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DNA methylation dynamics and effect of DEMETER DNA demethylase during *Arabidopsis* regeneration

애기장대 재생과정 동안 DNA 메틸레이션 역동성과 DNA 디메틸라제 DEMETER의 영향

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전 윤 지

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Yunji Jeon

Chair Youbong Hyun (Seal)

Vice Chair Yeonhee Choi (Seal)

Examiner Pil Joon Seo (Seal)

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지도교수 최 연 희

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위 원 장 현 유 봉 (인)

부위원장 최 연 희 (인)

위 원 서 필 준 (인)

Abstract

DNA methylation dynamics and effect of DEMETER DNA demethylase during *Arabidopsis* regeneration

Yunji Jeon

School of Biological Sciences

The Graduate School

Seoul National University

Plant regeneration, a process that undergoes de-differentiation from already specialized tissue followed by re-differentiation, involves the remarkable process of cell fate transition, requiring DNA methylation reprogramming. This study explores DNA methylation dynamics during regeneration across leaf, callus, and de novo shoot stages in *Arabidopsis* Ler wild type. Differentially methylated regions (DMRs) were identified, with distinct patterns observed in different cytosine contexts, (CG, CHG, and CHH) during these cell fate changes. The effect of DEMETER (DME), a DNA demethylase involved in endosperm-specific hypomethylation compared to embryo, was also investigated in each stage. Intriguingly, *dme-2* mutants

exhibited increased callus and *de novo* shoot formation, accompanied by consistently lower methylation levels compared to the wild type, especially in non-CG contexts. Gene expression analysis revealed reduced expression of some key RNA-directed DNA methylation(RdDM) pathway genes in dme-2 mutants, potentially contributing to decreased DNA methylation during regeneration. This study sheds light on the role of DNA methylation during *Arabidopsis* regeneration, particularly the role of DME in this process.

Keywords : *Arabidopsis* regeneration, DNA methylation, DEMETER (DME), de-differentiation, re-differentiation

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Abbreviations

CIM	Callus-inducing medium
SIM	Shoot-inducing medium
RIM	Root-inducing medium
DAG	Days after germination
DAC	Days after incubation on CIM
DAS	Days after incubation on SIM
WGBS	Whole genome bisulfite sequencing
RdDM	RNA-directed DNA methylation
DMR	Differentially methylated region
TE	Transposable element

BACKGROUD

1. Plant regeneration in *Arabidopsis*

Plants have the ability to heal wounds by regenerating new tissues at the site of injury. Plant regeneration refers to the process by which plants are able to repair, renew, or replace damaged or missing tissues and organs (Sugimoto et al., 2011). It is based on the principle of totipotency, which means that every cell in a plant has the potential to develop into any type of cell or tissue (Long et al, 2022). Plant regeneration can occur through different mechanisms such as dedifferentiation, organogenesis, and somatic embryogenesis (Fehér et al., 2019). Dedifferentiation involves the reversion of specialized or differentiated cells to a less specialized state, allowing them to differentiate into new tissues or organs. Organogenesis involves the development of new organs from undifferentiated cells, while somatic embryogenesis involves the development of whole plants from single somatic cells (Ohtani, Misato et al., 2015). Plant regeneration plays an essential role in plant growth, development, and response to environmental stresses and is therefore of great significance in agriculture and horticulture.

2. DNA methylation in *Arabidopsis*

Epigenetics is the study of reversible changed in the phenotype of organisms that are inherited but not caused by alterations to the genetic code itself (Smulders et al., 2011). DNA methylation, along with histone modifications, is a major focus of research in understanding epigenetic phenomena (Acharjee et al., 2023). DNA methylation is a key epigenetic mechanism that involves the attachment of a methyl-group to the fifth carbon at the cytosine base (Moore et al., 2013). While not conserved among eukaryotes, it plays a crucial role as a regulatory mechanism in many organisms. *Arabidopsis,* a model organism for flowering plants, has a complex DNA methylation system that includes various cytosine methylation contexts, CG, CHG, and CHH (H=A, T or C), and specific DNA methyltransferases and demethylases (Dubin et al., 2015). *Arabidopsis* has several methyltransferases including METHYLTRANSFERASE 1 (MET1), DOMAINS REARRANGED METHYLASE 1/2 (DRM1/2), CHROMOMETHYLASE 2 (CMT2), and CHROMOMETHYLASE 3 (CMT3) (Cao et al., 2002). It also has active DNA demethylases such as DEMETER (DME), Repressor of transgene silencing 1 (ROS1), DEMETER-LIKE 1 (DML1), and DML2 (Schumann et al., 2019). This system allows tight regulation of gene expression and transposable element (TE) silencing (Gehring and Henikoff, 2007). The regulation of genebody methylation and TE methylation differs. While genebody methylation is associated mainly with

CG context, TE methylation involves all cytosine contexts and dynamic changes are observed in non-CG methylation levels that vary by cell type and genomic environment (Muyle et al., 2022). In mammals, DNA methylation primarily occurs at the CG context, with global demethylation observed during primordial germ cell and early embryonic development. In both plants and mammals, DNA methylation levels can be reprogrammed during reproduction (Greenberg et al., 2019). In *Arabidopsis,* this involves the reduction of CHH methylation levels in early embryos, followed by gradual increase through the RNA-directed DNA methylation (RdDM) pathway(Papareddy et al., 2020).

3. DMETER, DNA demethylase

DEMETER (DME), a DNA glycosylase domain-containing protein (1,879 aa), is primarily expressed in the central cell of the female gametophyte and in the vegetative nucleus of the bi-cellular pollen. DME plays a significant role in early female and male gametophyte development and seed development (Choi et al., 2002). As an active DNA demethylase, DME can only remove 5-mC in all contexts but is most efficient in 5meCG, whether the DNA strands are both methylated or hemi-methylated (Gehring et al., 2006). DME is unique to the plant lineage, and its family has been extensively characterized in vitro, along with REPRESSOR OF SILENCING1 (ROS1). The HhH-GPD DNA glycosylases are the largest class of glycosylases among all organisms, and the DME family is distinct from them (Gehring and Henikoff, 2008). A conserved aspartic acid residue and an invariant lysine are present in HhH-GPD DNA glycosylases, and when either of these residues is mutated in recombinant DME, 5-mC DNA glycosylase activity is lost (Choi et al., 2002; Choi et al., 2004; Gehring et al., 2006; Morales-Ruiz et al., 2006).

To eliminate 5-mC from DNA through base excision repair, it is necessary to have both DNA glycosylase activity and AP lyase ctivity that can break the bond between the base and sugarphosphate, as well as AP endonuclease activity that can remove the sugarphosphate (Lee et al., 2014). In *Arabidopsis,* the enzymes that act as DNA demethylases have both DNA glycosylase and

AP lyase activity. This bi-functional enzyme creates an AP site, which is then repaired by DNA polymerase beta, resulting in the replacement of 5-mC with cytosine (Kress et al., 2006).

I. Introduction

Plants have the ability to heal wounds by regenerating new tissue, a process known as vegetative propagation. Through this process, plants can regenerate entire organisms from a few cells or tissues (Sugimoto et al., 2011). Plant regeneration induced by injury starts from undifferentiated cell masses called callus. Callus can differentiate into new organs, and by regulating the concentration of auxin and cytokinin, callus formation and desired organogenesis can be induced. When differentiated plant sections such as leaf explant are cultured in an callus-inducing medium (CIM; auxin-rich), calluse can be induced, and the callus is subsequently cultured in a shoot-inducing medium (SIM; cytokinin-rich) to form *de novo* shoot. Culturing those shoot in root-inducing medium (RIM; auxin-rich) results in the formation of regenerated plants that can grow into complete organisms (Ikeuchi et al., 2013).

Cell fate transitions require overall reprogramming of DNA methylation, and significant changes have been observed both within and between species (Lee and Seo, 2018). Accordingly, the global methylation pattern during callus and *de novo* shoot formation also varies among different plant species, and even within the same species. Furthermore, the distribution and changes in DNA methylation patterns differ depending on the tissue used for explant (Lee and Seo, 2018). Therefore, research using diverse tissues from various plant species is needed to fully understand the role of methylation in plant

dedifferentiation and redifferentiation processes. Studies on plant regeneration, using the model plant *Arabidopsis thaliana,* are also continuously being conducted to shed light on this topic. Currently, however, studies on methylation dynamics during plant regeneration are limited to the transition from leaves to callus in *Arabidopsis thaliana* (Shim et al., 2021). In addition, research on plant regeneration is limited because direct mutagenesis such as gamma irradiation or ethylmethane sulphonate (EMS) in callus cannot be obtained (Ramakrishna et al., 2018). Therefore, I examined methylation patterns at each developmental stage and conducted detailed analysis of methylation dynamics.

Recently, it has been reported that DEMETER (DME) also plays a crucial role in sporophytic development, including the regulation of transposable element (TE) expression, seed length and germination rate, and the process of dedifferentiation and *de novo* shoot formation (Kim at al., 2021). Specifically, DME affects callus and *de novo* shoot formation, as the *dme-2* mutant induces more callus and *de novo* shoot formation compared to the *Ler* wild-type ecotype (Kim et al., 2021). Based on this previous result, I investigated the epigenetic effect caused by mutation in *DME* gene during plant regeneration process. My study could provide valuable insights into how methylation dynamics are involved in *Arabidopsis thaliana* regeneration. This study will also provide an important clue about how to manipulate DNA methylation to improve regeneration efficiency for other plant species including crop plants.

II. Materials and methods

1. Plant Materials and Growth Conditions

The *Ler* ecotype was used to investigate the genome-wide distribution of DNA methylation in this study. The *Ler* wild-type and *dme-2* were used in this study. Seeds were germinated on MS (Murashige and Skoog) medium at 22℃ under long-day conditions (16 hours of light, 8 hours of dark) with cool white fluorescent light (100 μmole/m2/s). True leaf of seedlings at 14 days after germination (DAG14) was used. Callus was induced from leaf explants of true leaves of DAG14 seedlings on CIM (callus-inducing medium) (MS medium supplemented with 0.5 μg/ml 2,4-dichlorophenoxyacetic acid [2,4-D] and 0.05 μg/ml kinetin). The plates were incubated at 22℃ for 7 days continuous dark conditions. To get *de novo* shoot, callus (incubated on CIM for 7 days) was transferred to SIM (shoot-inducing medium) (MS medium supplemented with 0.9 μmol/l 3-indoleacetic acid, 2.5 μmol/l 2-isopentenyl adenine), and then cultured for up to 2 weeks at 22℃ under long-day conditions. The types of samples used and the quality check results are shown in the following table (Table 1, Table 2, and Table 3).

Table 1. Quality of wild-type and *dme-2* leaf samples.

	WT leaf								*dme-2* leaf							
Sample number	1		2		3		4		1		2		3		4	
Paired end	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Mapping efficiency (%)	49.2	49.9	46.9	47.8	49.8	50.5	48.8	49.4	43.3	44.0	43.0	43.3	44.3	44.9	43.5	44.2
C coverage	8.53x		8.21x		8.50x		8.12x		9.00x		9.98x		8.29x		9.00x	
[CG] plastid methylation ratio	0.606		0.556		0.575		0.574		0.543		0.538		0.569		0.539	
[CHG] plastid methylation ratio	0.542		0.485		0.515		0.515		0.473		0.482		0.516		0.504	
[CHH] plastid methylation ratio	1.791		1.647		1.659		1.695		1.808		1.783		1.792		1.956	

Table 2. Quality of wild-type and *dme-2* callus samples.

	WT callus						*dme-2* callus					
Sample number	1		2		3		1		2		3	
Paired end	1	2	1	2	1	2	1	2	1	2	1	2
Mapping efficiency (%)	53.3	53.5	56.7	57.6	51.8	52.5	54.1	54.6	57.2	58.0	53.6	54.7
C coverage	8.46x		9.71x		7.9x		9.94x		10.89x		7.88x	
[CG] plastid methylation ratio	0.678		1.054		1.146		1.115		1.162		0.997	
[CHG] plastid methylation ratio	0.633		0.69		0.751		0.803		0.814		0.741	
[CHH] plastid methylation ratio	1.571		2.209		1.458		2.163		2.355		1.744	

Table 3. Quality of wild-type and *dme-2 de novo* shoot samples.

	WT de novo shoot								*dme-2* de novo shoot							
Sample number	1		2		3		4		1		2		3		4	
Paired end	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Mapping efficiency (%)	47.5	48.1	44.1	44.7	43.8	44.4	48.9	49.5	43.2	43.9	44.9	45.3	39.3	39.8	43.3	43.8
C coverage	7.14x		8.03x		8.98x		10.57x		8.78x		8.23x		7.8x		9.05x	
[CG] plastid methylation ratio	0.572		0.568		0.619		0.679		0.538		0.531		0.626		0.608	
[CHG] plastid methylation ratio	0.558		0.502		0.567		0.631		0.519		0.503		0.561		0.533	
[CHH] plastid methylation ratio	1.775		2.359		1.829		2.097		1.988		1.9		2.101		2.147	

2. DNA methylome library construction

All samples were frozen rapidly using liquid nitrogen and stored at a temperature -80°C. DNA from all samples was extracted by small amount DNA extraction. For the Whole-genome bisulfite sequencing (WGBS) libraries, the Pico Methyl-Seq Library Prep Kit (Zymo research) and EqiTech Bisulfite by Macrogen (Seoul, South Korea) were used.

3. Sequencing data processing

The HiSeq2000 platform by Macrogen (Seoul, South Korea) was used to perform the sequencing procedure, generating 101 bp paired-end reads. All the reads were trimmed sequentially using Trim Galore and Trimmomatic. Bismark with bowtie2 was then used to map the Whole-genome bisulfite sequencing (WGBS) reads of *Ler* wild-type and *dme-2* to the TAIR10 genome, without allowing any mismatches. The mapped reads were then deduplicated, and cytosine information was collected using Bismark script (deduplicate_bismark and bismark_methylation extractor) under default conditions.

4. Measurement of DNA methylation levels

To calculate the fractional methylation within 50 bp windows, the mean level of each cytosine was determined without any overlapping. Informative windows that contained at least 3 cytosines with a minimum of 5 reads aligned per cytosine context as a probe were used.

5. Identification of differentially methylated region

The difference in methylation levels of each 50 bp window at each stage and genomic feature was calculated. Regions of the genome where the methylation level differed by more than a standard deviation from the global mean at each stage were identified as stage-specific differentially methylated regions (stage DMRs). Regions where the methylation levels differed between wild-type and *dme-2* were defined as dme DMRs.

6. RNA preparation and Real time PCR

Total RNA was extracted from *de novo* shoot using Qiagen RNeasy Kit (Qiagen, Germany). After extraction, cDNA was synthesized using QuantiTect Reverse Transcription kit (Qiagen, Germany) according to manufacturer's recommendations. Quantitative real time PCR was performed with primers listed (Table 4).

Table 4. List of primers used in real-time PCR

ACT2-F	5’-CCTTTAACTCTCCCGCTATGT-3’
ACT2-R	5’-GTAAGGTCACGTCCAGCAAG-3’
AGO1-F	5’-TCAAGCCCATCTATTGCTGC-3’
AGO1-R	5’-ATCATGCCACCAGTCACCAC-3’
AGO4-F	5’-TGGATGGTAAAGAGTTTGCT-3’
AGO4-R	5’-CCATCACTTGGACTTTCATT-3’
NRPD2-F	5’-GATGCTAGATATCCGCACCCC-3’
NRPD2-R	5’-CAGCTCTTCCATTCCACAAGC-3’
DRM1-F	5’-TAGAGCAATTGAAGAAACCGC-3’
DRM1-R	5’-CATTCGTGATCTCTCCCACATCT-3’
DRM2-F	5’-AAAATGTGGATATTGCAGAG-3’
DRM2-R	5’-TCCTATCATTGGATTTGGTA-3’
NRPE1-F	5’-CATCCGTCTGCGTACCCTG-3’
NRPE1-R	5’-TCAACCGTGATGAAGTCAACG-3’

III. Results

Much more callus and *de novo* shoot were induced in *dme-2* mutants

Previous study reported that the formation of callus and *de novo* shoot increased in *dme-2* mutant than in wild-type *Ler* (Kim et al., 2021). To independently validate these findings, I induced callus by incubating leaf explants from wild-type (*Ler*) and *dme-2* mutant on CIM for two weeks. For *de novo* shoot formation, firstly I incubated leaf explants on CIM for a week and then the induced DAC7 (7 days after incubation on CIM) callus were excised and incubated ont SIM for two weeks. Consistent with the previous observation, I confirmed that much more callus and *de novo* shoot were generated in *dme-2* mutant compared to wild-type (Figure 1).

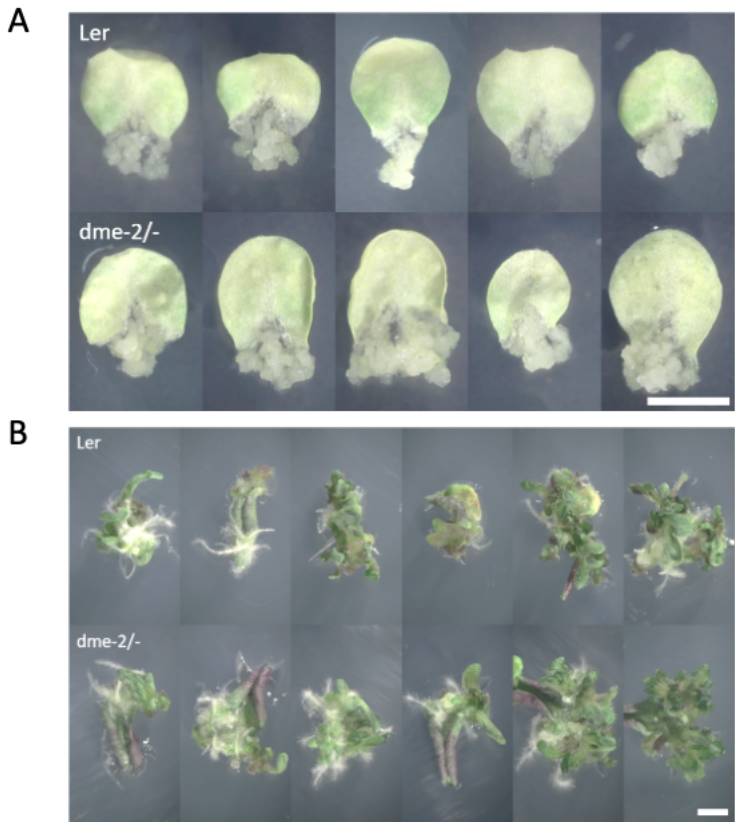

Figure 1. Callus and de novo shoot formation in wild-type and *dme-2*

(A) callus formation in wild-type *Ler* and *dme-2.* (B) *de novo* shoot formation in wild-type and *dme-2.* Scale bar: 0.5cm.

DNA methylation dynamics during WT *Arabidopsis* regeneration process

To investigate the methylation dynamics during wild-type plant regeneration process, whole genome bisulfite sequencing (WGBS) was performed on *Ler* leaf explants, DAC7 callus, and *de novo* shoot, and the data were processed and analyzed as in the Methods.

In overall, the average CG methylation levels remained constant during the WT regeneration process (Figure 2A). However, I observed a gradual increase in CHG methylation levels, especially in TEs. CHH methylation levels of TEs decreased when callus were induced from leaf explant, then increased when *de novo* shoot were induced from callus (Figure 2A). The observed patterns were evident in genebody and transposable elements (TE) regions (Figure 2B). These results indicate that while the average CHG methylation levels gradually increase during regeneration process of *Ler*, CHH methylation levels show fluctioation; decrease during callus formation and increase when differentaion occurs thereby organs are formed from callus.

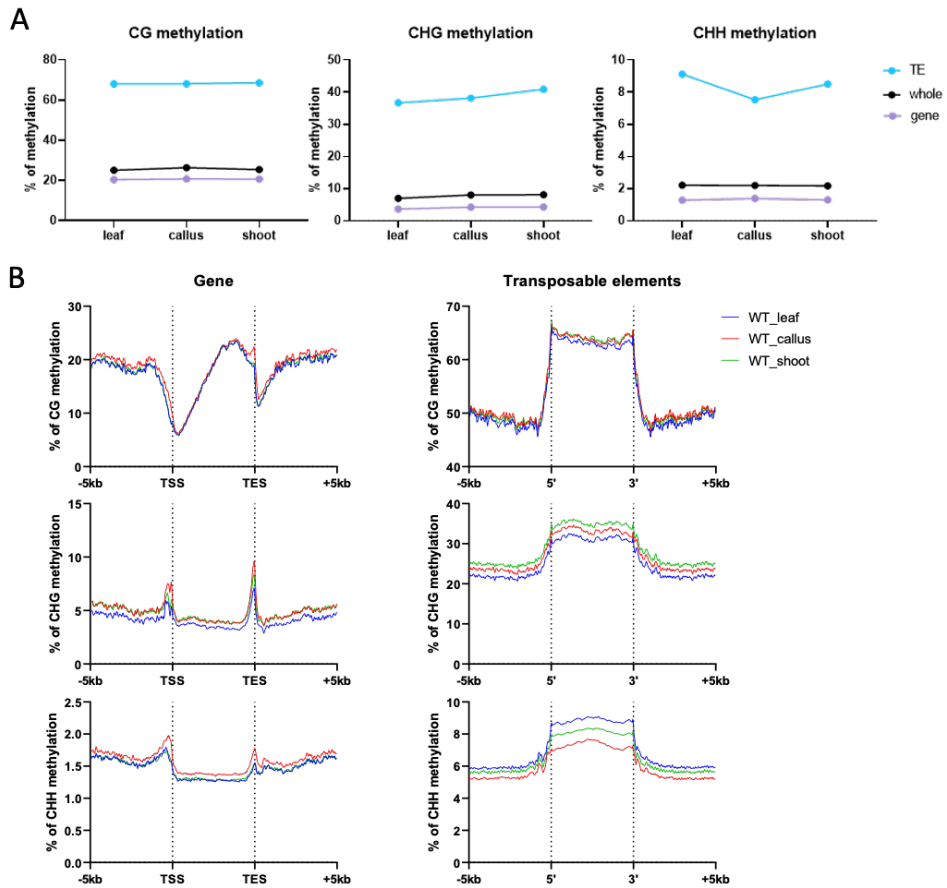

Figure 2. DNA methylation during *Arabidopsis* regeneration in *Ler* wild-type

(A) Average DNA methylation levels (%) of *Ler* wild-type were shown separately in whole genome (black), gene (purple), and transposable elements (sky blue) during regeneration process. (leaf, callus, and *de novo* shoot). **(B)** DNA methylation level of gene, transposable elements, and surrounding regions were analyzed for each cytosine context (CG, CHG, and CHH).

Although I observed constant average CG methylation levels during plant regeneration, it is unclear whether the CG methylation levels are not changed at specific genomic regions during regeneration. It is also possible that some regions show hyper CG methylation and some other regions show hypo CG methylation, thereby the total average CG methylation levels remain the same. To test this possibility, I calculated the difference in methylation levels of each 50 bp window at each stage and each of the genomic feature. Then, I identified regions of the genome that showed significant methylation differences between different stages, by considering those regions where the methylation level differed by more than a standard deviation from the global mean at each stage. These regions were then defined as stage-specific differentially methylated regions (stage DMRs). Subsequently, I defined callus DMRs as the regions showing significant methylation differences between leaf and callus, and *de novo* shoot DMRs as the regions exhibiting significant methylation differences between callus and *de novo* shoot.

While the global CG methylation levels remained relatively stable during callus formation, a significant number of callus hyper (14,815) as well as hypo (16,282) CG DMRs were observed (Figure 3A). Interestingly, genic regions are enriched in both hyper and hypo CG DMRs (Figure 3B). By contrast, for CHH methylation, a significant more hypo DMRs (31,936) were observed (Figuer 3A) and they were enriched in TE (including genes and TE) (Figure3B).

From callus to *de novo* shoot formation process (differentiation), methylation levels increase largely in TE (Figure 2A). Again, although the global CG methylation levels did not show a significant difference during shoot formation, the number of both hyer (26,926) and hypo (26,793) DMRs were more than those of callus DMRs. Non-CG methylation increase in TE was striking (Figure 2A). The number of hyper CHH DMRs (29983) was greater than hypo CHH DMR (16059), suggesting that the increase in the number of DMRs undergoing hypermethylation contributes to the overall increase in global methylation levels during the transition from callus to *de novo* shoot. In addition, genes were enriched for the demethylated regions (hypo DMRs), especially for CG and CHH contexts (Figure 3B).

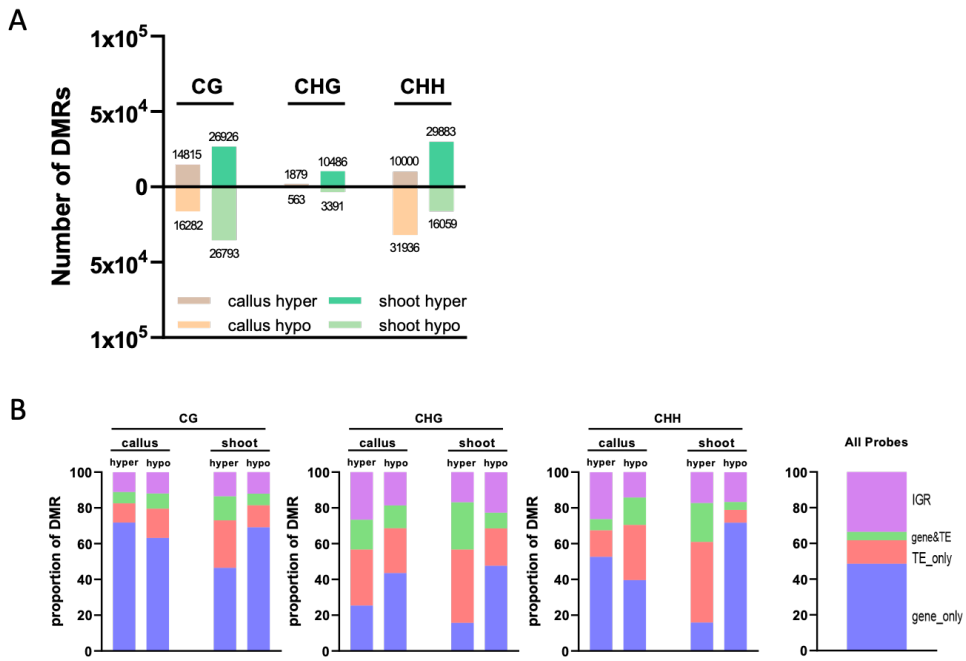

Figure 3. The number and the composition of stage DMRs during plant regeneration

The size of a window is 50 bp with overlap between two stages. **(A)** The total number of stage DMRs **(B)** The composition ratio of stage DMRs (gene, gene & TE, TE, and IGR)

To gain insight into the location where these DMRs are present, I examined the distribution of stage DMRs across *Arabidopsis* chromosomes. During de-differentiation (leaf to callus), both CG hyper and hypo DMRs were similarily distributed on chromosomal arms except for pericentromeric regions. CG Hypo DMRs were more concentrated on pericentromeric regions where as CG Hypo DMRs were absent in these regions (Figure 4, CG). This is consistent with my previous result (Figure 3), in that genes were more enriched in CG DMRs. CHG hyper DMRs, although the number is small, were concentrated in pericentromeric regions. In contrast, there were very few CHG hypo DMRs during callus induction (Figure 4, CHG). For CHH methylation, hypo DMRs were highly enriched in centromeric region (Figure 4, CHH).

During *de novo* shoot differentiation, pericentromeric hyper methylation was the most distinct pattern observed in all cytosine contexts, whereas hypomethylation was observed mainly on chromosomal arm only for CG context. Taken together, these analyses suggest that during de-differentiation, pericentomeric regions undergo hypomethylation primarily at the CG context and to a less extexnt at non-CG context. During differentiation and organogenesis, centomeric regions undergo methylation accumulation across all cytosine contexts.

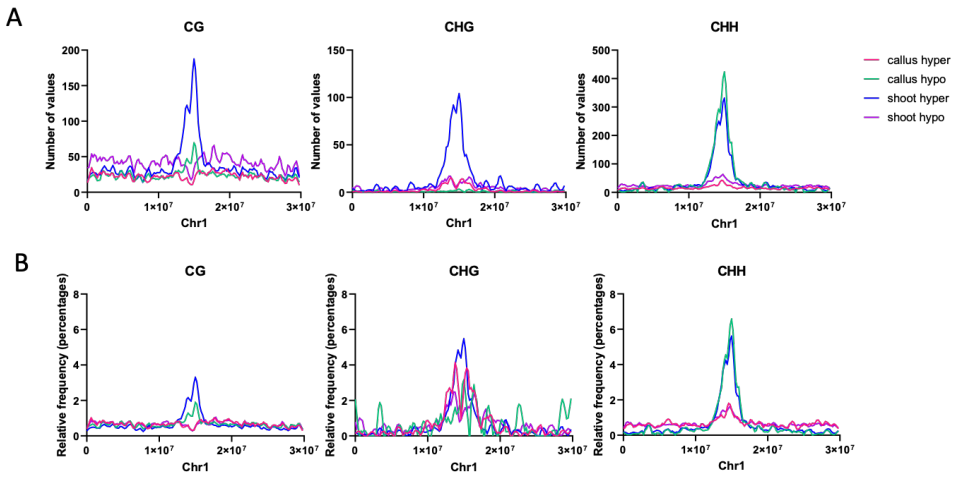

Figure 4. Positions of each stage DMRs on chromosome 1

The positions of stage DMRs (callus hyper, callus hypo, shoot hyper, and shoot hypo) was analyzed in chromosomal view. **(A)** Number of values of stage DMRs on chromosome 1 **(B)** Relative frequency (%) of stage DMRs on chromosome 1

Decreased methylation levels during regeneration in *dme-2* compared to wild-type

I found that callus and *de novo* shoot formation were increased in *dme-2* mutant (Figure 1). To find out the effect of DME (DEMETER) during plant regeneration, I examined DNA methylation dynamics in the leaves, callus, and *de novo* shoot of *dme-2* and compared to those in WT. TE showed slight hypo CG methylation both in callus and *de novo* shoot of *dme-2* globally (Figure 5A, CG). Genic region showed slight hyper methylation in *dme-2* during callus induction (Figure 5B, Gene). In CHG methylation, while wild-type exhibited gradual increase especially in TE, *dme-2* did not, resulting in hypo CHG methylation (Figure 5A, CHG). Wild-type CHH methylation decreased during de-differentiation (callus induction) and then regains CHH methylation during differentiation (*de novo* shoot induction), especially in TE (Figure 5A, CHH). *dme-2* also showed global CHH methylation decrease as wild-type (with slight hypo than wild-type) during de-differentiation and then it failed to regain, resulting in further hypo CHH methylation compared not only to wild-type shoot, but also to *dme-2* callus (Figure 5A, CHH).

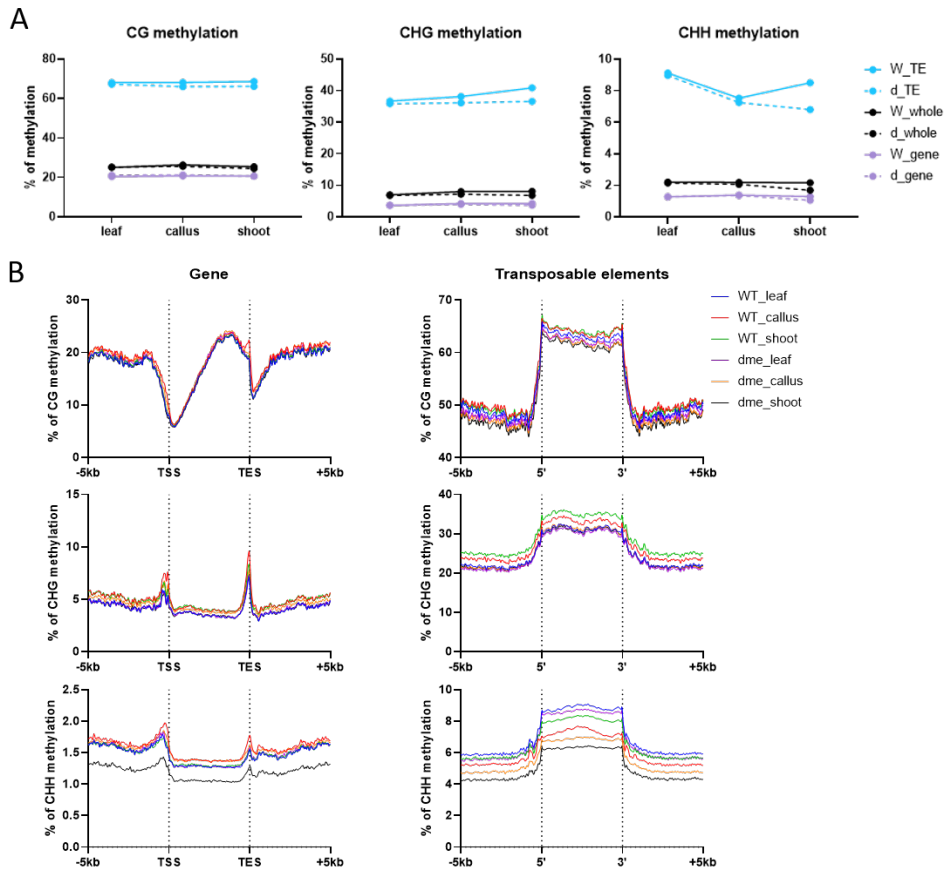

Figure 5. DNA methylation during *Arabidopsis* regeneration in wild-type and *dme-2*.

(A) Average of DNA methylation level (%) of *Ler* wild-type (lined) and *dme-2* (dotted) was calculated separately for whole genome (black), gene (purple), and transposable elements (blue) during regeneration process (leaf, callus, and *de novo* shoot). **(B)** DNA methylation level of gene, transposable elements, and surrounding regions were analyzed for each cytosine context (CG, CHG, and CHH).

To investigate the DMRs between wild-type and *dme-2* in each tissue (leaf, callus, and *de novo* shoot), I defined regions where the methylation values differed between wild-type and *dme-2* as dme DMRs for each stage. Intriguingly, although the global CG methylation was not significantly changed during entire regeneration processes (Figure 5A), the number of both hyper and hypo dme CG DMRs were large in *dme-2* (Figure 6A) for all stages. Since DMR functions as a DNA demethylase, having many CG hyper DMRs in *dme* mutants is reasonable. However, there are also a significant number of *dme* CG hypo DMRs. The hypomethylation exhibited in the absence of DME is more distinct in non-CG contexts, especially in *de novo* shoots. This is likely from an indirect effect caused by *dme* mutation.

To examine the genomic features of dme DMRs, I classified DMRs into gene, TE, gene & TE, and IGR categories. As a result, genes were highly enriched both in dme hyper and hypo CG-DMRs for all stage , as TE being present more in shoot hypo CG DMRs compared to all other CG DMRs (Figure 6B). In contrast, TEs were enriched in dme non-CG DMRs (Figure 6B). This suggests that genic regions are directly affected by *dme* mutation in CG context, while TEs are affected more in non-CG methylation, the indirect effect by *dme* mutation.

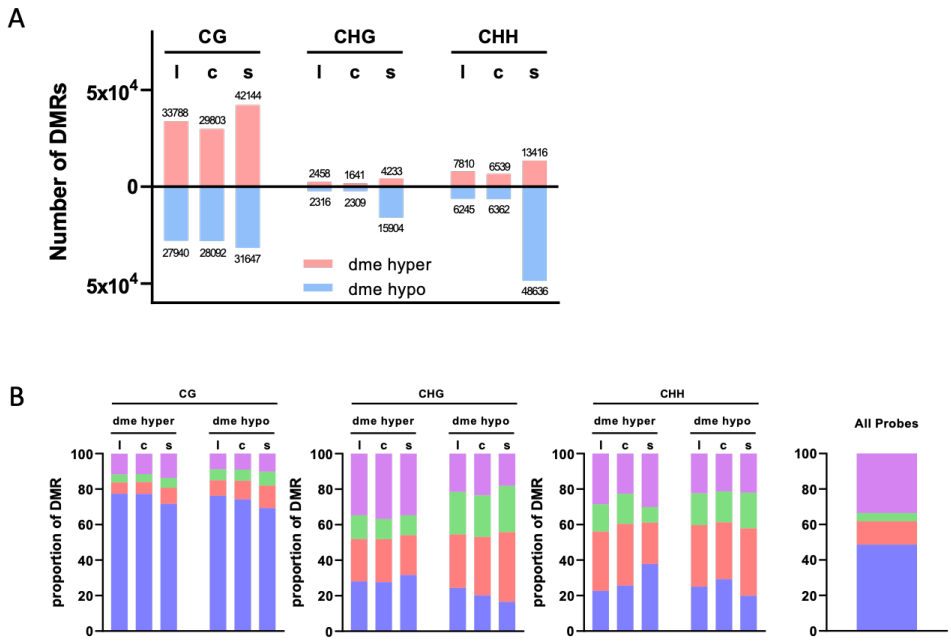

Figure 6. The number and the composition of dme DMRs

The window size is 50 bp with overlap between wild-type and *dme-2.* **(A)** The total number of dme DMRs **(B)** The composition of dme DMRs (gene, gene & TE, TE, and IGR) (l is leaf explant, c is callus, and s is *de novo* shoot).

To gain insight into the chromosomal location of the *dme* DMRs, I mapped those DMRs to *Arabidopsis* chromosome 1. Consistent with the result in that genes were enriched in dme CG DMRs (Figure 6B), dme CG hyper DMRs in three tissues were distributed throughout chromosomal arms, except for the pericentromeric regions where the TEs are enriched (Figure 7A and B, CG dme hyper DMR). In contrast, dme CG hypo DMRs were located more in pericentromeric regions in all tissues (Figure 7A and B, CG dme hypo DMR). Considering that DME is a DNA demethylase, CG hypomethylation in the absence of DME observed in pericentromeric regions is likely an indirect effect, suggesting that DME is required for CG methylation maintenance in the pericentromeric regions although indirect. For both hyper and hypo non-CG DMRs, most of the them were concentrated in pericentromeric regions, as *dme* hypo DMRs of the shoot being most distinct (Figure 7A and B, CHG and CHH dme hypo DMRs).

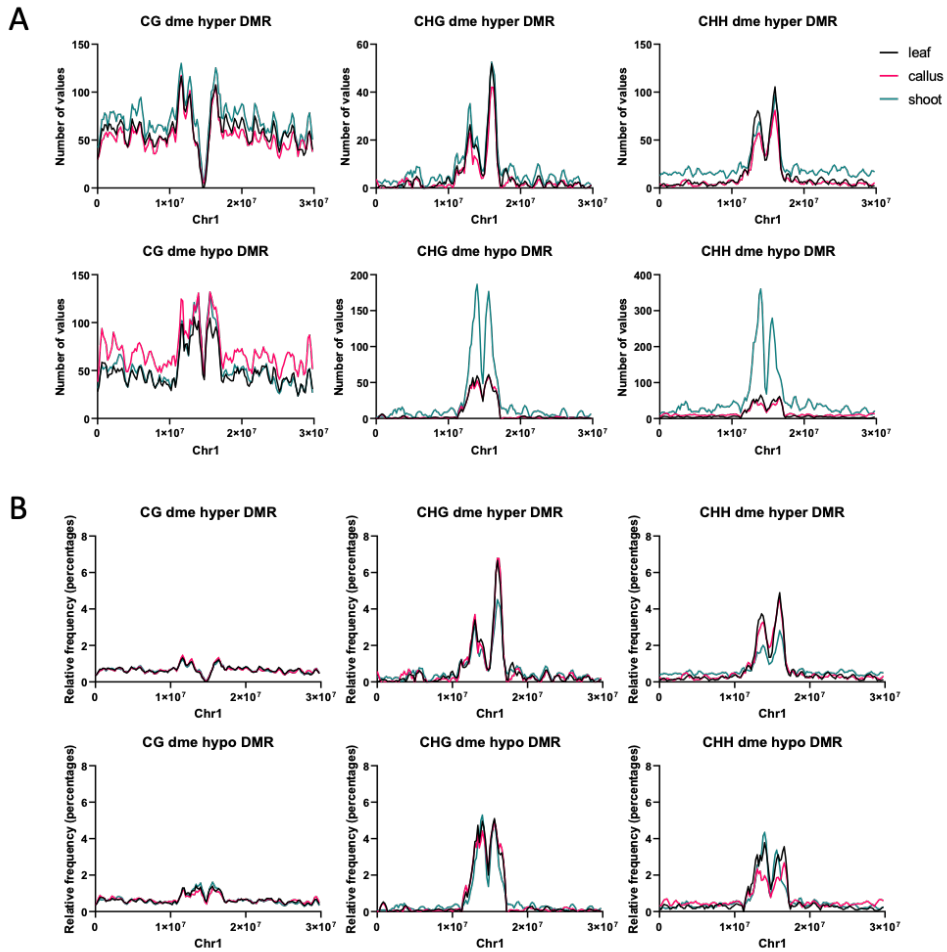

Figure 7. Positions of each dme DMRs on chromosome 1

The positions of dme DMRs (dme hyper and dme hypo in each stage) was analyzed in chromosomal view. **(A)** Number of values of dme DMRs on chromosome 1 **(B)** Relative frequency (%) of dme DMRs on chromosome 1.

Taken together from these results, it is possible that *dme* mutation might cause an inefficient RNA-directed DNA methylation (RdDM) effect, which is a key mechanism of non-CG methylation (Papareddy et al., 2020). To check this possibility, I examined RNA expression of the several key genes involved in RdDM pathway. AGO1, AGO4, and NRPD2 are involved in siRNA generation and DRM1, DRM2, and NRPE1 are related to *de novo* methylation setting. Both qRT-PCR and transcriptome analysis displayed decrease in RNA expression of those genes (Figure 8). In addition, expression of MET1 methyltransferase in *dme-2* shoot was also reduced (Figure 8B). This result, at least partly, could explain non-CG hypomethylation observed in *dme-2.*

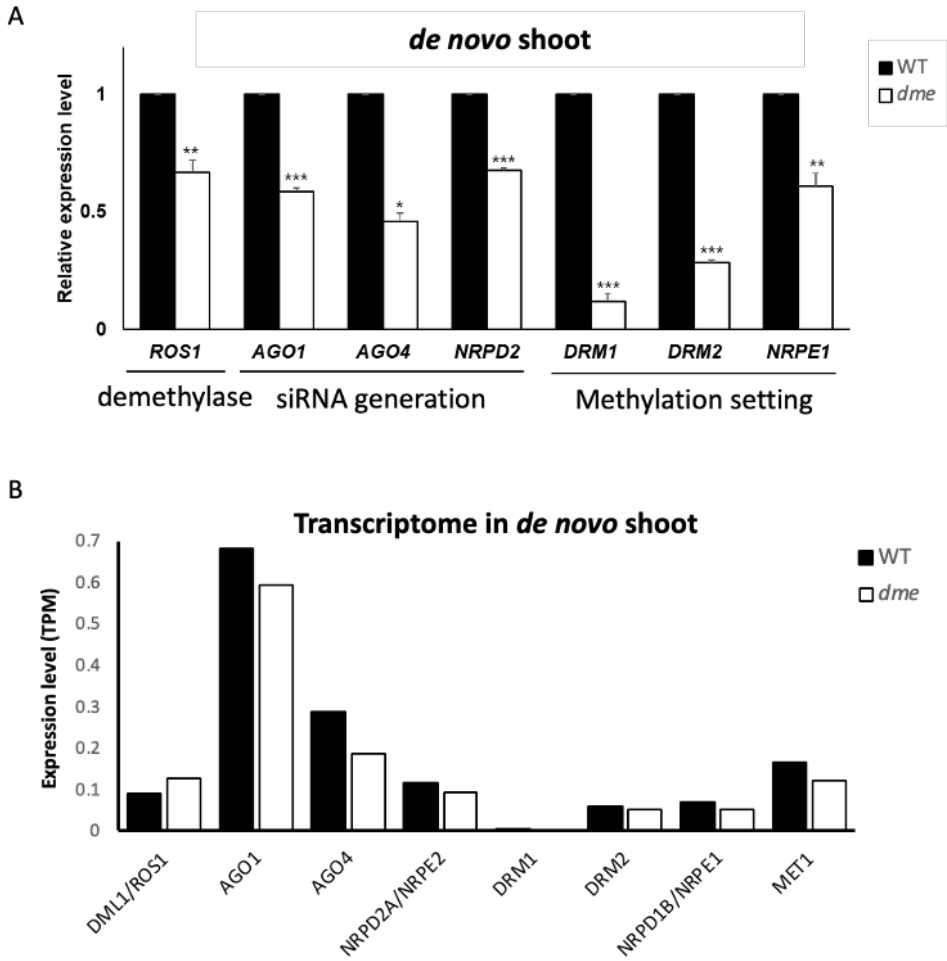

Figure 8. Expression levels of RdDM related genes in *de novo* shoot

(A) The relative espression level of RdDM related genes in *de novo* shoot (DAC7-DAS14) detected by real time PCR. The vertical axis shows the relative gene expression levels as the means SE (n = 3) relative to wild-type. Statistical significance was assessed using Student's t-test (*p value <0.01, **p value <0.005, ***p value <0.001). **(B)** Expression levels of RdDM related genes in *de novo* shoot predicted by transcriptome. The reference gene ACT2 is used for normalization.

IV. Discussion

During plant regeneration, I observed that the global CG methylation level remained relatively stable, while the global CHG methylation level increased gradually, and CHH methylation level decreased and then increased (Figure 2). However, when examining the number of stage DMRs, I found that the majority were CG hyper and hypo DMRs. This indicates that the CG methylation level is not maintained during plant regeneration, but rather, there are many regions that undergo both hypermethylation and hypomethylation during callus induction and shoot regeneration. These findings have important implications for understanding the complex regulation of DNA methylation during plant development and suggest that CG methylation may play a dynamic role in plant regeneration, rather than remaining static.

I analyzed the overall DNA methylation changes during plant regeneration, the extent of DNA methylation differences in different cytosine contexts, and the distribution of DMRs on chromosomes in this study. However, further studies are needed to elucidate the specific mechanisms underlying these observations. It would be possible to compare the DMRs that are maintained during regeneration process, as well as the regions that are DMRs when transitioning from leaf to callus but not when transitioning from callus to shoot. As previously mentioned, the methylation patterns of callus and *de novo* shoot can vary depending on the plant species and the tissue used as the explant. Therefore, based on the analysis of DNA methylation dynamics

during plant regeneration in this study, it would be possible to apply this knowledge to other plant species and compare and analyze the methylation dynamics during regeneration in various plant species. This could lead to the development of more efficient methods for producing various substances or crops in different fields that utilize tissue culture techniques in plants.

I hypothesized that mutation in the DNA demethylase DME would lead to impaired DNA demethylation in *dme-2* plants, resulting in increased DNA methylation levels. However, contrary to my prediction Therefore, it was hypothesized that dme DMRs may affect the RNA-directed DNA methylation (RdDM) effect. To test this possibility, RNA expression levels of several key genes involved in the RdDM pathway were investigated. It was found that the RNA expression of some key genes in the RdDM pathway was reduced in *de novo* shoot of *dme-2* compared to WT. To further investigate this, transcriptome sequencing was performed to examine differentially expressed genes (DEGs) and to determine the changes in RdDM pathway-related genes during *dme-2* regeneration that led to the lower methylation levels.

In this study, I analyzed DNA methylation during plant regeneration in *Arabidopsis thaliana* to provide fundamental knowledge for future applications of tissue culture in various fields such as crop production and antibody production. Based on the results, the use of tissue culture in plants could lead to the development of more efficient methods for the production of various substances or crops. Therefore, this study provides important

insights into the use of tissue culture in plants and its potential applications in different fields.

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국문초록

식물은 전형성능력을 가지고 있어서 이미 분화가 된 조직으로부터 탈분화와 재분화 과정을 거쳐 다시 새로운 개체를 형성할 수 있고, 이를 식물 재생이라고 한다. 대표적인 모델식물인 *Arabidopsis thaliana* 에서의 식물 재분화 과정에 대한 연구는 잎에서 캘러스로의 탈분화 전이 과정에 한정되어 있는 편이다. 본 연구는 식물 재생 과정 동안의 DNA methylation 역동성을 알아보고자 한다. 잎, 캘러스, *de novo* shoot 세 단계의 식물 재생 과정 동안 DNA methylation 변화는 cytosine context (CG, CHG, CHH) 별로 양상이 다르게 나타났다. 이를 differentially methylated regions (DMRs)을 계산을 통해 hypermethylation 된 지역과 hypomethylation 된 지역의 비율을 알아보았다. 추가적으로 DEMETER (DME)의 돌연변이인 *dme-2* 에서 WT 보다 캘러스와 *de novo* shoot 형성이 많이 일어나는 것을 DNA methylation 과 연관지어 알아보고자 한다. 식물재생 과정동안 wild-type 에 비해 *dme-2* 의 모든 과정에서 methylation level 이 감소하였다. 이는 *dme-2* 가 비효율적인 RNA-directed DNA methylation (RdDM) 효과를 일으킬 가능성이 있다. 이를 확인하기 위해 RdDM pathway 와 관련된 주요 유전자들의 RNA 발현을 확인해보았다. *dme-2* 의 *de novo* shoot 에서 RdDM 관련 유전자들의

발현이 감소한 것을 확인했고, 이는 *dme-2* 의 식물재생 과정에서 DNA methylation 이 증가한 것과 연관이 있을 수 있다. 나는 추가적인 연구를 통해 식물재생 과정동안의 DNA methylation 변화의 의미와, *dme-2* 에서 methylation 변화와 표현형의 연관성에 대해 알아보고자 한다.

주요어: 애기장대 재생, DNA 메틸레이션, 캘러스, *de novo* shoot, DEMETER (DME)

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