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DNA methyltransferase activity of chromomethylase CMT3 was regulated by E3 SUMO ligase AtSIZ1

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초록

크로마틴 리모델링은 유전자 기능 발현을 조절하는 새로운 개념이다. 크로마틴 리모델링이 일어나기 위해 진핵세포에서는 여러 유전자 발현 단계의 조절을 거친다. 그중 메칠화는 후성유전이 일어나기 위해 일어나는 여러 단계의 조절 기작 중 하나이다. 그 중 메칠화에는 두 가지 기작이 있는데 히스톤 메칠화와 디엔에이 메칠화가 있다. 히스톤 메칠화는 히스톤 잔기에 메칠화가 일어나는 현상을 말하며 디엔에이 메칠화는 디엔에이 염기서열 중 C에 메칠화가 일어나는 현상을 말한다. 동물의 크로마틴 리모델링 기작에서는 디엔에이 메칠화 기작에는 CG메칠화와 CHH 메칠화 두가지가 있으며 이 중 수모화가 CG 메칠화의 조절에 기여한다고 알려져 있다. 수모화가 되어 디엔에이 메칠화 효소의 활성이 증가한다고 알려져 있으며 이는 식물에서도 디엔에이 메칠화와 수모화가 연관이 있을 수 있다는 것을 말해준다.

그리고 번역 후 단백질 변이 기작에는 여러가지 기작이 있다. 이 중 수모화는 수모 단백질이 결합하여 단백질의 기능을 조절하는 기작으로서 다양한 생물학적 기작에 관여하는 중요한 생물학적 기작 중 하나이다. 선행연구에 따르면 질소동화 단백질이 수모화가 일어난 다는 것이 밝혀져 있다. 그리고 진핵세포의 생물 기작은 수모화에 의해 다양한 단백질의 기능이 강화되거나 안정화가 된다고 알려져 있다.

따라서 이 논문에서는 질소 환원 유전자에 대한 수모화와 디엔에이 메칠화의 영향에 대해 조사를 하였다. 디엔에이 메칠화 효소 중 CMT3, CHG 메칠화 효소가 수모화가 일어나는지 아닌지에 대해 증명을 하였고 또한 우리는 수모화가 된 CMT3 디엔에이 메칠화 효소가 수모화 여부에 따라 애기장대에서 암모늄 처리시 CMT3의 메칠화 효소의 활성이 어떻

게 달라지는지 알아보았다.

주요어: CHG DNA methylaion, E3 SUMO ligase, AtSIZ1, Nitrate reductase 1, 2 , Transposable element, CMT3.

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ABSTRACT

Chromatin remodeling was a new concept for controlling of gene function. And In eukaryotes, gene expression was controlled at multiple levels, and methylation, one of several epigenetic mechanisms. those were regulated by DNA and histone methylation by DNA and histone methyltransferases, respectively. In animal epigenetic system, DNA methyltransferase with CG methylation activity was modified by SUMO conjugation and then its activity was increased, which means that the activity of DNA methyltransferase was modulated by sumoylation. and Sumoylation was an important post-translational-modification regulating various biological processes. Accumulated data in eukaryotic system was proved that the stability and activity of various proteins were enhanced by sumoylation. And we known that protein stability was more stable by sumoylation already. Therefore we investigated to effect of nitrate reductase gene to DNA methylatransferase and sumoylated DNA methylatransferase. we want to know whether the activity of plant-specific DNA (cytosine-5)-methyltransferase (CMT3) is regulated by sumoylation or not. And we want to know that how different to sumoylated CMT3 activation in Arabidopsis and ammonium supply.

Keywords : CHG DNA methylaion, E3 SUMO ligase, AtSIZ1, Nitrate reductase 1, 2 , Transposable element, CMT3.

Student number : 2013-21101

CONTENTS

1. LIST OF TABLES

2. LIST OF FIGURES

3. INTRODUCTION

DNA methylation is a major mechanism of former transcription and epigenetic regulation in eukaryotes. It was methylated at cytosines that are followed by CpG, CpHpG, CpHpH. In mammals, the maintenance methyltransferase DNMT1(Lee B and Muller MT, 2009) and responsible for DNA methylation, which occurs primarily at CG methylation (Goll and Bestor, 2005). In plants, heterochromatin domains are determined in part by methylation of cytosines at the 5 position of the pyrimidine ring. This modification was methylated by methyltransferase enzymes using S-adenosyl methionine, methyl donor, which was added to cytosines in appropriate regions of the genome after DNA replication. The methylated 5 position in cytosine was exposed in the major groove of B-form-DNA, where it could make recognition DNA-binding proteins. The modification of covalent cytosine methylation was inherited on one of the two strands after DNA replication, providing an epigenetic mark inherited to the original methylation mark. Such as hemi-methylated DNA, the methylation mark, can be re-established on the newly DNA strand using the pre-existing methyl groups on the complementary strand. Thus, once methylation was established, it could be perpetuated by maintenance indefinitely.

In *Arabidopsis thaliana*, there were the DNMT1 homolog MET1 maintains CG methylation and the DNMT3a/b homologs DRM1/2 de novo CHH methylation and the plant-specific methyltransferase CMT3

maintains CHG methylation were responsible for methylation(Chan et al, 2005). Plant-specific methyltransferase CMT3(Chromomethylase 3) was recruited by H3K9me2 and associated with gene silencing. And previous research, in mammals, Post-translation modification interaction with DNA methyltransferase. Small ubiquitin-related modifier (SUMO) was interacted with DNMT1 and enhanced methyltransferase activity (Lee B and Muller MT, 2009).

SUMO constitute a highly conserved protein family that found in all eukaryotes and were required for viability of most eukaryotic cells. In multi-cellular organisms, SUMO conjugation takes place in all tissues at all developmental stages. And sumo protein covalently bound to target protein, which included the androgen receptor, histone deacetylases (HDACs), DNA methyltransferase, p53, and other proteins that participate in transcription, DNA repair, nuclear transport, signal transduction, and the cell cycle. Most SUMO-modified proteins that have been characterized in mammalian systems involved in transcription, which was often repressed by SUMO conjugation.

And Sumoylation was a key regulator of biological processes, was covalently conjugated to a lysine residue in a substrate protein via a reversible posttranslational modification process that was facilitated by E3 SUMO ligases. Also in other eukaryotes in plants, SUMO modification has been implicated in numerous basic cellular processes, such as stress and defense responses and the regulation of flowering. *Arabidopsis thaliana* had two known SP-RING E3 ligases, AtSIZ1 and HPY2 and encodes two related proteins with SP-RING domains, PIAL1

and PIAL2. Among SUMO E3 ligase, AtSIZ1 catalyze the transfer of ubiquitin or ubiquitin-related proteins from an E2 enzyme to a target lysine residue. And SUMO E3 ligase AtSIZ1 had an SP-RING-finger protein that contains a SAP domain and a zinc-finger domains. *siz1-2* dwarf plants with smaller leaves indicates that AtSIZ1 also had a role in vegetative growth and development. And sumo E3 liagase AtSIZ1 was a key regulator of signaling pathways that mediate responses to nutrient deficiency and environmental stresses.

Nitrogen was one of the most important inorganic nutrients and a major component of nucleic acids, proteins, various cofactors and secondary metabolites. The *Arabidopsis* genome contains two NR genes, *NIA1* and *NIA2*. Nitrogen assimilation was a fundamental biological process that had a marked effect on plant productivity, biomass and crop yield. And previous results indicated that sumoylation could regulate diverse biological processes, NR was a key enzyme in a plant's nitrogen assimilation pathway by sumoylation(Park et al, 2011).

And Transposon elements(TEs) were a source of endogenous small RNAs in animals and plants. Those TEs-derived small RNAs have been treated as functionally distinct from gene-regulating such as miRNAs. Two recent reports in Drosophila and Arabidopsis have blurred the lines of this distinction. In both reports, TE expression produce gene-regulating small RNAs. In the Drosophila early embryo, maternally deposited TEs derived PIWI-interacting piRNAs. In Arabidopsis, when Athila retro-transposons were epigenetically

activated, their transcripts were processed into small RNAs, which directly target the 30UTR of UBP1b mRNA. So we suggest small RNA is upstream of DNA methylation mechanism and control DNA methyltransferase specific activity to target gene.

So The diversity of methods investigated that sumoylation controlled *NIA2* DNA methylation after ammonium treatment. and research AtSIZ1 affect to CMT3. We examined a possible interaction between Arabidopsis E3 SUMO ligase AtSIZ1 and CMT3 and showed that CMT3 was modified by SUMO with E3 ligase activity of AtSIZ1, suggesting that the methyltransferase activity of CMT3 can be regulated by sumoylation. *NIA2* DNA methylation revealed that CMT3 had DNA methyltransferase activity in ammonium supply and its activity was stimulated by SUMO conjugation.

4. MATERIALS AND METHODS

Construction of recombinant plasmids

To produce His_6-CMT3, the cDNA encoding full-length CMT3 was amplified by PCR and inserted into the pET28a vector(Novagen). For the maltose-binding protein(MBP)–AtSIZ1-haemagglutinin(HA) fusion, a cDNA encoding full-length AtSIZ1 was amplified by PCR using a primer tagged with HA and inserted into the pMALc2x vector (New England Biolabs). For GST–CMT3 production, cDNA encoding full-length CMT3 was amplified by PCR using primers and inserted pGEX4T-1, respectively. The SUMO1 full-length cDNA was amplified by PCR with gene-specific primers and inserted into pET28a to produce the His_6-AtSUMO1-GG, SUMO E1 and E2 enzyme-encoding constructs were kindly provided by DrH.-P. Stuible(Colby ,2006). All constructs were transformed into strain R2 cells. The transformed cells were treated with IPTG(isopropyl-β-d-thiogalactoside) to induce fusion protein expression. All the constructs were verified by automatic DNA sequencing to ensure that no mutations were introduced.

Purification of recombinant proteins

All of the recombinant proteins were expressed in strain R2 and were purified in accordance with the manufacturer's instructions. Briefly, for His_6-AtSAE1b, His_6-AtSAE2, His_6-AtSCE1, His_6-AtSUMO1, His_6-CMT3 purification, bacteria were lysed in 50mM NaH_2PO_4 (pH8.0), 300mM NaCl, 1% TritonX-100, 1mM imidazole, 5mM dithiothretiol(DTT), 2mM phenyl methyl sulphonyl fluoride(PMSF), and a proteinase inhibitor cocktail(Roche), and purified on Ni^{2+}- nitrilotriacetate(Ni^{2+}-NTA) resins(Qiagen). For GST, GST–CMT3 purification, bacteria were lysed in PBS buffer (pH 7.5) containing 1% Triton X-100, 2mM PMSF, and a proteinase inhibitor cocktail (Roche), and purified on glutathione resins (Pharmacia).

Plant material and culture conditions

The *Arabidopsis thaliana* Columbia-0 (Col0) ecotype (WT) and the T-DNA insertion mutant plants *siz1-2*, *cmt3* were used. Plants were grown in fully automated growth chambers under 16 h illumination either on 0.6% agar media containing Murashige and Skoog (MS) salts, 0.5 g Mes and 10 g sucrose, or on soil. Plants were maintained at 22 °C during the light period and at 20 °C during the dark period. To WT and mutant seeds were germinated into soil. And solution containing (NH4)2SO4 treat 12 h before bolting, after 28 days.

In vitro pull down assay

To carried the *in vitro* binding of MBP–AtSIZ1 to His_6-CMT3, 5μg of full-length MBP–AtSIZ1 and 5 μg of full-length His_6-CMT3 prey were added to 1ml of binding buffer[50mM TRIS-HCl(pH7.5), 100mM NaCl, 10mM $MgCl_2$, 1% TritonX-100, 0.5mM β-mercaptoethanol]. Pre-incubation at 4 °C for 1h and the reaction mixtures were incubated with a glutathione resin for 1h. before washing six times with buffer 50mM TRIS-HCl (pH 7.5). Absorbed proteins were analysed by 7% SDS–PAGE and detected by western blotting using an anti-His antibody (Santa Cruz Biotechnology).

Sumoylation assays

In vitro sumoylation was performed in 30 μl of reaction buffer (20 mM HEPES (pH 7.5), 5 mM $MgCl_2$,2mMATP) with 1ug of MBP-SIZ1, 200ng of His_6-AtSAE1b, 200ng of His_6-AtSAE2, 200ng of His_6-AtSCE1, 5μg of His_6-AtSUMO1-GG and 500ng of GST-CMT3. After incubation for 3ht at 30°C, the reaction mixtures were separated on an 7% SDS–polyacrylamide gel. Sumoylated GST-CMT3 was detected by immunoblot analysis using an anti-GST antibody (0.2 μg ml − 1, Santa Cruz Biotechnology).

Effect of sumoylation on DNA methylatransferase activity

Full length CMT3 CDS was cloned into pGEX4T-1 vector and sumoylated CMT3 purified. DNA methyltransferase activity assay was

carried out at room temperature for 3 hour in a total volume of 50 ul containing 2ul of S-adenosyl-l- [methyl-3H] methionine (SAM) (55-85 Ci/mmol, American Radiolabeled Chemicals (ARC)), 4 mM H3K9me2 peptide, 200 ng substrate DNA and 500 ng, 1ug, 2ug CMT3 protein in assay buffer (50mM Tris–HCl, pH 8.0, 5mM NaCl and 5% glycerol) and stopped by adding 2 ml of Proteinase K. To purify each reaction was applied onto DE81 paper (Whatman) and washed two times with 200 mM ammonium bicarbonate, two times with water and two times with 70% ethanol. Next, paper was dried and placed into liquid scintillation cocktail (GE Healthcare). and measured in liquid scinallation counter. H3K9 peptide used to contains amino acid 1 to 21 of the N-terminal sequences of histone H3 (64624-025, Anaspec) with di-methyl K9 modifications at N-termini and biotin labeling at C-terminal sequences and using Oligos (28-mers) 5'-ATTCAGTCAGATCTGATCAGTACTGATT-3' contained methyl CHG. Single-stranded oligos were annealed previously described (Bacolla et al., 1999).

RT-PCR

For real-time PCR, the cDNA encoding and cDNA synthesis, primer design and SYBR Green I RT-PCR were carried out. In brief, cDNA was synthesized using random hexamers using 1μg of each total RNA sample. RT-PCR amplification mixtures (20μl) contained 50ng template

cDNA, 2x SYBR Green I Master Mix buffer (10μl) (Enzynomics) and 10pcM forward and reverse primer. The cycling conditions comprised 10 min polymerase activation at 95°C and 40 cycles at 95°C for 15 sec and 60°C for 60 sec. and RT-PCR was carried out using cDNA. mRNA was isolated from rosette leaves of 4-weeks -old. and cDNA synthesize using cDNA synthesize premix(Enzynomics) and carried out using target primer for amplified PCR products.

Bisulfite Sequencing

gDNA was isolated from rosette leaves of 4-week-old, soil-grown plants. Bisulfite treatment and recovery of samples were carried out with the EpiTect Bisulfite kit (QIAGEN cat. 59124) by following the manufacturer's instructions. 2 μg DNA in 20 μL volume was used for each reaction and mixed with 85 μL bisulfite mix and 35 μL DNA protect buffer. Bisulfite conversion was performed as follows: 99°C for 5 min, 60°C for 25 min, 99°C for 5 min, 60°C for 85 min, 99°C for 5 min, 60°C for 175 min and 20°C in definitely. The bisulfite-treated DNA was recovered by EpiTect spin column and subsequently sequenced to confirm the efficiency of bisulfite conversion. And primer design using online software meth-primer (http://www.urogene.org/methprimer/). And coversion cytosine analyze using online software(http://katahdin.mssm.edu/) After Carried out PCR and sequencing.

McrBC-PCR

McrBC-PCR analysis was performed as described previously (Ding et al.,2007). A total of 500ng of genomic DNA from 7-days old, 28-days old Col, *siz1-2*, and *cmt3* plants, growing in long-day conditions, was digested with 20 units of McrBC endonuclease (catalogue no. M0272S; New England Biolabs) for 3h at 37 ℃. Following the McrBC treatment, subsequent PCR was used to analyze the methylation status. All analysis carried out using target primer for amplified PCR products and compared to each sample. For PCR, 50 ng of digested genomic DNA was used in a 20ul reaction and the PCR products was confirmed by agarose gel electrophoresis.

Mapping and peak calling of Me-DIP sequencing

500ng of MBD-enriched DNA and construct DNA libraries used to according to the Illumina protocol. The libraries were sequenced on the Illumina Genome Analyzer platform, and reads were used to identify the MBD-bound fraction of the *Arabidopsis thaliana* genome. Trimmomatic with Option is ILLUMINACLIP:TruSeq3-SE:2:30:10 and TRAILING:20 MINLEN:36. And FastQ Quality Encoding used Sanger / Illumina 1.9 encoding format. Sequenced reads were mapped to the *Arabidopsis thaliana* genome (BSgenome. Athaliana. TAIR. TAIR9) the using Bowtie2. Peaks for each sample was called using Peak Analyzer with TAIR10 GTF information(FDR < 0.05). Sequenced reads

were used as a control for each type and categorized methylation sequence coverage gene.

Chromatin IP

two-week-old plants were infiltrated CMT3-$FLAG_4$ and treated $(NH_4)_2SO_4$ and harvested after 3days. DNA and protein extraction IP buffer using Equal amounts of plants samples. 50mM HEPES[pH7.5], 150mM NaCl, 1mM EDTA, 1% TritonX-100, 0.1%SDS, 1mM PMSF, protease inhibitor cocktail tablets(Roche14549800)). DNA was sheared by sonication to approximately 500-1000-bp fragments. After centrifugation 10 min at 13,000 rpm, 4 ℃ and the supernatents were precleared with agarose A. after preclearing, added FLAG antibody 10ul(Sigma) for immunoprecipitations and rotate 2hr in 4℃. After binding antibody , added agarose A and rotate 1hr in 4℃ and Wash 5times during 3min using 1% SDS, 0.1 M NaHCO3. Centrifuge to Agarose A(3000rpm) and lysis DNA using 300ul of lysis buffer in 6hr, 65℃ (1% SDS, 0.1M NaHCO3 and 12ul 5M NaCl) And followed by phenol/chloroform/ isoamyl alcohol extraction and ethanol precipitation. Pellets were washed with 70% EtOH and elute 30ul H_20. And 1 ul of DNA was used for PCR.

5. RESULTS

DNA Methylation was decreased when after ammonium treatment in only 28days old *siz1-2* and *cmt3*

Estimation of DNA methylation of *transposon elements gene* in WT and *siz1-2* mutant(Figure 1). In 7days old plants, after treat K_2SO_4, KNO_3, $(NH_4)_2SO_4$, methylation didn't decrease dramatically and didn't shown any different methylaion level in WT and *siz1-2*. but All methylation dramatically decreased in only 4weeks old *siz1-2* mutant. And $(NH_4)_2SO_4$ treatment samples had same DNA methylation pattern with no treatment samples.

Assessment of AtSIZ1 affected on the DNA methylation level analysis at the endogenous transposable elements in mature plants (28-day-old). And relative transcript levels of transposable elements compared in wild-type and *siz1-2* mutant at mature stage.

We thus carried out next investigation that the DNA methylation of *siz1-2* and *cmt3* mutants by methyl binding domain sequencing (MeDIP)(table 1) and gene analysis (Figure 2,3,4). CG-enrich analysis showed that the genome of *siz1-2* and *cmt3* mutants was less methylated than that of wild-type plants. In addition, genome methylation of *siz1-2* and *cmt3* mutants was dramatically decreased after ammonium(NH_4^+) treatments while genome methylation of wild-type plants was not changed after ammonium(NH_4^+) treatments(Table 1).

CpG coverage analysis showed that CpG coverage of *siz1-2* and

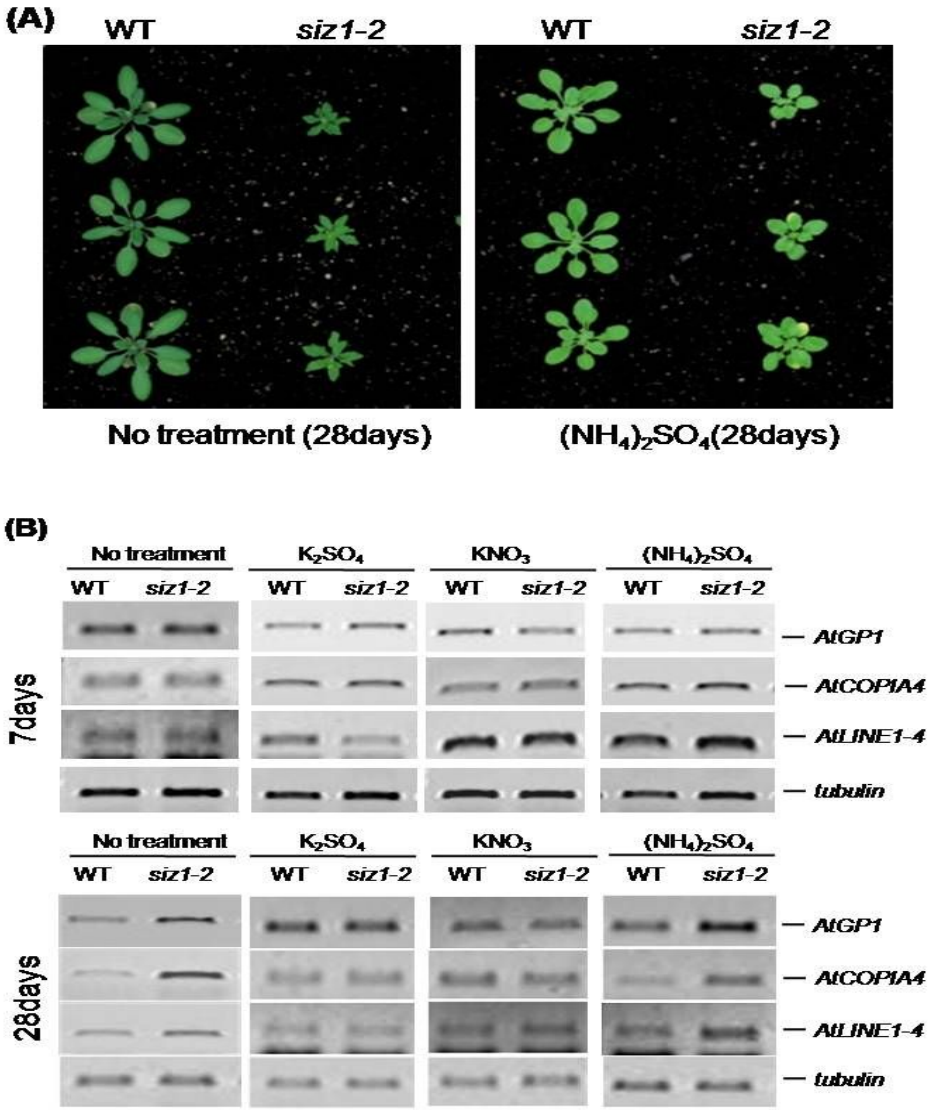


Figure 1. DNA methylation of transposable elements was decreased in *siz1-2* mutants. (A) photographs of WT, *siz1-2* plants which were grown for 7 or 28 days in soil or LB Base. To check the effect of ammonium, samples were treated with 5 mM of $(NH_4)_2SO_4$. (B) Genomic DNA was isolated from each samples and DNA methylation

of transposable elements, *ATGP1*, *ATCOPIA4* and *ATLINE1-4*, was assessed by PCR with gene-specific primers after treatment of McrBC. *Tubulin* was also used as a control.

cmt3 mutants was a little decreased coverage than that of wild-type plants. Interestingly, CpG coverage of *siz1-2* and *cmt3* mutants was significantly decreased after ammonium (NH_4^+) treatments whereas it was abit changed in wild-type plants(Figure 2). In particular, DNA methylation (CpG coverage) of exon and intron parts of both *siz1-2* and *cmt3* mutants were greatly decreased compared to that of other regions. this data mean AtSIZ1 and CMT3 was main controlling facter and ammonium was another facter in DNA methylation pathway(Figure 3).

Methylation coverage showed degree of methylation in genome. That coverage pattern was changed in mutants and decreased methylation after ammonium treatment. we carried out Methylation pattern analysis using another method, which sequence pattern coverage and frequency were more dept in lower counts. those were showed methylation coverage decrease in ammonium treatment and each *siz1-2, cmt3* mutants. For each data, the number of peak calls carried out enough the number of MeDIP-Sequencing reads and estimated saturation correlation.

we carried out to determined pearson correlation using peak calling analysis(Figure 4). For compared to each sample, sequencing reads were counted after pull down CpG region. Scatter plots and correlation coefficients were calculated on a test set(FDR<0.05). Data shown were for the *Arabidopsis thaliana*, and Scatter plots and correlation coefficients, which related of methylation coverage were excluded. This figure showed coverage genes differents between each mutants and

Table 1. CpG and C enrichment analysis of WT, *siz1-2* and *cmt3* plants by methylated DNA Immuno-Precipitation (MeDIP) sequencing. Genome methylation was decreased in *siz1-2* and *cmt3* mutants compared to WT Plants. In particular, genome methylation was significantly decreased in *siz1-2* mutants. In addition, genome demethylation was induced by exogenous $(NH_4)_2SO_4$ supply in all of WT, *siz1-2* and *cmt3* plants.

	No treatment			$(NH_4)_2SO_4$		
	WT	*siz1-2*	*cmt3*	WT	*siz1-2*	*cmt3*
CG regions	15,407,837	8,924,729	12,271,936	13,311,909	3,629,978	7,611,452
C regions	105.277.236	60,661,714	84,371,718	98,704,801	24,018,136	48,214,609
Total base (bp)	3.101.970.144	3,161,486,736	3,524,753,514	3,938,852,604	3,317,072,283	3,607,390,293
Alignment rate (%)	79.67	82.43	73.74	91.72	72.28	73.23

treatment or No treatment samples. After treatment, correlation was more decreased than no treatment. the results mean that ammonium affect to DNA methylation.

All of data indicate that the methyltransfearse activity of CMT3 was regulated by sumoylation through E3 ligase activity of AtSIZ1 and CMT3 mediated gene expression and plant development were tightly controlled by E3 SUMO ligase activity. Besides, our data also suggest that ammonium(NH_4^+) can stimulate AtSIZ1 and CMT3 mediated DNA methylation.

NIA2 DNA methylation was more decreased in _siz1-2_, _cmt3_ than WT when after ammonium treatment.

DNA methylation status was assessed using samples that methylation-specific recognition restriction enzyme McrBC treat 3hr in 37℃ and amplification of *NIA1*, *NIA2* by PCR(Figure 4). Methylation status were decreased in *siz1-2, cmt3* and ammonium treatment only. Comparison of relative transcript levels of transposable elements in wild-type and *siz1-2, cmt3* mutant at 28day-old-sample (Figure 5). After ammonium treatment, *NIA2* expression level was decreased. But *NIA1* DNA methylation status didn't changed any mutant or treatment samples.

And Total RNAs were isolated from 28day-old-leaves and WT and mutants(Figure 6). Likewise DNA methylation status, *NIA1* relative transcription levels didn't changed any mutant or treatment. Results

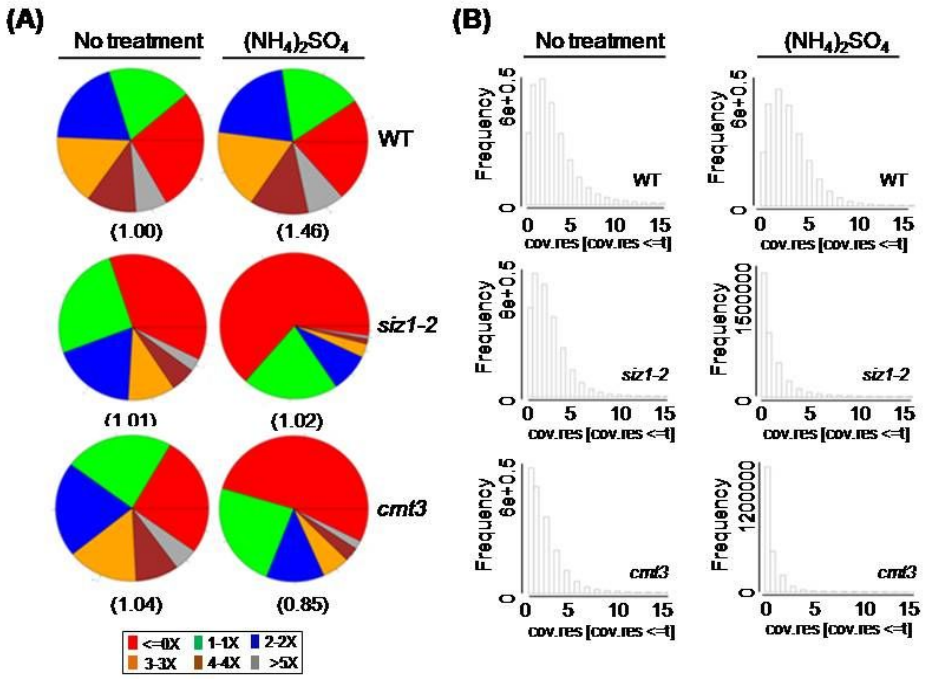


Figure 2. Genomic coverage by MeDIP-seq in *siz1-2* and *cmt3* mutants. Genomic coverage was quantified by the number of DNA methylation measurements that overlap with CpG islands (A) CpG coverage was shown by counting of the number of reads. CpG islands were calculated using the program MEDIPS. Numbers below diagrams indicate " Do not cover of pattern". (B) Methylation pattern was displayed by another method, a sequence pattern coverage. Parameter t indicates the maximal coverage depth to be plotted (here: 15 reads per sequence pattern). Both presentations indicate that CpG coverage was decreased in *siz1-2* and *cmt3* mutants compared to WT, and it was more decreased after treatment of $(NH_4)_2SO_4$ in both mutants.

showed as means that *NIA2* transcription level was increased in

ammonium treatment and *siz1-2*, *cmt3.*

And To Show were bisulfite sequencing results after bisulfite treatment (Table. 2). *NIA2* methylation was controlled by ammonium and AtSIZ1, CMT3. after treatment, methylation ratio was decreased and methylation ratio more decreased in *siz1-2* than WT. To show no treatment result, WT was more methylated to *NIA2* than mutant. That mean *NIA2* was controlled by CMT3 that enhanced by AtSIZ1 and when after ammonium treatment. bisulfite sequencing data showed that methylation ratio of ammonium treatments samples were less than no treatment samples data. when ammonium treatment, only CHG, CHH methylation was decreased in all plants, CG methylation was only decreased WT*, siz1-2*. all methylation ratio was decreased after ammonium treatment.

The data show *NIA1* didn't controlled by ammonium and AtSIZ1, CMT3. *NIA1* methylation status didn't changed after treatment ammonium but *NIA2* methylation status were decreased in *siz1-2, cmt3* and ammonium treatment. This data showed *NIA2* expression controlled by CHG DNA methylation and AtSIZ1 and DNA methylation status were decreased in ammonium treatment.

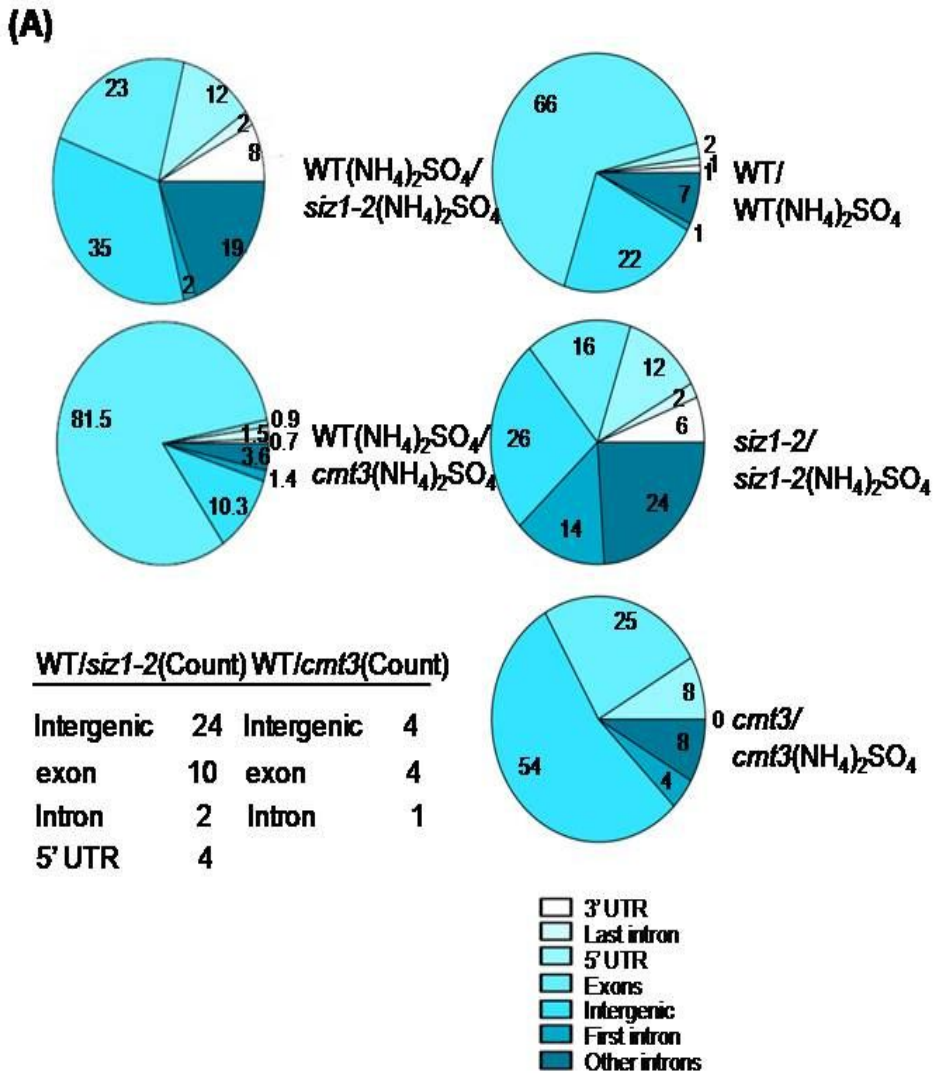


Figure 3. AtSIZ1 and CMT3 are associated with CpG methylation of coding regions. MeDIP sequencing was performed using non- or $(NH_4)_2SO_4$-treated samples. Genomic features of methylated CpGs were comparatively analyzed as indicated and the proportions of each genomic features were presented by pie charts. Pie size corresponds to relative ratio of different genomic elements. When the methylation of

genomic elements was analyzed, there was no big difference between WT and *siz1-2* or between WT and *cmt3*. However there was big difference between WT and *siz1-2* or between WT and *cmt3* after ammonium treatments.

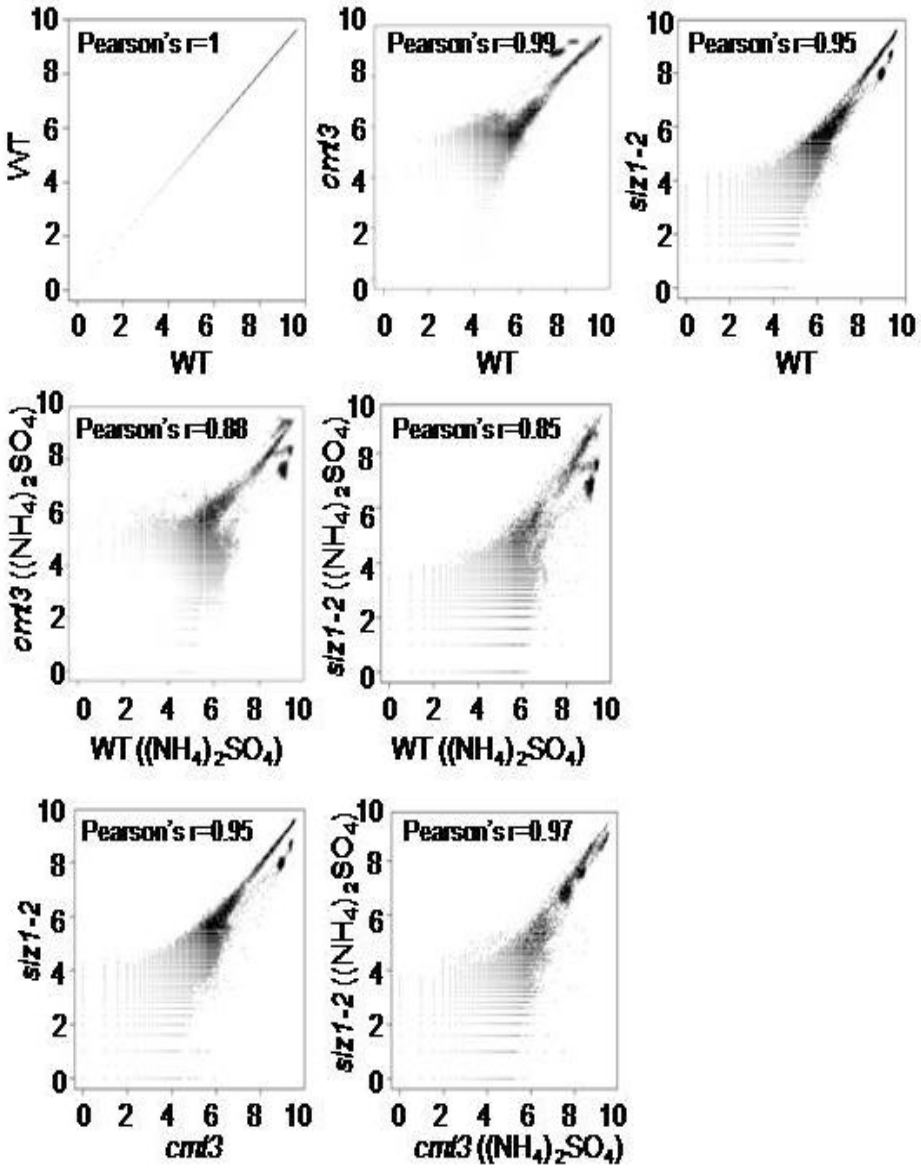


Figure 4. Scatter plots for correlation between each samples. Pairwise correlations between WT/WT, WT/*siz1-2*, WT/*cmt3*, *siz1-2*/*cmt3*, WT/WT($(NH_4)_2SO_4$), *cmt3*/*cmt3* ($(NH_4)_2SO_4$), *siz1-2*/*siz1-2* ($(NH_4)_2SO_4$), WT($(NH_4)_2SO_4$)/*siz1-2*($(NH_4)_2SO_4$), WT($(NH_4)_2SO_4$)/*cmt3*($(NH_4)_2SO_4$) and

siz1-2($(NH_4)_2SO_4$)/*cmt3* ($(NH_4)_2SO_4$). Pearson's correlation coefficients (r) are shown. Scatter plots were drawn by the program MEDIPS and correlation coefficients were calculated using the program MEDIPS with a test set (FDR<0.05). Coverage genes were different from between WT and each mutants or between ammonium-treated WT and -each mutants and/or *siz1-2* and *cmt3*. Coverage genes were especially different from between ammonium-treated WT and -each mutants. $(NH_4)_2SO_4$ treatment induced less correlation between WT and mutants than no treatment while there was no difference on coverage genes between *siz1-2* and *cmt3.* This result indicates that correlation ammonium affects on DNA methylation.

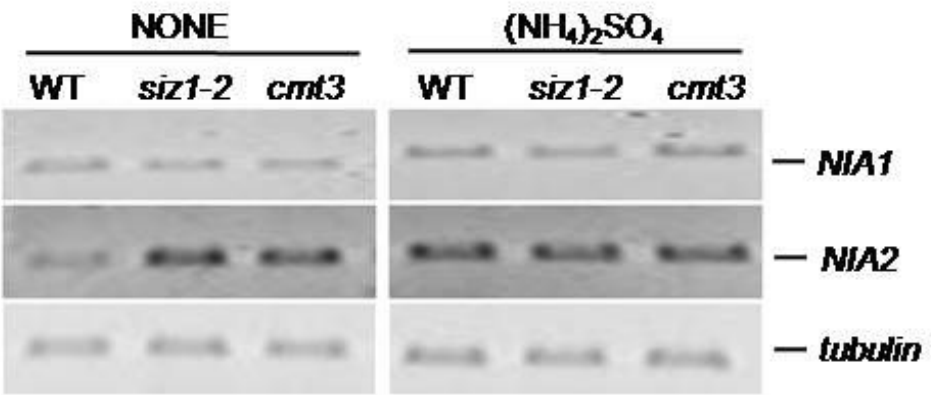


Figure 5. NIA2 Methylation decrease in mutant and ammonium treatment.

methylation-specific recognition restriction enzyme McrBC treat 3hr in 37 ℃ and amplification of target *NIA1, NIA2* sites. *NIA1* methylation status do not changed after treatment ammonium but *NIA2* methylation status decreased in *siz1-2, cmt3* and ammonium treatment.

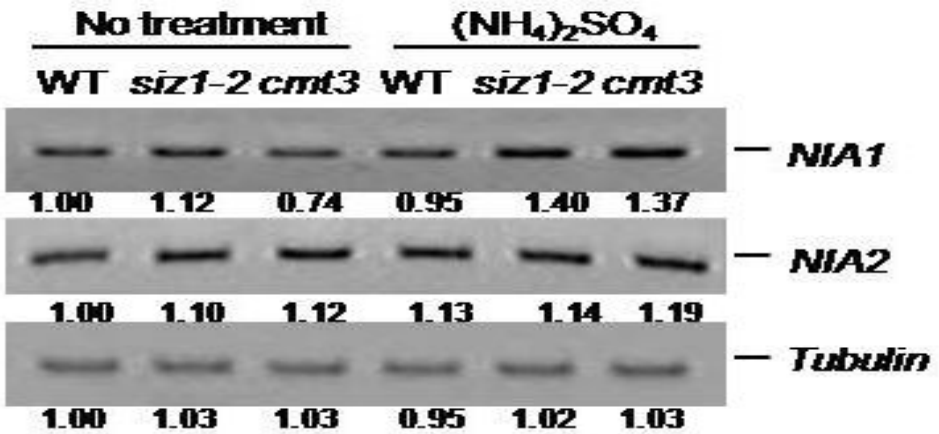


Figure 6. NIA2 genes expression increase in mutant and ammonium treatment.

Total RNAs were isolated from the leaves and WT and *siz1-2, cmt3* plants. The mRNA levels were examined by RT-PCR with gene-specific primers. NIA1, NIA2(nitrate reductase). Results are expressed as means NIA2 transcription level is increased in ammonium treatment and *siz1-2*, *cmt3*. the data show NIA1 didn't controled by ammonium and AtSIZ1, CMT3.

(A) . *NIA1*

	WT (No treatment)		WT ($(NH_4)_2$ SO_4)	
	Methylated (%)	Non-methylated (%)	Methylated (%)	Non-methylated (%)
CG	10.00	90.00	5.55	94.44
CHG	0.00	100.00	0.00	100.00
CHH	0.00	100.00	1.28	98.71
All	1.89	98.11	1.90	98.10

	siz1-2 (No treatment)		*siz1-2* ($(NH_4)_2$ SO_4)	
	Methylated (%)	Non-methylated (%)	Methylated (%)	Non-methylated (%)
CG	5.55	94.44	5.00	95.00
CHG	0.00	100.00	0.00	100.00
CHH	3.89	96.10	2.63	97.36
All	3.85	96.15	2.86	97.14

	cmt3 (No treatment)		*cmt3* ($(NH_4)_2$ SO_4)	
	Methylated (%)	Non-methylated (%)	Methylated (%)	Non-methylated (%)
CG	0.00	100.00	0.00	100.00
CHG	0.00	100.00	0.00	100.00
CHH	2.56	97.43	2.77	97.22
All	1.92	98.08	2.13	97.87

(B) *NIA2*

	WT (No treatment)		WT ($(NH_4)_2$ SO_4)	
	Methylated (%)	Non-methylated (%)	Methylated (%)	Non-methylated (%)
CG	11.11	88.88	5.88	94.11
CHG	11.11	88.88	0.00	100.00
CHH	16.66	83.33	0.00	100.00
All	14.81	85.19	1.01	98.99

	siz1-2 (No treatment)		*siz1-2* ($(NH_4)_2$ SO_4)	
	Methylated (%)	Non-methylated (%)	Methylated (%)	Non-methylated (%)
CG	15.78	84.21	5.55	94.44
CHG	5.55	94.44	0.00	100.00
CHH	9.72	90.27	2.89	97.10
All	10.09	89.91	2.91	97.09

	cmt3 (No treatment)		*cmt3* ($(NH_4)_2$ SO_4)	
	Methylated (%)	Non-methylated (%)	Methylated (%)	Non-methylated (%)
CG	11.11	88.88	11.76	88.23
CHG	0.00	100.00	0.00	100.00
CHH	3.03	96.96	1.61	98.38
All	4.05	95.95	3.13	96.88

Table 2. methylation analysis of *NIA1, NIA2* genes by bisulfite sequencing. Methylation levels of both genes were decreased in *siz1-2* and *cmt3* mutants. *NIA1* gene was not methlated in the CHG region

of both *siz1-2* and *cmt3* mutants, which suggests that *NIA1* gene is not methylated by CMT3. In case of *NIA2* gene, it was methylated in the CHG region of *siz1-2* mutants but not in the CHG region of *cmt3* mutants, which indicated that *NIA2* gene is methylated by CMT3 and CMT3 activity is low in *siz1-2* mutants because CMT3 activity is stimulated by sumoylation. CG methylation of *NIA2* gene was not affected by lose of CMT3 because CMT3 has methyltrasnferase activity CHG and CHH regions.

NIA1, 2 DNA and promoter was bound to CMT3 that was enhanced by ammonium.

in bisulfite data, we showed that CMT3 have CHG DNA methyltransferase activity to only *NIA2*. so we test whether CMT3 bound to *NIA1, 2* gene or not. in plants, *35S-CMT3-FLAG*$_4$, constructs were infiltrated into the leaves of WT and after treat ammonium(Figure 7A). The CMT3 was immunoprecipitated by anti-FLAG antibody. And *NIA1, 2* gene and promoter were detected by PCR.

CMT3 bound to double strained oligo, hemi-methylated oligo and fully-methylated oligo(Figure 7B). this data showed that CMT3 could bind to all of DNA and methylated status of oligo didn't effect to CMT3 DNA binding. and we tested CMT3 binding site in genomic DNA and promoter. DNA was sheared by sonication to approximately 500–, 1000-bp fragments before carried out cromatin IP.

after IP and PCR, we showed that CMT3 bound *NIA1, NIA2* promoter(Figure 7C) and gene(Figure 7D). but CMT3 could't bind to some genomic DNA and promoter, 1001bp to 2000bp in *NIA2,* 2001bp to 2500bp in *NIA1* and –1501bp to –3000bp in *NIA2 promoter*, sequence. so we had a question that how CMT3 was methylase speicific activity to *NIA2.*

CMT3 CHG methyltransferase activity was dependent on small RNA.

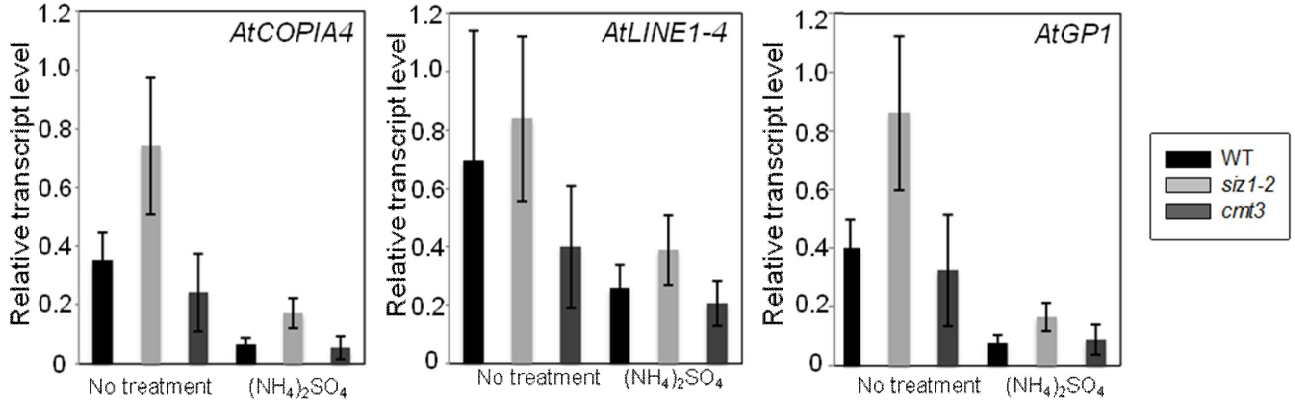
in privious study, DRM2 DNA methyltrasferase activity was dependent on hcsiRNA with AGO4(Ruiqiang Ye et al. 2012). and CMT3 CHG methylation was occurred after DRM2 CHH *de novo* methylation. also transposon element expression effect to siRNA expression. this results suggest that how CMT3 had specific acitivity to *NIA2.* we thaught AGO4/siRNA depedent DRM2 DNA methylation was preceded to occur CMT3 CHG methylation. so we predicted siRNA in *NIA1, NIA2 intron*.

NIA1 had three *introns* and *NIA2* had two *introns*. the siRNA prediction result showed that siRNA expression in *NIA1 intron1, intron2, intron3,* and *NIA2 intron1*(Figure 8). so Total RNAs were isolated from 28day-old-leaves and WT and *siz1-2, cmt3* plants and after treatment samples and treated Dnase 1hr. each *intron* was analyzed using RT-PCR and amplification of *Tubulin* was used for equilibration. only *NIA2 intron1* express in all samples. to compare siRNA prediction data and RT-PCR data, we knew siRNA prediction site was matched to RNA expression of *NIA2 intron1*(Figure 8).

and we compareed transposon elements transcription levels in WT, *siz1-2, cmt3* in no treatment and after ammonium treatment(Figure 9). we thaught that transposon elements expression was increased in ammonium treatment samples. but despite DNA methylation decreased when after ammonium treatment, transposon elements expression was decreased in ammonium treatment. this data suggest

Figure 8 . small RNA suppressed by decrease of transposon elements transcription

Transposon elements(TEs) are a source of endogenous small RNAs in plants . Transposon elements expression decreased in ammonium treatment samples . That mean small RNA expression decreased because transposon elements expression decreased by ammonium . Comparison of relative transcript levels of transposable elements in wild-type and *siz1-2*, *cmt3* mutant at 4weeks old sample . After ammonium treatment, transposon elements expression level is decreased . Several endogenous transposable elements were analyzed using real-time PCR and amplification of *Tubulin* was used for equilibration . Figure 7 showed transposon elements expression level decrease after ammonium treatment .



that ammonium effect to DNA methylation by decreased of siRNA. 8

but we didn't know that why transposon element expression was increased in siz1-2 mutants yet. we suggest reason of transposon elements level in siz1-2, which because AtSIZ1 affected to RDR6, DCL4, DCL2. those genes was related siRNA expression(Andrea D. McCue and R. Keith Slotkin 2012). so transposon elements expression was increased in siz1-2 and we thaught that AtSIZ1 was main gene in epigenetic pathway and affected to siRNA expression, DNA methylation, and histone methylation pathway.

NIA2 DNA methylation was contolled by CMT3 and sumoylated CMT3 that was facilitated by AtSIZ1

then we had another question how AtSIZ1 enhanced CMT3 methyltranferase activity. Our results implied that AtSIZ1 may modulate the activity or stability of CMT3 by acting as an E3 SUMO ligase. The deduced amino acid sequences of the CMT3 revealed that proteins had putative sumoylation sites (ψKXE), which were located at lysine 400(K400), lysine 431(K431), lysine 437(K437)(Figure 10A). Therefore, to evaluate the possible interaction between AtSIZ1 and CMT3, glutathione sulphur transferase HIS-tagged CMT3 and Maltose- tagged AtSIZ1 were over expressed in *Escherichia coli* and purified with glutathione and agarose resins, respectively. *In vitro* pull-down of HIS_6-CMT3 with GST and MBP-AtSIZ1 revealed that AtSIZ1 interacts with HIS_6-CMT3, whereas the resin or GST alone did not interact with

either HIS_6-CMT3(Figure 10B,C).

On the basis of the interaction between AtSIZ1 and CMT3, we examined the possibility that the CMT3 could be substrates for AtSIZ1. HIS_6-AtSAE1b, HIS_6-AtSAE2, HIS_6-AtSCE1, HIS_6- AtSUMO1, MBP-AtSIZ1, GST-CMT3 were over expressed in *E. coli* and purified with Ni^{2+}-NTA or glutathione, agarose resins(Figure 10D). Then, the recombinant proteins were applied to the *in vitro* sumoylation reactions. CMT3 was sumoylated by AtSIZ1 in a reaction that was dependent on E1 and E2 activities (Figure 10E).

Most of the proteins that were post-translational modified by small or large molecules are functionally regulated. We investigated to the effect of the sumoylation of CMT3 on their CMT3 activities. So we estimated the effect of sumoylation that based on CMT3 methylates hemimethylated Oligomer. hemi methylated Double strained oligo was synthased and to estimated CMT3 bound on hemi methylated Double strained oligo(Figure 11C). The results revealed that CMT3 methylates to hemimethylated oligo(Figure 11B).

And To examine CMT3 methyltransferase activity was changed whether sumoylated or not. the effect of sumoylation on the CMT3 activities was assessed by measuring the Counts per minute by liquid scintillation counter after the *in vitro* sumoylation reactions(Figure 11D). DNA methylation by Sumoylated CMT3 was increased significally in the reaction mix containing non-sumoylated proteins (Figure 11E). These results indicated that SUMO conjugation increased the CMT3 methyltransferase Activities and *NIA2* DNA methylation was controlled

by AtSIZ1 through CMT3 sumoylation.

(A)

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MAPKRKRPATKDDTTKSIPKPKKRAPKRAKTVKEEPVTVVEEGEKHVARF   50
LDEPIPESEAKSTWPDRYKPIEVQPPKASSRKKTKDDEKVEIIRARCHYR  100
RAIVDERQIYELNDDAYVQSGEGKDPFICKIIEMFEGANGKLYFTARWFY  150
RPSDTVMKEFEILIKEKRVFFSEIQDTNELGLLEKKLNILMIPLNENTKE  200
TIPATENCDFFCDMNYFLPYDTFEAIQQETMMAISESSTIISSDTDIREGA  250
AAISEIGECSQETEGHKKATLLDLYSGCGAMSTGLCMGAQLSGLNLVTKW  300
AVDMNAHACKSLQHNHPETNVRNMTAEDFLFLLKEWEKLCIHFSIRNSPN  350
SEEYANLHGLNNVEDNEDVSEESENEDDGEVFTVDKIVGISFGVPKKLLK  400
RGLYLKVRWLNYDDSHDTWEPIEGLSNCRGKIEEFVKLGYKSGILPLPGG  450
VDVVCGGPPCQGISGHNRFRNLLDPLEDQKNKQLLVYMNIVEYLKPKFVL  500
MENVVDMLKMAKGYLARFAVGRLLQMNYQVRNGMMAAGAYGLAQFRLRFF  550
LWGALPSEIIPQFPLPTHDLVHRGNIVKEFQGNIVAYDEGHTVKLADKLL  600
LKDVISDLPAVANSEKRDEITYDKDPTTPFQKFIRLRKDEASGSQSKSKS  650
KKHVLYDHHPLNLNINDYERVCQVPKRKGANFRDFPGVIVGPGNVVKLKE  700
GKERVKLESGKTLVPDYALTYVDGKSCKPFGRLMWDEIVPTVVTRAEPHN  750
QVIIHPEQNRVLSIRENARLQGFPDDYKLFGPPKQKYIQVGNAVAVPVAK  800
ALGYAIGTAFQGLAVGKDPLLTLPEGFAFMKPTLPSEIA             839
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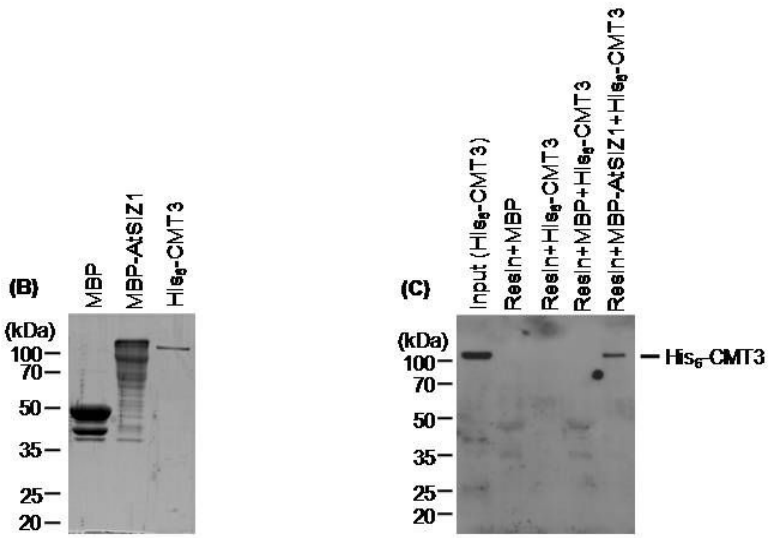

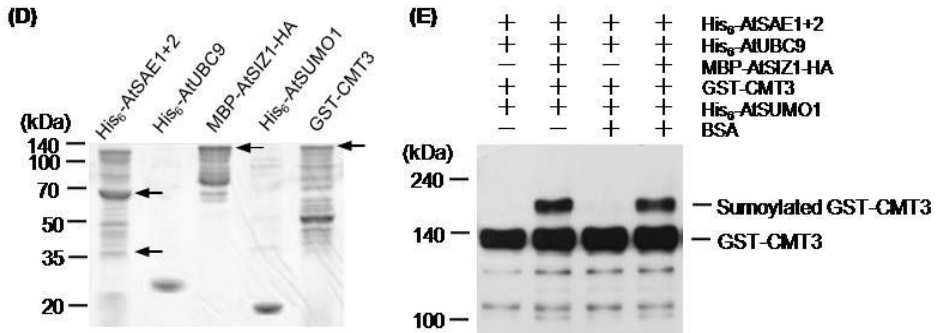

Figure 8. CMT3 is sumoylated by AtSIZ1 *in vitro*. Predicted amino acid sequence of the CMT3 protein. (A) putative sumoylation site (ΨKXE), identified using the SUMOplot Analysis Program, is indicated in bold type and underlined. Sumoylation of CMT3 was examined in vitro(E). AtSIZ1 physically interacts with CMT3. MBP, MBP-AtSIZ1 and His_6-CMT3 were overexpressed in E.coli and purified with amylose and nickel resins, respectively. Purified proteins were separated by 11% SDS-PAGE(B). CMT3 was pull-downed with full-length AtSIZ1. His_6-CMT3 absorbed with MBP or MBP-AtSIZ1 was detected using western blotting with anti-His antibody(C). His_6-AtSAE1b, His_6-AtSAE2, His-AtUBC9, MBP-AtSIZ1, His-SUMO1, and GST-CMT3 were overexpressed in E.coli and purified with amylose and nickel resins, respectively(D). E3 ligase activity of AtSIZ1 for CMT3 was assayed in the presence or absence of His-AtSAE1+2, His-AtUBC9, MBP-AtSIZ1, His-SUMO1 and GST-CMT3. After the reaction, sumoylated CMT3 was detected using western blot with anti-GST antibody(E).

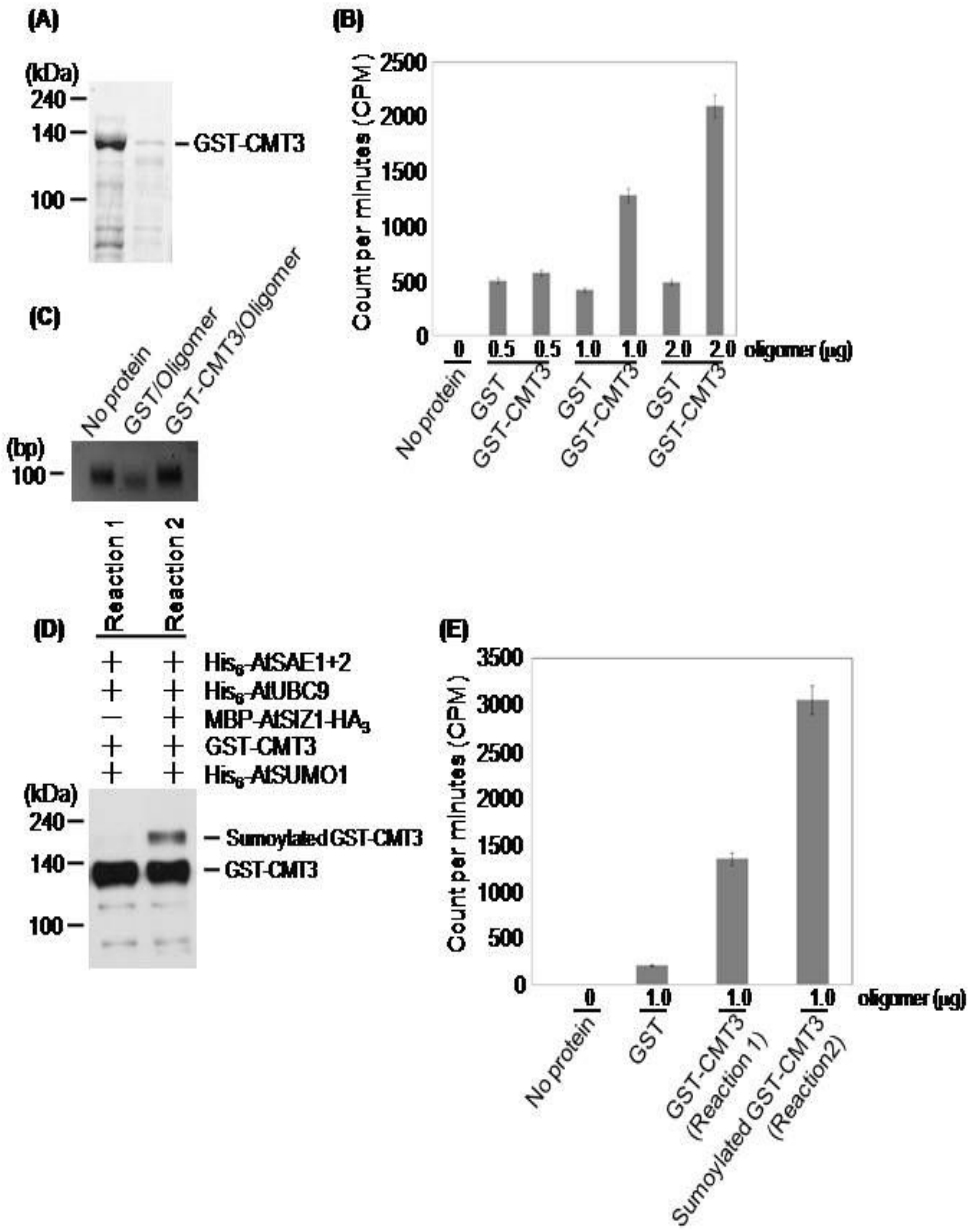

Figure 9. Methyltransferase activity of CMT3 was stimulated by sumoylation *In vitro.* (A) GST-CMT3 protein was overexpressed in E.coli and purified with glutathion column. Purified GST-CMT3 was separated by10% SDS-PAGE. (B) Methyltransferase activity of

GST-CMT3 was examined in vitro. GST or GST-CMT3 was mixed with 1 or 2ug of oligomer and methyl donor ^{3}H-*S*-adenosyl-methionine (^{3}H-SAM). After reaction, methylated oligomers were purified and then incorporation of ^{3}H-methyl group was counted by LSC. (C) Sumoylation reactions of GST-CMT3 were carried out without (Reaction 1) or with (Reaction 2) AtSIZ1. (D) Methyltransferase activity of sumoylated GST-CMT3 was examined. One microgram of oligomer and ^{3}H-SAM were added into reaction products 1 and 2 in (C). After reaction, methyltransferase activity was estimated by measurement of incorporation of ^{3}H-methyl group by LSC.

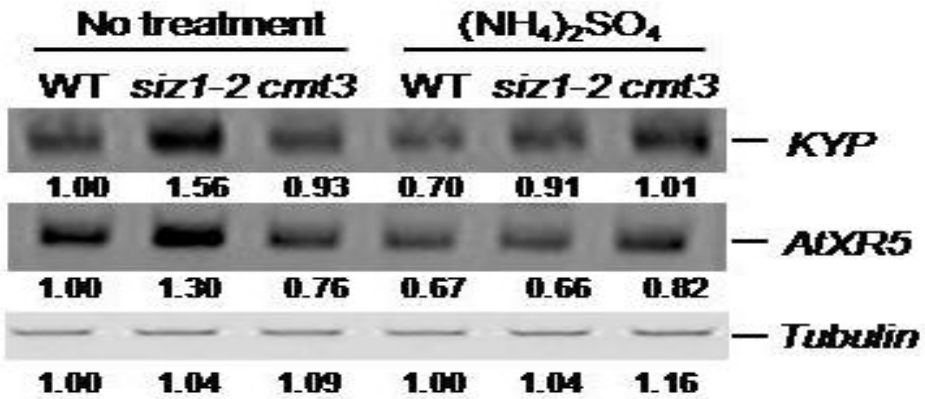

Figure 10. H3K9, H3K27 methyltransferase expression were decreased by ammonium supply.

Total RNAs were isolated from the leaves and WT and *siz1-2, cmt3* plants. The mRNA levels were examined by RT-PCR with gene-specific primers. *KYP*(H3K9 methyltransferase), *AtXR5*(H3K27 methyltransferase). Results are expressed as means histone methylation is decreased in ammonium treatment.

KYP(H3K9, _AtXR5_(H3K27) methyltrasnferase were controlled by ammonium and AtSIZ1 for _NIA2_ CHG DNA methylation.

And Total RNAs were isolated from the leaves and WT and *siz1-2, cmt3* plants. *KYP, AtXR5* were analyzed using RT-PCR and amplification of *Tubulin* was used for equilibration. *KYP, AtXR5* was Histone3 methyltransferase and those controlled CMT3 activation. CMT3 methylatrasferase needed both H3K9 and H3K27 to DNA Methylation. *KYP, AtXR5* expression level was decreased after ammonium treatment(Figure 12). These results indicated that the CMT3 methylates *NIA2* DNA that controlled by ammonium through H3K9, H3K27 methyltransferase expression. and we showed those transcription levels were increased in *siz1-2* than WT. we suggest that KYP, AtXR5 were stablilized by sumoylation through E3 ligase AtSIZ1. so in siz1-2 mutant, KYP, AtXR5 were unstabled and those transcription levels were increased for maintain their methylase activity.

6. DISCUSSION

CMT3 was CHG specific DNA methyltransferase in plants. CHG methylation was occurred in only plants. AtSIZ1 led to CMT3 sumoylation and enhanced CMT3 methyltrasnferase activity. those were very important result to epigenetic mechanism of plants. Also, small RNA(siRNA) pahway was important epigenetic mechanism. this pathway induced to de novo DNA methylation and RNA siliencing. Nitrogen, potassium and phosphate were absolutely required for plant growth as essential nutrients. the effect of these fertilizers on the growth of the mutant plants was examined because of the severe dwarfism of *siz1-2, cmt3* plants.

Previous Studies in some eukaryotes had shown that sumoylation can directly regulated methyltransferase the activities of target proteins. SUMO modification enhances DNMT1 of activity(B Lee et al, 2009). However, no study had shown that sumoylation to target proteins modulated the activity of enzymes that increased of DNA methyltransferase activity in plants. Therefore we carried out research that nitrogen nutrients affect to DNA methylation and post-translational modification(sumoylation) enhance DNA methylation.

so we studied DNA methylation in *Arabidopsis* enhanced by AtSIZ1. And ammonium enhanced *NIA2* expression by decreased of DNA methylation. but we didn't know relationship in ammonium and sumoylation by E3 sumo ligase AtSIZ1 yet. so we suggest that ammonium affected to sumoylation or directed effect to AtSIZ1, CMT3.

and sumoylation and ammonium had antagonisme activity each other.

and AtSIZ1 was expressed in almost all plant tissues where it regulates cell expansion and proliferation and responds to nutrient deficiency, various stresses and several developmental processes. In addition, massive identification studies of SUMO conjugates in *Arabidopsis* had suggested that AtSIZ1 must be a central coordinator of numerous events, including basic gene expression and metabolism.

The phenotypes were recovered to WT phenotypes with only exogenous ammonium, but not with nitrite and NR activity were low in *siz1-2* plants, while the nitrate level was high(Park et al, 2011). The results showed that reason of decreased DNA CHG methylation in *siz1-2* mutants. and when after ammonium supply then *NIA2* DNA methylation decreased(Table 3). nitrate amounts was high in *siz1-2* mutants. so *NIA2* transcription level was increased in *siz1-2* and NIA1/NIA2 protein was increased in *siz1-2* mutants. while in *cmt3* mutants, only NIA1/NIA2 protein level was increased in ammonium treatments status. we suggest this reason. beacause antibody detected both of protein NIA1 and NIA2. and RT-PCR data(Figure 6) showed that *NIA1* transcription level was decreased in cmt3 no treatment sample. so we thaught that decreased of NIA1 protein expression level was more higher than increased of NIA2 protein expression level. in this reason, NIA1 or NIA2 protein level was decreased in *cmt3* mutants.

and This finding led us to think that the reason of *siz1-2* phenotype recovered when ammonium supply, that was related of Plants DNA

methylation, especially *NIA2* DNA CHG methylation. To analyze the DNA methylation used McrBC PCR and bisulfite sequencing, functional regulation of *NIA2* by ammonium supply. And *NIA2* methylation wasn't occurred in *cmt3* mutants and decreased in *siz1-2* mutants. That means AtSIZ1 enhanced *NIA2* DNA methylation and *NIA2* methylation controlled by CMT3. And after ammonium supply, transposon elements expression level were decreased and *NIA2* methylation was decreased(Figure 1,5).

then we had a question that how CMT3 had DNA methyltrasferase activity to *NIA2.* we search *NIA1, NIA2* transcription level previous data in tair. data showed that *NIA2* transcription level was more higher than *NIA1* transcription level. that mean *NIA2* was more affect to nitrate reductase pathway than *NIA1* in plants. so we thaught only *NIA2* was regulated by DNA methylation efficiently. and CMT3 bound to different region both *NIA1, NIA2* promoter and DNA(Figure 7C, D). and small RNA prediction site wasn't accorded to CMT3-*NIA2* DNA binding site(Figure 8). That means non-coding siRNAs expression was competed by CMT3 for DNA binding. so siRNA dependent DNA methylation was inhibited by CMT3 DNA binding. we suggest that siRNA affected to CMT3 methyltrasferase activity to target DNA and specific DNA region. and siRNA expression was interferenced by CMT3. In this reason, CMT3 could methylate to specific DNA. but we didn't tested siRNA expression in vivo, whether *NIA2* target siRNA or not and to interact any methyltrasnferase yet. we needed to get more experiment to know that a factor of determination CMT3 target

specificity.

And Pull-down and in vitro sumoylation assays showed strong interactions between AtSIZ1 and CMT3 (Figure 9). The *in vitro* DNA methylation assay (Figure 11) demonstrated that both CMT3 and sumoylated CMT3 occured methylation and enhanced their activity by sumoylation depended on the E3 SUMO ligase AtSIZ1. The first time of these finding in plants suggest that the DNA methylation were controlled by sumoylation.

And sumoylated NIA1 and NIA2 could form homodimers, and the sumoylation of NIA1 and NIA2 by AtSIZ1 dramatically increased their nitrite production(Park et al, 2011). Those results showed that AtSIZ1 enhanced protein activity usually and controlled CMT3 activity for maintenance *NIA2* expression and controlled NIA2 activity, that showed AtSIZ1 was regulator in nitrogen metabolites by control NIA2, CMT3 activity.

And we can cautiously that the early flowering phenotype of *siz1-2* plants came from the low DNA methylation, which was the result of decreased histone methylation due to decrease of CMT3 activity by effect of ammonium(Figure 12). and this data showed that *KYP, AtXR5* transcription level was increased in *siz1-2* mutants. it suggest AtSIZ1 was affect to KYP, AtXR5 through by sumoylation or DNA binding. in this reason, *NIA2* DNA methylation decreased in *siz1-2.* Those results mean that *NIA2* DNA methylation controlled by CMT3 through sumoylation of CMT3 and ammonium.

Nitrogen could regulate secondary metabolism, hormone metabolism

and transport, protein synthesis, signal- transduction pathways, pathways for the production of reducing equivalents and nitrate assimilation by controlled of NR activity. also DNA methylation was decreased in ammonium supply and increased to plants growth and developments highly. In this reason, DNA methylation was controlled by ammonium and sumoylation.

In conclusion, our study showed that CMT3 sumoylated by AtSIZ1 stimulates CMT3 activities, and that enhanced *NIA2* DNA CHG methylation. And supply of ammonium that *NIA2* DNA CHG methylation was decreased. therefore Sumoylation was a critical protein modification for the regulation of Arabidopsis DNA CHG methylation, and sumoylation of target proteins by AtSIZ1 had an essential role in plant DNA methylation. And ammonium enhanced plants developments and growth thorough by decreased of plant DNA methylation. Those pathway important to plants developments and growths.

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