

2013 International Symposium on Environmental Science and Technology (2013 ISEST)

## DNA methylation changes in response to sulfur dioxide stress in *Arabidopsis* plants

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### Abstract

Sulfur dioxide (SO<sub>2</sub>) exposure causes alterations of DNA cytosine methylation in *Arabidopsis* genome. Analysis of methylation-sensitive amplified polymorphism (MSAP) revealed that SO<sub>2</sub> induced methylation changes of cytosine in CCGG sites, in which hypermethylation is more than hypomethylation. Bisulfite sequencing showed that the methylation levels of cytosine in promoter regions of *Arabidopsis* genes encoding 1-aminocyclopropane-1-carboxylic acid synthase 6 and nitrilase 2 decreased accompanying with enhanced transcript levels. The global genome hypermethylation and locus-specific hypomethylation in *Arabidopsis* cells, which contribute to genome stability and gene regulation, can be considered as a very important regulatory mechanism for plants adaption to environmental stress.

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Selection and peer-review under responsibility of Beijing Institute of Technology.

*Keywords:* Sulfur dioxide; *Arabidopsis*; DNA methylation; Gene expression; Genome stability

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### 1. Introduction

DNA methylation is one of the most important epigenetic modifications in eukaryotes. In plant cells, DNA cytosine methylation exists in CG, CHG and CHH sites (where H is A, C or T). The genome-wide DNA methylation map of *Arabidopsis* reveals that many genes are methylated within their promoters or transcribed regions, meanwhile the DNA methylation status of genes are highly correlated with their transcription levels [1]. DNA methylation contributes to the transcriptional silencing of transposon and foreign DNA, maintaining genome stability and controlling gene expression across development. When

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plant is challenged by environment stress, epigenetic modification including DNA methylation will be occurred to initiate plant adaptation to the environmental challenges [2-4].

Sulfur dioxide (SO<sub>2</sub>) is one of the most common and harmful air pollutants. High concentrations of SO<sub>2</sub> can cause leaf chlorosis and necrosis, growth inhibition, DNA damage and plant death. Epigenetic mechanisms such as DNA methylation are crucial to appropriate plant reactions to stress [5], but little is known about the general pattern of DNA methylation linked with plant responses to SO<sub>2</sub>.

Cytosine methylation analysis in plants has been approached by studying global levels of methylated cytosine using methylation-sensitive amplified polymorphism (MSAP) method, or by examining the levels of methylated cytosine in some specific regions using bisulfite sequencing. In this study, we found that SO<sub>2</sub> induced an increase in DNA methylation in the entire *Arabidopsis* genome accompanying by locus-specific hypomethylation. The overall increase in DNA methylation could favor genomic stability, whereas the decreased methylation of stress-response genes could lead to enhanced transcription. This report may help to reveal a possible role of the epigenetic mechanism in plant adaption to environmental stresses.

## 2. Materials and methods

### 2.1. Plant materials

Plants of *Arabidopsis thaliana* (L.) ecotype Columbia (Col-0) were grown in a controlled growth chamber at 22°C ± 1°C with a 16 h photoperiod per day, 70% relative humidity and a photosynthetic photonflux density of 140 μmol/m<sup>2</sup>s. Four-week-old plants were exposed to 30 mg/m<sup>3</sup> SO<sub>2</sub> [6], or to filtered pollutant-free air (control) for up to 72 h in fumigation chambers. The gas was released through a tube from a cylinder and continuously sampled and measured by para-rosaniline hydrochloride spectrophotometry to monitor SO<sub>2</sub> concentrations [7].

### 2.2. Methylation-sensitive amplification polymorphism (MSAP) analysis

Genomic DNA was isolated from *Arabidopsis* shoots by using a modified CTAB method [6]. MSAP analysis was performed as described by Cervera et al. [8]. The methylation-sensitive isoschizomers *Hpa*II (NEB) and *Msp*I (NEB) were employed as frequent-cutter enzymes, while *Eco*RI (NEB) was used as rare-cutter enzyme. The number and type of bands in lanes H (*Eco*RI/*Hpa*II) and M (*Eco*RI/*Msp*I) were summarized for each sample. The polymorphic bands were scored '+' for the presence and '-' for the absence.

### 2.3. Bisulfite sequencing analysis

According to the procedure described by Jacobsen et al. [9], methylation status of two SO<sub>2</sub>-responsive genes *ACS6* (At4g11280) and *NIT2* (At3g44300) [6] was analyzed by using bisulfite sequencing. The sequence of the clones was analyzed with the software CymATE [10].

### 2.4. RNA isolation and RT-PCR

Total RNA were isolated from *Arabidopsis* shoots using Trizol reagent (Invitrogen). cDNA were synthesized from RNA using PrimeScript<sup>TM</sup> RT-PCR Kit (Takara). Primers for *ACS6* gene were 5'-TAACTTCCAAATCTCAACA-3' and 5'-GAATAACTCCGTCAGGTC-3'. Primers for *NIT2* were 5'-

TTCCTCATGCCA TCCTCCGTCTT-3' and 5'-CAGCGATACCTGAGAACATAGTGG-3'. *Actin2* gene was used as a control for RT-PCR analysis.

### 3. Results

#### 3.1. SO<sub>2</sub>-induced changes in global DNA methylation in *Arabidopsis* genome

By using twelve primer pair combinations, a total of 703 clear and reproducible fragments were amplified in *Arabidopsis* shoot cells (Table 1).

Table 1. Changes of DNA methylation in *Arabidopsis* plants under SO<sub>2</sub> stress.

	Band pattern				Number	Percentage
	Control		Treatment			
	H	M	H	M		
Type A Hypermethylation						
A1	+	+	+	–	15	
A2	+	+	–	+	21	
A3	+	+	–	–	21	20.6%
A4	–	+	–	–	44	
A5	+	–	–	–	44	
Subtotal					145	
Type B Hypomethylation						
B1	–	–	+	+	10	
B2	+	–	+	+	21	
B3	–	+	+	+	14	11.8%
B4	–	–	+	–	21	
B5	–	–	–	+	17	
Subtotal					83	
Type C Uncertain						
C1	+	–	–	+	13	
C2	–	+	+	–	19	4.6%
Subtotal					32	
Total for polymorphic loci					260	37.0%
Type D No-change						
D1	+	+	+	+	378	
D2	–	+	–	+	44	63.0%
D3	+	–	+	–	21	
Total for monomorphic loci					443	63.0%

The number and percentage of methylation change in CCGG sequences. +, band present; –, band absent. H and M are the combinations of enzymes of *EcoRI/HpaII* and *EcoRI/MspI*, respectively.

Among all these bands, 38.1% of CCGG sites were methylated, and full methylation of internal cytosines occurred more frequently than hemimethylation of external cytosines, whether in control or in treatment samples. After SO<sub>2</sub> exposure, the percentage of total number of methylated bands increased to 39.8%, indicating that SO<sub>2</sub> induced an increase in the global DNA methylation level in *Arabidopsis* genome. The different banding patterns between control and SO<sub>2</sub>-fumigated plants were further classified into two groups. The group I was polymorphic sites, which mean a change of banding patterns after SO<sub>2</sub> fumigation. In this group, type A and B represented hypermethylation and hypomethylation sites in SO<sub>2</sub> treatment as compared to the control, which respectively accounted for 20.6% and 11.8% of the total bands. The results showed that SO<sub>2</sub> induced more hypermethylated sites than hypomethylated ones, suggesting that the global DNA methylation level of *Arabidopsis* genome were obviously promoted after SO<sub>2</sub> exposure. Type C referred to uncertain methylation sites that were interpreted as the potential conversion of methylation types (from external cytosine to internal cytosine or vice visa). The group II was monomorphic sites that showed no difference between the control and SO<sub>2</sub> treatment. The number of group II bands was 443 (Table 1), which accounted for 63% of the total bands, indicating that most methylation sites were stable and did not change under SO<sub>2</sub> stress.

### 3.2. SO<sub>2</sub>-induced hypomethylation in promoter regions of stress-responsive genes

To find out DNA methylation changes under SO<sub>2</sub> stress, we used bisulfite sequencing technique detecting the cytosine methylation in genes *ACS6* and *NIT2*, which were stress-responsive genes [6].

For *ACS6* gene, we examined methylation pattern of cytosine within 6863217~6863618 (402 bp) in promoter region that contains 45 cytosines, including 13 CG sites, 5 CHG sites and 27 CHH sites. After SO<sub>2</sub> exposure, hypomethylation occurred at seven CG sites, two CHG site and twelve CHH sites in *Arabidopsis* shoot cells. The methylation levels of cytosine in CG and CHH sites were reduced, whereas methylation levels at CHG sites was nearly similar to the control. The methylation levels at cytosine in *ACS6* promoter region was lower in SO<sub>2</sub>-fumigated plants (26.3%) compared with the control (30.2%).

For *NIT2* gene, we examined methylation pattern of cytosine within 15993439~15993888 (450bp) in promoter region that contains 31 cytosines, including 9 CG sites, 2 CHG sites and 20 CHH sites. After SO<sub>2</sub> exposure, hypomethylation occurred at seven CG sites and eight CHH sites in *Arabidopsis* shoot cells. The methylation level of cytosine decreased in CG and CHH sites, but increased a litter at two CHG sites. The methylation levels of cytosine in *NIT2* promoter region was lower in SO<sub>2</sub>-fumigated plants (32%) compared with the control (37%).

### 3.3. SO<sub>2</sub>-induced transcriptional activation of *ACS6* and *NIT2*

RT-PCR was used for detecting transcript levels of *ACS6* and *NIT2* genes in *Arabidopsis* shoots.

Relatively lower expression of *ACS6* and *NIT2* was observed in the untreated control, while higher expression was seen in SO<sub>2</sub>-fumigated samples (Fig. 1). These results indicated that SO<sub>2</sub> exposure induced both *ACS6* and *NIT2* gene transcription in *Arabidopsis* shoot cells.

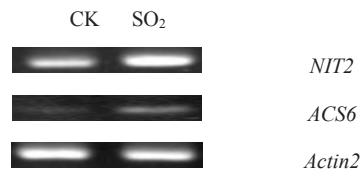


Fig. 1. RT-PCR results of *NIT2* and *ACS6* in *Arabidopsis* shoot cells.

#### 4. Discussion

Epigenetic mechanism such as DNA methylation/demethylation is critical for maintenance or formation of heterochromatin, and its alteration will result in a serious modification of genome stability and gene expression [11]. We examined the alterations of DNA methylation in *Arabidopsis* plants exposed to SO<sub>2</sub>. The MSAP data showed that SO<sub>2</sub> induced extensive variation in methylation at CCGG sites, with more hypermethylation sites and less hypomethylation sites, suggesting a high global level of cytosine methylation in *Arabidopsis* genome under SO<sub>2</sub> stress. Our result is consistent with previous reports that environmental stresses such as salt and osmotic stress tend to cause high level of cytosine methylation in the plant genome [12,13]. DNA hypermethylation, together with specific histone modifications, prevent recombination events and stabilize the genome [14]. Genome stability is crucial for cell growth and cell survival. In this case, SO<sub>2</sub>-induced hypermethylation could be considered as a stress response that results in genome stability contributing to the plant adaptation.

DNA methylation plays important roles in the regulation of gene transcriptional activity [1,15]. In the present study, we found that SO<sub>2</sub> induced cytosine hypomethylation in the promoter regions of *ACS6* and *NIT2* genes associated with enhanced transcription levels. The fact that *ACS6* and *NIT2* overexpressed in the null methyltransferase mutants suggests that SO<sub>2</sub>-induced demethylation of cytosine in the promoter regions was correlated with the increase of gene transcription. *ACS6* and *NIT2* are considered as key regulatory enzymes in the biosynthesis of ethylene and auxin [16,17]. The up-regulation of *ACS6* and *NIT2* will cause increases in ethylene and auxin production, which may play important roles in plant physiological process under SO<sub>2</sub> stress. Our observations provide clear evidence that SO<sub>2</sub>-evoked alteration of DNA methylation participate in the regulation of gene transcriptional activity in plant response to environmental stress. The SO<sub>2</sub>-induced methylation/demethylation in global genome is expected to have contributed greatly to regulating genome-wide gene expression in response and adaptation to SO<sub>2</sub> stress.

#### 5. Conclusions

In conclusion, SO<sub>2</sub> induced global genome hypermethylation and locus-specific hypomethylation in *Arabidopsis* shoot cells. These changes in DNA methylation were correlated with genome stability and gene expression, and the epigenetic modification was expected to have contributed greatly to plant response to SO<sub>2</sub> stress. We provide the first experimental evidence that cytosine methylation in the plant genome changes in plant response to SO<sub>2</sub> stress, which can shed some light on the possible roles of epigenetic mechanisms in plant adaptation to environmental stress.

#### Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant Nos. 30470318 and 30870454), Research Fund for the Doctoral Program of Higher Education of China (Grant No. 20070108007) and Shanxi Scholarship Council of China (Grant No. 2009022).

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