – – – + +
kyp – – – + + + – +
23 25 57 3 11 59 60 27
Sau3A I
Bgl II
undigest
B
ddm1 cmt3 (6th G)
ddm1 CMT3 (6th G)
DDM1 cmt3 (6th G)
DDM1 CMT3 (6th G)
ddm1 kyp (6th G)
ddm1 KYP (6th G)
DDM1 kyp (6th G)
DDM1 KYP (6th G)
(1028bp)
100 (%)
50
0
CpG
CpHpG
Asymmetric
100bp

C

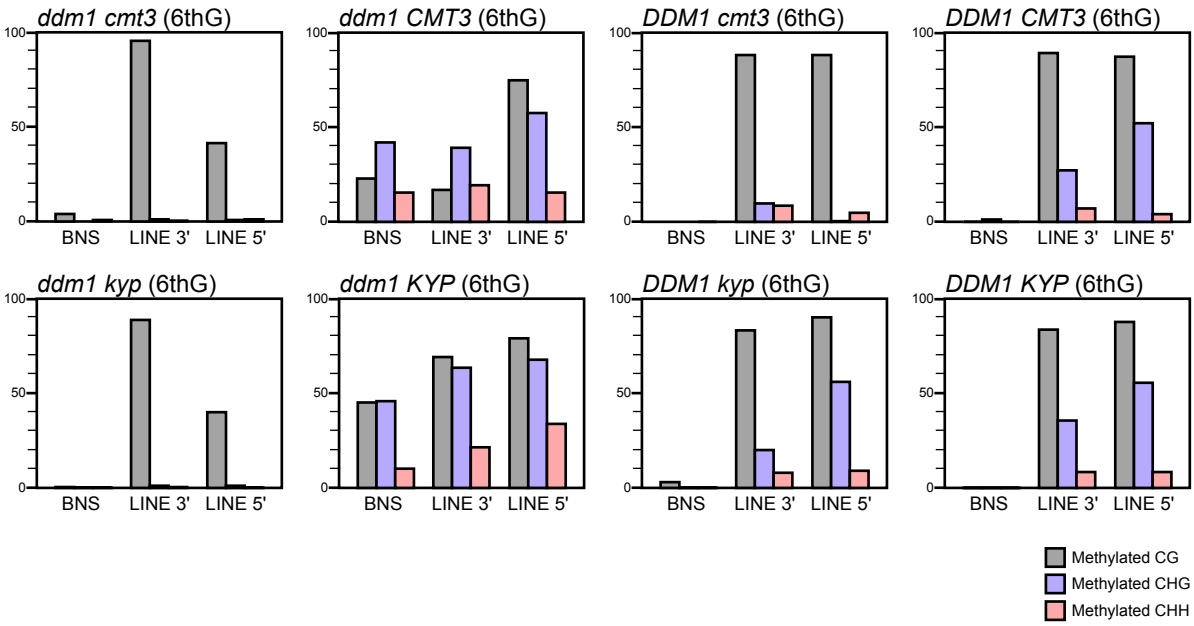
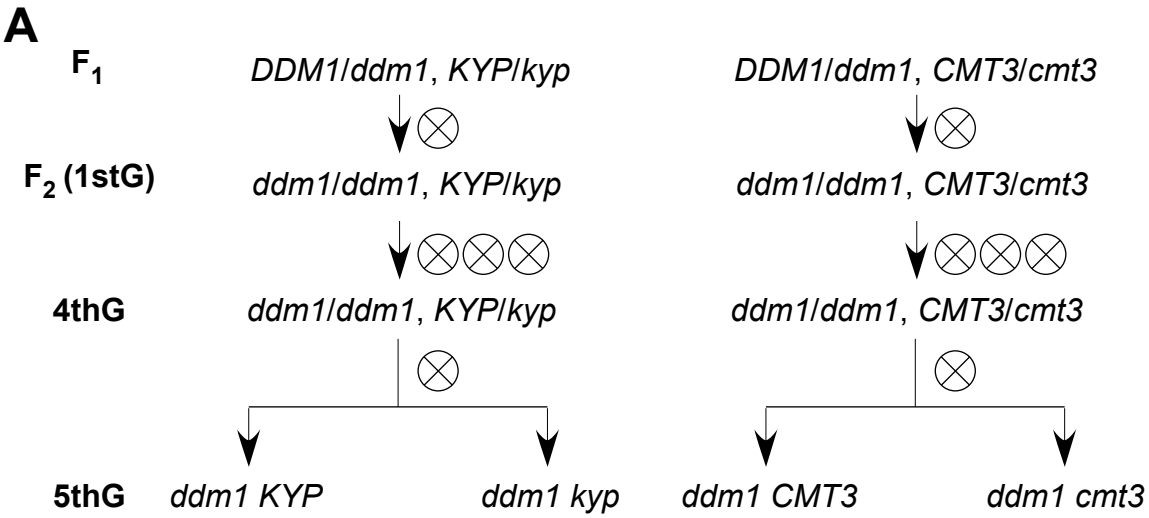

ddm1 cmt3 (6thG)
ddm1 CMT3 (6thG)
DDM1 cmt3 (6thG)
DDM1 CMT3 (6thG)
ddm1 kyp (6thG)
ddm1 KYP (6thG)
DDM1 kyp (6thG)
DDM1 KYP (6thG)
100
50
0
BNS
LINE 3'
LINE 5'
Methylated CG
Methylated CHG
Methylated CHH

Figure 4 Suppression of the *ddm1*-induced *BNS* methylation in *kyp* and *cmt3* mutants. DNA methylation states in *BNS* locus of 6G *ddm1 cmt3* siblings (upper) and *ddm1 kyp* siblings (lower) were detected by restriction digestion (**A**) and bisulfite-sequencing analysis (**B**). Samples indicated as * were used for bisulfite-sequencing analysis. (**C**) Percentage of methylated cytosines in *BNS* locus. Data were based on bisulfite-sequencing analysis shown in (**B**).


A
F1
DDM1/ddm1, KYP/kyp
DDM1/ddm1, CMT3/cmt3
F2 (1stG)
ddm1/ddm1, KYP/kyp
ddm1/ddm1, CMT3/cmt3
4thG
ddm1/ddm1, KYP/kyp
ddm1/ddm1, CMT3/cmt3
5thG
ddm1 KYP
ddm1 kyp
ddm1 CMT3
ddm1 cmt3

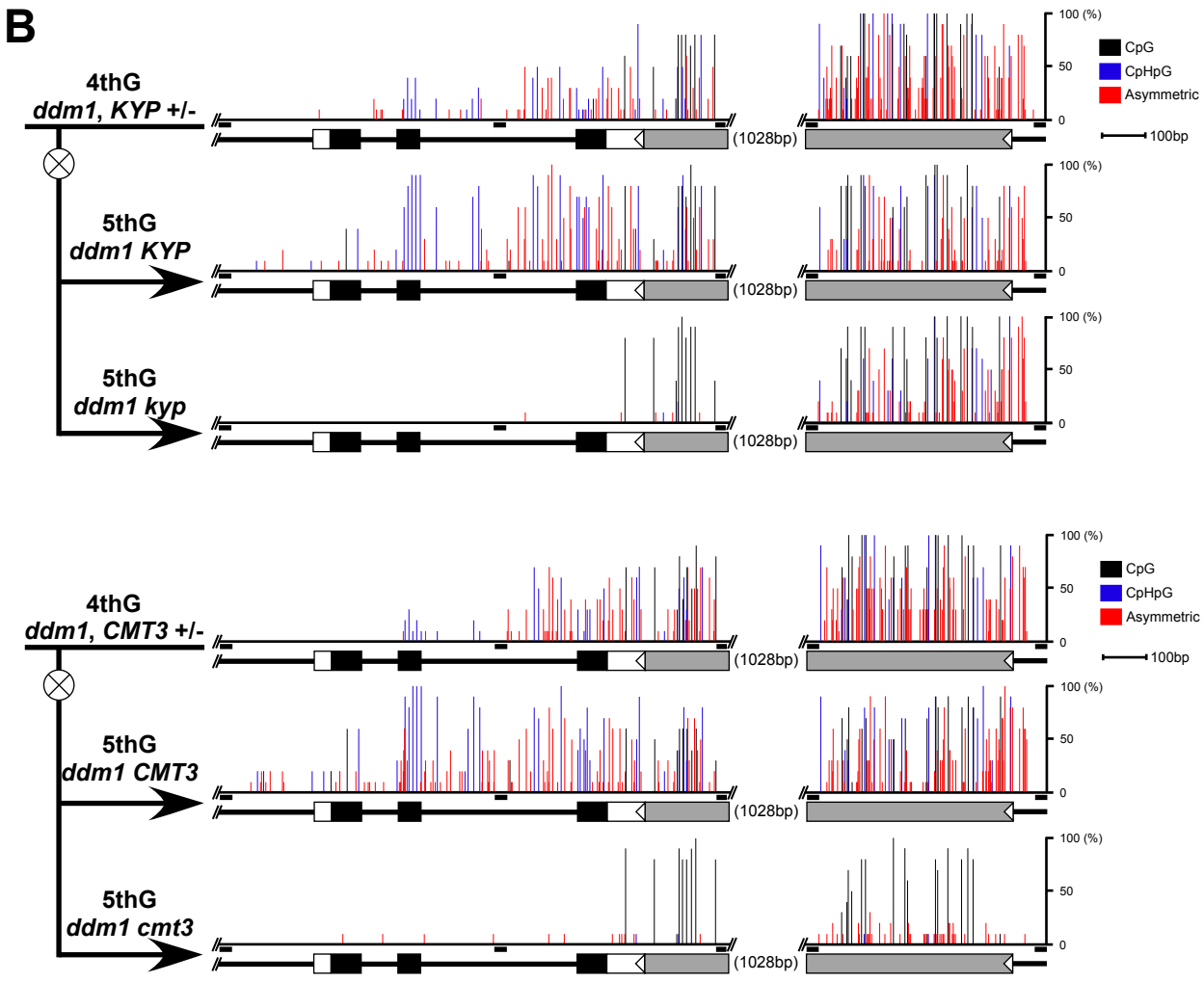
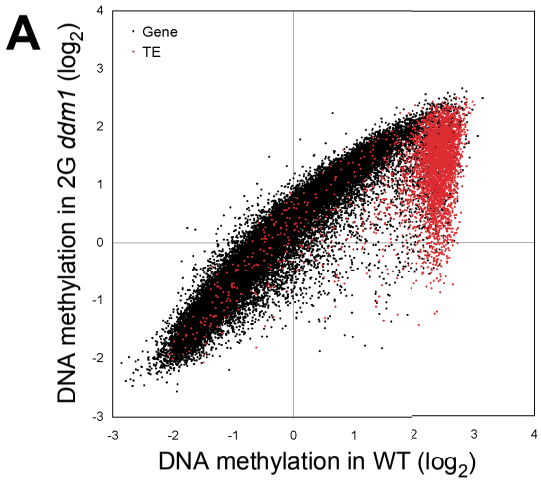
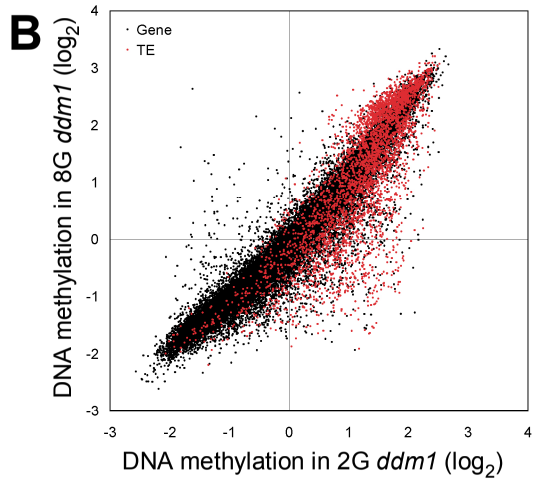
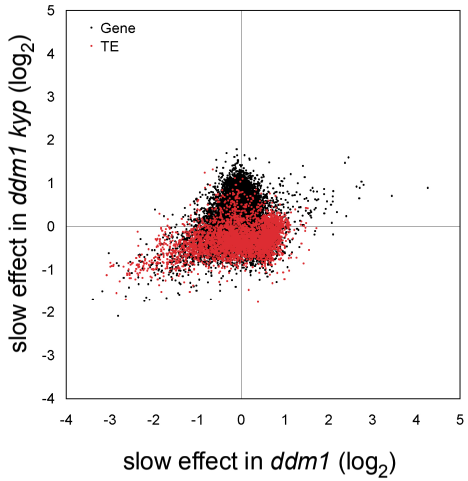
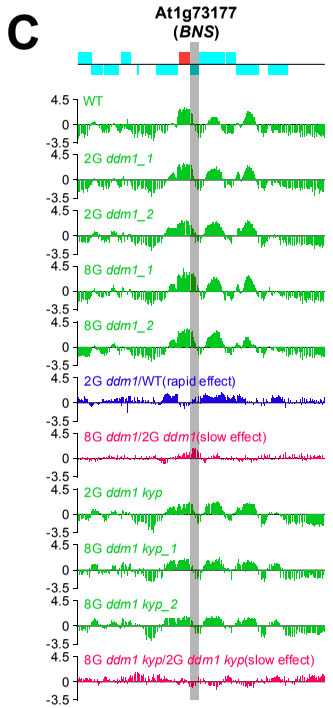

B
4thG
ddm1, KYP +/-
5thG
ddm1 KYP
5thG
ddm1 kyp
4thG
ddm1, CMT3 +/-
5thG
ddm1 CMT3
5thG
ddm1 cmt3
(1028bp)
100 (%)
50
0
CpG
CpHpG
Asymmetric
100bp

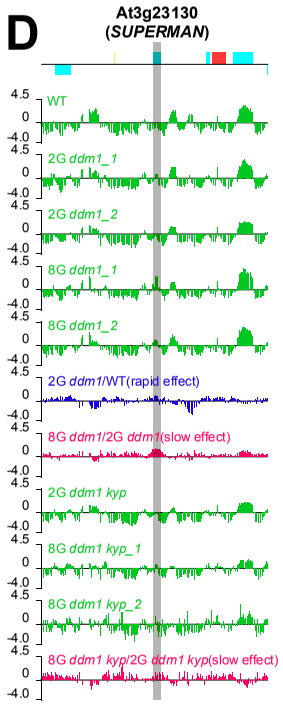
Figure 5 Effect of KYP and CMT3 on the maintenance of DNA methylation in *BNS* locus. (**A**) Genetic scheme used to create plants, which are hyper-methylated in *BNS* locus with heterozygous in *kyp* or *cmt3* mutation. After that *BNS* locus was methylated (4thG), DNA methylation states in segregated *ddm1* single mutants and *ddm1 kyp*/*cmt3* double mutants (5thG) were analyzed. ⓧ indicate a self-pollination. (**B**) DNA methylation states in 4G *ddm1* and self-pollinated 5G plants, including *ddm1* single mutant and *ddm1 kyp*/*cmt3* double mutant.

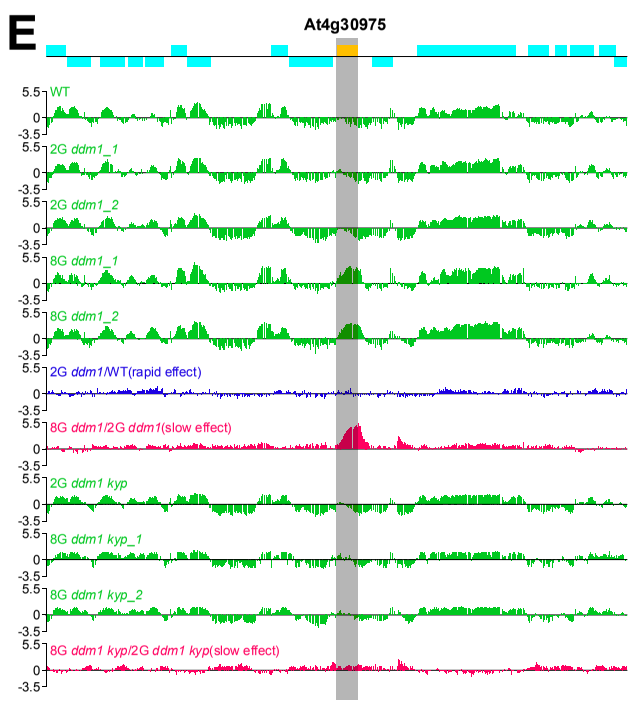

A
Gene
TE
DNA methylation in 2G ddm1 (log2)
DNA methylation in WT (log2)


B
Gene
TE
DNA methylation in 8G ddm1 (log2)
DNA methylation in 2G ddm1 (log2)


G
Gene
TE
slow effect in ddm1 kyp (log2)
slow effect in ddm1 (log2)


C
At1g73177
(BNS)
WT
2G ddm1_1
2G ddm1_2
8G ddm1_1
8G ddm1_2
2G ddm1/WT(rapid effect)
8G ddm1/2G ddm1(slow effect)
2G ddm1 kyp
8G ddm1 kyp_1
8G ddm1 kyp_2
8G ddm1 kyp/2G ddm1 kyp(slow effect)


D
At3g23130
(SUPERMAN)
WT
2G ddm1_1
2G ddm1_2
8G ddm1_1
8G ddm1_2
2G ddm1/WT(rapid effect)
8G ddm1/2G ddm1(slow effect)
2G ddm1 kyp
8G ddm1 kyp_1
8G ddm1 kyp_2
8G ddm1 kyp/2G ddm1 kyp(slow effect)


E
At4g30975
WT
2G ddm1_1
2G ddm1_2
8G ddm1_1
8G ddm1_2
2G ddm1/WT(rapid effect)
8G ddm1/2G ddm1(slow effect)
2G ddm1 kyp
8G ddm1 kyp_1
8G ddm1 kyp_2
8G ddm1 kyp/2G ddm1 kyp(slow effect)

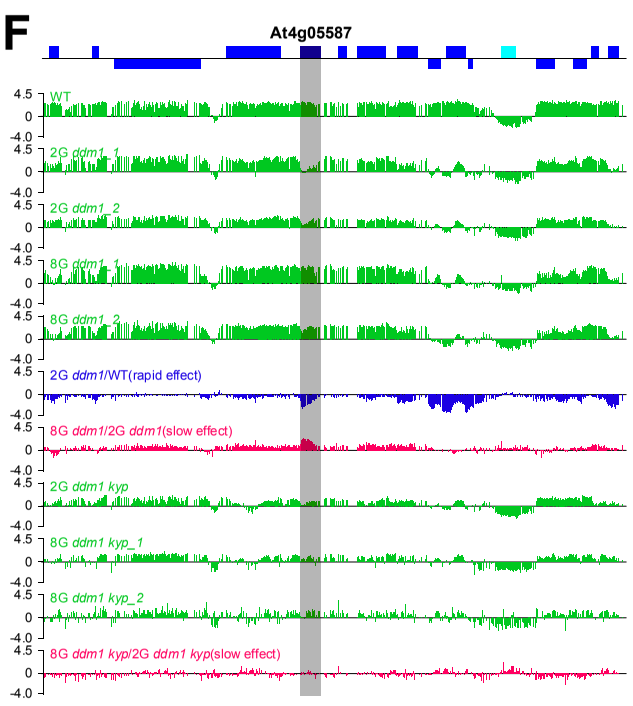

F
At4g05587
WT
2G ddm1_1
2G ddm1_2
8G ddm1_1
8G ddm1_2
2G ddm1/WT(rapid effect)
8G ddm1/2G ddm1(slow effect)
2G ddm1 kyp
8G ddm1 kyp_1
8G ddm1 kyp_2
8G ddm1 kyp/2G ddm1 kyp(slow effect)

Figure 6 MeDIP-chip analysis in *ddm1* mutant. (**A**, **B**) Scatter plot of DNA methylation in WT (horizontal axis) and 2G *ddm1* (vertical axis) (**A**) and that in 2G *ddm1* (horizontal axis) and 8G *ddm1* (vertical axis) (**B**). (**C**, **D**) Tiling microarray data in previously reported hyper-methylated loci in *ddm1* mutant, *BNS* (**C**) and *SUPERMAN* (**D**). Each vertical green bars represent $\log_2$ signal of immunoprecipitated DNA divided by input control. Blue bars represent rapid effect calculated as [2G *ddm1* / WT] ratio of signals. Magenta bars represent slow-effect calculated as [8G *ddm1* / 2G *ddm1*], and [8G *ddm1 kyp* / 2G *ddm1 kyp*] ratio of signals. Light blue, blue, red, and yellow boxes in gene model represent genes, transposable elements, pseudogenes, and other sequences, respectively. (**E**, **F**) Tiling microarray data in newly identified *ddm1*-induced hyper-methylated loci in gene-rich region (**E**: At4g30975) and TE-rich region (**F**: At4g05578). (**G**) Scatter plot of slow-effect in *ddm1* (vertical axis) and *ddm1 kyp* (horizontal axis). Black and red dots indicate gene and transposable element, respectively.

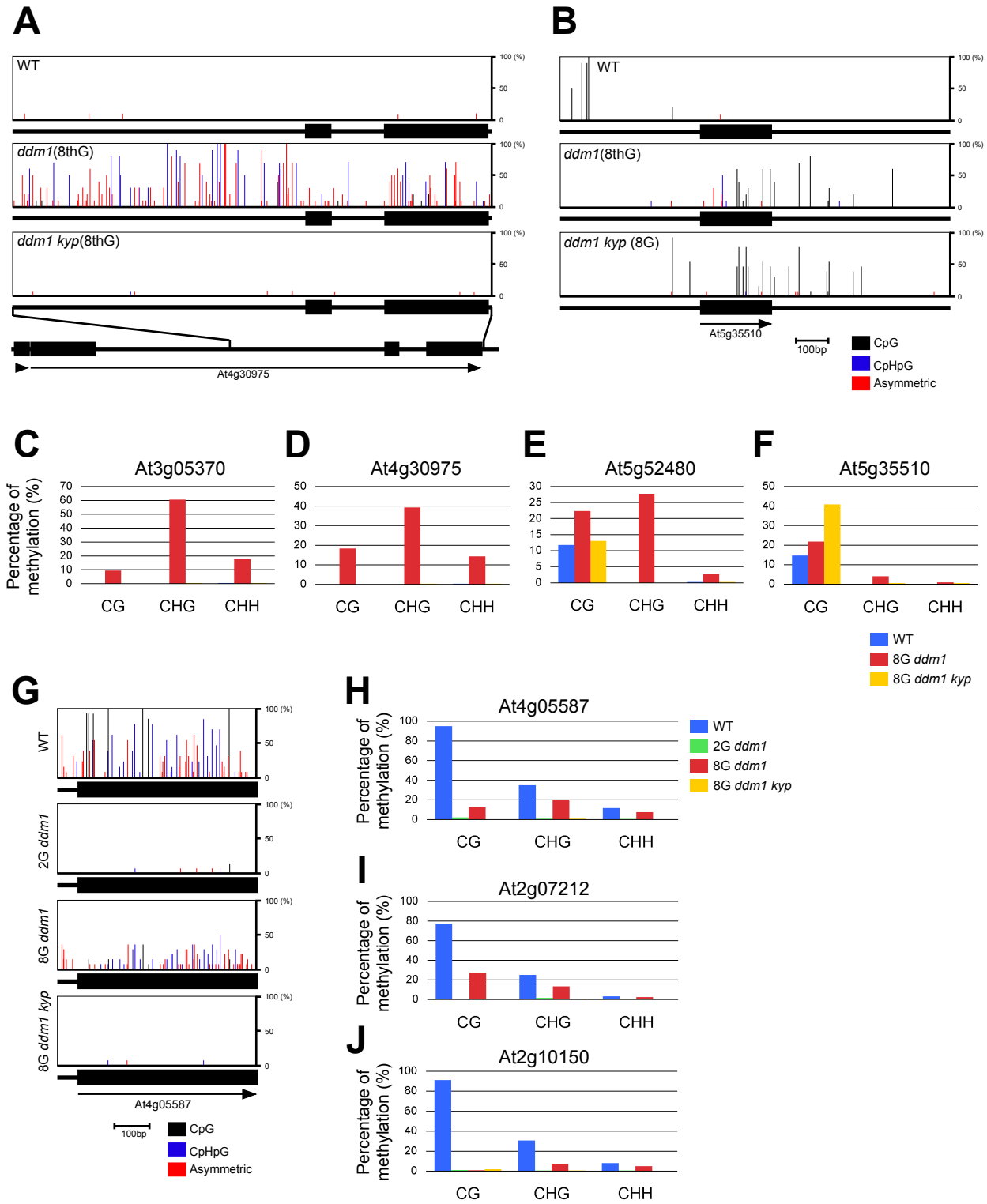
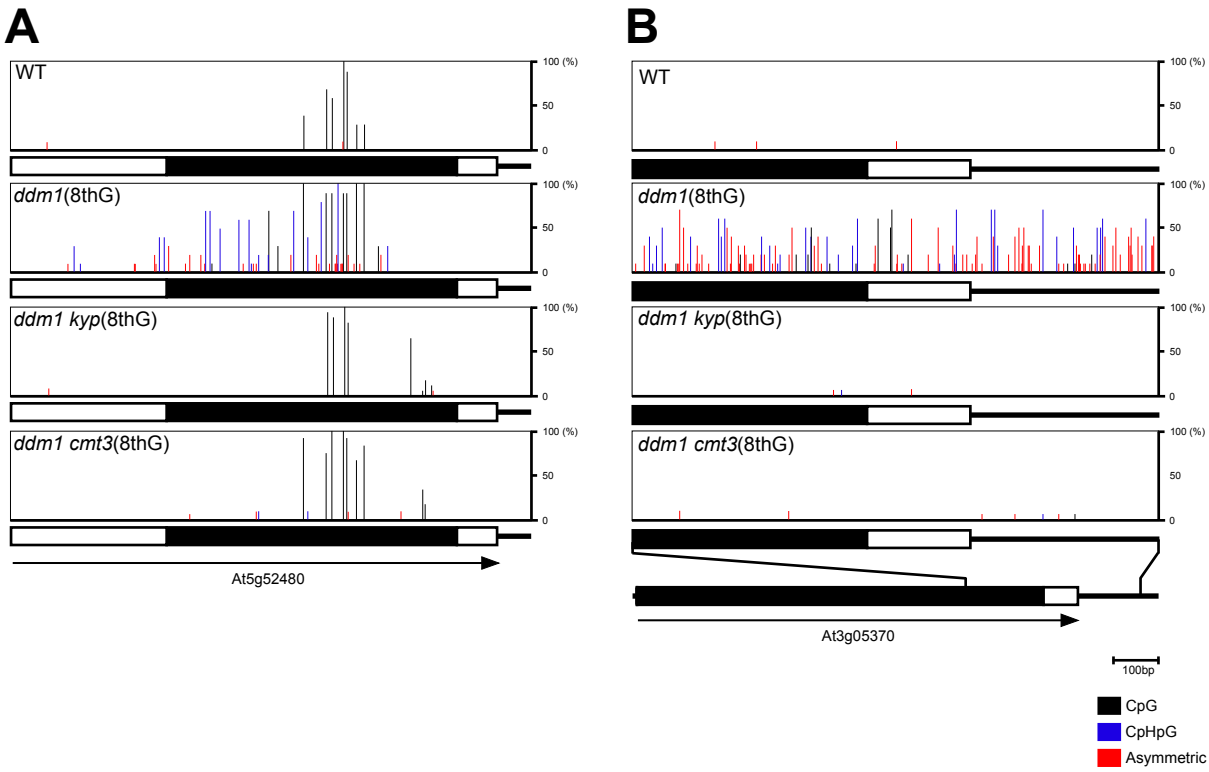

A
WT
ddm1(8thG)
ddm1 kyp(8thG)
100 (%)
50
0
At4g30975
B
WT
ddm1(8thG)
ddm1 kyp (8G)
At5g35510
100bp
CpG
CpHpG
Asymmetric
C
At3g05370
D
At4g30975
E
At5g52480
F
At5g35510
Percentage of methylation (%)
CG
CHG
CHH
WT
8G ddm1
8G ddm1 kyp
G
WT
2G ddm1
8G ddm1
8G ddm1 kyp
At4g05587
100bp
CpG
CpHpG
Asymmetric
H
At4g05587
2G ddm1
I
At2g07212
J
At2g10150

Figure 7 DNA methylation states of *ddm1*-induced hyper-methylated loci in genic regions (**A**-**F**) and transposable elements (**G**-**J**). Proportions of DNA methylation detected by bisulfite-sequencing analysis in WT, 8G *ddm1*, and 8G *ddm1 kyp* mutant within genic region (**A**, **B**) and those in WT, 2G *ddm1*, 8G *ddm1*, and 8G *ddm1 kyp* mutant within transposable element (**G**) which showed high slow effect. The percentages of methylated cytosines in genic regions (**C**-**F**) and transposable elements (**H**-**J**) detected by bisulfite-sequencing analysis. **A**, **D**: At4g30975 (see also **Figure 6E**), **B**, **F**: At5g35510, **C**: At3g05370, **E**: At5g52480, **G**, **H**: At4g05587 (see also **Figure 6F**), **I**: At2g07212, **J**: At2g10150


A
WT
ddm1(8thG)
ddm1 kyp(8thG)
ddm1 cmt3(8thG)
100 (%)
50
0
At5g52480
B
WT
ddm1(8thG)
ddm1 kyp(8thG)
ddm1 cmt3(8thG)
100 (%)
50
0
At3g05370
100bp
CpG
CpHpG
Asymmetric

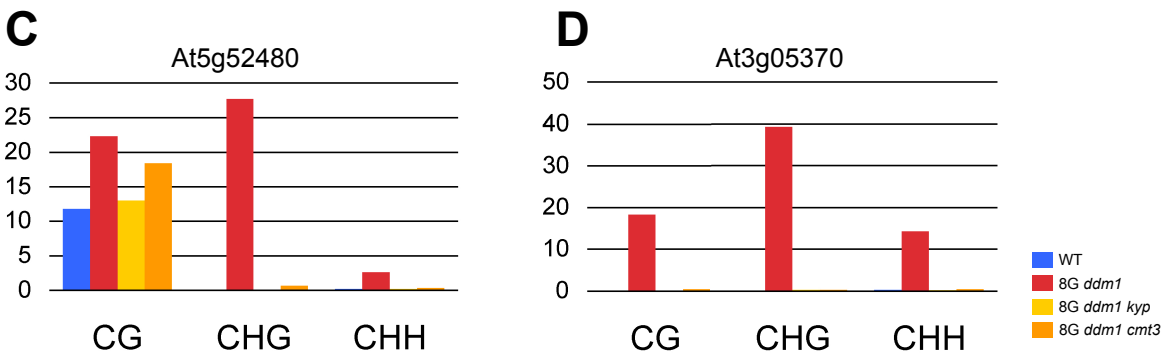

C
At5g52480
30
25
20
15
10
5
0
CG
CHG
CHH
D
At3g05370
50
40
30
20
10
0
CG
CHG
CHH
WT
8G ddm1
8G ddm1 kyp
8G ddm1 cmt3

Figure 8 Effect of KYP and CMT3 on the *ddm1*-induced DNA hyper-methylation. Proportions of DNA methylation detected by bisulfite-sequencing analysis (**A**, **B**) and percentage of methylated cytosines (**C**, **D**) in WT, 8G *ddm1*, 8G *ddm1 kyp*, and 8G *ddm1 cmt3* within target genes of *ddm1*-induced DNA hyper-methylation (**A**, **C**: At5g52480 , **B**, **D**: At3g05370).

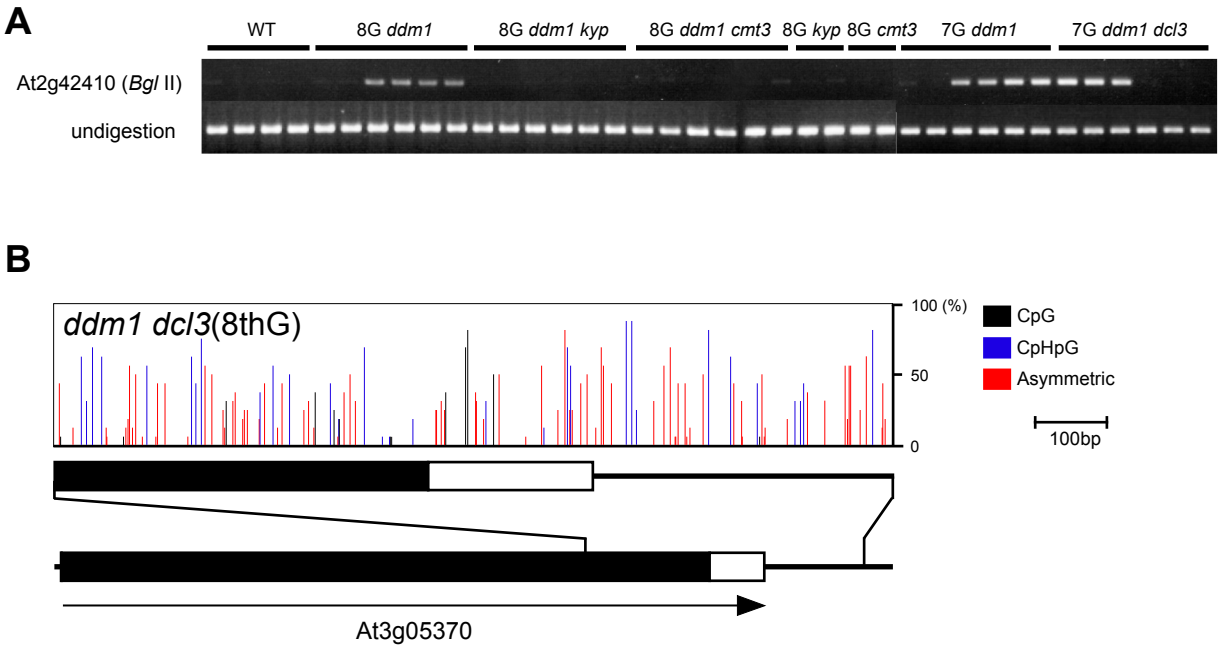

A
WT
8G ddm1
8G ddm1 kyp
8G ddm1 cmt3
8G kyp
8G cmt3
7G ddm1
7G ddm1 dcl3
At2g42410 (Bgl II)
undigestion
B
ddm1 dcl3(8thG)
100 (%)
50
0
CpG
CpHpG
Asymmetric
100bp
At3g05370

Figure 9 *ddm1*-induced DNA hyper-methylation in *dcl3* mutant. (**A**) DNA methylation states in At2g42410 were detected by restriction digestion by *Bgl* II and subsequent PCR. (**B**) Proportion of DNA methylation in At3g05370 detected by bisulfite-sequencing analysis (see **Figure 8B** for the proportion of DNA methylation in WT and 8G *ddm1*).

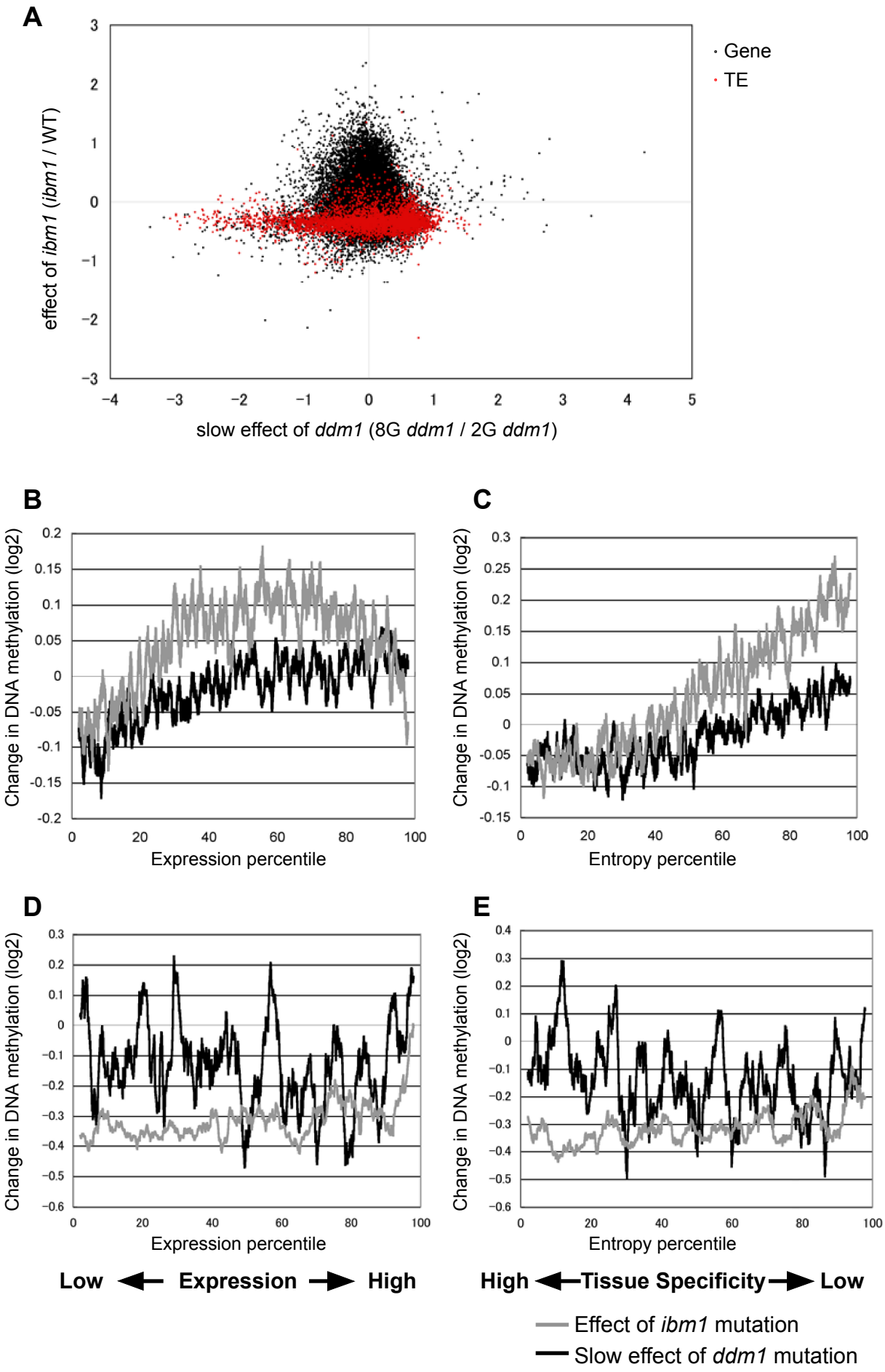

A
Gene
TE
effect of ibm1 (ibm1 / WT)
slow effect of ddm1 (8G ddm1 / 2G ddm1)
B
Change in DNA methylation (log2)
Expression percentile
C
Change in DNA methylation (log2)
Entropy percentile
D
Change in DNA methylation (log2)
Expression percentile
Low Expression High
E
Change in DNA methylation (log2)
Entropy percentile
High Tissue Specificity Low
Effect of ibm1 mutation
Slow effect of ddm1 mutation

Figure 10 Different target specificity of DNA hyper-methylation between *ddm1* and *ibm1*. (**A**) Scatter plot of slow effect of *ddm1* mutation (vertical axis) and effect of *ibm1* mutation (horizontal axis). Slow effect of *ddm1* mutation was calculated as [8G *ddm1* / 2G *ddm1*], and effect of *ibm1* mutation was calculated as [*ibm1* / WT]. Data used in this analysis were from previous study analyzing the genome-wide DNA methylation states in *ibm1* mutant (Miura *et al*. 2009). (**B**, **D**) Relationship between transcriptional activity and ectopic induction of DNA methylation in *ddm1* and *ibm1*. All genes (**B**) and transposons (**D**) were rank-ordered and binned based on the sum of expression in all of *Arabidopsis* tissues. The vertical value shows mean methylation signal for genes in each percentile. (**C**, **E**) Relationship between tissue-specificity of expression and ectopic induction of DNA methylation in genes (**C**) and transposons (**E**). Same as (**B**, **D**), except that the genes were rank-ordered based on tissue specificity of expression measured by entropy level.

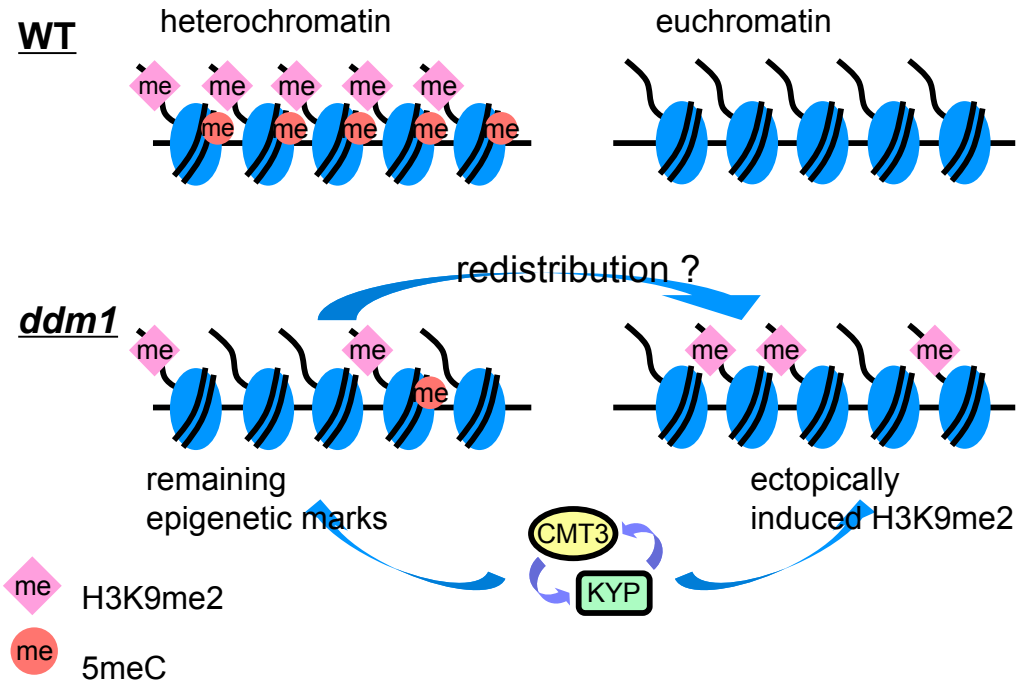

WT
heterochromatin
euchromatin
me
redistribution ?
ddm1
remaining
epigenetic marks
ectopically
induced H3K9me2
CMT3
KYP
H3K9me2
5meC

Figure 11 Possible model of *ddm1*-induced DNA hyper-methylation. In *ddm1* mutant, heterochromatic H3K9me2 modification might be reduced and redistributed throughout the genome including euchromatic regions. Genes ectopically modified by H3K9me2 might become targets of KYP-CMT3 mediated self-reinforcement mechanism of DNA and histone methylation during self-pollinations of *ddm1* mutant. In transposons, DNA and histone methylation might be decreased in *ddm1* mutant, but remaining epigenetic marks might be targets of KYP-CMT3.

Table 1 List of primers used in this study.

Primers for restriction amplification		
Target	Primers	Sequence (5' to 3')
BNS	BNS F2	GCT AGA GGT TTT TAG TTC TCT G
Bgl II	BNS R3	TTC CTT ATG ACA TTT CAA GGT C
BNS	BNS F3	GTA ATG GAG ACA CAT ACG TCA C
Sau3A I	LINE RB1	TGT TTA TAG AGA AGC TAA TCA
At2g42410	At2g42410-F	CAC ATA GTA ACT CCT CCG TAG
	At2g42410-R	AAG GCT CAA GAT GAG CTG TTG

Primers for Bisulfite-sequencing		
BNS 5'	meBNS RF1	AGY TTT TTT TAT AAG YTA GAG GTT TTT AGT
	meBNS RR1	ACC TAT ATA TRA ATA TCT AAA ACA ATC TAC
BNS middle	meBNS RF3	TTG TTT TAG ATA TTY ATA TAT AGG TTA TTG
	meBNS RR3	TCT CCA TTA CTT TCC TTA TRA CAT TTC A
BNS 3'UTR	meBNS RF2	TTG AAA TGT YAT AAG GAA AGT AAT GGA GA
	meBNS RR5	TTA RCT AAC TAT RCT TTT CTT TTA CC
BNS LINE	meBNS RF4	TTY ATA YYT TYT GTG ATA TAY GGY GTA AG
	meBNS RR6	CAR CRA CAA ATT CTT AAA ATT TAT RCC ACC
At3g05370	At3g05370BisF1	GAA YAA AGG AGT AGA GAY GGA GTT TAA G
	At3g05370BisR1	CTA TTC CCR CRR CTA TCC ART TAA TCA C
	At3g05370BisF2	GAT TTG TYT GAG YYG GAA GAA YAT GTG
	At3g05370BisR2	AAC TTC CTC ATC ATT TTR TTC TRC AAC C
At4g30975	At4g30975BisF3	GAA AAG TYT TTG YGT TTG AGT TTG AAG
	At4g30975BisR3	CAT CTC TAT ARC TCT ATC CCA CTT CAC C
	At4g30975BisF4-2	AAT TAA AYT TYA GGT GAA GTG GGA TAG AG
	At4g30975BisR4	TTA RAT ATA TCC TTR AAA TRT CTT CCC AC
	At4g30975BisF5	GAA AGA TGA TAT YAA ATA TYA TGT GGG
	At4g30975BisR5	CAA RTA RAT RCT ARA ACT RTA ACT CAC C
At5g35510	At5g35510BisF1	GTT AAT GTG GAT TTA ATG TAG GAA G
	At5g35510BisR1	CAC TRT CCA CTT CAA AAT ATC TAT CTC
	At5g35510BisF2	GAY AGA GAT AGA TAT TTT GAA GTG G
	At5g35510BisR2	RCA AAT CCA CTA TTA TTC AAA CTT CAC
At5g52480	At5g52480BisF1	YYG AYG GAT TTG ATG TGT TTG ATT YTG
	At5g52480BisR1	CAR CCC TRC AAR TTT CAT ARA CTC TCC
	At5g52480BisF2	GGT TTY AGG AAT ATY ATT GTY AGG AGA G
	At5g52480BisR2	CAT TTT AAT CCT ACT TRT TTC TRC ATR C

Primers for restriction amplification

Target	Primers	Sequence (5' to 3')
At2g07212	At2g07212BisF1-2	TTT GTT GGT YAT YAG GGT GAG AYT G
(TE)	At2g07212BisR1-2	CAC ACA AAA TTC ARA ACT TAT ART TAR TC
At2g10150	At2g10150BisF1-2	GTA AYT GGT YGA GTA GAG AAT YGA GTA GG
(TE)	At2g10150BisR1-2	CTT TCT TTR CAA CCT TAA RAT RTT CCC C
At4g05587	At4g05587BisF1	AAT TYT TGA GAA YAA TTY YGG TTT YAT AG
(TE)	At4g05587BisR1	CTC AAR AAT CCC AAT CCT TCA CTT TCC

Materials and Methods

Plant Materials

The isolation of *ddm1-1*, *cmt3-i11*, *kyp*, *met1-3*, *rdr6-11*, *ago1-25*, *ago4*, *drm2*, and *sde3-1* were reported in previously (Vongs *et al.* 1993, Bartee *et al.* 2001, Saze *et al.* 2003, Peragine *et al.* 2004, Morel *et al.* 2002, Zilberman *et al.* 2003, Cao *et al.* 2002, Dalmay *et al*.2001). *rdr2* (SALK_059661), *dcl3* (SALK_005512), *nrpd1* (SALK_128428), *nrpd1b*/*nrpe1* (SALK_029919), *nrpd2a*/*nrpe2a* (SALK_046208), *rdr1* (SALK_112300), and *dcl2* (SALK_095060) T-DNA insertion lines were obtained from the *Arabidopsis* Biological Resource Center. Arabidopsis plants were sawed on soils and grew at 24°C in 16hr-8hr light cycle.

DNA methylation analysis

Genomic DNA was isolated with the illustra Nucleon Phytopure Genomic DNA Extraction Kits (GE Healthcare). DNA methylation analysis by methylation-sensitive restriction enzyme was performed as described by Saze & Kakutani (2007). 50 ng of genomic DNA was digested with methylation-sensitive restriction enzymes *Sau*3A I or *Bgl* II in 20µl reaction mix. After the restriction digestion for 5 hours at 37°C, 1µl of digested sample was used as a template for PCR. Primers used for restriction amplification were listed in Table 1.

DNA bisulfite sequencing was performed as described by Paulin *et al*. (1998). 250ng of genomic DNAs were double-digested in 30 µl of the reaction mixture. After ethanol precipitation, DNAs were dissolved in 20 µl of water. After being heated at 94°C for 10 minutes

and then cooled on ice, DNAs were denatured by the addition of 2.2 µl of 3 N NaOH and incubated at 37°C for 15 minutes. The denatured DNAs were dissolved in 208 µl of urea/bisulfite solution (7.5 g of urea (Wako, Osaka, Japan) and 7.6 g of sodium metabisulfite (MERCK, Germany) dissolved in 20 ml of water, adjusted to pH 5.0) and 12 µl of 10 mM hydroquinone (SIGMA, USA) and overlaid with mineral oil. The samples were subjected to 30 cycles of 95°C for 30 seconds and 55°C for 15 minutes, followed by 55°C for 15 hours in a PCR instrument. After the reaction, DNAs were purified using the Gene Clean Kit (Q-BIOgene, USA) and eluted with 20 µl of water. For the desulfonation, 2.2 µl of 3 N NaOH was added and the DNAs were incubated at 37°C for 15 minutes. After ethanol precipitation, DNAs were eluted with 20 µl of water. PCR was performed in 50 µl of reaction mixture containing 5 µl of DNA as a template. The PCR condition was 94°C for 1 minutes followed by 40 cycles of 94°C for 15 seconds, 50°C for 30 seconds, and 72°C for 1 minute, and a final extension at 72°C for 10 minutes. Primers used for bisulfite sequencing was listed in Table 2. The PCR products were gel-purified and cloned with the pGEM-T easy Vector Systems (Promega). Ten to sixteen independent clones were sequenced.

Immunoprecipitation and microarray analysis of methylated DNA

The genomic DNA was sonicated to produce fragments ranging in size from 100 to 1,000 bp. Immunoprecipitation using anti-5-methylcytosine antibody (Calbiochem) was performed as described by Waber *et al*. (2005). Immunoprecipitated DNA was amplified using GenomePlex Complete Whole Genome Amplification Kit (SIGMA) and purified using QIA Quick PCR Purification Kit (QIAGEN). 2µg of amplified DNA and input DNA were labeled with

Cy5 and Cy3, respectively.

Array design and data analysis

The NimbleGen 3×385K array (Zilberman *et al.* 2007), which covers entire *Arabidopsis* genome with an intervals of ~100bp, were used. The methylation signal for each probe was represented as $\log_2$ signal ratio of immunoprecipitated DNA to input DNA. The Arabidopsis genome is covered by three 385K chips of NimbleGen. The first chip covers entire chromosome 1 and part of chromosome 2. The second chip covers entire chromosome 3 and parts of chromosome 2 and 4. The third chip covers entire chromosome 5 and part of chromosome 4. In order to integrate the results from three chips, the IP/input signal values of each chip was normalized using average of one chromosome for each chip: chromosome 1, 3, and 5 for chip 1, 2, and 3, respectively. Values for part of chromosomes were not used, in order to avoid bias caused by the different proportion of methylated pericentromeric regions and arm regions in the part of chromosome. Methylation of each accession is represented by the average of the $\log_2$ signal for all probes covered by each accession. Annotation information was based on TAIR8.

Relationship between DNA methylation and gene expression

Data used in the analyses of gene expression levels and tissue specificities were from a previous study analyzing the genome-wide expression pattern in various developmental stages and tissues of *Arabidopsis* (Schmid *et al*. 2005). For Figure 10 B, D, sum of expression in all of stages and tissues was used. For tissue specificity (Figure 10 C, E), entropy levels of

each gene were calculated as described by Zhang *et al*. (2006).

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